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Review

INTESTINAL MICROBIOTA MUTUALISM AND GASTROINTESTINAL DISEASES

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Summary

The purpose of this work is to investigate the link between an altered intestinal mcrobiota or dysbiosis and chronic inflammatory disorders, in particular inflammatory bowel disease (IBD). Along with probiotics, faecal microbiota transplantation (FMT) opts to be a promising therapeutic treatment for restoring the bacterial homeostasis of the human intestine and reducing the risk of colorectal carcinogenesis. Microbiota is the complex microbial flora that resides in the gut establishing a mutually beneficial relationship. Alteration of the microbiota's composition, termed as dysbiosis, may lead to pathological conditions. Treatment with probiotics can restore the normal commensal flora in IBD. Intestinal microbiota affects the circadian rhythm which in turn regulates the expression of different genes in GALT (gut associated lymphoid tissue) playing a role in the prevention of inflammation and colorectal cancer (CRC) progression. This article highlights the involvement of different microbial strains in the pathogenesis of dysbiosis and in the creation of a carcinogenic milieu caused by an altered stimulation of the immune system. Therapies targeting the equilibrium of the microbiota to switch off chronic inflammation and prevent the progression to CRC seem to be a promising therapeutic tool for a variety of inflammation-associated diseases.

Introduction

Microbial involvement in Inflammatory Bowel Diseases (IBDs) and Colon-Rectal Cancer (CRC) is nowadays well established. Technological advances allowed assessing a

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considerable amount of data concerning possible genetic susceptibility to Crohn's disease rather than Ulcerative Colitis and Colon-rectal cancer. The development of newer molecular tools for the global assessment of the gut microbiome and the identification of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) in 2001 and other susceptible genes for Crohn's disease in particular has led to better understanding of the aetiopathogenesis of IBDs. The microbial studies projected towards a much deeper elucidation about normal composition of the gut microbiome and its perturbations in the setting of IBDs. Condition of "altered" microbiome is called "dysbiosis" and represents a key player in the protracted course of inflammation in IBDs and, possibly in CRC. Numerous genome-wide association studies have identified further genes involved in gastrointestinal innate immunity to better elucidate the relationship of the local innate immunity with the adjacent luminal bacteria. This knowledge has also spurred the search for specific pathogens, which may have a role in the metamorphosis of the gut microbiome from a symbiotic entity to a putative pathogenic one. Here we review advances in our understanding of microbial involvement in IBD and CRC pathogenesis to shape over therapeutic management of gastro-intestinal diseases in the coming years.

Epidemiology and clinical manifestation of IBD

IBDs mean the inflammatory bowel diseases with chronic recurring character. IBDs are a group of pathologies encompassing Crohn's Disease (CD) and Ulcerative Colitis (UC) with a significantly augmented prevalence and incidence in industrialized countries [1]. It seems that the way of life together with the eating habits of the people living in the most industrialized countries are more susceptible to the onset of IBDs. Worldwide prevalence and incidence of IBDs are summarized in Table 1.

In UC mucosal lesions appear in the rectum and extend to the entire colon with a hyperemic mucosa and in severe cases, bloody and ulcerated with pseudo-polyps. On the other hand, CD can affect any part of the gastrointestinal tract from the mouth to the anus. These chronic inflammatory pathologies involve systemic clinical manifestations ranging from orthopedics (arthritis) [2], cardiovascular (endocarditis) [3], endocrinological (thyroiditis) [4], ocular (conjunctivitis, episcleritis, scleritis and uveitis) [5], and cutaneous
(pyoderma gangrenosum, acne,
and suppurative hidradenitis) [6] involvement.

In patients affected by IBD and secondary arthritis, bacterial antigens and genetic material, often belonging to gram-negative, have been found in synovial fluid.

Furthermore, several studies have shown that aberrant migration of intestinal lymphocytes or mononuclear cells is responsible for the onset of joint inflammation [7]. This phenomenon is probably due to the penetration of saprophytic commensal microflora through damaged tight mucosal joints with consequent loss of impermeability. Genetic polymorphisms

WORLDWIDE PREVALENCE	
Crohn's disease	**Ulcerative colitis**
26 to 199 cases on 100.000 people	*37 to 246 cases on 100.000 people*
201 on 100.000 adults	*238 on 100.000 adults*
WORLDWIDE INCIDENCE	
Crohn's disease	**Ulcerative colitis**
3.1 to 14.6 cases on 100.000 people	*2.2 to 14.3 cases on 100.000 people*

Table 1. Data obtained from Center for Disease Control and Prevention website.

of HLA-B27 and of the receptor for interleukin 23 increase both the risk and susceptibility of developing both IBD and arthritis [8, 9]. Since among the microorganisms detectable in CD patients there is *Klebsiella,* infection by this bacterium in the bowel may cause ankylosing spondylitis, through the production of anti-*Klebsiella* antibodies. The latter, can also bind to cross-reactive self-antigens like HLA-B27 and collagen fibers in the joints, with a release of further new antigens on the surface of damaged tissue. These new antigens are responsible for prolonged or continuous production of autoantibodies and further damages to the articular tissues with a perpetuation in the disease process. Recurrent *Klebsiella* infections could explain the characteristic trend present in patients affected by CD and ankylosing spondylitis, consisting in remission/exacerbation features, observed frequently in patients with these diseases [10]. Moreover, in serum and colonic biopsies derived from IBD patients, elevated levels of Hsp60, Hsp10, Hsp70 and Hsp90have been detected. The latter, present molecular structures very similar to those detectable in the microbiome counterpart, supporting the hypothesis of an exacerbated GALT activation in response to these self -antigens through the phenomenon of molecular mimicry at the base of IBD etiopathogenesis [11, 12].

Biological circadian regulation of colonocytes gene expression in response to microbiota and dysbiosis conditions

The microbial associated molecular patterns (MAMPs) are responsible for the activation of immune system through interacting with pattern recognition receptors (PRRs) and subsequent triggering of inflammatory processes. Recent evidences support the great importance of sleeping processes to prevent several pathological conditions and also inflammatory, even CRC [13]. The circadian rhythm switches on/off different genes in GALT (Gut Associated Lymphoid Tissue), among them TLR1, TLR5, TLR9 and NOD2. Several homeostatic intestinal processes such as nutrient absorption, cell proliferation, gut motility and metabolic activities are known to be regulated in a circadian manner [14]. Components of circadian clock, like BMAL1, are required for the correct functionality, in mouse small intestine, of some TLR genes expressed in a circadian manner [15]. The gene encoding for NOD2 receptor, which belongs to the group of NLR intracellular receptors genes [16], was the first susceptible gene to be linked to Crohn's disease. Moreover, NLR is important for the release of antibacterial compounds, like Cryptidins by Paneth cells; for this reason microbiota-depleted mice show a major intestinal susceptibility to inflammation and colitis [17]. Thus, it is very important to highlight that the dialogue between PRRs expression and bacterial MAMPs is highly regulated. The absence of microbiota precludes PRR-mediated signaling, as well as the function of the clock, thus impairing genic expression in colocytes, dependent on both PRRs and clock components, representing the base for the breakage of the delicate equilibrium involved in regulation of gut innate and adaptive immunity. Furthermore, the expression of several genes involved in gut innate immunity (Angiogenin 4, TSLP, and Claudin2 and Claudin12) is microbiota-vitamin D3 dependent, since a defective vitamin D3 receptor signaling has been shown to increase the susceptibility to IBD [18]. RORα transcription factor seems to be involved in the circadian activation of Bmal1 expression in colonic nuocytes [19]. Moreover, short chain fatty acid receptor FFAR3 (GPR41), involved in intestinal motility control [20], appears to be regulated by RORα in a ZT0 > ZT12 circadian manner. In fact, in antibiotic-induced microbiota-depleted mice, FFAR3 is significantly decreased. Of note, rhythmic activation of IKKb and JNK represents a very important factor for correct timing of colocytes homeostatic functions dependent on genes activated by AP1 and NF-kB, showing a circadian activation pattern at diurnal times ZT20- ZT4 which correspond to the mouse ''active phase''. Furthermore, the circadian rhythm of IKKb and JNK activation prevents the inappropriate activation of RevErba by

PPARα. Together these mechanisms ensure that, during the same ZT20-ZT4 active phase, the transactivating (RORα and Bmal1/Clock) and transrepressing (RevErba/E4BP4) molecular clock components can adequately control the temporal expression of RORE- and E-box-containing genes encoding colonic homeostatic functions. Importantly, microbiota derived MAMPs maintain the circadian clock through activation of RevErba by PPARα, and also controlling proper repression mediated by E4BP4, thereby allowing, at diurnal times ZT8–ZT16 (rest phase) [21], the expression of the numerous D-box-containing genes encoding intestinal epithelial cells homeostatic functions [22]. These conclusions suggest that the dialogue between microbiota and the circadian system may have different effects on the development of IBD.

Complementary treatment of IBDwith probiotics

Nowadays, it is well established that inflammatory pathologies affecting gastrointestinal tract are narrowly correlated to dysmicrobism and other various factors such as genetic background and diet. Since now it is well established that dysbiosis is a characterizing condition of IBD, a question remains to be answered: Is dysbiosis a cause of IBD or just a secondary phenomenon? Research on IBD onset and development is oriented towards the investigation of the molecular mechanisms underlying the instauration and the perpetuation of GALT activation. Increasing evidences suggest that the intestinal microbiota play a role in initiating, maintaining, and determining the severity of IBD. The precise role of the microbiota in the etiology consists in continuous antigenic stimulation that has the potential to activate pathogenic T cells and, subsequently, cause chronic intestinal injury. Together, the above mentioned factors concur to the typical alterations of GALT, characterized in IBDs [23]. Mutations in genes encoding for PRRs, such as Nod2/CARD15, significantly contribute to loss of immune tolerance [24,25]. Children with altered microbial flora have a higher incidence of developing IBD during adulthood. Approaches based on mucosal bacterial isolation show increased concentrations of *Bacteroides vulgatus* and *Enterobacteriaceae*, especially *E coli* and decreased concentrations of *Bifidobacteria* species, in subjects affected by CD [26, 27]. Mucosal specimens derived from CD patients revealed a highly significant presence of *Mycobacterium avium,* suggesting a potential role of this enteric pathogen in disease causation [28]. Dysbiosis involves the decrease in microbiome biodiversity, with underrepresentation of the phyla *Bacteroidetes* and *Firmicutes* in feces/mucosa-associated among IBD patients [29]. In truth, a differentiated approach should be used in the study of microbiome since, there is a difference between fecal and mucosa adherent bacteria. Indeed, Swidsinski group demonstrated thick layers of adherent mucosal associated bacteria in both UC and CD patients with higher bacterial concentrations in CD [30]. Immunological studies conducted on patients with IBD revealed the presence of specific antibodies and T cell subsets in both serum and tissue. In particular, significantly higher systemic antibody responses were found in UC towards *Peptostreptococcus anaerobius*, in parallel with higher recovery rates of this strain from the colonic mucosa [26]. The employment of lactic acid-producing organisms, firstly discovered in the beginning of the 20th century by Metchnikoff, revealed a successful tool for ameliorating inflammatory background [31]. Indeed, fermented milk contains specific compounds and microorganisms, known as probiotics, beneficial to human health. Probiotics counteract the activation of Nf-κB, maintaining it bound to IκB in the cytoplasm, thus inhibiting pro-inflammatory cytokines production. Hegazy group investigated the effect of *Lactobacillus delbruekii* and *Lactobacillus fermentum* administration on thirty patients with mild to moderate UC, and evaluated their potential immune-modulating effects. Results derived from this study revealed that 8 weeks of administration significantly ameliorated the inflammation by decreasing the colonic concentration of IL-6, expression of TNF-

α and NF-kB p65, leukocyte recruitment, as demonstrated by a decrease in colonic MPO activity, and the level of fecal calprotectin compared to sulfasalazine group and the control [32].Moreover, *Lactobacillus plantarum* has been shown to inhibit the degradation of IκB and, consequently, the activity of NF-κB in vitro [33]. Decreased amount of *Faecalibacterium prausnitzii* have been shown to predict high risk for early reactivation of ileal Crohn's disease [34]. Probiotic based approaches based on the administration of *Lactobacilli* and *Bifidobacteria* probiotics have been shown to improve clinical symptoms of IBDs through GALT immune modulation. In particular, the beneficial effects of probiotics have been observed in the activation of T_{reg} cells through an immunoregulatory response involving IL-10 and TGF-β [35]. The study of T cell subsets in IBD patients indicated a predominance of T helper 17 cells (Th17). Precisely, IL-17A and IL-17F are abundantly found in inflamed IBD mucosa, suggesting their pivotal role in IBD [36].Interestingly, a subpopulation of Th17 (supTh17) cells exhibits immune suppressive properties because it expresses high levels of both CD39 and FOXP3 and consequently produces extracellular adenosine. Longhi group reported reduced levels of the above mentioned lymphocyte population in IBD patients [37].

Administration of *Lactobacillus casei* and *Bifidobacterium lactis* in mice with TNBS induced colitis led to a significant reduction of inflammation in the colonic mucosa, reversing malignant changes and exerting a potential role in cancer prevention. Benefic effect of probiotic treatment has been observed in the restoration of the goblet cells number back to normal. Some of the diverse mechanisms of action consist in competing with other luminal bacteria, preventing them from reaching the lamina propria. Moreover probiotics modulate expression of genes encoding junction proteins in colocytes to ameliorate the epithelial layer structure of intestinal mucosa and stimulate the mucosal immune system in the patient's intestinal tract to secrete protective immunoglobulins as secretory IgA and protective defensins and bacteriocidins in the colonic lumen [38].

Microbiota transplantation for IBD treatment: state of the art

Nowadays, the role of the gastrointestinal microbiota in driving chronic inflammation in IBD is well established, thus treatments based on microbiota manipulation resulted of great interest in clinical practice, with variable evidence for their efficacy. So, an additional alternative treatment for IBD management is represented by faecal microbiota transplantation (FMT). The principal of FMT for this indication is predicated on the concept that antibiotic therapy disrupts the normal microbial homeostasis, allowing pathogen colonization. FMT rational consists of the transfer of gastrointestinal microbiota from a healthy donor to IBD patient by duodenal infusion of liquid stool suspension. In rodent models, FMT offers both an investigational tool to study the role of microbes in disease development and treatment response, as well as a new therapeutic intervention. The gained credibility in the clinical world on FMT is subsequent to the first publication on the effectiveness of this treatment for antibiotic-resistant C difficile–induced diarrhea [39]. Re-establishment of microbial homeostasis has been demonstrated by significant increase in *Bacteroidetes* species and *Clostridium* species clusters IV and XIVa and a decrease in *Proteobacteria*, according to healthy donor profiles. Recently, Suskind DL group enrolled nine patients, aged 12 to 19 years with mild-to-moderate Crohn’s disease, to undergo FMT by nasogastric tube opting for reducing the intestinal inflammation by altering the fecal dysbiosis. Follow-up evaluations at 2, 6, and 12 weeks, considering PCDAI parameters, showed an improvement in mean PCDAI score at 2 weeks to 6.4 ± 6.6 and at 6 weeks to 8.6 ± 4.9. Results revealed a late remission in patients who did not receive any treatment of engraftment [40]. FMT therapeutic approaches used for IBD treatment are reported in Table 2.

Microbiota, dysbiosis and coloncancer

Studies conducted on initiation and promotion of colon-rectal carcinogenesis revealed the crucial role of the rupture in the physiological equilibrium between the commensal bacteria inhabiting colonic mucosa. Indeed, some bacterial strains may "drive" initial pathological changes in colocytes behavior and immune system responses. Physiologically, the mutualistic relationships between commensal bacteria and epithelium, promote colonic health counteracting meanwhile pathogen infections, opposing the creation of favorable conditions for developing CRC. Although >80% of intestinal bacteria cannot be cultured, identification of all bacteria has become possible by using high technology to perform whole DNA genome sequencing. With the evolvement of phylogenetic analysis of bacterial 16 S rRNA genes this goal has been achieved. In fact, *Firmicutes, Bacteroidetes*, and *Proteobacteria* were reported as the most dominant phyla in bacteria adherent to precancerous adenomatous polyps [49].

Thus, once mucosal integrity is destroyed, other bacteria can pass in the injured zone and support CRC development. The creation of a carcinogenic environment may be caused by a decrease in levels of butyrate-producing species such as *Ruminococcus* and *Roseburia* species relative to controls [50].The gram-negative bacterium*Fusobacterium*

	Outcome	Author
Mild to moderate Crohn's disease	7 of 9 patients in remission at 2 weeks and 5 of 9 in remission at 6 and 12 weeks without additional therapies	*[40] Suskind DL et al 2015*
IBD patient with two Clostridium Difficile infections in 18 months	Microbiota remodeling towards the donor's sample composition coinciding with symptom resolution at 18 months follow up	*[41] Brace C et al. 2014*
Crohn's disease	CD related improvement was not reported	*[42] Grehan et al. 2010*
Crohn's disease combined with Clostridium Difficile infection	Two cases accepted the second FMT due to CDI recurrence, but the efficacy of FMT on CD was not reported	*[43] Hamilton et al. 2012*
Crohn's disease	Documented clinical remission for more than 9 months	*[44]Zhang et al. 2013*
Ulcerative colitis combined with Clostridium difficile infection	UC relapse 9 days after FMT	*[45] De Leon et al. 2013*
Ulcerative colitis combined with Clostridium difficile infection	Diarrhea improved or resolved 3 mo after FMT	*[46] Patel et al. 2013*
Ulcerative colitis	Documented improvement from 1 to 36 months	*[47] Borody et al. 2012*
Ulcerative colitis	Documented improvement	*[48] Kump et al. 2013*

Table 2. Therapeutic approaches for IBD treatment

nucleatum binds to E-cadherin through its membrane protein FadA, activating β-catenin signaling, triggering inflammatory and oncogenic responses [51]. Moreover, high levels of *Fusobacterium nucleatum*result prevalent in stool derived from subjects affected by CRC, suggesting a potential role of this microrganism in initiation and progression processes [52]. Increased levels of *Akkermansia muciniphila* and *Citrobacter farmer* have been reported in CRC cases and, depletion of the first mentioned strain, results associated with IBD development. On the other hand, depletion of *Bifidobacterium longan*, *Clostridium clostridioforme*, and *Ruminococcus* species have been reported in CRC cases [50]. Thus, bacterial metabolites evoke an immune response characterized by increased levels of IL-17, supporting cancer progression [53]. On the other hand, innate immunity activated by bacterial PAMPs, has been reported as a very important factor for tumor progression in murine models. Indeed TLR2 and TLR4, play a crucial role in tumor formation, especially in presence of specific human genetic polymorphisms such as TLR4 299Gly [54]. In colitis-associated CCR, TLR signaling activates Epiregulin, responsible of ERK activation and then, actively supporting tumor growth [55]. Studies conducted on murine models, reveal that dysbiosis "alone" is able to induce CCR formation in presence of polymorphisms responsible of reduced activity of NOD2 [56]. Molecular dynamics at the base of dysbiosis induced CCR encompass production of genotoxic metabolites from different bacterial strains such as Escherichia coli, Enterococcus faecalis, and B.fragilis. In particular, cyclomodulins produced by groups B2 and D of Escherichia Coli, exert detrimental effects in the mechanisms responsible of cellular differentiation, apoptosis, and proliferation control [57]. A direct damage of DNA integrity is caused by B.fragilis toxin, in particular through a mechanism involving the polyamine catabolism [58]. Sobhani group reported significantly increase of bacteria belonging to *Bacteroides/Prevotella* group in CRC patients, compared to healthy independently from age and BMI [59]. The great importance of colonic microbiome in CRC development is substantiated by experiments of stool transfer from individuals with colon cancer and healthy germ-free mice. Follow up to 6 weeks revealed that composition of microbiota in mice's stools was of human type and remained stable over time. However, cell proliferation and aberrant crypt foci increased in the colons of mice given the cancerous stools [60]. Regular probiotics intake may actively prevent the initiation and development of CRC. In fact, Hatakka group reported a significant lowering in putative pro-carcinogenic enzymatic activities such as β-glucosidase, β-glucuronidase and urease after *Lactobacillus Rhamnosus* administration [61]. Recently, it has been tested on HT-29 (colon tumor cell line) a particular bacterial strain derived from vaginal secretions of adolescent and young adult women, belonging to the *Lactobacillus plantarum* species. The isolated strain, exhibited probiotic properties such as low pH and antimicrobial activity against some pathogenic bacteria. Moreover, *Lactobacillus plantarum 5BL* strain exhibited desirable remarkable anticancer activity against the tested human cancer cell line showing favorable potential as a bioactive therapeutic agent [62, 63].

Conclusions

Ulterior clinical investigations on the mutualistic relationship microbiota-colonic mucosa are useful to clarify the physiological, biochemical and immunoregulatory dynamics. Treatment of IBDs with probiotic based therapies may significantly improve life quality and reduce risk of progression towards the onset of CRC. It results very interesting the discovery of supTh17 since, until now, T lymphocytes producing IL17 have been usually addicted as detrimental for the immune homeostasis in colonic mucosa. Therapies oriented towards the equilibrium of microbial may represent the key strategy to switch off chronic inflammatory processes hitting colonic mucosa preventing at the same time the onset of CRC. Data reported in this review could prompt research on IBD and CRC to

deepen the knowledge about microbiota, not intended exclusively as colonic, and to discover potential biological weapon useful to both switch off chronic inflammation and actively prevent carcinogenesis.

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