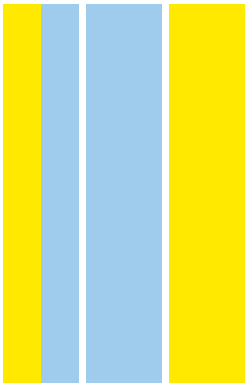


BIOLOGIA BASICA E APLICADA

Diet-microbiota interaction in health and disease: role of high salt diet in inflammatory bowel disease

Pedro Miguel Miranda

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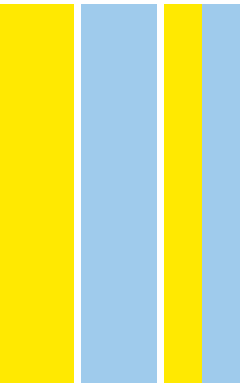
Pedro M. Miranda Diet-Microbiota Interaction in Health and Disease: Role of High Salt Diet in Inflammatory Bowel Disease



D.ICBAS 2018

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Pedro Miguel Miranda



PEDRO MIGUEL GOMES DA SILVA MIRANDA

**Diet-microbiota interaction in health and disease: a role
of high salt diet in inflammatory bowel disease**

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Abstract / Resumo

Abstract

Changes in hygiene and dietary habits, including increased consumption of foods high in fat, simple sugars, and salt that are known to impact the composition and function of the intestinal microbiota, may explain the increase in prevalence of chronic inflammatory diseases. High salt consumption has been shown to worsen autoimmune encephalomyelitis and colitis in mouse models through p38/MAPK signaling pathway. However, the effect of high salt diet (HSD) on gut microbiota and on intestinal immune homeostasis, and their roles in determining vulnerability to intestinal inflammatory stimuli are unknown. Here, we investigate the role of gut microbiota alterations induced by HSD on the severity of murine experimental colitis.

Compared to control diet, HSD altered fecal microbiota composition and function, reducing *Lactobacillus* sp. relative abundance and butyrate production. Moreover, HSD affected the colonic, and to a lesser extent small intestinal mucosal immunity by enhancing the expression of pro-inflammatory genes such as *Rac1*, *Map2k1*, *Map2k6*, *Atf2*, while suppressing many cytokine and chemokine genes, such as *Ccl3*, *Ccl4*, *Cxcl2*, *Cxcr4*, *Ccr7*. Conventionally raised mice fed with HSD developed more severe DSS- (dextran sodium sulfate) and DNBS- (dinitrobenzene sulfonic acid) induced colitis compared to mice on control diet, and this effect was absent in germ-free mice. Transfer experiments into germ-free mice indicated that the HSD-associated microbiota profile is critically dependent on continued exposure to dietary salt.

Our results indicate that the exacerbation of colitis induced by HSD is associated with reduction in *Lactobacillus* sp. and protective short-chain fatty acid production, as well as changes in host immune status. We hypothesize that these changes alter gut immune homeostasis and lead to increased vulnerability to inflammatory insults. This study provides an example of how the gut microbiota plays an essential intermediary role between environmental factors, such as diet, and host physiology. Furthermore, this thesis indicates the high salt consumption as a potential risk factor for inflammatory bowel diseases.

Resumo

Mudanças nos hábitos higiênicos e alimentares, incluindo o consumo de alimentação com alto teor de gordura, açúcares simples e sal - que se sabe exercerem um impacto na composição e função da microbiota intestinal - podem explicar o aumento da prevalência de doenças imunes crônicas. Foi recentemente demonstrado que o consumo de alto teor de sal tem um efeito adverso no desenvolvimento de encefalomielite autoimune e colite em ratinhos através da cascata de sinalização p38/MAPK. No entanto, ainda é desconhecido o efeito da dieta com alto teor de sal (DS) na estrutura da microbiota intestinal (ou flora intestinal) e na homeostasia do sistema imune; bem como o seu papel na vulnerabilidade para responder a estímulos imunológicos no intestino. Neste estudo, investigamos o papel que as alterações na microbiota intestinal induzidas por DS, exerce na severidade do desenvolvimento de colite em ratinhos.

Comparado com a dieta controle, a DS induz a alteração da composição e função da microbiota fecal, nomeadamente a redução da abundância relativa de *Lactobacillus sp.* e produção de butirato. Além disso, DS afetou a imunidade da mucosa intestinal no cólon e, de uma maneira menos proeminente, do intestino delgado. DS induziu a expressão de genes pro-inflamatórios como por exemplo Rac1, Map2k1, Map2k6, Atf2, e suprimiu genes de várias citocinas e quimiocinas, dos quais Ccl3, Ccl4, Cxcl2, Cxcr4, Ccr7. Ratinhos alimentados com DS desenvolveram uma colite mais severa usando os modelos de colite induzida por DSS e DNBS, quando comparados com ratinhos em dieta controle. Este efeito esteve ausente quando usados ratinhos desprovidos de bactérias (Germ Free mice). Experiências de transferência de microbiota para ratinhos desprovidos de bactérias indicaram que a microbiota associada a DS precisa duma exposição continuada a DS para manter o seu perfil.

Os nossos resultados indicam que o agravamento da colite induzido pela DS está associada à redução de *Lactobacillus sp* e à produção de butirato, bem como a mudanças no status imunológico do hospedeiro. Nós propomos que estas mudanças alteram a homeostasia da imunidade nos intestinos, o que leva

ao aumento da vulnerabilidade aos insultos inflamatórios. Este estudo apresenta-se como um exemplo do papel essencial que a microbiota intestinal desempenha na intermediação entre factores ambientais, como a dieta, e a fisiologia do hospedeiro. Além disso, esta tese indica o consumo elevado de sal como um potencial factor de risco em doenças intestinais inflamatórias.

List of abbreviations

5-HT	Serotonin
ASD	Autism spectrum disorder
BDNF	Brain-derived neurotropic factor
BMI	Body mass index
<i>B. fragilis</i>	<i>Bacteroides fragilis</i>
<i>C. difficile</i>	<i>Clostridium difficile</i>
CD	Crohn's disease
CDI	<i>Clostridium difficile</i> infection
CID	Chronic immune disorders
CVD	Cardiovascular disease
DA	Dopamine
DNBS	Dinitrobenzene sulfonic acid
DSS	Dextran sulfate sodium
EAE	Experimental autoimmune encephalomyelitis
FMT	Fecal microbiota transplant
GALT	Gut-associated lymphoid tissues
GF	Germ-free
GWAS	Genome-wide association studies
HFD	High fat diet
HSD	High salt diet
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IL	Interleukin
LPS	Lipopolysaccharide
MAMP	Microbial-associated molecular pattern
MLN	Mesenteric lymph node
MS	Multiple sclerosis

NA	Noradrenaline
NaCl	Sodium chloride (salt)
NFAT5	Nuclear factor of activated T-cells 5
NLR	Nod-like receptors
NMDA	N-methyl-D-aspartate
P38/MAPK	p38 mitogen-activated protein kinase
PP	Peyer's patches
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
SCFA	Short-chain fatty acid
SFB	Segmented filamentous bacteria
SGK1	Serum/glucocorticoid-regulated kinase 1
SNP	Single nucleotide polymorphism
TLR	Toll-like receptor
TMAO	Trimethyl amine N-oxide
TNF	Tumor necrosis factor
UC	Ulcerative colitis
WD	Western diet

I – General Introduction

I – General Introduction

1. Chronic inflammatory disorders: incidence in the “Western world”

The incidence of chronic diseases, namely related to some form of immune disorder, such as inflammatory bowel disease (IBD), multiple sclerosis (MS), diabetes, asthma, rheumatoid arthritis (RA), and others has been increasing over the past 50 years, namely in the “Western” (industrialized) regions of the planet [1,2]. Although many of these chronic immune disorders (CID) have been linked to genetic susceptibility factors, genetic drift alone can not explain the population incidence rising of CID over the last half-century [3]. Moreover, a relatively low concordance rate for most of autoimmune diseases between monozygotic twins suggests non-genetic factors as important triggers of disease [4]. Several of these non-genetic factors have been proposed to explain the rise of chronic immune related disorders, including decreased incidence of infectious diseases (the so called “hygiene hypothesis”) [1,5] (Fig. 1), as well as environmental factors related to changes in life-style observed in the western world, in particular dietary and hygiene factors [6].

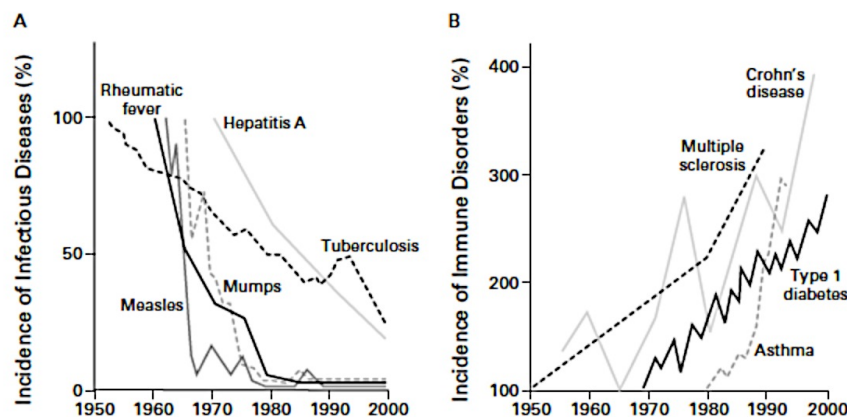


Figure 1. Hygiene hypothesis. Inverse relationship between the incidence of prototypical infectious diseases (Panel A) and the incidence of chronic immune disorders (Panel B) from 1950 to 2000. (Figure from Bach JF, 2002[1]).

2. “Western diet”

2.1. General features and implications in chronic inflammatory disorders

Diet have undergone significant changes over the last few decades, that have been considered too quick on an evolutionary time scale, for the human genome to adjust [7]. The Western Diet (WD) is generally characterized by high content of saturated fats, red meats, refined sugars and salt, while low in fresh fruits and vegetables with consequent low fiber content [7,8]. There is growing evidence from human and animal studies implicating WD factors in the development of CID. Epidemiological studies revealed a strong correlation of dietary factors such as low fiber [9] and high fat [10] consumption with CID, in particular IBD, diabetes and asthma [8]. However, careful approach must be taken when interpreting diet-disease epidemiological studies because of the multifactorial etiology of chronic diseases, and the complexity and often subjective nature of self-reported dietary patterns [11]. Interventional studies have uncovered a link between high fat and low fruit and vegetables consumption and worse asthma outcomes [12,13]. Although population studies have demonstrated a correlation between diet and CID, the mechanisms involved in diet-mediated inflammatory processes are largely unknown. Feeding mice with high fat diet (HFD) was shown to spontaneously increase the circulating proinflammatory cytokines $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 in the plasma, and to induce macrophage infiltration and inflammation in the adipose tissue, in a TLR4 signaling pathway dependent way [14]. Moreover, it was shown in rodent models that a high-fat diet exacerbates models of IBD [15], experimental autoimmune encephalomyelitis (EAE, a rodent model mimicking aspects of MS) [16] and collagen-induced arthritis (CIA) [17].

2.2 Salt: a new player in chronic inflammatory disorders

One dietary feature of WD that has been overlooked for its potential role in contributing for CID is its high salt (sodium chloride, NaCl) content. The consumptions of salt has increased in the Western world societies, mainly driven by the increased intake of processed foods and “fast food” in the industrialized societies, which can contain more than 100 times the salt found in similar home made meals [7,18,19]. In addition to well-documented effects of excess salt intake in increased blood pressure, cardiovascular disease and stroke [20], salt was recently shown to modulate aspects of the immune system and was linked to EAE severity in mice [21,22]. Increased NaCl concentration was able to induce the differentiation of a pathogenic form of Th-17 T cells from human and rodent naïve CD4⁺ T cells *in vitro*. This process was dependent on the activation of p38 mitogen-activated protein kinase (P38/MAPK), nuclear factor of activated T-cells 5 (NFAT5) and serum/glucocorticoid-regulated kinase 1 (SGK1) [21,22]. Th17 cells are a group of CD4⁺ROR γ t⁺ T cells that secrete interleukin-17 (IL-17A and IL-17F) and IL-22, and play an important role in protecting against bacterial and fungal pathogenic infections, particularly at mucosal surfaces [23]. Pathogenic IL-23-dependent Th-17 cells have been shown to be critical for the development of EAE in rodents and autoimmune diseases [24]. Indeed, mice receiving a salt-enriched diet (high salt diet, HSD) developed an exacerbated form of EAE, thus showing for the first time a link between salt consumption and a CID in an animal model [21,22]. Moreover, an observational clinical study using regression analysis, found a positive correlation between sodium intake and increased disease activity in a cohort of relapsing-remitting patients with MS followed for two years [25]. Interestingly, in a longitudinal experiment performed under highly controlled spaceflight simulation program, human subjects were exposed to fixed salt intake changes and their immune system was assessed. Subjects, when exposed to HSD, displayed increased numbers of circulating monocytes and pro-inflammatory cytokines IL-6 and IL-23, along with decrease of anti-inflammatory cytokine IL-10 [26].

A possible mechanism by which dietary factors modulate physiological processes and in particular the immune system, is through interaction with gut microbiota. In fact, it is well established that diet determines the composition and function of the gut microbiota, with repercussions for health and disease [27].

3. Gut Microbiota

3.1. Overview

Accumulating data suggest that gut microbiota plays an essential role in host health and disease. It consists of a living consortium of different microorganisms such as Bacteria and Archaea, Eukarya and Viruses inhabiting the gastro-intestinal tract (GI tract), that have co-evolved with the host over thousands of years into a complex symbiotic mutualistic relationship. While the host provides a protected habitat and the necessary substrate, the gut microbiota contributes by controlling a vast enzymatic repertoire, essential for gut physiology, metabolic, immune and neuronal functions.

Bacteria numbers in the human body were estimated to account for $\approx 10^{14}$, thus exceeding human body cells by 10:1. These numbers were however recently revisited, after the original estimation was traced back to an old “back-of-the-envelope calculations” published in the 1970’s [28,29]. In the new proposed estimation, the number of total bacteria was estimated to $\approx 3.8^{13}$, resulting in a $\approx 1:1$ ratio of bacteria to human cells [29,30]. Even more relevant, the largest human gut microbiome (the sum of all genes in the microbiota) metagenomics analysis to date [31], has identified around 10 million non-redundant genes from a multi-continental human consortium including Europeans, Chinese and American subjects, comprising of an average of $\approx 750,000$ genes per sample. These numbers completely dwarf the 19,000-20,000 protein-coding genes that comprise the human genome [32], indicating that the gut microbiome gene pool is 500x larger than human genome, and that an

average human gut microbiome contains 40x more genes than the human host genome itself.

Microbiota populates the entire GI tract, from mouth to rectum, but the density and composition of bacteria varies greatly along the gut. While the upper GI tract contains low density of predominantly aerobic bacteria, the lower GI tract harbors higher density numbers of anaerobic and facultative anaerobic bacteria, ranging from 10^3 - 10^4 bacteria/ml in the stomach and duodenum, to 10^{11} bacteria/ml in the colon, which harbors the vast majority of the body's bacterial load [33]. This heterogeneous pattern is a reflection of several determinant factors such as pH and oxygen level, as well as the content flow speed through the tract. Low pH and faster content flow, typical of the stomach and the upper part of small intestine impose a harsh environment that limits bacteria colonization, and vice-versa. This diversity makes the GI tract a continuous collection of different micro ecosystems, with specialized functions and microbiota-host interactions.

The development of culture-independent technologies based on the sequencing of gut microbiota genetic information, started a new era in our understanding of the role of microbiota in host's health and disease, by allowing a deeper monitoring and quantification of microbiota composition, function and dynamics, in a relatively fast and low cost manner. Perturbations in gut microbiota composition and function, known as dysbiosis, have been extensively associated to multiple diseases, ranging from intestinal disorders to metabolic, hepatic, neuronal, respiratory and immune-related diseases. In fact, gut microbiota has gained tremendous attention as a potential key mediator between the rapid changes occurring in environmental factors and life-style and the rise of the incidence of several diseases, as this cannot be explained by the human population genetic drift alone.

This thesis will be focus on the bacteria that inhabits the GI tract. So for simplicity purposes, I will be referring to bacteria when mentioning microbiota.

3.2. Development of the human gut microbiota

Historically, the newborn infant was considered to be sterile until the mother has given the initial microbiota inoculum at birth, from the vaginal or skin microbiota, at natural birth or C-section respectively. However, this dogma was challenged by DNA-based studies that shown undeniable evidence of the microbial presence in the healthy placenta, amniotic fluid [34,35], umbilical cord [36] and meconium [37,38] (the first stool of a newborn). It is still unclear how bacteria penetrate the intrauterine environment but in one experiment, specific labeled bacteria fed to pregnant mice has been detected in the meconium of the offspring, suggesting that intrauterine bacteria can be transmitted from the mother's GI tract [37].

After birth, the GI tract becomes rapidly colonized, with the neonatal gut microbiota composition being determined by several factors such as the mode of delivery (vaginal or C-section), gestational age, diet (breast milk or milk formula) and early antibiotic treatment [39–42]. Early assembly of gut microbiota is a non-random process with different species appearing at different time points, beginning with the establishment of oxygen tolerant bacterial species mainly from Firmicutes and Proteobacteria phylum, which reduce the GI tract environment allowing the further establishment of strict anaerobic species such as *Bifidobacterium* and *Bacteroides* [40,41,43]. Over time, the microbiota diversity rapidly increases until an adult-like microbiota community dominated by the bacteria phylum Bacteroidetes and Firmicutes becomes established at around 2-4 years old [40,41,43]. In the elderly, the gut microbiota suffers a general loss of diversity, a reduction of beneficial bacteria such as *Bifidobacterium spp* and changes in metabolic pathways such as the decrease of Short Chain Fatty Acid (SCFA) production, a class of bacteria metabolites essential for intestinal function, that will be discussed below [44–46].

Several studies have attempted to define healthy gut microbiota enterotypes (defined types of microbial communities) [47,48] based on diet habits or geographical distribution of the population. However, and despite the efforts this concept remains controversial due to the disparities found across multiple studies and the sensitivity of the clustering methods applied in

defining microbiota enterotypes [49,50]. In the past decade, two major population-scale studies, The Human Microbiome Project (HMP, [51,52]) and the Metagenomics of the Human Intestinal Tract (MetaHIT, [31]), helped shedding light on the structure and diversity of healthy human microbiota. These large population studies confirmed that the human gut microbiota is clearly dominated by two bacteria phyla, Bacteroidetes and Firmicutes; accompanied by bacteria from Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia phyla and others. Overall, mice share the same gut core microbiota as humans, with a strong dominance of Bacteroidetes and Firmicutes phyla, although the proportions of some phyla and genera can be distinct [53,54].

3.3 The gut microbiota functions in health

The beneficial properties that the gut microbiota provides to the host are diverse and include breaking down diet-derived molecules and generating new essential metabolites with beneficial functions to the host, contributing to intestinal barrier function, development and maturation of the host's immune system, protecting against pathogens and even playing a role in the central nervous system function.

Studies using animals deprived of any microbes (germ-free) and studies using antibiotics to induce dysbiotic gut microbiota, provide an important proof-of-principal to understand the functions and benefits of the microbiota. GF animals are reared in sterile isolators in order to control for the exposure of any microorganism. These animals can be studied in their sterile condition (germ-free animals) or be colonized with a defined microbiota mixture (gnotobiotic animals). When compared to conventional mice harboring normal microbiota, GF mice suffer from a generalized metabolic inefficiency, as demonstrated by the need to consume more food to maintain the same body weight [55] or the resistance of these mice to diet-induced obesity [56]. GF mice are also deficient in some vitamins and completely lack secondary bile acids [57,58]. Moreover, GF mice have an immature IS, with extensive deficits in the development of the gut-associated lymphoid tissues (GALT), and in the production of antibodies,

defensins and other anti-microbial proteins. GF mice contain abnormal immune cell levels and have deficiencies in immune responses [59]. Defects in the gut barrier were also observed of GF mice, including thinner mucus layer, lower expression of proteins important for gut epithelial integrity and a general increased permeability [59]. Finally, GF mice were shown to have altered responses to stress, behavioral baseline and brain chemistry when compared to conventional mice [60].

Similarly to studies in GF animals, the action of antibiotics in disrupting the gut microbiota has helped shedding light on the beneficial role of gut microbiota. Most notably, antibiotics have been shown to be associated with intestinal pathogenic infections such as *Clostridium difficile*, reinforcing the function of commensal microbiota in the protection against pathogens [61]. Likewise, the use of antibiotics during development is associated with detrimental health consequences such as increased risk of infections, asthma, allergies, diabetes and obesity [62], supporting the role of gut microbiota in the development of the IS and metabolic function of the host.

3.3.1. Microbiota role in host metabolism

The gut microbiota controls a vast enzymatic repertoire that exceeds by a large scale the host enzymatic capacity. This bacterial enzymatic machinery play an essential role complementing the digestion of the host's diet, as well as by providing the host with a vast diversity of metabolites that are important for host's health function. The large quantities of complex carbohydrates (fiber) that escape digestion and absorption by the small intestine are assimilated by colonic bacteria and fermented into different SCFA, representing the major flow of carbon from the diet, through the microbiome to the host [63]. SCFA are fatty acids with two to six carbon atoms that are small enough to be absorbed by the epithelial cells in the GI tract, and play an important function in host physiology as sources of energy and signaling molecules that act as regulators of cellular processes such as gene expression, chemotaxis, differentiation, proliferation and apoptosis [64]. The three major SCFA are acetate, propionate and butyrate

and they differ in their potential function in host physiology. While most anaerobic bacteria can produce acetate, which invariably achieves the highest concentrations among the SCFA, propionate and butyrate are produced by specific groups of gut bacteria that use distinct metabolic pathways [64]. Propionate is produced by members of Bacteroidetes phylum, such as certain *Bacteroides* and *Prevotella* bacteria, whereas butyrate is mostly produced by members of Firmicutes phylum, mainly by bacteria of *Lachnospiraceae* and *Ruminococcaceae* families, but also by Actinobacteria species [64]. Butyrate has received much attention for its many roles in health and disease and potential therapeutic value [65]. Butyrate serves as the preferred metabolic substrate of colonic epithelial cells (colonocytes), where it is oxidized to CO₂ in the presence of circulatory O₂, resulting in the formation of ATP [66]. Butyrate has been shown to have anti-inflammatory properties, mainly via the inhibition of nuclear factor κB (NF-κB) in colonocytes [67]; and anti-cancer activity via the ability to induce apoptosis in colonic cancer cells [65]. Butyrate has also been shown to contribute to intestinal barrier integrity and to regulate the intestinal pH [68]. A decrease in butyrate-producing bacteria has been found in IBD patients [69] and is associated with expansion of pathogenic bacteria such as *Salmonella* [70], supporting the view that butyrate plays a critical role in disease resistance.

Vitamins are another class of beneficial metabolites that can be synthesized by gut microbiota. These include vitamin K and several B group vitamins such as biotin (B8), cobalamin (B12), folate (B9), pyridoxine (B6), riboflavin (B2) and others [57]. In fact, a study analyzing the biosynthesis pathways of eight different B-vitamins in the human gut microbiome, estimated that the gut microbiota contributed with over a quarter of the suggested dietary intake of four B vitamins [71]. Colonic bacteria can also metabolize bile acids that escape the enterohepatic circulation, into secondary bile acids. These metabolites play an important role in dietary digestion and as a microbial community selective pressure due to its anti-microbial properties [72].

3.3.2. Microbiota role in host immune system

The co-evolution of the immune system and the gut microbiota created a symbiotic relationship, where the immune system maintains a tolerant stand towards the microbial communities inhabiting the intestine while providing microbial population control that ultimately benefits the community. In turn, the microbiota promotes and calibrates multiple aspects of the immune system [73]. The communication between the gut microbiota and the immune system is mainly mediated by the recognition of conserved microbial-associated molecular patterns (MAMPs) such as lipopolysaccharide (LPS) and peptidoglycans, by the host cells. These molecular effectors can be recognized by pattern recognition receptors (PRRs) in epithelial and immune cells, such as toll-like receptors (TLRs) and Nod-like receptors (NLR).

Studies in GF mice have revealed that these mice have fewer and smaller Peyer's patches (PP) and mesenteric lymph nodes (MLNs), reduced numbers of CD4⁺ T cells, IgA-producing plasma cells and reduced expression of antimicrobial molecules and TLRs; thus illustrating the key role that microbiota plays in the development of host's immune system [59]. The role of microbiota in the immune system starts at birth, when the first members of the microbial community start to colonize the GI tract and interact with the naïve IS. This early interaction is vital to promote the healthy colonization of the microbial community and benign relationship between microbiota and the immune system, while tuning a healthy responsive mucosal and systemic immune system for the long-term. In fact, early antibiotic interventions are associated with increased risk for diseases related to hyperreactivity of the IS such as asthma, allergies, Crohn's disease, Type 1 and Type 2 Diabetes [62,74].

One of the first described direct effects of a specific commensal bacteria on the host immune system was the role of polysaccharide A (PSA) produced by the commensal *Bacteroides fragilis* in stimulating the expansion of CD4⁺ T cells and development of lymphocyte follicles in the spleen [75]. Observations in GF mice and antibiotic studies have shown that the differentiation and expansion of Th17 populations is highly dependent on the gut microbiota [76]. Indeed, Ivanov *et al* observed that mice from a specific animal commercial vendor

facilities presented abnormally low numbers of Th17 cells, which could not be attributed to genetic differences but instead to the gut microbial composition [76]. Moreover, the authors showed that a particular bacteria, the segmented filamentous bacteria (SFB), induced differentiation and expansion of Th17 cells in intestinal *lamina propria* [77].

3.3.3. Microbiota role in promoting epithelial barrier

An example of the mutualistic dialogue between the gut microbiota and the immune system is the team effort to limit bacteria contact with the epithelial cell surface. This is an essential strategy for maintaining the homeostasis between the two parts, and is accomplished by the combination of several factors including the epithelial cell monolayer, production of mucus, IgA, antimicrobial peptides and immune cells, a phenomenon that has been referred as the “mucosal firewall” [78]. Microbiota derived molecules such as LPS and SCFA stimulate different types of host cells to produce this panoply of immune factors that will limit microbial translocation to *lamina propria* and tissue inflammation, which could result in damaging consequences for both the microbial community and the host health [79]. Deficiencies in this balance are suggested to play a role in the development of IBD. Indeed, patients with IBD have increased numbers of epithelial cell surface-associated bacteria [80] and patients with PRR NOD2 defects, one of the first identified IBD risk allele, have lower antimicrobial α -defensin production [81].

Several bacteria species have been linked to promoting epithelial integrity. *Akkermansia muciniphila* and *Lactobacillus plantarum* were shown to upregulate tight junction proteins and reduce gut permeability [82,83]. In addition, *Faecalibacterium prausnitzii* and *Bacteroides thetaiotaomicron* species have been shown to modulate the production of mucus glycans and the development of goblet cells [84].

3.3.4. Microbiota role in protection against pathogens

Gut microbiota plays an essential role in protecting the host against potential pathogens by limiting pathogen colonization through competing for metabolites and ecological niches within the intestine habitat, in a process referred to as colonization resistance [85]. External factors such as diet and antibiotics can disrupt the microbiota ecological balance and release competitive pressure that will benefit pathogens [85]. This microbiota function is well illustrated by the antibiotic-induced *Clostridium difficile* infection (CDI), which prevalence and severity have been increasing in the past two decades [86]. CDI can be resolved by fecal microbiota transplantation (FMT) with remarkable efficacy, likely by restoring microbial diversity and altering the metabolic environment in the intestine of recipient [87]. In fact, specific commensal bacteria belonging to XIVA cluster of Clostridia were shown to be sufficient to confer resistance to CDI by synthesizing *C. difficile*-inhibiting metabolites from host-derived bile acids [88].

3.3.5. Microbiota role in central nervous system

Gut microbiota is in constant communication with the enteric and central nervous system, through bidirectional neural, immune and endocrine pathways, the so-called microbiota-gut-brain axis [89]. Over the last decades, a tremendous body of evidence has demonstrated the essential role of gut microbiota in the development and function of the host brain, and host behavior [89]. The earliest descriptions that the gut microbiota could impact brain function and behavior arise from the observation that administration of oral antibiotics can reverse hepatic encephalopathy [90]. GF mice exhibit altered brain chemistry, including the expression of neurotransmitters such as noradrenaline (NA), dopamine (DA) and serotonin (5-HT); NMDA and 5-HT receptors, as well as brain-derived neurotrophic factor (BDNF) [91,92]. Additionally, GF mice exhibit an increased response to stress and exploratory behavior, as well as learning, memory and sociability deficits [91–94]. Treating

mice with antibiotics was also sufficient to trigger changes in brain chemistry and behavior, and these changes were reversible upon normalization of microbiota [95], or treatment with probiotics and physical exercise [96]. Moreover, in an important proof-of-principal experiment demonstrating the influence of gut microbiota on host's behavior, Bercik *et al* were able to modulate the behavior of different mice strains, the timid BALB/c mice and the exploratory NIH Swiss mice, by transplanting the gut microbiota from one strain to the other. When the microbiota of NIH Swiss was transplanted to the GF BALB/c mice, the recipient mice acquired a more exploratory behavior characteristic of the NIH Swiss donor. However, GF NIH Swiss mice become less exploratory when received microbiota from BALB/C mice [95]. Studies with specific probiotics have also showed the dynamics of the microbiota-gut-brain axis. Feeding mice with specific beneficial bacteria was shown to modulate brain chemistry and ameliorate stress, anxiety and depressive-like behaviors, promote social behavior and improve cognitive function [97]. Gut microbiota has been also implicated in neuropsychiatry disorders including depression and anxiety, autism spectrum disorder (ASD), schizophrenia and Alzheimer's disease [98].

In addition to CNS, gut microbiota was shown to be important for the normal post-natal development of the enteric nervous as demonstrated by a decreased in nerve density and abnormal neurochemical composition and function of enteric neurons in the small intestine of GF mice [99].

3.4. Factors influencing gut microbiota

A multitude of endogenous host factors and external factors contribute to shaping the gut microbiota composition. Host factors such as genetic background [100], gender, body mass index (BMI) [101], age [102], the immune response [103], gut function (e.g. gut motility and pH) [33], central factors (e.g. stress, mood, psychiatry disorders) [104] and even diurnal rhythm [105] have been described to exert influence on the composition of gut microbiota. Likewise, environmental factors such as diet [106], xenobiotics (e.g. antibiotics) [107], probiotics/prebiotics [108], FMT [109] and life style factors (e.g. smoking)

[110], and mode of delivery of a neonate [102] were reported to have an impact in shaping microbiota composition and function.

3.4.1. Genetics

In the last few years, several studies have attempted to analyze the role of host genetics shaping gut microbiota. In particular, three independent reports using different large population cohorts published in the same issue in *Nature Genetics* (Nov 2016) have found association between host single nucleotide polymorphisms (SNPs) and genomic loci and individual microbiota taxonomies and functional pathways [111–113]. However, the fact that even highly heritable microbiome traits identified associations have small effect size, and recently described concerns about the statistical associations of these studies, have raised the question of the true weight of host genetic in modulating the microbiota [114–116]. Indeed, a recent study has found limited evidence of microbiome-genetic association after analyzing the previous published cohorts, and using a new independent cohort [116]. The study showed that sharing a common household, not host genetic relationship, determines microbiome similarity, and concludes that environmental factors dominate over host genetics in shaping human gut microbiota [116].

3.4.2. Lifestyle, geography and diet

To determine how lifestyle, including geographic, ethnic factors and long-term diet, impacts the gut microbiota, scientists have employed comparative population studies between rural communities and industrialized/westernized societies. These studies have revealed that industrialization had a profound impact on gut microbiota including a decrease in the diversity within the individual (α -diversity) and an increased diversity between the individuals (β -diversity) [117,118], the acquisition and loss of specific gut microbes [119], and major shifts in gut metabolic patterns [120]. These differences were mainly

attributed to major changes in long-term dietary habits between rural and industrial societies. In particular, the high fiber plant-based diet of rural communities was shown to favor *Prevotella* bacteria while low-fiber animal based diet typical of industrialized societies favors *Bacteroides* [117,120–122].

Short-term diet-intervention studies in humans and animals have helped corroborating the impact of diet and specific dietary ingredients on gut microbiota composition and function. The content of fiber, protein, fat, artificial sweeteners, and emulsifying agents have all been reported to modulate the diversity and specific microbes of the GI tract [27,123–126].

3.4.3. Microbe-microbe interaction

Gut microbes live in close interaction with each other, either in co-operative or competitive relationships. For example, certain carbohydrate fermentation products such as lactate, succinate and 1,2-propanediol, are not usually found in high levels in the human colon of healthy adults, because they can serve as substrates for other bacteria, including propionate and butyrate producers, a mechanism called microbial cross-feeding [64]. On other hand, microbes compete for specific substrates and niches in the GI tract. Indeed, specific perturbation of gut microbiota by the depletion (e.g. antibiotics, dietary factors) and addition of specific microbial species (e.g. probiotic and FMT interventions) will impact the composition of microbiota [127].

3.5. Diseases associated with microbiota

Considering the important role of the gut microbiota in the host's health, it is not surprising that association studies in humans and animals have revealed a wide range of dysbiosis-related chronic diseases. These include IBD, IBS, celiac disease, colorectal cancer, metabolic disorders (such as diabetes and obesity), atherosclerosis, multiple sclerosis, asthma, chronic kidney disease, and autism spectrum disorder [128]. Although there has been certain demonstrated

causality in some of these associations, many are still based on epidemiological and observational studies, with the mechanisms linking the microbiota and the host disorders still largely unknown. Adoptive transfer experiments, where the gut microbiota is transferred from a human or animal to germ free animals, have provided a major contribution to understand causality of the effect of microbiota in chronic disorders. Different studies were able to demonstrate that transplanting patient microbiota in mice was sufficient to transfer the human phenotypes related to obesity [129], Crohn's disease, Ulcerative colitis [130], IBS [131] and MS [132].

4. Diet-microbiota interaction in health and disease

As described above, diet exerts a major influence on gut microbiota composition and function. The specific dietary components that arrive as substrate to gut microbes, will determine the metabolic pathways that the microbes will favor among its enzymatic repertoire. The cocktail of metabolites generated will, in turn, have an impact on the dynamics of that specific microbe and its “neighbors”, as well as on the host physiology (Fig. 2).

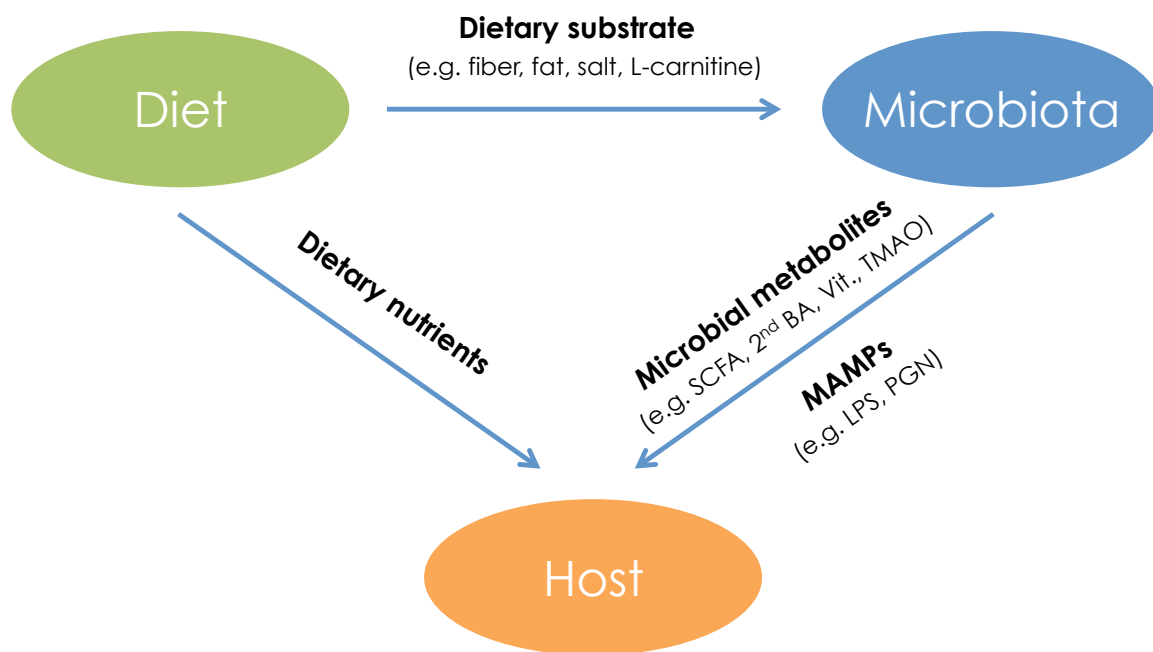


Figure 2. Diet-microbiota-host interaction. Diet provides substrate to gut microbiota, determining microbial metabolic pathways and composition. Host health and disease is affected by dietary nutrients and microbial metabolites and MAMPs. SCFA (short-chain fatty acids), 2nd BA (Secondary bile acids), Vit. (vitamins), TMAO (Trimethyl amine N-oxide), LPS (Lipopolysaccharide), PGN (Peptidoglycan).

Gut microbiota can be described an intermediate catalyzer between diet and the host, with the ability to generate *de novo* molecules such as SCFA, vitamins and secondary bile acids, which directly benefit or exert negative

influences on the host. The beneficial diet-microbiota-host interaction is well illustrated by the induction of SCFA-producing bacteria with high fiber diet, and consequent production of SCFA, such as butyrate and acetate, with major implications for gut homeostasis [6]. Diet-microbiota-host interaction can also have a detrimental impact on the host. Trimethyl amine N-oxide (TMAO) is a microbial metabolite generated from dietary lecithin and L-carnitine, molecules that are enriched in foods such as red meats, eggs, milk and some fish. TMAO was found to accelerate atherosclerosis in mice [133,134], and associated with increased risk of cardiovascular disease (CVD) in humans [134]. Moreover, vegans and vegetarians do not only have lower levels of red-meat derived TMAO compared to omnivores, but their microbiota also has a lower capacity to synthesize it from ingested carnitine [134]. In a study analyzing the glycemic response to semi-standardized diet, Zeevi *et al*, observed a high inter-personal variability for the same foods [135]. Using an algorithm that integrated the glycemic response data with clinical and microbial features, the authors were able to demonstrate that gut microbiota is an important predictor of the glycemic outcome to specific dietary factors. Moreover, the authors were able to integrate the microbiota information with other clinical features and design personalized diets with beneficial glycemic outcomes [135]. These and other examples of diet-microbiota-host interaction (Table 1) provide a strong evidence of how gut microbiota plays a key role in mediating the outcomes of diet in host health and disease.

Article	Year	Model	Diet/Prebiotic Intervention	Microbiota/metabolic shift	Phenotype on the Host	Others/Comments
Koeth RA et al. Nat. Med. [134]	2013	Human and Mice	L-carnitine supplemented diet	Increased TMA/TMAO levels	Accelerated atherosclerosis and inhibition of reversed cholesterol transport in a microbiota dependent fashion	Reveals a microbiota-dependent pathway linking dietary red meat ingestion with CVD
Zeevi D et al. Cell [135]	2015	Humans	Personalized Diet	Microbiota modulation is very consistent and highly predictive of the PPGRs outcome	Predicted "Good" diet improved Postprandial Glycemic Response	
Dey N et al. Cell [152]	2015	Mice	Different world diets were tested sequentially to mimetize a "travel" scenario	Microbiota shifts accordingly to diet with consequences on bile acid metabolite concentrations	Diet-induced microbiota and bile acid shifts determine gut motility phenotypes	
Devkota S et al. Dig. Dis. [148]	2015	Mice	High saturated (milk derived)-fat diet (MF) and n-6 polyunsaturated (Gafflower oil)-fat diet (SF)	MF: Promotes <i>Bifidophila wadsworthia</i> expansion and increase in taurocholic (TC) bile acid	<i>B. wadsworthia</i> bloom in MF promotes a Th1-mediated immune response and development of colitis in IL-10 ^{-/-} mice.	Possible explanation for the link between IBD and dietary-mediated selection of gut microbial pathobionts in genetically susceptible hosts
Perry RJ et al. Nature [213]	2016	Rats	High Fat Diet	Increased acetate turnover	Drives GSIS secretion in a microbiota dependent fashion. Acetate supplementation with normal diet was able to induce Obesity	Acetate was found to induce insulin via parasympathetic activation involving the vagus nerve. Authors described an acetate mediated microbiome-brain-β cells axis promoting metabolic syndrome
Devlin AS et al. Cell H&M [153]	2016	Mice	Fructo-oligosaccharides (FOS) diet	Favoured growth of indole-non-producing Bacteroides species, leading to reduction of indole	Reduction in indoxyl sulfate, which can be of critical importance in patients with chronic kidney disease	bacteria metabolites were modulated using a combined approach of diet intervention and antibiotics
Thaiss CA et al. Nature [204]	2016	Mice	High Fat Diet (HFD)	Increase levels of flavonoid-converting bacteria, leading to a chronic decrease levels of the certain flavonoids	Impairment of energy expenditure in brown adipose tissue (BAT), leading to susceptibility to accelerated weight regain upon HFD	By use of post-biotics flavonoides, authors reverted the HFD-induced microbiota-dependent phenotype
Geirnaert A et al. Sci. Rep. [205]	2017	Human microbiota/ in vitro	Butyrate-producing bacteria supplemented <i>in vitro</i> to Crohn's disease (CD) patient microbiota	Increased butyrate levels	Enhanced intestinal epithelial barrier integrity	Pilot study using microbiota of 10 CD patients that provide a proof-of-concept data with therapeutical potential

Abbreviations: TMA (Trimethylamine); TMAO (Trimethylamine-N-Oxide); CVD (Cardio Vascular Disease); GSIS (Glucose Stimulated Insuline Secretion); IBD (Inflammatory Bowel Diseases)

(See table on previous above)

Table 1. Evidence of diet-microbiota-host interaction. This table provides examples of studies demonstrating microbiota-mediated host phenotypes using diet or pre/probiotic interventions.

5. Inflammatory Bowel Disease

5.1. Epidemiology and etiologies

The inflammatory bowel diseases, Crohn's disease (CD) and ulcerative colitis (UC), are chronic idiopathic disorders causing inflammation of the gastrointestinal tract. CD can involve any part of the GI tract in a non-continuous fashion and is commonly associated with complications such as strictures, abscesses and fistulas. UC is characterized by inflammation that is limited to the colon and which usually starts in the rectum. The microscopic features of CD include transmural inflammation with thickened submucosa, while in UC inflammation is limited to the mucosa and submucosa tissue, with cryptitis and crypt abscesses [136].

The prevalence of IBD rapidly increased in the Western world in the second half of the twentieth century and is becoming more common in different parts of the world that are adopting a Western lifestyle [137]. Epidemiological observations indicate that there are strong environmental influences on IBD, which is corroborated by the relatively low concordance rate in identical twins (50% for CD, and 10% for UC) [138]. Nevertheless, genome-wide association studies (GWAS) have found associations between specific genes and genetic loci with IBD, many of them involved in intestinal barrier function, epithelial restitution, innate and adaptive immune regulation and microbial defense [139,140]. The etiology of IBD remains an enigma but it is hypothesized that IBD results from an adverse interaction between environmental, host factors and genetic susceptibility with gut microbiota playing a key role mediating the pathology [136] (Fig. 3).

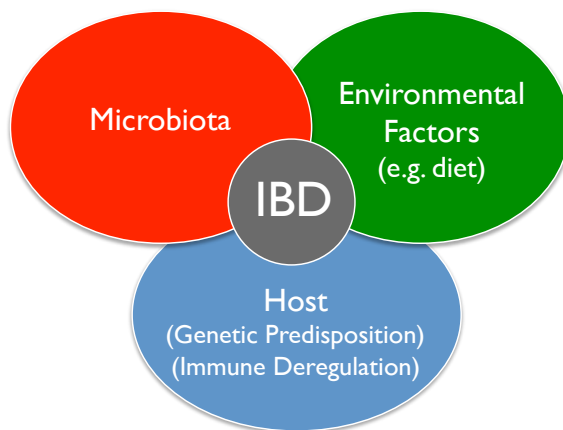


Figure 3. Interaction of various factors contributing to chronic intestinal inflammation in a genetically susceptible host.

Indeed, despite some disparate results, studies assessing gut microbial features of IBD patients support the concept that there is a generalized reduction in biodiversity (α -diversity) and altered representation of several specific bacterial taxa [141]. Moreover, some of the genes with the strongest association to IBD, such as NOD2 and genes involved in the differentiation of Th17 cells (such as IL-23 and IL-23R), play a key role in gut microbial surveillance [142,143]. Recently, it has been shown that transplanting gut microbiota from patients with CD to GF mice induced a pro-inflammatory gene expression profile in the mice gut that resembles the immunological signatures found in CD patients. Furthermore, microbiota of CD patients exacerbated experimental colitis in IL-10 deficient mice, a murine model of genetic susceptibility to colitis [130].

Diet has also been considered as an additional risk factor contributing to the etiology of IBD. While no effect was found for the intake of fiber, sugar, macronutrients, total energy, vitamin A, C, D, E and carotene on the risk of ulcerative colitis, n-6 polyunsaturated fatty acid was identified as a risk factor for UC, whilst n-3 polyunsaturated fatty acid may protect against UC. Furthermore, high animal protein diet was associated with increased risk of IBD and relapses [144].

5.2. Colitis models in mice

Experimental colitis models have widely served as useful tools for understanding the etiology of IBD and the preclinical evaluation of the efficacy of new drugs. Several models of acute and chronic intestinal inflammation have been developed to simulate various aspects of IBD in humans. Different models use very distinct strategies to induce GI inflammation including 1) disruption of epithelial barrier, 2) disruption of innate immunity, 3) excessive effector immune cell responses, 4) inadequate regulatory response, and others [145]. The methodology is also highly variable, including chemical-induced, genetic manipulation, pathogen inoculation, immune cells transfer and whole microbiota transfer [145]. Each experimental colitis model results in different phenotypic outcomes, including immunological and histological features, with various degrees of modeling human CD and UC (For a review in colitis models in mice, see [145]).

In this study, we used two different chemical-induced models, the dextran sulfate sodium (DSS) and the dinitrobenzene sulfonic acid (DNBS) models. Both models trigger a disruption of the GI tract epithelial layer although with different mechanisms of action and phenotypical outcomes.

5.2.1. DSS-colitis

DSS is delivered in drinking water of rodents and results in an inflammatory response in the GI tract with the recruitment of M1 macrophages and neutrophils to the mucosa. Histologically, DSS colitis induces submucosal erosions, inflammatory cell infiltration, erosion of the epithelium and mucosal hyperplasia. Mice with DSS colitis will show signs of acute colitis including body weight loss, bloody stools and diarrhea, accompanied with the increase in mucosal production of pro-inflammatory cytokines IL-6, TNF α and IL1 β [146]. The damage induced by DSS affects preferentially the colon and the cecum. The mechanisms of action are not fully understood but it has been proposed that DSS establishes linkages with medium-chain-fatty acids (MCFA) to form vesicles

that can fuse with colonocyte membranes, leading to a loss of barrier function, increased translocation of luminal antigens and activation of intestinal inflammatory signaling pathways [147]. The concentration and the number of cycles of DSS administration can be customized in order to induce different severity degrees of acute colitis and to trigger chronic colitis, respectively [146].

5.2.2. DNBS-Colitis

DNBS is a hapten reagent delivered rectally in the rodent colon which induces a localized colonic inflammation. DNBS induces significant body weight loss and bloody diarrhea. The colon damage is characterized by hyperplasia, excess of fibrotic tissue and lymphocytic pus, as well as areas of mucosal necrosis and typical visible ulcerations. Histological features include a transmural necrosis, loss of epithelium and diffuse leukocyte cellular infiltrate in the submucosa, namely neutrophils. DNBS is dissolved in a 50% ethanol solution to help break the mucosal barrier. The hapten properties will trigger a T cell response, including the production of Th1 profile cytokines (IFN γ , TNF α and IL12) [146].

5. AIMS

High salt consumption was described to have an impact in modulating the immune system in a pro-inflammatory manner, with a direct effect on the differentiation and expansion of Th17 cells, and to exacerbate the development of MS, both in rodents and humans.

Considering that gut microbiota and Th17 T cells play a pivotal role in the development of the pathogenesis of IBD, we hypothesized that a diet-enriched in salt could affect the development of colitis in mice, through the modulation of gut microbiota.

First, we characterized the effect of HSD on the composition and metabolic pathways of mouse gut microbiota. We proceeded by characterizing the impact of HSD on the mucosal immunity of intestinal *lamina propria*. After that, we assessed the effect of HSD on the development of gut inflammation using two different models of experimental colitis, DSS and DNBS colitis. Finally, using GF mice and gut microbiota transplant experiment, we sought to determine whether gut microbiota plays a role in the HSD-induced colitis severity.

These data open new insights on the potential mechanisms for the development of IBD, as well as reveal a new pathway of diet-microbiota-host interaction.

II – Research Work

High diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production

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High salt diet exacerbates colitis in mice by decreasing
Lactobacillus levels and butyrate production

The research work “High salt diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production” consists of a first-authorship publication. Here is clarified the contribution of each of the authors:

Publication:

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Pedro Miguel Miranda, the candidate for the PhD degree at the University of Porto, contributed to the paper by initially single-handed establishing the HSD-colitis mice model in the laboratory. Pedro also developed the study concept and design, with the supervision of the authors GDP and PB. Moreover, Pedro prepared and carried out all experiments that led to this publication, including DNA/RNA isolation, 16S rRNA gene library preparation, sequencing analysis, interpretation of microbiome/immune/colitis data, SPF mice experiments. Finally, Pedro wrote this manuscript with the supervision of the authors GDP and PB.

The contribution of each author to the publication is clarified as the following:

Authors contribution: GDP and PB contributed to develop the study concept and design, interpretation and discussion of the results. GDP performed all experiments with the GF mice. VS and JM assisted with all immune cell isolation experiments, cell-staining and flow cytometry procedures. JL assisted with all animal work, including blind colitis and histological assessments. MPLA carried out Q-PCR experiments. EFV helped coordinating all GF mice experiments. GDP, PB and EFV were major contributors to the critical review of the manuscript. All authors read and approved the final manuscript.

High salt diet exacerbates colitis in mice by decreasing *Lactobacillus* levels and butyrate production

Keywords: Salt, NaCl, Western Diet, Colitis, Microbiota, *Lactobacillus*, Butyrate, MAPK-pathway

1. Abstract

Background: Changes in hygiene and dietary habits, including increased consumption of foods high in fat, simple sugars, and salt that are known to impact the composition and function of the intestinal microbiota, may explain the increase in prevalence of chronic inflammatory diseases. High salt consumption has been shown to worsen autoimmune encephalomyelitis and colitis in mouse models through p38/MAPK signaling pathway. However, the effect of high salt diet (HSD) on gut microbiota and on intestinal immune homeostasis, and their roles in determining vulnerability to intestinal inflammatory stimuli are unknown. Here, we investigate the role of gut microbiota alterations induced by HSD on the severity of murine experimental colitis.

Results: Compared to control diet, HSD altered fecal microbiota composition and function, reducing *Lactobacillus* sp. relative abundance and butyrate production. Moreover, HSD affected the colonic, and to a lesser extent small intestine mucosal immunity by enhancing the expression of pro-inflammatory genes such as *Rac1*, *Map2k1*, *Map2k6*, *Atf2*, while suppressing many cytokine and chemokine genes, such as *Ccl3*, *Ccl4*, *Cxcl2*, *Cxcr4*, *Ccr7*. Conventionally raised mice fed with HSD developed more severe DSS- (dextran sodium sulfate) and DNBS- (dinitrobenzene sulfonic acid) induced colitis compared to mice on control diet, and this effect was absent in germ-free mice. Transfer experiments

into germ-free mice indicated that the HSD-associated microbiota profile is critically dependent on continued exposure to dietary salt.

Conclusions: Our results indicate that the exacerbation of colitis induced by HSD is associated with reduction in *Lactobacillus* sp. and protective short-chain fatty acid production, as well as changes in host immune status. We hypothesize that these changes alter gut immune homeostasis and lead to increased vulnerability to inflammatory insults.

2. Background

The prevalence of chronic inflammatory diseases such as multiple sclerosis (MS), rheumatoid arthritis, diabetes and inflammatory bowel disease (IBD), has been steadily increasing over the past half-century [1]. Although multiple genetic factors have been identified to contribute to the risk of developing these disorders, genetic drift alone cannot explain the rapid increase in prevalence within this relatively short time interval [3]. Therefore environmental factors, particularly related to dietary and hygiene habits, have been proposed as disease modifiers [5,6]. The relationship between dietary habits and the increased prevalence of intestinal inflammatory disorders is of particular interest. Consumption of highly-caloric processed foods, high in fat and simple carbohydrate content, and low in fiber content, has been steadily increasing in the “Western World”, where the incidence of chronic inflammatory diseases is the highest [7,8]. Strong epidemiological evidences support the concept that diet underpins the rise in chronic inflammatory diseases such as IBD, diabetes or asthma [6–8]. However, the exact mechanisms by which specific dietary changes increase susceptibility to inflammatory diseases, and whether they are indeed mediated by changes in microbiota composition or function, are still unclear. Previous studies have shown that a high fat diet may have a direct pro-inflammatory effect, increasing the production of pro-inflammatory cytokines TNF α , IL-1 β and IL-6 [14]. Others have shown direct interactions between animal fat consumption, altered bile acid metabolism and specific

alteration in the gut microbiota, leading to increased colitis severity [148]. However, one characteristic element in the Western diet that has been overlooked until recently is its high salt (Sodium Chloride, NaCl) content.

NaCl has been shown to induce pathogenic Th17 cells (IL-17-producing T-helper cells) in both human and mice naïve CD4⁺ cell cultures in vitro. Mice receiving a salt-enriched diet developed a more severe form of experimental autoimmune encephalomyelitis (EAE), an animal model of MS, mediated by IL-17A and ROR γ t expressing Th17 cells [21,22]. Interestingly, high NaCl intake has been associated with increased disease activity in MS patients [149]. Moreover, a longitudinal clinical study showed that high NaCl intake increases circulating monocytes and levels of plasma cytokines such as IL-6 and IL-23 [26]. A recent study has shown that exposing human *lamina propria* mononuclear cells (LPMC) to high concentrations of NaCl enhances TNF- α and IL-17A release in a p38-dependent manner, and that feeding mice a salt-enriched diet exacerbates experimental colitis [150]. While all these studies suggest a promoting role of high salt diet in driving a pro-inflammatory state, the underlying mechanism are still not fully understood.

We hypothesized that a high salt diet impacts inflammation via the interaction with the gut microbiota. In fact, it is well established that diet determines the composition [48,117,151] and function [152,153] of the gut microbiota. It has been recently shown that diet low in dietary fiber may be causing irreversible changes in gut microbiota with some species disappearing over generations [154]. Given that gut microbiota composition has been implicated in the pathophysiology of multiple immune-related disorders, such as IBD, diabetes or asthma[155], and taking into account that diet shapes the gut microbiota, we investigated the ability of high salt diet (HSD) to modulate gut microbial composition and metabolism, and to influence vulnerability to intestinal inflammatory stimuli.

3. Materials and Methods

Mice

Six- to eight-week old specific pathogen free (SPF) male C57BL/6 mice (obtained from Taconic) were housed at the animal facility of McMaster University under 12h light/dark cycles and standard conditions for temperature and humidity. All mice received irradiated control diet (7004, Teklad, with 20% of calories from protein, 29% from fat and 51% from carbohydrates, containing 0.4% Na and 0.7% Cl) and sterile tap water *ad libitum* upon arrival. Experiments started one week after arrival in the central animal facility, to allow the animals to habituate to the novel environment. Mice were then divided into 2 groups: 1) Control diet - mice were fed with control diet for the entire course of the experiment; 2) HSD (High Salt Diet) - mice received the same diet supplemented with 4% NaCl (custom ordered from Teklad), plus 1% NaCl sterile water, *ad libitum*, during 4 weeks. For germ-free (GF) mice experiments, C57BL/6 mice were re-derived in McMaster Axenic Gnotobiotic Unit (AGU) by two-stage embryo transfer and kept in dedicated isolators during the entire course of the experiment. For the microbiota-transfer experiment, GF mice were colonized by oral gavage with 200ml of cecal microbiota from a mouse fed with control diet or HSD and housed in dedicated individually ventilated racks.

DNA isolation and 16S rRNA Illumina sequencing

DNA was extracted from 100mg of fecal content samples using the MagMAX DNA Multi-sample kit and the MagMAX Express-96 well magnetic particle processor (ThermoFisher, Waltham, MA) to achieve an automatic and standardized DNA extraction across samples. Briefly, fecal samples were mechanically homogenized with 2.8 mm ceramic beads (MoBio, Carlsbad, CA, USA; an additional 0.2 g of 0.1 mm glass beads were added), 100 ml of guanidine thiocyanate-EDTA-N-lauroyl sarcosine and 800 ml of 200mM NaPO₄. Samples were homogenized for 3 min at 300 rpm and centrifuged 5 minutes at maximum speed. 200 ml of the extract was added to the MagMAX Express plate

for further DNA extraction procedures according to manufacturer's instructions. Isolated DNA was kept at -20° C (or -80° C for longer storage). Polymerase Chain Reaction (PCR) amplification of the variable 3 (V3) region of the 16S rRNA gene was performed as previously described [156,157]. Purified PCR products were sequenced using the Illumina MiSeq platform by the McMaster Genomics facility. Of note, we lost two samples during processing, one from Baseline group and one from 4 Weeks HSD group.

Illumina sequencing processing and analysis

Custom, in-house Perl scripts were developed to process the sequences after Illumina sequencing [158]. The sl1p pipeline is open source and publicly available at <https://bitbucket.org/fwhelan/sl1p>. Briefly, Cutadapt [159], was used to trim any over-read, and paired-end sequences were aligned with PANDAseq [160], with a 0.7 quality threshold. If a mismatch in the assembly of a specific set of paired-end sequences was discovered, they were culled. Additionally, any sequences with ambiguous base calls were also discarded. Operational taxonomic units (OTUs) were picked using AbundantOTU+ [161], with a clustering threshold of 97%. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier v.2.2 [162] against the Greengenes SSU reference database (2013 release) [163]. For all downstream analysis we used Quantitative Insights Into Microbial Ecology (QIIME) [164]. We obtained a total of 2,839,134 reads after quality filtering, with an average of 76,733.351 reads/sample (range: 34,470 to 144,746). OTU tables were filtered to exclude "Root" and any OTU with less than 10 sequences in all samples, and finally rarefied to 30,000 reads/sample. Calculations of within-community diversity (a-diversity), between-community diversity (b-diversity), relative abundance taxonomic summaries, and the different statistical analysis were performed using QIIME (scripts "alpha_rarefaction.py" and "beta_diversity_through_plots.py", respectively). The relationships between HSD and microbiota phylogeny were explored using Principal Coordinate analysis on unweighted unifracs metrics and abundance jaccard index ("beta_diversity_through_plots" script, QIIME), and the dissimilarity distance

between the diet groups was tested using ANOSIM statistical method (“compare_categories” script, QIIME). For relative abundance taxonomic summaries and further statistical analysis, OTUs with a representation of less than 0,1% in the community were excluded. The correlations between fecal microbiota composition (at the highest taxonomic levels (family and genus) and at OTU level) and the diet, were assessed by Spearman’s rank correlation coefficient with fisher z-transformation P-value assignment method, using the “observation_metadata_correlation” script (QIIME), based on the attribution of a ranked value to the variable “Weeks of NaCl in diet”, from “0” to “4”. The “Wash Out” group was ranked “0”. PICRUSt [165] was used to predict the altered KEGG pathways based on the 16S sequencing data. L3 KEGG pathways correlation with length of HSD was calculated based on Spearman’s correlation using Prism GraphPad 6 (GraphPad software). Heatmaps were generated using the Heatmaps2 package (gplot) for R (version 3.3.2). In the microbiota-transfer experiment, the OTUs were considered successfully transferred when present with an average of 2 reads or higher.

Short-Chain Fatty Acids (SCFA) quantification

SCFA were quantified as previously published [166,167]. Briefly, 20-50 mg of frozen cecal content was collected into a tube, previously treated with methanol. The cecal samples were diluted 1:1 (w:v) with HCl 3.7% (10X diluted) and homogenized. Then 10 ml of internal standards and 500 ml of diethyl ether were added to the samples, and the tubes vortexed for 15 min. The extract (400 ml) was transferred to a clean tube. This step was repeated until we obtained a total of 800 ml of diethyl ether-fecal extract, 60 ml of which was then transferred to a chromatographic vial with 20 ml of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA). The organic extract-MTBSTFA was incubated at room temperature for at least 1 hour, before being analyzed using the GC-MS Agilent 6890N GC coupled to Agilent 5973N Mass Selective Detector, with the column DB-17HT (30m x 0.25 mm ID, 0.15 mm film).

Experimental colitis

Additional groups of six- to eight-week old specific pathogen free (SPF) male C57BL/6 mice (obtained from Taconic) and NIH Swiss mice (obtained from Harlan) were housed at the animal facility of McMaster University as described above. After a period of 4 weeks on HSD or Control Diet, mice were given 3.5% Dextran Sodium Sulfate (DSS, MW 36-50kDa; MP Biomedicals, Santa Ana, CA) in drinking water for 5 days, followed by regular water for the next 2 days. HSD mice were switched to control diet before starting DSS administration. Body weight and general health condition were assessed daily. At day 7, mice were euthanized and the colon was blindly evaluated using a previously validated score [168,169]. Briefly, the colon was evaluated for muscle thickness, hyperemia and erythema, presence of blood in the stools, and stool formation (For details, see Supplementary Methods). Control mice (no DSS) received only water during the entire procedure. For germ-free (GF) mice, the DSS dose had to be lowered to a 2% solution due to their higher sensitivity to experimental colitis. For the microbiota adoptive transfer experiments in ex-germ free mice a 2.5% DSS solution was used, starting 5 days after colonization.

For Dinitrobenzene sulfonic acid (DNBS)-colitis, mice were anesthetized with isoflurane (3%) and injected with 1 ml of saline subcutaneously. A custom-made polyester catheter was introduced 3.5 cm into the rectum and 100 µl of 50% EtOH containing 3.5 mg of DNBS (MP Biomedicals, Santa Ana, CA) was injected. From that day, all mice received control diet with normal water containing 6% sucrose to prevent dehydration. At day 3 post DNBS, mice were euthanized and blindly evaluated using a validated DNBS-colitis macroscopic score [170]. The colon was evaluated for adhesion to surrounding tissue, thickness, hyperemia, erythema, and stool consistency. Mucosal ulcers were quantified and measured (for details see Supplementary Methods). Mice from control group (no DNBS-colitis) were rectally injected with a 50% EtOH solution.

Tissue processing

Blood was collected via orbital bleeding and centrifuged at 5000 rpm, for 10 minutes. The serum was separated and stored at -80° C. The colon (full

length, from the rectum to the cecum) was excised and measured. Colonic content was removed and 0.5-1.0 cm sections were dissected from mid-colon and either snap-frozen in liquid N₂ for MPO analysis, stored in RNA later (Ambion) for RNA extraction or fixed in 10% buffered formalin for histology assessment (from the border of an ulcer when present). MPO was performed on frozen tissues as previously described [171] and its activity expressed in units per milligrams of tissue. For histology, colonic sections were embedded in paraffin, 48 hours after being fixed in 10% formalin solution, and stained with haematoxylin and eosin (H&E). Inflammation was blindly evaluated following an inflammation histology scoring system (see Supplementary Materials).

Gene Expression

Total RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions, including the DNA removal step using the RNase-Free DNase Set (Qiagen). NanoString nCounter Gene Expression CodeSet for mouse inflammation genes (v2; 248 genes) and a custom CodeSet panel that included 68 genes related to immune, gut barrier and neurobiology functions (see Supplementary Materials); were run according to manufacturer's instructions (NanoString Technologies Inc., Seattle, WA). Data from nCounter was analyzed using nSolver 2.5 software (Nanostring Technologies Inc.). The log₂ ratios of gene expression were then uploaded into Ingenuity Pathway Analysis software (Qiagen) for further analysis. For Q-PCR gene expression, cDNA library was generated using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA), according to manufacturer's instructions, using Oligo-dT primers (Qiagen). Additional information on the primers is provided in supplementary materials.

Immune Cells Extraction

Mesenteric lymph nodes (mLN) were harvested aseptically and the cells were released from the nodes using a 70 mm strainer with the help of a syringe

plunger, and suspended in RPMI medium. After washing, the cells were kept in complete RPMI medium on ice until further use.

Colonic and small intestinal (SI) *lamina propria* (LP) cell extraction was performed using established protocols [172]. Briefly, colon and SI were removed and kept in cold sterile Dulbecco's phosphate buffered (DPBS) solution until further processing. All fatty tissue, content and the Peyer's patches were removed. Sections of intestine were open longitudinally, cut into 0.5-1 cm pieces and incubated in 2 mM Dithiothreitol (DTT)/Hank's solution (HBSS), on a horizontal shaker (240rpm) for 15 minutes at 37° C to remove the mucous layer. After vigorous vortexing, the tissues were strained with a sterile metal strainer and incubated with a 5 mM EDTA/HBSS solution for 10 minutes at 37°C (240rpm) to remove the epithelial layer. After vigorous vortexing, the tissues were strained once again. This epithelial removal step was repeated 3-4 times. After removing the EDTA solution, the tissues were digested in 0.1% Collagenase solution (Type VIII; Sigma-Aldrich, St. Louis, MO) and DNase (I recombinant, Grade I; Roche, Basel, Switzerland; 0.05mg/50ml of digestion solution) to aid the release of the LP cells (15 minutes for SI and 25 min for colon, 37° C). Supernatants were strained through a 40 mm cell strainer to separate LP cells from the remaining intestinal tissue, and washed thoroughly. The cell suspension was then purified by Percoll (GE Healthcare, Chicago, IL) density gradient method. Briefly, the cell suspension was re-suspended in 35% Percoll solution and underlayered with a solution of 70% Percoll. The tubes were centrifuged at 4° C, 670 G, for 30 minutes (without break and with slow acceleration). The purified cell suspension was removed from the interphase, washed and resuspended in complete RPMI. For LP cells gene expression, the cell suspension was resuspended in RLT buffer (RNeasy Mini Kit, Qiagen) and snap frozen in liquid nitrogen.

Flow Cytometry

Cells isolated from mLN, colon LP and SI LP were divided into two groups for intracellular staining: 1) anti-IL17A + anti-ROR γ t; and 2) anti-FoxP3. Cells of the first group were stimulated for 4-5 hours at 37° C with PMA (50 ng/ml),

ionomycin (750 ng/ml; both from Sigma-Aldrich), in the presence of 10mg/ml of Brefeldin A (BD Biosciences, San Jose, CA), to activate IL17A expression. After this period, all cells were washed with PBS solution and stained for cell viability with Fixable Viability Dye eFluor® 780 (1:1000; eBiosciences, San Diego, CA) for 30 minutes at RT. Cells were then stained with the following extracellular antibodies: anti-TCRb-PerCP-Cy5.5 (1:100; H57-597 clone; eBioscience) and anti-CD4+-Pacific Blue (1:200, RM4-5 clone, BD Biosciences), for 30 minutes at 4° C. Cells were fixed and permeabilized with FoxP3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), according to manufacturer's instructions, and stained for 90 minutes at 4° C, with the following intracellular antibodies: anti-IL17A-APC (1:100; TC11-18H10.1 clone, Biolegend, San Diego, CA) and anti-RORgt-PE (1:100; AFKJS-9 clone; eBioscience); or anti-FoxP3-FITC (1:100; FJK-16s clone; eBioscience). The data was acquired on LSR II cytometer (BD Bioscience) and analyzed with FlowJo software (10.2 version) (TreeStar, Ashland, OR).

Statistical Analysis

Data analysis was carried out using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Student's t-test, multiple t-test, one-way ANOVA or two-way ANOVA, were used as appropriate. For ANOVA, samples were corrected for multiple comparisons using Tukey's test. For Nanostring data, including heatmaps, the statistical analysis was carried out using nSolver 2.5 software (Nanostring Technologies). Heatmaps were generated using Euclidean distance metric based on group average. $P < 0.05$ was considered as statistically significant. Values are presented as means $\pm$ SEM.

4. Results

HSD alters gut microbiota composition

To investigate the impact of HSD on gut microbiota composition, we analyzed intestinal microbiota dynamics of mice fed HSD for 4 weeks, with a

follow-up at 1 week after the mice returned to the control diet. While fecal bacterial richness (observed species) and diversity (Chao1 diversity index) were not affected during the HSD, both α -diversity metrics decreased at 1 week follow-up (Supplementary Fig. 1). Despite some variability within groups, we observed a significant modulation of fecal bacteria composition with time exposure to HSD, based on the phylogenetic distance metric unweighted unifrac (UnwUF) and the non-phylogenetic abundance weighted Jaccard distance (AJac) (ANOSIM of UnwUF: $R=0.416$, $p=0.001$; ANOSIM of AJac: $R=0.283$, $p=0.003$) (Fig. 1A, Table 1). One week of HSD was not sufficient to significantly modulate the microbiota but 4 weeks of HSD led to a significant shift in the composition, as revealed by ANOSIM pairwise analysis (UnwUF: $R=0.480$, $p=0.004$; AJac: $R=0.036$, $p=0.008$; Table 1). Interestingly, 1 week after returning to the control diet, fecal microbiota composition partially reverted to its original composition, based on unweighted and weighted unifrac metrics (Fig. 1A, Table 1 and supplementary fig. 2).

Table 1: ANOSIM statistical analysis of distance metrics on the effect of HSD on fecal microbiota composition

	Unweighted Unifrac		Abundance Jaccard Index	
	ANOSIM R	ANOSIM p	ANOSIM R	ANOSIM p
All Groups	0.416	0.001	0.283	0.003
Baseline vs 1 Week HSD	0.144	0.133	0.211	0.108
Baseline vs 4 Week HSD	0.48	0.004	0.36	0.008
Baseline vs Wash Out	0.341	0.034	0.197	0.106
1 Week HSD vs 4 Week HSD	0.277	0.052	0.528	0.006
4 Week HSD vs Wash Out	0.525	0.007	0.075	0.237

High diet exacerbates colitis in mice by decreasing *Lactobacillus* levels and butyrate production

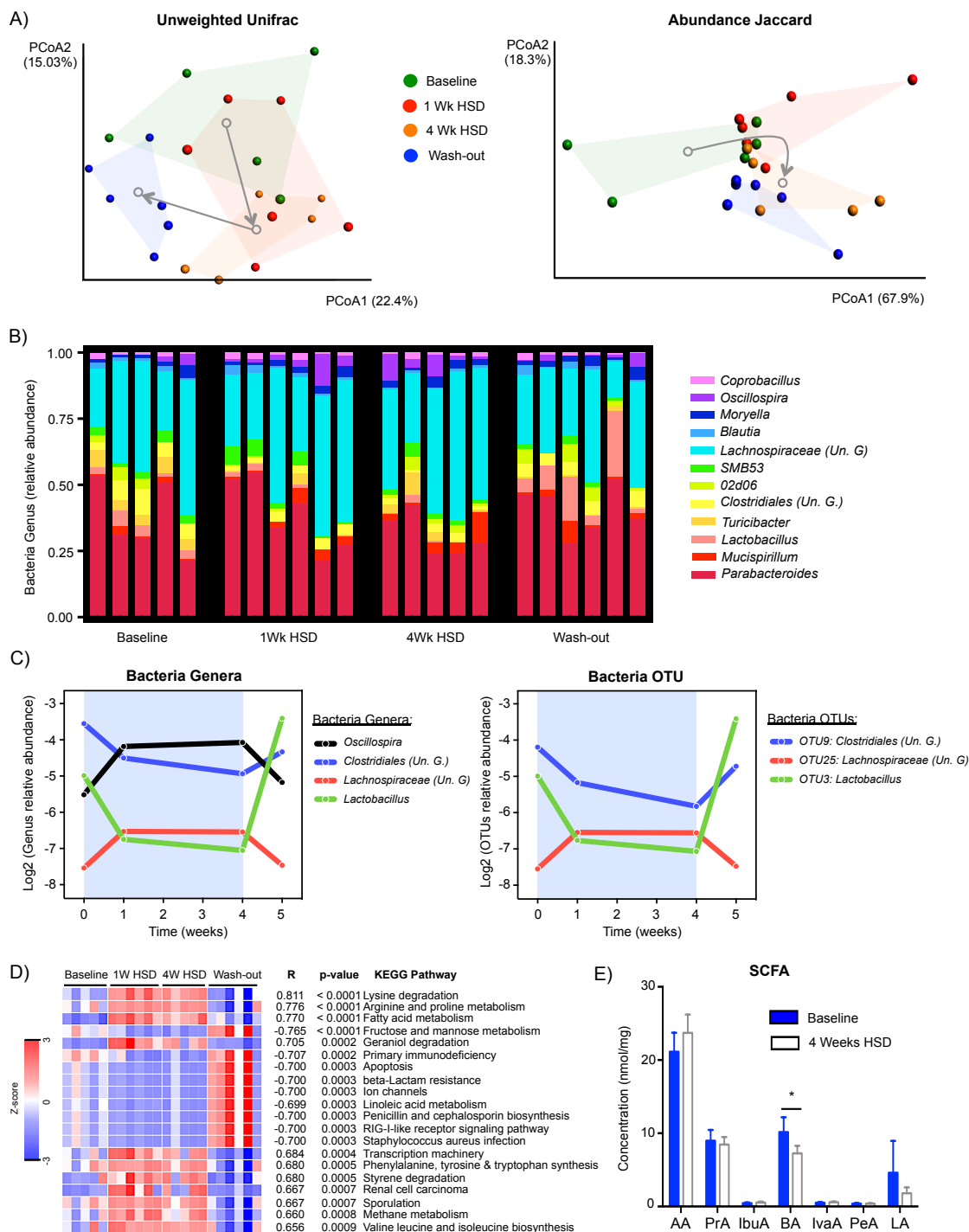


Figure 1. (See legend on next page)

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Figure 1. High salt diet alters gut microbiota composition and function. A: Principal coordinate analysis (PCoA) plots of unweighted unifrac metric (left panel) and abundance jaccard index (right panel) of fecal microbiota composition over time exposed to HSD. Grey arrows indicate the direction of the significant microbiota composition shifts between groups B: Summary of the relative abundance of microbial genera present in 99.9% of the community over time exposure to HSD. C: Relative abundance of fecal bacterial genera (left panel) and OTUs (right panel) that significantly correlate with HSD ($q < 0.05$). The blue background represents the period exposed to HSD. D: Heatmap of the 20 most significant KEGG pathways that correlate with HSD in fecal microbiota. E: SCFA quantification, at 0 and 4 weeks after HSD. Un. G (Unclassified Genus). AA (acetic acid), PrA (propionic acid), IbuA (isobutyric acid), BA (butyric acid), IvaA (isovaleric acid), PeA (pentanoic acid), LA (lactic acid). Values are presented as means $\pm$ SEM. $n = 5-6$ mice/group. * ($p < 0.05$).

In order to better understand the impact of HSD on the microbial community, we studied the correlation between HSD exposure and the taxonomic composition at genus and OTU level. Four bacterial genera were found to significantly correlate with HSD (Fig. 1B-C, Table S1). *Lactobacillus* genus showed the strongest correlation by decreasing 2-fold of its relative abundance after 4 weeks of HSD and quickly normalizing after returning to the control diet ($r = -0.703$, $q = 0.003$). An unclassified genus belonging to the order *Clostridiales* also decreased relative abundance over 1-fold after 4 weeks of HSD and partially normalized 1 week after returning to control diet ($r = -0.605$, $q = 0.017$). Finally, unclassified genera belonging to the *Lachnospiraceae* family and the *Oscillospira* genus showed a positive correlation with HSD ($r = 0.614$, $q = 0.017$ and $r = 0.548$, $q = 0.042$, respectively) (Fig. 1B-C, Table S1). Similarly, three fecal OTUs correlated with time of exposure to HSD (Fig 1C, Table S2): OTU #3, belonging to the genus *Lactobacillus* ($r = -0.707$, $q = 0.005$), OTU #9, belonging to the order *Clostridiales* ($r = -0.684$, $q = 0.005$), and OTU #25, belonging to the family *Lachnospiraceae* ($r = 0.609$, $q = 0.027$).

To test the stability of fecal microbiota and rule out spontaneous changes in microbial profiles during this age period, we analyzed microbiota composition in an additional group of control mice fed with control diet. There were no spontaneous changes in the microbiota genus affected by HSD such as

Lactobacillus, *Oscillospira* and unclassified genus from *Clostridiales* and *Lachnospiraceae*, between week 8 and 12, in mice on control diet (Supplementary Fig 3). However, the microbiota profiles of mice on HSD and control diet clustered separately at both time points, likely consequence of the cage effect and low number of mice (Supplementary Fig. 3).

HSD affects predicted microbial metabolic activity and luminal SCFA levels

To investigate whether the differences in fecal microbiota composition induced by HSD could impact its metabolic activity, we assessed inferred metagenomic KEGG pathways, using PICRUSt [165]. Multiple pathways appeared to be affected, with three of them being strongly upregulated by HSD, including “fatty acid metabolism”, “lysine degradation” and “arginine and proline metabolism”, while “fructose and mannose metabolism” was down-regulated (Fig 1D). As HSD altered the relative abundance of several OTUs and genera of major SCFAs producers such as *Lachnospiraceae*, *Clostridiales* and *Oscillospira*, and PICRUSt predicted changes in the metabolism of fatty acids, we sought to quantify luminal SCFA in mice on HSD. Out of the seven SCFA tested, only butyrate levels were significantly affected, showing a decrease after 4 weeks of HSD (Fig. 1E). We also observed a decrease in lactic acid concentration, however this change did not reach statistical significance. Taken together, these results show that HSD modulates both composition and function of the gut microbiota.

HSD modulates colonic and small intestinal immune gene expression

We subsequently tested whether the HSD-induced changes in gut microbiota were affecting host's gut mucosal homeostasis. We assessed gene expression profile of colonic *lamina propria* immune cells with nCounter Mouse Inflammation V2 Panel (Nanostring). HSD altered the expression of 35 genes ($p < 0.05$) in mouse colonic *lamina propria* cell extract, with 11 transcripts being significantly upregulated and 24 being downregulated (Fig. 2A-B; See Table S3 for the full list of affected genes). Some of these genes were selected for

validation by q-PCR (Fig. 2C). A considerable part of the affected transcripts were chemokines, cytokines or their receptors (Table S3). Among these, the cytokine IL-7 and the receptor IL-1 α were upregulated by HSD (0.47 and 0.19 log₂ ratio, respectively), while all other cytokines and chemokines were down-regulated. Of note, we observed the downregulation of several chemokines (and chemokine receptors) important for the chemotaxis of monocytes/macrophages, dendritic cells, neutrophils, and NK cells, such as Ccl3, Ccl4, Cxcl10, Cxcl2, Ccr7 and Cxcr4 (Table S3), indicating a possible impairment of their migration. The most significant change observed was the upregulation of Rac1 (Ras-related C3 botulinum toxin substrate 1) transcript (log₂ ratio=0.41, p=0.0001), a member of Rho family GTPases with broad functions, from innate immunity processes to leukocyte chemotaxis and barrier function.

In order to identify possible functional pathways affected by HSD we integrated our gene expression data with publicly available databases results using Ingenuity Pathway Analysis (IPA) bioinformatics platform. The upregulation of the genes Map2k6, Map2k1, Mapk3, Atf2 and IL1 α was predicted by IPA to stimulate several pro-inflammatory canonical pathways such as “Acute phase response signaling”, IL-6 and Mapk signaling (Table S4). The role of Macrophages and IL-17 signaling were also strongly predicted to be affected by HSD (Table S7). Even though IL-17A cytokine itself was not found to be significantly affected by HSD in *lamina propria* colonic cell extracts, IPA identified multiple genes related to IL-17 signaling to be affected, such as Mapk3, Map2k1, Map2k6 and the transcription factor Atf2 (all upregulated), as well as Cxcl10, Hras and Ptgs2 (all downregulated). When analyzing functions affected by HSD in the colon, IPA predicted downregulation of leukocyte migration and movement (Fig. 2D, Table S5). This was expected since HSD suppressed the expression of many chemokines and cytokines responsible for immune cells chemotaxis. On the other hand, the functions related to leukocyte viability and maturation were predicted to be upregulated, based on the expression of genes like IL-7, Map2k1 and Mapk3 (Fig. 2D, Table S5).

High diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production

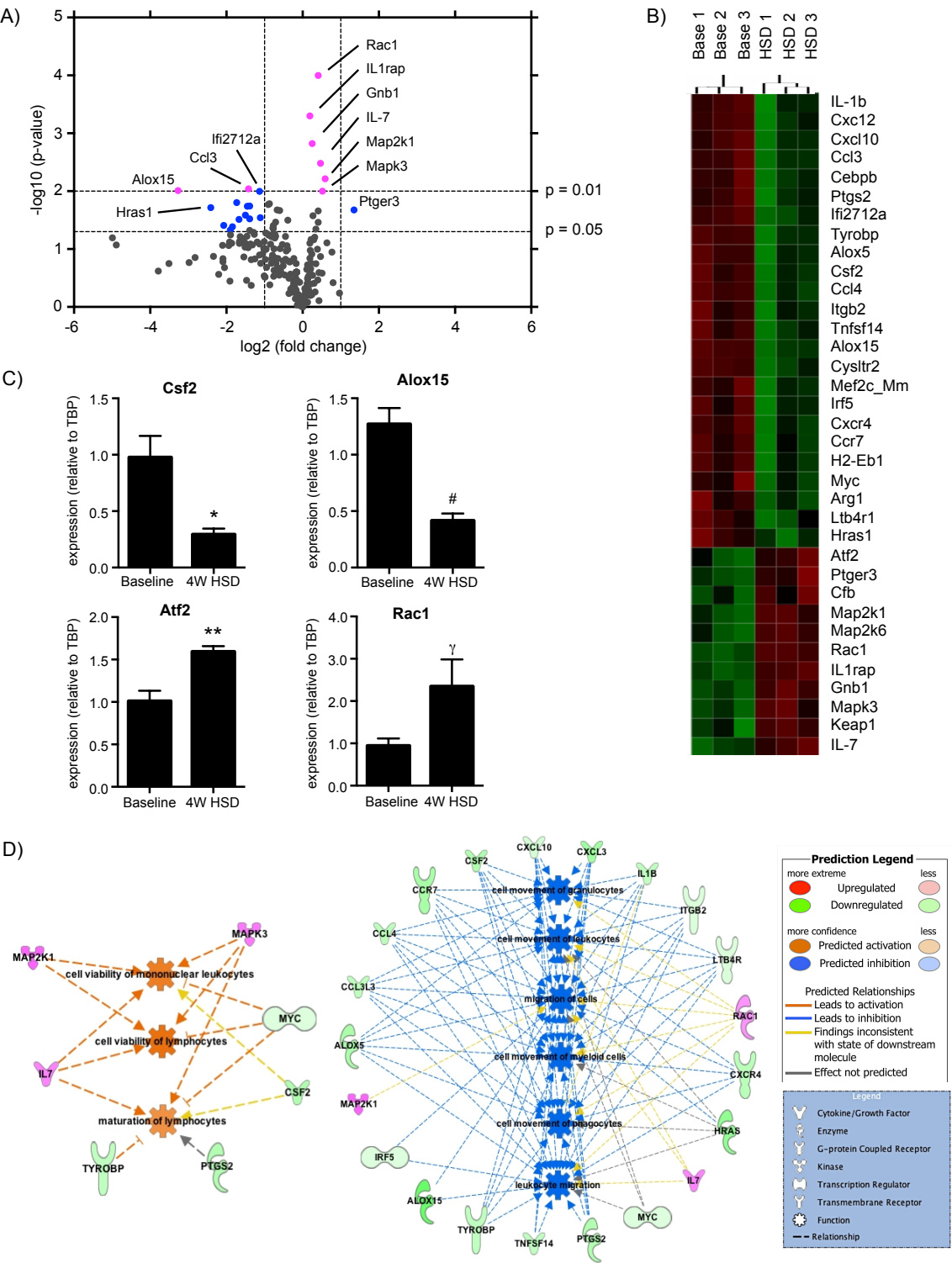


Figure 2. (See legend on next page)

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Figure 2. High salt diet modulates immune expression in colonic *lamina propria*. A: Volcano plot of gene expression in colonic *lamina propria* total cell extract of mice receiving HSD or control diet for 4 weeks, n=9 mice/group. Pink dots: genes with $p < 0.01$. Blue dots: genes with $0.01 < p < 0.05$, fold change > 2 . B: Heatmap of immune genes that changed with HSD ($p < 0.05$). Based on groups average, euclidean distance metric. C: qRT-PCR analysis of differentially expressed genes in the two groups. Values are presented as means $\pm$ SEM; n= 5-6 colon/group; * ($p < 0.05$) ** ($p < 0.01$) # ($p < 0.001$) g ($p = 0.065$). D: Gene-cell function networks obtained from Ingenuity Pathway Analysis. On the left, network with the genes that contribute to the predicted inhibition of functions related to immune cell migration, and, on the right, network with the genes that contribute to the predicted upregulation of the immune cell viability and maturation.

Although the focus of our study was the effect of HSD on colonic microbiota and immune function, we also assessed its effect on small intestine recognizing that that most of the Na^+ and Cl^- is absorbed in this compartment. We observed a significant change in the expression of 11 genes ($p < 0.05$), including the upregulation of IL17A and Defa-rs1, as well the downregulation of IL12a and Cxcr4 (Supplementary Fig. 4, Table S6). Only 2 genes (Cxcr4 and Mef2c-Mm) were affected similarly in both colonic and small intestinal *lamina propria* cell extracts, emphasizing the distinct nature of the immune milieu of each gut compartment. IPA identified three canonical pathways related to bacteria recognition, toll-like receptors and IL-17 signaling to be most affected in the small intestine (Table S7). While “recruitment of leukocytes” ($z = 0.196$, $p = 4.01 \times 10^{-5}$) was upregulated, “proliferation of lymphocytes” ($z = -2.398$, $p = 5.44 \times 10^{-6}$) was downregulated.

HSD increases RORgt⁺IL17⁺ T-cells in mLNs but not in intestinal lamina propria

To better understand the effect of HSD on lymphocyte function, we phenotyped T cell populations in the small intestinal and colonic *lamina propria* and in mesenteric lymph nodes (mLNs) using flow cytometry. HSD led to a 2-fold

increase of RORgt⁺IL17A⁺ cells in the mLNs but not in small intestinal or colonic *lamina propria* (Supplementary Fig. 5A). However, we consistently observed a mild but not statistically significant increase of this cell population in the colon across all experiments. No change was observed in the expression of IFN γ by CD4⁺ T cells in mice on HSD (data not shown), nor in the frequency of CD4⁺FoxP3⁺ T cells, even though, for the latter, we noticed a small increase of this cell type across tissues in all experiments (Supplementary Fig 5B). Taken together, these results show that HSD affects the gut mucosal immune compartment.

HSD exacerbates DSS colitis

To investigate how the changes in gut microbiota and immune function affect vulnerability to inflammatory insults, we used DSS and DNBS colitis models. We confirmed that mice on HSD developed a more severe DSS colitis when compared to mice on control diet, as previously described[150], characterized by an increased rectal bleeding, diarrhea, and general inflammation of the colonic tissue (Fig. 3A). HSD induced higher weight loss on day 5 of DSS administration, when compared to DSS mice on control diet, a difference that was maintained until the end of the experiment, on day 7 (Fig. 3B), indicating an accelerated onset and increased severity of colitis. Colon shortening, normally used as indicator of colitis severity, was greater in HSD mice compared to control diet mice (Fig 3C). MPO activity, reflecting neutrophil infiltration, was increased 2-fold in the colon of mice on HSD after DSS administration, when compared to mice on control diet (Fig. 3D). Furthermore, there was a significant difference in mortality between the two groups, with 5% of mice on control diet meeting the endpoint criteria prematurely, while this number increased to 19% in mice on HSD (Fig. 3E).

To better understand the immune responses during DSS colitis, we assessed the expression of a group of genes related to immune and barrier function using a customized Nanostring gene expression codeset (See list of genes in supplementary methods). HSD altered the expression of 9 genes in the

colon of mice on DSS, when compared to DSS mice on control diet (Fig. 3F). HSD decreased expression of genes involved in the maintenance of mucosal barrier such as Tjp1 (tight junction protein ZO-1), Muc2 (Mucin 2), Tff3 (trefoil factor 3) and Ocln (Occludin). On other hand, HSD mice had an increased expression of MMP9 (Matrix Metalloproteinase 9) and Cnlp (Cathelicidin-related antimicrobial peptide), genes expressed by innate immune cells such neutrophils and macrophages and previously described to be over-expressed in gut inflammatory conditions [173,174]. HSD also reduced expression of two genes strongly expressed by epithelial cells: F2rl1 (protease activated receptor 2) and 5-HT (serotonin). This finding might be a consequence of the severe epithelial damage observed in colitis. Thus, the altered gene expression profile supports the notion that HSD exacerbates DSS colitis.

These results were reproduced using another mouse strain; NIH Swiss mice also developed a more severe DSS colitis after 4 weeks of HSD, confirming that HSD induces susceptibility to experimental colitis in multiple mouse strains (Supplementary Fig. 6).

HSD exacerbates DNBS-colitis

To confirm the increased sensitivity to colonic inflammation in mice on HSD, we used the DNBS model. Similar to the DSS colitis model, we chose a concentration of DNBS that would induce a mild form of colitis (3.5mg of DNBS/mouse), in order to appreciate the effect of diet on worsening of experimental colitis. Similar to the results obtained with DSS-induced colitis, mice that were previously exposed to a 4-week HSD developed more severe colitis after rectal injection of DNBS (Fig. 4A). HSD mice had higher number of ulcers that were larger in size, when compared to control diet mice. Fig. 4B-C shows that HSD mice developed ulcers that affected the entire colonic wall, up until the serosa with large clusters of cellular infiltrates, and, in some cases, a total loss of the epithelial layer. Strikingly, while none of the control diet mice met the endpoint criteria to be euthanized at this mild DNBS concentration, the mortality of HSD mice after DNBS administration reached 25% (Fig. 4D). Taken together, these results show that HSD exacerbates both, DSS- and DNBS-colitis.

High diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production

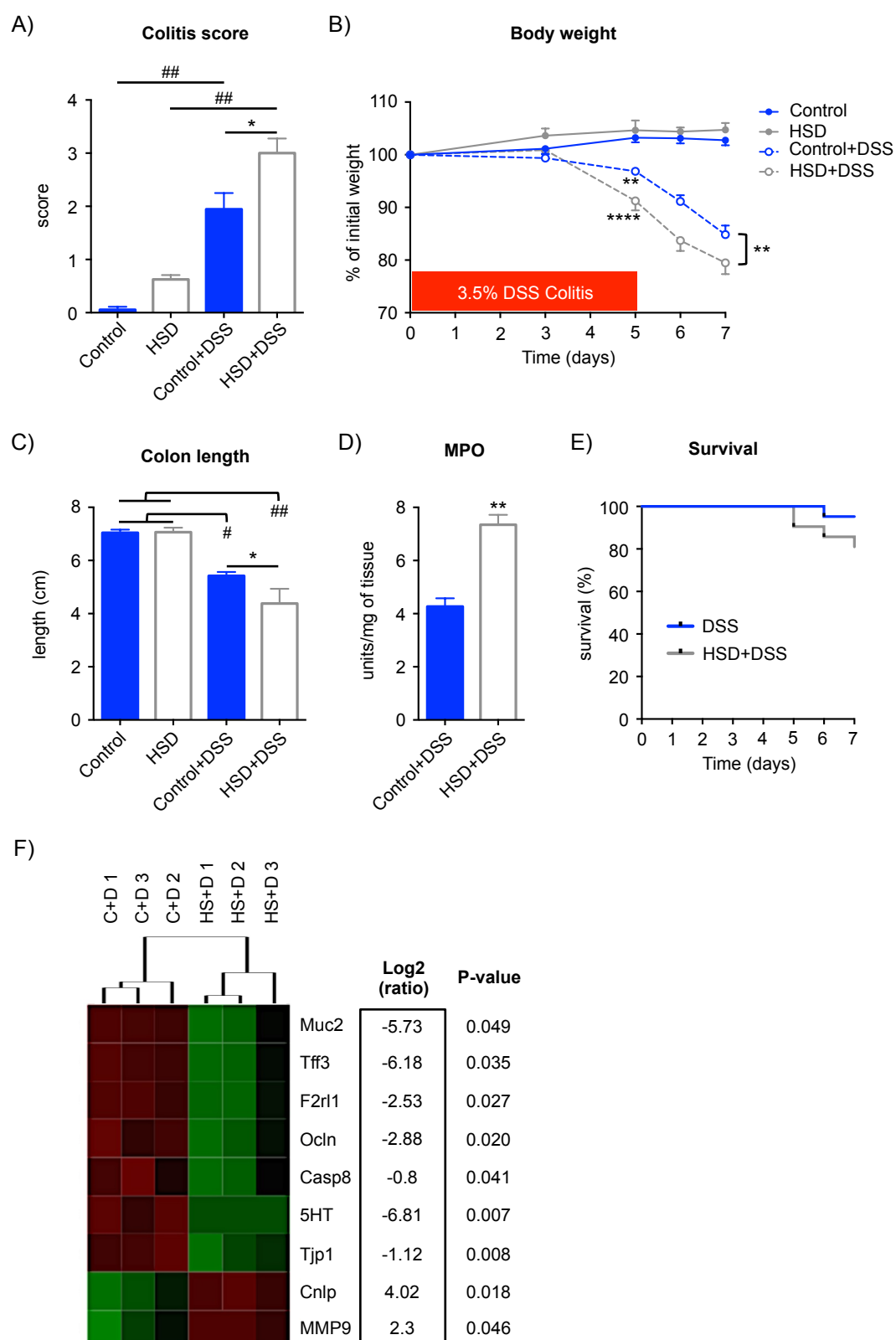


Figure 3. (See legend on next page)

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Figure 3. High salt diet exacerbates DSS colitis. A: DSS colitis macroscopic scores at endpoint (day 7) in four groups of mice: Control (control diet, no DSS), HSD (high salt diet, no DSS), Control+DSS (control diet, DSS in water), HSD+DSS (high salt diet, DSS in water). B: Body weight changes during DSS administration. C: Colon length at endpoint. D: Colonic MPO of the two groups of mice receiving DSS. E: Survival curve of the two groups of mice with DSS colitis. F: Heatmap of differentially expressed genes ($p < 0.05$) in the colon of mice with DSS colitis: C+D (Control diet+DSS), HS+D (HSD+DSS). The heatmap was generated based on group average, using Euclidean distance metric. Colitis scores, weight change, colon length and MPO are presented as means $\pm$ SEM; $n = 8-9$ mice/group. * ($p < 0.05$) ** ($p < 0.01$) # ($p < 0.001$) ## ($p < 0.0001$).

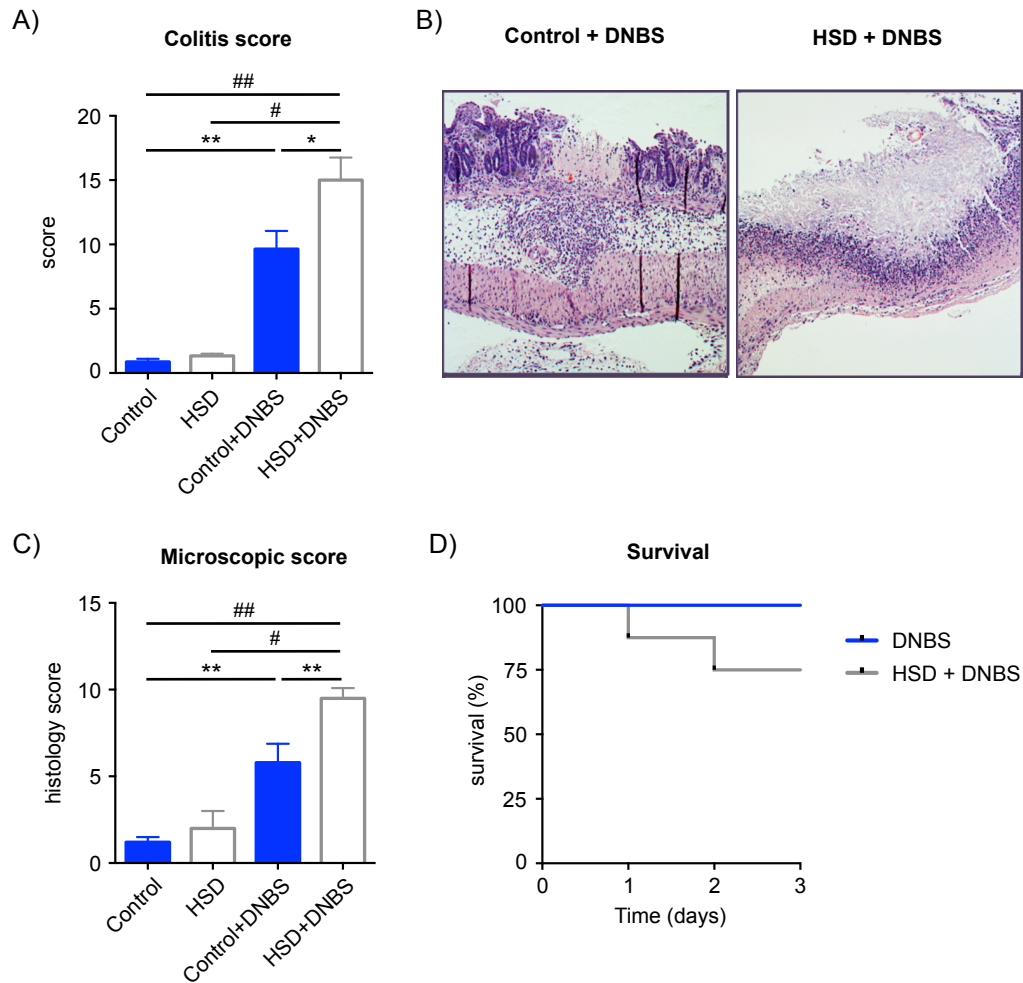
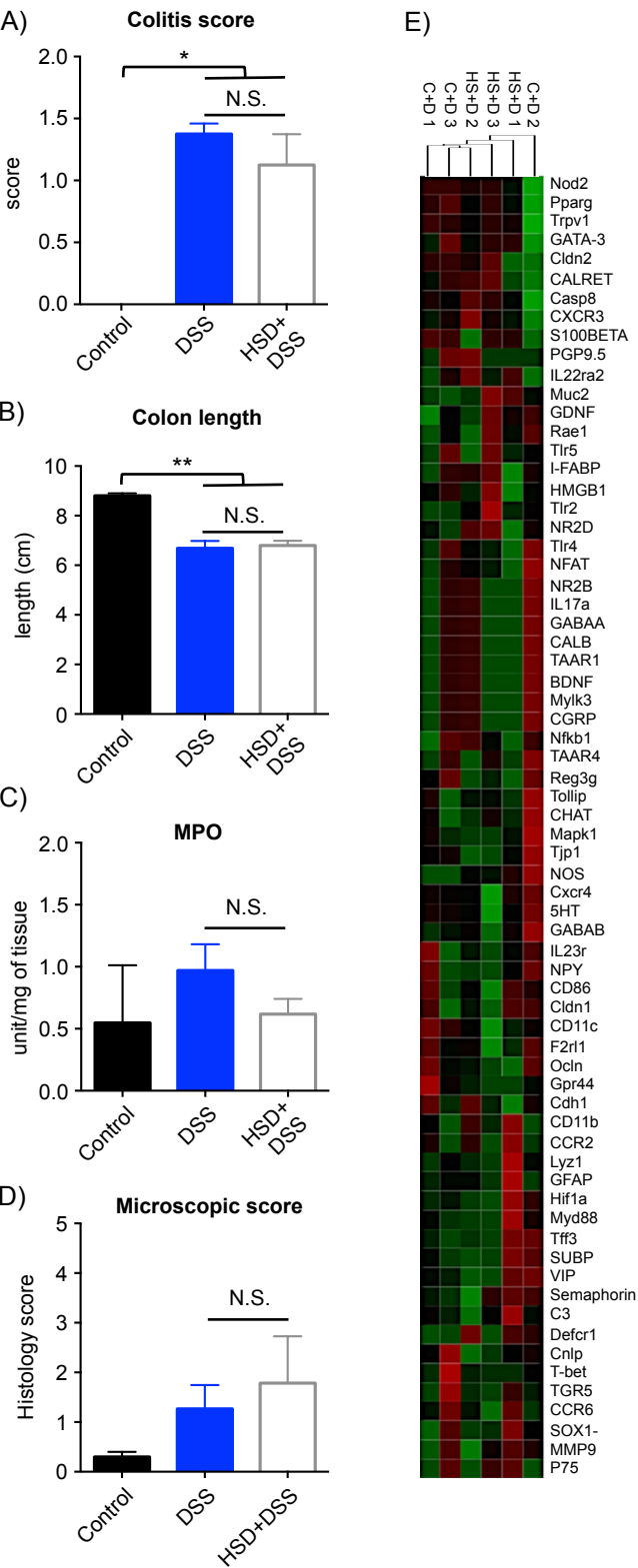


Figure 4. High salt diet exacerbates DNBS colitis. A: DNBS colitis macroscopic scores at endpoint (day 3) in four groups of mice: Control (control diet, 50% EtOH vehicle), HSD (high salt diet, 50% EtOH vehicle), DNBS (control diet, DNBS in 50% EtOH vehicle), HSD+DNBS (high salt diet, DNBS in 50% EtOH vehicle). B: Representative H&E-staining of colonic section of DNBS mice highlighting the ulcers in the mucosa. C: Histological scores in colonic sections. D: Survival curve of the two groups with DNBS colitis. Colitis and histology scores are presented as means $\pm$ S EM; n= 5-9 mice/group. * (p<0.05) ** (p<0.01) # (p<0.001) ## (p<0.0001).

HSD exacerbates experimental colitis in a gut microbiota-dependent manner



We hypothesized that HSD exacerbates colitis through induction of gut dysbiosis as it decreases levels of beneficial bacteria, such as *Lactobacillus*, and decreases butyrate, a SCFA important for gut immune homeostasis and previously shown to be protective in colitis. To test this hypothesis, we administered 2% DSS to germ-free (GF) mice after 4 weeks of HSD. DSS administration induced colitis in GF mice (Fig. 5A-B), with increase in macroscopic scores and decreased colon length compared to control mice not exposed to DSS. However, there were no differences in macroscopic scores, colon length, MPO or histology scores between GF mice on HSD and those on control diet after DSS-colitis (Fig. 5 A-D). We assessed the expression of a group of relevant genes using a customized Nanostring gene expression codeset.

Figure 5. (See legend on next page)

(See figure on previous page)

Figure 5. The effect of high salt diet on exacerbation of colitis is dependent on gut microbiota. A: DSS colitis macroscopic scores at endpoint (Day 7) in three groups of mice: Control (no DSS colitis), DSS (control diet, DSS in water), HSD+DSS (High salt diet, DSS in water). B: Colon length of mice at endpoint. C: Colonic MPO. D: Histological scores of the DSS colitis. Values are presented as means $\pm$ SEM; n= 3-6 mice/group. * (p<0.05) ** (p<0.01). E: Heatmap of the gene expression of 68 genes related to immune, gut barrier and neural function, in the colon tissue of germ-free mice after DSS administration, on control diet or HSD: C+D (control diet + DSS), HS+D (HSD+DSS). The heatmap was generated based on group average, using Euclidean distance metric.

DSS administration altered expression of 25 genes in GF mice on control diet (Table S8) that were related to immune, barrier and neural function such as Mapk1, Nfkb1, CD11c and GABA B, confirming that the administration of DSS altered gut physiology, even in the absence of microbiota. However, out of the 68 tested genes, only one gene, Reg3 γ , was found to be differentially expressed between HSD and control diet groups after DSS administration (log2 ratio=-1.15; p=0.032). These results suggest that the colonic microbiota is a key factor in exacerbation of colitis by HSD.

To test whether HSD-associated microbiota alone can confer increased vulnerability to inflammatory insult, we transferred the microbiota from mice on HSD or control diet to GF mice. The recipient mice received control diet during the entire course of the experiment and were exposed to 1 cycle of DSS, starting at day 5 post-colonization (Fig 6A). We observed no differences in colitis severity, including weight loss, colon length and macroscopic scores between the two groups of mice (Fig 6F-H). However, when analyzing the fecal microbiota profiles we found that prior to DSS administration, both groups of recipient mice had similar microbial profiles, closer to control donor, based on abundance jaccard index (Fig 6B-C). Further analysis has confirmed that there were no differences between the microbiota genera of both recipient groups (Table S10), including genera affected by HSD such as Lactobacillus (Fig. 6D). When analyzed in detail, the OTUs successfully transferred from control diet-microbiota accounted for 98.0% of the entire donor's community, while the OTUs from HSD-microbiota accounted for 75.9% of the donor's microbiota

community (Table 2). From the 15 most abundant OTUs of each microbiota-type, that accounted for 99% of control diet microbiota and 95% of HSD microbiota, we verified that only 7 OTUs from HSD microbiota were detected in the recipient mice, while from control diet-microbiota 12 OTUs were present (Fig 6E, Table 2). These results demonstrate that a significant proportion of the HSD OTUs did not successfully colonize the gut of recipient mice when these were fed control diet, suggesting that a constant exposure to high levels of NaCl in the diet is necessary to maintain the HSD signature microbiota profile. Taken together, similar colitis severity observed in the two microbiota-similar recipient groups supports our hypothesis that microbiota composition and function is a major determinant of colitis severity.

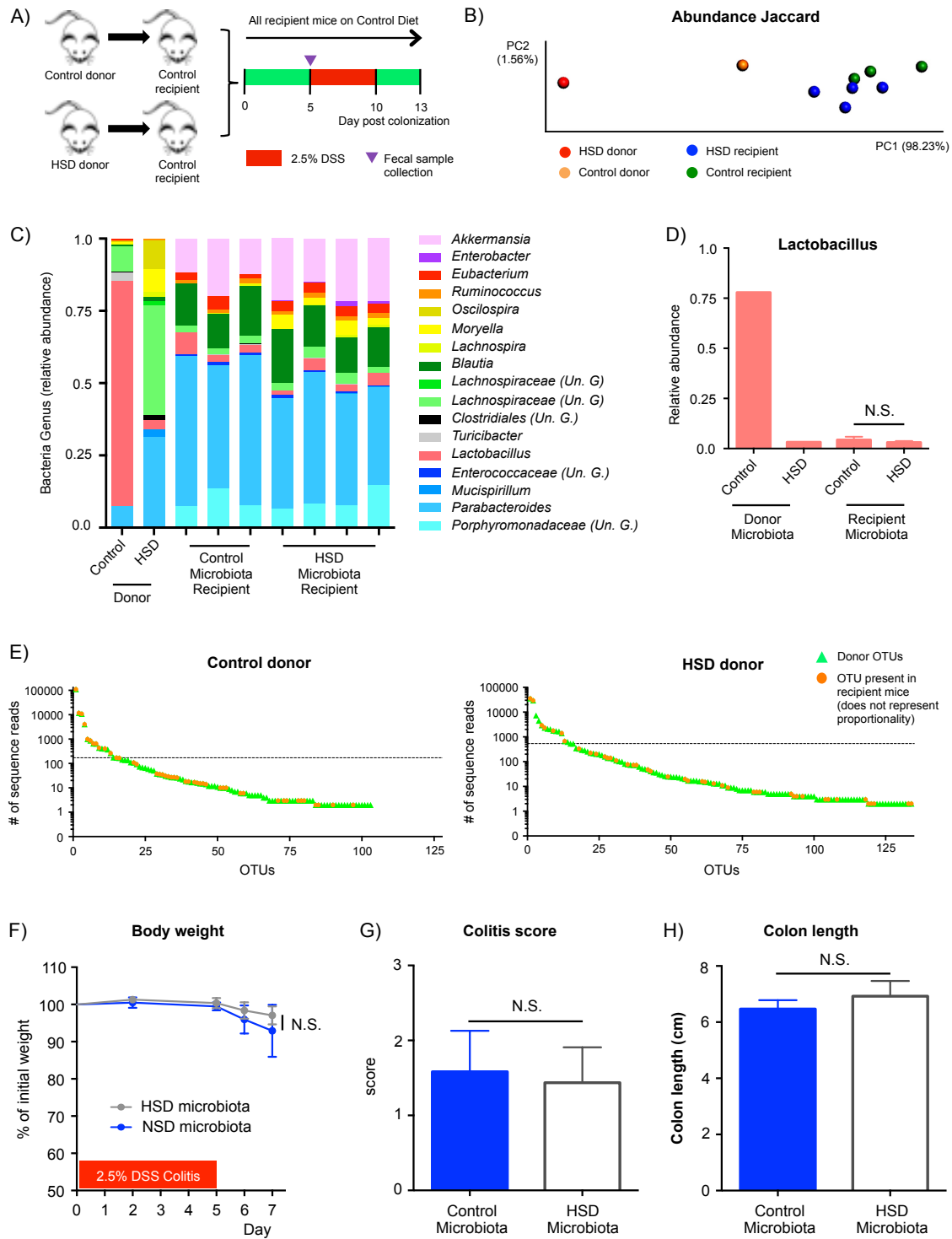
Table 2: OTU count analysis of Microbiota Transplant Experiment

	# of OTUs		
	Total From Donor	Successfully Transferred to Recipient	Transferred From 15 Most Abundant OTUs
CTL Microbiota	103	43 (98.0%)*	12
HSD Microbiota	134	43 (75.9%)*	7

*Coverage of the donor microbiota community based on read counts

Figure 6 (see figure on page below). **HSD-signature microbiota requires constant exposure to high salt diet for its maintenance.** A: Experimental design. B: Principal coordinate analysis (PCoA) of abundance Jaccard index from fecal microbiota composition data. C: Summary of the relative microbial genera present in 99.9% of the community. Un. G (Unclassified Genus) D: Relative abundance of *Lactobacillus* genus E: Total read count of each OTU present in the indicated donor fecal microbiota (in green triangles). Orange dots indicate the OTUs that were present in recipient mice at day 5 post-colonization. The horizontal dashed line separates the 15 most abundant OTUs from the others. F: Body weight during DSS administration. G: Macroscopic scores of DSS colitis at endpoint. H: Colon length at endpoint.

High diet exacerbates colitis in mice by decreasing *Lactobacillus* levels and butyrate production



5. Discussion

In addition to host genetic make-up, environmental factors such as diet and microbiota composition have been shown to play a crucial role in the development of inflammatory diseases. Here we show that high content of NaCl in diet modulates gut microbiota composition and function, namely decreasing the relative abundance of *Lactobacillus* spp and levels of the SCFA butyrate, promoting a pro-inflammatory state in the gut. We demonstrate that the gut microbiota is a key mediator in the exacerbation of experimental colitis by HSD, supported by the fact that the detrimental effect of HSD was not observed in GF mice.

To understand the impact of HSD in the modulation of gut microbiota, we fed mice with a diet supplemented with 4% NaCl for 4 weeks and analyzed fecal microbiota dynamics during and after HSD. The NaCl concentration employed here simulates the concentration of a typical high salt diet[18,150], which was shown to exacerbate the development of several immune disorders in mice[21,22,150]. Importantly, 4 weeks of 4% NaCl diet do not affect mouse blood pressure[21]. HSD altered gut microbiota composition and function, mainly decreasing colonic *Lactobacillus* spp. relative abundance and SCFA butyrate levels, two factors that have been implicated in the maintenance of a healthy gut physiology. This is in agreement with a very recently published study by Wilck et al showing that high salt diet alters gut microbiota, mainly by decreasing levels of *Lactobacillus murinus*, leading to induction of T helper 17 cells[175] and that supplementation with *L. murinus* can abrogate the pro-inflammatory effect of HSD. In addition to butyrate, lactic acid also appeared to decrease after HSD, although not significantly. Interestingly, it has been shown that lactic acid is used by some butyrate-producing bacteria for the production of high concentrations of butyric acid[176], thus it is plausible that the decrease observed in *Lactobacillus* spp. is, at least in part, indirectly responsible for the significant decrease of butyric acid. Several species of *Lactobacillus* genus have been shown to play a role in host's gut mucosal immune and barrier function[177]. A recent study showed that *Lactobacillus reuteri* ameliorates

colitis in mice by increasing the colon mucus thickness[178]. Butyrate plays an essential role in gut homeostasis as it serves as the primary energy source for colonocytes and is important for maintaining tissue barrier function[179]. Moreover, it has been directly implicated in colonic Tregs' differentiation[180,181], intestinal macrophage function[182], and colonic inflammation down regulation[183]. Furthermore, a recent study has shown that butyrate deficiency is directly associated with colitis[184], while small clinical studies have shown that butyrate treatment improves colitis[185,186].

We hypothesized that the changes in gut microbiota driven by HSD, in the absence of any additional inflammatory insult, would affect intestinal immune homeostasis. Our data revealed a strong impact of HSD on mucosal immunity, both in the colon and small intestine. Even though Na⁺ and Cl⁻ ions are thought to be absorbed in the small intestine, HSD had a stronger impact in the colon, when considering the number of affected genes (35 genes in the colon and 11 genes in the small intestine). This is in line with a recent study showing that HSD increased sodium luminal concentration in the colon but not in the small intestine[187], which may partially explain the different impact of HSD on small intestinal and colonic mucosal immunity.

Many of the altered genes were related to immune cell trafficking, cell viability and signal transduction elements. Rac1 gene, which was increased by HSD, has been associated with ulcerative colitis in many studies. Several polymorphisms that lead to the increase of Rac1 expression have been shown in patients with UC[188] and Rac1 is the target of a commonly used IBD treatment azathioprine[189]. Moreover, mice with a Rac1 conditional deletion in neutrophils and macrophages are resistant to DSS-colitis, showing reduced neutrophil migration and reduced levels of IL1 β and KC[188]. Furthermore, the genes Mapk3, Map2k1, Map2k6, Atf2 and Hras1, together with Rac1, were also integrated by IPA to be part of the upregulated "LPS-stimulated MAPK signaling" as well as part of other MAPK-related canonical pathways (Table S7). This is of particular interest, not only because MAPK signaling pathway has been shown to be induced by NaCl[21,22,150], but also because microbiota-derived molecules such as LPS, flagellin, DNA, RNA, and others, can lead to the activation of MAPK signaling pathways by selectively binding to various pattern recognition

receptors on innate immune cells[136,190]. In agreement with previous reports, we found that IL-17 signaling pathway was predicted to be affected by HSD in both colonic and small intestine tissues[21,22,150].

Surprisingly, we found that HSD downregulated the expression of multiple chemokines, cytokines and its receptors in the colonic *lamina propria*. This was unexpected considering that previous studies have shown that NaCl induces macrophage chemotaxis *in vitro*[191], and that macrophages infiltrate the skin of rodents fed with HSD in response to the storage of Na⁺ in this organ, a mechanism important to maintain interstitial electrolyte and volume homeostasis[192]. However, several other studies have shown downregulation of the expression of chemokine receptors in neutrophils and other granulocytes, upon TLR engagement in a p38-MAPK signaling dependent manner, with cells remaining partially functionally competent; a potential mechanism regulating cell positioning and activation[193,194]. Considering that HSD induced changes in colonic microbiota and upregulation of MAPK signaling pathways in the colon, it is conceivable that the observed downregulation of chemokine receptors and other molecules is a result of mucosal granulocytes TLR engagement. Downregulation of cytokines and chemokines could compromise the colonic immune barrier, and promote a pro-inflammatory response or impaired resolution of inflammation after the intestinal injury induced by DSS and DNBS.

To investigate the effect of HSD induced alterations in mucosal immune function and gut microbiota, we used two different models of experimental colitis. We confirmed that HSD exacerbates colitis in mice [150] and showed enhanced colonic barrier disruption after DSS in mice fed HSD. In fact, Ocln and ZO-1, two components of the tight junction (TJ) structure, were significantly downregulated, as well as Muc2, a protein secreted by goblet cells (GC) and essential for the structure of the mucus layer in the intestine, and Tff3, also mostly expressed by GC, with a key role in mucus layer stability and mucosal repair [195]. On other hand, Mmp9 and Cnlp were upregulated in HSD mice after DSS-colitis. Even though Cnlp has been proposed to have a protective role [196], the level of this peptide has been shown to be elevated in DSS colitis and patients with Ulcerative colitis [197,198]. Mmp9 is markedly elevated in intestinal tissues of IBD patients and it has been proposed to be a biomarker of

disease activity [199–201]. *Mmp9* is also elevated in DSS colitis and has been shown to contribute to the severity of colitis by increasing intestinal epithelial TJ permeability [201]. *Cnlp* and *Mmp9* are highly expressed by active neutrophils, therefore their upregulation could also reflect the increased infiltration of these cells in the inflamed colonic tissue of HSD mice after DSS administration. We reproduced the colitis results in a different mouse strain, and showed that HSD leads to a similar exacerbation of DSS colitis in NIH Swiss as in C57BL/6 mice, suggesting that HSD-mediated exacerbation of experimental colitis is independent of mouse genotype. Further supporting our results, several studies have recently reported increased severity of colonic inflammation not only in models of chemically induced colitis[150,202], but also in IL-10 KO mice, which spontaneously develop colitis, and in *Salmonella typhimurium* infected mice, while on HSD[187].

In order to test whether gut microbiota plays a role in the HSD-dependent exacerbation of gut injury, we induced DSS colitis in GF mice. While DDS induced colitis in both groups of germ-free mice, there was no worsening of the colonic inflammation in mice fed a HSD. This suggests that the increased susceptibility to colitis induced by a HSD is critically dependent on the presence of the microbiota. In subsequent experiments, we found no difference in colitis severity when transferring gut microbiota from SPF mice on a HSD into germ-free recipients. Although seemingly counterintuitive, the results are in accordance with our previous data as we found that at the beginning of DSS administration (five days post-colonization) the microbiota of all recipient mice had converged to a similar profile, regardless of the donor microbiota. This is in sharp contrast with our SPF mice experiment, where we observed a marked dysbiosis prior to commencing DSS and subsequently worse colitis in mice previously on high salt diet. In our transfer experiment, most of the abundant OTUs from the control diet donor were transferred to the recipient mice while only a minority of abundant HSD donor OTUs were detected in the recipient mice. These differences in microbiota profiles could be partially attributed to technical issues, including freezing and thawing, and exposure to oxygen, as we did not use fresh cecal content samples for colonizations. However, we believe that the major factor accounting for this difference was the fact that recipient

mice were given control diet, which did not allow establishment of HSD microbiota profiles. Thus, the microbiota transfer experiment supports the notion that HSD-induced dysbiosis, and its altered metabolic activity, is a major determinant of the sensitivity to inflammatory insult in this model and that a constant exposure to HSD is necessary to maintain the “pro-inflammatory” microbiota profile.

Even though it has been previously shown that NaCl can directly affect immune cells *in vitro*, we hypothesize that these direct effects do not play a major role in our *in vivo* model. Taking into consideration that i) dysbiosis, including the depletion of *Lactobacillus* and butyrate, is known to affect mucosal immune homeostasis, and that ii) the mucosal milieu in the colon, which harbors the highest microbial density along the GI tract, was more affected by HSD than small intestine, and that iii) multiple immune pathways affected by HSD are strongly modulated by microbial products, we hypothesize that changes in the immune system are a consequence of the disruption of the gut microbiota by HSD. This notion is further supported by the lack of changes in colonic mucosal immune milieu in germ-free mice on HSD (Table S9).

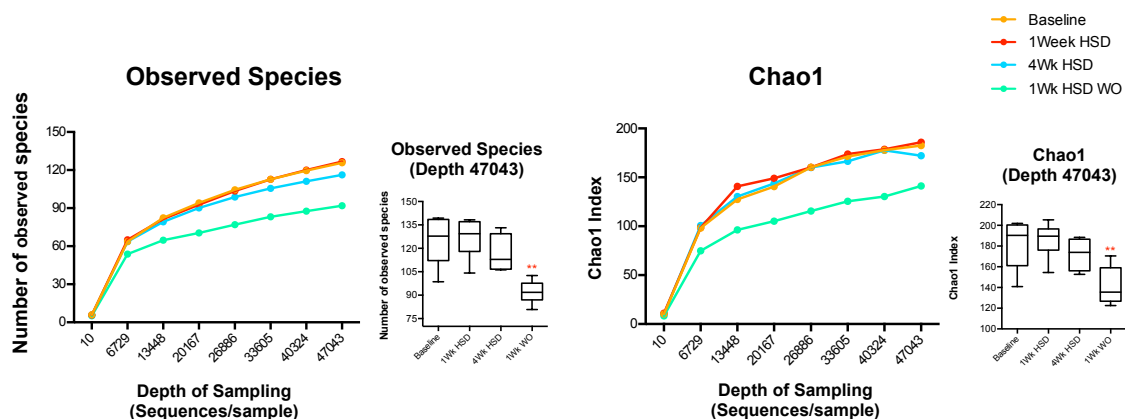
Taken together, this study provides an example of how environmental factors, such as diet, play a key role in shaping gut microbial communities that influence host physiology and predisposition to disease.

6. Conclusions

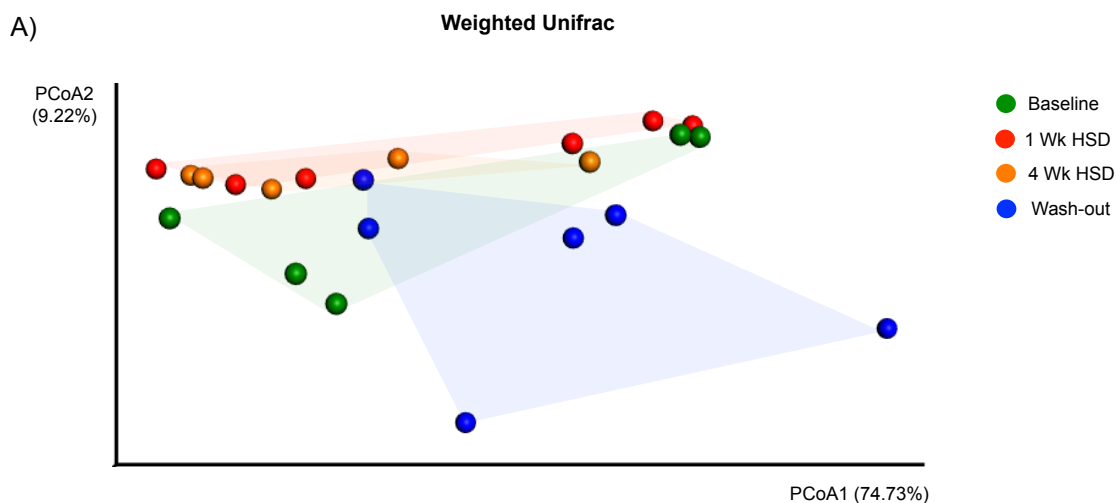
We have demonstrated that consumption of a diet with high salt content results in alteration of gut microbiota composition and function that may be pro-inflammatory by virtue of the loss of *Lactobacillus* spp. and reduced production of the protective SCFA butyrate. Our findings indicate that the maintenance of the pro-inflammatory microbiota is dependent on a continued high salt intake, and is associated with changes in gut immune homeostasis, predisposing mice to the development of a more severe form of experimental colitis. Our study suggests that high concentrations of dietary salt, similar to those found in the Western Diet, contribute to the increased prevalence of

chronic inflammatory disorders through microbiota dependent mechanisms. This finding has implications for the management of patients with IBD and other inflammatory disorders and should be further investigated, as it provides an easy candidate for dietary therapeutic interventions.

7. Supplementary Figures

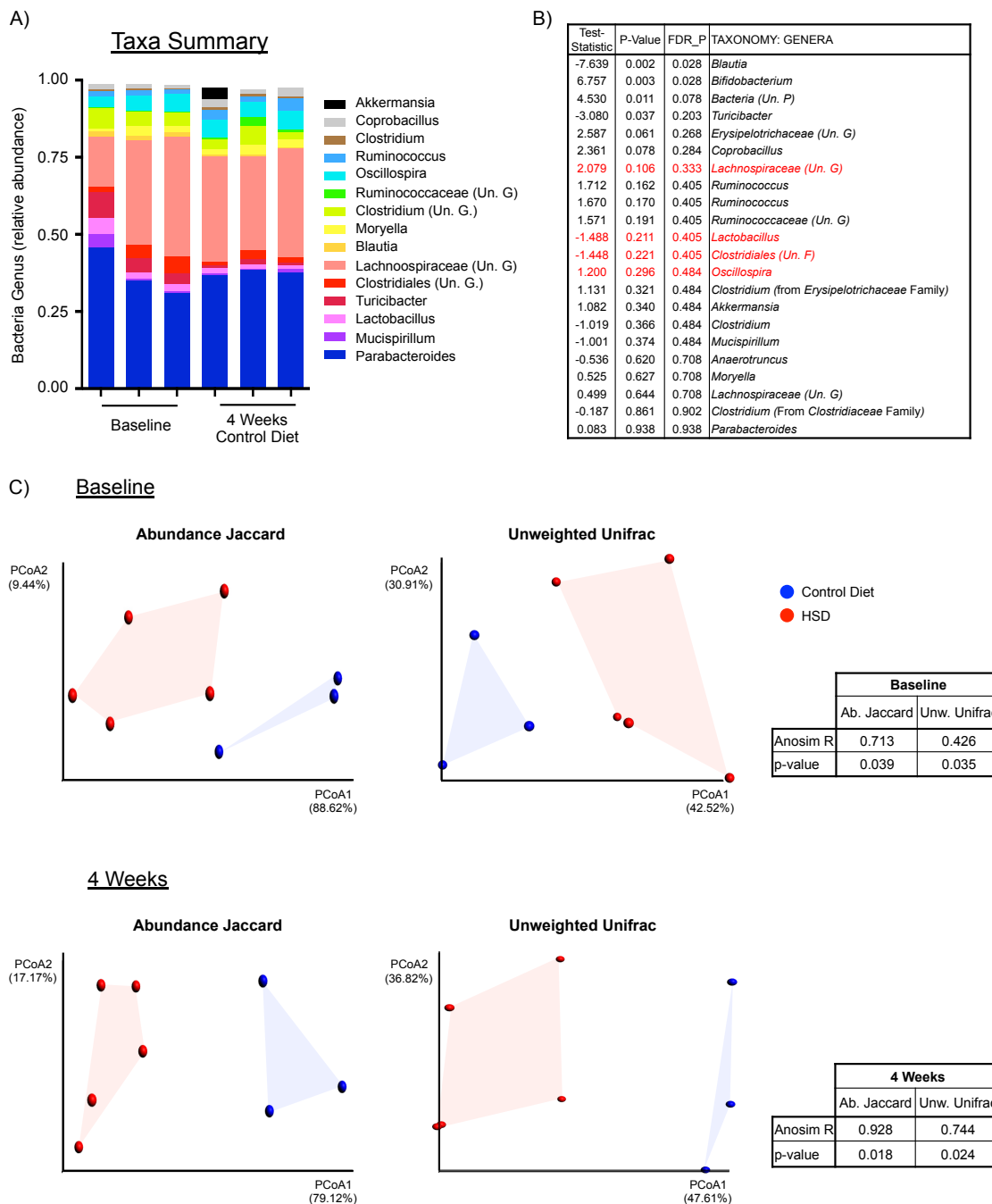


Supplementary Figure 1. HSD affects richness and diversity of fecal microbiota. Observed species and Chao1 diversity index of fecal microbiota at the indicated time points after introduction of HSD.

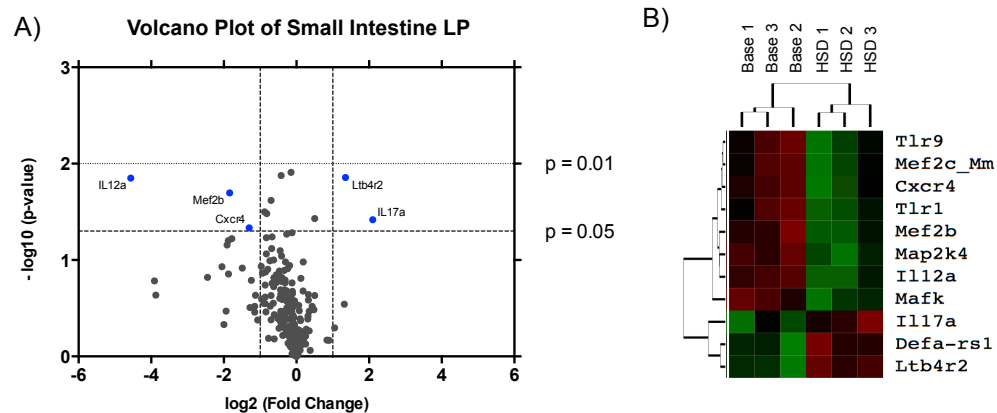


Supplementary Figure 2. Weighted Unifrac distance metrics of gut microbiota of mice fed with HSD. PCoA plot of weighted unifrac metric of fecal microbiota composition over time exposed to HSD.

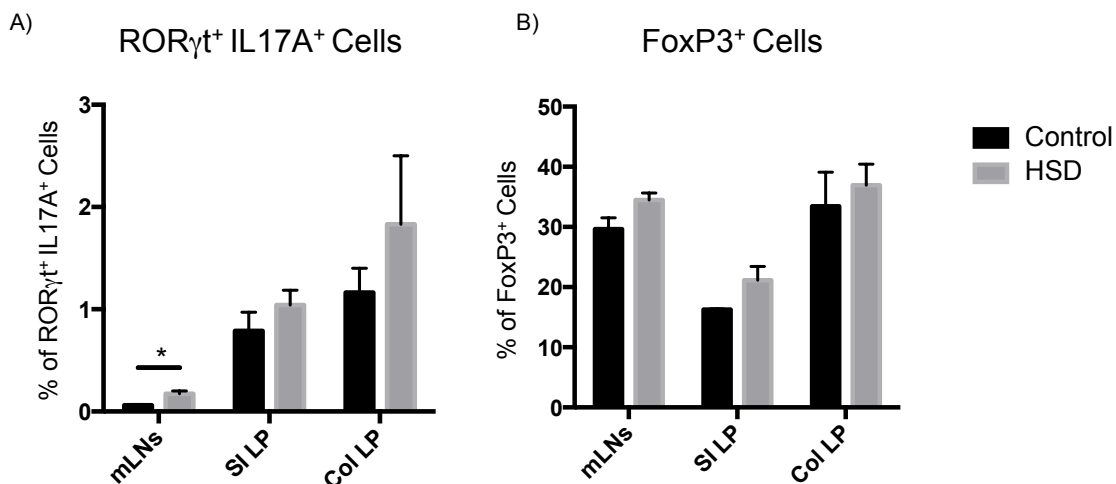
High diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production



Supplementary Figure 3. Gut microbiota is stable in mice on control diet during week 8-12 A: Summary of the relative abundance of microbial genera present in 99.9% of the community before and after 4 weeks on control diet. (Un. G: Unclassified Genus) B: Summary of the t-test statistical analysis of microbiota genera before and after 4 weeks on control diet. Highlighted in red are the taxa that were shown to correlate with HSD. C: PCoA plots of abundance Jaccard index (left panels) and unweighted unifrac metric (right panels) of fecal microbiota composition from mice on control and high salt diet, at the beginning of the experiment (Baseline) and 4 Weeks of diet. Anosim statistical analysis of respective distance metrics.

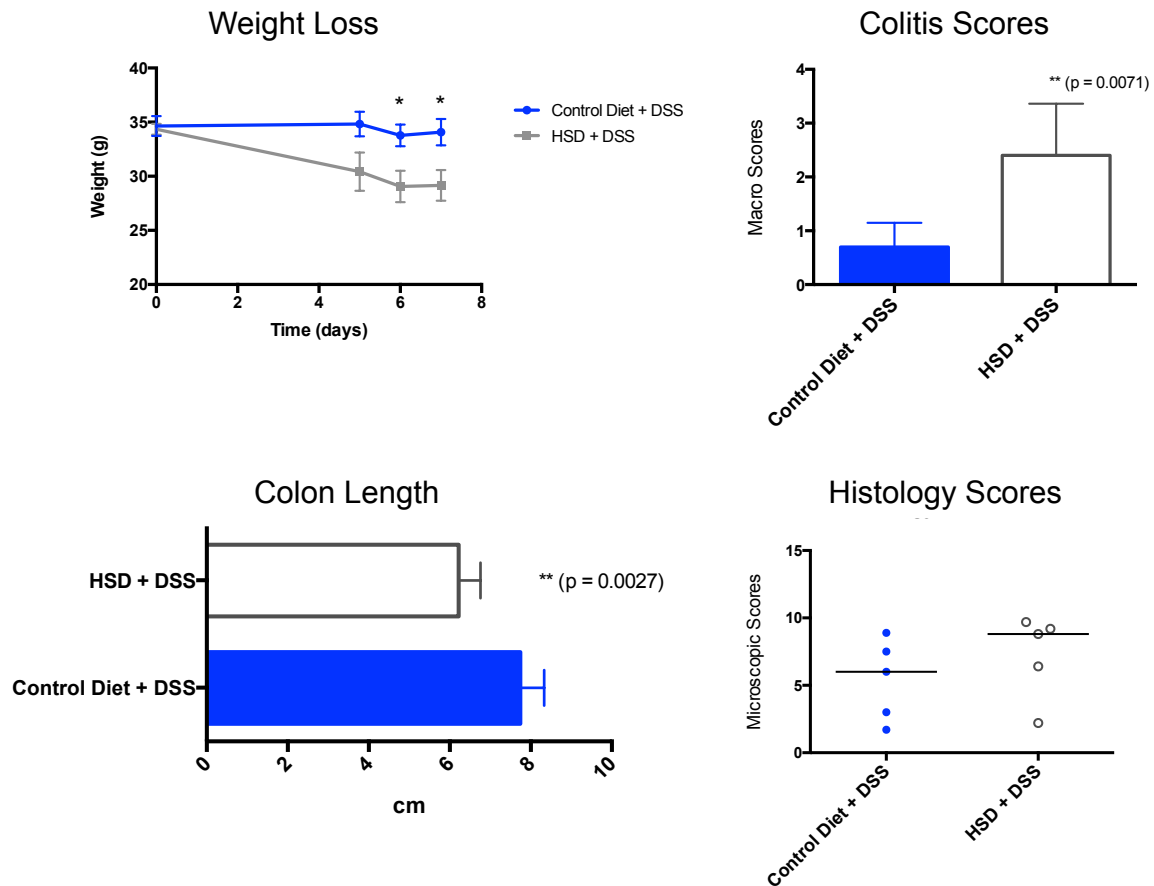


Supplementary Figure 4. HSD modulates immune expression in small intestine *lamina propria*. A: Volcano plot of gene expression in small intestine *lamina propria* total cell extract of mice receiving HSD or control diet for 4 weeks, n=9 mice/group. Blue dots: genes with p < 0.05, fold change > 2. B: Heatmap of immune genes that changed with HSD (p < 0.05). The heatmap was generated based on group average, using Euclidean distance metric.



Supplementary Figure 5. HSD increases ROR γ t⁺ IL17⁺ Cells in mLN but it doesn't affect FoxP3 cells. Total cell extract of mesenteric lymph nodes (mLNs), small intestine *lamina propria* (SI LP) and colonic *lamina propria* (Col LP) was performed on mice fed HSD for 4 weeks (and control diet). Cells were analyzed with flow cytometry. Indicated % are from viable cells gated on TCRb⁺ CD4⁺. This is a representative experiment of 3 other different independent experiments. Values are presented as means $\pm$ SEM; n = 6-9 mice/group. * (p < 0.05).

High diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production



Supplementary Figure 6. HSD increases susceptibility to DSS colitis in NIH Swiss mice. Mice, previously fed HSD or control diet, were exposed to 3.5% DSS in drinking water during 5 days. DSS (Control diet, DSS in water), HSD+DSS (High salt diet, DSS in water). A: Body weight changes during DSS treatment B: Clinical assessment of DSS colitis at endpoint (Day 7). C: Colon length at endpoint. D: Histology score of colonic sections. Values are presented as means $\pm$ SEM; n = 5 mice/group. * (p<0.05) ** (p<0.01).

8. Supplementary Tables

Table S1

Table S1 - Fecal microbiota genera that significantly correlates with HSD

Taxonomy (Genus)	Spearman r	p-value	q (FDR)
<i>Lactobacillus</i>	-0.703	1.39E-04	0.003
<i>Lachnospiraceae (Un. Genus)</i>	0.614	0.002	0.017
<i>Clostridiales (Un. Genus)</i>	-0.605	0.002	0.017
<i>Oscillospira</i>	0.548	0.007	0.042

Table S2

Table S2 - Fecal microbiota OTU that significantly correlates with HSD

Taxonomy (OTUs)	Spearman r	p-value	q (FDR)
OTU3: <i>g__Lactobacillus</i>	-0.707	1.22E-04	0.005
OTU9: <i>o__Clostridiales</i>	-0.684	2.62E-04	0.005
OTU25: <i>f__Lachnospiraceae</i>	0.609	0.002	0.027
OTU10: <i>g__Oscillospira</i>	0.482	0.022	0.214
OTU24: <i>f__Lachnospiraceae</i>	0.454	0.033	0.214
OTU16: <i>f__Lachnospiraceae</i>	-0.454	0.033	0.214
OTU2: <i>f__Lachnospiraceae</i>	0.427	0.047	0.260

High diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production

Table S3

Table S3 - List of the genes that are significantly altered by HSD in colonic lamina propria

Gene Code	Gene Name	Accession #	log2(ratio)	P value	Type
<u>Cytokines, Chemokine and Receptors</u>					
Ccl3	<i>C-C motif Chemokine Ligand 3</i>	NM_007482.3	-1.42	0.0091	Chemokine
Ccl4	<i>C-C motif Chemokine Ligand 4</i>	NM_144938.2	-1.45	0.0182	Chemokine
Cxcl10	<i>C-X-C motif Chemokine Ligand 10</i>	NM_009777.2	-0.9	0.017	Chemokine
Cxcl2	<i>C-X-C motif Chemokine Ligand 2</i>	NM_016960.1	-2.07	0.039	Chemokine
Ccr7	<i>C-C motif Chemokine Receptor 7</i>	NM_013653.1	-1.9	0.047	Chemokine Receptor
Cxcr4	<i>C-X-C motif Chemokine Receptor 4</i>	XM_356827.6	-1.5	0.0259	Chemokine Receptor
Csf2	<i>Colony Stimulating Factor</i>	NM_007465.2	-1.73	0.0157	Cytokine
Ifi2712a	<i>Interferon Alpha Inducible Protein 27 Like 2</i>	NM_009744.3	-1.13	0.0101	Cytokine
Il1b	<i>Interleukin 1 Beta</i>	NM_013654.2	-1.39	0.0476	Cytokine
Il7	<i>Interleukin 7</i>	NM_009662.2	0.47	0.0033	Cytokine
Tnfrsf14	<i>Tumor Necrosis Factor Superfamily Member 14</i>	NM_023143.3	-1.39	0.0181	Cytokine
Il1rap	<i>Interleukin-1 Receptor Accessory Protein</i>	NM_007440.4	0.19	0.0005	Cytokine receptor
Cysl2r2	<i>Cysteinyl Leukotriene Receptor 2</i>	NM_133882.2	-1.11	0.0287	Leukotrienes Receptor
Ltb4r1	<i>Leukotriene B4 Receptor 1</i>	NM_019577.4	-0.78	0.043	Leukotrienes Receptor
<u>Transcription Factors and Regulators</u>					
Atf2	<i>Activating Transcription Factor 2</i>	NM_146148.1	0.27	0.0265	Transcription Factor
Cebpb	<i>CCAAT/Enhancer Binding Protein Beta</i>	NM_007572.2	-0.87	0.0165	Transcription Factor
Irf5	<i>Interferon Regulatory Factor 5</i>	NM_011888.2	-0.71	0.0343	Transcription Factor
Mef2c_Mm	<i>Myocyte-specific Enhancer Factor 2C</i>	NM_009778.2	-0.67	0.021	Transcription Factor
Myc	<i>V-Myc Avian Myelocytomatosis Viral Oncogene Homolog</i>	NM_011332.2	-0.39	0.0316	Transcription Factor
Keap1	<i>Kelch-like ECH-associated Protein 1</i>	NM_013652.1	0.35	0.045	Regulatory Element
<u>Signal Transduction Elements</u>					
Ptgs2	<i>Prostaglandin-endoperoxide Synthase 2 (aka COX-2)</i>	NM_011330.3	-1.67	0.0307	Signal Transduction Enzyme
Map2k1	<i>Mitogen-Activated Protein Kinase Kinase 1</i>	NM_009704.3	0.59	0.0061	Signal Transduction Kinsases
Map2k6	<i>Mitogen-Activated Protein Kinase Kinase 6</i>	NM_016704.2	0.41	0.0219	Signal Transduction Kinsases
Mapk3	<i>Mitogen-Activated Protein Kinase 3</i>	NM_009743.4	0.52	0.01	Signal Transduction Kinsases
Ptger3	<i>Prostaglandin E Receptor 3</i>	NM_009779.2	1.35	0.021	Signal Transduction Receptor
Gnb1	<i>G-Protein Subunit Beta 1</i>	NM_009660.3	0.25	0.0015	Signaling Transduction
H2-Eb1	<i>Histocompatibility 2, class II antigen E Beta</i>	NM_011124.4	-0.77	0.0394	Signaling Transduction
Hras1	<i>GTPase Hras (aka Transforming Protein p21)</i>	NM_013484.2	-2.41	0.0192	Signaling Transduction
Rac1	<i>Ras-related C3 botulinum toxin substrate 1</i>	NM_007425.2	0.41	0.0001	Signaling Transduction
Tyrobp	<i>TYRO Protein Tyrosine Kinase Binding Protein</i>	NM_013485.1	-1.39	0.03	Signaling Transduction
<u>Other</u>					
Alox15	<i>Arachidonate 15-lipoxygenase</i>	NM_001025093.1	-3.27	0.0098	Lipid Oxidation Enzyme
Alox5	<i>Arachidonate 5-lipoxygenase</i>	NM_009137.2	-1.84	0.0411	Lipid Oxidation Enzyme
Arg1	<i>Arginase1</i>	NM_011413.2	-0.6	0.0216	Enzyme
Itgb2	<i>Integrin Subunit Beta 2 (aka CD18)</i>	NM_011337.1	-0.77	0.0444	Integrin
Cfb	<i>Complement Factor B</i>	NM_011333.3	0.41	0.0346	Complement Factor

*From IPA analysis

Table S4

Table S4 - Most significant canonical pathways altered by HSD in colonic lamina propria

Canonical Pathways	Genes involved	p-value	Ratio	z-score
Colon				
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	MAP2K6,MYC,MAPK3,RAC1,HRAS,IL1B,CEBPB,CSF2,MAP2K1,IL1RAP,IL7,ATF2	1.07E-12	4.03E-02	NaN
Role of IL-17F in Allergic Inflammatory Airway Diseases	CXCL10,CCL4,MAPK3,IL1B,CSF2,MAP2K1,ATF2	3.22E-12	1.71E-01	0
IL-17 Signaling	CXCL10,MAP2K6,MAPK3,HRAS,CEBPB,PTGS2,MAP2K1,ATF2	1.05E-11	9.52E-02	NaN
Communication between Innate and Adaptive Immune Cells	CXCL10,CCL4,CCL3L3,IL1B,CSF2,HLA-DRB5,CCR7	6.07E-10	8.33E-02	NaN
Acute Phase Response Signaling	MAP2K6,MAPK3,CFB,HRAS,IL1B,CEBPB,MAP2K1,IL1RAP	1.34E-09	5.23E-02	0.707
Eicosanoid Signaling	ALOX15,LTB4R,PTGER3,PTGS2,ALOX5,CYSLTR2	4.57E-09	9.68E-02	NaN
IL-6 Signaling	MAP2K6,MAPK3,HRAS,IL1B,CEBPB,MAP2K1,IL1RAP	8.45E-09	5.74E-02	0.378
LPS-stimulated MAPK Signaling	MAP2K6,MAPK3,RAC1,HRAS,MAP2K1,ATF2	3.36E-08	6.98E-02	1.633
p38 MAPK Signaling	MAP2K6,MYC,IL1B,MEF2C,IL1RAP,ATF2	1.48E-07	5.45E-02	0

*From IPA analysis

Table S5

Table S5 - Most significant functions or diseases altered by HSD in colonic lamina propria

	Functon Or Disease	Genes involved	p-value	Activation
Colon				
Top 5 (Lowest P-values)	adhesion of immune cells	ALOX15,CCL3L3,CCL4,CCR7,CSF2,CXCL10,CXCL3,CXCR4,HRAS,IL1B,IL7,ITGB2,LTB4R,MAP2K1,PTGS2,RAC1	3.18E-18	-2.268
	cellular homeostasis	ARG1,CCL3L3,CCL4,CCR7,CSF2,CXCL10,CXCL3,CXCR4,HRAS,IL1B,IL7,IRF5,ITGB2,LTB4R,MAP2K1,MAPK3,MEF2C,MYC,PTGS2,RAC1,TYROBP	8.01E-17	-1.409
	leukocyte migration	ALOX15,ALOX5,CCL3L3,CCL4,CCR7,CSF2,CXCL10,CXCL3,CXCR4,HRAS,IL1B,IL7,IRF5,ITGB2,LTB4R,MYC,PTGS2,RAC1,TNFSF14,TYROBP	9.04E-17	-3.291
	cell movement of leukocytes	ALOX5,CCL3L3,CCL4,CCR7,CSF2,CXCL10,CXCL3,CXCR4,HRAS,IL1B,IL7,ITGB2,LTB4R,MYC,PTGS2,RAC1,TNFSF14,TYROBP	1.64E-15	-3.017
	inflammatory response	ALOX5,CCL3L3,CCL4,CCR7,CEBPB,CSF2,CXCL10,CXCL3,CXCR4,IL1B,ITGB2,LTB4R,PTGS2,RAC1,TNFSF14,TYROBP	4.47E-15	-3.311
Bottom 5 z-score	Inflammatory Response	ALOX5,CCL3L3,CCL4,CCR7,CEBPB,CSF2,CXCL10,CXCL3,CXCR4,IL1B,ITGB2,LTB4R,PTGS2,RAC1,TNFSF14,TYROBP	4.47E-15	-3.3
	Leukocyte Migration	ALOX15,ALOX5,CCL3L3,CCL4,CCR7,CSF2,CXCL10,CXCL3,CXCR4,HRAS,IL1B,IL7,IRF5,ITGB2,LTB4R,MYC,PTGS2,RAC1,TNFSF14,TYROBP	9.04E-17	-3.29
	Cell Movement of myeloid Cells	ALOX5,CCL3L3,CCL4,CCR7,CSF2,CXCL10,CXCL3,CXCR4,HRAS,IL1B,ITGB2,LTB4R,PTGS2,RAC1,TYROBP	2.69E-14	-3.26
	Migration of Cells	ALOX15,ALOX5,CCL3L3,CCL4,CCR7,CSF2,CXCL10,CXCL3,CXCR4,HRAS,IL1B,IL7,IRF5,ITGB2,LTB4R,MAP2K1,MYC,PTGS2,RAC1,TNFSF14,TYROBP	8.76E-15	-3.2
	Cell Movement of phagocytes	ALOX5,CCL3L3,CCL4,CCR7,CSF2,CXCL10,CXCL3,CXCR4,HRAS,IL1B,ITGB2,LTB4R,PTGS2,RAC1,TYROBP	3.99E-14	-3.01
Top 5 z-score	Cell Viability of lymphocytes	IL7,MAP2K1,MAPK3,MYC	5.98E-04	1.925
	Cell Viability of mononuclear leukocytes	CSF2,IL7,MAP2K1,MAPK3,MYC	5.01E-05	1.478
	Maturation of Lymphocytes	CSF2,IL7,MAPK3,MYC,PTGS2,TYROBP	9.96E-08	1.109
	Quantity of Lymphoid tissue	ARG1,CCR7,CXCR4,IL7,ITGB2,MAPK3,RAC1,TYROBP	1.28E-06	1.06
	Cell Cycle Progression	CSF2,HRAS,IL1B,IL7,MAP2K1,MAP2K6,MYC,PTGS2	9.25E-05	0.599

*From IPA analysis

Table S6

Table S6- List of the genes that are significantly altered by HSD in small intestine lamina propria

Gene Code	Gene Name	Accession #	log2(ratio)	P value	Type
<u>Cytokines, Chemokine and Receptors</u>					
Il12a	<i>Interleukin 12A</i>	NM_008351.1	-4.56	0.0141	Cytokine
Il17a	<i>Interleukin 17A</i>	NM_010552.3	2.1	0.0381	Cytokine
Cxcr4	<i>C-X-C motif Chemokine Receptor 4</i>	NM_009911.3	-1.3	0.0464	Chemokine receptor
Ltb4r2	<i>Leukotriene B4 Receptor 2</i>	NM_020490.2	1.35	0.0139	Leukotrienes Receptor
<u>Transcription Factors and Regulators</u>					
Maik	<i>V-Maf Avian Musculoaponeurotic Fibrosarcoma</i>	NM_010757.2	-0.42	0.0133	Transcription Factor
Mef2b	<i>Myocyte-specific Enhancer Factor 2B</i>	NM_001045484.1	-1.84	0.0201	Transcription Factor
Mef2c_Mm	<i>Myocyte-specific Enhancer Factor 2C</i>	NM_025282.2	-0.88	0.0316	Transcription Factor
<u>Signal Transduction Elements</u>					
Map2k4	<i>Mitogen-Activated Protein Kinase Kinase 4</i>	NM_009157.4	-0.15	0.0123	Signal Transduction Kinsases
<u>Innate Immunity System</u>					
Tlr1	<i>Toll-Like Receptor 1</i>	NM_030682.1	-0.7	0.024	Receptor
Tlr9	<i>Toll-Like Receptor 9</i>	NM_031178.2	-0.82	0.033	Receptor
Defa-rs1	<i>Alpha-Defensin-Related Sequence 1</i>	NM_007844.2	0.5	0.0371	Defensin

*From IPA analysis

Table S7

Table S7 - Most significant canonical pathways altered by HSD in small intestine lamina propria

Canonical Pathways	Genes involved	p-value	Ratio	z-score
Small Intestine				
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	<i>MAP2K4, IL12A, TLR1, TLR9, IL17A</i>	9.01E-09	3.79E-02	NaN
Toll-like Receptor Signaling	<i>MAP2K4, IL12A, TLR1, TLR9</i>	8.59E-08	5.56E-02	NaN
IL-17 Signaling	<i>MAP2K4, TLR9, IL17A</i>	1.76E-05	3.57E-02	NaN
Communication between Innate and Adaptive Immune Cells	<i>IL12A, TLR1, TLR9</i>	1.76E-05	3.57E-02	NaN
p38 MAPK Signaling	<i>MAP2K4, MEF2C, MEF2B</i>	3.95E-05	2.73E-02	NaN

*From IPA analysis

High diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production

Table S8

Table S8 - List of genes that are statistically altered by DSS in Germ-Free mice

Gene expression was acquired using Nanostring technology, from a customized gene panel

Gene Name	Gene Function	DSS*	DSS*	DSS*	Control*	Control*	Control*	log2ratio	p-value
GABAB	Neuro	616.07	735.68	632.93	189.68	172.53	168.23	1.9	0.0001
Tjp1	Barrier	2192.85	2423.08	2210.85	1410.66	1233.45	1305.33	0.79	0.0005
Cdh1	Barrier	18558.07	17560.81	16639.28	11999.26	12128.77	10918.29	0.59	0.0009
Mapk1	Immune	4223.89	4414.01	4148.45	3550.86	3416.6	3458.43	0.29	0.0016
Nfkb1	Immune	1645.58	1844.32	1874.55	1240.21	1098.16	1217.55	0.59	0.0019
NFAT	Immune	1502.39	1691.27	1592.82	1127.83	1105.94	1061.49	0.54	0.0023
Muc2	Barrier	57291.14	61718.74	55687.45	15352.86	10675.6	12250.46	2.21	0.0027
SEMAPHORIN	Neuro	198.83	234.09	189.73	86.14	83.96	112.14	1.15	0.0032
Tollip	Immune	1157.57	1243.7	1072.79	872.77	888.89	801.4	0.44	0.0059
GDNF	Neuro	63.05	119.63	97.31	31.85	16.8	21.11	2.01	0.0064
Tff3	Barrier	93229.47	109376.23	89628.82	61487.63	60132.8	47410.46	0.79	0.0076
P75	Neuro	156.04	154.35	198.64	96.24	80.07	107.27	0.84	0.0079
NOS	Neuro	266.32	375.56	266.57	163.16	154.04	108.89	1.09	0.0115
Il23r	Immune	52.35	43.74	23.81	6.59	2.2	8.11	2.95	0.019
Tlr4	Immune	867.07	964.61	940.27	446	537.53	621.77	0.8	0.0202
F2rl1	Immune	2594.45	2590.28	2353.39	2218.76	2074.39	1941.75	0.27	0.0204
Tlr5	Immune	73.74	83.61	101.76	52.05	59.63	40.62	0.77	0.0229
Hif1a	Immune	2278.43	2987.69	2397.93	4239	3347.49	3746.16	-0.57	0.023
CHAT	Neuro	46.59	74.61	31.61	17.96	6.09	5.67	2.49	0.0236
Rae1	Barrier	823.45	926.03	874.57	757.87	753.6	671.35	0.27	0.0248
Ocln	Barrier	3872.49	3750.37	3223.08	2606.39	2924.1	2203.47	0.49	0.0321
CALRET	Neuro	18949.8	16364.71	20667.03	11789.66	14783	14612.45	0.44	0.0376
CD11C	Immune	140.4	118.34	125.15	67.2	44.05	76.38	1.07	0.0377
Il22ra2	Immune	265.49	249.52	315.57	434.63	350.65	519.36	-0.64	0.0384
SOX10	Neuro	53.17	69.47	86.17	24.27	44.05	19.49	1.31	0.0441

* Normalized transcript counts

High diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production

Table S9

Table S9 - Effect of HSD in Germ Free mice colonic gene expression

Gene expression was acquired using Nanostring technology, from a customized gene panel

Gene Name	Accession #	%CV**	Control Diet*	Control Diet*	Control Diet*	HSD*	HSD*	HSD*	log2ratio	p-value
NFAT	NM_010901.2	5.04	1204.52	1181.14	1133.67	1224.86	1299.2	1278.44	0.11	0.0366
Hif1a	NM_010431.2	22.36	4527.23	3575.11	4000.88	3097.03	2266.78	3356.97	-0.49	0.0874
Mapk1	NM_011949.3	4.56	3792.3	3648.91	3693.58	3373.96	3418.93	3649.66	-0.09	0.0998
Tff3	NM_011575.2	16	65668.45	64221.5	50634.1	49948.18	51113.29	43974.82	-0.31	0.1062
I-FABP	NM_007980.2	30.2	1342.06	1255.99	1019.95	2324.59	1504.1	1427.85	0.51	0.1339
Cldn2	NM_016675.3	7.69	1854.49	1969.08	1868.05	1578.47	1713.06	1863.82	-0.15	0.159
CALRET	NM_007591.3	11.63	12591.29	15788.17	15606.02	15721.58	17905.79	17265.02	0.22	0.1688
Muc2	NM_023566.2	19.3	16396.77	11401.49	13083.42	16955.2	14441.21	19648.53	0.33	0.1804
Tollip	NM_023764.3	10.42	932.12	949.33	855.89	868.96	838.99	698.17	-0.19	0.1805
Tlr5	NM_016928.2	18.4	55.59	63.68	43.38	49.6	40.53	42.17	-0.29	0.2171
P75	NM_033217.3	13.79	102.79	85.51	114.56	94.23	80.37	85.15	-0.21	0.2214
T-bet	NM_019507.1	90.07	5.69	1.04	2.58	1.14	1	1.02	-1.24	0.2229
GFAP	NM_001131020.1	28.26	82.56	61.6	45.98	45.03	54.35	40.12	-0.42	0.226
Cldn1	NM_016674.3	13.83	114.92	105.26	114.56	78.21	95.82	111.76	-0.24	0.2416
SOX10	NM_011437.1	29.76	25.92	47.05	20.81	45.03	38.9	44.21	0.54	0.2645
Cdh1	NM_009864.2	6.01	12815.14	12953.46	11660.67	11229.98	12488.97	11543.17	-0.09	0.2665
GATA-3	NM_008091.3	25.39	32.66	15.86	32.09	37.02	35.65	37.05	0.52	0.2697
GABAB	NM_019439.3	9.52	202.58	184.26	179.67	224.69	182.82	215.12	0.13	0.2893
Cnlp	NM_009921.2	67.37	9.74	1.31	3.45	11.84	3.94	12.49	1.24	0.2908
5HT	NM_010484.2	18.19	271.35	250.79	236.09	207.52	263.32	158.83	-0.3	0.293
VIP	NM_011702.2	24.32	729.84	897.36	507.79	584.02	567.41	504.75	-0.33	0.3007
NPY	NM_023456.2	99.59	1.35	5.47	1	1	1	1.02	-0.95	0.3356
CD11C	NM_021334.2	32.58	71.77	47.05	81.57	82.79	121.03	60.59	0.38	0.3703
NOS	NM_008712.2	17.69	174.26	164.51	116.3	205.24	163.31	154.74	0.21	0.3971
TGR5	NM_174985.1	106.61	5.69	1.04	1	1	1	1.02	-0.84	0.415
Myd88	NM_010851.2	16.97	437.22	504.43	610.22	477.59	682.06	604.02	0.18	0.4244
CALB	NM_009788.4	104.92	5.69	1.04	1	1.14	1	1	-0.79	0.4404
NR2B	NM_008171.3	5.43	1	1.04	1	1.14	1	1.02	0.06	0.4445
CCR6	NM_001190333.1	69.96	70.42	25.22	131.92	56.47	220.22	89.24	0.75	0.4559
HMBGB1	NM_010439.3	7.16	1478.26	1496.11	1251.72	1325.56	1367.5	1303	-0.08	0.4572
Gpr44	NM_009962.2	58.53	3	1.04	1	1.14	1	1.02	-0.47	0.4606
CD11b	NM_001082960.1	35.93	25.92	32.5	69.42	40.45	60.86	52.4	0.38	0.4792
Rael	NM_175112.5	6.4	809.4	804.84	717	707.61	725.97	800.51	-0.06	0.4798
Pzrl1	NM_007974.4	7.77	2369.62	2215.44	2073.78	2242.19	2206.61	1880.2	-0.08	0.4898
NfkB1	NM_008689.2	8.8	1324.53	1172.83	1300.33	1182.52	1365.87	1475.95	0.08	0.5125
Casp8	NM_009812.2	13.47	580.16	612.54	700.5	476.45	590.18	688.96	-0.12	0.5382
Il23r	NM_144548.1	43.88	7.04	2.35	8.66	8.41	10.45	4.3	0.47	0.547
Tlr4	NM_021297.2	26.33	476.32	574.07	664.04	452.42	899.15	697.14	0.21	0.5536
SUBP	XM_006505028.1	27.15	487.11	451.41	276.02	376.89	426.75	230.47	-0.24	0.5603
BDNF	NM_007540.4	88.97	1.65	1.04	1	4.97	1	1.02	0.52	0.571
Reg3g	NM_011260.1	54.64	40.76	8.59	41.64	22.14	63.3	29.89	0.51	0.6009
CGRP	NM_007587.2	12.48	1.35	1.04	1	1.14	1	1.02	-0.09	0.6038
Defcrl	NM_010031.2	12.48	1.35	1.04	1	1.14	1	1.02	-0.09	0.6038
GABAA	NM_010252.4	12.48	1.35	1.04	1	1.14	1	1.02	-0.09	0.6038
Il17a	NM_010552.3	12.48	1.35	1.04	1	1.14	1	1.02	-0.09	0.6038
PGP9.5	NM_011670.2	12.48	1.35	1.04	1	1.14	1	1.02	-0.09	0.6038
TAAR1	NM_053205.1	12.48	1.35	1.04	1	1.14	1	1.02	-0.09	0.6038
SEMAPHORIN	NM_011352.2	17.37	92	89.67	119.77	96.52	134.85	96.41	0.11	0.6128
S100BETA	NM_009115.3	49.2	11.09	8.59	9.53	17.56	11.26	2.25	-0.34	0.7414
NR2D	NM_008172.2	18.51	42.1	36.65	26.89	41.59	29.15	41.14	0.09	0.7462
Il22ra2	NM_178258.5	40.16	464.19	374.49	554.67	312.81	899.97	484.28	0.17	0.7512
CCR2	NM_009915.2	30.86	175.61	206.09	306.4	152.6	308.04	181.35	-0.13	0.7612
CXCR3	NM_009910.2	26.02	42.1	52.25	42.51	40.45	63.3	28.86	-0.11	0.7684
MMp9	NM_013599.2	33	92	72	36.44	73.64	51.91	47.28	-0.14	0.7757
Pparg	NM_011146.1	25.97	25.92	16.9	18.21	24.43	12.07	21.7	-0.11	0.7901
CHAT	NM_009891.2	73.29	19.18	6.51	6.05	23.28	1	14.53	-0.39	0.8176
trpv1	NM_001001445.1	40.53	11.09	7.55	6.05	11.84	3.13	10.44	-0.13	0.8585
Cxcr4	NM_009911.3	41.77	449.35	222.72	328.97	233.85	604.82	283.69	0.1	0.8623
Mylk3	NM_175441.5	123.04	12.44	1.04	1	1.14	6.38	1.02	-0.26	0.8677
TAAR4	NM_001008499.1	69.65	7.04	2.35	5.18	3.83	1.5	11.46	-0.13	0.9037
Lyz1	NM_013590.3	45.08	7008.48	7605.24	11796.09	5960.21	17006.51	7054.53	0.06	0.9136
Tlr2	NM_011905.2	35.18	100.09	84.47	162.3	81.65	178.76	102.55	0.04	0.9289
Tip1	NM_009386.1	4.42	1506.58	1317.32	1394.09	1414.82	1381.32	1429.9	0	0.9409
C3	NM_009778.2	41.53	5196.09	2381.76	3592.89	3200.02	6301.34	2355.06	0.03	0.9555
Nod2	NM_145857.2	66.67	7.04	6.51	26.89	6.12	10.45	17.61	-0.04	0.9591
GDNF	NM_010275.2	38.74	34.01	17.94	22.55	41.59	17.76	19.65	0.03	0.9593
Ocln	NM_008756.2	17.75	2783.61	3122.92	2353.3	2423	3586.42	2406.23	0.01	0.9652
Cd86	NM_019388.3	38.99	62.33	33.54	63.35	35.87	89.32	43.19	0.02	0.9685

* Normalized transcripts counts

** Coefficient of variation percentage

Table S10

Table S10 - Fecal microbiota genera comparison between recipient mice groups

TAXONOMY: GENERA	Test-Statistic	p-Value	q (FDR)
Moryella	-3.738	0.013	0.229
Parabacteroides	2.577	0.050	0.419
Enterobacter	-2.255	0.074	0.419
Akkermansia	-1.768	0.137	0.525
Mucispirillum	1.630	0.164	0.525
Turicibacter	1.535	0.185	0.525
Lachnospira	-1.387	0.224	0.544
Oscillospira	-1.198	0.285	0.545
Ruminococcus	-1.187	0.289	0.545
Lachnospiraceae (Unclassified Genus)	-1.098	0.322	0.548
Lactobacillus	0.794	0.463	0.655
Eubacterium	-0.772	0.475	0.655
Clostridiales (Unclassified Genus)	0.725	0.501	0.655
Blautia	-0.173	0.869	1.000
Porphyromonadaceae (Unclassified Genus)	0.094	0.929	1.000
Enterococcaceae (Unclassified Genus)	-0.064	0.952	1.000

9. Supplementary methods

Macroscopic evaluation of DSS colitis in mice

Presence of pellets in proximal colon	yes	0
	no	0.5
Presence of pellets in distal colon	yes	0
	no	0.5
Pellet consistency	normal	0
	soft	0.5
	diarrhea	1
Blood in anus	yes	0.5
	no	0
Blood in cecum	yes	0.5
	no	0
Blood in colon	yes	0.5
	no	0
Bloody stool	yes	0.5
	no	0
Pale and rigid distal colon	yes	0.5
	no	0

Macroscopic evaluation of DNBS colitis in mice

Ulcer	# of ulcers and size (cm)
Diarrhea	(0-3)
Adhesions	(0-3)
Thickness	(0-3)
Hyperemia	(0-3)
Inflammation	(0-3)

Ulcer scoring:

2 points for each ulcer < than 0.5cm

3 points for each ulcer 0.5cm < x < 1cm

4 points for each ulcer => than 1cm

Histology scores

Loss of architecture	(0-3)
Cellular infiltrate	(0-3)
Goblet cell depletion	(0-1)
Ulcers	(0-1)
Edema	(0-1)
Muscle thickening	(0-2)
Crypt abscess	(0-1)

Primer's list

All primers used for Q-PCR analyses were QuantiTech Primer Assay primers (Qiagen). Mm_Csf2 (Ref: 249900), Mm_Alox15_1 (Ref: 249900), Mm_At2_1 (Ref: 249900), Mm_Rac1_1 (Ref: 249900). As reference gene we used the TATA-box binding protein (Tbp) as previously described[203].

High diet exacerbates colitis in mice by decreasing Lactobacillus levels and butyrate production

Nanostring custom genes panel

Official Symbol	Name	Accession #
5HT	Serotonin	NM_010484.2
BDNF	Brain-derived neurotrophic factor	NM_007540.4
C3	Complement component 3	NM_009778.2
CALB	Calbindin 1	NM_009788.4
CALRET	Calreticulin	NM_007591.3
Casp8	Caspase 8	NM_009812.2
CCR2	C-C chemokine receptor type 2	NM_009915.2
CCR6	C-C chemokine receptor type 6	NM_001190333.1
CD11b	Integrin subunit alpha M	NM_001082960.1
CD11C	Integrin subunit alpha X	NM_021334.2
Cd86	CD86	NM_019388.3
Cdh1	E-cadherin	NM_009864.2
CGRP	Calcitonin gene-related peptide	NM_007587.2
CHAT	Choline acetyltransferase	NM_009891.2
Cldn1	Claudin 1	NM_016674.3
Cldn2	Claudin 2	NM_016675.3
Cnlp	Cathelin-related antimicrobial peptide (CRAMP)	NM_009921.2
Cxcr3	C-X-C motif chemokine receptor 3	NM_009910.2
Cxcr4	C-X-C motif chemokine receptor 4	NM_009911.3
Defcr1	Defensin-related cryptdin peptide-1	NM_010031.2
F2rl1	Protease activated receptor 2 (PAR2)	NM_007974.4
GABA A	Gamma-aminobutyric acid A	NM_010252.4
GABAB	Gamma-aminobutyric acid B	NM_019439.3
GATA-3	GATA Binding Protein 3	NM_008091.3
GDNF	Glial Cell Derived Neurotrophic Factor	NM_010275.2
GFAP	Glial Fibrillary Acidic Protein	NM_001131020.1
Gpr44	Prostaglandin D2 Receptor 2	NM_009962.2
Hif1a	Hypoxia Inducible Factor 1 Alpha Subunit	NM_010431.2
HMGB1	High Mobility Group Box 1	NM_010439.3
I-FABP	Fatty acid-binding protein 2	NM_007980.2
Il17a	Interleukin-17A	NM_010552.3
Il22ra2	Interleukin 22 Receptor Subunit Alpha 2	NM_178258.5
Il23r	Interleukin 23 receptor	NM_144548.1
Lyz1	Lysozyme 1	NM_013590.3
Mapk1	Mitogen-Activated Protein Kinase 1	NM_011949.3
MMP9	Matrix Metalloproteinase 9	NM_013599.2
Muc2	Mucin 2	NM_023566.2
Myd88	Myeloid Differentiation Primary Response 88	NM_010851.2
Myk3	myosin light chain kinase 3	NM_175441.5
NFAT	Nuclear factor of activated T-cells	NM_010901.2
Nfkb1	Nuclear factor NF-kappa-B p105 subunit	NM_008689.2
Nod2	Nucleotide-binding oligomerization domain-containing protein 2	NM_145857.2
NOS	Nitric Oxide Synthase 1	NM_008712.2
NPY	Neuro peptide Y	NM_023456.2
NR2B	Glutamate Ionotropic Receptor NMDA Type Subunit 2B	NM_008171.3
NR2D	Glutamate Ionotropic Receptor NMDA Type Subunit 2D	NM_008172.2
Ocln	Occludin	NM_008756.2
P75	Nerve Growth Factor Receptor	NM_033217.3
PGP9.5	Ubiquitin C-Terminal Hydrolase L1	NM_011670.2
Pparg	PPAR-gamma, peroxisome proliferator activated receptor gamma	NM_011146.1
Rae1	Ribonucleic Acid Export 1	NM_175112.5
Reg3g	Regenerating Family Member 3 Gamma	NM_011260.1
S100BETA	S100 Calcium Binding Protein B	NM_009115.3
SEMAPHORIN	Semaphorin	NM_011352.2
SOX10	SRY-Box 10	NM_011437.1
SUBP	Substance P	XM_006505028.1
T-bet	T-box 21	NM_019507.1
TAAR1	Trace amine associated receptor 1	NM_053205.1
TAAR4	Trace amine associated receptor 4	NM_001008499.1
Tff3	Trefoil factor 3	NM_011575.2
TGR5	G-protein coupled bile acid receptor 1	NM_174985.1
Tjp1	Tight junction Protein 1 (ZO-1)	NM_009386.1
Tlr2	Toll-like receptor 2	NM_011905.2
Tlr4	Toll-like receptor 4	NM_021297.2
Tlr5	Toll-like receptor 5	NM_016928.2
Tollip	Toll Interacting Protein	NM_023764.3
trpv1	transient receptor potential cation channel, subfamily V, member 1	NM_001001445.1
VIP	Vasoactive Intestinal Peptide	NM_011702.2

10. Declarations

Ethics approval and consent to participate

All experiments were approved by the McMaster University animal ethics committee and conducted under the Canadian guidelines for animal research.

Consent for publication

Not applicable

Availability of data and materials

All data generated during this study are included in this manuscript [and its supplementary information files]. The raw data has been deposited under the umbrella Bioproject PRJNA394874.

Competing interests

The authors declare no competing interests.

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Authors' contributions

PMM, GDP and PB develop the study concept and design. PMM carried out all experiments, including DNA/RNA isolation, 16S rRNA gene library preparation, sequence analysis, interpretation of microbiome/immune/colitis data, SPF mice experiments, and wrote the manuscript. GDP performed all experiments in the Germ-Free mice and contributed to the interpretation of the microbiome and immune data, guided statistical analysis, as critically reviewed the manuscript. VS and JM assisted with all immune cell isolation experiments, cell-staining and flow cytometry procedures. JL assisted with all animal work, including blind colitis and histological assessment. MPLA carried out Q-PCR

experiments. EFV helped coordinating all Germ-Free mice experiments. EFV, SMC and PB were major contributors to the critical review of the manuscript. All authors read and approved the final manuscript.

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III – General Discussion

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Main findings:

- High salt diet impacts gut microbiota composition and function:
 - Lactobacillus genera is reduced by HSD and this effect is reversible
 - Metabolic pathways shift, with altered metabolism of lysine, arginine, proline and fatty acids.
 - SCFA butyrate is reduced by HSD.
- High salt diet modulates mucosal immune system in the colon and, to a lesser extent, in the small intestine:
 - In the colon, Rac1, IL1rap, Atf2, IL-7 and several Map kinase genes were upregulated whereas several chemokines and chemokine receptor genes were downregulated.
 - IL-6, LPS-stimulated MAPK and Acute phase response signaling pathways were upregulated in the colon; while leukocyte migration functions were downregulated.
 - In the small intestine, IL17A, Defa-rs1 and Ltb4r2 genes were upregulated whereas IL12a, Cxcr4 and Mef2b downregulated.
 - IL-17, TLR and p38 MAPK signaling was altered in the small intestine.
- High salt diet increases ROR γ ⁺IL17⁺ cells in MLNs but not in intestinal *lamina propria*.
- HSD exacerbated DSS and DNBS colitis in SPF mice , this effect was absent in germ-free mice.
- Transferring gut microbiota from mice on HSD into GF mice revealed that maintenance of the HSD-microbiota signature is dependent on constant exposure to HSD.

There has been extensive effort to understand the rise of chronic inflammatory disorders (CID), observed in the western world over the last 50 years, and more recently in regions of the world adopting western-like economical and social style. Because five decades, or even a century, represent a very short time period for a major human population genetic shift, it has become clear that the increase of CID must be driven by changes in external/environmental factors, resulting from altered lifestyle from societies in developed and industrialized regions. The changes observed in dietary habits of the western world have been proposed as an important driver of the CID epidemic. Moreover, gut microbiota is seen as a key mediator between environmental factors such as diet, and the host health and disease.

Diet-microbiota interaction in health and disease

Given the major impact that gut microbiota has on host health and disease, and the influence that diet exerts on microbiota composition and function, it is possible that the increased incidence of CID in the western world may be, at least partially, explained by changes in dietary factors that ultimately impact the gut microbiota. Indeed, several studies have demonstrated that the effect of dietary factors on gut microbiota can have profound consequences for the host's health and disease (Table 1).

Devlin *et al.* observed that different *Bacteroides* species differ in their ability to produce indole, the precursor to the toxin indoxyl sulfate, and this capacity was dependent on the expression of tryptophanase, an enzyme that converts tryptophan into indole. Other *Bacteroides* are adapted to use fructans instead of tryptophan as substrate. Taking advantage of this metabolic difference between the *Bacteroides* species, the authors used a Fructo-oligosaccharides (FOS) diet to boost the level of indole-non-producing *Bacteroides*, which outcompete indole-producing *Bacteroides*, with consequent decrease in toxic indoxyl sulfate. In a nutshell, the authors were able to shape

microbiota composition and metabolic outcome by favouring the genetic tools of specific bacteria using dietary intervention[153].

Furthermore, in a HSD-induced model of recurrent obesity, mice previously exposed to obesity cycles showed enhanced susceptibility to metabolic phenotype complications, and this was related to the perpetual microbiota dysbiosis from previous HSD cycles[204]. Two flavonoids, apigenin and naringenin, were shown to be persistently decreased post obese phase because of a combination of low dietary flavonoid availability during HFD and the long-standing presence of flavonoid-degrading bacteria. Transplanting healthy microbiota or treating mice with flavonoids ameliorated the rate of secondary weight regain[204]. This not only shows the long-term influence that dysbiotic microbiota can have on host physiology but also that it is possible to beneficially modulate the host physiology downstream of the microbiota by usage of post-biotics.

In our study, we showed that the effect of HSD on exacerbating colitis was lost in germ-free mice, demonstrating that gut microbiota is essential for the pathological effect of HSD. Moreover, we observed that all recipient mice in the microbiota transplant experiment had similar microbial profiles prior to the induction of DSS colitis, irrespective of the donor, and at the end of the experiment had the same histological and clinical level of colitis. In this experiment, we wanted to study whether the dysbiotic HSD-microbiota was able to transfer the HSD-colitis phenotype by itself. For that reason it was important to maintain both recipient mouse groups, control and HSD-gnotobiotic mice, on control diet. Unexpectedly, HSD microbiota profile converged to control one within 5 days thus demonstrating that the maintenance of the HSD-microbiota profile requires a constant exposure to HSD, and that the microbial changes induced by HSD are key for the determination of the colitis phenotype.

Moreover, we observed a decrease of the bacterial product butyrate in mice fed with HSD, a feature that has been linked to the development of IBD [184]. In a recent study, supplementing butyrate-producing bacteria to fecal microbiota of Crohn's disease patients enhanced the intestinal epithelial barrier integrity in an in vitro system stimulating the mucus- and lumen-associated microbiota[205], thus supporting the role that the decrease of butyrate

observed in our study may have on a barrier integrity loss model in this model of colitis.

A recent study has revealed a new potential role of high salt intake, showing that HSD promotes neurovascular and cognitive dysfunction in mice through a gut-initiated Th17 response[206]. Although the role of the microbiota was not assessed in that study, given the effect of HSD on the microbiota, the microbiota-Th17 axis and the microbiota-gut-brain axis, we speculate that microbiota must be an important element linking HSD and cognitive dysfunction. Interestingly, the effect of HSD on cognitive dysfunction was reversible after mice returned to control diet, similar to the reversible “behavior” of microbiota profiles.

Is high salt diet a risk factor for IBD?

Much like other CID, the incidence of IBD has been increasing, especially in the western regions or recently developing countries. Occurrence of IBD in new immigrants to countries with the high prevalence of the disease along with an increasing number of the disease with industrialization and westernization in area with previously low rate prevalence such as Iran, India, Lebanon, and South Korea[207], all highlight the significant effect of environmental triggers in IBD. The strongest environmental associations that have been identified in IBD are cigarette smoking and appendectomy, although neither factor alone explains the worldwide incidence of IBD[207]. Western diet, with high content of fat, sugar and salt, has been proposed to play a role in the IBD[208]. The consumption of salt has increased over the past decades, especially in the western world, reflecting the increased consumption of processed foods in a society with increasingly less time to dedicate to home cooking[18,19] (Fig. 1).

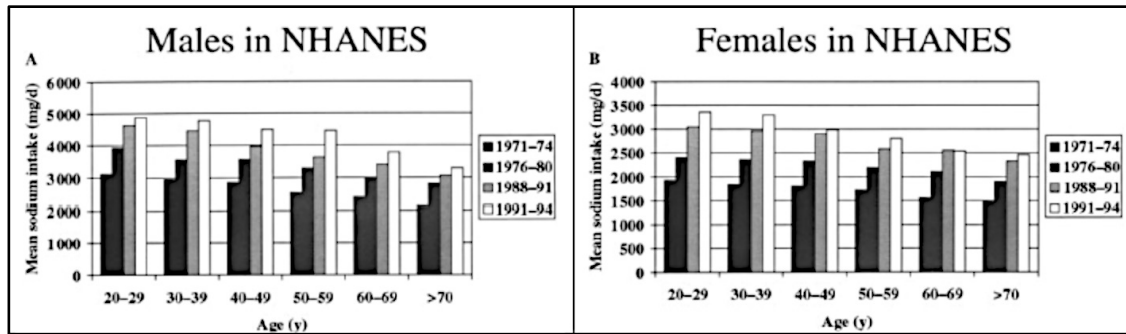


Figure 1. Self reported survey on mean sodium intake among (A) males and (B) females in the USA National Health and Nutrition Examination Surveys (NHANES) (Adapted from Loria CM *et al*, 2001[209]).

In our study, we used two models of colitis by inducing damage in the epithelial barrier which leads to a pathological immune response. We observed that the impact of HSD on gut microbiota and immune system primes the host to develop an exacerbated inflammatory response following the loss of intestinal integrity. Although the damage to the intestinal epithelium in our models was acute and to some degree artificial, the impairment of intestinal barrier function is a hallmark of IBD. Indeed, many of the genes that are associated with IBD are involved in several aspects of gut barrier function, such as hepatocyte nuclear factor 4 α (HNF4 α), E-cadherin (CDH1), meprin 1A (MEP1A), and others[210]. Moreover, colitis has been shown to be exacerbated by HSD in other murine models. Tubbs *et al*, tested the effect of HSD in a genetically susceptible model that develops spontaneous colitis, the IL-10 KO, and in an infectious model of colitis, with *Salmonella typhimurium*. HSD exacerbated intestinal inflammation and proinflammatory cytokines in the colons of IL-10 KO mice. HSD also worsened the expression of proinflammatory cytokines but not the histological features of *S. typhimurium* colitis[187].

Until today, the role of the increased intake of sodium as a risk factor for the development of IBD has not been assessed clinically. Salt consumption was associated with hypertension and cardiovascular disease since 1960[211]. There is, however, very limited data on cardiovascular comorbidity in IBD. On one retrospective cohort, IBD patients were found to have significantly higher incidence of hypertension than controls[212].

Taken all together, we hypothesize that HSD consumption is a risk factor for the development of IBD and it is a major determinant of the clinical course of the disease. This hypothesis is now being tested in a pan-canadian study led by McMaster University and supported by Canadian Institutes for Health Research, in which 8,000 individuals, including 4,000 patients with IBD, will be studied during 5 years (<http://imaginespor.com>). In this study, microbiota composition and metabolomic profiles together with detailed dietary habits will be assessed and linked to diseases course and severity. If the association found in our mouse experiments is confirmed, then a dietary intervention study will be initiated.

I hope this thesis has contributed to the increasing perception of the impact that the interaction between diet and the gut microbiota can have in the host's health and disease.

Future perspectives

Several open questions remain. Here is a list of future experiments and studies as a follow up of this thesis:

- To isolate the lactobacillus bacteria and use them to supplement mice on HSD in order to improve the colitis severity.
- To explore the role of butyrate and butyrate-producing bacteria on the HSD-exacerbated colitis model.
- Test different genetically knock-out models that have impaired intestinal barrier function.
- Using bacterial cultures to assess sensitivity of individual bacterial strains to high salt environment.
- Prospective monitoring salt consumption and disease progression in IBD patients (currently being in process).
- Retrospective or Case control study to evaluate the correlation between salt consumption and IBD prevalence/incidence.

IV – Publications

IV - Publications

The following scientific article is the result of the PhD. research work portrayed in this dissertation:

Pedro M. Miranda^{1,2}, Giada De Palma¹, Viktoria Serkis¹, Jun Lu¹, Marc P. Louis-Auguste¹, Justin McCarville¹, Elena F. Verdu¹, Stephen M. Collins¹, Premysl Bercik^{1*}, 2018. High salt diet exacerbates colitis in mice by decreasing *Lactobacillus* levels and butyrate production. *Microbiome Journal* (published)

V – References

V – References

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