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Human GI Tract Microbiota in Health and Disease: A Focus on Inflammatory Bowel Diseases

Clarissa Santos Rocha[1], Marcela de Azevedo[1], Daniela Santos Pontes[2], Pamela Mancha Agresti[1], Vanessa Bastos Pereira[1], Camila Prósperi de Castro[1], Vanessa Pecini da Cunha[1], Alberto Fernandes de Oliveira Junior[1], Cassiana Severiano de Sousa[1], Tessália Diniz Luerce Saraiva[1], Tatiane Melo Preisser[1], Bianca Mendes Souza[1], Meritxell Zurita Turk[1], Vasco Azevedo[1], Maarten van de Guchte[3], Anderson Miyoshi[1]* and Rodrigo Dias de Oliveira Carvalho

[1]Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil

[2]Departamento de Ciências Biológicas, Campus V, Universidade Estadual da Paraíba, Brazil

[3]Institut national de la recherche agronomique, France

***Corresponding author:** Anderson Miyoshi, Laboratório de Genética Celular e Molecular (LGCM), Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (ICB/UFMG), CP 486 CEP 31270-901, Belo Horizonte-MG, Brazil, Tel: 005531 3409 2610; Email: miyoshi@icb.ufmg.br

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ABSTRACT

The study of bacteria-host interactions and their importance for human health is an area of growing interest. While much progress has been made in the understanding interactions between pathogenic bacteria and the host and their relation with human health, an increasing interest for commensal bacteria that reside in the human gastrointestinal tract, constituting the densest bacterial population known to date, has emerged. Recent application of molecular biology techniques, including high throughput sequencing of bacterial genomes, have allowed to identify a more representative description of the gastrointestinal tract's microbiota members and the functions encoded by these organism. A growing number of studies indicate that the intestinal microbiota influences host energy balance and immune responses, and contributes to gut homeostasis. More recently, it has been indicated that correlations between disturbed/

imbalanced microbial composition (dysbiosis) and diseases, such as inflammatory bowel diseases, obesity, diabetes, cancer and several extra intestinal diseases, exist. However, little is still known about the molecular mechanisms or the bacterial effector molecules involved in this bacteria-host dialogue. Here we provide an up-to date review of the present knowledge of the impact of gut microbiota to human health, as well as an overview of the main factors identified so far in the host-bacteria interactions in the gastrointestinal tract. Finally, we discuss the use of probiotics as new tools to prevent and treat inflammatory bowel diseases.

Keywords: Inflammatory Bowel Diseases; Mucosal Immune System; Intestinal Microbiota; Gastrointestinal Tract; Probiotics.

ABBREVIATIONS

ASF-Altered Scheidlre Flora; B-B-lymphocytes; *Card15*-Caspase Recruitment Domain Family member 15; CD-Crohn's Disease; CLA-Conjugated Linoleic Acid; COPD-Chronic Obstructive Pulmonary Disease; COX-2-Cyclooxygenase 2; DCs-Dendritic Cells; DSS-Dextran Sulfate Sodium; FIAF-Fasting-Induced Adipocyte Factor; GALT-Gastrointestinal Associated-Lymphoid Tissue; GALT-Gastrointestinal-Associated Lymphoid Tissue; GF mice-Germ Free mice; GI–Gastrointestinal; GWS-Genome Wide Scanning; HeLa Cells-Human Epitheloid Cervix Carcinoma Cell Line; HT29 Cells-Human Colorectal Adenocarcinoma Cell Line with Epithelial Morphology; IBD-Inflammatory Bowel Diseases; IBS-Irritable Bowel Syndrome; IECs-Intestinal Epithelial cells IECs; IELs-Intraepithelial Lymphocytes; IFLs-Lymphoid Follicles; IFN-α/β–Interferon-α or Interferon-β; IgA-Secreted Immunoglobulin A; IHMC-International Human Microbiome Consortium; IKK Complex-IKappaB Kinase Complex; IL–Interleukin; IP-10-Interferon-Inducible Protein; IRAK-IL-1 Receptor Associated Kinases; IRAK4/IRAK1 - IL-1 Receptor-Associated Kinases; LAB-Lactic Acid Bacteria; LPL-Lipoprotein Lipase; LPS–Lipopolyssacharide; LTA-Lipoteichoic Acid; M-Microfold cells; MAMPs-Microbe-Associated Molecules; MAPKs-Protein Kinase; MDP-Muramyl Dipeptide; MDP-Muramyl-Dipeptide (MDP); MEKKs-Protein Kinase Kinases; *Meso* DAP- *Meso*-Diaminopimelic Acid; MHC I-Class I Major Histocompatibility Complex; MHC II-Class II Major Histocompatibility Complex; MLN-Mesenteric Lymph Nodes; NEC-Necrotizing Enterocolitis; NF-κB-Nuclear factor κB; NOD1-Pattern Recognition Receptor NOD1; PGN-Peptidoglycan (PGN); PP-Peyer Patches; PPARγ-Peroxisome Proliferator-Activated Receptor γ; PSA-Polysaccharide A; Regulatory T cells-Treg Cells; SCFA-Short Chain Fatty Acids; SED-Subepithelial Dome; SNP-Single Nucleotide Polymorphism; T84 Cells-Colonic Adenocarcinoma Cell Line; TAK1-Factor-β Activated Kinase; TGF-β-Transforming Growth Factor- β; TH1-T Helper 1 Cells; TH17-T Helper 17 Cells; TH2-T Helper 2 Cells; TLR-Toll Like Receptors (TLR); TNBS-2,4,6-Trinitrobenzenesulfonic Acid; TNBS-2,4,6-Trinitrobenzenesulfonic Acid; TNF-α–Tumorous Necrosis Fator; TRAF6-TNF

Receptor Associated Factor; TSLP-Thymic Stromal Lymphopoietin; UC-Ulcerative Colitis; WGS-Whole Genome Shotgun; γδ T Cells-T Gamma Delta Lymphocytes.

INTRODUCTION

The study of bacteria - host interactions and their role in human health is an area of growing interest. While much progress has been made in the understanding of interactions between pathogenic bacteria and the host, there is an increasing interest in understanding the relationship of the host and commensal bacteria that reside in the human gastrointestinal (GI) tract, constituting the densest bacterial population known to date, and which under normal conditions live in a symbiotic relationship. A growing number of studies indicate that the intestinal microbiota influences host energy balance and immune responses, and contributes to gut homeostasis. It has been shown that the intestinal microbiota also influences the immunologic development of the host, the immune responses and the human susceptibility to a variety of diseases. A disturbed/imbalanced microbial composition (dysbiosis) has been associated with diseases, such as obesity [1,2], diabetes [3], cancer [4,5], several extra intestinal diseases [6-9] and inflammatory bowel diseases (IBDs) [10-13].

It has been well established in animal models and humans that some components of the microbiota are an essential requirement for full expression of IBDs. Patients with IBDs have shown to present an abnormal luminal microbiota with disruption of microbial diversity, presenting a reduced bacterial diversity, bacteria from the *Clostridium* groups IV, XIV a (*Faecalibacteriumprasnitzii*), Bifidobacteria and lactobacilli with an increase in mucosal bacterial number, *Mycobacterium aviumparatuberculosis, Clostridium difficile, Ruminococcusgnavus* and Enterobacteriaceae. It has also been suggested that a lack of bacteria with anti-inflammatory properties could be a key factor in the persistence of inflammation [14-24].

IBDs, including ulcerative colitis (UC) and Crohn's disease (CD), are complex intestinal diseases characterized by spontaneous and chronic inflammation in the gastrointestinal tract (GIT). Despite much research, the exact etiology and pathogenesis of these diseases remain unclear; however, it is widely accepted that IBDs are caused by a deregulation of the mucosal immune system toward the native intestinal microbiota in genetically predisposed individuals [25], leading to the inappropriate and excessive activation of the intestinal immune system causing pathogenic gastrointestinal inflammation and tissue damage [26]. Four factors contribute to the development of IBDs: microbiota, genes, immunity and environment, being none alone is sufficient for the development of this disease.

Current treatments for IBDs are restricted to the use of anti-inflammatory drugs, immunesupressors and antibiotics, which have the objective of controlling the development of inflammation. However, these drugs are associated with serious collateral side effects, such a fever, allergic reactions and liver problems [27,28], and their therapeutic effect is quite limited,

as such, research has been now, for many years, focusing on developing new strategies for the treatment of IBDs.

Probiotics have been capable at preventing and treating some intestinal inflammatory and allergic diseases due to their immunestimulatory properties with anti-inflammatory effects; as such, probiotics have also been widely used in the attempt of treating other diseases [29]. Several probiotics capable of showing protective effects have been already proposed for IBD treatment and tested in animal models of experimental colitis and for some also in human clinical trials [30,31]. However, the exact mechanisms underlying the probiotic effects are still poorly understood and it is believed that the administration of probiotic bacteria may restore the balance of the disrupted microbiota.

Here we present a review of the present knowledge on bacteria - host interactions in the GI tract with a special focus on IBDs.

THE GASTROINTESTINAL TRACT MICROBIOTA

The human gastrointestinal (GI) microbiota is composed of bacteria, archea, microbial eukaryotes (fungi and protozoan) and viruses, but bacteria are the most abundant and diversified group. The human GI tract harbors a diverse and complex bacterial community that is composed of up to 1014 bacteria exceeding ten times the number of somatic human cells [32]. The symbiotic relationship between human and complex microbial communities is part of a co-evolution process [2]. Under normal conditions, the gut microbiota contribute and supply essential metabolic and protective functions in the human GI tract, playing an important role in the host's normal development and physiology. It is known that the GI bacteria are involved in carbohydrate and protein metabolism, fat storage, inflammatory immune responses and homeostasis of human body [33-36]. It is increasingly clear the importance of these gut microbial communities in human health. Therefore, most of human gut bacteria and their activity remain unknown. The study of commensal bacteria - host interactions is an area of growing interest and we are only beginning to appreciate the diversity and function of the gut microbiota. This knowledge is essential for a better understanding of the impact of the microbiota on our health and well being, and to optimize the role that food or probiotic bacteria may play.

In humans, our knowledge on the composition of the GI tract microbiota has long been based on microscopic observations and culturing of bacteria present in fecal samples. In this way some hundred species associated with the GI tract have been distinguished [37]. The human GI bacterial community composition is diverse and differs between different individuals, challenging microbiota composition approach. Most of the bacteria cannot be cultured *in vitro* in the laboratory standard conditions and most of the GI tract bacteria remain uncultivable. The development of sequencing technology, including the target gene sequencing (16S rRNA genes), and metagenomic, metatranscriptomic and metaproteomic and metabolomic techniques, allowed a more representative description and function of the GI tract microbiota [38-40]. Today several

research programs worldwide, such as MetaHIT (METAgenomics of the Human Intestinal Tract, http://www.metahit.eu), NIH Human Microbiome project (http://commonfund.nih.gov/hmp/) and International Human Microbiome Consortium (IHMC) (http://www.human-microbiome.org/), aim to characterize the human GI tract microbiota through large-scale sequencing of fecal DNA samples and individual bacterial genomes, thus providing an impression of the metagenome of the human GI tract environment and the functions encoded therein. In the following paragraphs we will present an overview of the composition of the human gut microbiota based on these data.

Composition of the Intestinal Microbiota

The GI tract is the major microbial colonized human organ and approximately 70% of all the microbes in the human body are present in the colon [41]. Colonization of the human gut starts at birth when infants are exposed to an environmental microbial population during passage through the birth canal. The infant's evolving GI microbiota present different composition, which is certainly influenced by the delivery mode. Accordingly, infants delivered though cesarean section show a different microbiota composition and a greater susceptibility to atopic diseases compared with vaginally delivered infants [5]. After the initial establishment of the intestinal microbiota and during the first year of life, the microbial composition of the mammalian intestine is relatively simple and varies with time to become more stable afterwards [6,4]. *Escherichia coli* and streptococci are the most commonly isolated organisms from the upper digestive tract immediately after birth [4]. These species appear to be responsible for the creation of an environment favorable for the establishment of anaerobic bacteria, where *Bifidobacterium* and *Bacteroides* that colonize the digestive tube of neonates within days after birth are predominant. During the first two years of life, an increase of lactobacilli, eubacteria, clostridia, and fusobacteria is observed. After weaning, the composition of the intestinal microbiota in infants gradually evolves and becomes similar to the microbiota observed in adults [4], where strictly anaerobic bacteria predominate over facultative anaerobes [37].

The composition and density of bacterial populations of healthy adults vary along the length of the GI tract [9]. Relatively low numbers (up to 103) of bacteria are present in the upper GI tract (stomach, duodenum, jejunum and proximal ileum) where the presence of acid, bile and pancreatic secretions hamper bacterial colonization [7,8]. This part of the GI tract is populated by acid-tolerant bacteria such as streptococci and lactobacilli [42]. Much higher numbers of bacteria reside in the lower compartments of the GI tract, where bacterial populations reach 1011 - 1012 per gram of intestinal content, the highest density for any microbial habitat known to date [41].

The majority approaches to identify GI human bacterial and archeal diversity is based on sequencing 16S rRNA genes. The human gut bacterial species are classified into two major phyla: the Gram-negative *Bacteroidetes* and the Gram-positive *Firmicutes*, whereas *Actinobacteria, Fusobacteria, Proteobacteria, Verrucomicrobia, Cyanobacteria* phyla are minor representatives

of the GI microbiota [43,44]. Most of the *Bacteroidetes* sequences are classified as *Bacteroides vulgatus, Prevotellaceae, Bacteroides thetaiotaomicron, Bacteroides caccae, Bacteroides fragilis*, and *Bacteroides putredinis* phylotypes confirming the abundance and variety of the genus *Bacteroides* across samples [43,44]. Among the *Firmicutes, Clostridia* represent the majority of the bacterial sequences (up to 82%), while *Mollicutes* and *Bacilli* classes are less representative [3,37,43]. Most of *Clostridia* sequences belong to the genera *Eubacterium (Eubacterium rectale, Eubacterium hadrum), Ruminococcus (Ruminococcus torques, Ruminococcus gnavus), Dorea (Dorea formicigenerans, Dorea longicatena), Butyrivibrio, Coprococcus* and *Faecalibacterium* [43]. Among the *Bacilli*, the genera *Streptococcus, Gemella,* and *Lactobacillus* are the most abundant. *Lactobacilli* constitute about 1 % of the fecal bacteria samples [3,42,43]. Contradictory results have been reported for the *Actinobacteria*, with large variation of frequencies, which they are found in human GI [3,43,45]. Irrespective of this variation, it appears that the majority of GI tract associated *Actinobacteria* correspond to the genera *Actinomyces, Corynebacterium, Rothia* and *Bifidobacterium* [3,43,46].

A Metagenomic research represents a powerful alternative to rRNA sequencing for the analysis of complex microbial communities [44,47,48]. The MetaHIT consortium recently published a metagenome sequence analysis of the human gut microbiota from fecal samples of 124 adult individuals [49]. The data were used to establish a catalogue consisting of 3.3 million non-redundant human intestinal microbial genes. This core of genes exceeds 150-fold the number of human genes. Essentially all of the genes identified (99,1%) are from bacterial origin, the remainder being mostly archaeal, with only 0.1% of eukaryotic and viral origins. The authors showed that almost 40% of the genes from each individual are shared with at least half of the other individuals analyzed. They also identified 75 species common to >50% of individuals and 57 species common to >90%. This large number of shared genes and species supports the view that the prevalent human microbiota is of a finite and not overly large size [49]. A comparative metagenomic study was published revealing conserved functional groups of genes present in most or all GI bacterial species and probably required for the maintenance of gut ecosystem reflecting the minimal human gut metagenome. There were identified functions essential for any bacterium to survive and thrive in the host gut as general housekeeping functions involved in the main cell metabolism and required in all bacteria and those specific for the gut including degradation of complex polysaccharides, synthesis of short chain fatty acids (SCFA), amino acids and vitamins. Moreover, the minimal metagenome encompasses many genes of unknown function, rare in sequenced genomes and possibly specifically required for the functioning of the gut ecosystem [22]. Most recently, another comparative metagenomic study was published to obtain information about variation between GI human microbiome across different populations [39] (samples from 6 different nationalities) confirming, by phylogenetic analyses, that the majority of the dominant GI human microbiota are composed by *Bacterioidetes* and *Firmicutes* species [44]. To get an overview of species variation Arumugam and collegues [44] determined the existence

of three different bacterial enterotypes, species clusters based on its bacteriological ecosystem in the gut microbiome, using data of several nations and continents. Each of these enterotypes was identifiable by the variation in the levels of one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) or *Rumminococcus* (enterotype 3). These enterotypes indicated that intestinal microbiota variation is generally stratified, that indicates the existence of a limited number of well-balanced host-microbial symbiotic states that may respond differently to diet and drug intake. Therefore, it does not exist a consensus on the analytical basis for enterotypes and on the interpretation of these results. The methods used to access and clustered the human GI bacterial species on enterotypes are strongly influenced by clustering methodology, distance metrics and taxonomical level. Moreover OTU-picking approaches, sequencing depth, data type (whole genome shotgun) vs 16S rRNA gene sequence data), and 16S rRNA region can also affect enterotyping. Researchers interested in the enterotype concept have to standardize enterotyping methodologies for the concept to gain usefulness [50,51].

Metatrancriptomic approach also revealed that the principal roles of human gut microbiota were carbohydrate metabolism, energy production and synthesis of cellular components [39]. Exploring the impact of variation patterns in gut human microbiome it was also observed that individual-specific strains remain almost unchanged for extended periods of time and that an individual might present a metagenomic variation profile that could be unique, which open new perspectives for human health [52].

Autochthonous and Transiting Bacteria

Within a given intestinal habitat, some microbial members can be classified as true residents or autochthonous species [53]. These microorganisms have a long-term association with the particular habitat and form a stable community. Human colon-associated autochthonous bacteria are enriched for the families Lachnospiraceae and Ruminococcaceae representants [54]. Autochthonous bacteria have been proposed to be closely associated with the intestinal mucosa. Other bacteria, classified as allochthonous, transit with the food and under normal conditions do not colonize the GI tract [53], are thought to be located in the central lumen as part of the fecal stream and play a role that is different from autochthonous bacteria [54]. Fermented food products like yogurt and cheese are important sources of transiting bacteria, typically delivering 109 to 1010 live bacteria per serving. Studies aiming to reveal true resident and transient species are still emerging, and recent reports focus on the group of lactic acid bacteria [55] that are widely used in food fermentation [53,56]. Within this group, species such as *Lactobacillus plantarum, L. casei, L. paracasei, L. buchneri, L. brevis, L. rhamnosus, L. fermentum* and the thermophilic dairy lactobacilli *Lactobacillus delbrueckii* and *L. helveticus* appear not to form stable communities and can be classified as allochthonous species. In contrast, other species of *Lactobacillus* as *L. reuteri* and *L. mucosae*, have been reported to be autochthonous to the human GI tract [57]. Some controversial results have been reported for *Bifidobacterium* species. The presence and persistence of *B. adolescentis, B. longum* and *B. bifidum*, largely vary between individuals [56],

making it difficult to draw more definite conclusions about which species are autochthonous or in transit. The survival of allochthonous bacteria in the GI tract depends on several factors among which their resistance to gastric acid, bile salts and pancreatic juice [58].

Allochthonous bacteria can have important effects on their hosts, both in a negative and in a positive way. Transiting bacteria include several of the enteric food-borne pathogens, which, in contrast to commensal microorganisms, may invade and disrupt the intestinal epithelial barrier [2,56]. The most frequent bacterial pathogens associated with human GI tract infections include species belonging to the *Campylobacter, Salmonella, Shigella* and *Listeria*, genera, as well as some other species among which *E. coli*, *Yersinia enterocolitica, Clostridium botulinum* and *Clostridium perfringens*, and five species from the genus *Vibrio: Vibrio vulnificus, V. mimicus, V. hollisae, V. fluvialis*, and *V. furnissii*. These pathogens are sensed by the host leading to the release of proinflammatory mediators, causing inflammation and contributing to diarrhea. On the other hand, transiting bacteria also include species that are now recognized as probiotics. Probiotics and their beneficial effects for the human health will be discussed later.

GASTROINTESTINAL TRACT MICROBIOTA AND HUMAN HEALTH

Commensal bacteria affect many aspects of human health. They provide components necessary for the digestion of food. They are responsible for the synthesis of nutrients, such as some vitamins, and other metabolites that are essential for the maintenance of host health. Metabolic activities of the gastrointestinal microbiota improve dietary energy yield and also play a role in detoxification of certain food compounds, enhancing the function of others. Moreover, accumulating evidence indicates that they have an essential role in the development and functioning of the immune system.

Carbohydrate Metabolism

Evidence that gastrointestinal bacteria aid their hosts in extracting maximal nutritional value from the diet came from studies showing that conventional rats require 30% less caloric intake to maintain their body weight than their germ-free counterparts. Indeed, the microbiota that resides in the intestine of mammals are adept to metabolize glycans and polysaccharides, including those in dietary plants (starch, hemicellulose and pectin), animal-derived cartilage and tissue (glycosaminoglycans and *N*-linked glycans), and host mucus (*O*-linked glycans) [59]. While mammals actively transport simple dietary sugars like glucose and galactose in the proximal regions of the small intestine [60,61] and can hydrolyze certain disaccharides (e.g., sucrose, lactose, and maltose), they are limited in their capacity to hydrolyze and utilize other polysaccharides that is present in the diet. Undigested polysaccharides such as cellulose, xylan, undigested starch and host derived glycans thus reach the colon where several members of the microbiota are able to digest these polymers, releasing monosaccharides that are subsequently fermented by many other bacteria [60-62]. The large catabolic capacity of some of the gut bacteria is illustrated by the genome of the commensal *Bacteroides thetaiotaomicron* which encodes around 400 enzymes

involved in the transport, binding, and digestion of complex sugars, including a well-studied starch utilization system [63].

The major end products of bacterial fermentation in the gut are short chain fatty acids (SCFA). There are many types of SCFAs, including formic acid, acetic acid, propionic acid, isobutyric acid (also know as 2-methylpropanoic acid), butyric acid, isovaleric acid (3-methylbutanoic acid) and Valeric acid (pentanoic acid). Several tissues in the human body are able to oxidize SCFA for energy generation, and the bacterial formation of SCFA thus enables the host to stock energy that would be lost otherwise. The three-major SCFA produced in the gut are acetate, propionate and butyrate, the main fuel for enterocytes [60,64,65].

In addition to their nutritional value, SCFA have important effects on other aspects of gut physiology. For example, SCFA are the predominant anions in the colon, and the absorption of water is coupled with sodium chloride as well as SCFA transport [60]. Interestingly, butyrate, and important type of SCFA, has been reported to play a role in the prevention of diseases, such us ulcerative colitis (UC) and colorectal cancer, and a number of observations indicate its ability to inhibit the genotoxic activity of nitrosamides and hydrogen peroxide in human colonic cells [60,64,65].

Different bacterial species contribute to the production of SCFAs. A number of *Ruminococcus, Lactobacillus, Enterococcus* and *Bifidobacterium* species are the main producers of acetate. *Faecalibacterium prausnitzii, Eubacterium halii, Eubacterium uniforme, Eubacterium formicigenerans, Eubacterium ventriosum, Eubacterium rectale, Eubacterium ramulus, Coprococcus eutactus, Roseburia* spp. and *Clostridium* species are involved in the production of butyrate, and propionate is mainly produced by *Bacteroides, Roseburia inulinivorans* and some other species belonging to the *Clostridia* group [65].

Amino Acid Metabolism

Basically all dietary components that escape digestion in the small intestine, including proteins, are potential substrates for bacteria present in the intestine [64]. It is likely that bacteria have evolved the capacity to digest peptides because they can use it as carbon or nitrogen source or to gain energy [66]. Experimental evidences suggest that the microbiota from the GI tract is able to degrade and synthesize amino acids. Using a method in which microbial amino acids were specifically labeled with N^{15}, Metges et al. showed that 1-20% of circulating plasma lysine and threonine in humans is derived from the GI tract microbiota [67]. It has been shown that intestinal bacteria can hydrolyze proteins originated from various sources (including gastric and pancreatic secretory products) by using extracellular proteases and peptidases into amino acids and peptides.

Microbiota can also participate in the catabolism of some indispensible amino acids and a number of anaerobic species has been reported to use this material for fermentation processes, releasing at the end organic acids, ethanol and some gazes. For instance, bacterial degradation of

the amino acids cysteine and methionine results in the formation of H2S [64]. H2S is considered to be toxic for human being and, moreover, is able to inhibit butyrate oxidation in colonocytes. As butyrate metabolism is impaired in patients with UC, it was subsequently proposed that H2S accumulation could contribute to the disease [64,68].

Microrganisms from the gastrointestinal tract also plays a key role in nitrogen recycling in the intestine. Basically, amine and polyamines can be rapidly metabolized by some bacterial species to SCFAs. Urea generated by host tissues, for example, passes into the gastrointestinal and is hydrolyzed to ammonia and carbon dioxide by microorganisms [69]. The importance of the endogenous microbiota in the digestion of urea is evidenced in studies with germ-free rats, where high concentrations is found in their colons [70]. The generated ammonia can be then utilized as nitrogen source or be excreted [66].

A large number of bacteria from the GI tract are amino acid fermenters, such as members belonging to the genera *Fusobacterium, Bacteroides, Propionibacterium, Actinomyces,* and some gram-positive cocci (*Peptococcus, Streptococcus, Megasphaera*). Numerous sulfate reducing bacteria (SRB), that can degrade organic compounds and are major sources of H2S, can also be isolated from human feces, mainly belonging to the *Desulfovibrio* genus [71].

Vitamin Biosynthesis and Mineral Metabolism

The ability of gastrointestinal bacteria to synthesize vitamins was recognized many years ago. It was demonstrated that germfree rodents require vitamin K and certain B vitamins (e.g. B12, biotin (vitamin B7), folic acid (B9) and pantothenate (B5) in their diets, in contrast to conventionally raised rodents [72]. These vitamins are produced by several genera of gastrointestinal bacteria, including *Bacteroides, Eubacterium, Propionibacterium,* and *Fusobacterium* [73].

Minerals and essential elements are crucial components of enzymes, structural proteins and redox transport chains. Cobalt, zinc, copper and iron are elements that can be exchanged in the gastrointestinal tract between bacteria and their hosts thus increasing the availability of these elements for our utilization [74].

Calcium metabolism is also highly influenced by our microbiota. SCFAs produced by gastrointestinal bacteria have the ability to induce expression of the vitamin D receptor on eukaryotic cells [75] and vitamin D is a key regulator of calcium metabolism in mammals and ensures calcium absorption from dietary intake, intracellular storage and deposition in bone and teeth [76].

Detoxification

GI tract bacteria are involved in detoxification of certain food compounds that may be harmful. A number of plants such as spinach, beet, peanuts and legumes contain oxalates [77], the deprotonated, charged form of oxalic acid or an ester of oxalic acid. Food oxalates are absorbed in the stomach and in the small and large intestines and may result in kidney disease due to the

formation of calcium oxalate stones (urolithiasis). A number of intestinal microorganisms have been reported to degrade oxalate. *Oxalobacter formigenes* is able to transform oxalate to carbon dioxide and formate and a reduced presence of *O. formigenes* has been correlated with urolithiasis [78]. The oral uptake of *O. formigenes* by human volunteers reduced the urinary oxalate excretion and resulted in oxalate-degrading activity in the feces [79].

Activation of Bioactive Food Components

Recent reports demonstrated that gastrointestinal bacteria are involved in the metabolism of food compounds with antioxidant activity, transforming them into more, or less, active compounds. For example, the human diet contains a large variety of plant-derived nonnutritive substances such as isoflavonoids, which belong to the large group of polyphenols. Gastrointestinal bacteria can convert isoflavonoids to isoflavones which have been associated with a number of effects, such as the prevention of hormone-related cancers, breast cancer, atherosclerosis, and osteoporosis and in the alleviation of menopausal symptoms [80]. The soybean isoflavone daidzein has been studied extensively for anti-breast cancer activity because of its estrogen receptor antagonist and agonist activities [80]. Interestingly, it has been shown that daidzein may undergo transformation by intestinal bacteria toform products with enhanced biological activity like equol [64,80].

Lipid Metabolism

Results of recent studies indicate that intestinal microorganisms also directly affect the metabolism of the host and energy storage in adipose tissue. In a series of experiments with mice, Backhed et al., [81] found that conventional animals had a 40 % higher body fat than germfree mice. When the distal gastrointestinal microbiota from the normal mice was transplanted into the germfree mice, this resulted in a 60 % increase in body fat. The authors revealed that the implanted microbiota promoted the absorption of monosaccharides in the gastrointestinal tract inducing hepatic lipogenesis in the host. Additionally, it was observed that this lipid accumulation was due to the suppression of fasting-induced adipocyte factor (FIAF; also known as ANGPTL4), a member of the angiopoietin-like family of proteins, in the presence of enteric bacteria, leading to a microbiota-induced deposition of triglycerides in adipocytes. In addition, the storage of triglycerides in these cells resulted in (i) an increase of lipoprotein lipase (LPL) activity, (ii) an increase in the absorption of triglycerides, and (iii) an increased storage of triglycerides in the adipocytes increasing globally the body fat in the mice [81,82].

BACTERIAL-DERIVED MOLECULES UNDERLYING PROBIOTIC EFFECTS

As discussed above, bacterial effectors of different nature have been implicated in beneficial effects. These include DNA, lipoteichoic acids, lipoproteins, lipopolysaccharides, flagelin and peptidoglycan, all of which bind to eukaryotic receptors inducing distinct patterns of gene expression in the host cell that guide the activation of innate immunity and initiate the development of antigen-specific acquired immunity [83]. Bacterial surface proteins have also been implicated

in the binding of bacteria to epithelial host cells, mucus or fibronectin [84]. The picture of these interactions is far from complete, however.

DNA Effect

CpG DNA from VSL3# probiotic was shown to inhibit IL-8 secretion, reduce p38 MAPK and NF-κB activation in IECs [85]. Rachmeliwitz et al. [86] demonstrated that administration of genomic DNA of probiotic and non-probiotic bacteria (*E. coli* DH5α, a strain developed in the laboratory) can affect the development of chemically induced ulcerative colitis in mice using dextran sulfate sodium (DSS). DNA of the probiotic VSL3# when given intragastrically or subcutaneously ameliorated colitis symptoms in mice treated with the chemical. The administration of the probiotic DNA resulted in marked inhibition of various symptoms in the DSS-induced model of colitis. The subcutaneous administration leaded to the same result in the TNBS (2,4,6-trinitrobenzenesulfonic acid)-induced model of colitis as well. Surprisingly, the same data were obtained in the spontaneous model of colitis using IL-10-knockout mice [86].

Secreted Factors

Several studies reported probiotic effects for which direct cell contact was not required. Soluble peptides of the probiotic mixture VSL#3 were shown to inhibit the degradation of IκB, a member of the proinflammatory NF-kB pathway (described later in this chapter), blocking the formation of signals needed for the expression of proinflammatory cytokines. Moreover, this probiotic was shown to induce heat shock proteins through proteasome inhibition [87]. VSL#3 was also reported to stabilize tight junctions between instestinal epithelial cells (IECs), improving GI physical barrier, and induce mucins in IECs, augmenting thus their protection against bacteria diluted in the intestinal lumen, by a large (>50 kDa) but unidentified proteinaceous soluble factor [88].

Metabolic product with anti-inflammatory properties has been observed in many probiotics [19]. Two lactic acid bacteria: *B. breve* and *S. thermophilus* CM showed this characteristic in intestinal epithelial cell line. It is known that these active metabolites have molecular weights below 3000 Da and are non-proteinous. And probably do not share the same active metabolites, since there discordance has been observed in experiments designed to better understand the mechanisms of the inhibitory effect. Active metabolites released by probiotic bacteria during intestinal transit may cross the intestinal layer to exert anti-inflammatory effects [89].

Secreted proteins of *L. rhamnosus* GG (p40 and p75) stimulated activation of Akt, promoted epithelial cell growth and inhibited TNF-α-induced epithelial cell apoptosis. These two proteins show similarity with putative cell wall-associated hydrolases or cell wall-modifying enzymes and are abundantly present in *L. rhamnosus* GG culture supernatants [90,91]. Recently, Kaci et al. showed that small soluble factors (<3000 Da) of *Streptococcus salivarius* were able to inhibit TNF-α-induced NF-κB in IECs [92].

Some bacterial metabolites have been shown to exert inhibitory effects on NF-kB activation, as well as the short chain fatty acids (SCFAs), including butyric, acetic and propionic acids. Bacterial metabolites produced by fermentation of polysaccharide, oligosaccharide, proteins, peptide, and glycoprotein precursors in the colon have shown its potential for development of probiotic products [89,93,94].

Conjugated linoleic acid and linolenic acid, considered to be beneficial functional lipids, for instance, the conjugated linoleic acid (CLA) [95,96], that have been intensively inhibits rat mammary tumorigenesis, initiation of mouse skin carcinogenesis, atherosclerosis and contributes for fat loss and lean gain [97-102]. Cis-9, trans-11-18:2, the main beneficial CLA isomer [103], can be produced by *Bifidobacterium* and *Lactobacillus* species and, thus, dairy products are major natural sources because these groups of bacteria are used for the preparation of cheeses, yogurts and fermented milk.

Cell Surface Structures

Bacterial surface proteins have also been implicated in the binding of bacteria to epithelial host cells, mucus or fibronectin [84]. The picture of these interactions is far from complete, however.

L. reuteri was reported to inhibit IL-8 secretion by T84 and HT29 cells and to block the translocation of NF-κB to the nuclei of HeLa cells, inhibiting the activation of the NF-κB pathway and, thus, blocking the expression of proinflammatory cytokines (discussed later). The authors showed that *L. reuteri* must be preincubated with IECs and be adherent and alive to induce its inhibitory effect. The effect was not reproduced by conditioned media, bacterial lysates or heat-killed or gamma-irradiated bacteria [104]. *L. acidophilus* ATCC 4536 was able to mediate anti-inflammatory and anti-apoptotic effects only in direct contact with epithelial cells, by the activation of MAPK and the prevention of NF-κB activation [91,105,106].

Some important cell surface factors from lactobacilli involved in immune modulation effects have been identified. For instance, lipoteichoic acid (LTA) from *L. johnsonii* La1 and *L. acidophilus* La10 inhibited lipopolyssacharide (LPS) induced IL-8 release by HT29 cells, a cytokine that can favour inflammation in the intestinal mucosa [107]. Polysaccharide A (PSA) from *B. fragilis* is protective against colitis [108]. Peptidoglycan (PGN), a component of the bacterial cell wall, is among the main surface structures of Gram positive bacteria recognized by the innate immune system [109]. While most research has highlighted the role of PGN in the pathogenesis of various bacteria [110], relatively few papers reported beneficial effects of this bacterial compound. Shida et al. showed that PGN from *L. johnsonii* JCM 2012 and *L. plantarum* ATCC 14917 inhibited IL-12 production, a proinfalmmatory cytokine, by macrophages [111]. Recently, Fernandez et al. show that PGN can downregulate pro-inflammatory genes. Once PGN-derived muropeptides can be interact with receptors of the innate immune system [112]. For exemple, the muramyl-dipeptide (MDP) of *Lactobacillus salivarius* Ls33 that is recognized by the cytosolic pattern-recognition receptor *nod2* (NOD2 receptor) from IECs, and are related with local production of IL-10 in

murine TNBS-induced colitis model. Augmentation of interleukin-10 (IL-10) has been related to reduction of mucosal inflammation and amelioration of IBD-related symptoms. Bleau et al. reported that exopolysaccharides (EPS) from *L. rhamnosus* RW-9595M increase IL-10 production by macrophages [113].

Surface proteins can also interact with host cells, mainly by toll like receptors (TLR) pathways, as has been demonstrated mainly for pathogenic bacteria [114,115]. Our research group reported that surface proteins of *L. delbrueckii* are involved in its anti-inflammatory effects in HT-29 cells [116]. Hoermannsperger et al. showed that cell surface proteins of the *L. casei* strain present in the probiotic mix VSL#3 were responsible for the inhibition of the proinflammatory cytokine TNF-α-induced secretion of T-cell chemokine interferon-inducible protein (IP-10) in Mode-K cells, demonstrating that cell surface proteins of *L. casei* are able to elicit anti-inflammatory effects in IECs [117].

The examples described above illustrate that multiple cell surface and secreted factors as well as DNA of probiotics seems to exert protective effects *in vitro* and in animal models of colitis. However, the effector-receptor interaction still needs to be studied in most cases. The identification of other bioactive probiotics molecules will provide new insights for the development of probiotic-based drug treatment for IBD.

Maturation and Modulation of the Immune System

The GI tract of mammals is sterile at birth, and is subsequently colonized by the intestinal microbiota. This colonization plays an important role in the maturation and shaping of both the mucosal and systemic immune systems [118,119]. Germ-free animals provided important insights into how the microbiota affects the host immune system.

The role of the microbiota for the development of the immune system is evidenced in the defective gastrointestinal-associated lymphoid tissue (GALT), the first line of defense for the intestinal mucosa, from germfree mice. They present fewer and smaller Peyer's patches, fewer and smaller mesenteric lymph nodes and isolated lymphoid follicles, and a less developed lamina propia of the small intestine relative to conventional animals with a microbiota [118,120-123]. Besides, germfree mice exhibit a reduced expression of Toll-like receptors (TLR) and the class II major histocompatibility complex (MHC II), which is involved in microbial sensing and antigen presentation, respectively, in the intestinal epithelial cells (IECs) [124,125]. The number of IgA-producing cells is reduced, as are the levels of secreted immunoglobulins (IgA and IgG) [118]. They also exhibit irregularities in cytokine levels and profiles and are impaired in the generation of oral tolerance [2,126].

Another altered structure in germfree mice is the intraepithelial lymphocytes (IELs), which are reduced in these animals. IELs, in particular γδ T cells (T gamma delta lymphocytes), are interspersed with enterocytes, thus having direct contact with foreign antigens derived from the gastrointestinal lumen, and play a key role in the immune responses toward these antigens and

in pathogenesis of a variety of diseases [127,128]. Germfree mice also have reduced numbers of CD4+ T cells in the lamina propria and in the spleen, which leads to a lower expression of some important cytokines involved in the protection against pathogens or allergic diseases [129,130]. The development of lymphoid follicles (specialized intestinal structures composed of dendritic cells (DCs) and B cell aggregates) is also disturbed [129].

Beyond the development and maturation, the microbiota also influences functional aspects of our immune system. It was demonstrated that germfree mice are more susceptible to infectious agents, such as *Shigella flexneri* and *Leishmania* [119]. Therefore, the contribution of the microbiota to the development and function of the immune system appears to be crucial. Several reports indicated that the conventionalization, or even the monocolonization with specific bacterial species are able to correct several defects found in the immune system of germ-free mice. Bouskra et al. [129] showed that the colonization of germfree mice with a cocktail of bacterial strains called Altered Scheidlre Flora (ASF) including *E. coli* and some other Gram negative (*Mucispirillum schaedleri* ASF457and *Bacteroides distasonis* ASF519) and Gram positive strains (*Clostridium propionicum* ASF 356, *Eubacterium plexixaudatum* ASF 492, *Firmicutes sp.* ASF 500, *Clostridium sp.* ASF 502, *Lactobacillus acidophilus* ASF 360 and *Lactobacillus murinus* ASF 361) was able to significantly increase the number of isolated lymphoid follicles (IFLs) in the ileum and colon, demonstrating that a restricted set of commensal bacterial species, devoid of invasive bacteria or pathogens, can induce the formation of ILFs. Umesaki et al. [131] showed that colonization of germfree mice with segmented filamentous bacteria (SFB) increased the total number of IELs in the small intestine, the expression of MHC II molecules in IECs and also the number of IgA-producing cells in the small intestine. Mazmanian et al. [132] demonstrated that the monocolonization of germ-free mice with *B. fragilis* was sufficient to correct several immunologic defects found in the absence of a bacterial microbiota. The authors focused on systemic T cell development, and in particularly CD4+ T cells that are important for proper immune function. These cells are of two general subtypes, T helper 1 (TH1) and T helper 2 (TH2), each carrying out distinct and opposing activities [133]. A proper TH1/TH2 balance is critical for human and animal health, and over- or underproduction of either response is associated with immunologic disorders [132,133]. A reduced proportion of CD4+ T cells, which are skewed towards TH2, is observed in germfree mice [132]. The authors showed that colonization with *B. fragilis* restores the proportions of CD4+ T cells in the spleen and TH1/TH2 balance to the levels that are observed in conventional mice [132]. Moreover, it was shown that the purified polysaccharide A (PSA) from *B. fragilis* was able to induce CD4+ T cell expansion in GF (germ free) mice while a PSA negative mutant of *B. fragilis* did not restore these immunologic functions [132].

Many cell types are influenced by our microbiota and, several reports indicate that commensals play an important role in CD4+ T cell differentiation. Induction of each lymphocyte subset may be regulated by a distinct component of the microbiota. For instance, SFB strongly induce TH17 cells, which play a role in host resistance against intestinal pathogens and promote systemic

autoimmunity [134-136]. Naive CD4+ T cells can also adopt a regulatory phenotype (Treg cells). Again, the microbiota may be critically involved in the differentiation of some gastrointestinal Treg subsets. Induction of Tregs is an important target for treating both autoimmune and atopic disorders as these cells can suppress the activity of effector cells by inducing IL-10 production. Several gastrointestinal bacteria, such as *Bifidobacteria infantis* and *F. prausnitzii*, have been shown to induce Tregs and IL-10 production in the GI tract [19,137].

Clarke et al. [138] showed that peptidoglycan (PGN) from the microbiota systemically primes the innate immune system, enhancing killing of bacteria by bone-marrow-derived neutrophils, crucial component of the innate immune system, after infection with *Streptotococcus pneumoniae* and *Staphylococcus aureus*. This effect was dependent on signaling via the pattern recognition receptor *nod1* (nucleotide-binding, oligomerization domain-containing protein-1) which recognizes *meso*-diaminopimelic acid (*meso* DAP)-containing peptidoglycan mainly found in Gram-negative bacteria [138].

Numerous other aspects of the immune system are influenced by GI microbiota. For instance, several reports indicate that commensal bacteria can interfere with important signaling pathways of their host, thus stimulating the production of anti-inflammatory mediators, while at the same time inhibiting the secretion of pro-inflammatory cytokines.

Classical NF-κB Pathway (Myd88-dependent)

One of the central transcription factors mediating immune and inflammatory responses is nuclear factor κB (NF-κB). NF-κB is required for the transcriptional activation of a number of pro-inflammatory effectors, including IL-8, TNF-α, IL-6, Cox2, iNOS [139]. The NF-κB family of transcription factors consists of five members (subunits), p50, p52, p65 (RelA), c-Rel, and RelB [140]. In most cells, NF-κB form dimers that primarily resides in the cytoplasm, in an inactive complex associated with a member of the family of inhibitory IκB proteins (IkBα, IkBß and IκBε) or the precursor proteins p100 and p105 [140]. After engagement of TLRs by their respective ligands (MAMPs), TLR dimerizes and undergo conformational change required for the recruitment of downstream signaling molecules [83]. Signaling to NF-κB continues through intracellular adaptor proteins belonging to RIP and TRAF families. For example, the adaptor molecule myeloid differentiation primary-response protein 88 (Myd88) is recruited to all TLRs, with the exception of TLR3, and is essential for activation of NF-κB pathway and thus for the production of inflammatory cytokines induced by those TLRs [141]. It recruits the IL-1 receptor-associated kinases (IRAK4, IRAK1). IRAK activation results in an interaction with TNF receptor associated factor (TRAF6) which leads to the activation of mitogen-activated protein kinase (MAPKs), transforming growth factor-β activated kinase (TAK1) and mitogen-activated protein kinase kinases (MEKKs). These kinases activate the IkappaB kinase complex (IKK complex/IKKα, IKKß, IKKγ [also termed NEMO]) that phosphorylates IκBα, the most extensively studied member of the IκB family, and targets it for proteosomal degradation (Figure 1) [83,140,142]. Therefore,

MyD88 functions as an adaptor linking TLRs with downstream signaling molecules [83]. The degradation of IκB allows the translocation of active NF-κB from the cytoplasm to the nucleus where it regulates several events that result in auto-regulation of inflammatory cytokines (for example expression of chemokines [monocyte chemotactic protein-1 - MCP-1], IL-8, TNF-α, ICAM and cyclooxygenase 2(COX-2) [140,142].

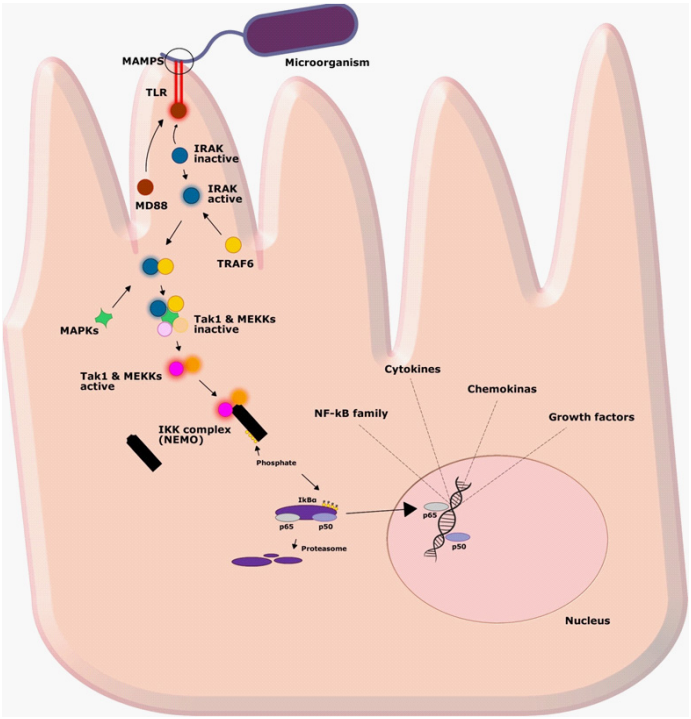

Figure 1: NF-κB classical pathway Myd88-dependent. After engagement of TLRs by their congnate MAMPs, Myd88 recruits IRAK4 and IRAK1. IRAK activation results in an interaction with TRAF6, which leads to the activation of MAPKs, TAK1 and MEKKs. These kinases activate the IKK complex that phosphorylates IκB and targets it for proteosomal degradation. Free NF-κB migrates to the nucleus where it will activate the transcription of several inflammatory mediators.

In the body, activation of NF-κB may be beneficial in suppressing toxic/septic shock, host and acute inflammatory reactions, acute phase response, and radiation damage [143]. Actually, inflammation is essential for immune defense, nevertheless, exaggerated or persistent inflammation leads to tissue injury [144]. In the intestine, multiple lines of evidence suggest that NF-κB activation has a dual role (detrimental and beneficial). NF-κB is reported to contribute to the development and maintenance of intestinal inflammation and was found to be over activated in mucosal cells of patients suffering from intestinal inflammatory diseases, such as IBD. Pharmacological inhibition of NF-κB activity seemed to ameliorate intestinal inflammation in mouse models of colitis [145]. Administration of antisense oligonucleotides to p65 (one of

the subunits of NF-κB) reduced the severity of colon inflammation in both chemically induced models and in the IL-10 knockout model of colitis [146-148]. Taken together, these studies suggest that excessive NF-κB activation may contribute to intestinal inflammation and that NF-κB inhibition could have therapeutic effects in IBD. Nevertheless, it has been reasoned that NF-κB activity has more diverse functions than initially anticipated, governing both protective and destructive response, which depend on the cell types involved [149]. A number of studies showed that inhibition of NF-κB activation, specifically in the intestinal epithelium, causes severe inflammation. For instance, mice lacking NEMO (one of the subunits of the IKK complex) in IECs developed severe chronic colitis characterized by epithelial ulceration, elevated expression of proinflammatory mediators and infiltration of immune cells [150]. Here, the complete abrogation of Myd88-dependent NF-κB activation caused colon inflammation, demonstrating that IKK/NF-κB signaling performs essential homeostasis preserving functions in the colonic epithelium [150]. The crucial role of NEMO-dependent NF-κB signaling in the maintenance of intestinal homeostasis is also supported by studies in patients carrying NEMO mutations. These patients usually suffer from severe immunodeficiency and developmental skin defects and some of them also develop colitis [151-153]. NF-κB has a protective function under acute inflammatory injuries as well. It was demonstrated that loss of RelA (p65 transcription factor) in intestinal epithelial cells increased acute colitis and enhanced mortality in mice previously treated with DSS [154]. These data suggest that an interference on the NF-κB signaling pathway compromises normal intestinal homeostasis [149]. The studies mentioned above highlighted the dual role of NF-κB in the maintenance of intestinal immune homeostasis. As the GI tract epithelium is in close contact with commensals, there is robust mechanism necessary to control excessive NF-κB activation and maintain its steady state activation.

In order to prevent exaggerated inflammation and host tissue damage manifested in inflammatory diseases, the body has evolved some strategies to regulate TLR-mediated proinflammatory-signaling pathways [144]. Actually, TLR signaling and subsequent events are tightly regulated by inhibitors of TLR signaling to maintain immune balance and avoid over activation of NF-κB pathway. Several inhibitors of TLR signaling pathways have been characterized, including IRAK-M, TOLLIP, zinc finger protein A20 and Peroxisome Proliferator-Activated Receptor γ (PPARγ) [155]. PPARγ is a member of a nuclear receptor family that has been proposed as a therapeutic target in IBD as it is able to regulate colonic inflammation [156]. It was shown that activation of PPARγ in the colon inhibited mucosal production of inflammatory cytokines, mainly through downregulation of the NF-κB signaling pathway [157]. Another research work demonstrated that signals from TLR4 and luminal bacteria were able to regulate PPARγ expression leading to the inhibition of NF-κB [158]. Ogawa et al. [159] showed that PPARγ and other nuclear receptors repress overlapping but distinct subsets of TLR inflammatory response genes, while Kelly et al. [160] demonstrated that PPARγ directly associates with the RelA subunit of NF-κB and is responsible for its nuclear export limiting the duration of NF-κB's action. Interestingly, in this same work, they showed that the anti-inflammatory effect of the

commensal *B. thetaiotaomicron* was correlated with its ability to increase PPARγ expression in a Caco-2 epithelial cell line.

A20 zinc finger protein (also termed ABIN1) has recently been linked with other inflammatory diseases, such as lupus erythematosus, psoriasis [144]. A20 is a tumor necrosis factor (TNF) - and interleukin 1 (IL-1)-inducible cytoplasmic protein that negatively regulates NF-kappaB-dependent gene expression. Usually it is induced by inflammatory stimuli and confers resistance to apoptosis [161]. A20-deficient mice are markedly more sensitive to LPS and TNF injection, suggesting that A20 plays a regulatory role in TLR signaling [162]. Actually, in 2011 Zhou and collaborators showed that ABIN1-deficient mice developed a progressive, lupus-like inflammatory disease revealing that ABIN1 as an essential anti-inflammatory component of TLR-signaling pathways [144].

As discussed above, *nod2* recognizes the muramyl dipeptide component of bacterial cell wall PGN and activates NF-κB. However, germline mutations of the LRR domain of *nod2* increase susceptibility to Crohn's disease [163,164]. Some recent observations suggest that *nod2* may act as an inhibitor of TLR2 signaling and those mutations reduces *nod2* function increasing TLR signaling by removing its inhibitory function [165]. Recently, it was shown that *nod2* signaling could also modulate the production of IL-10. Macho Fernandez et al. [112] demonstrated that the anti-inflammatory capacity of the *Lactobacillus salivarius* Ls33 is correlated with a local IL-10 production in a *nod2*-dependent way. It should also be noticed that, despite the ability of TLR2 to induce NF-κB, some studies demonstrated that TLR2 might have a protective effect in the GI tract. For example, Cario et al. [166] showed that TLR2 signaling enhances intestinal epithelial integrity through the rearrangement of tight junctions-associated ZO-1. The authors suggest that certain probiotic compounds may contain TLR2-specific immunostimulatory features leading to amelioration of colitis by restoring intestinal barrier integrity. These dual functions of *nod2* and TLR2 are still to be clarified.

IRAK-M is a member of the IL-1 receptor associated kinases (IRAK) family of adaptor molecules. IRAK-M blocks the formation of IRAK1-TRAF6 complexes, thereby inhibiting downstream activation of NF-κB. Berglund et al. [167] showed that IRAK-M-deficient mice are more susceptible to DSS-induced colitis compared with their wild-type counterparts. Tollip inhibits IL-1 and NF-κB activation through suppression of autophosphorylation and kinase activity of IRAK [168]. Transfection of Tollip in IECs resulted in a decreased responsiveness to stimulation with LPS and LTA [169]. As it will be discussed later, several commensal bacteria and probiotics are able to regulate TLR signaling responses. For example, they can inhibit NF-κB and MAPK pathways thus contributing to the homeostasis of the GI tract epithelium. Although some of the mechanisms that may enable the mucosa to control activation of innate immune response despite continuous exposure to commensal bacteria have been characterized, a number of questions remain unanswered. For example, it is not known how the inhibitory mechanisms are switched off during a pathogen infection.

INTESTINAL IMMUNITY

The intestinal epithelium has evolved in the presence of diverse enteric microflora. The contact of TLRs with these microorganisms is able to convert the recognition of microbe-associated molecules (MAMPs) in the GI tract into signals for anti-microbial peptide expression, barrier fortification, proliferation of epithelial cells and production of cytokines, as mentioned above [170]. The intestinal epithelium not only functions as a physical barrier but also makes part of the digestive tract's immune system which involves IECs and the gastrointestinal associated-lymphoid tissue (GALT), the largest immune organ in the human body [2,171]. The lymphoid tissue comprises Peyer's patches in the small intestine, and isolated lymphoid follicles embedded in the lamina propria all along the intestinal tract [172]. These sites of the GALT play a fundamental role in the induction of immune responses against pathogens that invade the epithelium and contribute to the maintenance of the balance between immunity and tolerance at the mucosal surface [2,171,173]. IECs known as M (microfold) cells that overlie Peyer's patches participate in the sampling of luminal content and microorganisms, and deliver these to the subepithelial dome (SED), an area that is populated by immune cells including professional antigen-presenting cells and a number of dendritic cell (DC, #53) subsets [171,173]. In addition, specialized intestinal DCs located in the lamina propria of the small intestine express tight-junction proteins that allow for direct luminal sampling through the extension of dendrites between IECs, while keeping the IEC barrier intact [174].

The recognition of bacterial signals by IECs is essential for mucosal immune homeostasis, implicating IECs as key modulators of inflammatory responses [129,166,175]. One mechanism by which IECs may regulate intestinal homeostasis is by influencing dendritic cells (DCs), macrophages and lymphocytes through the local expression of immunoregulatory cytokines, including thymic stromal lymphopoietin (TSLP), IL-10, transforming growth factor-β (TGF-β), prostaglandin E2, retinoic acid, and IL-25 [176-180]. Iliev et al. [181] showed that IECs promoted the differentiation of tolerogenic DCs able to drive the development of Foxp3+ regulatory T cells (Tregs). This effect was lost in Crohn's Disease (CD) patients and correlated with a reduced expression of tolerogenic factors by primary IECs.

IECs are in direct contact with intraepithelial lymphocytes (IELs) that express all the molecular machinery required for antigen processing and presentation, including major histocompatibility complex (MHC) class II (MHCII) molecules [182]. Some *in vitro* studies showed that rodent IECs could process and present antigens through the MHCII pathway [183]. Despite this ability to process and present antigens, IECs are also reported to lack costimulatory molecules, suggesting that they cannot prime naive T cells. However, the GI lamina propria contains a large population of memory/activated T cells that exhibit less stringent requirements for costimulation and therefore may be influenced by IEC-intrinsic antigen presentation [184,185]. In addition, IECs may deliver inhibitory or tolerogenic signals directly to T and B cells [186]. IEC-mediated recognition of

commensals thus results in the production of immunoregulatory signals that can control innate and adaptive immune cell functions.

DCs are key modulators of the adaptive immune system and provide a link between innate and adaptive immunity [187]. They are found throughout the intestine, including the lamina propria (LP, #61) of the small and large intestine, the isolated lymphoide follicles, the Peyer patches (PP) and the mesenteric lymph nodes (MLN) [172] and present specific phenotypic characteristics and perform distinct functions depending on their anatomical localization [188]. Particularly in the GI tract, a subset of DCs has been shown to preferentially drive the development of Tregs, the main effectors of tolerance [189].

CD4+ T cells are subdivided into T helper cells (TH1, TH2 and TH17 cells) and Tregs. TH1 cells are inflammatory cells that release IFN-γ and are involved in immunity against intracellular pathogens, whereas TH2 cells are primarily involved in B cell help as they release B cell growth factors, like IL-4. TH17 cells play a critical role in host defense against a variety of bacteria and fungi [190], but under pathological conditions such as autoimmunity, TH17 cells exacerbate inflammation [191]. Tregs suppress the function of effector T cells and are thus essential to counteract inflammatory responses [192].

Intestinal DCs regulate local T cell response in part by production of IL-12 and IL-23. IL-12 is a regulatory cytokine that induces TH1 cell differentiation [193] while IL-23 drives inflammatory TH17 responses [194]. They are tolerogenic compared with systemically circulating DCs, thus contributing to the generation of oral tolerance [181,195]. For example, stimulation of intestinal DCs with LPS (TLR4 ligand) resulted in increased production of the anti-inflammatory cytokine IL-10, while stimulation of systemic DCs resulted in pro-inflammatory activation [196,197]. The mechanism by which intestinal DCs may present a tolerogenic phenotype is not fully understood, but includes reduced TLR expression and negative regulation of the NF-κB pathway via *nod2*, for example. [165,179,197-199]. Intestinal DCs transport bacterial antigens to the mesenteric lymph nodes (MLNs) where they influence the local responses. For example, they promote conversion of naïve CD4+ T cells into Tregs in a retinoic acid- and TGF-β-dependent manner [189,200,201]. In conclusion, intestinal DCs are important to maintain mucosal homeostasis, however, the role of GI tract bacteria in promoting the tolerogenic profile of mucosal DCs remains to be studied.

In summary, healthy individuals have a tolerogenic response against the GI microbiota, where we found a balance between effector and regulatory T cells (Figure 2). When this balance is disrupted (Figure 2), either by an increase in the population of effector T cells, or by a decrease in the population of Tregs, the homeostasis is broken leading to mucosal inflammation. The loss of tolerance to the normal GI microbiota is observed in patients with IBD [202].

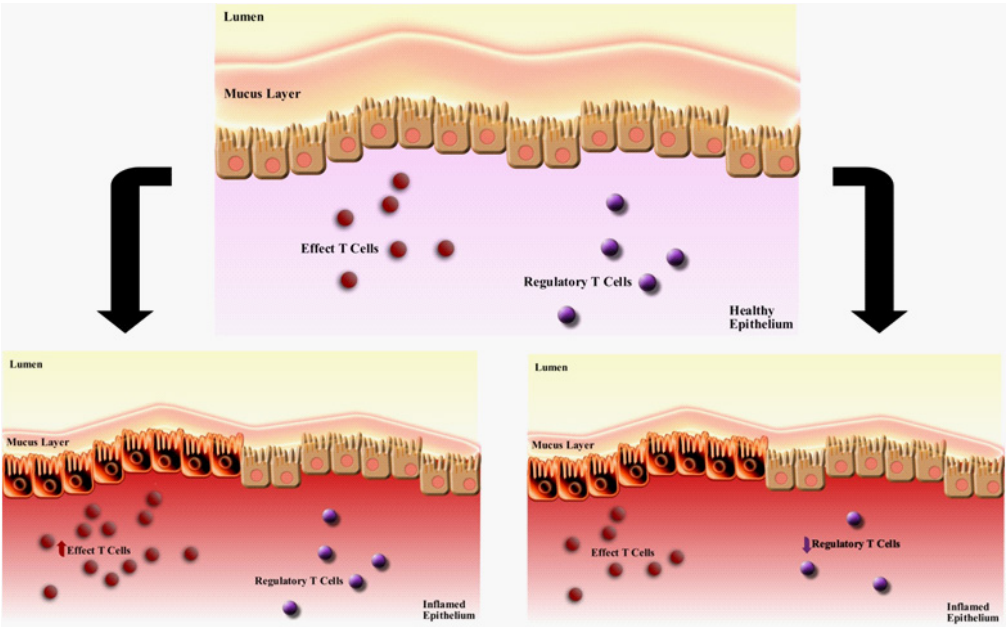

Figure 2: Breakdown of mucosal homeostasis. In healthy conditions there is a balance between effector and regulatory T cells. This balance can be disrupted either by an increase in the population of effector T cells, or by a decrease in the population of Tregs, leading to mucosal inflammation.

DISTURBED MICROBIOTA COMPOSITION AND DISEASE

A growing number of reports indicate correlations between disturbed microbiota composition (dysbiosis) and diseases such as inflammatory bowel diseases (IBD), obesity, diabetes, cancer and also several extra-intestinal diseases [203-206].

Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBDs) are chronic, relapsing, immunologically mediated disorders confined to the GI tract. The two major forms of IBDs are Ulcerative colitis (UC) and Crohn's disease (CD) [207,208]. The European continent and Canada reported the highest prevalence of IBDS. Nevertheless, Asia has a lower prevalence. Studies that investigated temporal trends reported that the incidence of IBD has an alarming increase in many regions of the world [209]. The incidence of both diseases is increasing [210]. In the developed countries they have became a main gastroenterological problem [210,211]. Another important details is that there has been an frightening increase in the traditional low-incidence areas, such as East Asia [212,213], the Indian subcontinent [214], the Middle East [215], Latin America [216], and Eastern Europe [209,217]. As can be seen, the increasing incidence of these diseases in different regions of the world, indicate the emergence of IBD as a global public health problem [209].

Ulcerative colitis is characterized by inflammation that is limited to the colon: it begins in the rectum, spreads proximally in a continuous fashion and frequently involves the periappendiceal region. Histologically, UC shows superficial inflammatory changes limited to the mucosa and submucosa with cryptitis and crypt abscesses. Crohn's disease, in turn, involves any part of the gastrointestinal tract-most commonly the terminal ileum or the perianal region — in a non-continuous fashion and, unlike ulcerative colitis, is commonly associated with complications such as strictures, abscesses and fistulas. The histological features of CD include thickened submucosa, transmural inflammation (where the whole mucosa layer is affected), fissuring ulceration and non-caseating granulomas. The main symptoms of the two diseases are similar: diarrhea, abdominal pain, and weight loss [202,218,219].

The pathogenesis of IBDs is not fully understood. Epidemiologic observations indicate that there are strong environmental influences in IBD. Their influence is confirmed by the relatively low concordance rate in identical twins (~50% for CD and ~10% for UC) [220]. These twin studies and their increased incidence of IBD in first-degree relatives of probands with either disease indicate that genetic factors are also involved [219]. Recent advances in understanding the genetics of human IBD originate from studies based on single nucleotide polymorphism (SNP) and candidate gene approaches, genome wide scanning (GWS) methods, and studies of mouse experimental colitis that used transgene and deletion (knockout) techniques [218,219]. Today exist solid proof that the most prominent genetic polymorphisms associated with IBD cause disease (or prevent disease) by affecting responsiveness of the mucosal immune system [206].

The first gene to be associated with CD was *nod2*, also known as *card15* (caspase recruitment domain family member 15). Three polymorphisms of this gene have been associated with the onset of CD: two missense mutations leading to amino acid replacements (Arg702Trp and Gly908Arg), and one frameshift mutation leading to a truncated protein through the introduction of a premature stop codon (Leu1007fsincC). These mutations are found in the region of *nod2* that encodes leucine-rich repeat which is responsible for bacterial recognition by the *nod2* receptor [163,164]. The leucine-rich repeat region of *nod2* binds muramyl dipeptide (MDP), which is a PGN constituent found in almost all bacteria [221]. The binding of MDP by *nod2* activates NF-κB, stimulating the transcription of multiple genes that encode pro-inflammatory molecules [219,222]. However, findings suggest that *nod2* may act as an inhibitor of TLR2 signaling and those mutations that reduce *nod2* function lead to increased TLR signaling by removing its inhibitory function [165]. It has been reported that *nod2* deletion in mice or CD-associated *nod2* polymorphisms in humans lead to amplified Toll-like receptor (TLR) responses, once such responses are regulated by prolonged or repeated stimulation of *nod2* [223].

This could be an explanation for the observed linkage between *nod2* mutations and IBD. Also, *nod2* is constitutively expressed in Paneth cells, the main source of secreted antimicrobial peptides such as the α-defensins [224]. Deletion of *nod2* in mice decreases the α-defensin production and

enhances susceptibility to experimental infections, such as *Listeria monocytogenes* infection after oral challenge [225]. These results are consistent with the decrease in α-defensin production seen in CD patients, especially those with *nod2* mutations [226].

Another gene that has been associated with IBD, especially with UC, is the *pparG* gene (peroxisome proliferative-activated receptor γ). PPARγ is a nuclear receptor that inhibts NF-κB activity and its expression is decreased in patients with active UC [158].

The two examples above highlight the importance of the NF-κB response in the pathogenesis of IBD. Other genes associated with the pathogenesis of IBD regulate immune responses, mucosal barrier functions and bacterial killing [219]. IBD patients show chronically activated innate (macrophages and neutrophils) and acquired (T and B cell) immune responses and loss of tolerance to the gastrointestinal microbiota [227,228]. In healthy subjects, tolerance is mediated mainly by regulatory T cells and also by B lymphocytes, natural killer T cells and DCs that secrete transforming growth factor (TGF)-β and interleukin (IL)-10, interferon (IFN)-α/β and prostaglandin J2 [219]. In IBD patients, DCs and macrophages present in the lamina propria are increased in number and have an activated phenotype. Most of the pro-inflammatory cytokines and chemokines are overexpressed in patients with UC and CD, however, TH1 and TH17-related cytokines (IL-12, IL-23, IL-27, IFN-γ) are selectively activated in CD patients while UC seems to be a TH2 disorder with increase of IL-13 (table 1) [219].

Table 1: Cytokines associated with IBDs [219].

Cytokines	Crohn'sdisease	Ulcerativecolitis
Innate immune responses		
IL-1β	↑	↑
TNF	↑	↑
IL-6	↑	↑
IL-8	↑	↑
IL-12	↑	-
IL-18	↑	↑
IL-23	↑	-
IL-27	↑	-
T-cell response IFN-γ	↑	-
IL-5	-	↑
IL-13	-	↑
IL-17	↑	-
IL-21	↑	-

(↑: increase; -: normal).

In addition to environmental and genetic factors, there is strong evidence that IBD are also caused by a dysfunctional interaction between the GI microbiota and the mucosal immune

system, in genetically disposed individuals [219]. Evidence for the role of the microbiota in the pathogenesis of IBD is provided by studies demonstrating that antibiotics can reduce or prevent inflammation, both in patients and in murine models of IBD [229,230]. Besides, ulcerative colitis (UC) patients that received a fecal transplantation with stool collected from healthy donors that exhibited disease remission within a week after the transplant, with a complete recovery after 4 months [231].

IBDs have been associated with decreased microbial diversity, decreased numbers of *Firmicutes* and *Bacteroidetes*, and increased numbers of *Proteobacteria* and *Actinobacteria* [232]. In addition, biopsies from IBD patients contain higher amounts of bacteria associated with the mucus layer and the epithelial surface when compared with tissues obtained from healthy subjects [202] but these studies do not support the presence of specific, IBD causing, pathogenic microorganism [20,202].

Some investigations suggest that the absence of specific (groups of) bacteria may be important [19,233]. Notably the Clostridial clusters IV and XIV are less abundant in patients with IBD than in healthy controls. Particular interest has been placed in the members of this group as they are known producers of short chain fatty acids (SCFAs) with potent anti-inflammatory properties [232,234,235]. For example, the species *Faecali bacteriumprausnitzii* - which belongs to the Clostridial cluster and shows anti-inflammatory effects *in vitro* and *in vivo* [19] - has been reported to be under represented in ileal Crohn's disease (CD) patients [233]. Together, these observations support its positive association with GI tract health and suggest that its depletion may disrupt host tolerance to the intestinal microbiota, which may precede or perpetuate intestinal inflammation.

In one view, the microbiota is both qualitatively and quantitatively normal and the disease defect lies within the mucosal immune system. In this case, the normal state of immunologic tolerance to microbial antigens in the GI tract is disturbed either by the presence of a defective mucosal effector T cell population that overreacts to usual microbial antigens or, alternatively, by the presence of a defective mucosal Treg cell population that underreacts to usual microbial antigens such that even normal effector T cells are not properly modulated. In another view, a fundamental abnormality exists in the GI microbiota, either in the number or type of organisms that constitute the gastrointestinal tract population or in the extent to which the organisms confront the mucosal immune system. In both views there is loss of tolerance to commensals, by either excessive effector T-cell function or deficient regulatory T-cell function, thus highlighting the importance of the GI microbiota in the pathogenesis of IBD. In line with this, it is known that IL-10 KO mice developed severe colitis when raised in a conventional setting and their GF counterparts failed to do so [236].

The presence of the GI microbiota and the presence of TLR2 are necessary to help the development of Tregs, it was thus established that innate TLR2 responses initiated by the microbiota are necessary for Treg development. However, the intestinal homeostasis, that is,

the noninflamed state of the normal intestinal, is dependent on Tregs induced by commensal microbiota that gain entry into the lamina propria. So in this way, the concept of IBDs; the inflammation of the GI tract defines these diseases must initially overcome two antiinflammatory barriers: the barrier imposed by Tregs induced by commensal microbiota and that created by Tregs that are generated by the inflammation itself [206].

The state of tolerance to the native microbiota is disturbed by the presence of a deregulated effector T cell population that reacts to the normal microbiota or, alternatively, by the presence of a defective Treg (regulatory T-cell) population that does not properly modulate the effector T cell responses [202].

In this context the efficacy of probiotic intervention, by administration, has been studied in a quantity of human diseases including IBD (CD, UC and pouchitis), irritable bowel syndrome (IBS), constipation, diarrhea, colon cancer, cardiovascular disease, necrotizing enterocolitis (NEC), allergic diseases, obesity and metabolic disorders and these have been the focus of systematic reviews [237-240].

Research about the *in vivo* effects of probiotics on the human host examined the influence of a probiotic microorganism on human duodenal mucosal gene expression. The authors showed that changes in gene expression patterns, especially in the NF-kB dependent pathways, induced by *Lactobacillus plantarum* WCFS1 could be linked to the establishment of immunotolerance in human adults [241].

Obesity and Diabetes

Obesity is a complex disease characterized by excessive body fat accumulation and has traditionally been considered as a disorder caused by nutritional surplus, which in some cases is associated with a genetic predisposition.

Several components of the classical inflammatory response in response to pathogens are involved in the inflammatory response provoked by obesity, which includes an increase in circulating inflammatory cytokines and acute phase proteins (e.g., C-reactive protein), recruitment of leukocytes to inflamed tissues, activation of tissue leukocytes, and generation of reparative tissue responses (e.g., fibrosis) [242].

Evidences for the role of the GI microbiota has offered new insights into the pathogenesis of obesity [1]. Studies in mice suggest that the composition of the gastrointestinal microbiota may be a determining factor in the caloric extraction of ingested food and in the determination of bodyweight. One of the first pieces of evidence linking obesity and microbiota came from studies performed by Bäckhed and colleagues [81]. They compared germ-free mice with germ-free mice that were colonized with the microbiota of conventionally raised mice and observed an increase of body fat content of the colonized mice despite reduced food intake.

Inflammatory and metabolic signals related to dynamics regulation of inflammatory cells and obesity converge in a multitude of situations. This communications offers news opportunities to comprehend the pathogenesis of many organ-specific diseases associated with obesity [243].

In addition to obesity, the gastrointestinal microbiota has been suggested to play a role in obesity-associated metabolic disorders, such as type 2 diabetes. Researches showed that the relative abundance of Firmicutes was significantly lower in diabetic patients compared with non-diabetic persons. In contrast, the Bacteroidetes and Proteobacteria were present in higher abundance [204].

Microbiota and Extra-Intestinal Diseases

Given the intimate relationship between the gastrointestinal microbiota and the intestine, it may not seem surprising that dysbiosis is implicated in intestinal diseases. However, the GI microbiota has also been associated with several extra-intestinal diseases, such as atopic and allergic diseases. It has been suggested that microbiota composition might be the underlying factor in allergic diseases such as atopic eczema (inflammation of skin) [203]. This hypothesis is based on examination of the microbiota composition in children from countries with a high or low prevalence of allergic diseases. Allergic children from all these countries shared a similar microbiota composition, with increased levels of aerobic microbes and decreased levels of anaerobic microbes and lactobacilli in comparison to non-allergic children [203].

Researchers showed that IBD and COPD (chronic obstructive pulmonary disease) share many similarities in epidemiological and clinical characteristics, as well as in inflammatory pathology [244,245]. An important environmental factor for these patients is an exposure to air pollution [246,247]. This situation seemed to be a direct association between increased environmental pollution and hospitalization among adult IBD patients [248]. A group of patients with nonspecific inflammatory disease of the colon developed severe, unexplained, chronic bronchopulmonary disease [249]. Hereafter numerous pulmonary manifestations including small and large airway dysfunction or obstruction, inflammation of pulmonary parenchyma and vasculature, as well as bronchopulmonary hyper-reactivity have been reported in IBD [204,250-254]. The variety of respiratory disorders occurring among patients with IBD is broad [204,253-255]. The studies described above indicate correlations between dysbiosis and several intestinal and extra-intestinal diseases. However, it remains to be seen whether these alterations in microbial communities are the cause or the consequence of such diseases.

CONCLUSION

Several studies indicate that the intestinal microbiota influences host energy balance and its immunologic development, contributing to gastrointestinal homeostasis. The composition and density of bacterial populations of adults vary along the GI tract. Streptococci and lactobacilli populate the upper part. Much higher numbers of bacteria reside in the lower compartments, where bacterial populations reach 10^{11}-10^{12} per gram of intestinal content, the highest density

for any microbial habitat known on earth. The majority of the dominant GI human microbiota is composed by *Bacterioidetes* and *Firmicutes* species. Alterations in the intestinal microbiota can cause human susceptibility to a variety of diseases, including cancer and inflammatory bowel diseases (IBDs) (ulcerative colitis and Crohn's disease). It was demonstrated that patients with IBDs present a reduced bacterial diversity and has a deregulation of the mucosal immune system, which respond toward the native intestinal microbiota. Probiotic, microorganisms that confers health benefit on the host, have been capable at preventing and treating some intestinal inflammation due to their immunestimulatory/anti-inflammatory properties. Probiotics covers a large family of microorganisms mainly belonging to the lactic acid bacteria (LAB) group. These bacteria can be found as residing the human gastrointestinal tract (commensals) or transiting the GI tract after being introduced with the diet. Even though experiments using animal models of intestinal inflammation demonstrated that probiotic can ameliorate symptoms manifested by IBD patients, the mechanisms underlying the probiotic effects still remains to be elucidated. However, it is believed that the administration of probiotic bacteria may restore the balance of the disrupted microbiota. Moreover, it seems that they are involved in host carbohydrate and protein metabolism, fat storage, inflammatory immune responses and homeostasis of human body. They can increase the amount of anti-inflammatory cytokines, improve gut physical barriers, and produce compounds such as short chain fatty acids (butyrate), synthetise vitamins that may have a role in the prevention of colitis and colorectal cancer. Moreover, they can be involved in detoxification of certain food compounds that may be harmful for the host and to hydrolyze proteins originated from various sources that are essential for the maintenance of host health. Some bacterial factors involved in the probiotic effect as well as the pathway by which bacteria exert its positive effect on the host have been identified such as bacterial DNA and multiple cell surfaces and secreted proteins. However, more details regarding the effector-receptor interaction still needs to be studied in most cases. We believe that the identification of other bioactive probiotic molecules may provide new insights for the development of probiotic-based drug treatment for IBDs. In fact, the study of commensal bacteria-host interactions is essential for a better understanding on the impact of the microbiota on our health, and to optimize the role that food or probiotic bacteria may play.

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