João Miguel da Silva Gonçalves

The Human Microbiome: A fine line between symbiosis and pathogenesis

Monografia realizada no âmbito da unidade Estágio Curricular do Mestrado Integrado em Ciências Farmacêuticas, orientada pelo Professor Doutor João António Nave Laranjinha e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Julho 2016

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Coimbra, 5 de Julho de 2016.

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(João Miguel da Silva Gonçalves)

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Abbreviations

AMP - Antimicrobial proteins

CD - Crohn's disease

DC – Dendritic cells

IEC – Intestinal Epithelial Cell

FMT - Fecal microbial therapy

GF - Germ-free

IBD - Inflammatory Bowel Disease

IgA - Immunoglobulin A

IkB - inhibitors of kappa light poly-peptide gene-enhancer B cells

IL - Interleukin

LRR ligands - leucine-rich repeat ligands

MAMP - Microbe associated molecular patterns

MAPK - Mitogen-activated protein kinase

MDP - Muramyl dipeptide

MUC2 - Mucin 2

MyD88 - Myeloid differentiation primary-response protein 88

NF-kB - Nuclear factor kappa B

NLR - NOD-like receptor

NOD - Nucleotide oligomerization domain

RIP - Receptor-interacting proteins

ROS - Reactive oxygen species

SCFA - Short-chain fatty acid

SOD - Superoxide dismutase

TNF – Tumor necrosis factor

TLR - Toll-like receptor

Treg cell - Regulatory T-cell

UC - Ulcerative colitis

WT - Wild type

Resumo

A evolução do ser humano e dos organismos que constituem o seu microbiota está intimamente relacionada e a sua relação de simbiose é essencial para o desenvolvimento de ambos. As vias de sinalização entre os diferentes organismos do microbiota e as células do hospedeiro ainda não são totalmente conhecidas. No entanto, estão a ser feitos esforços no sentido de compreender de que modo o microbiota influencia o desenvolvimento humano, e vice-versa. A alimentação, antibióticos e outros fatores ambientais, assim como o genoma humano desempenham papéis importante na regulação do microbiota. Certas moléculas que se pensava terem um efeito deletério para o ser humano, como as espécies reativas de oxigénio e azoto, demonstraram ser peças fundamentais deste puzzle. Este trabalho tem como objetivo demonstrar que a modulação destas vias de sinalização pode constituir um avanço importante no tratamento de doenças como a IBD.

Abstract

The evolution of human beings and the organisms that constitute their microflora is closely related, and their symbiotic relationship is essential for the development of both. The signalling pathways supporting the interaction between the different organisms of the microbiota and host cells are not yet fully understood. However, efforts have been made in order to understand the bidirectional relationship between the microbiota and human metabolism. Diet, antibiotics and other environmental factors, as well as the human genome play an important role in the regulation of the microbiota. Some molecules that were initially thought to have a detrimental effect for humans, such as reactive oxygen and nitrogen species, proved to be fundamental pieces of this puzzle. This work aims to demonstrate that modulation of these signalling pathways can be an important advance in the treatment of diseases such as Inflammatory Bowel Disease.

1. Introduction

Since the dawn of mankind we have lived in close association with bacteria, archae, viruses and unicellular eukaryotes, which are collectively known as microbiota or microflora. It is estimated that approximately 100 trillion (10^{18}) microrganisms colonise the exposed surfaces of the human body, being the gastrointestinal tract the home to the majority of these microbes. In a healthy individual, the gut is inhabited by approximately 10^{14} bacteria, a number that is 10 times higher than the number of eukaryotic cells in the human body; additionally, their collective genome is ca. 100 times greater than the human genome. Although there were characterised over 50 bacterial phyla, the human gut microbiota is predominantly composed by the Gram-negative *Bacteroidetes* (16.3%) and the Gram-positive *Firmicutes* (65.7%), and, to a lesser extent, by *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, Fusobacteria, *Spirochetes*, *Fibrobacteres*, *Cyanobacteria* and *Planctomycetes*. Since the human intestine harbours eleven of the 50 known bacterial phyla, one may suggest that the bacteria aforementioned have evolved and specialised to live in symbiosis within the mammalian intestine. Several studies have ascribed beneficial roles to intestinal bacteria, e.g. support to nutrition (vitamin synthesis and degradation of complex carbohydrates), energy extraction, epithelial homeostasis, regulation of barrier function and epithelial restitution, modulation of fat metabolism, promotion of angiogenesis, exclusion of pathogenic microrganisms and promotion of the normal development and regulation of the immune system (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Clemente *et al.*, 2012; Karczewski *et al.*, 2014; Sekirov *et al.*, 2010).

The mammalian gut is colonized by microbes immediately after birth. This conclusion is supported by the similarities between the infants gut microbiota and their mothers vaginal microbiota. Furthermore, infants delivered by caesarean section demonstrate an initial microbiota composition similar to the skin microbiota from their mother. The number and diversity of intestinal microbes increases during the first year of life, after which it begins to resemble the microbiota of a young adult and then stabilizes (Sekirov *et al.*, 2010).

Numerous international projects, like the "Human Microbiome Project" and the "Metagenomics of the Human Intestinal Tract", demonstrated that the collective microbiota's genome contains ca. 100 times more unique genes than the human genome (Karczewski *et al.*, 2014). These projects also suggested that human population may be categorised in three different enterotypes, defined by bacterial species and their genes. Nevertheless, intestinal microbiota composition is not static over time. The richness (i.e.

number of species per sample) and the evenness (i.e. the relative abundance of species) depend on a plethora of factors rather than the host genotype, such as diet, antibiotics intake, pathogen infections, hygienic habits and exposure to stressful daily events. Variations of these factors can lead to the outgrowth of pathogenic species or depression of the beneficial ones. This is known as "dysbiosis" and has been implicated in diverse inflammatory and autoimmune diseases, including inflammatory bowel diseases (IBDs), coeliac disease, rheumatoid arthritis, type I and type II *diabetes mellitus*, obesity, multiple sclerosis and allergies, among others. In order to determine the aetiology of human inflammatory diseases and to develop a preventive and/or therapeutic strategy, one needs to understand how intestinal bacterial signals modulate the human immune system (Karczewski *et al.*, 2014).

2. Interactions between the microbiota and the host immune system

Over millennia, humans and their microbiota have evolved together. Ergo, it is expectable that the microbiota may participate in the development of the human being and vice-versa. In fact, studies with germ-free (GF) mice have demonstrated the impact of the microbiota in the development of the host immune system. GF animals do not acquire microbiota since they are created in sterile conditions. These animals contain atypical numbers of several immune cell types and immune cell products, specifically reduced count of immunoglobulin A (IgA)-producing plasma cells and decreased percentage of CD4+ T lymphocytes (Macpherson and Harris, 2004). Moreover, GF mice present aberrant intestinal epithelial cells and lymphoid structures, such as poorly developed gut-associated lymphoid tissue, hypoplastic Peyer's patches and fewer and smaller lymphoid nodes. Additionally, GF conditions also produced systemic alterations, like the underdevelopment of the spleen and lymph nodes (Round and Mazmanian, 2014). Interestingly, GF animals were found to be more sensitive to certain bacteria, virus or parasite infection. For example, infection by *Shigella flexneri* had more severe implications (decrease immune resistance to infection and increased mortality) in GF mice when compared with wild type (WT). It was also shown that previous colonisation with specific commensal bacteria oppose *S. flexneri* infection (Maier and Hentges, 1972). Hence, one may conclude that some members of the microbiota may provide protection against intestinal pathogens.

Mucous Layer – The Intestinal Great Wall

The intestinal epithelium consists of a single layer of columnar cells organized into villi (projections) and crypts (invaginations). This epithelium is mainly constituted by absorptive enterocytes, which are cells specialized in metabolic and digestive functions. Nonetheless, the multifunctional role of the intestinal epithelium correlates with the additional specialized cells. Secretory intestinal, namely enteroendocrine cells, goblet cells and Paneth cells are scattered through the enterocytes. Enteroendocrine cells secrete numerous hormones and peptides (serotonin, enteroglucagon and somatostatin) that regulate the digestive function. Goblet cells are located in the villi and secrete mucin while Paneth cells are located in the crypts and produce antimicrobial proteins (AMP). Together, these cells are responsible for the development of a dynamic physical and biochemical barrier between the host and the microbiota (Kierszenbaum, 2007).

Mucins are highly glycosylated proteins that form the mucous layer. Intestinal mucous layer is mostly composed by mucin 2 (MUC2) and is considered the first line of defence against microbial invasion. The role of MUC2 is highlighted by the spontaneous development of colitis in MUC2-deficient mice (Van der Sluis *et al.*, 2006). Additionally, this model also demonstrated that lack of MUC2 may result in inflammation-induced colorectal cancer (Velcich, 2002). Goblet cells are also responsible for the secretion of trefoil factor 3 and resistin-like molecule β. Among other functions, trefoil factor 3 promotes epithelial repair, prevent apoptosis and induces mucin crosslinking, thereby increasing mucous viscosity. In turn, resistin-like molecule β regulates macrophage and adaptive T-cell responses during inflammatory events and enhance MUC2 secretion (Peterson and Artis, 2014).

The main AMPs released in the crypts of the intestinal epithelium are defensins, cathelicidins and lysozyme. These molecules disrupt microbial organisms by multifarious mechanisms. For example, defensins and cathelicidins are able to form pores in bacterial cell membrane whereas other AMPs target Gram-positive cell wall peptidoglycans (Mukherjee *et al.*, 2014). As it will be discussed, AMPs are able to regulate both pathogenic and commensal bacteria.

Interestingly, the combined antimicrobial activity of mucin and AMPs seems to be important to limit microbial communities: whereas the number of goblet cells increase from the duodenum to the terminal ileum, the production of AMP also differs along the intestinal tract, being higher in the distal intestine (Darmoul and Ouellette, 1996). Although more

research is needed, one may postulate that this may correlate with the differences of both intestinal microbiota composition and localization along the gut.

Microbiota-host immune system interactions

Studies from the last decades have shown that microbiota influences the host immune system through a multitude of factors that are collectively known as microbe associated molecular patterns (MAMPs). These factors include not only microbial components but also their metabolites as described in table I.

Table I. Examples of bacterial ligands, receptors and their immunologic effects.

Ligand	Receptor	Immunologic Effect
LPS	TLR4	• Activates NF-kB; • Activates DCs; • Inhibits mucosal DCs; • Promotes bacterial colonization during homeostasis; • Protects against colitis.
PSA	TLR2	• Promotes Th1/Th2 balance; • Suppresses intestinal IL-17 production; • Induces Treg cells; • Promotes bacterial colonization during homeostasis; • Protects against colitis.
Flagellin	TLR5	• Activates NF-kB; • Induces Treg cells; • Promotes colitis.
MDP	NOD2	• Activates NF-kB; • Promotes lymphoid tissue development; • Promotes antigen-specific immune responses; • Promotes tolerance to bacterial products; • Inhibits IL-12 production; • Regulates intestinal bacterial communities; • Protects against Crohn's disease and colitis.
Butyrate	GPR109A	• Suppresses NF-kB signalling in IECs; • Reduces production of: TNF-a, TNF-b, IL-6; IL-1b.

(Adapted from Karczewski *et al.*, 2014)

The host innate immune system is able to recognise those factors through receptors known as pattern recognition receptors. The main pattern recognition receptors are the Toll-like receptors (TLRs) and the NOD-like receptor (NLR) and the RIG-1-like receptor (Peterson and Artis, 2014).

Interactions mediated by Toll-Like Receptors

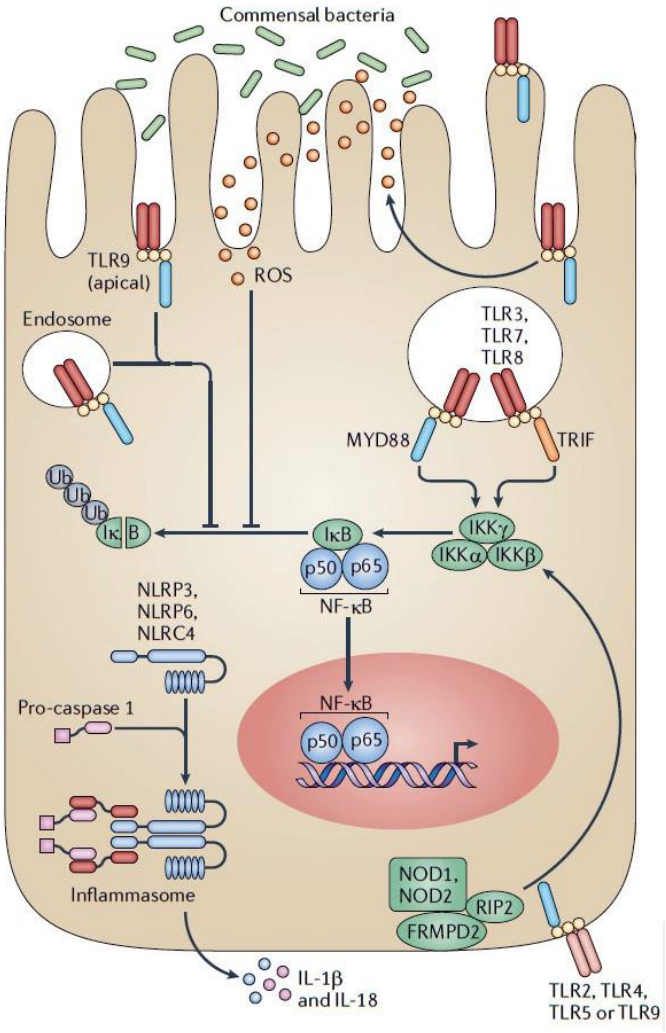
TLRs are a family of nine transmembrane receptors located on both, the apical and the basolateral sides of the cell membrane, recognizing external stimuli or microbial nucleic acids, respectively. Lee *et al.* have shown that the basolateral activation of TLR9 leads to the

expression of proinflammatory cytokines, but apical activation of the same receptor intercepts the proinflammatory response and promotes tolerance to the intestinal microbiota (Figure 1). The interaction between TLR and MAMPs at the basolateral side promotes the association of both the adaptors myeloid differentiation primary-response protein 88 (MyD88) and the TIR-domain-containing adaptor protein inducing interferon-β with the intracellular portion of the TLR, activating two distinct signal transduction pathways: 1) promotes the phosphorylation of the inhibitors of kappa light poly-peptide gene-enhancer B cells (IκB) kinase, which activates the nuclear factor kappa B (NF-κB) pathway and 2) induces the activation of the mitogen-activated protein kinase (MAPK) pathway. Both pathways culminate with the activation of transcriptional factors that promote the expression of proinflammatory genes and, consecutively, the production and activation of proinflammatory molecules, such as interleukin (IL)-2, IL-6 and tumor necrosis factor (TNF)-α (NF-κB pathway). The apical interaction between TLR and MAMPs (commensal bacteria) promotes the stabilization of IκB and thereby preventing NF-κB activation. Moreover, this apical signal is responsible for tolerance to further TLR stimulation (Lee *et al.*, 2006).

The protective role of the TLR has been studied using knockout mice, i.e. genetically modified mice that do not express one or more genes. TLR2, TLR4, TLR9 and MyD88 knockout mice are highly susceptible to dextran sulphate sodium-induced colitis (Araki *et al.*, 2005; Rakoff-Nahoum *et al.*, 2004). Furthermore, $TLR5^{-/-}$ mice have exhibit an abnormal microbiome, causing spontaneous colitis (Vijay-Kumar and Aitken, 2010). However, the constitutive TLR signalling must be perfectly regulated. Excessive activation of the NF-κB pathway may lead to harmful inflammation since the proinflammatory cytokines induced by this pathway, like the interferon-γ and the TNF-α, may interact with tight junction functions and increase the epithelial barrier permeability, especially in IBDs (de Kivit *et al.*, 2014). Thus, TLRs are regulated at four different levels: genetic, transcriptional, translational and post-translational. The regulation of TLRs is mostly achieved by protein post-translational modifications. Although the main post-translational mechanisms described in literature are phosphorylation, acetylation and methylation, ubiquitination has recently been found to regulate several TLR signalling molecules, such as TNF receptor-associated factors, receptor-interacting proteins, interleukin IL-1 receptor-associated kinases, IκB, IκB kinases and interferon response factors. A20, tumor suppressor cylindromatosis, otubain 1, otubain 2, Cezanne and Yopj antagonise ubiquitin by promoting the deubiquitination of the stated signalling proteins (Anwar *et al.*, 2013). The withdrawal of one negative TLR regulator leads to the exacerbation of TLR signalling despite it may have a positive influence in the

expression of the remaining factors. Thus, it seems that each negative TLR regulator is important to suppress TLR signalling and it may explain why they don't balance each other deficiency (Karczewski *et al.*, 2014).

Figure 1. Microbial recognition through TLR.



The basolateral interaction between TLRs and MAMPs promotes the activation of the NF-κB and the MAPK pathway, with production of proinflammatory cytokines. Additionally, this interaction may induce the production and secretion of IL-1β and IL-18 from inflammasomes. On other hand, the apical interaction between TLRs and MAMPs prevents the activation of the NF-κB pathway (adapted from Peterson e Artis, 2014).

Finally, the integrity of the epithelial cell barrier is important to an appropriate regulation of TLRs. Constant bacterial translocation through intestinal epithelia seems to lead to chronic TLR activation, and thus to an overexpression of proinflammatory cytokines. Consecutively, epithelial destruction is enhanced, culminating in chronic intestinal inflammation (Welz *et al.*, 2011).

Host-microbiota interaction via TLRs is also important for the production of IgA, which is essential not only to define the qualitative and the quantitative composition of the microbiota but also to limit the immune response towards commensal organisms. IgA secreted to intestinal lumen is composed by a dimer (or tetramer), a J-chain polypeptide and the secretory component. Since it is polymeric, secretory IgA is able to cross-link antigens with multiple epitopes (Kindt *et al.*, 2007). Bacterial and viral surface antigens form complexes with IgA, thus, preventing the attachment of pathogens to epithelial cells. It's noteworthy that secreted IgA displays a dual role in host-microbiota interaction. First, it prevents the overgrowth of microbial species, which could promote dysbiosis. Second, it minimizes the interaction between microbiota and mucosal immune system, preventing an exacerbated inflammatory response (Peterson *et al.*, 2007). IgA-producing B lymphocytes may be produced in Peyer's patches through a T-cell-dependent manner. Microfold cells mediate the sampling of luminal contents and are responsible for antigen presentation to the mucosal immune system. T-cells detect the antigens and promote the differentiation of IgA-producing B cells. These cells migrate into draining mesenteric lymph nodes, where they differentiate into IgA-secreting plasma cells that, in turn, spread along the intestinal *lamina propria*. However, the main source of IgA-producing B lymphocytes are isolated lymphoid follicles, through a T-cell-independent process. Symbiotic microbiota plays an important role in this mechanism given that it is required for the normal development and maturation of isolated lymphoid follicles. In addition, GF mice hold few IgA-secreting plasma cells in the intestinal mucosa in comparison with WT, corroborating this idea (Jacobs and Braun, 2014).

The development of T-cells is modulated by TLR signalling, as it is demonstrated by the lack of Th17 in the intestinal mucosa of GF mice. Th17 cells are an important subset of T-helper cells that are implicated in the production proinflammatory cytokines (like IL-17A, IL-17F, IL-21, IL-22 and TNF-β) as well as in regulation of granulopoiesis, neutrophil recruitment and induction of diverse antimicrobial peptides. This T-cell subset has also been implicated in the maintenance of epithelial homeostasis and in the prevention of microbial epithelial translocation, which is observed during infections that promote de destruction of Th17 lineage (HIV and SIV) (Karczewski *et al.*, 2014).

Interactions mediated by Nucleotide Oligomerisation Domain 1 and 2

NOD1 and NOD2 receptors are located inside the epithelial cell and participate in the recognition of pathogenic microrganisms that are able to invade and multiply in cytoplasm. Once activated, NOD receptors stimulate signal transmission through receptor-interacting protein 2, activating NF-κB and MAPK pathways (Franchi *et al.*, 2008).

NOD receptors are one of the major components of inflammasomes, multiprotein complexes responsible for the maturation and secretion of several proinflammatory cytokines. Nlrp3, also known as Nalp3, Cryopyrin, CIAS1, PYPAF1 and CLR1.1, is an example of an inflammasome that may be found in intestinal epithelial cells. It is composed by a NOD motif, an array of 12 leucine-rich repeat (LRR) ligands and a pyrin domain. The LRR ligands modulate Nlrp3 activity and interact with microbial signals. The pyrin domain allows the cooperation with the adaptor protein apoptosis-associated speck-like protein containing a CARD, which allows the interaction with the caspase-1. This enzyme is responsible for the activation of the proinflammatory cytokines IL-1β and IL-18. Besides their role in the inflammatory response, the latter molecules induce the production of grown factors and promote the epithelial repair and healing process (Zaki *et al.*, 2011).

Mechanisms of inflammatory tolerance

Besides the recognition and elimination of pathogenic microrganisms, the human immune system must avoid deleterious reactions to the host. In order to achieve self-tolerance and immune homeostasis, the human immune system developed several mechanisms, like the production of regulatory T (Treg) cells. This T-cell subset is responsible for suppressing immune responses towards self, quasi-self (like autologous tumour cells) and non-self cells (like intestinal bacteria). The generation of Treg cells may occur in the thymus, due to the interaction between forkhead box P3 and T-cell precursor, or in peripheral tissues, due to the stimulation of naïve T-cells by TGF-β, IL-2 and retinoic acid. Once activated, Treg cells supress the proliferation and differentiation of naïve T-cells *in vivo*, as well as the function of several immune cells, like natural killer cells, natural killer T-cells, B cells, macrophages and dendritic cells. *In vitro* reports have stated that Treg cells may also suppress the proliferation and cytokine production. One of the mechanisms that may explain Treg-mediated suppression is the immunosuppressive cytokines secretion by Treg cells. For example, IL-10 and TGF-β promote suppression of the inflammatory process in IBD (Sakaguchi *et al.*, 2008).

In summary, exogenous stimuli, such as commensal bacteria interaction with the intestinal epithelia, may promote the activation of Treg cells and, consequently, negative regulation of T-cell responses.

3. Intestinal Bowel Diseases – When microbiota-host interaction goes too far

IBDs is a heterogeneous group of chronic, relapsing, immune-mediated inflammatory disorders of the gastrointestinal tract. There are some records of IBD observations since ancient times, but only in 1875 Wilks and Moxon brand the term "Ulcerative Colitis" (UC) to describe a case of a young woman, who died from severe diarrhoea and presented ulceration and inflammation of the colon. After this report, doctors' awareness to inflammatory bowel diseases grew and in 1932 Crohn *et al.* published a paper describing a distinct inflammatory disease, which is known nowadays as Crohn's Disease (CD). The main differences between UC and CD are summarised in table 2.

Table 2. Main differences between UC and CD.

	Ulcerative Colitis	**Crohn's Disease**
Affected regions	Colon and rectum	Mostly ileum, but may involve any other intestinal region
Inflammation process	Restricted to the mucosa	Involves the full thickness of the bowel
Presence of pathological artefacts	Mostly absent	Granulomas, strictures and fistulas are often present
*Environmental factors (smoking)**	Higher incidence among non-smokers and former smokers	Higher incidence among smokers

*Other environmental factors, such as diet, antibiotic use and hygiene, as well as the genetic component of IBD are discussed below.

Published studies have highlighted a decreased qualitative and quantitative composition of both mucosa and fecal-associated bacteria in patients with IBD. As reviewed by Daniela Serban, the table 3 summarizes the main results of published studies.

The incidence of IBD is remarkable worldwide, especially in western countries and, in Europe alone, ca 2.5 and 3.0 million people suffer from the disease. These gastrointestinal disorders are mainly diagnosed in adolescence and early adulthood (Loftus, 2004) and, since they are associated with low mortality, the prevalence of IBD tends to increase over the years. As consequence, they can represent a significant economic burden to the health-care system.

The geographical disparity of IBD's incidence may be explained by various factors: 1) there is a genetic predisposition for the development of the disorder, 2) the environmental factors and their consequences vary between populations, 3) there are differences between the health-care facilities and medical technology, and 4) there is a discrepancy in the methods used to diagnose and control the progress of IBD between develop and developing countries (Kaplan, 2015).

Table 3. Bacteria associated with IBD.

Potentially Harmful	**Potentially Protective**
Adherent-invasive Escherichia coli • ↑in mucosa of ileal CD, colonic CD and UC *Fusobacterium spp.* • ↑in active UC pouchitis • *Fusobacterium varium*: ↑in UC • *Fusobacterium nucleatum*: ↑in mucosa of IBD adults and newly diagnosed CD children *Campylobacter concisus* • ↑in pediatric and adult CD and UC *Desulfovibrio spp.* • Associated with less sulphated mucin and correlated with mucosal inflammation in UC *Klebsiella spp.* • Associated with CD *Enterohepatic Helicobacter* • ↑in mucosa in UC and CD *Ruminococcus gravus* (controversial): • ↑in feces in CD • ↓in mucosa of newly diagnosed CD children *Clostridium difficile* • ↑risk of colonization/infection in IBD • Significantly ↑prevalence in IBD children at diagnose *Veillonella spp.* • ↑in mucosa of newly diagnosed CD children and associated with worse clinical outcome • ↑in post-surgical recurrence in CD	*Faecalibacterium prausnitzii* • ↓ in ileal mucosa in newly diagnosed pediatric CD • ↓ in feces in adult CD, active IBD and adult UC • ↓ in ileal mucosa in post-operative recurrence of CD • ↓ in mucosa of healthy siblings of CD patients *Clostridium clusters IV and XIVa* • ↓ in ileal mucosa in CD and in feces in active CD and UC • *Roseburia spp.*: ↓ in mucosa of adult CD and UC of newly diagnosed CD children *Bacteroides spp.* • ↓ in mucosa in adult CD and UC, active UC pouchitis and newly diagnosed pediatric UC and CD *Bifidobacterium spp.* • ↓ in CD (newly diagnosed children, in mucosa) and UC (in mucosa and feces) • *Bifidobacterium adolescentis*: ↓in fecal samples in CD *Anaerostipes spp.* • ↓in current or former smokers *Dorea spp., Butyricicoccus spp., Coriobacteriaceae spp.* • ↓in patients receiving antibiotics

(adapted from Serban, 2015)

Genetic Component of IBD

Recent studies point to 163 loci as a susceptibility region for the development of IBD. The main genes described in literature are the *NOD2* gene and the *ATGG16L1* (Jostins *et al.*, 2012).

The *NOD2 gene*

The NOD2 gene, also known as caspase recruitment domain family member 15, was the first gene to be associated with CD. There are reports of three main mutations in this gene, all targeting the region that encodes a LRR. This region is responsible for binding MDP, present in the cell wall of most bacteria. The interaction between MDP and LRR promotes the activation of the NF-κB pathway, leading to the production of proinflammatory molecules. Then, *NOD2* mutations may lead to defective MDP binding. Additionally, Watanabe *et al.* demonstrated that TLR2 downregulates NF-κB in *NOD2*-defective cells, which could be responsible for the development of a nonregulated inflammatory response by means of an inefficient downregulation of innate immune response, ineffective clearance of intracellular bacterial infection and proliferation of commensal bacteria (Watanabe *et al.*, 2008).

It is estimated that 25 to 35% of CD patients with European ancestors hold one of *NOD2* mutations, but they were not found in Asian nor African American CD patients (Kaplan, 2015).

The *ATG16L1* gene

The *ATG16L1* gene is correlated with autophagy, an essential mechanism of cellular homeostasis. Mice with low expression of *ATG16L1* exhibit Paneth cells with morphological and genetic abnormalities, which is also perceptible in CD patients with this genetic mutation. Furthermore, ATG16L1 seems to regulate the release of IL-1β, a cytokine responsible for the inhibition of inflammatory response (Abraham and Cho, 2009).

The influence of antibiotics

Several studies have pinpointed antibiotics as one of the main causes of dysbiosis. Up to 30% of the gut bacterial species can be affected by the use of broad-spectrum antibiotics and these disturbances may persist months or even years after ceasing the treatment. Studies by Fouhy et al and Tanaka et al demonstrated that the administration of antibiotics in infants led to the development of an abnormal microbiota with reduced bacterial diversity

(Fouhy *et al.*, 2012; Tanaka *et al.*, 2009). Additionally, the utilization of the "omic" techniques demonstrated that gene expression, protein activity and the metabolism of the microbiota were also affected by antibiotics (Franzosa *et al.*, 2015).

One of the effects induced by antibiotic administration is the increased susceptibility of the patient to intestinal infections. As already mentioned, commensal bacteria prevent the adhesion of pathogenic microbes to the human gut mucosa by competitive exclusion. Once antibiotics reduce the quantity of microbiota, this mechanism does not occur. Moreover, pathogens may overgrow commensal bacteria, leading to systemic infection (Francino, 2016).

The microbiota is indispensable for the development and homeostasis of human immune system. Hence, alterations induced by antibiotics may have important long-term repercussions, especially if occurring in the first years of life (Francino, 2014). There are several indications of a link between atopic diseases (e.g. asthma), inflammatory diseases (e.g. IBD) and autoimmune diseases (type 1 diabetes mellitus) and changes in microbiota composition during childhood. Additionally, there are reports associating the development of obesity and dysbiosis, namely modifications at the phylum level, reduction of bacterial diversity and alterations of bacterial metabolic pathways (Bäckhed *et al.*, 2004; Turnbaugh *et al.*, 2009, 2006).

Although it is far beyond the scope of this work, antibiotics affect the microbiota by increasing the amount of resistant organisms. Importantly, antibiotic resistance genes have no only been detected in adults but also in children and infants. There are reports of antibiotic resistance genes detected in feces of 1-week-old babies and even in meconium, which indicate that resistances may be vertically inherited (Francino, 2016).

Hygiene Hypothesis

The fact that autoimmune disorders (like allergies) were less commonly observed in people who were raised in rural areas or who belong to numerous families, motivated Strachan to develop the hygiene hypothesis in 1989 and to improve it in the following decades. According to this hypothesis, excessive hygiene during childhood hampers exposure to a wide range of antigens, leading to dysbiosis and thus preventing the regular development of the immune system (Strachan, 2000).

Diet – we are what we eat

Diet has been considered one important factor in the development of IBD. This association has been hypothesized in the twentieth century but the first strong evidence

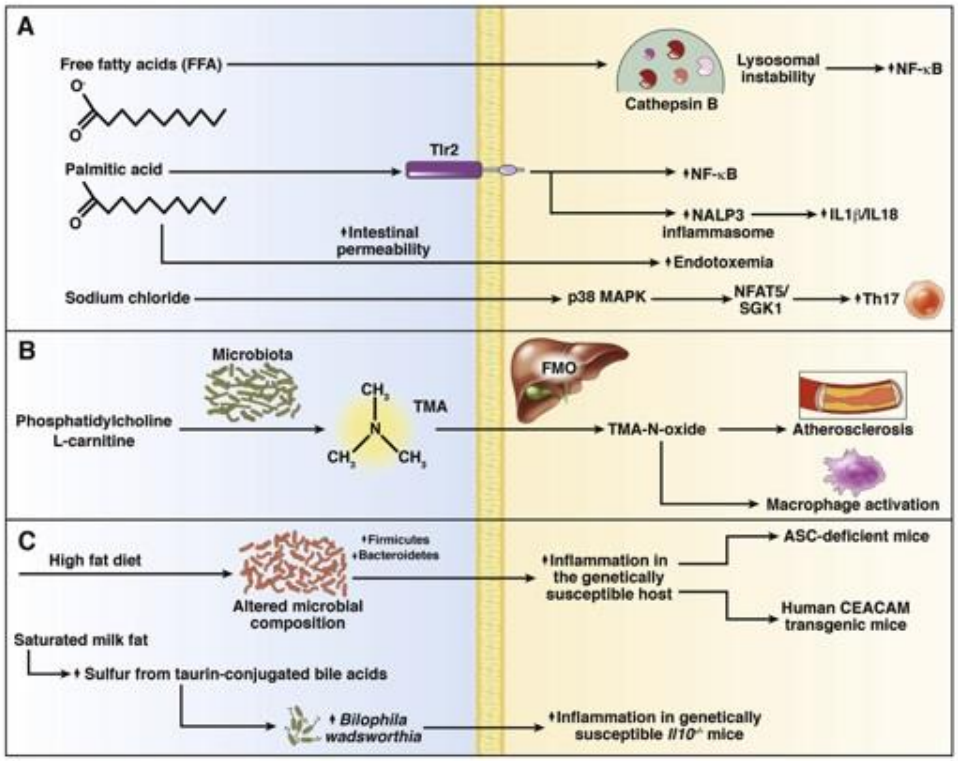
came from a comparative study of the microbiota of European children and rural African children. The investigators observed that stools from African children, whose diet consists of foods with high fibre content, showed less *Firmicutes* and *Enterobacteriaceae*, but more *Provetella* in comparison with the European children, whose diet is dominated by animal proteins, in particular meat and saturated fats. Besides the alterations of the microbiota composition, diet is also used as substrate for microbial metabolism, which may affect the host immune system (De Filippo *et al.*, 2010).

One of the main elements of the development of IBD is the high fat content of the western diet. Fatty acids promote inflammation through numerous mechanisms. Free fatty acids induce lysosomal instability, which leads to the activation of the NF-κB pathway. Moreover, palmitic acid may interact with TLR2, activating the NALP3 inflammasome thereby increasing intestinal permeability and aggravating inflammatory processes. Overall, a high fat diet seems to contribute to the initiation or exacerbation of intestinal inflammation. Also, western diet is dominated by a high amount of animal proteins and salt. Phosphatidylcholine and L-carnitine are metabolized by the gut microbiota, resulting in accumulation of trimethylamine, which may be further converted in trimethylamine N-oxide by hepatic flavin mono-oxygenase. Trimethylamine N-oxide are correlated with adverse cardiovascular events, such as atherosclerosis and activates macrophages (Tilg and Moschen, 2015). These mechanisms are summarized on Figure 2.

On the other hand, there are protective outcomes on the relationship between diet, microbiota and host immune system. A major component of the anti-inflammatory effect induced by diet is promoted by the aryl hydrocarbon receptor, which is expressed by immune and epithelial cells. Activation of the aryl hydrocarbon receptors by exogenous ligands promotes the expression of several genes that encode proteins enrolled with immunity and inflammation, such as *IL22*. The importance of the aryl hydrocarbon receptor in the homeostasis of the intestinal immune system has been established by studies in mice lacking the aryl hydrocarbon receptor, which were more susceptible to severe colitis, and by the fact that IBD patients presented reduced expression of aryl hydrocarbon receptor (Kiss *et al.*, 2011; Li *et al.*, 2011). Another important mechanism that promotes gut homeostasis is the production of short-chain fatty acids (SCFAs), particularly acetate, propionate and butyrate, by intestinal bacteria. SCFAs are obtained by dietary-fibre metabolism, what explains why African children stools are particularly rich in these metabolites when compared with European children (De Filippo *et al.*, 2010). Acetate, propionate and (mainly) butyrate ensure gut homeostasis by several mechanisms: 1) they stimulate mucous and IgA

production, both important for the development of the host-microbiota barrier, 2) they potentiate Treg cells responses, increasing immune tolerance to xenobiotics and antigens, 3) SCFAs maintain epithelial integrity through inflammasome activation and production of IL-18 and 4) they inhibit the NF-κB pathway, reducing the production of proinflammatory cytokines and endothelial cell adhesion molecules (Thorburn *et al.*, 2014). A recent systematic review corroborates these findings, since it describes a positive relation between the risk of developing UC or CD and high intake of fat. Contrastingly, the regular intake of vegetables (fruit and high fibre products) was associated with a decrease in the risk of developing UC or CD (Hou *et al.*, 2011).

Figure 2. Inflammatory mechanisms of food components.



(A) Certain dietary components, like free fatty acids and palmitic acid, can activate inflammatory pathways directly. (B) Other dietary components, namely phosphatidylcholine and L-carnitine, may induce inflammation indirectly, after modification by the commensal microflora. (C) Certain diets alter the intestinal microbiota to one that promotes inflammation (Tilg & Moschen. 2015).

One of the clinical hallmarks of IBD is chronic intestinal bleeding, with poor outcomes in terms of quality of life scores. These haemorrhages frequently lead to iron deficiency. Thus, supplementation of iron is important for these patients in order to avoid the development of anaemia. However, oral iron supplementation may exacerbate the inflammatory process and aggravate the symptoms. The mechanism underlying iron proinflammatory properties are related with the formation of toxic reactive oxygen species (ROS), which may activate the NF-κB pathway and exacerbate the inflammatory state

(Weiss, 2011). Although this deleterious action is described in the literature, ROS have an important role in intestinal homeostasis that will be extensively detailed in the next section.

4. Reactive Oxygen Species

ROS may be broadly defined as short half-life and electrophilic molecules, which result from the incomplete reduction of molecular oxygen. This group of molecules may be divided in radical forms (e.g. superoxide radical, O_2^-) or non-radical forms (e.g. hydrogen peroxide, H_2O_2).

Traditionally, ROS were seen as an undesirable side products of cellular metabolism. The scientific community shared the belief that their production was not regulated and oxidant species might target intracellular molecules (lipids, proteins or DNA) randomly. This would lead to the accumulation of damaged biomolecules which, in turn, were responsible for numerous disorders, including neurodegenerative diseases, atherosclerosis and ageing (Holmström and Finkel, 2014). In order to reduce the deleterious effects of ROS, the cell accommodated several antioxidant enzymes, like the superoxide dismutase (SOD), that converts superoxide into hydrogen peroxide, and catalase, which are responsible for the conversion of latter in water. Along with these enzymatic scavengers, other proteins such as glutathione, thioredoxins and peroredoxins may also reduce the intracellular steady state concentration of these evanescent molecules (Jones *et al.*, 2012).

However, the paradigm is changing and in the recent years the notion of "redox signalling", the fine tuning of endogenous signalling pathways by oxidizing species, has emerged.

ROS cellular sources and main classical effects

One may consider two main sources of ROS within the cell: mitochondria and NADPH oxidases. Other enzymes, such as xanthine oxidase, nitric oxide synthase, cyclooxigenases cytochrome P450 enzymes and lypoxygenases can also produce ROS and their contribution to the total amount of oxidants varies according to cell type (Holmström and Finkel, 2014).

The mitochondria is responsible for the oxidative phosphorylation in eukaryotes. This process is characterized by the generation of ATP in an oxygen-dependent manner due to the electrons flow in the respiratory chain, culminating with the reduction of oxygen to

water in mitochondrial complex IV. Several steps during this process have the potential to generate ROS due to the transition of one electron to the oxygen molecule, resulting in the formation of a superoxide radical (Murphy, 2009). As already mentioned, superoxide radical is further converted into hydrogen peroxide by SOD. In contrast to superoxide radical, hydrogen peroxide may diffuse through mitochondrial membranes into the cytoplasm and through the plasmalemma into the extracellular milieu. Due to the presence of ferrous and cuprous ions, hydrogen peroxide might be reduced to the hydroxyl radical, an extremely reactive species, which oxidizes proteins, lipids and nucleic acids (Pelletier *et al.*, 2012).

Alternatively, ROS may be produced by NOX enzymes. This family of proteins is composed by seven members: NOX1-5, dual oxidase (DUOX) 1 and DUOX 2. NOX2, was first described in neutrophils, phagocytic cells. In this process, microrganisms are confined in a specialized compartment called the phagosome, where they are degraded due to the action of enzymes and ROS (Holmström and Finkel, 2014).

Lastly, there are several enzymes that may generate ROS as a byproduct of their normal activity. The oxidants obtained by these enzymes have been connected with inflammatory responses. One example is the xanthine oxidase, which promotes purine degradation, and uric acid production. Under certain conditions, like hypoxia, xanthine oxidase produce superoxide and hydrogen peroxide. These oxidants can then induce early growth response-1, a transcription factor important for fibrosis and inflammation (Pelletier *et al.*, 2012).

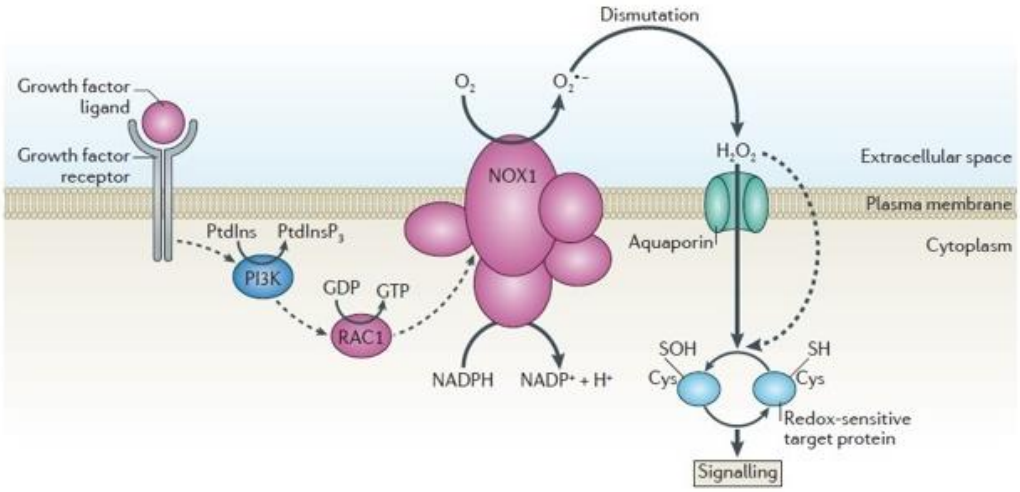
The physiological role of ROS – a bright new world

Despite the antimicrobial effect and the biochemical impact on proteins, lipids and nucleic acids, reports are emerging on the signalling function of ROS, especially hydrogen peroxide.

Mitochondrial ROS are important in sterile inflammation mediated by cytokine IL-1β. The increased production of mitochondrial ROS stimulates inflammasome priming by inactivation of MAPK phosphatases (and, consequently, to sustained MAP kinase activity) or accumulation of hypoxia inducible factor 1. These molecules lead to the transcription of pro-IL-1β and NLRP3 (Chandel *et al.*, 2000, 1998). Additionally, this factor is one of the main players in the metabolic adaptation to hypoxic situations. Since macrophages lacking the α subunit of hypoxia inducible factor 1 present metabolic defects, like reduced ATP and aggregation problems, mitochondrial ROS may be involved in cellular metabolism (Cramer *et al.*, 2003).

Early studies supporting a physiological role for ROS in signal transduction reported that there was an increase of ROS generation preceding the increase of tyrosine phosphorylation that follows stimulation of growth factors (like the epidermal growth factor) (Rhee *et al.*, 1997). This increase of ROS generation was not harmful but it was important for downstream signalling. Later studies demonstrated the mechanisms underlying these observations: for instance, the activation of Nox enzymes, leads to the production of superoxide radical, which is further converted in hydrogen peroxide. Hydrogen peroxide may then diffuse back to the cell where it interacts with reduction-oxidation-sensitive targets like the cysteine residues of tyrosine phosphatases (Figure 3) (Meng *et al.*, 2002). The resulting oxidized cysteine residues may be reversibly converted by peroxiredoxin. As a result of the reversibility if this mechanism, due to the effect of a cellular enzyme, the physiological role of ROS is reinforced.

Figure 3. Reactive oxygen species can function as mediators of intracellular signalling.



Upon activation via a signalling pathway triggered by receptor activation (e.g., GFr), membranar NADPH oxidase uses reducing equivalents from NADPH to produce superoxide radical extracellularly that, in turn, may dismutate to H_2O_2. Because H_2O_2 is able to permeate cell membranes (or might cross via aquaporins), might activate intracellular signalling pathway by reversible oxidation of –SH groups in cysteine residues in regulatory proteins (Holmström & Finkel, 2014).

One of the models that has recently been introduced to the study of host-microbiota interactions is the *Drosophila* model. *Drosophila* presents a simple commensal community as well as a limited genetic pool. Additionally, its immune system is well known and it's relatively easy to generate gnotobiotic animals.

As reviewed by Sung-Hee Kim and Won-Jae Lee, ROS produced by *Drosophila* Duox participate in host-microbiota interactions, being implicated in microbial clearance,

discrimination between commensal bacteria and pathogens, cross-linking of biomolecules, intestinal epithelial cell renewal and redox-dependent modulation of signalling pathways.

1) The activation of Duox leads to the production of non-phagocytic ROS in the epithelial cells of the intestinal tract (Kim and Lee, 2014). ROS generated this way may have an antimicrobial effect similar to phagocytic ROS in mammals. This fact is confirmed by DUOX-knockdown models, which presented enhance susceptibility to develop gut infections by numerous microrganisms (Ha *et al.*, 2009).

2) Duox plays also an important role in the discrimination between commensal bacteria and pathogens due to the recognition of a uracil nucleobase that is secreted only by allochthonous bacteria. The interaction between Duox and uracil promotes the release of ROS with ensued antimicrobial activity (Lee and Brey, 2013).

3) Duox is involved in the cross-link of biomolecules, which is important to maintain the integrity of gut barrier function. In *Anopheles gambiae*, the hydrogen peroxide produced by DUOX acts as a substrate of a peroxidase, which catalyzes the protein cross-linking in the mucin layer (Kumar *et al.*, 2010).

4) As described by Buchon *et al.*, flies with impaired activity of Duox are not able to induce the regeneration of intestinal epithelium after infection due to reduce ISC proliferation and differentiation (Buchon *et al.*, 2009).

5) ROS produced by Duox may be important for signal transduction. A relevant report of signal transduction was the inability to express TLR-downstream target genes in epithelial cells due to the absence of Duox-dependent hydrogen peroxide (Joo *et al.*, 2012).

Recently, Jones *et al.* studied the role of *Lactobacillus spp,* in *Drosophila* and Mice gut. In the *Drosophila* model, the commensal *Lactobacillus spp.* induced the production of endogenous ROS leading to cellular proliferation. Administration of *Lactobacillus rhamnosus* strain GG induced the Nox1-dependent ROS generation in enterocytes, which was responsible for cellular proliferation in the gut. In a mice model, *Lactobacillus*-induced ROS were responsible for the inactivation of protein-tyrosine phosphatase and Src homology phosphatase 2, which are known regulators of focal adhesion kinases phosphorylation. Furthermore, commensal bacteria were responsible for the increase of cell migration velocity *in vitro* and for the recovery of the barrier function after infection *in vivo* (Jones *et al.*, 2013).

5. Therapeutic interventions on IBD

One of the recent hallmarks of IBD pathophysiology is dysbiosis. So, therapies targeting the microbiota have been under extensive study. The main advantage of this approach, when compared with traditional pharmacological therapies, is the increased safety of the method.

Gene's therapeutic approach

The "Human Microbiome Project", the "Metagenomics of the Human Intestinal Tract" and other studies allowed the determination of several bacterial genes responsible for the survival of microbiota. The target genes are usually involved in the metabolism of carbohydrates, amino acids, xenobiotics, and also and biosynthesis of methane, vitamins and isoprenoids. Usually, the target genes are non-existent in the human genotype, which constitutes a major advantage of this approach, since it may have virtually no side effects. Some examples of targets are the genes involving in the bacterial synthesis of thiamine, folic acid and methionine (Belizário and Napolitano, 2015).

It is likely that this approach may be more commonly used in the near future due to the introduction of metagenomics data, which will extend our database of possible targeting genes.

Antibiotic resistance genes

The microbiota profile is maintained due to the intercellular signalling between microbes themselves and the host, a mechanism known as quorum sensing, which not only ensures bacterial communication but also synchronize responses, like gene expression, to external stimuli (Wright, 2010).

Antibiotic resistance genes may be acquired due to mobile genetic elements, including conjugative transposons that are transferred between bacteria through plasmids or bacteriophages. One of the approaches has been the transposon-aided capture, which consists in the trap of particular plasmids that contain antibiotic resistance genes (Mullany, 2014). Thus, this technique might allow the withdrawal of specific genes that encode resistance to antibiotics, in order to manipulate the microbiota through an antibiotic approach.

Quorum sensing systems approach

Quorum sensing systems are indispensable for the microbiota community since this mechanism is responsible for the synthesis of several products as well as virulence factors. The unbalance of quorum sensing systems might be an alternative approach to control microbiota, promoting the growth of commensal bacteria and inhibiting the increase of pathogenic bacteria. In order to this approach to succeed, it is necessary to identify the main molecules responsible for the inter-bacteria signals, their receptors and the mechanisms underlying quorum sensing. More research is needed, but metabolomics studies might help to reveal the main mediators of bacteria communication (Belizário and Napolitano, 2015).

Fecal Microbial Therapy

Fecal microbial therapy (FMT) consists in the transplantation of stool from a healthy donor into the gut of an unhealthy patient in order to cure a specific disease. This procedure has been successfully used in patients with *Clostridium difficile* colitis, even in cases of refractory pseudomembranous colitis. The mechanisms underlying this approach are: 1) competition for nutrients, which prevents *C. difficile* colonization; 2) direct inhibition of *C. difficile* development; 3) modulation of some metabolic pathways, like the degradation of bile salts, impairing the life cycle of *C. difficile*; and 4) enhance of host-microbiota interaction, with development of the immune system, and consequently prevention of *C. difficile* colonization and its recurrence.

The use of FMT for IBD was first reported in late 80's. The transplantation of stool from a healthy donor by retention enema was able to recede the symptoms of UC 6 months after FMT. A systematic review and meta-analysis from 2014 including 18 studies (9 cohort studies, 8 case studies and 1 randomized controlled trial) suggested that FMT was a safe treatment for IBD but its efficacy was very variable. Moayyedi *et al.* performed a randomized controlled trial for evaluation of FMT efficacy in remission of UC in adults in 2015. After 6 weeks of treatment, remission was achieved in 24% of the patients receiving FMT vs 5% of patients receiving placebo. Additionally, analysis to stool samples suggested an increase in microbial diversity in stool from patients who received FMT when compared to the placebo group (Moayyedi *et al.*, 2015). Interestingly, the randomized controlled trial from Rossen *et al.* showed no significant difference in clinical and endoscopic remission between patients who received stools from healthy donors and those who received their own stool (Rossen *et al.*, 2015). These 2 trials presented several differences between them, such as the route of delivery and the dose schedule, which may, in part, explain the different results. Grispan and

Kelly reviewed both studies and concluded that FMT is a promising treatment strategy for IBD, but for now it should remain in clinical trials and not clinical practice (Grinspan and Kelly, 2015). Furthermore, there are some concerns regarding potential side effects of this approach, like the transmission of infections from the host to the patient.

In future clinical trials it should be considered several clinical conditions, such as patients' clinical characteristics, pre-treatment preparation, frequency, dosage and duration of FMT, route of delivery and identification of most beneficial microbes in the donor stools.

There are 8 randomized, controlled trials of FMT in IBD registered on clinicaltrials.gov (NCT02487238, NCT02272868, NCT02291523, NCT02391012, NCT02154867, NCT01847170, NCT01896635 and NCT02390726) that may give additional information on this subject.

Dietary approaches

Given that diet is one of the main factors in the maintenance and disruption of microbiota homeostasis, there were some attempts to control IBD with the addition and/or the exclusion of certain nutrients.

One adopted approach was the exclusive enteral nutrition. This approach consists in the administration of liquid formulas as unique source of nutrition. Enteral formulas include reduced amounts of allergenic factors and anti-inflammatory lipids without nucleotides and food additives. Despite it is not known the exact mechanism of action, exclusive enteral nutrition demonstrates anti-inflammatory properties, as well as the ability to restore the epithelial barrier. Recent reviews have hypothesized that these effects could take place due to the ability of liquid formulas to induce host-immune responses against gut microbiota, which could correct the dysbiosis (Serban, 2015).

A more promising approach to modulate the microbiota consists in the use of probiotics. Probiotics are non-pathogenic live organisms that have several benefits on gut microbiota when administrated in certain amounts. The main organisms used so far are from *Lactobacillus* and *Bifidobacterium* genera. Probiotics can reverse the inflammatory process in the gut through numerous mechanisms, such as blockage of pathogen adhesion to epithelial cells, regulation of epithelial permeability by inducing the formation of epithelial tight-junctions, stimulation of mucous production and promotion of immune tolerance due to downregulation of innate immune receptors (Hill *et al.*, 2014). Additionally, as it was stated, probiotics may induce the production of ROS by Nox1, which contributes to cellular

proliferation, epithelial renewal and recovery of the barrier function (Jones *et al.*, 2013). Future trials may study the administration of other beneficial bacteria, such as *Faecalibacterium prausnitzii* and *Clostridium* clusters IV and XIVa.

6. Conclusion

"IBD is a modern disease of modern times"(Kaplan, 2015). Alterations of microbiota's composition and function seem to be one of the major aspects of IBD, implying that microbiota modulation is a promising approach to revert IBD's symptoms. The modulation of microbiota is, however, a complex task. As discussed in this work numerous endogenous (e.g., host immune system) and exogenous factors (e.g., diet, ROS) exert disparate, antagonist, additive and synergistic effects in the modulation of microbiota. In view of the promising results it might be expected that future progress in disease therapy will depend on the development of the understanding of microbial signalling and of the mechanisms underlying intestinal immune homeostasis.

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