Dissertation

Microbiota in immune-mediated diseases

submitted by

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Statutory declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz.

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Abbreviations

MAMPs	microbe-associated molecular patterns
Tregs	T regulatory cells
DCs	Dendritic cells
SCFAs	Short-chain fatty acids
IECs	Intestinal epithelial cells
MHC	Major histocompatibility complex
GALT	Gut-associated lymphatic tissue
MALT	Mucosa-associated lymphatic tissue
PP	Peyer´s Patches
ILFs	Isolated lymph follicles
M cell	Microfold cell
SED	Subepithelial dome
LP	Lamina propria
MLNs	Mesenteric lymph nodes
MadCAM-1	Mucosal adressin cell adhesion molecule-1
HEV	High endothelial venules
APCs	Antigen-presenting cells
IL	Interleukin
TGF-beta	Transforming growth factor-beta
Th	T helper cell
CTL	Cytotoxic T lymphocyte
CD	Cluster of differentiation
Ig	Immunoglobulin
sIg	Secretory Immunoglobulin

SFB	Segmented filamentous bacteria
PPR	Pathogen recognition receptors
PAMPs	Pathogen associated molecular patterns
TLRs	Toll-like receptors
CCR	Chemokine receptors
TNF-alpha	Tumor necrosis factor-alpha
ENS	Enteric nervous system
SMP	Submucosal plexus
GABA	Gamma-aminobutyric acid
PD	Parkinson's disease
Ach	Acetylcholine
MS	Multiple sclerosis
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
IBS	Irritable bowel syndrome
IBS-D	Diarrhea-predominant irritable bowel syndrome
FACS	Fluorescence-activated cell sorting
EDSS	Median Expanded Disability Status Scale
QIIME	Quantitative Insights Into Microbial Ecology
LDA	Linear discriminant analysis
HLA	Human leukocyte antigen
HPLC	high-performance liquid chromatography
Buk	Butyrate kinase
But	Butyryl-CoA: acetate CoA-transferase
IBS-SSS	Irritable bowel syndrome severity scoring system

Zusammenfassung

Hintergrund: Eine Vielzahl von immun-mediierten Erkrankungen wie zum Beispiel die Multiple Sklerose (MS), eine entzündliche Erkrankung des Zentralnervensystems oder das Reizdarmsyndrom vom Durchfalltyp (IBS-D), eine funktionelle Magen-Darm-Erkrankung, werden kürzlich immer mehr mit Veränderungen der Darmflora in Zusammenhang gebracht. Eine veränderte Komposition der Flora, welche in weiterer Folge auch die Produktion von Metaboliten, die Struktur der Darmbarriere und auch das mukosale Immunsystem verändert, könnte dabei eine zentrale Rolle spielen. Leider sind humane Daten noch rar. Probiotika und zu einem geringeren Anteil auch Synbiotika sind in der Behandlung des IBS-D evidenzbasiert, obwohl die Mechanismen noch nicht vollständig verstanden sind. In dieser Dissertation wird darum einerseits der Magen-Darm-Trakt (Immunzellen, kurzkettige Fettsäuren (SCFAs) und Flora) von MS Patienten im Vergleich zu gesunden Kontrollen untersucht. Andererseits wird der Effekt einer vierwöchigen oralen Synbiotika-Therapie auf verschiedene Regionen des Magen-Darm-Traktes untersucht.

Material und Methoden: Biopsien aus der Schleimhaut des Colons von fünfzehn unbehandelten MS Patienten und zehn Kontrollen werden während einer Ileokolonoskopie gesammelt. Zusätzlich werden Stuhlproben gesammelt um den Gehalt von SCFAs (mittels GC-MS) und die fäkale Darmflora zu bestimmen (mittels 16S rRNA Gen- Analyse).

Das Magen-Darm-System in zehn IBS-D Patienten wird zusätzlich vor und nach einer vierwöchigen Therapie mit einem oralen Synbiotikum untersucht. Eine endoskopische Untersuchung des oberen und unteren Magen-Darm-Traktes wird hierbei verwendet, um Biopsien für eine FACS-Analyse der Schleimhaut des Duodenums und des proximalen Colons zu bekommen. Zusätzlich wird eine 16S rRNA Gen-Analyse (aus Schleimhautproben von Magencorpus, Duodenum und proximalem Colon) durchgeführt und eine Analyse des Gehaltes von SCFAs und Zonulin (mittels ELISA-kit) im Stuhl.

Resultate: Das Darm-Ökosystem von MS Patienten zeigte deutliche Veränderungen im Bereich von Immunzellen, Metaboliten und Florakomposition im Vergleich zu gesunden Kontrollen. Die FACS-Analyse der Schleimhaut des Colons zeigte eine geringere Anzahl von Dendritischen Zellen (DC), CD103+ tolerogenen DCs und CD4+CD25+127- regulatorischen T-Zellen (Tregs) im distalen aber nicht proximalen Colon von MS Patienten.

GC-MS Messungen von Stuhlproben zeigten eine Reduktion von SCFAs (Acetat und Butyrat) bei MS Patienten. Zusätzlich konnten Veränderungen in der Komposition der fäkalen Flora vor allem im Bereich der *Clostridiales* gezeigt werden.
Bei der Untersuchung des Effekts einer oralen Synbiotika-Therapie bei Patienten mit IBS-D zeigten sich Veränderungen auf verschiedenen Ebenen. FACS-Analyse der Schleimhaut zeigte eine Reduktion von CD4+ T Zellen und einen Trend in Richtung Erhöhung von doppel-negativen T Zellen im proximalen Colon. Die Synbiotika-Therapie erhöhte ebenfalls die Biodiversität in Schleimhautproben des Magens und des Duodenums und auch das Vorkommen von *Lactobacillaceae* im Stuhl. Zusätzlich zeigten sich im Stuhl erhöhte SCFA Levels (Acetat und Butyrat) und eine reduzierte Zonulin Konzentration, welche mit einer Reduktion von Symptomen assoziiert war.

Schlussfolgerung: Die Daten dieser Dissertation zeigen Hinweise auf ein regional-regulatorisches Defizit von mukosalen DCs und Tregs im distalen Colon von MS Patienten. Diese Veränderungen sind assoziiert mit einer Reduktion von SCFAs und Veränderung der Komposition der Flora im Stuhl. Diese Daten unterstreichen eine mögliche Rolle des Darms in der Entstehung der MS. Orale Synbiotika zeigten eine Vielzahl von Einflüssen auf den Magen-Darm-Trakt auf verschiedenen Ebenen bei IBS-D Patienten. Orale Synbiotika zeigten eine Vielzahl von Einflüssen auf den Magen-Darm-Trakt auf verschiedenen Ebenen bei IBS-D Patienten und weitere Studien müssen den kausalen Charakter dieser Beobachtungen untersuchen.

Abstract

Background: A variety of immune-mediated diseases comprising multiple sclerosis (MS), a disabling autoimmune disease of the central nervous system and diarrhea-predominant irritable bowel syndrome (IBS-D), a functional gastrointestinal disorder, have been, intriguingly, recently linked to structure and function of the gastrointestinal microbiota. Thereby, an altered microbial composition of the intestinal microbiota affects metabolite production, intestinal barrier and mucosal immune function but human data remain to be scarce. In IBS-D, probiotics and to a lesser extent synbiotics are an evidence-supported treatment. Mechanisms of action are not fully understood. This dissertation therefore investigates the gastrointestinal tract (mucosal immune cells, short-chain fatty acids (SCFAs) and fecal microbiota) of patients with MS compared to heathy controls. Furthermore, the investigation of the effects of a four-week oral synbiotic treatment on gastrointestinal mucosa-associated immune cells, mucosal and fecal microbiota, fecal short-chain fatty acids and fecal zonulin in ten patients with IBS-D was performed.

Methods: Mucosal specimens were collected during ileocolonoscopy from fifteen untreated MS patients and ten controls for FACS analysis. Furthermore, stool samples were collected for measurement of fecal short-chain fatty acids (SCFAs) using GC-MS and for bacterial community profiling using 16S rRNA gene analysis.

The gastrointestinal microenvironment in ten patients with IBS-D was compared before and after a four-week synbiotic treatment. Endoscopic evaluation of the upper and lower gastrointestinal tract to collect mucosal specimens for FACS analysis (duodenum and proximal colon) and mucosal 16S rRNA gene analysis (gastric corpus, duodenum, proximal colon) was performed. Moreover, analysis of fecal SCFAs using GC-MS, fecal zonulin using ELISA and fecal 16S rRNA gene analysis was performed.

Results: The colonic microenvironment of MS patients exhibits differences at the levels of mucosal immune cells, bacterial metabolites and the composition of the intestinal microbiota compared to controls. FACS analysis of colonic mucosal specimens showed decreased numbers of total dendritic cells (DC), CD103+ tolerogenic DCs and CD4+CD25+127- regulatory T-cells (Tregs) in the distal but not proximal colon of MS patients. GC-MS measurement of fecal samples depicted reductions of fecal SCFAs (especially acetate and

butyrate) in MS patients. Additionally, fecal 16S rRNA gene analysis revealed alterations of bacterial strains predominantly belonging to the *Clostridiales* order.
The gastrointestinal microenvironment of ten IBS-D patients treated with open-label oral synbiotics for four weeks depicted changes in various sites and on different levels of the gastrointestinal tract after treatment. FACS analysis of mucosal immune cells showed a treatment-induced reduction of CD4+ T cells in the proximal colon. Synbiotics increased diversity in gastric and duodenal mucosal specimens and increased fecal abundance of *Lactobacillaceae* after treatment. Synbiotics induced fecal SCFAs (acetate and butyrate) and reduced fecal zonulin concentration accompanied by a reduction in symptom severity.

Conclusion: The data of this dissertation indicate a regional regulatory deficiency of mucosal DCs and Tregs in the distal colon of MS patients being associated to a reduction of fecal SCFAs triggered by specific microbial alterations. Even though these observational data are not implying causality, the observed findings strengthen a role of intestinal immune system in MS.
In IBS-D patients, oral synbiotics may influence the human gastrointestinal tract on different levels and sites. The observed effects substantially differ between regions and may influence distinct members of the gastrointestinal ecosystem driving improvement of symptoms in patients. While not implying causality, these findings warrant further investigation of synbiotic treatment in IBS-D.

1 Introduction

1.1 The Human Microbiota

1.1.1 General Introduction

Life on earth started approximately 3.7 billion years ago after the formation of the earth`s crust. Bacteria started to colonize oceans by synthesizing hydro-carbonated compounds, photosynthesizing oxygen and using respiratory oxygen consumption without requiring organic nutrients. Thereby, Cyanobacteria are assumed to be the first bacterial inhabitants of the planet and could be the origin of free oxygen gas in the earth´s atmosphere. This may link microbial chemistry with eukaryotic aerobic respiration. (2) Today, Cyanobacteria are vastly abundant in oceans, fresh water, soil and on rocks. They are involved in vital ecological functions such as carbon fixation, oxygen production and nitrogen cycles. Therefore, it is of utmost importance to understand that microbial communities on our planet massively impact every aspect of life on earth. (3) As the human body developed through evolutionary processes and is a multi-cellular eukaryotic organism, the awareness of the presence of microbial communities in and on it starts to shine new light on basic physiologic processes. Since scientists around the world are currently investigating the chronic microbial colonization of the human body and its role in shaping human physiology, we are about to enter a new era of understanding the complexities of human health. (4-6) Therefore, the human body cannot be seen as an isolated organism but more as an open ecological system in permanent cross-talk to its environment. (4) If one is to understand the impact of structural and functional changes on ecosystems in and on the human body, one must also include basic ecological mechanisms, phenomena and disturbances in the ecosystems surrounding humans worldwide. We are living in a time of massive biodiversity losses called the sixth mass extinction. This current extinction of earth´s biota is far above the background extinction rates seen in the other five mass extinction on earth and is largely caused by human emissions of greenhouse gases, agriculture, deforestation and overfishing. (7) Changes and dysfunctions of the human microbiota may be connected to these interruptions worldwide. The awareness of this interconnection between the environment and the human body brings human microbiota research together with public health and other disciplines. Furthermore, it strengthens bio-psycho-social endeavors in understanding basic processes and interventions to maintain and promote human health and raises the appeal towards a sustainable live of humans on earth. (8)

1.1.2 Biology of Prokaryotic Cells

As our inhabitants in and on our body are mainly bacteria, their characteristics have to be understood to comprehend their impact on human health. Bacteria are together with archaea prokaryotes, revealing no cellular nucleus, membrane-bound organelles or mitochondria. Their genetic information lies shelterless as chromosomal DNA and plasmid structures in the cytoplasm. The small size of their genome compared to eukaryotic cells forces them to interdependence between species for survival and makes them vulnerable when isolated, but strong in symbiotic populations and ecosystems. This multispecies community appearance is their natural existence and accounts for their resistance against stressors such as temperature and nutrient deprivation. In this way, their complex multispecies "body" behaves like a single organism. Mechanisms involved in resilience strategies are genetic diversity and plasticity, functional redundancy and metabolic cooperation. Furthermore, cell-to-cell signaling and thereby coordinated collective behavior may improve the ability to respond to environmental challenges. Consequently, this ability to maintain structure and function over adaptation and evolution brings a multifunctional resilient organism, that can recover from habitat alterations quickly. (3, 9, 10)

1.1.3 General Concepts of Host-Microbe Symbiosis

Vertebrates are known to live in constant symbiosis with a magnitude of prokaryotic organisms, whereas invertebrates are often in a binary host-symbiont association. A famous model of investigating this complex symbiotic relationship is the symbiosis of the Hawaiian squid *Euprymna scolopes* with the marine bacterium *Vibrio fischeri*, known to be capable of auto-luminescene. As the squid uses the luminescent activity of Vibrio fischeri to counterilluminate predators, *Vibrio fischeri* colonizes a distinct niche (crypts of the light organ of the squid) that no other microorganism is capable to colonize. (11) Thereby, communication and interaction in the crypt epithelial fields are mediated by microbe-associated molecular patterns (MAMPs) such as peptidoglycan and lipopolysaccharides. These bacterial-specific envelope components are best known for their role in systemic inflammatory processes and sepsis in humans, where they mediate pathogenesis and inflammation and are associated to critical immune responses in septic shock. (12, 13) Intriguingly, over stimulation of Toll-like receptors which mediate in turn gene transcription, these MAMPs in the context of the squid-vibrio symbiosis might be involved in basic symbiotic processes thereby facilitating morphogenesis rather than pathogenesis. (11) This concept shines new light on basic communicational processes between the host and

microbial partners living in a symbiotic relationship. As the human microbiota depicts a typical host-microbe symbiosis, different microbial surface molecules might be of relevance in mediating basic symbiotic interactions and impact also human physiology. It has been proposed that the mammalian genome does not encode for all the necessary information required for a healthy immunological development but that the interactions with their microbiotas is essential to their function and regulatory capabilities. Thereby, microbial surface molecules (e.g. polysaccharide A of *Bacteroides fragilis*) might stimulate the immune system to induce regulatory mechanism for antigen-specific tolerance being involved in different immune-mediated diseases. (4, 14, 15) T regulatory cells (Tregs) are playing an essential role in this mechanism, also being involved in the "old friends" hypothesis, describing "friendly" organisms that are being recognized by different Toll-like receptors on the surface of antigen-presenting cells called dendritic cells (DCs) (e.g. CARD 15 and toll- like receptor 2). (16) The binding to the Toll-like receptors by these antigens promotes the maturation of regulatory DCs which stimulate an anti-inflammatory response by Tregs. Thereby, old friends are able to permanently induce a background suppression of the immune system to tolerate organisms and may also mediate tolerance to self- and foreign antigens. This process might be involved in the basic symbiotic mechanism in humans driving specific immune regulation. (17) In sum, symbiosis is an ancient mechanisms rooting in basic evolutionary processes including human evolution. The relationship between the host and its microbial symbiont is beneficial for both and includes molecules former being related only to pathogenesis. As the human gastrointestinal tract harbors a vast amount of microbial antigens possibly interacting with mucosal cells, the microbiota may be involved in regulating basic immunological processes. (11)

1.1.4 Structure and Analysis of the Human Microbiota

The term human microbiota is used to describe the microbial community in and on the human body, whereas microbiome is used to describe the collective genome of the flora. Interestingly, the microbiome outweighs the human genome by far and is highly involved in the production of vital nutrients such as vitamin k and b12. (18) In general, the human body is host for around 10^{14} microorganisms (bacteria, viruses, fungi and parasites) and its evolutionary biology is evidently linked to the development and presence of cooperative strategies with these inhabitants. The gastrointestinal tract is by far the most populated area with up to 1000 bacteria species residing in a maximum number of 100 trillion (distal gut). (18-20) This complex ecosystem is mainly build by two different types of microorganisms. Autochthonous organisms reside and colonize a distinct ecological niche, whereas allochthonous organisms are passing through the gut and have more transitory effects. This reflects an important property of the human microbiota due to the possible impact of bacterial species from the environment which may impact structure and function in humans. The environment can thereby play an important role in shaping microbial patterns. (11, 21-23) In general, bacteria (prokaryotes) represent the most prominent domain in the human microbiota. Prokaryotic cells need biodiversity for growth and since up to 80 % of bacteria in fecal samples cannot be grown in cultural media, the establishment of molecular-biological techniques made it possible for the first time to properly investigate complex microbial communities. These techniques use differences in nucleotides of microbial genes for identification and could show that initial analysis of microbial genes in human fecal samples revealed previously undescribed microorganisms. (24) This reflected the limitations of the techniques used before to describe microbial communities with microscopic examination and culturing. For example, a very powerful possibility to analyze microbial communities is metagenomics, meaning the analysis of all genetic material recovered from environmental samples. All these new approaches bypass the need of culturing and isolating microbial community members and are therefore of utmost importance in the understanding of micro-ecology. (24) Metagenomic technology is using DNA from a certain community to gather information regarding community profiles (which organisms are living there, e.g. bacteria, archaea, viruses, eukaryotes) and genetic/ metabolic capacities to investigate the activity of the community. Combined with complex tools in bioinformatics, meta-transcriptomics and metabolomics are opening new possibilities to investigate structure and function in microbial communities. (25)

Using these new techniques, the human fecal microbiota could be analyzed. 16S rRNA gene analysis showed that two predominant bacterial inhabitants of the gut mainly belong to the phyla *Bacteroidetes* and *Firmicutes*. The high inter-individual variability of the human microbiota with the most abundant genera *Bacteroides*, *Faecalibacterium* and *Bifidobacterium* shows potent difficulties in interpretation and comparison between individuals. Intriguingly, the analysis of fecal samples revealed that individuals form three distinct robust clusters, so-called enterotypes. These enterotypes consist of variations in one of the three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3). These enterotypes may represent distinct ecological patterns, where host-microbe interaction is operative and symbiotic patterns are in balance. (3, 26, 27) Other scientists believe that this distinction of enterotypes is too strict and that the model of enterogradients is more useful. (28) Although over 90 % of all known phylotypes belong to the two mentioned main phyla *Bacteroidetes* and *Firmicutes,* other phyla have been found regularly in human distal gut samples too. *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* were the most important. Furthermore, other species of the domain *archaea* were found (e.g. *Methanobrevibacter smithii*) in the distal gut. (19) If comparing the human intestinal microbiota to other ecosystems on earth such as soil or ocean waters, diversity is much lower with an estimated reduction of 20 phyla. Interestingly, over 60 % of the phylotypes originally detected were unknown species and most of them have never been cultivated, showing the enormous advances in using new molecular-biological techniques. (18, 19)

The structural anatomy of the human intestinal microbiota shows different phyla as mentioned above. These phyla incorporate certain bacterial strains, which may have distinct functions in the human intestinal ecosystem by producing metabolites (e.g. short-chain fatty acids, SCFAs such as acetate, butyrate, propionate) and thereby influencing their environment. As the *Firmicutes* represent the most numerous and diverse phylum in the human gut, it harbors very important strains known to be of vital importance to human physiology. (3) For example, the *Clostridium* cluster XIVa contains some important butyrate-producing strains such as *Roseburia,* which have been shown to be of importance in stimulating mucosal Tregs. (29, 30) The degradation of starch and inulin to butyrate could thereby be an important mechanism and links diet to microbial production of metabolites. (31) Furthermore, *Faecalibacter prausnitzii*, belonging to the Clostridium cluster IV, is known to be a fermenter of starch and inulin to butyrate and reduction in human samples

have been associated to the pathogenesis of inflammatory diseases in humans. (32) *Bacteroides*, belonging to the *Bacteroidetes* phylum, are known to be capable of degradation of starch and structural polysaccharides and are involved in mucin-related metabolic procedures. (3) Intriguingly, polysaccharides from *Bacteroides fragilis* are involved in anti-inflammatory processes by stimulating regulatory DCs and Tregs in the lymphatic system and might be therefore of systemic immunological relevance. (15) From the *Actinobacteria* phylum, *Bifidobacteria* are the best known and represent mostly starch, inulin and mucin degraders. As lactate is their main fermentation product and is mostly converted into butyrate by secondary fermenters, *Bifidobacteria* have strong effects on butyrate production in humans and might therefore play a role in inflammatory diseases. (33, 34) The *Proteobacteria* phylum comprises classes such as *Gamma-Proteobacteria*, where *Enterobacteriacea* such as *Escherichia*, *Klebsiella*, *Proteus* and *Enterobacter* occur in low abundances in the human gut microbiota. *Akkermansia muciniphila*, a mucin-degrading strain belonging to the *Verrucomicrobia* phylum, is currently under immense investigation due to its possible influence on human health. Not only has increased abundance been linked to remission in Crohn´s disease patients and improved glucose tolerance in humans but also with an altered microbiota signature in eczema. (35-37) Further studies will show the potential benefits of *Akkermansia* in human health. Taken together, the human intestinal flora is a highly complex ecosystem, where different phyla of bacteria and their classes and species play important roles in human physiology and thereby health and disease. But if one is to understand the effect of the composition of the intestinal microbiota on the host, one must consider the complexity of the involved ecology. The inter-individual difference and variance of the microbiota is high and studies show that over 70% of the identified phylogenetic types are unique to each person. (38) Thereby, a personal distinct pattern of microbial diversity and composition is present, depending on the host genotype and initial microbial acquisition at birth via vertical transmission. (39) Intriguingly, the microbiota seems to be stable over time in individuals, showing less difference intra-individually than inter-individually. (38) Furthermore, different habitats of the body from oral cavity to stool form different community structures, which have very little variability over time. (40) The colonic ecosystem exhibits regional differences, where *Enterobacteriacea*, *Bifidobacteria* and *Lactobacilli* are more abundant in the proximal colon, whereas *Bacteroidales* and *Clostridia* are more prominent in the rectum. (41) Certain influences have been detected to impact the microbiota over time. Three main life-long legacy effects on the microbiota linking bio-psycho-social factors to microbiology have been detected. Gender, breast

feeding history and most intriguingly the educational level of the individuals could be used to distinguish microbial clusters. This may represent first hints on how certain socioeconomic factors and gender impact dynamics of microbial communities and thereby influence certain types of immune-mediated diseases. (42) Generally, diet, cold exposure, travel, antibiotics and acute diarrhea can change the composition of the microbiota, but normally are transitory. (43) The recovery time of a healthy ecosystem has been proposed to be a marker of stability, where compromised ecosystems need longer recovery time. (44) Since the human distal gut microbiota is less impacted by modifications and hosts specific species, autochthonous microbes may represent the majority of colonizing microbes. If so, allochthonous organism would be superseded. In contrast, allochthonous microbes have strong influences on microbial composition in the upper gastrointestinal tract reflecting different anatomical and functional abilities of gastrointestinal regions. (45)

Looking at the abundances of bacteria along the human gastrointestinal tract, variations at different sites are common. The oral cavity and the colon host the highest number of microorganisms. In contrast, the stomach and duodenum have very low populations of microbes due to the fast transit time mediated by propulsive motor activity, excretions such as bile, acid and pancreatic secretion which impair microbial growth and survival. (3) Nevertheless, it should not be forgotten that the proximal gut contains a vast amount of mucosal lymphoid structures and the small intestine can be of high relevance in immune responses. (46) The bacterial count steadily increases towards the distal gut and reaches highest levels in the colon, where the colonic microbial community depicts the highest cell densities recorded in any ecosystem (**Figure 1**). (3, 47)

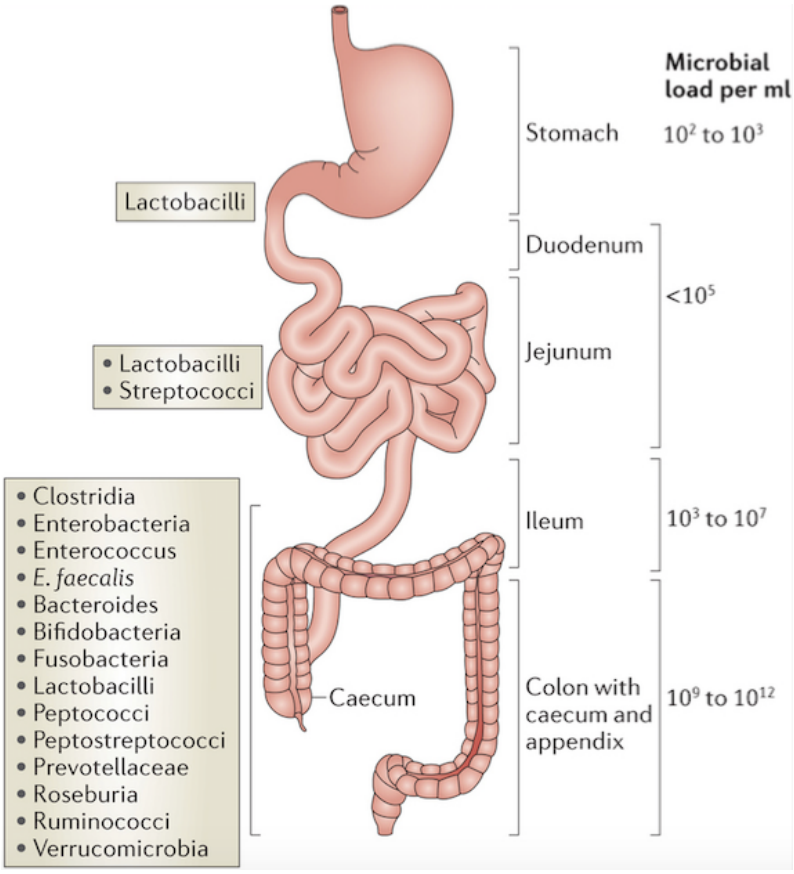

Figure 1 | Abundance of commensal microbes along the gastrointestinal tract. The stomach and duodenum are less populated by microbes due to fast transit time mediated by propulsive motor activity, excretions such as bile, acid and pancreatic secretion. (3) The microbial abundance steadily increases towards the distal gut and reaches highest levels in the colon. (3, 47) Microbial diversity is the highest in the caecum and the lowest in the distal colon. *Reprinted and adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology](48), copyright (2014)*

1.1.5 Metabolites of the Human Gastrointestinal Microbiota

The human microbiota produces a vast amount of different membrane components and metabolites reaching from MAMPs and SCFAs to hormone-like metabolites, representing the metabolome of the human microbiota. The role of these metabolic transmitters is currently under firm investigation but involvement in a variety of human diseases including immune-mediated diseases such as multiple sclerosis and rheumatoid arthritis is suspected. The unique enzymatic capability of degrading complex polysaccharides derived from the diet is an outstanding property of the human microbiota. Thereby, gut commensals process nutrients from food to ensure survival but concomitantly generate side-products that maintain gut homeostasis and downregulate systemic inflammatory responses. In general, these metabolites can be distinguished by their biological function. SCFAs, medium-chain fatty acids (MCFAs), bile acids, choline and L-carnitine may thereby play the most known role and may be of relevance regarding immune-mediated and other diseases. (49)

1.1.5.1 SCFAs

SCFAs are volatile fatty acids produced by the colonic microbiota as a result of the saccharolytic fermentation of non-digestible carbohydrates escaping the small intestine. They are characterized by having fewer than six carbons in straight-or branched-chain structure, from acetic acid (C2), propionic acid (C3) to butyric acid (C4). The conjugate bases, called acetate, propionate and butyrate thereby represent the most abundant SCFAs in the human colon covering up to 95 % of the total SCFA content. Small amounts of acetate and propionate are also produced by fermentation of amino acids but this contributes for just 5 % of the total content. Furthermore, lactate, although not belonging to the SCFAs, is also a byproduct of fermentation and is mainly produced by lactic acid bacteria and *Bifidobacteria*. As lactate can be further processed to acetate, propionate and butyrate, it represents a source for SCFA production. (50) Acetate is the most abundant SCFA in the human colon and accounts for around 50 % of the colonic SCFA content. The greatest amount comes out of carbohydrate fermentation by colonic bacteria. (50) In contrast, as the production of acetate is widely distributed among microbial species in the human colon, the synthesis of butyrate and propionate is reserved for specific strains. *Akkermansia muciniphila* is known to produce propionate, whereas *Faecalibacterium prausnitzii* and *Roseburia* are typical butyrate-producers. (51)

The propionate production by colonic bacteria is accomplished by different pathways termed succinate-, acrylate- and propanodiol- pathway. Different families and phyla are involved in certain pathways, including *Bacteroidetes*, *Lachnospiraceae* and subclasses of *Firmicutes*. (50) As the abundance of *Bacteroidetes* is associated to fecal propionate levels in humans, factors influencing microbial patterns and microbiota structure (e.g. diet, food additives, antibiotics) might also contribute to fecal propionate deficiency seen in immune-mediated diseases. (52)

Butyrate is produced, as mentioned above, by specific strains that comprise the genetic structure of synthesizing butyrate. At least two different pathways are involved, the butyrate kinase (buk) and butyryl-CoA: acetate CoA-transferase (but) pathway. The buk pathway is less common in the human microbiota, only species such as *Coprococcus* use it. The but pathway is in contrast used by most butyrate producers in the human microbiota such as *Faecalibacterium*, *Eubacterium* and *Roseburia*. (50)

The overall production of SCFAs is tightly linked to dietary patterns, where the consumption of high-fiber is associated to a favorable profile of increased SCFA levels compared to an animal-based diet. In general, a high-fiber plant-based diet produces higher amounts of SCFAs compared to a westernized diet rich in animal fats. (53, 54) SCFAs levels are positively associated to a variety of beneficial effects regarding inflammation and neoplastic growth. (55) This might reflect the power of dietary interventions in mediating health improving effects in general. (56) SCFAs are known to play a vital role in maintaining intestinal homeostasis, barrier function and immune regulation. (50) Moreover, activation and regulation of anti-inflammatory cells such as mucosal Tregs and DCs is depending on the presence and extent of SCFAs. (29, 57) It is therefore of the utmost interest in current scientific investigations if the amount of SCFAs produced by the microbiota is linked to the etiopathology of immune-mediated diseases such as multiple sclerosis. (58)

1.1.6 Acquisition and Shaping of the Human Microbiota

1.1.6.1 Birth

The human gastrointestinal tract at birth is sterile and starts to get colonized in delivery during exposure to vaginal microbes. (45) The vagina is host to an abundant ecosystem and represents a vertical transmission pathway in delivery, where at this time point, the birth canal mostly harbors *Lactobacillus* and *Prevotella* species. (59) The fetal immune system is instructed by maternal immune cells in utero that cross the placenta to favor the development of Tregs allowing the inoculation by maternal microbial communities. (60) Eventually, the skin and oral microbiota depict the mother´s vaginal microbial signature and is also present in the meconium in vaginally delivered infants. (61) However, only a few species will be able to sustainably colonize the gastrointestinal tract and will thereby contribute to a distinct microbiota in later adolescence. (40) In contrast, cesarean section-delivered infants harbor the mother´s skin microbiota dominated by *Staphylococcus*, *Corynebacterium* and *Propionibacterium spp*. As these communities are not closer to the skin of the mother than to skin of other woman, influences of other caring people in the first days of life are suspected. Furthermore, infants have in contrast to their mothers an undifferentiated microbiota across their whole body, harboring the species of their delivery mode. (61) This reflects the strong impact of delivery on the shaping of the human microbiota. Increasing incidences of immune-mediated diseases might be associated to the increased use of cesarean sections as delivery modus, whereas probiotic therapy reduces allergic diseases cesarean-born infants. (62-64) Intriguingly, the composition of fecal bacteria is influenced by the duration of hospitalization in neonates, where colonization from the hospital environment is evident. (65)

1.1.6.2 Host Genetics

The birth´s impact on the microbiota is followed by a time of a chaotic and extraordinary transformation process involving a replacement of the acquired strains (vaginal and skin) by strains of less known origin. In general, the diversity increases steadily and rapidly, facultative anaerobes are starting first, followed by strict anaerobes. (3) After 12 months, fecal microbial communities become more stable and depict the classic structure in adults, represented mainly by *Firmicutes* and *Bacteroidetes*. A very important factor that influences the composition in early years might be the genetic background of the host, as monozygotic twins show higher similarity of fecal microbiota composition than unrelated individuals. (66) However, the microbiota of monozygotic twins differs not from the microbiota of dizygotic twins, although family members in general were similar to each other, reflecting the importance of environmental influences on microbial shaping. Moreover, family members show differences in bacterial lineages but overall genes stay similar, suggesting a core microbiome. Changes in diversity, phylum level, gene representation and metabolic pathways are thereby associated with obesity. (67)

1.1.6.3 Breastfeeding

Human milk is a complex mixture of nutrients such as oligosaccharides and glycans, microorganisms, growth factors, cytokines, lysozymes, immunoglobulins and digestion enzymes. Its composition is influenced by gestational age at parturition, body mass index, weight gain during pregnancy, lactation period and diet. (68, 69) Breastfeeding itself has been linked to a variety of health benefits for the infant including protection against allergy, obesity and celiac disease. (70) These effects may be partially mediated through the early colonization of the infant´s gastrointestinal tract by *Bifidobacteria*, as breastfed infants depict a microbiota signature richer in *Bifidobacteria* compared to formula-fed infants. Thereby, *Bifidobacteria* accounted for more than seventy percent of the fecal bacteria in breastfed infants, including species such as *B.longum, B.breve, B.bifidum*. (69, 71) Gene analysis of different *Bifidobacteria* strains suggests that these bacteria have co-evolved with the human lactation process. The ability of processing complex sugar structures (e.g. human milk oligosaccharides, HOM), indigestible to the infant, might therefore reflect the evolutionary adaption of these strains in maintaining symbiotic patterns. (72) Especially, *B. infantis* is able to outcompete other Bifidobacteria in co-culture digesting milk glycans. This mechanism might allow *B. infantis* to overgrow other colonic bacteria in vivo and be partly responsible for the beneficial effects attributed to breastfeeding in general. (71, 73) The source of colonizing *Bifidobacteria* inhabiting the infant gastrointestinal tract is unknown. Colonies living in the human milk as an endogenous source of lactic acid bacteria have been proposed, as milk harbors a small but viable colony of bacteria (10^3 colony forming units/milliliter), to impact and seed the infant intestinal microbiota. (74) In contrast, strains acquired during birth could also be of importance since vaginally delivered neonates had high levels of Bifidobacteria compared to cesarean delivered infants. (75) The mixture of human milk contains prebiotic nutrients as well such as oligosaccharides, glyopeptides and glycolipids which are capable of stimulation growth of certain bacteria, especially *Bifidobacteria* and also being able to hamper growth of pathogenic bacteria. (76) Furthermore, high concentrations of lysozyme, lactoferrin and secretory immunoglobulin A (acquired from the mother´s IgA pool mainly against digestive tract pathogens) contribute to the known antibacterial and immune-modulating properties of human milk. In sum, human milk represents the perfect nutrition for infants in their first months of life, providing all the ingredients for adequate development, growth and immune function and might play a key role in shaping a healthy balanced microbiota. (69)

1.1.6.4 Diet

Diet shapes the human microbiota in various ways and is thereby an important modulator connecting humans to their environment and circumstances of living. Since detailed investigations shed light on different mechanism involved, different patterns of modulation have been observed. (77)

First, massive diet modifications are able to impact and change the composition of the human microbiota rapidly. (53) Consumption of an animal-based diet (rich in meats, cheeses and eggs) changes the composition of the fecal microbiota in one to two days depicting an increased abundance of *Bacteroides, Bilophila and Alistipes,* all bile-tolerant microorganisms. In particular, *Bilophila wadsworthia*, a sulphite-reducing pathobiont, increased after animal-based diet consumption, especially long-term diary intake, in fecal samples of healthy subjects. (53) Interestingly, *Bilophila wadsworthia* induced intestinal inflammation after intake of dietary milk-derived fats in a mouse model of inflammatory bowel disease. By promoting changes in host bile acid composition and altering the gut microbiota, a western-type diet high in saturated milk-derived fats promoted gut inflammation in genetic susceptible mice. This observation might link the increasing incidence of immune-mediated diseases such as inflammatory bowel disease in genetically susceptible hosts in westernized countries to western dietary habits. (78) In this context it is worth mentioning that the intake of L-carnitine over red meat consumption produces a microbiota-dependent metabolite (trimethylamine-N-oxide, TMAO) that is an independent cardiovascular risk factor (hazard ratio 2.1) in humans, linking the microbiota to the established association between red meat consumption and cardiovascular risk. (79) Subjects consuming the animal-based diet depicted lower levels of the SCFAs acetate and butyrate but higher levels in iso-valerate and iso-butyrate, whereas changing to a plant-based diet (rich in legumes, grains, fruits and vegetables) mirrored the effects with higher levels of acetate and butyrate and lower levels of iso-valerate and iso-butyrate. (53) All in all, large dietary changes can rapidly modify the human microbiota structure and metabolic activity in days, reflecting the dynamic properties of our microbiota and possible consequences for human health. (77)

Second, long-term dietary habits are of great importance in shaping the intestinal microbiota and enterotypes are associated to these dietary habits, despite the described rapid changes and responses to short-term diet. Thereby, the *Bacteroides* enterotype is associated to an

animal-fat and protein diet, whereas the *Prevotella* enterotype is linked to carbohydrate consumption. The *Ruminococcus* enterotype could not be linked to dietary patterns. Most importantly, rapid changes in diet do not affect the overall enterotype and might therefore reflect the impact of long-term imprinting on the human gut microbiota. (80) Interestingly, children in the European Union show a reduction of fecal Bacteroidetes and increased Firmicutes associated to a reduction in SCFAs in fecal samples compared to rural African children. The *Prevotella* enterotype in African children is thereby associated to a high-fiber diet in contrast to the *Bacteroides* enterotype of European children, linked to a typical western diet rich in sugar, animal protein and fats. Further studies have to evaluate if the *Bacteroides* enterotype is involved in certain diseases increasing in westernized countries and if long-term modulation of dietary habits can impact the course of diseases. (77, 81)

Third, changes in diet can have variable influences on individuals, since the amount and responses of overall taxonomic changes differs in individual subjects. Intriguingly, obese men show massive increases in the abundance of *Ruminococcus bromii* after a resistant-starch diet, but these increases are depending on the initial composition of the gut microbiota. (82) Thereby, individuals lacking *Ruminococcus bromii* strains had no increases after diet intervention. (83) These personalized morphologies of the human gut microbiota will have to be taken into account in future studies. (77)

The above mentioned dietary influences might also help to explain why specific metabolic inputs are able to shape intestinal microbial communities and metabolite production. Thereby, structural and metabolic adaption to environmental challenges (e.g. changes in diet) might display evolutionary patterns of shaping microbial adaption to environmental offers. Supplementation of L-carnitine in mice harboring microbiotas with a low activity of producing trimethylamine changed intestinal microbial abilities to achieve high production of trimethylamine. (79) In humans, the consumption of seaweed is associated to the occurrence of genes coding for carbohydrate-active enzymes such as porphyranases and agarases in the microbiome of Japanese individuals. These enzymes are able to digest the polysaccharide porphyrin from marine red algae *Porphyra* and are not found in North American individuals. This transfer of important digestive enzymes from marine bacteria living on the algae to the human microbiome might indicate how non-sterile foods impact the human microbiota. (84)

1.1.6.5 Prebiotics

Gibson et al. 2004 defined prebiotics as *"a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health."* (85) Thereby, three specific criteria have been proposed to describe prebiotics. The ingredient proposed: *"(1) resists gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (2) is fermented by the intestinal microflora; (3) stimulates selectively the growth and/or activity of intestinal bacteria associated with health and wellbeing."* (85) Prebiotics are therefore functional foods taking an intermediate stage between drugs and foods and are mainly represented by oligosaccharide carbohydrates such as fructooligosaccharides, galactooligosaccharides, mannooligosaccharides and xylooligosaccharides. (86) Typical prebiotics are inulin, oligofructose, transgalacto-oligosaccharides, lactulose and lactosucrose. (85) There are multiple proposed mechanisms of how prebiotics may exert beneficial effects. First, prebiotics change the composition of the intestinal microbiota including selective growth stimulation of potentially beneficial strains such as *Bifidobacteria* and *Lactobacilli*, inhibiting adhesion and invasion of pathogenic bacteria. Second, prebiotics are able to impact the production of metabolites produced by the intestinal microbiota. SCFAs, a diversity of antimicrobial compounds and vitamins can beneficially impact host health, immunity and metabolism. Third, prebiotic treatment affects intestinal barrier directly by stimulating mucus production and indirectly by promoting the production of SCFAs such as butyrate. Fourth, prebiotics mediate a variety of immune modulatory effects including the reduction of pro-inflammatory cytokines, stimulation of anti-inflammatory cytokines and immunoglobulin A production. Fifth, prebiotics improve nutrient absorption by increasing villi height and crypt depth. (86) Taken together, prebiotics impact the human microbiota on different sites and levels mediating possible beneficial effects on host health. Further investigation of specific mechanisms of action may impact the therapeutic range and potentials in a variety of human diseases including immune-mediated diseases.

1.1.6.6 Probiotics

Probiotics are defined as "*live organism, that when administered in adequate amounts, confer a health benefit to the host."* (87) First reports about potential beneficial effects were made by Nobel laureate Ilya Metchnikoff suggesting that longevity is associated to the consumption of probiotic bacteria. The term "probiotic" was first used by Lilly and Stillwell around four decades ago. (11) Most of the probiotic strains used today are common members of the human intestinal microbiota and are belonging to the genera of *Lactobacillus* and *Bifidobacterium.* Additionally*,* selected lactic acid bacteria genera such as *Streptococcus* and *Enterococcus*, some genera from *Bacillus* and *Propionibacterium*, some gram-negative strains (e.g. *E. coli Nissle*) and yeast (*Saccharomyces*) are therapeutically used in probiotic mixtures. (88) Beneficial effects of oral probiotic treatment on human health have been described and mechanisms of action show three different levels. First, probiotics influence directly or indirectly the intestinal microbiota inter alia by reducing intestinal luminal pH, production of antimicrobials, superseding of pathogenic bacteria and induction of host antimicrobials (e.g. defensins). The modulation of the composition of the intestinal microbiota is not limited to the given oral probiotic strains but may also increase the overall microbial diversity and composition reflecting a species independent effect. Strain-specific alterations of microbial patterns remain elusive. Second, probiotics influence the intestinal mucosa by interacting with mucus layer and the intestinal epithelium and thereby influence intestinal barrier function and integrity. Probiotics are thereby also interacting with the mucosa-associated immune system and are involved in mediating local anti-inflammatory signals and cells. (88) Although the health-beneficial effect of probiotics are mostly thought to be due to the immune modulatory effects on mucosa-associated immune cells, human data remain contradictory with inconclusive results. The lack of coherent and comparable interventional studies contributes to these results, as well as the lack of knowledge of strain-specific modulatory properties. A magnitude of in vitro and animal studies show that probiotic effector molecules are directly involved in immune regulation and may even have a prominent role in the regulation of the human immune system. (89) In healthy humans, oral administration of *Lactobacillus spp.* revealed host-specific mucosal reactions and comparable transcriptional responses in duodenal mucosa specimens, underlining strain-specific immuno-modulatory capacities coherent to mechanisms seen in preliminary animal models. (90, 91) Different mucosal immune cells are inducible by probiotics reaching from tolerogenic DCs to Tregs. The induction of these anti-inflammatory cells that play important

roles in a diversity of human immune-mediated diseases and may be of systemic relevance and therapeutic potential. (92) Third, probiotics are interacting with mucosa-associated immune-, epithelial and nerve cells influencing other organ systems including the host immune system, liver or central nervous system. (88, 93) The above mentioned mechanisms might explain the growing evidence for probiotic treatment in multiple intestinal but intriguingly also extra-intestinal human diseases including inflammatory bowel disease, irritable bowel disease, antibiotic-associated diarrhea, obesity, allergic diseases and colon cancer. (88) Taken together, probiotics have a variety of influences on the human microbiota and systemic physiology. Further studies have to elucidate which strains can be used in specific diseases and if administration of probiotics to healthy individuals has beneficial disease-preventing properties.

1.1.6.7 Antibiotics

Antibiotics have, by their nature of action, a very powerful effect on the composition and function of the human microbiota. The bacterial abundance is greatly affected including diversity and evenness after administration of broad-spectrum antibiotics. Treatment-stop is associated to a certain resilience of the microbiota, although the state before the treatment is usually not attained. These impacts of antibiotics can therefore be of long-term nature and may persist even for years. Antibiotic treatment in children or the mother before delivery affects negatively the abundance of *Bifidobacteria* and increases *Proteobacteria*. Furthermore, antibiotic application is strongly linked to alterations in the function of the overall ecosystem influencing gene expression and metabolism that can mimic different disease-associated patterns as for example antibiotic-related changes in enzymatic activities for carbohydrate degradation are similar to changes observed in obese subjects. It appears that antibiotic treatment might therefore greatly influence structure and metabolic function of the human microbiota (including intestinal and other biotas such as skin and vaginal). Antibiotic-induced changes could thereby be linked to different diseases such as obesity, type 2 diabetes, allergic disease and also autoimmune diseases such as type 1 diabetes. In type 1 diabetes, the use of different antibiotics was associated to development of disease in humans and mouse models show that antibiotic alterations of the microbiota can induce the disease. This reflects how altering the microbiota can impact host metabolism, immune regulation and inflammatory responses. Mechanisms of action could thereby also be changes in the repertoire of MAMPs affecting toll-like receptor stimulation and consequently the differentiation and maturation of mucosal immune cells (see chapter 1.2 for further explanations of involved immune processes). (94) Moreover, since SCFAs play essential roles in regulating host immunity and metabolism, antibiotic-induced dysbiosis affecting SCFA production, thereby influencing type and amount of SCFAs, is likely to be involved in mediating alterations in immune responses and metabolic activities. (95) A major problem of antibiotic use in humans is the possibility of generating antibiotic-resistant strains that are not reacting on any kind of antimicrobial treatment. Thereby, the human gut microbiota has been marked as a major source of antibiotic-resistant bacteria with highest abundance of resistant strains for antibiotics being long-term used in the market and approved for animal use (e.g. tetracycline). The higher abundances of resistant bacteria in fecal samples from Southern Europe compared to samples from Northern Europe correlated with total outpatient antibiotic use, reflecting that exposure to antibiotics increases the possibility of stimulating

antibiotic-resistant strain growth. Taken together, the discussed effects of antibiotic use on the human microbiota can negatively affect health and even promote different diseases, antibiotic-resistant bacteria and infections. This warrants extreme caution in prescribing antibiotics in general without evidence-based indications in humans and most importantly questions the general use of antibiotics in animal farming. (94)

1.1.6.8 Food Additives

The European Food and Safety Authority (EFSA) defines food additives as "substances added intentionally to foodstuffs to perform certain technological functions, for example to color, to sweeten or to help preserve foods." (96) Thereby, E numbers are used to identify specific food additives and food products are mandatorily labeled with an ingredient list depicting the function of the additive and the specific substance used. Common food additives used in the European Union are antioxidants, colors, gelling agents, emulsifiers, stabilizers, thickeners, preservatives and sweeteners. In the United States, the Food and Drug Administration lists these substances as "generally recognized as safe" (GRAS). (96, 97) The effects of these additives on health have been focused on acute toxicity and cancer development using animal models but specific health impacts of long-term consumption on microbiota structure and function is lacking. Recent studies suggest that some food additives might have distinct influences on the intestinal microbiota structure and function. Oral administration of the emulsifiers carboxymethylcellulose or polysorbate-80 via drinking water in mice induced a reduction of microbial diversity, protective mucus layers and stimulate low-grade inflammation, colitis and obesity. Moreover, emulsifier treatment induced a massive reduction in SCFAs (butyrate). (98) As the administration of carboxymethylcellulose and polysorbate-80 promoted neoplastic growth by altering the microbiota via the induction of low-grade gut inflammation, inflammatory responses might be involved in tumor growth too. (99) Thus, dietary emulsifiers trigger pro-inflammatory responses and tumor growth due to microbial changes in the microbiota accompanied by metabolic changes and might therefore be involved in the raising of inflammatory diseases, obesity and even colonic cancer in the last century. (98) Non-caloric artificial sweeteners are also commonly used in typical westernized diets and were introduced over a century ago to sweeten foods without elevating energy content. These substances (e.g. saccharin, sucralose and aspartame) are considered safe and beneficial due to assumed health benefits such as weight and blood sugar reduction. Recent investigations show that the oral administration of saccharin in mice induces glucose intolerance through induction of compositional and functional changes in the intestinal microbiota. (99) Taken together, food additives are necessary for modern food processing and conservation. However, some additives such as carboxymethylcellulose, polysorbate-80 or saccharin are able to influence microbiota composition, structure and function and may thereby be involved in multiple human diseases reaching from immune-mediated diseases to obesity and cancer. Thus, precise and rigorous

evaluation of food additives should be performed considering effects on the human microbiota. In general, until proven otherwise, the use and consumption of these substances should be strictly limited considering the possible negative impacts on human health.

1.2 The Mucosal Immune System

The gastrointestinal tract harbors a vast amount of immune cells situated in the local mucosa ready for interaction in the complex interplay between host and microbiota. About 80 % of antibodies produced in the human body arise from the gastrointestinal tract. As the massive gastrointestinal surface is in permanent contact with bypassing antigens comprising pathogens, food antigens and the commensal microbiota, its mucosa harbors specialized structures to cope and process this huge diversity of immunological signals. (3) Thereby, discrimination of reactions to pathogens as opposed to tolerance to food antigens and commensal microorganism is crucial for gut homeostasis since misguided reactions can result in local tissue damage, food allergies, inflammatory bowel disease or even systemic inflammatory responses. (14, 15, 100) Studies in germ-free animals show the importance of the host-microbiota interactions for the development of the immune system. Germ-free mice are prone to get opportunistic infections, are immune-deficient and lack the ability of establishing tolerance for oral antigens. (3) These important immunological imprints happen on the outer layer of the mucosa where intestinal epithelial cells (IECs) are in close relationship with luminal contents. These cells are immunologically active and harbor major histocompatibility complex II (MHC-II) on their basolateral surface but are lacking co-stimulatory molecules. (11) Sensing of luminal content is crucially mediated by membrane-bound receptors (toll-like receptors) and IECs are able to produce immunologically active substances such as prostaglandins and leukotrienes to actively recruit immune cells and foster immune responses. Furthermore, DCs are influenced by the presence of IECs and produce anti- but not pro-inflammatory cytokines indicating that IECs might be involved in tolerogenic responses. (11) As contact to epithelial surface is a major step for invading pathogens, limited contact possibilities are mediated through production of mucus and secretion of antimicrobial substances (e.g. defensins and cathelicidins). A major function of IECs is also the communication of apically received luminal signals (e.g. from MAMPs) to underlying mucosal immune cells. They can thereby transmit luminal signals by secreting pro-or anti-inflammatory cytokines and can directly affect immune cells in the lamina propria by for example inducing lymphocyte apoptosis. They represent thereby a major component of the mucosal innate response and are involved in orchestrating acquired immunity. (3, 11)

1.2.1 Gut-Associated Lymphatic Tissue

A vast amount of immune cells is located in the intestine sitting in gut-associated lymphatic tissue (GALT), mucosa-associated lymphatic tissue (MALT) or lamina propria. Furthermore, single cells are also found on the surface of the epithelium. (3) The GALT is an subepithelial organized lymphoid aggregation of mucosal or submucosal lymph follicles along the gut comprising Peyer`s patches (PP), the appendix and isolated lymph follicles (ILFs). Covered by apical follicle associated epithelium missing the typical mucosal structure (e.g. glycocalix and mucus), GALT contain so-called microfold cells (M cells). These are specialized structures for antigen transport into the underlying DC-rich subepithelial dome (SED) consisting of T cell and B cell areas surrounding a germinal center (**Figure 2**). M cells represent also a major entrance for pathogenic microorganism in the gastrointestinal tract. (48) The general structural organization of the mucosal immune system requires different stages of regulating immune responses, inductive sites (e.g. Peyer`s patches, mesenteric lymph nodes) and effector sites (e.g. lamina propria, LP) (**Figure 2**). Anatomically, Peyer´s patches are found in more densely populated areas of the gastrointestinal tract such as distal ileum after birth, although other lymphoid aggregates have been described to appear in the whole gastrointestinal tract. (11) They are formed during embryotic life but germinal centers are induced just after birth. Thereby, the total number of PP is increasing from 50 in the late trimester to 100 at birth and 250 in young adults depicting the importance of environmental signals crucial for development. (3) Their size and density increases along with microbial abundance in the small intestine from jejunum to ileum. As Peyer´s patches feature typical structural properties of GALT they comprise M cells and SED with hundreds of non-encapsulated B cell follicles surrounded by T cell areas also containing a germinal center. Germinal centers provide a continuous immune stimulation important for humoral activation such as immunoglobulin A making Peyer´s patches the major source for small intestine-homing immunoglobulin A plasma cells. Similar structures containing M cells are also found in the large intestine around the ileocaecal valve (caecal patches, crucial for immunoglobulin A production) and continuously along the colon and rectum (colonic patches). Furthermore, ILFs, smaller lymphoid structures of the GALT are also distributed consistently in the human gastrointestinal tract with increasing density from the small to the large intestine reaching a total number of 30.000 in the human intestine. (11) In contrast to PP, ILFs are arising after birth. (3) Mesenteric lymph nodes (MLNs) depict specialized structures independent of

growth factors important for other GALT structures (e.g. PP, M cells). They are larger than other lymph nodes in the human body and are important centers for lymphocytes which enter them via surface receptors such as $\alpha 4\beta 7$ integrin binding to mucosal adressin cell adhesion molecule-1 (MadCAM-1) on high endothelial venules (HEV). As L-selectin is known to guide lymphocytes to peripheral tissues and most lymphocytes express both L-selectin and MadCAM-1, GALT-driven immune response might influence the whole body. (11)

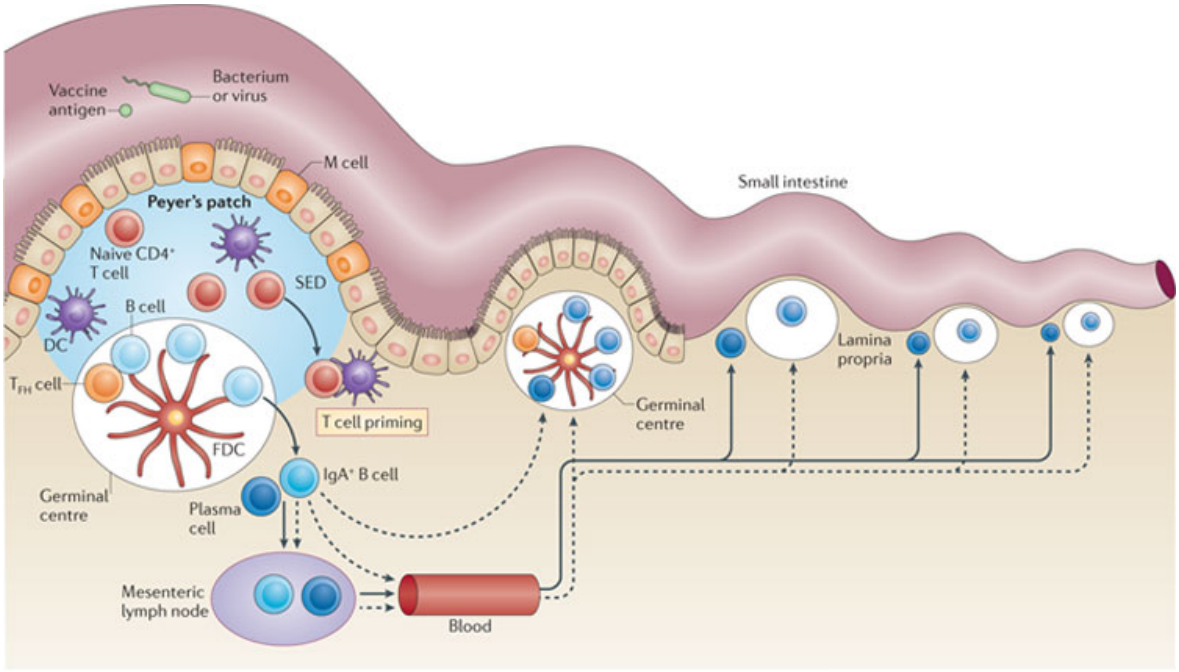

Figure 2 | Gut-associated lymphatic tissue (GALT) structure and immune cells. Luminal antigens (e.g. microbe-associated molecular patterns (MAMPs) of commensals, food antigens, vaccines) are processed by microfold cells (M cells) and interact with immune cells of the subepithelial dome (SED) such as dendritic cells or naïve CD4+ T cells. B cells leave after stimulation in the germinal center via efferent lymph vessel into mesenteric lymph nodes and enter the bloodstream to re-enter lamina propria effector sites in the small and large intestine. (101) As the lymphatic system communicates throughout the whole body, intestinal luminal information transmitted to immune cells in the mesenteric lymph nodes is likely to reach other organs such as the central nervous system. (102, 103) *Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] (101), copyright (2012)*

1.2.2 Components of the Mucosal Immune System

1.2.2.1 Dendritic Cells

Mucosal immune cells are able to shape immune responses in various ways and are thereby able to promote either mucosal tolerance or inflammation. Antigen-presenting cells (APCs) play a very important role since they are processing antigens and decide which immune reaction and pathway has to be taken to react adequately to the stimulus (antigen) given. DCs are professional APCs found abundantly in different subsets distributed along the human gastrointestinal tract and are massively involved in establishing intestinal immune tolerance. (11) The antigen-sampling role via expression of Toll-like receptors allows DCs to determine different types of immune reactions in response to a variety of luminal intestinal antigens (**Figure 2 and 3**). Lamina propria DCs thereby interact with luminal antigens by recognizing, processing and internalizing them. The antigen-loaded DCs are then translocating to the MLNs and PP stimulating residing naïve T cells and mediating homing advices to specific tissues. Of particular importance is the impact of mucosa-residing DCs on gut homeostasis since DCs in their steady-state produce anti-inflammatory cytokines such as interleukin-10 (IL-10) therewith inducing a profound Treg response. Moreover, by remaining hyporesponsive to toll-like receptor ligation, DCs are able to control inflammation and exacerbated immune responses. Distinct well-defined subsets of DCs are known to exist in the murine intestine, whereas the DCs of human intestine are still not fully understood and investigated. (104) Different groups around the world use different classification panels to analyze the DCs in the human intestine. In this dissertation we used a negative lineage cocktail comprising CD3, CD14, CD16, CD19, CD20, CD56, CD34 as previously described. (105) Furthermore, using HLA-DR, lamina propria DCs were identified as lineage negative and HLADR+ cells. As different subsets are of interest regarding immune regulatory properties, we used CD103 and CD11c markers for DC subset staining. (105) CD103+ DCs are known to have anti-inflammatory, tolerogenic properties being able to induce T regulatory cells and thereby promoting intestinal homeostasis via the production of IL-10, transforming growth factor-beta (TGF-beta) and retinoic acid. (106) CD103+ DCs in type 1 diabetes are not fully able to promote Treg induction compared to healthy individuals and their transfer has been linked to improvement of intestinal graft versus host disease. (107, 108) As DCs are shaped by the intestinal microenvironment such as microbial metabolites and cytokines, they may represent a cell line possibly influenced in a therapeutic manner. (57, 109)

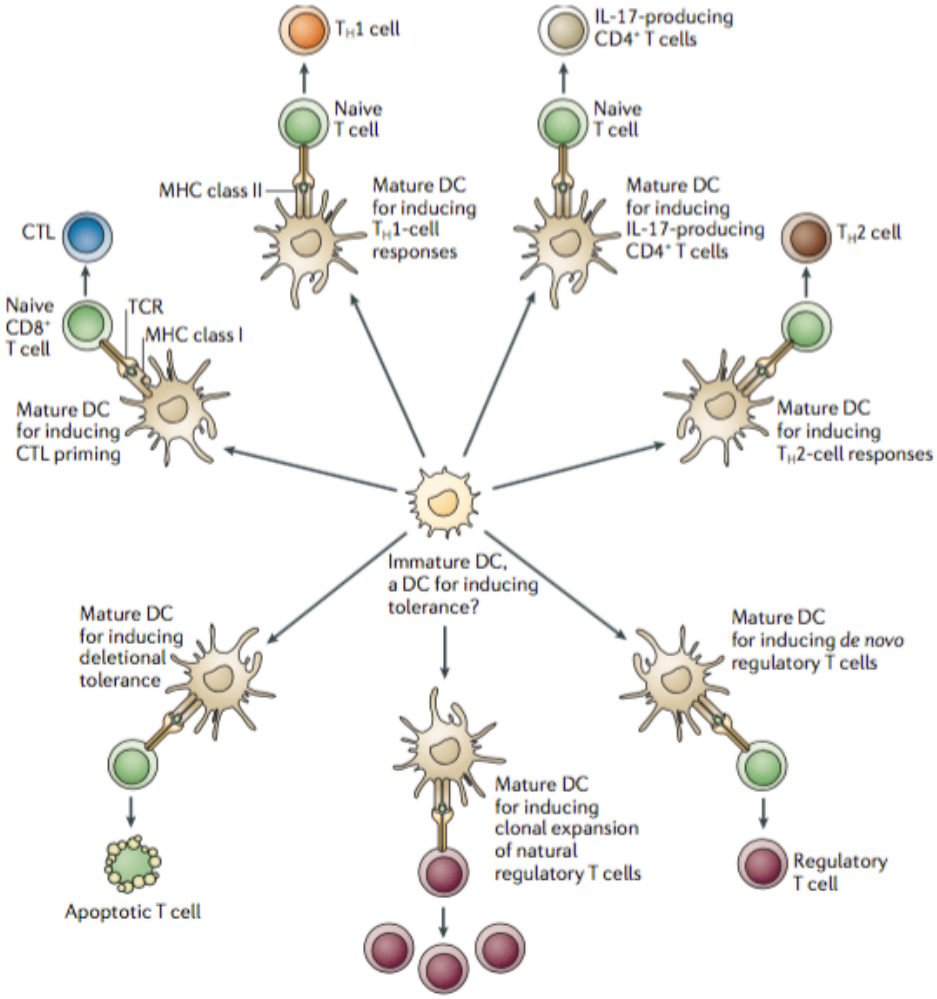

Figure 3 | The role of dendritic cells in shaping different immune responses. Immature dendritic cells (DCs) are able to mature into different types of effector DCs to stimulate T cell fates reaching from the induction of T regulatory cells and pro-inflammatory Th-17 cells to inducing cytotoxic T lymphocytes (CTL). Mature DCs are evolving thereby out immature DCs in response to exogenous (e.g. microbe-associated molecular patterns, MAMPs) or endogenous (e.g. cytokines) environmental signals. This reflects the importance of the quality and origin of the triggering signal in mediating host immune responses. (110) *Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] (110), copyright (2006)*

1.2.2.2 T Cells

Mucosal DCs interact with a number of different T cell types. CD4+ and CD8+ T cells are found in the lamina propria of the human gastrointestinal tract in a two to one ratio and are thought to arise of out secondary lymphatic organs. (48) CD4+ T helper cells (Th) are the biggest pool of interacting T cells which can differentiate, depending on the given stimulus, to certain types of effector T cells including Th1, Th2 or Th17 cells. (111) Mucosal T cells can vary between gastrointestinal regions in the human intestine with enriched Treg numbers in the appendiceal orifice region and the proximal colon and CD8+ T cells in the gastric mucosa. (112) Thereby, higher proportions of Th17 cells have been observed in the lamina propria of the colon and the ileum compared with the jejunum. The caecum may depict higher numbers of Th17 cells and Tregs compared with the terminal ileum and more distal regions of the large intestine. Th1 and Th2 cells were not described to vary in the human intestine. (48) In the GALT, Tregs depict by far the most important T cell line promoting tolerogenic immune reactions. In contrast, Th17 cells are able to drive inflammatory responses and tissue damage and are thereby involved in different autoimmune diseases such as inflammatory bowel disease and multiple sclerosis. (11, 111) Tregs are a specialized T cell line able to induce immunosuppression in T cells and other immune cells. The Treg pool comprises natural Tregs arising in the thymus and peripheral Tregs induced by certain stimuli (e.g. on mucosal surfaces by for example microbial metabolites such as short-chain fatty acids). (57) Thereby, expression of the transcription factor foxp3 is essential for Treg function and Tregs are defined as CD4+CD25+foxp3+ T cells. (111) In this dissertation, T regulatory cells are defined as CD4+CD25+ CD127 low cells, as the usage of foxp3 as a marker needs intracellular staining and makes assays with living cells impossible. The surface marker CD127 has thereby been shown to be effective in marking Tregs. (113, 114) The expression of CD25 (interleukin 2 receptor) is a common feature of Tregs and depicts one mechanism of Treg-induced immunosuppression, as this receptor can mediate a metabolic disruption including the high-affinity CD25-dependent cytokine- deprivation-mediated apoptosis. Using this mechanism, Tregs are thought to consume local IL-2 levels by high expression CD25 and therefore contribute to actively starve effector T cells. Other mechanisms of Treg-induced immunosuppression consist of inhibitory cytokine release (IL-10 and TGF-beta), suppression by cytolysis and modulation of function and maturation of DCs. (115) Tregs depletion and malfunction is associated to multiple human immune-mediated diseases including allergic diseases such as asthma, inflammatory bowel disease

and also multiple sclerosis. (14, 15, 116) The peripheral Treg pool appears to be modifiable by different bacterial metabolites such SCFAs and certain MAMPs. Probiotic mixtures, oral gavage of polysaccharide A and oral gavage of SCFAs such as butyrate induced mucosal and systemic Tregs and thereby ameliorated disease-models of multiple sclerosis, inflammatory bowel disease and food allergy. As the intestine harbors a vast amount of naïve T cells that can be differentiated into Tregs by adequate stimuli, the induction of Tregs is of the utmost interest regarding therapeutic interventions in multiple human immune-mediated diseases. (14, 15, 29, 57, 100)

1.2.2.3 B cells and Antibodies

Intestinal B cells are important producers of antibodies such as immunoglobulin (Ig) A and M. They are widely distributed mostly in the proximal and most distal part of the gastrointestinal tract and over 90 % of B cells in the colon produce IgA, whereas only 75 % of duodenal B cells. The rest of the antibody production consists mainly of immunoglobulin M. (48) Three grams of secretory immunoglobulin A (sIgA) are produced every day in humans on mucosal surfaces by plasma-B cells. sIgA comprises two IgA molecules bound by a J chain and has anti-inflammatory binding-capabilities of innocuous antigens and complement-activating abilities. In contrast, serum IgA is mainly of inflammatory value. In the gastrointestinal tract, dimers are the most common formation and represent a major mechanism to protect mucosal surfaces from invading pathogenic bacteria. Furthermore, sIgA is involved in mucosal homeostasis being spontaneously induced by the presence of commensals. Human IgA deficiency is linked to immune-mediated diseases such as inflammatory bowel disease and most intriguingly, also to elevated levels of segmented filamentous bacteria (SFBs). (11) SFBs are involved in triggering inflammatory diseases such rheumatoid arthritis and might influence GALT immune reactions. (117)

1.2.2.4 Toll-like Receptors

As mentioned above, APCs, especially DCs have the ability to discriminate between different microbial patterns and antigens. This property is mainly conducted by pathogen recognition receptors (PPR) that are able to differentiate between MAMPs and pathogen associated molecular patterns (PAMPs). Thereby, toll-like receptors (TLRs) are the most famous of PRR located on the surface of DCs which are able to mediate contacts to microbes initiating either anti- or inflammatory responses. There are different TLRs involved in

sensing antigens as for example TLR 4 binds to lipopolysaccharides and TLR 5 to flagellin. The human gastrointestinal tract harbors a vast amount of TLRs and pathogenic bacteria as well as commensals are able to bind and interact with them. Certain mechanisms are involved in preventing inflammatory immune responses to commensal bacteria, from sequestration of commensals to hyporesponsiveness of DCs. (11)

1.2.3 Gut Immune Responses and Regulatory Patterns

Homeostasis in the gut has to be tightly regulated by a complex interplay between host and microbiota. The structure and function of the mucosal immune system, as mentioned above, is thereby adjusted to maintain tolerogenic immune patterns and intestinal homeostasis. In getting contact to the antigen, immune cells can trigger typical pathways to either tolerogenic or inflammatory responses effecting local mucosal sites but also systemically the whole body and other organs. The antigen uptake can be facilitated in different routes. First, DCs in the PP can get stimulated by antigens processed by M cells. Loaded DCs then either present to local PP-T cells or migrate via draining lymph to the MLNs. Second, direct stimulation of underlying DCs or MHC-II-expressing enterocytes through the epithelium in the LP which then migrate to MLNs. Third, DCs are able to extend their dendrites through the epithelium of the LP into the gut lumen (tight junction protein-mediated) to start sampling antigens (**Figure 4, a**). All routes then mediate immune signaling to the MLNs where further immune responses are initiated. DCs are major APCs in the GALT and are responsible for the communication between inductive (PP, MLNs) and effector sites (LP) in the human gastrointestinal tract. This is mediated by surface receptor that are able to facilitate recruitment and guidance of DCs to different immune sites. Chemokine receptors (CCR) such as CCR 2 (mediates DCs migration to the LP) and CCR7 (mediates migration to MLNs) are important surface markers that promote the migration and translocation of DCs. The steady-state migration of commensally exposed DCs to MLNs is crucial for tolerogenic responses (mice lacking CCR7 are not able to promote oral tolerance) by inducing Tregs and inhibiting inflammatory T cell responses (**Figure 4**, **b**). (11)

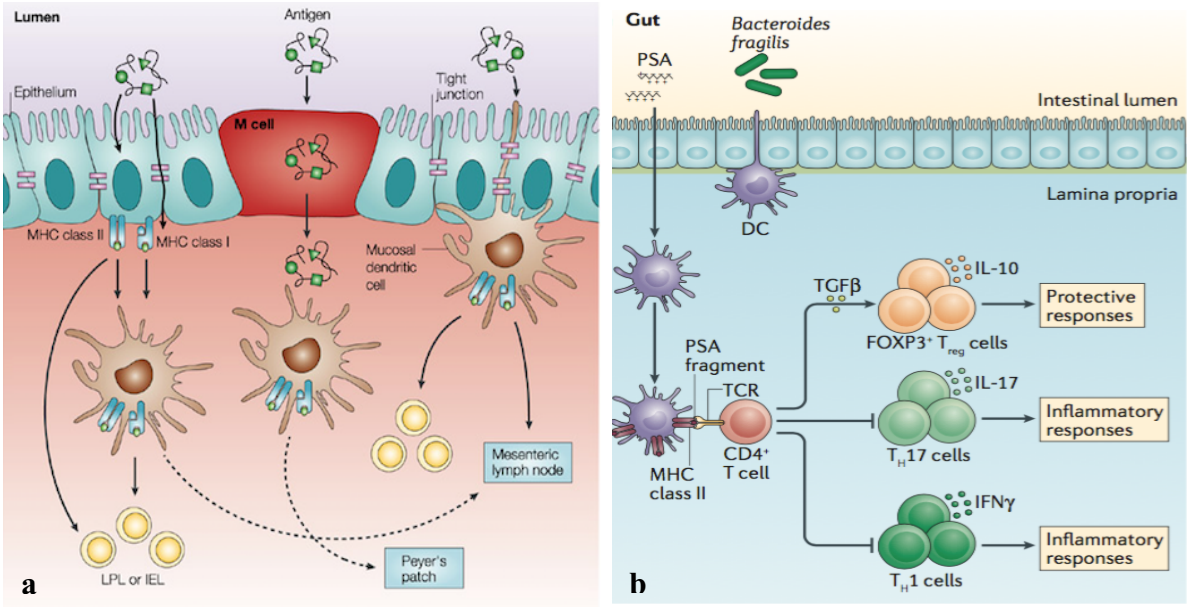

Figure 4 | Antigen processing in the intestinal immune system.

a | Dendritic cells (DCs) are able to process and get contact to luminal antigens by different routes: direct stimulation via the epithelium and MHC-II expressing enterocytes, M cell-mediated processing and presentation to underlying DCs and direct luminal antigen sensing by DCs facilitated by tight junction proteins. DCs are then able to stimulate lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) or directly migrate to Peyer`s patches or mesenteric lymph nodes to further interact with other immune cells (e.g. T cells). (118) **b |** Commensal antigens (e.g. polysaccharide A, PSA of *Bacteroides fragilis*) are processed by DCs (by routes described in Figure 4 A) and are presented to naïve CD4+ T cells. Different cytokines such as transforming growth factor-beta (TGFβ) or interleukin-10 (IL-10) induce the expansion of anti-inflammatory foxp3+ regulatory T (Treg) cells consequently suppressing the activity of inflammatory Th1 and Th17 cells. (119) *Reprinted and adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology] (118) (119), copyright (2004 and 2016)*

The major signals involved in the induction of Tregs are different cytokine such as TGF-beta or IL-10. Furthermore, this anti-inflammatory environment stimulates B cell-immunoglobulin-switching from IgM to IgA. The effector lymphocytes primed by DCs are then entering the MLNs for further differentiation and are then migrating to the bloodstream via the thoracic duct to migrate to their effector sites (e.g. LP). CRR9 expression and loss of L-selectin promotes lymphocyte-homing to their effector sites such as LP and is responsible for the vast amount of different immune cells in the LP such as hyporesponsive Tregs, primed T cells, DCs and B cell blasts (which turn into mature IgA-producing B cells in the LP). (11) Different subtypes of T cells can be differentiated out of a naïve CD4+ T cell pool instructed by loaded DCs. Th1 cells are promoting cell-mediated immunity and protection against intracellular pathogens (also complex pathogens such as *Mycobacterium*

tuberculosis) in releasing Th1-typical cytokines such as interferon-gamma and tumor necrosis factor-alpha (TNF-alpha). In contrast, Th2 cells are primarily working against extracellular pathogens and helminths excreting cytokines such as IL-4, 5 and 13 and thereby regulating other immune cells such as eosinophils, basophils and mast cells. Th2 cells are thereby also involved in allergic diseases such as asthma and atopic dermatitis. Th17 cells, secreting primarily the cytokines IL-17 and TNF-alpha, are involved in early-phase inflammatory responses and recruitment of neutrophils. (17) Th17 cells are known to play an important role in autoimmune reactions and tissue destruction of autoimmunity. (120) Controlling the differentiation of naïve CD4+T cells is therefore a powerful mechanism in regulating immune responses in the gut. (17)

1.2.3.1 Hygiene Hypothesis and "Old Friends"

The term "Hygiene Hypothesis" was first used by David Strachan in 1989 as he observed that having older siblings reduces the risk of allergic diseases. In general, the term is used to describe the possible effects of modern life and sanitation on the development of immune-mediated diseases. Epidemiological data point to a steady increase in the incidence of immune-mediated disease in westernized countries in the last decades of the 20th century. Asthma prevalence became very high in Europe, type 1 diabetes incidence raised in Britain from a prevalence of 0.04/1000 in the nineteenth century to 1.3/1000 children by 1970 and multiple sclerosis incidence in Germany doubled in over 15 years. These epidemiological patterns observed can only be explained by environmental factors leading to these rapid changes observed over decades. (17) Together with epidemiological data suggesting protective clues such as orofaecal transmission, keeping a dog, economic deprivation, living on a farm and exposure to helminth infection, the hygiene hypothesis could be investigated on an immunological level trying to understand possible backgrounds. (121) Thereby, the lack of beneficial organisms in daily life coming from soil, rotting vegetation, untreated water, helminth infection and accompanying changes in the composition of the microbiota associated to modern life might be responsible for the raising of immune-mediated diseases since the 20th century. These microorganisms which can be beneficial but can also act as pathogens were termed "old friends" comprising saprophytic *Mycobacteria*, *Bifidobacteria*, *Lactobacilli* and helminthes. Old friends are recognized by immature mucosal DCs as commensals and thereby innocuous. This recognition through activation of PRRs (e.g. CARD 15 and TLR-2) promotes the maturation of regulatory DCs which produce transforming growth factor-beta or IL-10 to stimulate an anti-inflammatory response of Tregs. (16) As molecular techniques developed to further investigate the human microbiota, a "microflora hypothesis " was developed stating that next to the known effects of missing old friends on immune regulation, further effects of general changes in the overall composition of the human microbiota, especially intestinal microbiota were evident. Thereby, changes in the intestinal microbiota were evident in patients with allergic diseases compared to healthy individuals. Intriguingly, these changes observed prior to the onset of atopy as well, linking compositional alterations of the human microbiota causally to allergic diseases. (11) Disease-specific dysbiosis (alterations in composition of the microbiota) is under current investigation in many immune-mediated diseases and researchers are seeking for microbial "fingerprints" that can explain disease-specific immunological patterns.

Recently, a distinct pattern of dysbiosis has been shown in patients with multiple sclerosis depicting a reduction of butyrate-producing bacteria belonging to the clostridial cluster XIVa. Further studies will elucidate if an “unhealthy” microbiota is causally involved in immune-mediated diseases and if reestablishing a healthy microbiota has any value in prevention and treatment of these diseases. (122)

1.3 Enteric Nervous System

The enteric nervous system (ENS) as is complex and interconnected system that involves as many nerves cells as the spinal cord (100 million neurons). (123) This system connects these neurons overseeing digestive functions even when disconnected from the central nervous system, although the ENS is not autonomous as reflexes are involving through local, sympathetic ganglia and the central nervous system. The role of the ENS is currently under intensive investigation and historical views that limit its functions to just digestive regulation (e.g. control of gastric acid secretion, movement of fluids and local blood flow) is overthrown by a growing evidence of enrollment in immune regulation, endocrine functions and even intestinal barrier of the gut. (124) The microanatomy of the ENS depicts different ganglia that are interconnected by fiber tracts forming continuous ganglionated plexuses along the length of the gastrointestinal tract (**Figure 5**).

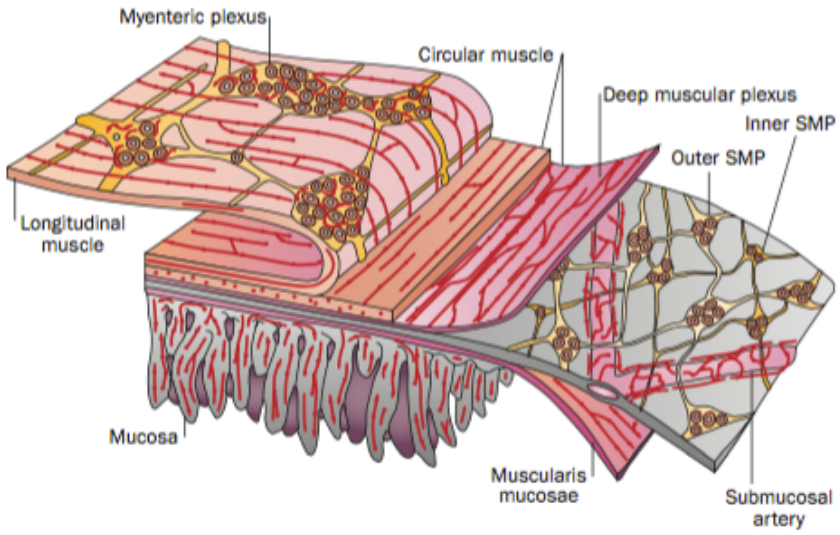

Figure 5 | Anatomy of the enteric nervous system (ENS) in humans.

The ENS comprises two ganglionated plexuses. First, the myenteric plexus (Auerbach´s plexus), which is situated between the longitudinal and circular stratum of the external musculature of most of the digestive tract. Second, the submucosal plexus (SMP), which is situated in the submucosal region between the circular muscle and mucosa comprising outer (Schabadasch`s plexus) or inner (Meissner´s plexus) elements. Furthermore, specified nerve fiber clusters are interrelating ganglia thereby forming plexuses that innervate other muscles (longitudinal muscle, circular muscle and muscularis mucosae) and other structures such as intrinsic arteries and the mucosa. (123, 124) *Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Gastroenterology and Hepatology] (124), copyright (2012)*

Thereby, two ganglionated plexuses are well known, the myenteric plexus (Auerbach´s plexus) and submucosal plexus (Meissner´s plexus). Plexus Auerbach is situated between the longitudinal and circular muscles stratums along most of the gastrointestinal tract. Motor neurons innervating circular and longitudinal muscles are situated in the myenteric plexus. In contrast, the submucosal plexus is situated between the circular muscle stratum and the mucosa and is most prominent in the small and large intestine. It forms outer (Schabadasch`s plexus) or inner (Meissner´s plexus) elements. Thereby, unlike autonomous ganglia, enteric ganglia have special abilities such as integration and procession of information normally attributed to nuclei in the central nervous system. (124)

1.3.1 Vagus Nerve and the Gut-Brain Axis

The vagus nerve is the main parasympathetic nerve of the autonomous nervous system and is known to be involved in metabolic homeostasis controlling various digestive functions and heart rate. (125) Ninety percent of vagal fibers are afferent bringing information from visceral organs to the central nervous system. (120) The intestinal endings of these afferents (termed intraganglionic laminar endings in myenteric ganglia and intramuscular arrays in smooth muscle) are in contact with the lamina propria and the mucosal epithelium, sensing luminal contents and translating information to the brain. (126) Manipulation of the intestinal microbiota via oral application of probiotics can influence expression of certain GABA receptors in mice brains and alter emotional behavior. These effects were not evident in vagotomized mice indicating that the vagus nerve represents a bidirectional pathway of translation and communication between the microbiota and the brain. This strongly contributes to the general phenomenon of gut-brain communication, termed the Gut-Brain Axis. (127, 128) This Gut-Brain Axis constitutes a communication between the microbiota and the brain mediated by neuronal (e.g. vagus), hormonal (e.g. noradrenaline), immunological (e.g. different cytokines) and metabolic signals (e.g. butyrate) affecting brain development, function and stress response. (129) Enteroendocrine cells are thereby also able to produce serotonin in great amounts. (126) The vagus nerve might therefore be involved in a variety of neurological diseases such as multiple sclerosis and severe neurodegeneration. (130) In this process the neurodegenerative misfolded protein alpha-synuclein, contained by neuronal inclusions termed Lewy Bodies linked to sporadic Parkinson’s disease (PD) and other synucleinopathies such as multiple system atrophy, could enter the central nervous system via enteric plexuses and preganglionic vagal afferents. (131) Human data show that

the intestinal barrier of patients with PD is compromised and that intestinal mucosa specimens (sigmoid) of patients contain more alpha-synuclein than healthy controls. Furthermore, mucosal staining for *E. coli* was elevated in patients accompanied with elevated tissue oxidative stress. As the formation of alpha-synuclein might depend on oxidative stress responses, the observed changes in the gut of patients with PD might represent the etiopathological origin of disease. (132) Mouse models support this theory by showing that microbial alterations in the gut can trigger motor deficits, microglia activation, and alpha-synuclein formation. Most intriguingly, colonization of the intestinal microbiota of mice by stool of PD-affected patients stimulates physical impairment, suggesting that alterations in the human microbiota represent a risk factor for PD and showing the enormous impact of the Gut-Brain axis on the development of neurological diseases. (133)

Next to the communicative role of the vagus, various studies show anti-inflammatory properties of the vagus nerve impacting the regulation of inflammation. Thereby, vagus activity mediated by acetylcholine release inhibits pro-inflammatory cytokine production (e.g. TNF-alpha, IL-1, IL-6 and IL-8) in macrophages via binding to an acetylcholine receptor (termed cholinergic anti-inflammatory pathway) (134, 135) Intestinal immune cells might be under vagal influence as well, as T cells, DCs and B cells residing in the mucosa/submucosa carry cholinergic receptors. (136) In fact, evidence that immune cells of the gut wall are under direct influence of the vagus is raising. As the human intestine is massively innervated by the enteric nervous system draining through the intestinal wall with its different layers (**Figure 5**), mucosal immune cells are controlled by the release of neurotransmitters (e.g. Ach). Thereby, the myenteric plexus offers a very special close relationship between enteric neurons and tissue-resident macrophages. Acetylcholine could locally influence macrophages stimulating anti-inflammatory responses and Treg induction. (137)

These findings are supported by clinical observations, where for example patients with inflammatory bowel disease depict low vagal activity correlating with high inflammatory cytokine levels showing a possible clinical relevance of vagal activity in intestinal inflammation. (138) Taken together, the vagus nerve is representing a direct bidirectional communicative structure in the human body between the microbiota and the brain. It is thereby involved in healthy brain development and function but also in stress responses and behavior that can influence the structure and function of the microbiota. (129) The vagus nerve might therefore be involved in a variety of human diseases and further studies need to elucidate possible therapeutic manipulations.

1.4. Multiple Sclerosis

Multiple sclerosis (MS) is a complex autoimmune disease of the central nervous system (CNS, brain and spinal cord) that is believed to arise in genetically susceptible individuals together with environmental factors such as viral infections, vitamin d deficiency or smoking that promote or may even trigger autoimmunity. (139-141) It constitutes a major source of disability in young adults and is therefore considered a major personal and socioeconomic burden. As the average age of onset is 30 years and 50 % of patients are bond to a wheelchair after 25 years, the burden of disease is considerable. MS presents clinically in various ways reaching from sensory and visual disturbances, motor impairments, fatigue, pain to cognitive deficits, correlating with the dissemination of lesions within the CNS. As the immune-mediated infiltrative lesions are the hallmark of multiple sclerosis, they represent the major pathological feature in MS patients featuring immune cells that penetrate across the blood–brain barrier. The influx of immune cells is thereby promoting inflammation and demyelination followed by gliosis and neuro-axonal degeneration. Consequently, the disruption of neuronal transmission results in clinical symptoms. Lesional immune cells infiltrating from the periphery consist mainly of CNS-directed autoreactive T cells of so far unknown origin. Of interest, relapses in the common relapsing-remitting multiple sclerosis can be stopped by anti-inflammatory treatment, whereas neuro-axonal degeneration is not affected, reflecting two different possible mechanisms driving relapses and disease progression. This theory is supported by the seldom form of primary progressive multiple sclerosis, where anti-inflammatory treatment has no effect on the course of disease. Since immune-modulatory treatments are influencing peripheral immune cells, this observation suggests the possibility of an additional inflammatory component residing in the CNS. This inflammatory process is likely to depict a continuous activation of innate immune cells, which have been found in demyelinated areas correlating with tissue damage. In contrast, these immune cells reside in normal white matter too. (140) Recently, speculations on an important role of the gut in this process became evident. (142) Thereby, changes of the intestinal microbiota´s composition and metabolism could participate in inducing autoimmune responses in the CNS. (143-146) Studies performed in animals using the experimental autoimmune encephalomyelitis (EAE) in mice showed that dietary modifications or antibiotic treatments induced massive changes in the microbiota. These microbial alterations were associated to inflammatory patterns and influenced disease severity. (143, 147) As changes in microbiota composition and metabolite production affect

mucosal resident immune cells, different types of mucosal immune cells are of interest. (29) Intestinal DCs play a critical role as APCs and their CD103+ DC subtype is vital for the differentiation of Tregs, stimulating tolerogenic properties in mice and humans protecting against CNS demyelination in mice (via expansion in the gut mucosa and draining lymph nodes. (15, 58, 148, 149) SCFAs can stimulate anti-inflammatory cells and thereby inhibit CNS demyelination through endowing DCs with an enhanced ability to induce the differentiation of Tregs. (30, 57, 58, 150) Because of the existing animal experimental data, the aim of the first study in this dissertation was to investigate the colonic microenvironment (mucosal immune cells, fecal SCFAs and microbiota composition) of patients with early stage MS compared to healthy controls (see section 1.6).

1.5. Diarrhea-predominant Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a functional disorder of the bowel presenting itself with different clinical symptoms sub classified as IBS subtypes such as obstipation-type, diarrhea predominant-type and mixed-type. The subtype diarrhea-predominant IBS (IBS-D) reflects diarrhea as the major symptomatic burden for patients. After the exclusion of organic disease, IBS-D diagnosis is made by the symptom-based ROME-criteria. (151) The worldwide prevalence of IBS is estimated to be between 10-20% and constitutes thereby a major medical burden (152). In IBS-D, changes in the microbiota accompanied with alterations of mucosal inflammatory responses have been observed and are supposed to be related to the etiopathogenesis of IBS-D (in contrast to constipation-type IBS). (151, 153-155) In this process, elevated pro-inflammatory cytokines could stimulate nociceptive and non-nociceptive afferents innervating the colon by potentiating mechanosensory functions of colonic c-fibers being associated to pain symptoms and hypersensitivity in patients with IBS-D. Furthermore, increased humoral immunity was seen in IBS-D patients in jejunal specimens associated to disease activity and an impaired jejunal barrier was proposed as an organic background in IBS-D patients. (156, 157) These findings support a microbiota-dependent immune-mediated pathogenesis of IBS-D and underlines the pathogenic role of the gastrointestinal immune system in disease activity. (153) The current state of therapeutic interventions in IBS-D consists of nutritional interventions, psychological or medical therapies. (151) Thereby, probiotics are effective in relieving IBS-D symptoms. Mechanisms of probiotic action as well as strain-specific influences on the gastrointestinal microenvironment remain elusive. Probiotic interactions with mucosal immune cells such as DCs and Tregs but also direct impacts on mucosal barrier and the microbiota are assumed. (29, 33, 158, 159). Prebiotic or synbiotic effects in IBS-D remain to be uncertain, but might powerfully impact microbiota composition and function, as prebiotics can massively influence intestinal microbial patterns and may thereby shape metabolite production (e.g. SCFAs). (160, 161) SCFAs play an important role in gastrointestinal homeostasis and are in general affected by probiotics. (33) In IBS, fecal SCFA levels are of diagnostic relevance. (33, 162) The aim of the second study in this dissertation was therefore to investigate the effect of oral synbiotics on mucosal immune cells, fecal SCFAs and the fecal as well mucosal microbiota in patients with IBS-D.

1.6. Hypothesis and Aims of Dissertation

1.6.1 Hypothesis of Dissertation

Recent studies in animals show intriguing data regarding the possible role of the gastrointestinal tract in human autoimmunity. Thereby, microbiota-metabolites such as SCFAs might play a key-role in mediating intestinal immune tolerance. (58, 163) Therefore, one hypothesis of this dissertation is that patients with MS depict lower levels of different fecal metabolites accompanied with changes of colonic mucosal immune cells. Furthermore, since diarrhea-predominant irritable bowel syndrome is also known to have a possible microbiota-dependent inflammatory pathogenesis, another hypothesis is that oral synbiotic therapy can exert effects on different levels of the gastrointestinal tract in patients with IBS-D. (156, 157)

1.6.2 Aims of Dissertation

As the measurement of different mucosal immune cells of the gastrointestinal tract is still an ongoing challenge and no standard method in humans, especially regarding certain diseases such as multiple sclerosis, the first aim of this dissertation was to establish a mucosal FACS analysis panel to investigate mucosal DCs and T cell subtypes out of mucosal specimens. To date, no human data are available investigating the gastrointestinal immune system of patients with multiple sclerosis. Furthermore, effects of interventions on the intestinal immune system (e.g. oral synbiotic therapy) in humans remain to be scarce.

Further aims of this dissertation are:

- To apply the established FACS panel in the analysis of mucosal immune cells in patients with immune-mediated diseases (MS, IBS-D)
- To investigate possible differences in the gastrointestinal tract (immune cells, SCFAs, microbiota) of patients with multiple sclerosis compared to healthy individuals
- To create a rationale for microbiota-targeted therapies in MS (e.g. synbiotics, probiotics, fecal microbiota transplantation)
- To investigate possible effects of a synbiotic therapy on different regions of the gastrointestinal tract in patients with IBS-D
- To further elucidate a rationale for synbiotic treatment in influencing the human gastrointestinal tract (immune cells, SCFAs, microbiota) in patients with IBS-D

2 Methods and Materials

In this Dissertation, two separate studies were performed investigating the gastrointestinal microenvironment in patients with immune-mediated diseases. First, the colonic microenvironment in fifteen patients with early stage MS who are naïve to long-term immunomodulatory treatment was compared to ten healthy individuals (referred to as MS Study). Thereby, endoscopic evaluation of the colon to obtain mucosal specimens for FACS analysis of mucosal immune cells was performed. Furthermore, fecal SCFAs and mucosal and fecal microbiota analysis of 16S rRNA genes were investigated. The results from the MS study were published recently in the Journal N2 Neurology. (1) Second, the gastrointestinal microenvironment in ten patients with IBS-D was compared before and after a four-week synbiotic treatment (referred to as IBS-D Study). Thereby, endoscopic evaluation of the upper and lower gastrointestinal tract to obtain mucosal samples for FACS analysis (duodenum and proximal colon) and mucosal 16S rRNA gene analysis (gastric corpus, duodenum, proximal colon) was performed. Moreover, analysis of fecal SCFAs, zonulin and fecal microbiota 16S rRNA gene was performed. The results of the IBS study will be published soon; the manuscript is under preparation.

2.1 General Methods and Materials

2.1.1 Mucosal specimens

In general, endoscopy was performed with standard equipment (Olympus, Hamburg, Germany) in sedated subjects to obtain mucosal specimens for FACS analysis. In the MS study, specimens were obtained during colonoscopy by forceps biopsy in the proximal and distal colon during the retraction of the endoscope. Biopsies were separately taken from the proximal (caecum and proximal colon) and distal (distaland sigmoid colon) colon during the retraction of the colonoscope to investigate possible differences of colonic immune cells related to colonic compartments. (1, 164) In the IBS-D study, Gastroduodeno- and Ileocolonoscopy was performed and specimens were separately taken from the duodenum and proximal colon during the retraction of the endoscope and immediately processed for FACS analysis. The colonic biopsies were obtained between the right-colonic flexure and the caecum. Additionally, mucosal specimens from the gastric corpus, duodenum and proximal colon were obtained for mucosal microbiota analysis.

2.1.2 Isolation of lamina propria mononuclear cells

Mucosal biopsy specimens were immediately preserved in chilled RPMI medium (Sigma; supplemented with penicillin, streptomycin and amphotericin) and washed once with calcium- and magnesium-free HBSS (Life Technologies, Vienna, Austria). Then specimens were incubated in calcium- and magnesium-free HBSS containing 1 mM DTT and 5 mM EDTA at 37°C for 20 min with gentle agitation to remove mucus and epithelial cells. After a brief wash with calcium- and magnesium-free HBSS, tissue was digested with 1 mg/ml Collagenase A (Roche, Basel, Switzerland) and 5 units/ml DNase I (Roche, Basel, Switzerland) in HBSS at 37°C for 60 min on a shaker and then mechanically disrupted by gentle pipetting. The complete dissociation was then controlled by visual inspection. After using a passage through a 70 µm cell strainer the released cells were washed twice with RPMI complete medium (containing 10% FCS and 1% penicillin/streptomycin). Then the cell suspension was finally re-suspended in RPMI complete medium and kept on ice until further analysis. (1)

2.1.3 Flow cytometry

The cell suspension was washed once with staining buffer (PBS containing 3% FCS and 2 mM EDTA). The cells were then stained in 100 µl staining buffer for 20 min at room temperature in the dark. For counting of lamina propria dendritic cells (LPDCs), directly labeled monoclonal antibodies for the subsequent markers were used: lin (lineage) 1-FITC (CD3, CD14, CD16, CD19, CD20, CD56, CD34), HLA-DR-PerCP-Cy5.5, CD11c-APC, and CD103-PE. LPDCs were recognized as lin1-/HLADR+ cells. Tregs were determined by using anti-CD3-APC-Cy7, anti-CD4-V450, anti-CD8-FITC, anti-CD25-PE, and anti-CD127-Alexa Fluor 647 antibodies. Except for CD103-PE (eBioscience, San Diego, USA) all antibodies were purchased from BD Bioscience (San Jose, USA). FMO (fluorescence-minus-one) controls were engaged to set the boundaries for gating of positively stained cells. After the staining reaction, cells were washed once with staining buffer and re-suspended in 100 µl staining buffer. Dead cells were excluded by using propidium iodide (PI) added to the samples immediately prior to acquisition on a LSR II (BD Bioscience, San Jose, USA) flow cytometer. Furthermore, data files were then analyzed using FlowJo (FlowJo, LLC) software. (1)

2.1.4 Isolation of total genomics DNA, 16s library preparation and Illumina sequencing

Stool and mucosal samples were stored at -80°C. Frozen stool and mucosal specimens were used for total DNA isolation combining mechanical and enzymatic lysis with the MagnaPure LC DNA Isolation Kit III (Bacteria, Fungi) (Roche, Mannheim, Germany) according to manufacturer's instructions as described. (165) Special Modifications were made for stool and biopsy samples. Thereby, stool samples were homogenized in 500µl PBS and 250µl of the suspension was mixed with 250µl of bacterial lysis buffer and transferred to Magna Lyser green bead tubes (Roche, Mannheim, Germany). Mechanical lysis was used two times at 6500 rpm in a MagNA Lyser Instrument (Roche, Mannheim, Germany). Biopsy samples were prepared with bead beating for four times at 6500 rpm in 500µl lysis buffer and enzymatic lysis samples were mixed with 25µl lysozyme (100mg/ml) and incubated at 37°C for 30 minutes. Afterwards, samples were mixed with 30 µl Proteinase K and stool samples were incubated at 65°C for 1 hour. In contrast, biopsy samples were incubated overnight at 65°C. Enzymes were heat inactivated at 95°C for 10 minutes and then the remaining steps were performed according to Magna Pure DNA isolation kit III (Bacteria, Fungi) manufacturer's instruction. 250 µl of biopsy samples and 100 µl of stool samples were taken for DNA purification that was eluted in 100 µl. For target specific PCR amplification the primers 27f (AGAGTTTGATCCTGGCTCAG) and 357r (CTGCTGCCTYCCGTA) were taken as described by Baker *et al.* 2003 and synthesized at Eurofins (MWG, Ebersberg, Germany). (166) Then, 5 µl of total DNA from biopsy sample and 2 µl from stool sample extracts were taken over for a 25 µl PCR reactions as described. (165) In the following, triplicates were pooled and amplification was verified using a 1% agarose gel. The sequencing library was amplified, quantified and sequenced on a MiSeqII desktop sequencer (Illumina, Eindhoven, Netherlands) as described. (165) Version 3 600 cycles chemistry (Illumina, Eindhoven, Netherlands) was taken according to manufacturer's instructions to run the 6 pM library with 20% PhiX (Illumina, Eindhoven, Netherlands) and FASTQ files were taken for further data analysis.

2.1.5 GC-EI/MS of short chain fatty acids

SCFAs (acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid and valeric acid) were extracted from stool frozen at -80°C. SCFA concentrations were measured by a GC-MS equipped with a PEG DB-WAXetr. (30m; 0,25mm ID; 0,25µm film) column. SCFA were extracted from feces by sequential addition of 1 ml phosphoric acid (0.5%) and 1 ml methyl-*tert*-butyl-ether, 10 min shaking, 10 min centrifugation and removal of the upper organic layer. Before extraction 100 nmol of d-acetic acid, d-propionic acid, d-butyric acid and d-valeric acid were added as internal standards. Calibration curves by stable isotope dilution were performed from 0.1 – 2000 µM for acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid and valeric acid. A 7890B/5977A MSD GC-MS (Agilent, Waldbronn, Germany) equipped with a PEG DB-WAXetr. (30m; 0,25mm ID; 0,25µm film) column was used. Helium was used as carrier gas at 1.3 ml/min in splitless mode at 250°C injector temperature. The initial oven temperature of 60°C was held for 2 min and then the temperature first was ramped up to 150°C at a rate of 15°C/min. This was followed by a ramp of 5°C/min up to 170°C and 20°C/min up to 250°C where the temperature was held for another 2 min. The mass spectrometer was run in electron impact (EI) mode where the fatty acids were detected in SIM mode on *m/z* 60, 63, 73, 74, 76, 79 and 80. The source temperature was set to 250°C and the transfer line temperature was 280°C. Data analysis was performed by Mass Hunter (Agilent, Waldbronn, Germany). (1)

2.1.6 Statistics

Statistical analyses were carried out using SPSS 22 (IBM® Corporation USA) and GraphPad Prism® (GraphPad Software, Inc. USA). Values are presented as number (%), mean (standard deviation (SD)) or median (interquartile range) as appropriate. For the comparison of categorical variables we applied Fisher's exact test. Group differences of continuous variables were determined by Mann–Whitney *U* test or t-test depending on non-gaussian and gaussian data distribution. Boxplots are depicted according to Tukey. Correlation analyses were performed using Spearman`s rank correlation coefficient (ρ). (1)

2.2 Specific Methods and Materials for the MS study

2.2.1 Patients and controls of MS Study

Patients were consecutively recruited from the MS outpatient clinic of the Department of Neurology, Medical University of Graz during the years 2013 and 2014. Inclusion criteria were: 1) early MS as evidenced by a clinically isolated syndrome (CIS) suggestive of MS or relapsing remitting MS within the first year after diagnosis (167), 2) age between 18 and 60 years and 3) an informed consent. Exclusion criteria were a) a known gastrointestinal pathology, b) previous or ongoing disease modifying treatment of MS, c) evidence for a concomitant other autoimmune disease, d) gastrointestinal infection during the last 2 months before endoscopy , e) a steroid pulse < 30 days before endoscopy, f) antibiotic therapy during the last month before endoscopy g) clinically relevant coagulopathy f) Expanded Disability Status Scale (EDSS) ≥7 h) severe infectious disease, i) alcohol or drug abuse j) history of a prior technically impossible colonoscopy. As controls we included healthy individuals (recruited between 2013 to 2014 from the Department of Gastroenterology and Hepatology, Medical University of Graz) meeting the following inclusion criteria: 1) age between 18 and 60 years, 2) screening colonoscopy for colorectal cancer due to age, positive family history or patient request, 3) informed consent. The exclusion criteria were identical to those of the CIS / MS patients. None of the controls had signs of an acute illness or received any long-term medication. Fifteen MS patients and ten controls consented to the study. In the MS group, median age of 34.6 years and 8/15 patients were women. The interval between first symptom and colonoscopy was 0.5 years and the Median Expanded Disability Status Scale (EDSS) was 2 [IQR: 1.0-2.0] at the time of colonoscopy. (168) Prior to examination, eighty-seven percent of MS patients received glucocorticoid treatment. The median time interval between steroid withdrawal and colonoscopy was 2.7 [IQR: 1.5-3.5] months (see **Table 1**). Relevant comorbidities were seen in one patient with arterial hypertension and obesity and in one patient with atopic dermatitis. All subjects included in the study signed an informed consent prior to study inclusion and all protocols and informed consents were a priori waived by the local institutional review board (IRB number IRB00002556), vote 25-287 ex 12/13. (1)

2.2.2 Microbiota Data analysis and statistical methods

Sequencing data generated as paired end reads on Illumina MiSeq instrumentation was processed and analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.9.1.(169) In the data analysis pipeline the following steps were included: paired end reads joining with standard settings of Ea-utils;(170) Filtering with the maximum unacceptable Phred quality score of 30 and trimming of forward and reverse primer sequences; chimera check using usearch;(171) pick OTUs with open reference method using uclust and latest GreenGenes reference DB from August 2013, followed by PyNAST alignment of the representative sequences for building phylogenetic tree with FastTree.(172-174) Generated OTU table and phylogenetic tree were used in the downstream analysis for the alpha and beta diversity as well as taxonomic summary tables. In order to avoid the influence of the different total number of reads in the samples, subsampling to the smallest sample size of 17800 reads was performed. For the beta diversity beside UniFrac phylogenetic distance metric we also used Bray-Curtis distances in the Principle Coordinate Analysis.(175) PCoA and alpha diversity (estimated by the observed number of species and the Chao1 index) plots and Linear discriminant analysis (LDA) effect size (LEfSe) method was used to depict microbiota differences separating MS patients and controls. (1, 176)

2.2.3 Quantitative analysis of microbial butyrate kinase (*buk*) and butyryl-CoA:acetate CoA-transferase (*but*)

Gene analysis in stool samples of MS patients and controls was performed according to the protocol of Vital et al.(177). qPCR was performed with Power SYBR® Green Mix (Applied Biosystems, Carlsbad, USA) on LightCycler® 480 (Roche Life Science,Vienna, Austria). Genes analyzed: *buk* - G_buk_F/R (*Clostridium acetobutylicum, C. butyricum, C. perfringens*); *but* - G_Acida_F/R (*Acidaminococcus* sp.), G_Fprsn_F/R (*Faecalibacterium prausnitzii*), G_RosEub_F/G_Ros_R/G_Eub_R (*Roseburia* sp., *Eubacterium rectale*)(177); 16S rDNA - 1132F/1108R (universal).(178) Primers used (available in the reference):

G_buk_F	tgctgtWgttggWagaggYgga	G_buk_R	gcaacIgcYttttgatttaatgcatgg
G_Acida_F	cgcagaagaacattgacaagg	G_Acida_R	atggcagggttattgtctacataatc
G_Fprsn_F	gacaagggccgtcaggtcta	G_Fprsn_R	ggacaggcagatRaagctcttgc

G_RosEub_F	tcaaatcMggIgactgggtWga	G_Ros_R	tcgataccggacatatgccaKgag
G_Eub_R	tcataaccgcccatatgccatgag	1132F	atggYtgtcgtcagctcgtg
1108R	Gggttgcgctcgttgc		

2.3 Specific Methods and Materials for the IBS-D study

2.3.1 Patients of IBS-D Study

IBS-D patients, which were defined according to the definition of recent guidelines, were recruited consecutively from the outpatient clinics of the Department of Internal Medicine, Division of Gastroenterology and Hepatology, Medical University of Graz. Patients were included meeting the criteria of having an IBS according to recent guidelines, being aged 18 to 65 and having signed the informant consent. (179) The following exclusion criteria were used to select patients: 1) chronic inflammatory, immune- or neoplastic diseases 2) recent application of immune-modifying medication 3) pregnancy 4) alcohol or drug abuse. No patients received new medications and no changes in medication dose were made during the study. All medications used by patients are systemically compiled in **Table 2**. All patients included signed an informed consent prior to study inclusion and all protocols and informed consents were a priori waived by the local institutional review board (IRB number IRB00002556), vote 25-594 ex 12/13.

2.3.1 Study Protocol

During a screening visit, inclusion, exclusion criteria and physical status was evaluated. Patients fulfilling criteria and included into the study signed an informed consent and were scheduled for the baseline study visit 1. At the first study visit (study visit 1), endoscopy was performed, fecal samples and IBS-SSS were obtained and synbiotic formulation was handed out. Patients recorded the oral administration of synbiotic mixture twice a day for 4 weeks. At the second study visit after 4 weeks (study visit 2), all examinations were re-performed including endoscopy and obtaining of fecal samples and IBS-SSS. No patients received new medications or any changes in medication dose during the study. All medications used by patients are systemically compiled in **Table 2**.

2.3.2 IBS-SSS

The IBS-SSS (irritable bowel syndrome symptom severity score) questionnaire (German) was received from the Zentrum für klinische Ernährung (ZKES, Wollgrasweg 49b, 70599 Stuttgart, Germany) and used as described to quantify symptoms prior and after four weeks of synbiotic therapy. (180)

2.3.3 Synbiotic formulation

Patients received over a four-week period a commercially available multi-strain synbiotic mixture (OMNi-BiOTiC® Stress Repair, Institut Allergosan, Graz) consisting of corn starch, maltodextrin, inulin, fructooligosaccharides, potassiumchloride, magnesiumsulfate, mangansulfate and enzymes. Furthermore, 7.5 billion of each of the following bacterial strains were contained in the mixture: Lactobacillus casei W56, Lactobacillus acidophilus W22, Lactobacillus paracasei W20, Lactobacillus salivarius W24, Lactobacillus plantarum W62, Lactococcus lactis W19, Bifidobacterium lactis W51 and W52 and Bifidobacterium bifidum W23.

2.3.4 Zonulin

A ready-to-use solid-phase sandwich ELISA (Immundiagnostik AG, Bensheim, Germany) was taken to detect zonulin (zonulin Stool ELISA) in fecal samples. The tests were performed according to the manufacturer's instructions and stool sampling was performed with the Stool Sample Application System (Immundiagnostik AG, Bensheim, Germany) according to the manufacturer's manual.

2.3.5 Microbiota Data analysis and statistical methods

Quality filtering and analysis of raw 16S rRNA gene sequence data (hypervariable region V1-V2) was performed with mothur (version 1.22.0) according to the recommended standard operating procedure of mothur for Illumina MiSeq data (https://www.mothur.org/wiki/MiSeq_SOP, accessed June 2016) with additional removal of singletons (default settings and parameters were used, if not specified otherwise). (181, 182) Briefly: Paired reads were merged using mothur's make.contigs command whereby reads less than 200 bps were filtered out of the dataset. In addition sequences containing ambiguous bases or more than eight homopolymeres were removed together with chimeric sequences or sequences outside of the core alignment with the SILVA reverence database

(version 119). Furthermore noisy sequences were identified using pre.cluster and finally deleted from the dataset. Remaining pre-processed and filtered sequences were clustered by mothur's *de novo* OTU-picking strategies into OTUs at a distance of 0.03. Finally taxonomic classification was assigned by using the RDP Bayesian classifier (version 2.10.1, trainingsset 10/29.10.2014) with default settings and a classification confidence cutoff of 80%. (183) Subsequent OTU-based microbiota analyses were performed in QIIME (version 1.8.0), including core diversity analysis with rarefaction to a sampling depth of 9,538 reads per sample for all four locations (COR=corpus, COL=colon, FEC=feces, DUO=duodenum). (172) Unweighted UniFrac distance metrics as measure of between-sample (beta) diversity was calculated and applied for principal coordinates analysis (PCoA) to visualize patterns of diversity. (175) Within-samples (alpha) diversity was calculated using four different measures (i) Observed Species, (ii) ChaoI index, (iii) Shannon Index, and (iv) Faith's Phylogenetic Diversity. (184-186) Statistical significant differences between sample (alpha) diversity were assessed by a nonparametric two-sample t-test (P-values were determined by Monte Carlo permutations. Calculations are based on the greatest rarefaction depth. Bonferroni correction was used to account for multiple comparisons.). Differences in taxonomic microbiota compositions (differentially abundant features/genera) within the four locations and between treatments were determined using linear discriminant effect size analysis (LEfSe) on the filtered datasets at species level. If not otherwise specified p-values below 0.05 were considered as statistically significant. (176)

2.3.6 Extract preparation from specimens for multiplex cytokine assay

Specimens from the proximal colon and duodenum were obtained in cold RPMI1640 medium supplemented with penicillin and streptomycin and transferred to cryotubes, snap frozen and stored in liquid nitrogen until sample preparation. Afterwards, samples were individually thawed on ice and immediately disrupted in 300 µl extraction buffer for 2 min on ice with a pellet pestle (Kimble Kontes, USA). Thereby, the extraction buffer contained DPBS (Dulbecco's Phosphate Buffered Saline without calcium and magnesium, Lonza) and EDTA-free protease inhibitors (cOmplete mini, Roche). After disrupting the samples mechanically by pipetting, specimens were passed through a 70 µm cell strainer. Samples were incubated on ice for 5 min. Finally, supernatants were collected by centrifugation at 10,000 ×g for 10 min at 4 °C, snap frozen in liquid nitrogen and stored at −80 °C until analysis. The following cytokines were comprised in the analysis using Multiplex

immunoassay kits (ProcartaPlex) obtained from eBioscience which were run according to manufacturer's instructions using magnetic beads: IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-17A, IL-23 and TNF-α. The standards for each cytokine were assayed in duplicates to generate standard curves using the reference concentrations as given by the manufacturer. Samples were individually thawed on ice and wash steps were performed using a hand-held magnetic block. Data were obtained on a validated and calibrated Bio-Plex 200 system (Bio-Rad) using Bio-Plex Manager 6.1 software (Bio-Rad). BCA Protein Assay (Pierce) was used to analyse total protein concentration and to normalize cytokine concentrations for each sample. (187)

3 Results

3.1 MS Study

3.1.1 Patients and Controls

Fifteen patients with early stage MS and ten healthy controls consented to the study (**Table 1**). MS patients had a median age of 35 years. 8/15 were women. Controls were median 44 years and 4/10 were woman. Relevant comorbidities were seen in one patient with arterial hypertension and obesity and in one patient with atopic dermatitis. (1)

	MS-p [c]	Ctrl	p
Number of patients (% female)	15 (53)	10 (40)	ns [d]
Age at colonoscopy (years) [a]	35 (7)	44 (13)	<0.05 [e]
Age at disease onset (years) [a]	33 (7)	na	na
Disease duration at colonoscopy (months) [b]	7 (4 - 29)	na	na
EDSS at time of colonoscopy [b]	1.5 (1.0-2.0)	na	na
N (%) of patients with disease modifying treatment at colonoscopy	0 (0.0)	na	na

EDSS: Expanded Disability Status Scale; MS-p: multiple sclerosis patients; Ctrl: control patients; na: not applicable; ns: not significant.
Values are presented as number (%), [a] mean (standard deviation (SD) or [b] median (interquartile range); [c] the MS-p cohort consisted of 10 patients with clinically isolated syndromes and 5 patients with relapsing-remitting MS; [d] Fisher's exact test; [e] Mann–Whitney *U* test.

Table 1 | Data of the included 15 patients with MS (MS-p) and 10 healthy controls (Ctrl). (1)

3.1.2 Colonic mucosal immune cells in MS patients compared to controls

Proximal and distal colonic mucosal immune cell subsets (especially lin1-/HLADR+ DCs, CD103+ DCs and CD4+25+127- Tregs) were characterized by FACS analysis as previously described (**Figure 6 and 7**). No differences in cell numbers between MS patients and controls were observed in the proximal colon (**Figure 8 a,b**). MS patients showed significantly fewer total DCs and CD103+ DCs in the distal colon compared to controls (total DCs: 3.0 [2.2-3.8] vs. 5.0 [3.6-6.3], p=0.019 [‰ of living cells] and 255 [93-381] vs. 404 [335-592], p=0.026 [absolute numbers]; CD103+ DCs: 1.4 [0.9-1.8] vs. 2.5 [1.4-3.1], p=0.036 and 114 [38-174] vs. 183 [124-303], p=0.048 [absolute numbers], **Figure 8 c,d**).

Significant reductions of CD4+25+127- Tregs in MS patients were likewise only seen in the distal colon (Tregs: 3.1 [1.3-3.7] vs. 4.7 [3.4-6.1], p=0.032 [‰ of living cells]) and 153 [83-205] vs. 226 [136-510], p=0.023 [absolute numbers], **Figure 9 a-d**). CD4+, CD8+ T cells (**Figure 10 a-d**) and natural killer cells (**Figure 11 a-d**) depicted no difference between MS patients and controls. As MS patients and controls depicted a significant age difference, calculation of the correlations between age and total DCs, CD103+ DCs and Tregs was performed. Significant correlations were only observed between age and Tregs in both compartments (proximal Tregs: p=0.048, rho= -0.461; distal Tregs: p=0.029, rho= -0.489, **Figure 12 a-c**). As controls depicted an older age, the observed reduction of mucosal DCs and Tregs in patients would be influenced by an underestimation of the observed reduction. (1)

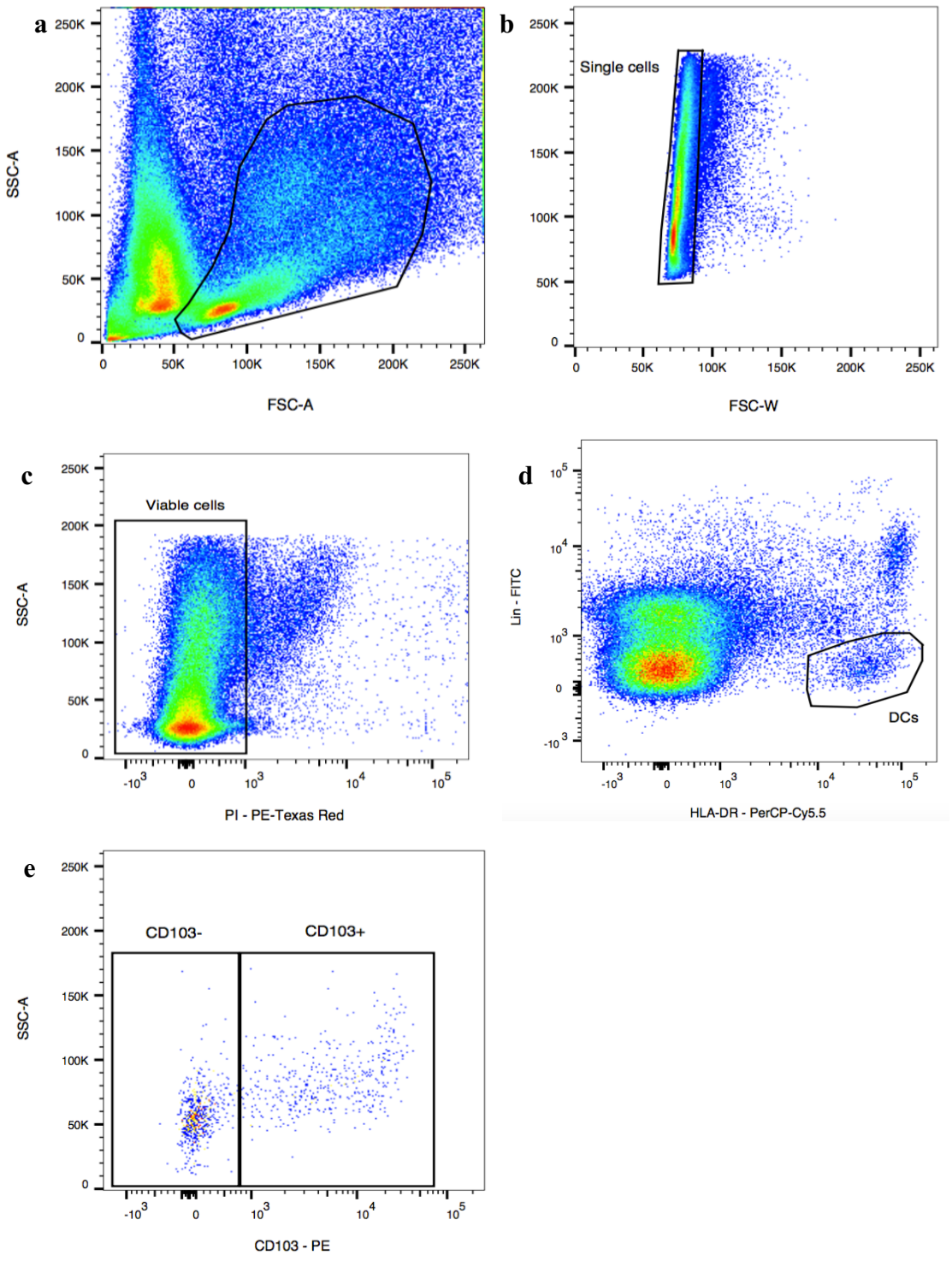

Figure 6 | FACS analysis of colonic mucosal Dendritic cells. Colonic specimens were obtained separately from the proximal and distal colon. Lamina propria mononuclear cells were isolated (**a**). Dendritic cells (DCs) were identified as single (**b**) live (**c**) cells that stain negatively for lin (lineage) 1 markers (CD3, CD14, CD16, CD19, CD20, CD56, CD34) and positively for HLA-DR (**d**). CD103 positive DCs were gated out of the DC population (**e**). (1)

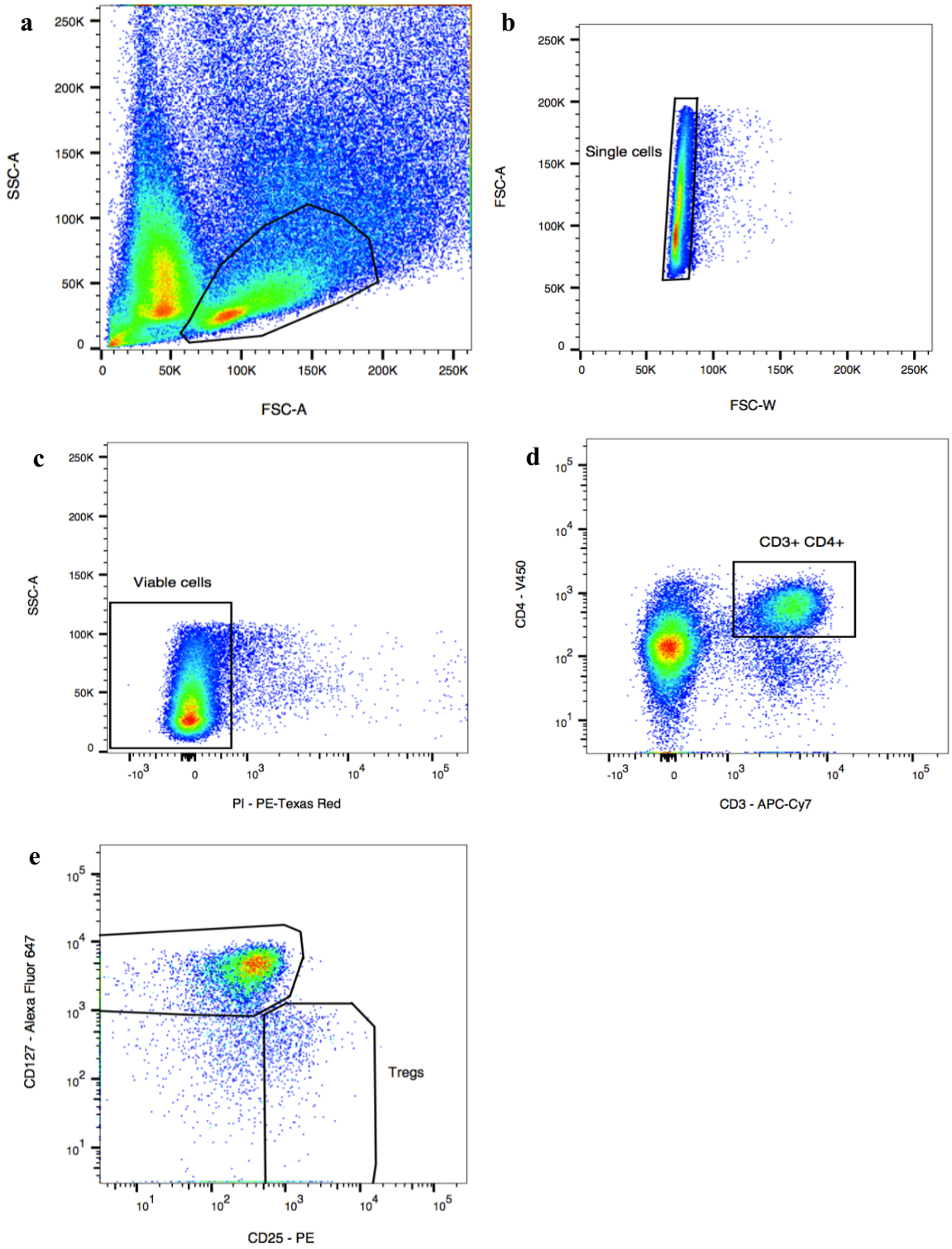

Figure 7 | FACS analysis of colonic mucosal T cells and regulatory T cells. Colonic specimens were obtained separately from the proximal and distal colon. Lamina propria lymphocytes were isolated (**a**). CD4+ T cells were identified as single (**b**) live (**c**) cells that stain positively for CD3 and CD4 (**d**). Tregs were identified as live single cells that stain positively for CD3, CD4, CD25 markers and low/negatively for CD127 (**e**). (1)

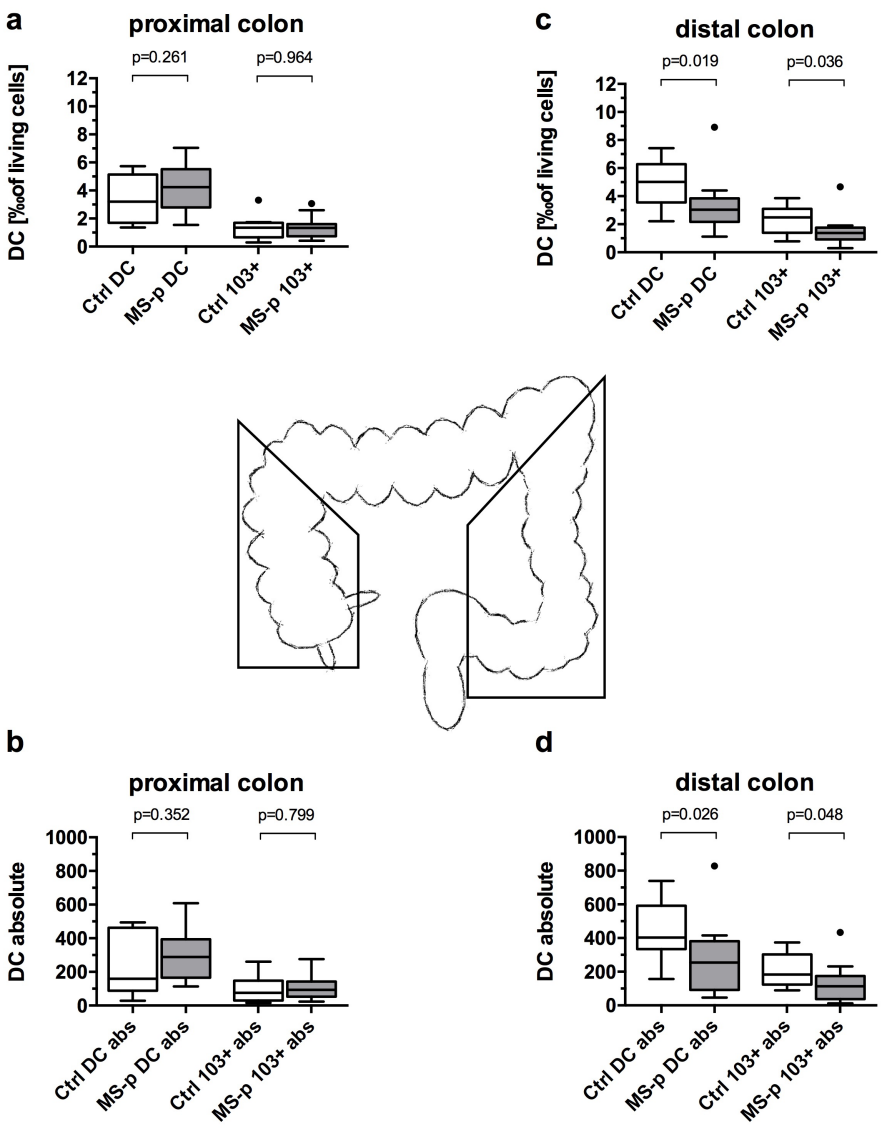

Figure 8 | Reduction of colonic mucosal total and CD103+ DCs in the distal colon of MS patients (MS-p). Total and CD103+ DC numbers of the proximal (a,b) and distal (c,d) colon. **a, b |** No significant differences in the number of total and CD103+ DCs were found in the proximal colon of MS-p compared to controls (Ctrl). **c,d |** Significant reductions of total and CD103+ DCs were observed in MS-p compared to Ctrl in the distal colon (total DCs: 3.0 [2.2-3.8] vs. 5.0 [3.6-6.3], p=0.019 [‰ of living cells] and 255 [93-381] vs. 404 [335-592], p=0.026 [absolute numbers]; CD103+ DCs: 1.4 [0.9-1.8] vs. 2.5 [1.4-3.1], p=0.036 and 114 [38-174] vs. 183 [124-303], p=0.048 [absolute numbers]. (Median [Q1-Q3]; Mann–Whitney U test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey). (1)

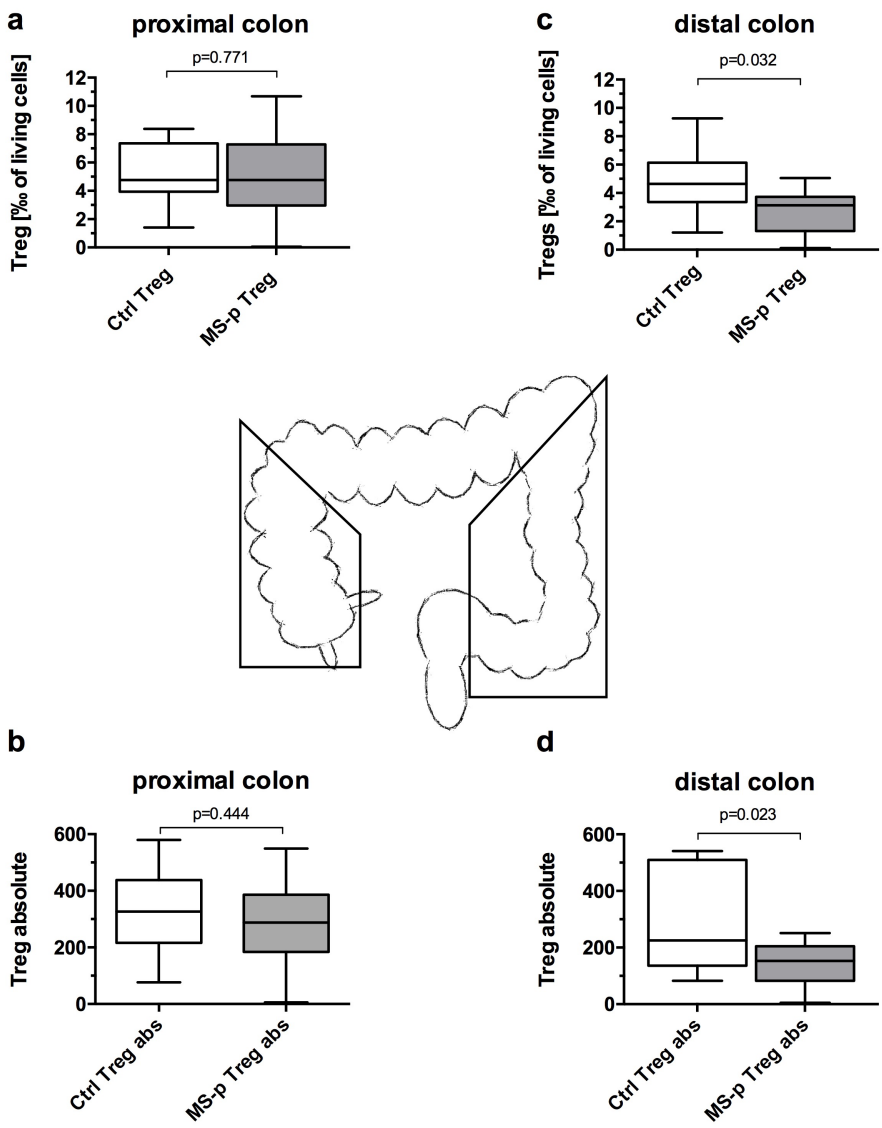

Figure 9 | Reduction of colonic mucosal Tregs in the distal colon of MS patients (MS-p).

CD4+25+127-Treg numbers from the proximal (**a,b**) and distal (**c,d**) colon. **a, b |** No significant differences in the number of Tregs were found in the proximal colon of MS-p compared to controls (Ctrl). **c,d |** Significant reductions of Tregs were observed in MS-p compared to Ctrl in the distal colon. (Tregs: 3.1 [1.3-3.7] vs. 4.7 [3.4-6.1], p=0.032 [‰ of living cells]) and 153 [83-205] vs. 226 [136-510], p=0.023 [absolute numbers]. (Median [Q1-Q3]; Mann–Whitney U test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey). (1)

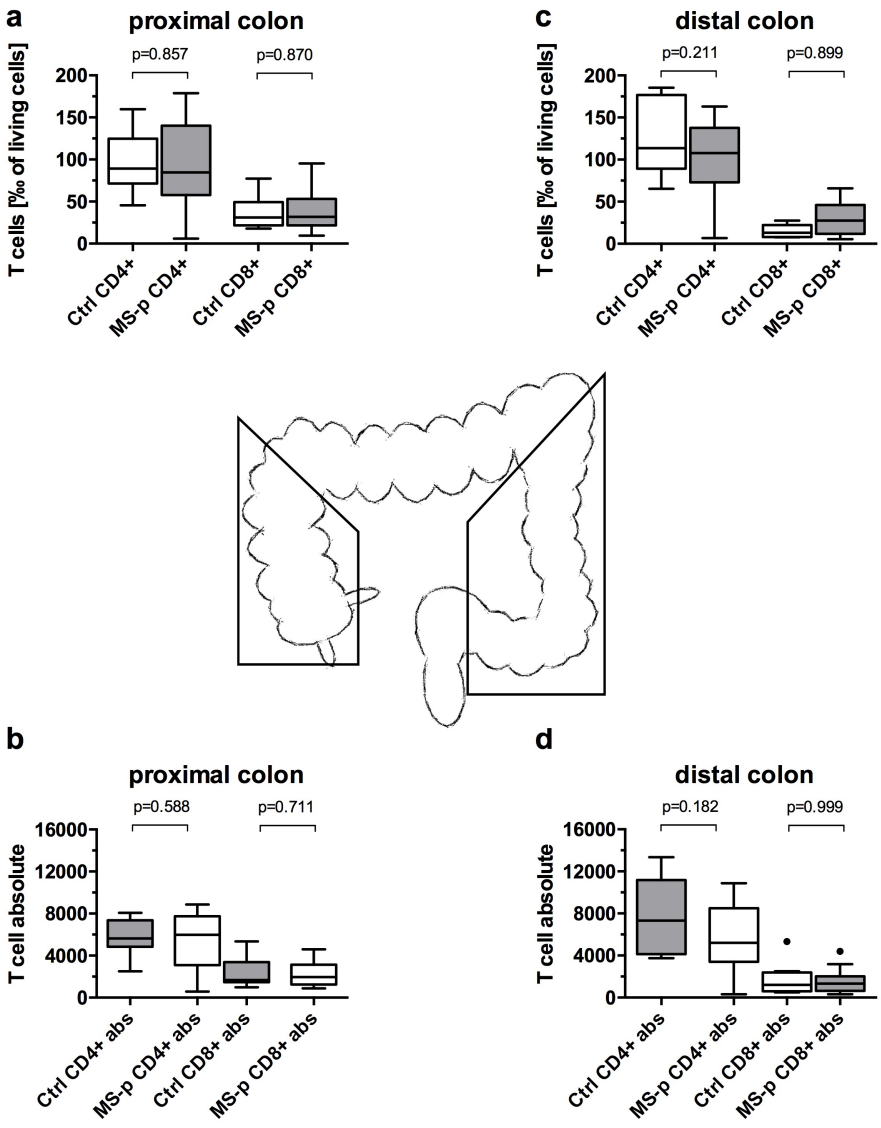

Figure 10 | Colonic mucosal CD4+ and CD8+ T cells in the proximal and distal colon of MS patients (MS-p) and controls (Ctrl). CD4+ and CD8+ T cells of the proximal (**a,b**) and distal (**c,d**) colon of MS-p were compared to Ctrl. No differences in the abundances of CD4+ or CD8+ T cells were found. (CD4+, distal: 108 [73-138] vs. 114 [89-177], p=0.211 [‰ of living cells] and 5208 [3403-8491] vs. 7326 [4129-11173], p=0.182 [absolute numbers], proximal: 85 [58-140] vs. 89 [71-125], p=0.857 [‰ of living cells] and 5977 [3105-7738] vs. 5636 [4843-7368], p=0.588 [absolute numbers]); (CD8+, distal: 28 [12-46] vs. 26 [13-38], p=0.899 [absolute numbers] and 1336 [644-2013] vs. 1210 [593-2384], p=0.999 [absolute numbers], proximal: 32 [22-53] vs. 31 [22-49], p=0.870 [‰ of living cells] and 1959 [1242-3145] vs. 1665 [1480-3377], p=0.711 [absolute numbers]). (Median [Q1-Q3]; Mann–Whitney U test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey). (1)

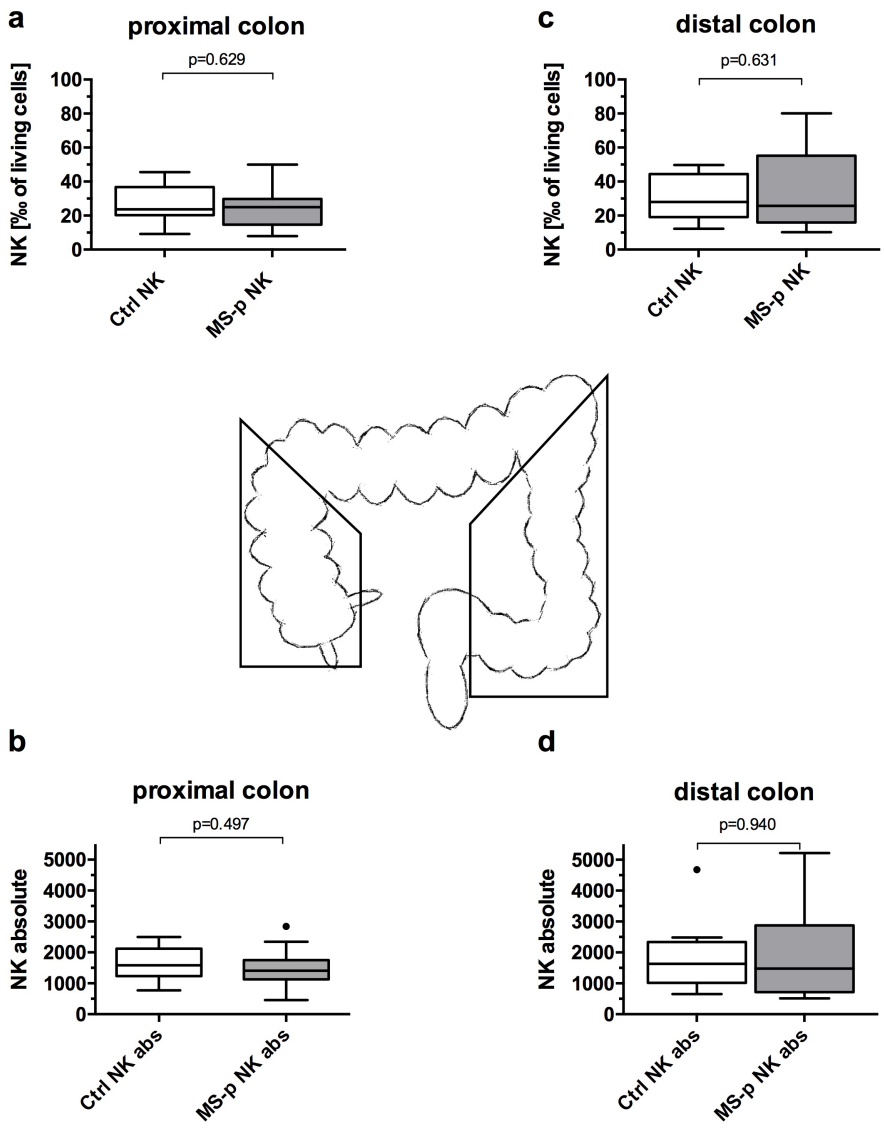

Figure 11 | Colonic mucosal natural killer (NK) cells in the proximal and distal colon of MS patients (MS-p) and controls (Ctrl). NK cells of the proximal (**a,b**) and distal (**c,d**) colon of MS-p were compared to Ctrl. No differences in the abundances of NK cells were found. (distal: 26 [16-55] vs. 28 [19-44], p=0.631 [‰ of living cells] and 1475 [713-2879] vs. 1632 [1019-2339], p=0.940 [absolute numbers], proximal: 25 [15-30] vs. 24 [20-37], p=0.629 [‰ of living cells] and 1408 [1131-1752] vs. 1580 [1231-2122], p=0.497 [absolute numbers]). (Median [Q1-Q3]; Mann–Whitney U test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey). (1)

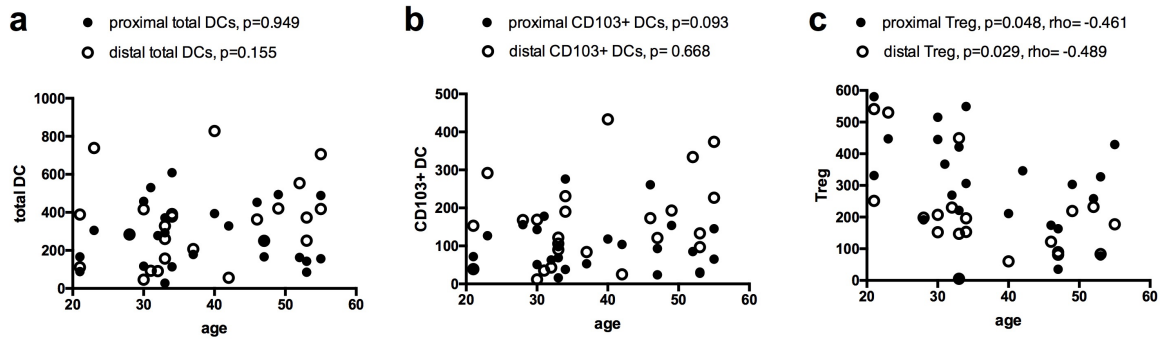

Figure 12 | Spearman`s rank correlations between age and mucosal total DCs, CD103+ DCs and Tregs of MS patients and controls. Correlations between age and total DCs **(a)** CD103+ DCs (**b**) and Tregs (**c**) were calculated and significant correlations were observed only between age and Tregs in both proximal and distal colon. (proximal: p=0.048, rho= -0.461; distal: p=0.029, rho= -0.489) only. (Spearman´s rank correlation). (1)

3.1.3 Fecal SCFA levels in MS patients compared to controls

SCFA levels in fecal samples of MS patients and controls were investigated by high-performance liquid chromatography (HPLC). Levels of total SCFAs, acetate and butyrate of MS patients were significantly reduced by 50-60% compared to controls. (total SCFAs, 252 [148-354] vs. 121 [82-162]; p=0.017), (acetate 180 [85-227] vs. 70 [45-96]; p=0.025), (butyrate 33 [18-48] vs. 14 [8-21]; p=0.018). Propionate and valerate were reduced in MS patients compared to controls as well but did not reach statistical significance (**Figure 13**). The sequence of absolute abundances of single SCFAs was similar in MS patients and controls (Acetate > Propionate > Butyrate > Valerate > Iso-Valerate > Iso-Butyrate). Furthermore, strong positive correlations between acetate and propionate as well as butyrate (ρ=0.91 and ρ=0.93, respectively. p<0.0001 for both) over both groups were evident (**Figure 14**). (1)

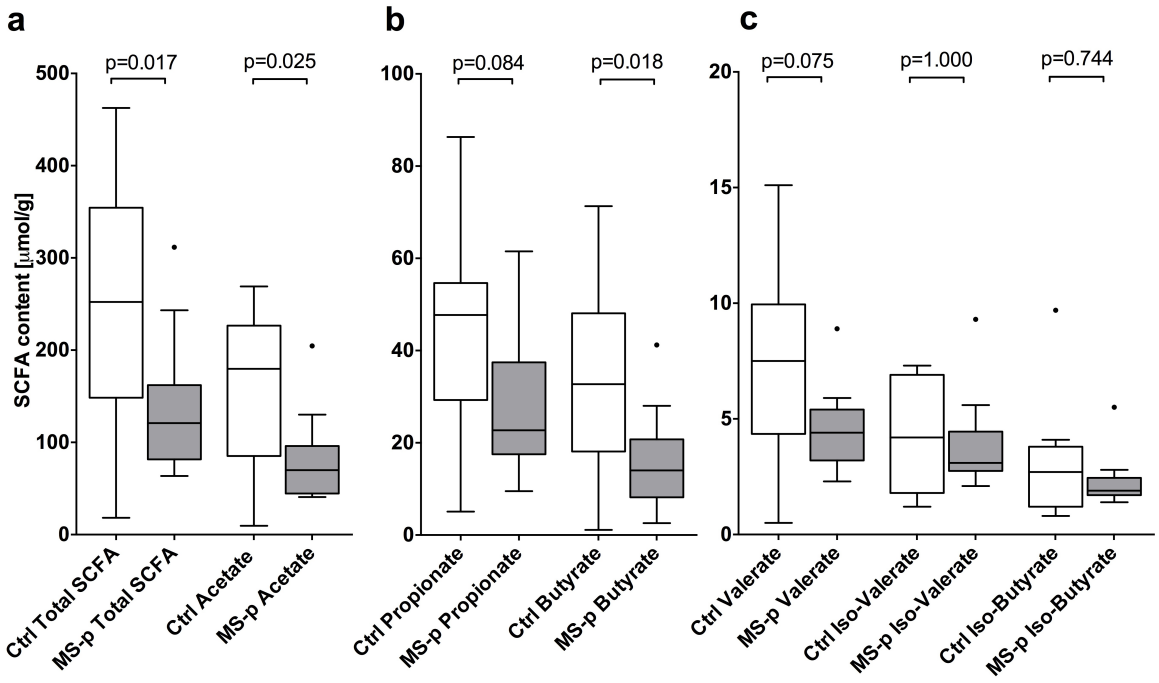

Figure 13 | Short-chain fatty acids in fecal samples of MS patients (MS-p) compared to controls (Ctrl). **a-c |** SCFA contents are shown in order of their absolute quantity in µmol/g (Total > Acetate > Propionate > Butyrate > Valerate > Iso-Valerate > Iso-Butyrate). A significant reduction in MS-p (grey) vs. controls (white) was found in total SCFA (252 [148-354] vs. 121 [82-162]; p=0.017), acetate (180 [85-227] vs. 70 [45-96]; p=0.025) and butyrate content (33 [18-48] vs. 14 [8-21]; p=0.018). Trends were observed in propionate (p=0.084) and valerate content (p=0.075). (Median [Q1-Q3]; Mann–Whitney *U* test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey). (1)

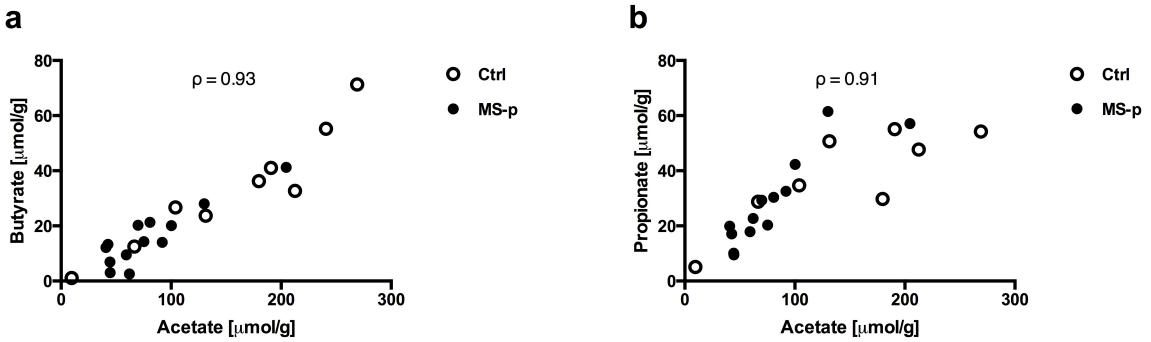

Figure 14 | Correlations of the fecal contents of acetate and butyrate as well as acetate and propionate in fecal samples of MS patients (MS-p) compared to controls (Ctrl). Levels in μmol/g. Spearman's rank correlation coefficient depict strong positive correlations between acetate and butyrate (ρ=0.93, p=0.0001) as well as acetate and propionate levels (ρ=0.91, p=0.0001) in MS-p and Ctrl.

3.1.4 Fecal microbial abundances of MS patients compared to controls

Bacterial community profiling was applied to fecal samples. Taxa belonging to the order *Clostridiales* were significantly different in the MS cohort compared to controls (**Figure 15**). *Roseburia spp*, a bacterial genus belonging to the *Clostridia* cluster XIVa, was reduced in MS patients (p=0.049). Furthermore, elevated *Oscillospira spp* (p=0.042) and *Ruminococcacceae* (p=0.007) in MS patients were evident. The *Prevotella* genus was reduced in MS patients compared to controls (p=0.049). We did not see any disease specific clustering in PCoA analysis and no difference in the bacterial diversity (**Figure 16**).

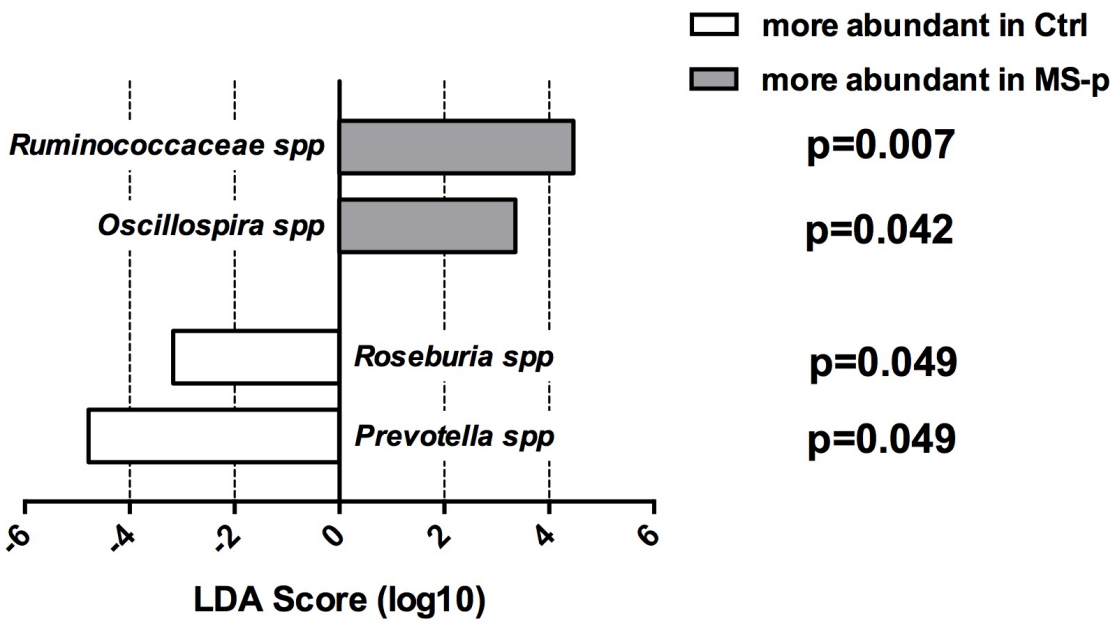

Figure 15 | Microbial abundances in fecal samples of MS patients (MS-p) compared to controls (Ctrl). Bacterial community profiling depicted as Linear Discriminant Analysis (LDA) indicated differences predominantly in bacteria belonging to the *Clostridiales* order. Negative scores depict higher abundance in Ctrl (white), positive scores higher abundance in MS-p (grey). Ctrl show a significant higher abundance of *Roseburia spp* (p=0.049) and *Prevotella spp* (p=0.049). MS-p fecal samples were richer in *Oscillospira spp* (p=0.042) and an undefined genus of *Ruminococcacceae spp* (p=0.007). (Kruskal-Wallis test).

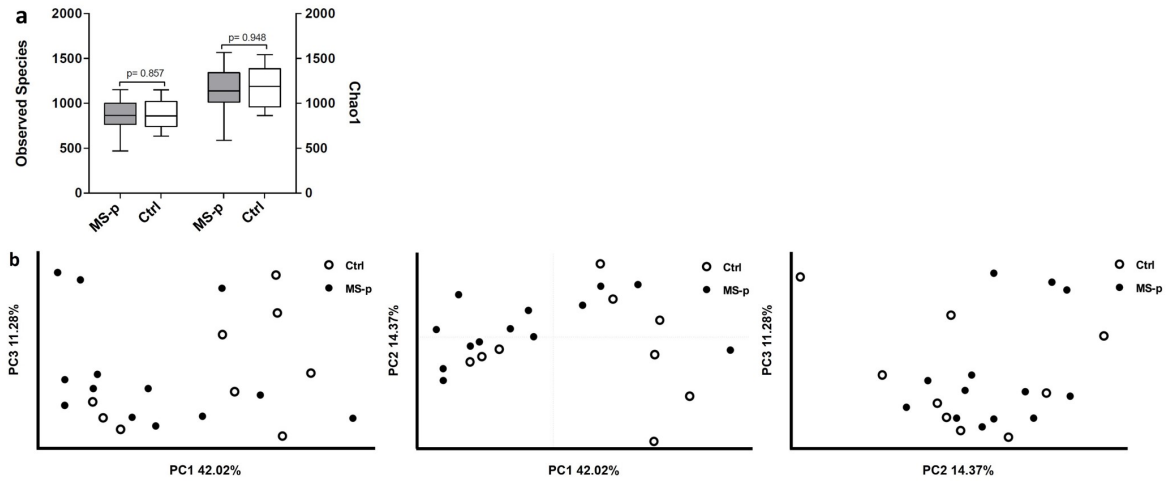

Figure 16 | Microbial alpha-diversity (Chao1) and PCoA-plots in MS patients (MS-p) compared to controls (Ctrl). a | Alpha-diversity depicted as observed species and Chao1 showed no difference between MS-p and Ctrl. **b |** Weighted UniFrac Principal Coordinate Analysis (PCoA, PC1-PC2, PC1-PC3, PC2-PC3) showed no significant differences and no apparent clustering of the gut microbiome between MS-p and Ctrl. Open and closed dots represent MS-p and Ctrl subjects respectively. (Unifrac phylogenetic distance, Bray-Curtis distances)

3.1.5 Quantitative analysis of microbial butyrate kinase (*buk*) and butyryl-CoA: acetate CoA-transferase (*but*) genes in fecal samples of MS patients compared to controls

Quantitative PCR analyses of the genes most abundantly involved in butyrate production (butyrate kinase [*buk*] and butyryl-CoA: acetate CoA-transferase [*but*]) in stool samples were performed. (177) No significant differences for *buk* and *but* genes between MS patients and controls could be observed (**Figure 17**).

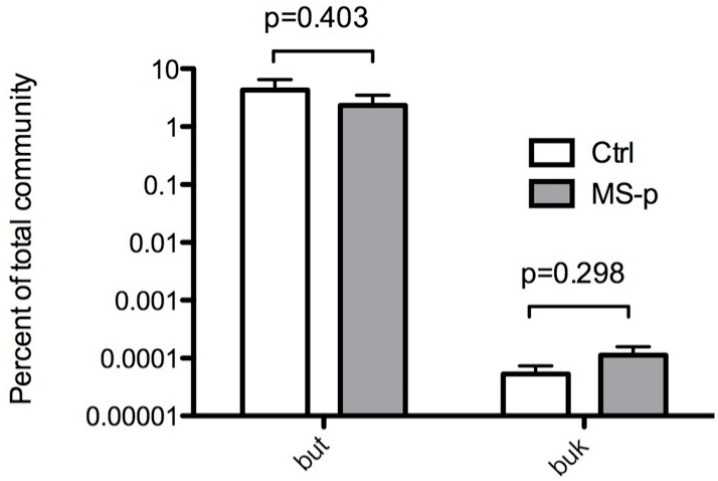

Figure 17 | Quantitative analysis of microbial butyrate kinase (*buk*) and butyryl-CoA: acetate CoA-transferase (*but*) genes in MS patients (MS-p) compared to controls (Ctrl). Quantitative PCR are depicted as percent of total community genes. Unpaired t-test showed no significant differences for *buk* and *but* genes between MS patient (MS-p) and control (Ctrl) stools. (Mean +/- SD).

3.2 IBS-D Study

3.2.1 Patients

Ten patients with IBS-D consented to the study. Their median age was 46 years and 5/10 were women. Four patients had no relevant medical comorbidities, those of the remaining six patients are compiled in **Table 2**.

Quantity of patients (% female)	10 (50)
Age at endoscopy, years (median, [Q1-Q3])	46 [37-53]
BMI (median, [Q1-Q3])	23 [22-25]
IBS-SSS baseline (median, [Q1-Q3])	236 [129-256]
Relevant comorbidities (number of patients)	
	hypothyroidism (4)
	iron deficiency (2)
	depression (2)
	bronchial asthma (1)
	gastroesophageal reflux disease (1)
	arterial hypertension (1)
	osteopenia (1)
	osteoporosis (1)
Co-medications (number of patients)	thyroid hormones (4)
	vitamin d3 (3)
	proton pump inhibitor (2)
	calcium (2)
	antidepressant (2)
	H2-blocker (1)
	selective estrogen receptor modulator (1)
	beta blocker (1)
	benzodiazepine (1)
	atypical antipsychotic (1)
	antacid (1)
	antihypertensive (1)
	spasmolytic (1)
	prokinetic (1)
	folate (1)
	vitamin b complex (1)

IBS-D, diarrhea-predominant irritable bowel syndrome; BMI, body mass index; IBS SSS, irritable bowel syndrome severity scoring system

Table 2 | Clinical and demographic data of the 10 included patients with IBS-D.

3.2.2 Synbiotic effect on mucosal immune cell lineages in duodenum and proximal colon

FACS analysis of mucosal specimens collected during endoscopy of duodenum and proximal colon was performed and mucosal T- and dendritic cell subsets were characterized as previously described (107). Thereby, no apparent changes regarding T-cell subsets were found in the duodenum. In contrast, a significant reduction of mucosal CD4+ T cells (p=0.042) was observed after treatment in the proximal colon. (60 [57-65] vs. 55 [50-60], p=0.042) (**Figure 18**). Additionally, double-negative T cells (CD4- CD8- T cells) showed a trend towards elevation after probiotic treatment in the proximal colon. (10 [9-12] vs. 13 [8-25], p=0.078) No dendritic cells could be isolated from the duodenal mucosa, whereas isolated DCs from the proximal colon (total, CD11c+ or CD103+) were not affected by treatment. Results from mucosal immune cells of the proximal colon and duodenum are systematically compiled in **Table 3**. Furthermore, analysis of cytokine concentration in mucosal specimen extract was performed using a multiplex cytokine assay. Results are systemically depicted in **Table 4**. TNF-alpha levels showed a trend towards elevation after synbiotic treatment in the proximal colon. (0.2 [0-0.4] vs. 0.6 [0-1.1] pg/ml, p=0.0547) (**Figure 19**). No other cytokines were affected by synbiotic treatment.

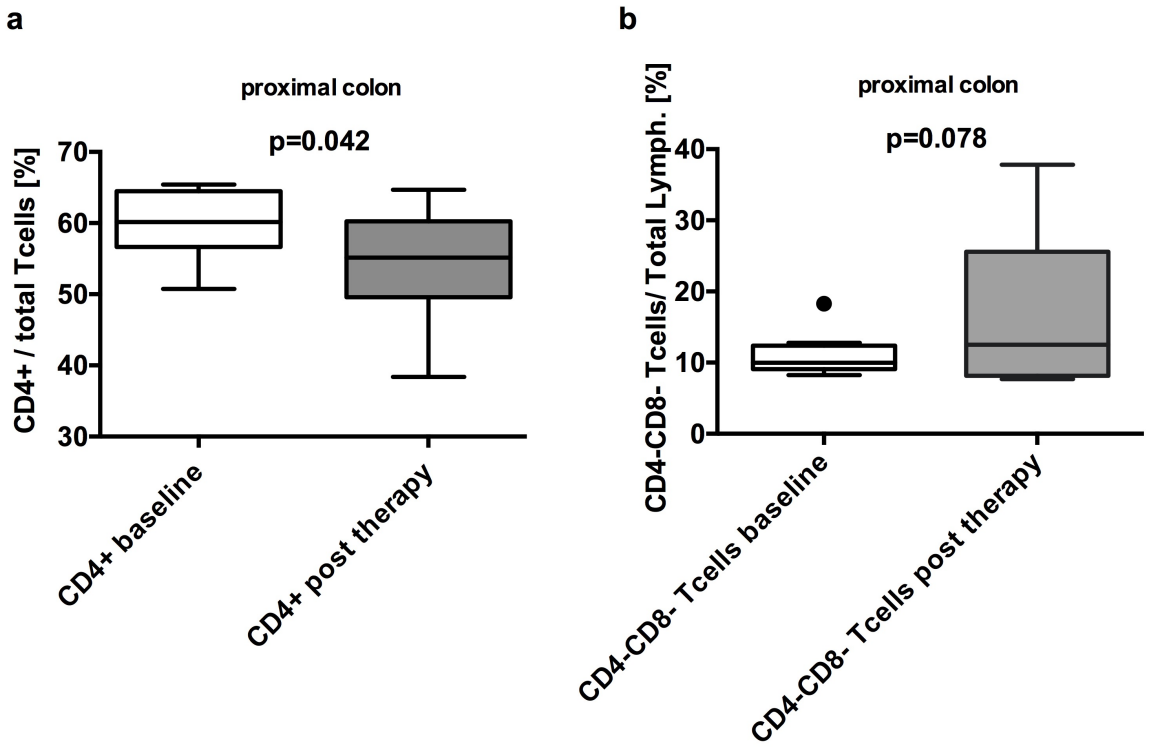

Figure 18 | Mucosal CD4+ T cells are reduced and a trend towards increased double-negative T cells is observed after synbiotic treatment in the proximal colon. Mucosal specimens from the proximal colon were analyzed by FACS analysis. **a |** CD4+ T cells [%] were compared at baseline (white) and after treatment (grey) and were significantly reduced after synbiotic intervention. (60 [57-65] vs. 55 [50-60], p=0.042) **b |** CD4-CD8- T cells [%] were compared at baseline (white) and after treatment (grey) and a trend towards increased numbers after synbiotic treatment was seen. (10 [9-12] vs. 13 [8-25], p=0.078) (Median [Q1-Q3]; Mann–Whitney *U* test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey).

	cell type	function	unit	median pre	median post	p
Duodenum						
	CD3+	T cell	[% l.c.[a]]	10.7	8.9	ns
	CD3+4+	T helper	[% CD3+]	19.4	23.8	ns
	CD3+8+	cytotoxic T lymphocyte	[% CD3+]	29.7	20.7	ns
	CD3+4+25+127-	Treg	[% CD4+]	1.2	1.5	ns
	CD3+4+8+	double-positive T cell	[% CD3+]	6.6	8.1	ns
	CD3+4-8-	double-negative T cell	[% CD3+]	22.9	23.4	ns
	CD3+56+16+	natural killer T cell	[% CD3+]	11.0	13.1	ns
	CD3-56+16+	natural killer cell	[% l.c.[a]]	4.5	3.0	ns
Proximal Colon						
	CD3+	T cell	[% l.c.[a]]	20.3	20.7	ns
	CD3+4+	T helper	[% CD3+]	59.1	54.7	0.042
	CD3+8+	cytotoxic T lymphocyte	[% CD3+]	21.8	18.8	ns
	CD3+4+25+127-	Treg	[% CD4+]	6.0	6.2	ns
	CD3+4+8+	double-positive T cell	[% CD3+]	1.9	2.3	ns
	CD3+4-8-	double-negative T cell	[% CD3+]	10.9	16.2	ns
	CD3+56+16+	natural killer T cell	[% CD3+]	2.6	2.0	ns
	CD3-56+16+	natural killer cell	[% l.c.[a]]	3.3	2.7	ns
	CD3-14-16-19-20-56-34-HLA-DR+	dendritic cell	[‰ l.c.[b]]	2.6	2.6	ns
	CD3-14-16-19-20-56-34-HLA-DR+103+	regulatory dendritic cell	[‰ l.c.[b]]	0.7	0.6	ns
	CD3-14-16-19-20-56-34-HLA-DR+11c+	11c+ dendritic cell	[‰ l.c.[b]]	2.4	2.3	ns

Table 3 | Mucosal immune cells of the duodenum and the proximal colon.

l.c.[a], living cells from the lymphocyte gate; l.c.[b], living cells of the lympho-monocyte gate; ns: not significant

	cytokine type	unit	median pre	median post	p
Duodenum					
	TNF alpha	[pg/ml]	2.4	2.8	ns
	IL12p40	[pg/ml]	6.7	6.8	ns
	IL12p70	[pg/ml]	1.4	1.3	ns
	IL6	[pg/ml]	10.3	9.4	ns
	IL23	[pg/ml]	0.3	4.2	ns
	IL17	[pg/ml]	0.5	0.7	ns
	IL10	[pg/ml]	0.2	0.3	ns
	IL1beta	[pg/ml]	3.3	3.0	ns
Colon					
	TNF alpha	[pg/ml]	0.2	0.6	0.0547
	IL12p40	[pg/ml]	11.2	5.9	ns
	IL12p70	[pg/ml]	0.6	0.8	ns
	IL6	[pg/ml]	0.0	0.0	ns
	IL23	[pg/ml]	0.0	0.0	ns
	IL17	[pg/ml]	0.0	0.0	ns
	IL10	[pg/ml]	0.1	0.1	ns
	IL1beta	[pg/ml]	4.2	3.5	ns

Table 4 | Mucosal extract cytokine levels of the duodenum and the proximal colon.

ns: not significant

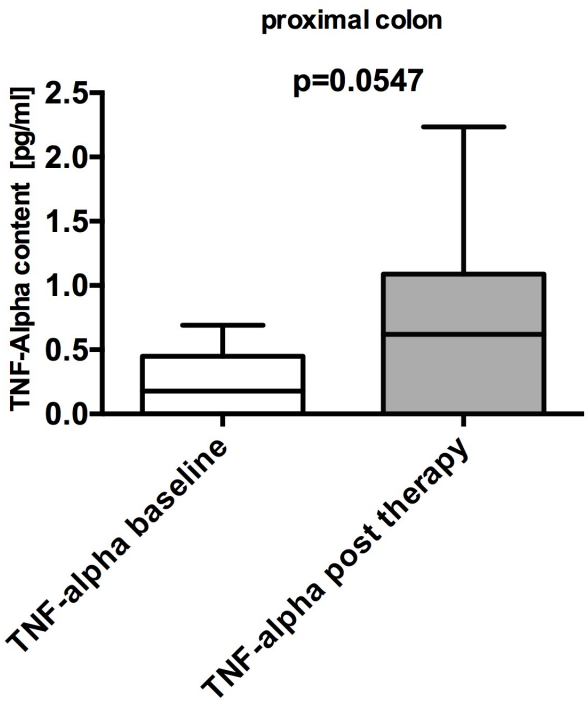

Figure 19 | TNF-alpha levels show a trend towards elevation in the proximal colon after synbiotic treatment. Mucosal extracts were analyzed by a multiplex cytokine assay before (white) and after synbiotic treatment (grey). TNF-alpha levels show a trend towards increased concentrations in the proximal colon after synbiotic treatment. (0.2 [0-0.4] vs. 0.6 [0-1.1] pg/ml, p=0.0547). (Median [Q1-Q3]; Mann–Whitney *U* test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey).

3.2.3 Synbiotic effect on phylogenetic diversity in the upper and lower gastrointestinal tract

The effect of synbiotic treatment on phylogenetic diversity in different regions of the human gastrointestinal tract (gastric corpus, duodenum, proximal colon, feces) was investigated. Significant elevations in phylogenetic diversity in the upper but not lower gastrointestinal tract were observed depicted by elevations in mucosal samples of the gastric corpus (p=0.008) and duodenum (p=0.025) and no increases in colonic (p=0.710) and fecal samples (p=0.358, **Figure 20**). All results of mucosal and fecal samples using phylogenetic diversity, observed species and Shannon Diversity index are systematically compiled in **Table 5**.

Region	PD	Observed Species	Shannon Diversity Index
gastric corpus	0.008	ns	ns
duodenum	0.003	0.011	ns
proximal colon	ns	ns	ns
feces	ns	ns	ns

Table 5 | Analysis of Mucosal Microbial Diversity using Phylogenetic Diversity, Observed Species and Shannon Diversity Index

PD: Phylogenetic diversity; ns: not significant

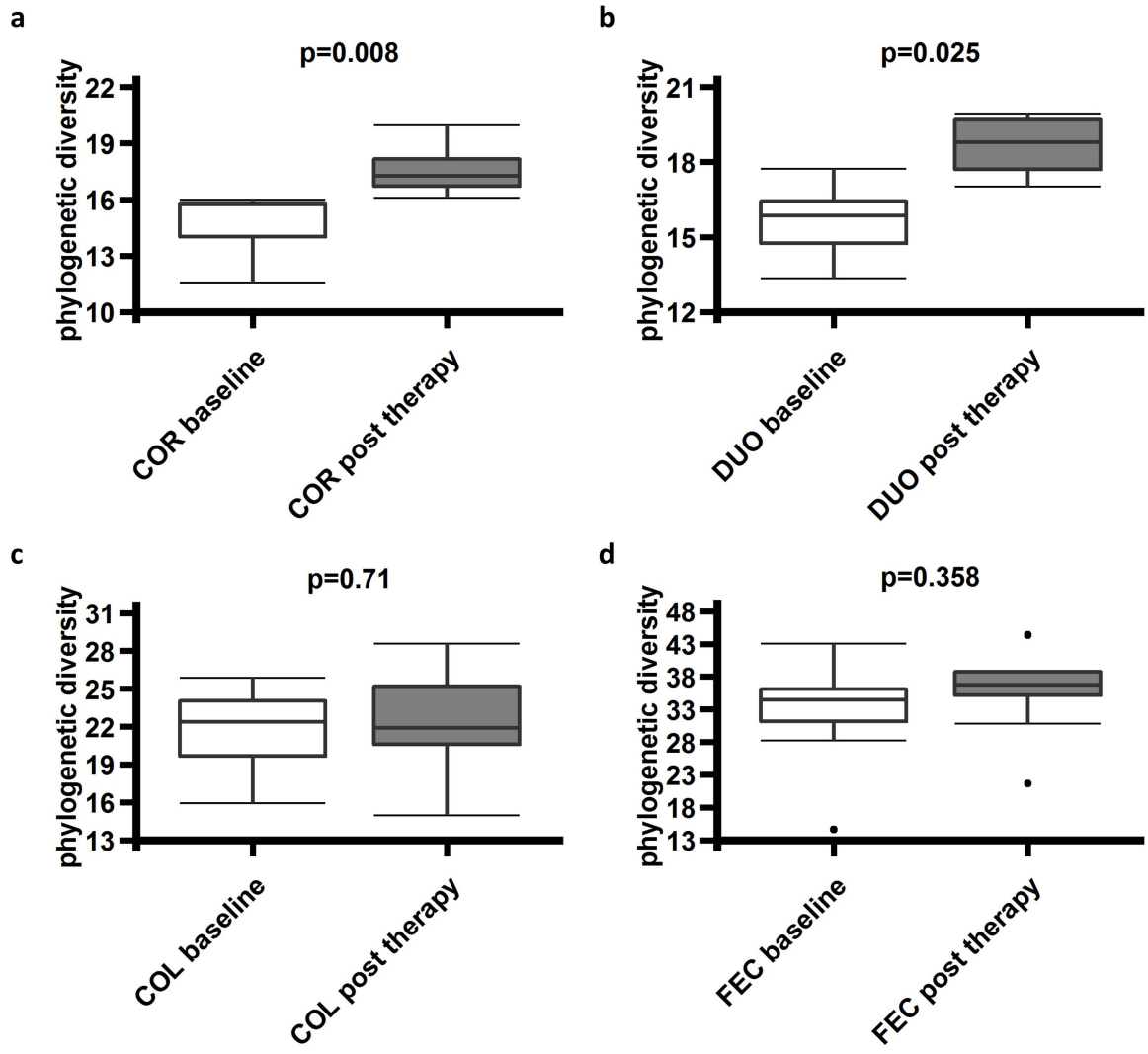

Figure 20 | Phylogenetic diversity increases in the mucosa of the upper but not lower gastrointestinal tract after synbiotic treatment. a,**b |** Phylogenetic diversity was analyzed in mucosal samples at baseline (white) and after synbiotic treatment (grey). Mucosal specimens from the gastric corpus (COR,p=0.008) and duodenum (DUO,p=0.025) revealed an increased phylogenetic diversity after synbiotic treatment. **c,d |** No differences in colonic samples (COL,p=0.710) and feces (FEC, p=0.358) were seen after treatment. (Faith's Phylogenetic Diversity, based on UniFrac phylogenetic distance)

3.2.4 Synbiotic effect on microbial abundances in the gastrointestinal tract

Bacterial community profiling was applied to upper and lower gastrointestinal mucosal specimens and fecal samples. Thereby, mucosal samples of the Gastric Corpus depicted increased relative abundances of unclassified *Halomonas* (p=0.007), unclassified *Neisseriaceae* (p=0.010), *Propionibacterium Acnes* (p=0.040) and *Clostridiaceae* (p=0.029) as well as reduced *Actinobacteria* (p=0.027) after synbiotic treatment. Mucosal samples of the duodenum showed elevated unclassified *Schwartzia* (p=0.013) and *Catonella* (p=0.029) as well as a reduction of an unclassified *Lactobacillus* after synbiotic treatment. No taxa showing significantly different relative abundances before and after treatment could be shown in colonic mucosal specimens. A higher abundance of unclassified *Lactobacillaceae* (p=0.006) could be found in fecal samples after synbiotic treatment. *Moraxella* (p=0.022) and *Moryella* (p=0.022) diminished after treatment in fecal samples (**Figure 21**)

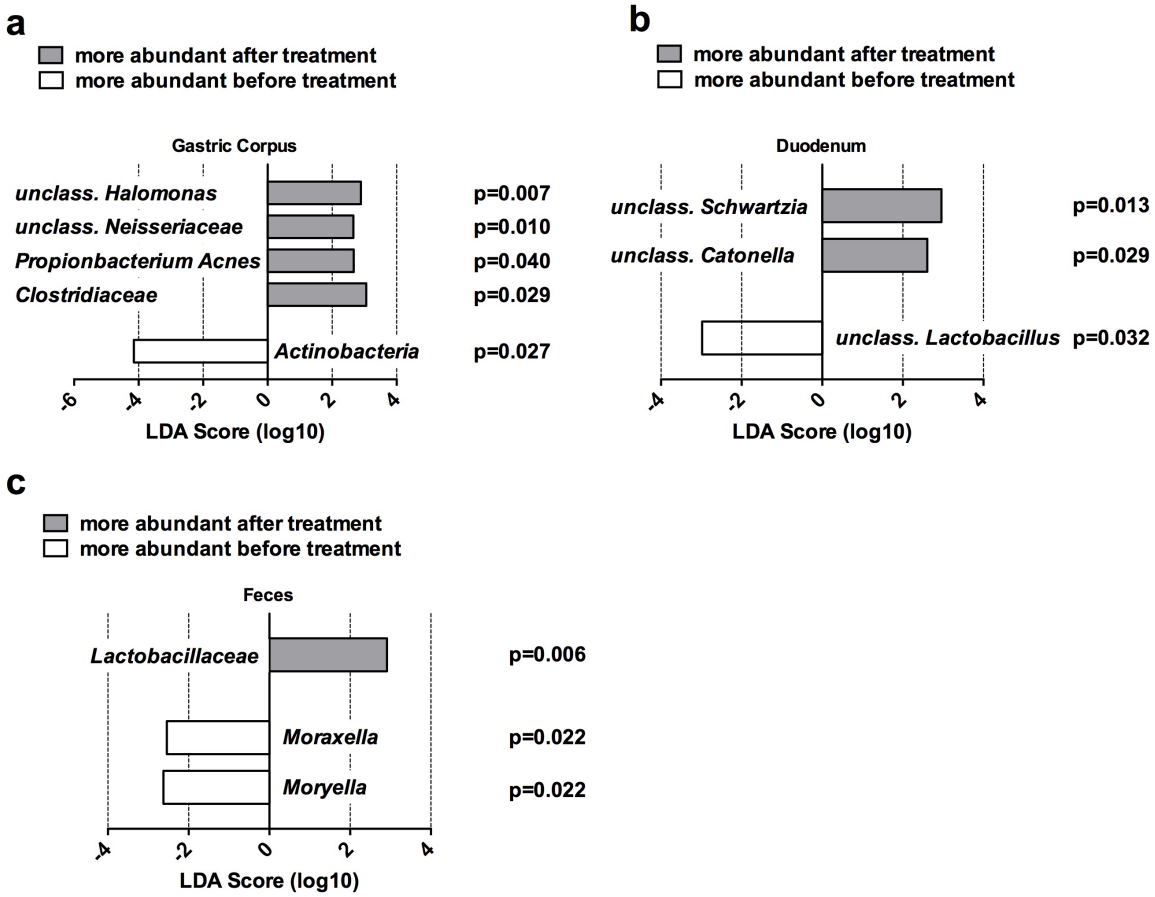

Figure 21 | Linear Discriminant Analysis of fecal and mucosal samples before and after synbiotic treatment. Linear Discriminant Analysis (LDA) was generated with LEfSe. **a |** Mucosal samples of the Gastric Corpus showed increased unclassified *Halomonas* (p=0.007), unclassified *Neisseriaceae* (p=0.010), *Propionibacterium Acnes* (p=0.040) and *Clostridiaceae* (p=0.029) as well as reduced Actinobacteria (p=0.027) after synbiotic treatment. **b |** Duodenal samples showed increased unclassified *Schwartzia* (p=0.013) and *Catonella* (p=0.029) as well as a reduction of an unclassified Lactobacillus after synbiotic treatment. (**c**) Fecal samples showed increased *Lactobacillaceae* (p=0.006) as well as diminished *Moraxella* (p=0.022) and *Moryella* (p=0.022) after synbiotic treatment.

3.2.5 Synbiotic effect on fecal SCFA levels

SCFAs in fecal samples were analyzed by high-performance liquid chromatography (HPLC). Synbiotic treatment significantly increased acetate and butyrate levels (**Figure 22**). (acetate, 101 [79-133] vs. 202 [68-252]; p=0.007) (butyrate, 27 [14-35] vs. 40 [25-67]; p=0.037). Synbiotic treatment did not affect propionate, iso-butyrate, iso-valerate and valerate levels.

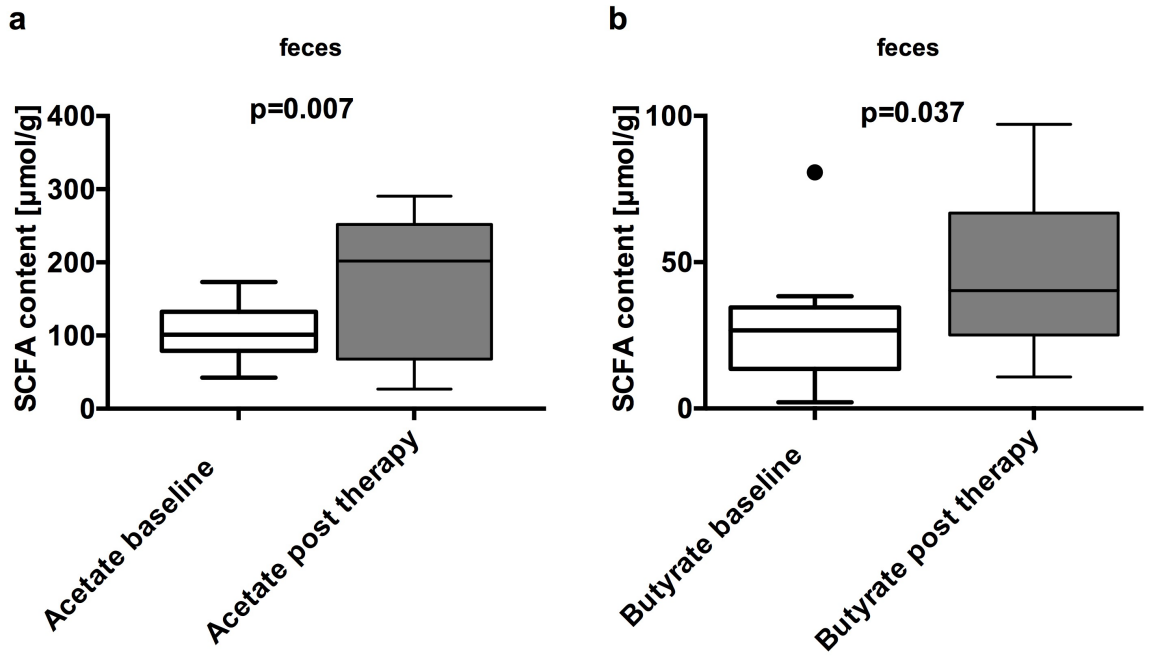

Figure 22 | Short-chain fatty acids are elevated in fecal samples after synbiotic treatment.
Fecal samples were analyzed by HPLC before (white) and after synbiotic treatment (grey). Synbiotics significantly increased fecal levels of acetate (101 [79-133] vs. 202 [68-252]; p=0.007) and butyrate (27 [14-35] vs. 40 [25-67]; p=0.037) [µmol/g]. (Median [Q1-Q3]; Mann–Whitney *U* test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey).

3.2.6 Synbiotic effect on fecal zonulin concentration

Zonulin concentration was analyzed in fecal samples by using competitive ELISA at baseline and after synbiotic treatment. Synbiotic treatment decreased fecal zonulin concentration [ng/ml] (**Figure 23**). (67 [38-92] vs. 36 [20-48]; p=0.035).

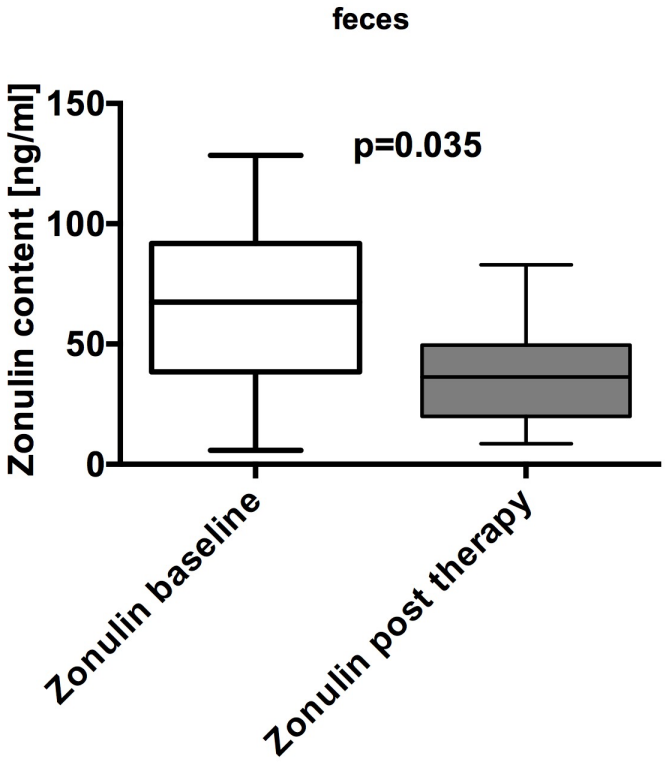

Figure 23 | Fecal zonulin concentration decreases after synbiotic treatment.

Fecal samples were analyzed by ELISA before (white) and after synbiotic treatment (grey). Synbiotic treatment significantly decreased fecal zonulin concentration [ng/ml]. (67 [38-92] vs. 36 [20-48]; p=0.035). (Median [Q1-Q3]; Mann–Whitney *U* test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey).

3.2.7 Synbiotic effect on symptom severity

The IBS-SSS (irritable bowel syndrome severity scoring system) was questioned to assess clinical effects of synbiotic treatment. Synbiotic treatment significantly improved symptoms in IBS-D patients (**Figure 24**). (237 [129-256] vs. 54 [12-158]; p=0.002).

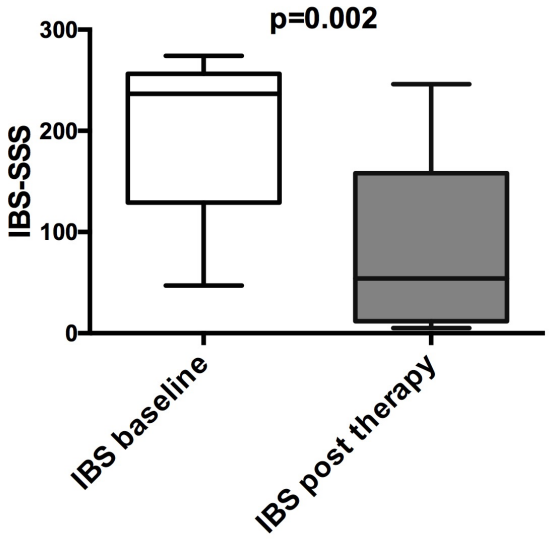

Figure 24 | Symptom severity decreases after synbiotic treatment.

Patients were questioned using the Irritable bowel syndrome symptom severity score (IBS-SSS) before (white) and after synbiotic treatment (grey). Synbiotic treatment significantly decreased symptom severity. (237 [129-256] vs. 54 [12-158]; p=0.002). (Median [Q1-Q3]; Mann–Whitney *U* test or t-test to compare non-gaussian and gaussian variables. Boxplots according to Tukey).

4 Discussion

4.1 MS Study

The focus of the MS Study was to investigate the colonic microenvironment of patients with early stage MS compared to healthy controls. As the role of the microbiota and the gastrointestinal immune system in patients with MS is still unknown, the MS Study tried to reveal differences in the colonic microenvironment which could link immunological observations in different animal EAE models to human MS. EAE studies in mice suggest that an altered composition of the intestinal microbiota influences metabolites (e.g. SCFAs such as butyrate) and thereby either trigger pro-inflammatory responses (e.g. Th17 cells) or reduce anti-inflammatory cells (e.g. Tregs) in the mucosal immune system mediating CNS directed autoimmunity. (58, 149, 188). As recent studies show a distinct pattern of microbial composition in fecal samples of MS patients, it is of the utmost interest if these observations in fecal samples also impact functional patterns of the microbiota and most importantly, the colonic mucosal immune system. (122) The promotion of Tregs in the colonic mucosa has been shown to be dependent on the presence and extent of certain SCFAs and other immune cells such as CD103+ DCs. (15, 57) Furthermore, Tregs can influence the course of EAE and might therefore be of clinical relevance in patients with MS. (15) We therefore investigated these known factors contributing in animal models of MS to disease initiation and progression (DCs, Tregs, SCFAs and microbiota composition) in patients with MS and compared them to healthy individuals. As the MS study is a Pilot study and only observational, no causal relationships can be concluded. Nevertheless, these findings could stimulate controlled interventional trails shaping the intestinal microbiota (e.g. fecal microbiota transplantation) which already have been described to affect the course of multiple sclerosis in single MS patients. (1, 189)

The MS Study reveals that MS patients have an altered colonic microenvironment depicted by a dysregulation of colonic mucosal immune cells (DCs, CD103+DCs and Tregs) and bacterial metabolites compared to healthy individuals. Moreover, these alterations are associated to a specific microbial composition. MS patients show reduced numbers of total DCs and CD103+DCs accompanied with a Treg deficiency only in the distal colon, despite normal levels of CD4+ and CD8+ T cells in both proximal and distal colon (**Figure 8-11**). (1) These alterations could provide evidence for a regional regulatory deficiency of the distal colon in MS patients, indicating that the colon might be have distinct immunological compartments and functions. (104, 164) These compartmental immune patterns along the

colon are also seen in other immune-mediated diseases (e.g. ulcerative colitis, Crohn's disease and intestinal graft-versus-host disease) also depicted by differences of mucosal dendritic cells in the proximal and distalcolon. (1, 164) As the proximal and distal colon differ in embryologic origin, gene-expression patterns, gene-methylation status, microbiota load and microbiota metabolic activity (e.g. SCFAs), these findings suggest a concept of colonic immune compartmentalization which could be mediated by altered amounts of bacterial metabolites along the colon. (190-192) These findings might contribute to a further investigation of the basic immunological role of the colon. (1, 104, 164) One might hypothesize that different immune effector cells could be released from alternate sites of the gastrointestinal tract to influence physiological immune tolerance and organ specific autoimmunity, as intestinal DCs act as antigen-presenting cells and are crucial in maintaining immune tolerance by interaction with numerous intestinal antigens. (148) Thereby, lamina propria DCs can transport bacterial antigens and products to the mesenteric lymph nodes, balancing between tolerance of the commensal microbiota and surveillance of invading pathogenic bacteria. (193, 194) As this tolerogenic effect is partly mediated by DCs via generation and maintenance of Tregs, alterations in number and function of mucosal DCs, as observed in the MS Study, could alter the ability of Tregs and hamper anti-inflammatory mechanisms. (1, 195) Thereby, CD103+ DCs are known to be most important for the differentiation of Tregs, mediating tolerogenic activities in mice and humans. (196, 197) As the results in the MS Study show a combined reduction of total DCs, CD103+DCs and Tregs only in the distal colon, one might hypothesize a mentioned regional regulatory deficiency, which in turn influences the pathogenesis of MS as Tregs are pivotal in protecting against CNS demyelination in mice and their expansion has been reported to occur in gut mucosa and draining lymph nodes of EAE protected germ-free mice. (1, 15, 149, 164)

The background of these regional differences of tolerogenic immune cells remains elusive. As altered amounts of bacterial metabolites along the colon are of relevance and SCFAs where shown to be critical for EAE development, luminal SCFA concentration might be the trigger of deciding number and properties of mucosal immune cells. (57, 58, 192) SCFAs are important for maintaining a tolerogenic mucosal environment and mediate protection from CNS demyelination (propionate and butyrate) through expansion of intestinal Tregs. (58) Furthermore, SCFAs endow dendritic cells with an enhanced ability to induce the differentiation of Tregs. (30, 57, 150) As MS patients in the MS Study showed massively reduced (by over 50%) levels of total SCFAs as well as acetate and butyrate compared to controls (**Figure 13**), it is tempting to hypothesize that the regional regulatory immune cell

deficiency of MS patients might be due to a lack of SCFA-induced regulatory immune cells. The cause of these regional differences might be due to alterations in the SCFA concentration along the colon as well as diverging SCFA availability to the colonic mucosa. (1, 104, 164, 192) Furthermore, as long-chain fatty acids can promote Th17 cells in the small intestine, varying regional impacts of bacterial metabolites on mucosa-associated immune cells may be of relevance. (58) Nevertheless, these data support the concept causally shown in animal models of EAE, where mucosal immune cells are influenced by SCFAs. Thereby, SCFA treatment improves CNS demyelination via long-lasting imprinting on lamina-propria-derived Tregs, demonstrating that intestinal-specific immune cells can subsequently impact central nervous autoimmunity. (58) As western lifestyle has been proposed to increase the incidence of MS and adherence to a Mediterranean diet elevates butyrate, acetate and propionate levels in healthy individuals, dietary habits might contribute to the observed dysregulation in altering the intestinal microenvironment. (1, 198, 199)

The origin of the observed changes of fecal SCFAs in MS patients, especially butyrate, are of great interest since recent findings support that compositional changes of the fecal microbiota in MS patients include a depletion of species belonging to *Clostridia XIVa* cluster. The *Clostridia* clusters XIVa are constituted by highly diverse bacterial species and many of them can produce short chain fatty acids (e.g. butyrate). (122) In the MS Study, taxa belonging to the order *Clostridiales* were significantly different in MS patients compared to controls including a reduction of *Roseburia spp*, a bacterial genus belonging to the Clostridia cluster XIVa (**Figure 15**). *Roseburia spp* are potent butyrate producers and are thought to be important in the homeostasis and integrity of mucosal barrier and immunity.(200) Furthermore, *Roseburia spp* were associated to decreased butyrate levels in autoimmunity. (30, 201) As the observed reduction of *Roseburia spp* merge with the recently described reduction of Clostridia XIVa clusters in MS patients, investigation of the impact of the observed taxonomic changes on quantity of butyrate-production genes in MS patients compared control stools was performed. Quantitative PCR analyses of the genes in stool samples most importantly involved in butyrate production (butyrate kinase [*buk*] and butyryl-CoA: acetate CoA-transferase [*but*]) showed no significant differences for *buk* and *but* genes between the microbial communities of MS patients and controls (**Figure 17**). (177) These results challenge the idea that specific compositional alterations of the gut microbiota (e.g. depletion of *Clostridia cluster XIVa* bacteria) in MS patients impact the genetic framework of butyrate production as proposed recently. (122) The MS Study results suggest that the observed microbial changes in MS patients do not explain the reduction of butyrate

and other SCFAs. As levels of several SCFAs (total SCFAs, acetate, butyrate and marginally not-significant propionate and valerate) are reduced to a comparable extent in MS patients compared to controls, the MS Study proposes a more generalized metabolic disturbance of SCFA production. This hypothesis is supported by the findings of strong positive correlations between acetate and propionate as well as butyrate in MS patients and controls, possibly depicting the interdependence of SCFA production. (**Figure 14**). Different factors contribute to intestinal SCFA production in general such as diet, substrate availability and synthesis capacities of bacteria. (192) Therefore, possible explanations for the reduction of fecal SCFAs in MS patients should further investigate the contribution of the observed microbial alterations and above mentioned factors to better understand the background of the generalized metabolic disturbance leading to reduced fecal SCFA levels in MS patients. Other differences observed in the composition of the fecal microbiota in MS patients compared to controls comprised elevated *Oscillospira spp* together with *Ruminococcacceae* and reduced *Prevotella* genus in MS patients (**Figure 15**). Increased abundance of *Oscillospira* was associated to autoimmunity in mice lacking Tregs linking *Oscillospira* overabundance to an inflammatory environment. (202). However, *Ruminococcacceae* are very potent butyrate producers. The elevation in MS patients could be of an compensational character to balance the lack of butyrate but remains to be explained . (203) Of interest, no disease specific clustering in PCoA analysis and no difference in the bacterial diversity could be observed (**Figure 16**).

The MS Study focused on patients with early MS. It is therefore of a speculative nature if the observed changes in the colonic mucosa are increasing in advanced disease stages, which needs to be acknowledged as a limitation of the study. Furthermore, as the study design also prohibited a closer matching of patients and controls, a significant age difference was observed. However, our correlation analysis of the effect of age on Tregs shows that the significantly higher age of controls should have led to an underestimation rather than an overestimation of observed differences (**Figure 12**). Further limitations of study could be that participating individuals may not be fully representational of patients and healthy individuals in general, although many exclusion criteria were applied to avoid any systematic bias. It is therefore of the utmost importance to replicate the study results and perform future studies on a larger number of patients. As the MS Study cannot serve to establish a causal relationship regarding the immunologic changes observed, a suggested interplay between alterations of colonic immune cells of the distal colon and impaired

microbiota structure and function has to remain speculative as our observations are based on cross-sectional data. (1)

4.1.1 Conclusion

The MS study delivers human data supporting animal models that imply systemic immunological changes driving CNS autoimmunity being linked to intestinal microbial composition and luminal SCFA content. These processes might thereby impact on the regulatory capacity of mucosal DCs and the formation of a colonic Treg pool. (57) Thereby, a disturbed luminal SCFA content (especially acetate and butyrate) jointly with an impaired number of total and tolerogenic DCs could consecutively lead to a regional regulatory deficiency in the distal colon depicted by a quantitative impairment of Tregs in MS patients. The observed mucosal immunological changes might be translated systemically throughout the whole body and reach the CNS. Manipulation of the intestinal ecosystem influenced the lymphatic immune system in mice stimulating CD103+DCs residing in cervical lymph nodes mediating attenuation of CNS autoimmunity. (15) As deep cervical lymph nodes were recently shown to communicate with a CNS associated lymphatic system in humans and the lymphatic system is known to communicate within the whole body, information from the intestinal lymphatic system is likely to reach the CNS. (102, 103)

In sum, the MS Study substantiates a disturbed colonic microenvironment in MS patients which may play a role in the immunopathogenesis of MS. Further studies should elucidate a possible causal role of the microbiota in MS and therapeutic interventions targeting the microbiota of MS patients. (1)

4.2 IBS-D Study

The IBS-D Study investigated the effect of oral synbiotic therapy on mucosal immune cells (in duodenum and proximal colon), SCFAs from stool and microbiota (stool as well as the mucosal microbiota from gastric corpus, duodenum and proximal colon) in ten patients with IBS-D. As the role of the gastrointestinal immune system and microbiota in IBS-D is still under investigation, the IBS-D Study tried to reveal possible sites and mechanisms of how synbiotics mediate a therapeutic effect in IBS-D. Specific compositional changes of the mucosal and fecal microbiota in IBS-D patients accompanied with a diminished microbial biodiversity in fecal samples have been shown. (204) The observed compositional changes could be involved in triggering mucosal inflammatory responses and may have a pathogenic role in IBS-D. (151, 153-155) Therapies influencing the composition and function of the intestinal ecosystem are therefore of interest although mechanisms of action of pro-and synbiotic therapies are not fully understood and remain to be elucidated. (88, 89, 205) Mucosal inflammatory processes present in IBS-D involve activated mast, B and plasma cells accompanied with an impaired barrier function in jejunal specimens associated to disease activity. This might reflect the importance of an intact intestinal microbiota in maintaining the intestinal barrier and protecting against exaggerated mucosal immune responses. (156, 157) The possible dysbiosis-triggered mucosal inflammation is characterized by elevated pro-inflammatory cytokines in mucosal samples of IBS-D patients interacting with colonic nociceptive and non-nociceptive afferents. Thereby, mechanosensory colonic c-fibers are sensitized and activated mediating pain symptoms in patients with IBS-D. (153) These microbiota-neuro-immunological interactions might therefore be involved in triggering intestinal hypersensitivity and pain suggesting that IBS-D has a microbiota-dependent immune-mediated pathogenesis. This is reflected by the fact that the grade of mucosal inflammation is associated to clinical disease activity underlining the pathogenic role of the gastrointestinal immune system IBS-D. (153, 156) Probiotics are effective in relieving IBS-D symptoms and interactions with mucosal immune cells (e.g. DCs and Tregs), direct impacts on mucosal barrier and the microbiota are suspected mechanisms of action. (29, 33, 158, 159). Synbiotic effects remain to be uncertain, but as prebiotics powerfully impact microbiota composition and function (e.g. through influencing intestinal microbial patterns), they may be able to shape metabolite production (e.g. SCFAs). (160)

The IBS-D Study shows that oral synbiotic treatment affects various sites and different levels of the gastrointestinal tract in IBS-D patients. Thereby, effects on immune function, biodiversity, metabolic activity and mucosal barrier could be responsible for clinical responses to treatment. As mentioned above, increased mucosal inflammation is associated with IBS-D disease activity and pathogenesis (in contrast to constipation-type IBS, where the fecal microbial composition mediated mucosal anti-inflammatory responses). (206) Moreover, elevated numbers of T cells have been shown in jejunal specimens of IBS-D patients. (153, 207) Oral synbiotic treatment reduced the number of CD4+ T cells in the proximal colon (**Figure 18**). Naïve mucosal CD4+ T cells are a major pool and source of inflammatory cells of the gastrointestinal immune system and are thereby involved in variety of inflammatory responses. (48) The treatment-induced reduction of CD4+ T cells might therefore influence the general inflammatory state in the mucosa of IBS-D patients and may lead to decreased mucosal inflammation (109). Furthermore, the treatment-induced reduction of CD4 + T cells (CD3+ CD4+CD8-) led to a trend towards an induction of double-negative T cells (which are CD3+ CD4- CD8-). The function of these cells is not well understood, although anti-inflammatory properties have been proposed similar to Tregs. The possible origin of double negative T cells is still unclear but studies have shown that CD4+ T cells can convert to double negative Tregs in vitro and in vivo (which is supported by the IBS-D Study). Double negative T cells can thereby suppress CD8+ T cell, CD4+ T cells , B cells and DCs. (208) The observation of a trend towards treatment-induction of double negative T cells might therefore be associated to anti-inflammatory patterns and of clinical interest. In sum, synbiotics influenced the number of mucosal CD4+ T cells and double-negative T cells (Tregs) in the proximal colon but no other cell line such as classical Tregs or DCs in neither duodenum nor proximal colon was affected. These observations might be involved in driving anti-inflammatory patterns to modulate IBS-D.

Mucosal bacterial composition and diversity is linked to gastrointestinal immune reactions and diseases such as celiac disease. Thereby, oral exposure to helminth antigens increased duodenal mucosal diversity in humans. (209) Increased treatment-induced mucosal microbial diversity can stimulate SCFA production and promote Tregs which can dampen inflammatory responses in immune-mediated diseases. (210) As probiotics induced tolerance genes in duodenal mucosa specimens in humans after oral administration, assessment of direct mucosal influences of oral synbiotic therapy was investigated in mucosal specimens of the gastric corpus, duodenum and proximal colon. (90) Furthermore, fecal samples were analyzed as well. Remarkably, synbiotic treatment influenced mucosal

diversity in the upper but not the lower gastrointestinal tract showing pronounced increases of diversity of the microbiota in gastric and duodenal mucosa, whereas diversity in colon and feces remained unaffected (**Figure 20**). The observed elevations of diversity in gastric and duodenal specimens were not chaperoned by increases in the orally administered probiotic strains, which is a known phenomenon in probiotic therapy. Oral probiotics might nevertheless catalyze the effect of boosting mucosal diversity. (88) As *Lactobacillaceae* could only be found elevated in fecal samples but not in any mucosal specimens after treatment, it can be hypothesized that probiotic strains need specific environmental circumstances being prone to region-specific populating factors (**Figure 21**). The backgrounds of region-dependent populating factors may include host-, microbiota and strain-specific properties. Fecal samples depicted a notably reduction of *Moraxella* and *Moryella* but the importance of this observations remains to be elusive. Taken together, synbiotic therapy induced an increased microbial diversity in mucosal specimens of the upper but not lower gastrointestinal tract. Orally administered probiotics could only be found in fecal samples but not in any mucosal habitat. If the observed treatment-induced changes are due to the probiotic or prebiotic substances in the administered mixture remain uninvestigated but since prebiotics are known to influence intestinal microbial composition and production of SCFAs in humans, the observed modulations by synbiotic treatment may also be due to its prebiotic components (86).

SCFAs are known to be pivotal for intestinal homeostasis and tolerogenic mucosal immune reactions. (50) In general, IBS patients show decreased fecal SCFA levels which may contribute to mucosal inflammation in IBS-D since SCFAs can interact and stimulate Tregs and other immune cells. (57, 162) In the IBS-D Study, synbiotic treatment elevated fecal acetate and butyrate levels (**Figure 22**). Since *Lactobacillaceae* mediate SCFA production, increased abundance observed in fecal samples after treatment could promote SCFA levels and be responsible for elevated treatment-induced fecal acetate and butyrate levels (211). Probiotic bacteria are metabolically active depending on the host microbiota and initial fecal butyrate level in healthy individuals. Thereby, the impact of probiotics on fecal butyrate level was highest in individuals with low butyrate concentration before treatment. As IBS patients are known to depict low fecal SCFA levels as mentioned above, probiotics might exert powerful abilities in elevating SCFAs in IBS-D patients. (33, 162, 212). Intestinal SCFAs are also thought to stabilize intestinal barrier function even after chemical disruption and might therefore be of importance in prophylaxis and treatment of gut leakiness. (213, 214) IBS-D patients depict abnormalities in their modulation of epithelial barrier function.

(157, 215) Therefore, investigation of fecal zonulin concentration, a surrogate for intestinal barrier, was performed to analyze the effect of synbiotics on intestinal barrier function. (30, 216) Synbiotics significantly decreased fecal zonulin concentration (**Figure 23**). Thus, synbiotic treatment is likely to influence intestinal barrier function in IBS-D. The impacts of stabilizing the epithelial barrier could also influence mucosal inflammation and known intestinal permeability disruptions in IBS-D. These effects might therefore be associated to clinical responses of oral synbiotic therapy seen in the IBS-D Study, depicted by a significantly lower symptom severity score after treatment (**Figure 24**). The limitation of the IBS-D study is the study design without controls or placebo-controlled design. These findings must be therefore further investigated in a double-blind placebo-controlled study to causally link the observed effects to oral therapy. In sum, synbiotic therapy elevated fecal acetate and butyrate levels accompanied by a reduction of fecal zonulin. These observations indicate possible effects of synbiotic therapy on microbiota metabolism and intestinal barrier function.

4.2.1 Conclusion

The IBS-D Study reveals potential mechanisms of action of oral synbiotic therapy in IBS-D, although no causal explanations can be concluded due to study design. The observed effects comprise influences on mucosal immune cells, elevations of mucosal microbiota diversity, induction of fecal SCFA levels and stabilization of intestinal barrier function. Studies in IBS-D suggest that altered microbial composition associated to mucosal inflammation, fecal SCFA depletion and disrupted intestinal barrier might play pivotal roles in the pathogenesis of IBS-D, factors that may be influenced by oral synbiotic therapy as depicted by observations in the IBS-D study. As the observed effects correlate with a reduction of symptom severity and probiotics are effective in improving IBS-D symptoms, the elucidated mechanisms might contribute to clinical efficacy. (217) These findings must be evaluated in double-blinded controlled trials to understand causal relations and elucidate mechanisms for optimizing therapeutic possibilities of synbiotics in IBS-D.

4.3 General Conclusion

The MS Study and IBS-D Study investigate the gastrointestinal ecosystem comprising intestinal mucosal immune cells, microbiota and metabolites in two different diseases. As general conclusions about possible similarities transferring results from the MS Study to the IBS-D Study are not scientifically valid, one might hypothesize that the treatment effects seen in the IBS-D Study could be of importance in MS. Probiotics are under current discussion as an unconventional new treatment option for MS, although no positive clinical study outcome are published to date. (218, 219) In contrast, animal models of EAE and other immune-mediated diseases show influences of oral probiotic therapies on Tregs and other immune cells being involved in CNS autoimmunity thereby ameliorating inflammatory responses. (220-222) In the MS Study, a regional regulatory deficiency of the distal colon was evident in MS patients being associated to reduced fecal SCFA levels. As synbiotic therapy stimulated fecal SCFA levels in IBS-D patients, oral synbiotic intervention could hypothetically elevate fecal SCFA levels in MS patients as well. If the observed regional regulatory deficiency of the distal colon in MS patients is rooted in compositional changes of the intestinal microbiota influencing SCFA levels, interventions comprising oral pre-or probiotics, diet changes, antibiotic therapy or even fecal microbiota transplantation could influence colonic mucosal immune cells numbers and function. Further double-blind randomized controlled studies are needed to investigate possible effects of the above mentioned interventional possibilities on the course of MS or other immune-mediated diseases. As the first study comprising human data of the gastrointestinal immune system in MS patients to date, the MS Study reveals indications that may be used as a ratio for further investigative studies in MS patients. The IBS-D Study warrants a comprehensive view of interventions targeting the gastrointestinal microbiota in human diseases, as effects of therapies might substantially differ between regions and influence distinct members of the gastrointestinal ecosystem (e.g. microbiota composition, mucosal immune cells, and metabolites). (1)

5 References

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