From gut microflora imbalance to mycobacteria infection: is there a relationship with chronic intestinal inflammatory diseases?

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The gut of a healthy adult harbours a myriad of different microbial species. It is estimated that approximately 10 [14] are present in total bacterial colony forming units (CFU). Each colony colonizes a specific intestinal tract.
In healthy adult, the main control of intestinal bacterial colonization occurs through gastric acidity but also other factors can influence the intestinal microenvironment such as pH, temperature, competition among different bacterial strains, peristalsis, drugs, radiotherapy and much more.
Impaired microbial homeostasis leads to an alteration of the permeability of tissue, together with the activation of the intestinal immune system MALT (mucosal associated lymphoid tissue). In this regard we discuss the increasing experimental evidences of the role of commensal microbiota in the activation of specific intestinal immunocompetent cells.
The aforementioned micro-environmental changes provide the substrate for the etiopathogenetic outbreak of numerous pathologies of gastro-intestinal tract, such as intestinal chronic inflammation (Crohn's disease and Ulcerative Colitis), together with a miscellany of extra intestinal disorders.
This article is an overview of the latest scientific findings about the close causal relationship between intestinal microbial flora and inflammatory bowel diseases or other extra-intestinal diseases; it is also mentioned the possible relationship between mycobacteria and Chron's disease. Finally we analyse the beneficial role of probiotics.

KEY WORDS: Chronic intestinal diseases, Intestinal microflora Imbalance, Intestinal immune system, Mycobacteria, Probiotics.

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are two major healthcare problems of the digestive tract, commonly known as inflammatory bowel diseases (IBD), characterized by chronic and spontaneous inflammation due to a complex interaction of genetic, microbial, and environmental factors, which results in continuous activation of the mucosal immune system [1].

Increasing evidences indicate that changes in gut microbiota, with an increase of pathogenic bacteria and a decrease of health-promoting symbionts, play an important role in promoting and maintaining intestinal inflammation in IBD [2]. In this regard it has also been underlined the ability of specific components of the gut microbiota to activate intestinal immunocompetent cells [3,4].

The other side of the coin is represented by probiotics

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which are microorganisms that confer health benefits in different ways, including modulation of immune response [5]. Despite the evidence that some probiotics can represent a valid therapeutic approach in IBD treatment, the mechanisms underlying the protection by probiotics is still largely unknown. In addition, not all probiotic strains are able to reduce intestinal inflammation [6].

Intestinal microbiota and related diseases

Alteration of commensal microbiota composition and "gas-related syndrome"

The human gut harbours about 100 trillion bacteria and more than 500 different species are present in the colon, so global bacterial concentration can reach 9x10 [13] units. Until now, it remains a mystery how such a large number of bacteria can lodge and coexist in our intestine without causing damage to the host organism. Mucus seems to play an important role in protecting the intestinal mucosa. It is responsible for the integrity of the mucosa maintaining both a stable solution in the submucosal glands secretions (secretory IgA) and a healthy mucosal tissue tropism itself [7]. Also the commensal intestinal flora exerts protective functions infact the complex symbiotic relationship between it and host leads benefits to both parties. This interaction, for example, is the basis of a regular modulation of numerous physiological functions throughout the digestive system [8].
In healthy individuals, the main control of bacterial colonization in the digestive tract occurs through the gastric acidity, because it can neutralize the transit of unwanted bacteria. Among other factors involved in controlling intestinal bacterial flora composition, we recall pH, temperature, interaction between different bacterial strains, peristalsis, secretion of digestive and pancreatobiliary juices, mucous secretions of goblet cells immune responses of B and T lymphocytes to specific antigens, drugs (especially antibiotics), and the effect of radiotherapy. In addition epithelial cells are known to actively release antibiotic peptides that contribute to a biochemical barrier against microbial colonization. Probably the most important cells, in order to keep gut microbial flora under check and protect the neighboring stem cells from microbial insults, are the ileal Paneth cells, which have the task to release antimicrobials in the lumen of the intestine by regulated exocytosis. Therefore, an inherited defect of this mechanism could be considered a potential cause of dysbiosis [9].
The competitive interaction between different intestinal bacterial strains and the biological fermentation processes triggered by them lead to production of putrefactive gas and nitrogen compounds which perform a noxious action on the mucous membrane [10]. These biochemical processes are at the basis of a rich parade of symptoms characterized by abdominal bloating, flatulence, borborygm, abdominal distension, feeling of discomfort[11]. The Anglo-Saxon authors define, very sharply, these disorders "gas-related syndrome".

Commensal microbiota and inflammatory intestinal diseases

It seems now established a close interaction between commensal bacterial flora and intestinal immune system. This interaction plays an essential role in the onset and development of several diseases such as IBD (Crohn's disease and Ulcerative Colitis) and others. Among the various etiopathogenetic hypotheses proposed, the most striking one postulates that a change in the saprophytic microbial flora is the "primum movens" which causes mucosal damage [12,13]. Specifically, the microbiological imbalance (dysbiosis) leads to a modification of intercellular tight junctions responsible for the correct structure of the epithelial layer of intestinal mucosa. This inevitably leads to a worsening of mucosal permeability [14]. Consequently, an effective penetration of antigens takes place within the intercellular space leading to activation of the intestinal lymphatic system (MALT), with recruitment and transition of the inflammatory cascade elements (leucocytes, cytokines, TNF-α) and tissue damage [12].
Other evidences underline an etiopathogenetic role of metabolic components expressed by the gut microflora. Endoluminal accumulation of toxic compounds can infact exert a mutagenic action on intestinal mucosa. We then understand that the maintenance of proper homeostasis of microbial saprophytic organisms is essential in order to avoid the onset of inflammatory intestinal diseases, including cancer and extra intestinal diseases[15]. Infact 1-2% of all colorectal cancers develop from a background of inflammatory bowel diseases such as Crohn's disease and Ulcerative Colitis [16].

Experimental evidences for a role of commensal microbiota in the activation of intestinal immunocompetent cells

The composition of the microbiota has been suggested to influence susceptibility to IBD [17,18], which are mediated by both innate and adaptive arms of the host immune system [19]. It is thus possible that distinct members of the commensal microbiota engage specific components of the immune system and in such a way participate in the regulation of intestinal immune homeostasis [20]. This ability of specific intestinal microbiota has interesting clinical implications in the cases of SIBO (small intestine bacterial overgrowth) which is found in association to IBD [21] or during PPI (Proton Pump Inhibitor) therapy [22].
In particular whether specific commensal microorganisms regulate the homeostasis of effector T cells in the lam-

ina propria is an important question that is only now beginning to be addressed. For example, it has been reported that the gut commensal *Bacteroides fragilis* affects systemic Th1 responses through the action of the bacterial-derived polysaccharide A (PSA) [23]. The lamina propria of the small intestine at steady state contains two populations of CD4 T cells, Th17 cells and regulatory T cells (Treg) [24]; in particular the former has been assumed a role in Chron's disease (CD) and Ulcerative Colitis [25,26]. Interestingly, Ivanov *et al.* [3] found that Th17 cells could be induced in the small intestinal lamina propria in response to specific components of the commensal microbiota belonging to the Cytophaga-Flavobacter-Bacteroides phylum, suggesting that the composition of the intestinal microbiota is likely to influence intestinal immunity, tolerance, and IBD susceptibility. More recently Ivanov and coworkers [4] stressed that segmented filamentous bacteria (SFB) are potent inducers of Th17 cells in the small intestine lamina propria of mice. In particular SFB colonization induced production of serum amyloid A (SAA) in the terminal ileum and SAA acted on lamina propria dendritic cells to promote Th17 cell differentiation. Also the aforementioned CD4 Treg cells can be stimulated by commensal microbiota as evidenced by O'Mahony's research group [27] who showed in mice that the deliberate consumption of the commensal organism *Bifidobacterium infantis* 35624 resulted in the induction of Treg cells which protected the host from excessive inflammation during the course of infection caused by *S. typhimurium.* In particular the reduction of the flogistic response was achieved through the control of excessive pathogen-mediated activation of NF-kB, a transcription factor often involved in innate pro-inflammatory signaling in response to microbial exposure [28].

Also natural killer (NK) cells plays an important role in innate immune system and it has been provided evidence [29] that, in a germ-free mice, NKp46$^+$ IL-22 producing cells were strongly reduced, suggesting that an environmental niche, operative in the gut, generated these unique effectors cells. Interestingly, more recently Takayama and colleagues [30] conducted a clinical study which showed that NKp46$^+$ cells were predominant in intestinal mucosa of patients with CD compared with controls or patients with ulcerative colitis. Upon interaction with intestinal inflammatory macrophages these cells were also activated via IL-23 and produced γ-IFN.

Another interesting point concerning intestinal chronic diseases is the role of epithelial antimicrobial proteins as innate immune effectors; they likely play an important role in maintaining mutually beneficial host-microbial relationships by restricting contact between resident microbes and mucosal surfaces, and their deficiencies are associated with IBD [31]. In particular, using a germ-free mice model, it has been shown [32] that resident gut bacteria drive intestinal epithelial expression of a C-type lectin that binds peptidoglycan and has direct antimicrobial activity; interestingly the human counterpart of this protein (HIP/PAP) is usually overexpressed in intestinal mucosa of IBD patients [33] and it is also believed a biomarker of pancreatic ductal adenocarcinoma [34].

The emerging role of Paneth cells in regulating commensal flora composition.

Recently, another important factor has been recognized to be involved in microflora control: the activity of Paneth cells. These particular cellular elements of innate immunity are specialized ileal epithelial cells located at the base of small intestine mucosal invaginations, called crypts of Lieberkühn. Paneth cells regulates the intestinal microbiota composition via secretion of granule contents including antimicrobial peptides – α-defensins and secretory phospholipase A_2 (sPLA$_2$) – and lysozyme [35]. A defect in the autophagy pathway of intestinal epithelium is responsible for the Paneth cell pathology [36]. Autophagy is an evolutionarily conserved process, with several forms described to date [37]. However, the most studied form is "macroautophagy" whereby cytoplasm and cytoplasmic organelles are encapsulated in double-membrane-bound vesicles (autophagosomes) and delivered to lysosomes, where they are degraded and their constituents recycled [37-40]. This macroautophagic process is increased in response to cellular stress, such as starvation or growth factor withdrawal, for which the term "induced autophagy" has been suggested [41]. Autophagy is thought to protect the cell by eliminating or limiting the growth of bacterial pathogens, a process termed "xenophagy"; therefore dysfunction of xenophagy might lead to persistent infection [42]. Considering CD only, one susceptibility allele is in the predicted autophagy gene *ATG16L1* [43-46]. IRGM and LRKK2 are two additional autophagy genes associated with an increased risk to develop CD [43,47,48]. The mutant Paneth cells show defects in the exocytic pathway. They also have degenerating mitochondria and an abnormal endoplasmic reticulum, which may reflect the loss of organelle degradative capacity associated with the autophagy defect, since autophagy plays an important role in removing damaged or dysfunctional organelles. These defects correlate with the absence of lysozyme in the protective mucus layer of the ileum [36,49]. In addition to its role in maintaining the granule exocytosis pathway, Atg16L1 is an important brake for the expression of proinflammatory genes in Paneth cells and the regulation of endotoxin-induced inflammosome activation [50]. An increase in transcripts associated with Peroxisome Proliferator-Activated Receptor (PPAR) signaling, acute phase reactants, adipocytokine signaling and lipid metabolism is present when Atg16L1 is defective. Many of these genes are directly implicated in inflammation, and especially two of these transcripts, leptin and adiponectin, are known to be increased in CD patients [51,52]. Saitoh and al.

demonstrated that Atg16L1-deficient macrophages stimulated with the toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) gave as response an increased secretion of IL-1β, and IL-18 via TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN)-dependent activation of caspase-1 and that, probably, autophagy is the main controller of inflammasome activation and it limits the production of the inflammatory cytokines IL-1, and IL-18 [50]. Increased IL-1β, may, among other effects, increase epithelial barrier permeability [53], possibly enhancing microbial product translocation. However, the increased production of cytokines may reflect failure of an autophagic stress response and, as postulated by Kuballa et al., the net effect of ATG16L1 coding variation should depend upon the balance between the high microbial load in the gut and the ability of autophagy to mediate defense against invading pathogens and internalized self and non-self antigens [54]. Recent data support a "two-hit hypothesis" wherein host (or potentially environmentally)-mediated alterations in the intestinal microbiota may only induce dysregulated intestinal inflammation characteristic of CD (and IBD) when present together with a tendency to hyperrespond to microbial stimuli [55,56].

Controversial etiopathogenetic role of Mycobacterium paratuberculosis in Crohn's Disease

It is generally accepted that Crohn's disease (CD) results from deregulation of immune responses to luminal antigens in susceptible individuals, but the precise etiology of this inflammatory bowel disease is unknown [57].

Several bacteria have been suggested to be involved in CD pathogenesis [58] including *Escherichia coli* and *Mycobacterium avium* subspecies paratuberculosis (MAP). Invasive *E. coli* have been found in higher frequencies in ileal tracts of CD patients [59]. The data on the presence of MAP are not uniform, but two meta-analysis of several published studies have concluded that MAP is more often present in CD patients than patients with UC (Ulcerative Colitis) and non-inflammatory bowl disease (non-IBD) [60,61]. Also another study described MAP detection using PCR techniques in patients with CD who underwent biopsy and patients with CD whose samples were obtained during surgical resection [62].

It has been also postulated [63] that infection of adipocytes or endothelial cells by MAP, rather than epithelial intestinal cells, could determine the unique pathologic features of Crohn's disease. The abnormal proliferation of endothelial cells, for example, could cause a vessel obstruction increasing propensity to granuloma formation.

On the basis of the aforementioned studies we can then conclude that an association between MAP and CD have been derived, until now, from studies that employed molecular, serologic, and immunocytochemical techniques to determine the presence of MAP in patients with CD[64]; however little is known about the ability of the bacterium to contribute to the inflammatory response during Crohn's disease.

A decade ago Duchman *et al* [65] showed that both CD and ulcerative colitis (UC) patients had T cells with reactivity to various commensal bacteria, including *E. coli*, however no differences were found between the two groups. To get information about the relative importance of various bacteria in the ability to elicit an inflammatory T cell response, Olsen *et al* [66] chose to characterize the specificity of intestinal T cells from CD patients. They isolated T cells from intestinal biopsies of CD, UC and non-IBD patients and detected responses to some tested bacteria. CD patients had a higher frequency of MAP reactive T cells than the UC patients and also a higher frequency of response to MAP compared to other bacterial antigens. Furthermore these T cells produced inflammatory cytokines like IFN-gamma and IL-17. These data suggest a possible role of mycobacteria in CD immunopathology. In this regard Ren *et al* [67] found significant higher levels of interleukin IL-4 and IL-2 in MAP positive CD patients compared to MAP negative ones. In particular IL-4 secretion was correlated with IL-2 production in blood cultures in CD, consistent with a Th2 immune response. Also these data provide the evidence of altered T cell function linked to MAP infection in CD and stimulate a debate about the putative role of this bacterium in the onset of the inflammatory intestinal disease. In this regard two other studies showed, respectively, the ability of MPA to invade human small-intestinal goblet cells and elicit inflammation [68] and to cause early phase morphological lesion of bovine ileum [69]. In particular Golan and coworkers [68] provided, for the first time, evidence of deleterious effect of MPA infection in a so-called "humanized-mouse model", which is the only ethically acceptable experimental model in such a field of research, being the alternative the "infectious studies in children". In particular they transplanted human fetal small intestine or colon at a gestational age 12–16 weeks subcutaneously onto the backs of SCID (severe combined immunodeficiency) mice and infected by an intraluminal inoculation of MPA bacteria. Then, 3 days after infection, mice were euthanized, and the grafts were removed for histologic and immunohistochemical analyses and for detection of inflammatory mediators. In particular they found an increase in tissue levels of IL-6, IL-1β, and TNFα which has also been reported in CD [70].

Interesting findings, finally, regard the association between the risk of developing CD and polymorphisms in several genes that are involved in interaction with bacteria. In particular, NOD2 [71], which is an intracellular sensor of bacteria, and ATG16L1 [46] and IRGM [72], which are involved in autophagy, are believed genetic factors for CD. However currently it is unclear whether the CD associated variants of NOD2, ATG16L1 and IRGM influence the host response to particular bacteria or whether they have more general effects to a wide range of gut bacteria.

Towards a new era in the treatment of intestinal chronic diseases: the probiotics

The history of "probiotics": from definition to therapeutic use.

The correct and thorough knowledge of the mechanisms of microbial homeostasis could be the prelude to unexpected therapeutic or prevention scenarios of many diseases. In this regard numerous clinical trials have already shown the preventive and therapeutic action of probiotics in the treatment of digestive tract diseases [14,16].
For a long time the so-called "official medicine" ignored the therapeutic potential of probiotics preferring the use of intestinal disinfectants, antibiotics and anti-inflammatory drugs specific for the digestive tract. We know today that the indiscriminate use of broad spectrum antibiotics, immunosuppressive therapy and radiotherapy, eventually cause important changes in bacterial intestinal microflora, which often lead to a procession of symptoms particularly severe [73].
The term "probiotics" has been used for the first time in 1965 by Lilly and Stilwell [73]. They reported the observation that certain substances obtained from intestinal segments, if placed in vitro with organic tissue, stimulated their growth. Subsequently, further studies better defined the role of these substances, identifying them as commensal intestinal bacteria. Today we tend to identify with the term of probiotics microorganisms (usually bacteria) producing beneficial effects on the host. These are part of the normal intestinal microbial flora together with the commensal. This feature distinguish them from pathogenic bacteria, both exogenous (Salmonella, etc.) and residents (Bacilli, Clostridia, Klebsiella, Proteus, etc.). These aren't part of the normal flora and, when present, they are usually about 0.02% of total [74]. We know that administration of certain live bacteria can have beneficial effects thanks to the ability of restoring microflora intestinal balance. Today pharmaceutical industry, sensing the safe therapeutic potential of probiotics, has studied and marketed preparation of probiotic bacteria with sinergistical action. These generally include various types of bacteria as Lactobacilli, Bifidobacteria and Enterococci [73] and they have a therapeutic use in intestinal and extra intestinal pathologies. Among these pathologies we can mention diarrheal syndromes (including those from antibiotics), the necrotizing enterocolitis, the Clostridium Difficile colitis, Rotavirus enteritis, infection by *Helicobacter Pylori*, infection of uro-genital apparatus (especially in woman), chronic inflammatory diseases (Chron, Ulcerative Colitis), and finally probiotic bacteria find a use in the preventive treatment of cancers of the digestive tract [14,73-75]. *Rhamnosus GG*, for example, can be used in traveler's diarrhea, but also in "milk-induced" food allergy and in prostate cancer, diabetes and rheumatoid arthritis [74,75].
The scientific literature also assessed the anticarcinogenic role of probiotics. In this regard it is interesting a double-blind study, by Hatakka *et al* [75], which stressed the protective role of Rhamnosus (*Lactobacillus casei*) in colon cancer. In particular this action was carried out lowering the levels of some enzymes as beta-glucosidase, beta-glucuronodase and urease which are considered carcinogenic factors in colorectal cancer.

Interaction between probiotics and intestinal immune system.

In regard of colonic diseases, it has been shown that *Lactobacillus Rhamnosus GG* also interacts with intestinal immune system (MALT). For example it is able to increase the number of IgA and other immunoglobulins secreted by the intestinal glands [73]. In particular it modulates the antigen recognition by the intestinal lymphoid tissue at the level of Peyer's patches and it reduces the levels of proinflammatory cytokine TNF-α [14]. Further studies showed a role of *Rhamnosus* also in improving the permeability of intestinal barrier [76]. Recently another study[77] has provided evidence, for the first time, of a new ability of a mixture of two probiotics (*Lactobacillus acidophilus and Bifidobacterium longum*) to induce the expansion of a subtype of intraepithelial lymphocytes, but not of lamina propria lymphocytes; interestingly administration of the aforementioned probiotics was able to prevent the onset of a chemically-induced colitis in mouse. Dong and colleagues [78] also showed, in a murine experimental model, an unexpected role of intestinal bifidobacteria in promoting the maturation of dendritic cells and expression of IL-12 locally in the gut, in influencing the development of T cells in the thymus and in favoring the development of T-helper cell type 1 response. In addition, these bacteria enhanced antibodies synthesis by PBMCs (peripheral blood mononuclear cells), thereby affecting the development of both the gut and systemic immunity in early life. Another evidence of beneficial effects of probiotics is provided by Schmidt and coworkers [79] who showed that *Lactobacillus acidophilus* NCFM, *Bifidobacterium bifidum* BI-98 and BI-504 were able to improve the gut-associated inflammation which usually occurs in IBD. In particular these microorganisms could enhance the suppressive effect of APC (Antigen Presenting Cells) on regulatory T cells (Treg cells).

Riassunto

Nell'intestino di un individuo sano esistono miliardi di batteri e la loro presenza è condizionata da una serie di fattori quali l'acidità gastrica, la temperatura, la competizione tra i vari ceppi, la peristalsi, l'uso di antibiotici o i trattamenti radioterapici.

Alterazioni della composizione della flora intestinale possono condurre all'attivazione del sistema immunitario intestinale e conseguentemente all'insorgenza di malattie infiammatorie croniche quali il morbo di Crohn e la colite ulcerativa.
In questo articolo, partendo da un'attenta analisi dei più recenti dati presenti in letteratura, sarà messo in evidenza lo stretto nesso causale tra la disbiosi e le malattie infiammatorie croniche intestinali ed extraintestinali; inoltre si farà cenno alle evidenze sperimentali riguardanti la possibile, ma dibattuta, relazione tra micobatteri e morbo di Chron. Infine saranno sottolineate le potenzialità terapeutiche dei probiotici.

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