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Intestinal Microflora and Homeostasis of the Mucosal Immune Response: Implications for Probiotic Bacteria?

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Abstract

The intestinal microflora can be considered a post-natally acquired organ that is composed of a large diversity of bacteria that perform important functions for the host and can be modulated by environmental factors, such as nutrition. Specific components of the intestinal microflora, including lactobacilli and bifidobacteria, have been associated with beneficial effects on the host, such as promotion of gut maturation and integrity, antagonisms against pathogens and immune modulation. Beyond this, the microflora seems to play a significant role in the maintenance of intestinal immune homeostasis and prevention of inflammation. The contribution of the intestinal epithelial cell in the first line of defense against pathogenic bacteria and microbial antigens has been recognized. However, the interactions of intestinal epithelial cells with indigenous bacteria are less well understood. This review will summarize the increasing scientific attention to mechanisms of the innate immune response of the host towards different components of the microflora, and suggest a potential role for selected probiotic bacteria in the regulation of intestinal inflammation.

Introduction

The mammalian intestine is inhabited by a complex and diverse microbial community which is in intimate association with the host epithelium. The colonization of the gastrointestinal (GI) tract by complex bacterial societies is determined by different habitats and ecological conditions. Although there is still much ignorance concerning the composition and temporal dynamics of the microbial gut ecosystem, the development of nucleic acid-based methods has significantly contributed to the specific detection of bacteria independent of culturing. In fact, the analysis of 16S rDNA from human fecal samples revealed that a large proportion of intestinal bacteria have escaped description so far. Cultivation-independent PCR-TGGE (temperature gradient gel electrophoresis) or DGGE (denaturing gradient gel electrophoresis) analysis combined with measurements of ecological diversity can be applied for monitoring diet- and antibiotic-induced alterations of complex intestinal microbial ecosystems

(Zoetendal *et al.*, 1998; Vaughan *et al.*, 2000; Walter *et al.*, 2000).

Although in permanent interaction with environmental microorganisms, partially food derived, the indigenous microflora has a remarkably stable composition throughout most of the life span (Kimura *et al.*, 1997; Tannock *et al.*, 2000). A drastic change in microflora composition certainly occurs immediately after birth, when the so far sterile fetus becomes exposed to the external environment. In addition to the maternal bacteria encountered during delivery and skin contact during breast-feeding, environmental microorganisms contribute to the neonatal microflora (Moreau *et al.*, 1986). It has been observed that before attaining a steady state around weaning, there is a defined sequence of dominant bacterial genera. Nevertheless, it appears that also the type of neonatal feeding may influence the composition of the intestinal microflora as differences have been observed between breast-fed and formula-fed babies (Benno *et al.*, 1986). During adulthood, perturbations of the GI microflora equilibrium are rare and mainly associated with pathological conditions such as enteric infections, antibiotherapy or immune suppression. However, in the healthy elderly a significant reduction of potentially protective bacteria, such as bifidobacteria, has been reported (Hopkins *et al.*, 2001).

The composition of the gut microflora may transiently vary as a consequence of a major bacterial inoculum in the diet (Pochart *et al.*, 1992; Bouhnik *et al.*, 1992). Furthermore, administration of oligosaccharides, non-digestible by human enzymes but fermented by bacteria in the colon, was shown to result in an enhanced growth of bifidobacteria (Bouhnik *et al.*, 1999).

The mechanisms that lead to a dynamic equilibrium of the GI tract microflora and the host are not entirely known, but seem to comprise both host and microbial factors that may differ at different levels of the intestine. A recent example demonstrates that *Bacteroides fragilis* is able to modulate its surface antigenicity by producing at least eight different capsular polysaccharides. Based on the combination of different surface carbohydrates, the microorganism might escape from immunosurveillance and by this means maintain its ecological niche in the intestinal tract (Krinis *et al.*, 2001).

Microflora and the Secretory Immune Response

It is generally accepted that the microflora in the human intestinal tract has a major impact on gastrointestinal and mucosal immune functions (Cebra, 1999). Bacterial colonization of the gut by indigenous bacteria was shown to alter intestinal physiology of the host by modulation of genes, implicated in nutrient absorption, mucosal defense and xenobiotic metabolism (Hooper *et al.*, 2001). The lower exposure of the neonate and infant to intestinal microbial challenge in the last decades, as indicated by

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epidemiological data, has been associated with a higher incidence of allergic diseases (Anderson *et al.*, 2001; Matricardi *et al.*, 2000; Isolauri, 1997). Thus, it appears that bacterial challenge of the host is an important prerequisite for the development of homeostasis of the intestinal immune system and maintenance of oral tolerance (Weiner, 1997). There is also increasing evidence that the breakdown of tolerance to the microflora could lead to, or perpetuate, inflammatory bowel disease (Duchmann *et al.*, 1995). This may imply that immunosurveillance of the bacterial content of the intestine contributes to i) the development of immunological tolerance and control of severe inflammatory reaction, ii) the control of colonization and iii) the appropriate defense against external antigens, pathogenic bacteria or viruses.

Host defense against the autochthonous microflora is still poorly understood. It has been reported that indigenous bacteria can be recognized by the host immune system and elicit local and systemic antibody responses (Kimura *et al.*, 1997; Apperloo-Renkema *et al.*, 1993). The production of secretory immunoglobulin A [(s)IgA] is the best defined effector component of the intestinal mucosa. In cooperation with innate defense factors, such as mucus, sIgA in the intestinal lumen will accomplish 'immune exclusion' to protect the mucosal surface. This occurs in the absence of complement activation and is thus a non-inflammatory process.

To date, it is still unknown whether the secretory immune response plays a role in determining the composition of the microflora. While a proportion of cells of the resident microflora is covered by IgA antibodies, the remainder are devoid of antibody coating (Van der Waaij *et al.*, 1996). Interestingly, local or systemic antibody responses do not seem to lead to the elimination of indigenous bacteria from the intestine (Apperloo-Renkema *et al.*, 1993). There is recent experimental evidence in mice suggesting that most of the intestinal IgA against indigenous bacteria is specifically induced in response to their presence, and that its production is independent of T-cell and germinal centre participation (Macpherson *et al.*, 2000). This IgA, mainly directed against bacterial protein antigens, appears to be derived from B1 lymphocytes that develop in the subepithelial compartment and are spread throughout the lamina propria (Herzenberg *et al.*, 2000). The IgA antibodies protect the host from invasion by indigenous bacteria, but do not spontaneously appear in the serum. In case of bacterial infection, specific IgG can be produced by T cell-dependent pathways. It is hypothesized that specific T cell-independent IgA forms part of the normal mucosal response against the continuous antigenic load of indigenous bacteria and might represent an evolutionary ancient pathway of the immune system. However, these observations have not so far been confirmed in humans.

In case of failure of this first line of protection, penetrating antigens need to be removed from the lamina propria (LP) by antibodies locally produced by terminally differentiated B cells and T cells. Production and secretion of IgA in the LP has been shown to be regulated by (i) endogenous mediators, such as TGF- β , IL-5 and IL-10, mainly produced by regulatory T cells (Lebman *et al.*,

1990) and (ii) is associated with intestinal bacterial colonization (Kett *et al.*, 1995). The main IgA subclass of the human jejunum is IgA1; whereas IgA2 is predominant in the colon. This might reflect the distribution of food antigens versus bacterial antigens in the normal gut. In the case of bacterial overgrowth, the composition is changed with an increase of IgA2 in the small bowel, suggesting that LPS might play a role in antibody class switch (Kett *et al.*, 1995).

Innate Defenses of the Intestinal Mucosa

The single layer of epithelial cells lining the intestinal tract has to protect the underlying compartments from both the normal microflora and invading pathogens. Moreover, the intestinal mucosa has to cope with a large antigenic load, including dietary and bacterial antigens, without triggering constant and severe inflammation. The potential for cumulative damage might explain the rapid turn-over of intestinal epithelial cells (IEC) and the requirement for mechanisms of cytoprotection and repair to preserve barrier integrity. Trefoil peptides secreted to the apical surface of the epithelium interact synergistically with intestinal mucin glycoproteins to reinforce a physicochemical barrier (Kindon *et al.*, 1995). These peptides are also involved in reconstitution of the epithelium after injury. Many factors may modulate the production of intestinal mucins for innate defenses. A recent publication underlines the protective effects of *Lactobacillus* species by stimulation of intestinal mucin synthesis (Mack *et al.*, 1999).

Antimicrobial peptides, such as α -defensins secreted from Paneth cells, or β -defensins secreted by the epithelial cell itself, are abundantly found in host defense reactions in the gastrointestinal tract (Hecht, 1999). There is now strong evidence that in addition to constitutively secreted peptide antibiotics, others are induced upon contact with microorganisms or by pro-inflammatory cytokines. The characteristic local expression pattern of defensin might indicate that specialized surfaces express a characteristic antimicrobial peptide pattern which defines the composition of the microflora and the density of microorganisms present on that surface. Antimicrobial activity of defensins is based on pore formation, membrane depolarization and interference with bacterial metabolism. In addition, some defensins induce a secretory chloride response in IEC (Lencer *et al.*, 1997), others display chemotactic activity for T cells, serving as a link between innate and adaptive immunity (Chertov *et al.*, 1996; Lillard, Jr. *et al.*, 1999). Finally, among the peptides promoting restoration of the epithelium are transforming growth factor (TGF)- β , keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) produced by epithelial cells and subjacent mesenchymal cells or myofibroblasts (Dignass *et al.*, 1993; Goke *et al.*, 1998).

Microbial Recognition

The first recognition of microbial determinants is achieved by host cellular defense molecules, the so-called pattern

recognition receptors (PRR). PRRs are germ-line encoded and recognize molecular structures shared by a variety of bacteria (Stahl *et al.*, 1998). In the gut mucosa PRRs are found on macrophages and dendritic cells, which are widely distributed beneath the epithelial surface where they guard the sites of antigen entry. In addition, newly described PRRs are expressed by the intestinal epithelial cell.

A classical PRR is the mannose receptor (MR), expressed on tissue macrophages and immature dendritic cells (DC) (Fraser *et al.*, 1998). MRs recognize the pattern of carbohydrates that decorate the surface of Gram-negative and Gram-positive bacteria, yeasts, parasites and mycobacteria (Stahl *et al.*, 1998). Ligation of the MR results either in endocytosis or phagocytosis of the ligand-receptor complex and subsequent clearance of the infectious agent. The MR appears to play a critical role in the processing of microbe derived glycolipids in conjunction with CD1b (Park *et al.*, 2000). Thus, the MR is involved in both antigen clearance in the tissues and stimulation of clonal adaptive immune responses.

Another class of PRRs, the human Toll-like receptors (TLRs), is related to the *Drosophila* Toll protein, which is required for ontogenesis and antimicrobial resistance (Medzhitov *et al.*, 2000). Generally, TLRs are type I transmembrane receptors with cytoplasmic domains that resemble the mammalian IL-1 receptor (IL-1R). Ten TLR molecules have been described so far in mammals, and it is assumed that each of the TLRs recognizes a discrete subset of molecules widely shared by microbial pathogens. Reaction of bacterial products with TLRs results in cellular signaling leading to NF- κ B or AP-1 activation (Medzhitov *et al.*, 2000). TLR4 was shown to be essential for the recognition of Gram-negative bacteria, acting as a co-receptor for CD14 in the cellular response to LPS (Yoshimura *et al.*, 1999; Aderem *et al.*, 2000). TLR2 is involved in cell responsiveness to Gram-positive bacteria, including peptidoglycans (Yoshimura *et al.*, 1999), lipoteichoic acid and bacterial lipoproteins (Brightbill *et al.*, 1999). This suggests that different microbial agents might activate different Toll members, leading to the activation of different target genes. More recently the differential expression of TLR2, TLR3 and TLR4 on intestinal epithelial cell lines and activation of specific signal transduction pathways after stimulation of IEC with LPS has been reported (Cario *et al.*, 2000). The same group showed that upon stimulation of TLRs with LPS or peptidoglycan, TLRs selectively move from the apical surface through the cytoplasm towards the basolateral membrane (Cario *et al.*, 2002). Thus, TLRs positioned at the apical pole of the epithelial cell seem to monitor the sensitive balance of the luminal microbial community. These data provide further evidence, that IEC might play a key role in the recognition and transduction of signals derived from luminal bacteria.

Sensing the Danger of Infection

Bacterial recognition systems used by epithelial cells are likely to have developed to maintain mucosal surfaces in a state of homeostasis with the normal microflora. A

sophisticated system is required to discriminate between indigenous and enteropathogenic bacteria. Discrimination seems to depend on bacterial feature recognition by PRRs and the cellular compartment where the bacterial presence is detected. For instance, the recognition of flagellin in the basolateral membrane of the intestinal epithelium was shown to initiate a defense response, subsequent to TLR5 binding (Philpott *et al.*, 2001).

In addition to sensing bacterial presence via PRR it has been proposed that a second signal is required to initiate an appropriate response to pathogens (Matzinger, 1998). Bacterial invasion provides a signal to initiate inflammatory responses and often results in cellular damage, leading to activation of immunological defense. These are key elements for control of infection and clearance of the infecting microbe. Non-invading pathogens are also recognized by the epithelium, if cytopathic or enterotoxic effects are induced (Beatty *et al.*, 1999). The alarm signals sent by stressed, damaged or parasitized cells comprise constitutive and inducible molecules that can initiate different kinds of immunity in different tissues and to different pathogens. Endogenous 'danger signals' comprise heat shock proteins, nucleotides, extracellular matrix breakdown products or necrotic cell derived molecules. Exogenous 'danger signals' are LPS, peptidoglycans or unmethylated CpG sequences in microbial DNA. These molecules will activate *a priori* dendritic cells, necessary for the initiation of primary and secondary immune responses (Pulendran *et al.*, 2001).

Recognition of Indigenous Bacteria by Intestinal Epithelial Cells

The intestinal epithelium is increasingly recognized as a constitutive component of the innate and adaptive response of the host towards luminal bacteria (Molmenti *et al.*, 1993). Several groups have demonstrated that IEC may exert accessory function for antigen (AG) presentation. They express MHC class II molecules and may activate CD4⁺ T cells (Mayer, 1998; Hershberg *et al.*, 1998).

Classical MHC class-I molecules (HLA A, B, C) are expressed in co-association with β 2-microglobulin and they present peptides to CD8⁺ intraepithelial lymphocytes (IEL). Since peptides contained within the groove of the MHC class Ia molecules are predominantly derived from degradation of intracellular molecules, CD8⁺ IEL have important roles in monitoring deleterious intracellular events that might occur during viral infection, cellular stress or neoplastic transformation. In addition to polymorphic MHC class Ia molecules, non-classical MHC class-Ib molecules, such as CD1d (Blumberg *et al.*, 1991), HLA-G (Pazmany *et al.*, 1996), HLA-E, HLA-H (Hfe) (Parkkila *et al.*, 1997), MICA (Bauer *et al.*, 1999; Bahram *et al.*, 1994) or the human homologue of the rodent neonatal Fc receptor (FcRn), involved in bidirectional transport of IgG across the epithelium, are also expressed on human epithelial cells (Israel *et al.*, 1997). Restriction in expression to certain tissues and the lack of polymorphism in MHC class-Ib molecules suggests that

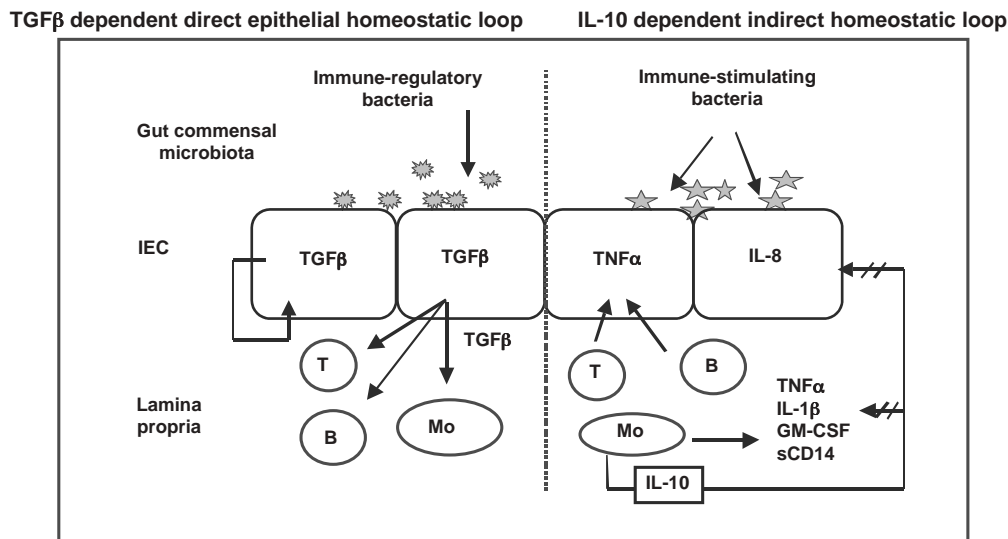


Figure 1. Proposed model for homeostasis regulation after activation of IEC by non-pathogenic bacteria. Left side: Direct TGF β -dependent epithelial homeostatic effect induced by immune-regulatory non-pathogenic bacteria. TGF β protects the barrier integrity. In addition, TGF β promotes regulatory T cells (Th₃). Right side: Indirect IL-10-dependent homeostatic loop induced by immune-stimulatory bacteria. The transient pro-inflammatory response is blunted by lamina propria (LP) cells, particularly LP macrophages, which predominantly secrete IL-10.

they bind a limited array of very distinct ligands. This is especially relevant for intestinal epithelial cells which express several MHC class-Ib molecules. CD1d, which is predominantly expressed on IEC of the upper crypt and villi, presents exogenous and/or endogenous lipid antigens to T cells. IELs which express CD1d in co-association with β 2-microglobulin were shown to induce secretion of IL-4 and IFN γ by NK-T cells, suggesting an important immunoregulatory function. Whether CD1d:ligand interaction on epithelial cells induces immunoregulatory cytokines needs further investigation. Expression of MICA on IEC is observed as a consequence of different stress signals and is thus thought to be involved in recognition of danger signals by CD94/NKG2 positive immune cells, leading to cytolysis of the damaged cell (Groh *et al.*, 1998).

It has also been shown that epithelial cells express complement factors (Andoh *et al.*, 1993), complement inhibitors (Guignot *et al.*, 2000), cytokine receptors (Bocker *et al.*, 1998) and can secrete cytokines and chemokines in response to pathogenic bacteria (Jung *et al.*, 1995; Eckmann *et al.*, 1993). More recently intracellular receptors for LPS have been described which were shown to be involved in the activation of NF- κ B, leading to the secretion of pro-inflammatory mediators (Bertin *et al.*, 1999; Beatty *et al.*, 1999).

Recent advances in the characterization of microbial-epithelial interactions suggest that the innate epithelial response can also recognize and discriminate between different indigenous bacteria. These observations confirm the frontline role of the intestinal epithelial cells (IEC) in the recognition of components of the intestinal microflora. However, it is obvious that bacterial signals need to be processed by a network of different mucosal immune cells, resulting in an integrated response that dictates the host reaction against a constantly changing microbial environment in the intestine.

Human *in vitro* co-cultures, produced by the co-cultivation of human intestinal epithelial cell lines, such as CaCO-2 or HT-29 cells, and human peripheral blood mononuclear cells (PBMC) using a transwell culture technique, proved to be useful models to investigate the molecular basis of microbial:epithelial interactions (Haller *et al.*, 2000). Our group recently demonstrated that IEC can recognize non-pathogenic bacteria in the presence of PBMC. Furthermore, a characteristic response to a given non-pathogenic indigenous strain could be observed distinguishing between two major cytokine/chemokine responses of IEC. Non-pathogenic Gram-negative *Escherichia coli* and certain Gram-positive lactobacilli triggered a NF- κ B mediated inflammatory response resulting in the production of TNF α , IL-1 β , IL-8 and MCP-1 (type 1). This initial epithelial pro-inflammatory reaction was only transient and self-limiting, as the PBMC in the basolateral compartment were able to switch off the initial inflammatory response of the epithelium. A detailed analysis of the role of different leukocyte subpopulations in the bacterial:mucosal cross-talk revealed that i) activation of epithelial cells by indigenous bacteria was promoted by T and B cells and that ii) macrophages acquired an immunosuppressive phenotype and were able to control inflammatory cytokine expression in IEC by the predominant secretion of IL-10, an inhibitory cytokine (Nathens *et al.*, 1995). Stimulation of CaCO-2/PBMC co-cultures with a non-invasive enteropathogenic *E. coli* (EPEC O111:H6) highlighted the differences in epithelial response to indigenous and pathogenic bacteria: whereas the pro-inflammatory cytokine induction was transient with the indigenous strains, inflammation was not inhibited in the case of EPEC treatment (Haller *et al.*, 2002).

A second class of lactobacilli, including *Lactobacillus johnsonii* La1 and *Lactobacillus gasseri*, induced the immunoregulatory cytokine TGF β in IEC in the absence

of any pro-inflammatory event (type 2). Of note, both indigenous bacterial strains were of human intestinal origin. TGF β is produced by both immune and non-immune cells and exhibits a broad range of functions, the most important being the modulation of immune responses. In the intestinal immune system, TGF β displays an important role in the maintenance of intestinal barrier integrity and induction of oral tolerance.

Both types of epithelial responses against non-pathogenic bacteria seem to indicate the importance of either a self-limiting (type 1) or non-inflammatory (type 2) cellular immune response in the context of the antigen rich intestinal environment. It appears that certain components of the gut microflora may contribute to maintain a low level of 'physiological' intestinal inflammation, whereas others directly promote the production of immunoregulatory cytokines (Figure 1). These examples of an integrated epithelial/lamina propria response strongly suggest a role for the indigenous microflora in homeostatic responses. Furthermore, these results provide direct evidence of the beneficial effects of specific probiotic strains on intestinal immune homeostasis.

Failure of Immunoregulation and Chronic Intestinal Inflammation

There is evidence that chronic intestinal inflammation, such as in Crohn's disease, is caused by excessive immune response to mucosal antigens and elements of the normal bacterial microflora, inappropriately controlled by the normal counter regulatory mechanisms. This includes the induction of T cell anergy or clonal deletion and the expansion of antigen specific regulatory T cells (CD4⁺CD25⁺) in the lamina propria that produce suppressor cytokines, such as TGF β (Th3 cells) or IL-10 (Tr1 cells) (Groux *et al.*, 1997; Singh *et al.*, 2001; Strober *et al.*, 2001b).

The development of intestinal inflammation in knockout and transgenic rodents has confirmed how genetic and environmental factors are both responsible for mucosal inflammation. Spontaneous inflammation of the GI tract has been demonstrated in transgenic rats expressing the human HLA-B27 transgene, in mice deficient for IL-2, IL-2R α , IL-10, TCR α , TGF- β 1 (Blumberg *et al.*, 1999) or CD4⁺CD45RB^{hi} reconstituted SCID mice (Leach *et al.*, 1996). The various models of mucosal inflammation are characterized by either the overproduction of key Th1 effector cytokines (IL-12, IFN γ or TNF α) or the underproduction of regulatory cytokines (IL-10, TGF β) (Strober *et al.*, 2001a). However, regardless of the immunological basis of experimental inflammation, the latter is dependent on the presence of the normal bacterial microflora, as inflammation does not develop under germ-free conditions (Bhan *et al.*, 1999). This suggests that inflammatory bowel diseases (IBD) are likely due to an abnormal response to the normal intestinal content rather than to intestinal pathogens (Duchmann *et al.*, 1995). Experimental animal studies demonstrated the importance of both IL-10 and TGF β in immunoregulation at mucosal sites, more recently suggesting that TGF β -

producing cells are the primary suppressor cells, but that IL-10 is necessary for these cells to expand in a Th1 dominant environment that would otherwise inhibit the expansion of these cells (Kitani *et al.*, 2000; Groux *et al.*, 1997). In addition to regulatory T cells, TGF β signaling in IEC plays a crucial role in maintaining mucosal immune homeostasis, as TGF β regulates expression of MHC class II molecules and activity of metalloproteinases (Hahm *et al.*, 2000). Inhibition of TGF β signaling due to overexpression of the Smad7 protein in target cells was shown to maintain the chronic production of pro-inflammatory cytokines (Monteleone *et al.*, 2001).

NF- κ B modulation has become an obvious target for anti-inflammatory therapy. It has been recently reported that some non-pathogenic bacteria can prevent NF- κ B activation through inhibition of I κ B- α ubiquitination. This may suggest that luminal microflora can send positive and negative signals to mucosal epithelial cells as part of the interactions with the host (Neish *et al.*, 2000).

Indigenous bacteria, which have the capacity to induce immune-regulatory cytokines, such as TGF β in the intestinal epithelium or IL-10 in lamina propria macrophages, may have the potential to modulate gastrointestinal inflammation. This could represent a nutritional strategy to contribute to the reconstitution of intestinal homeostasis in the case of its impairment due to genetic defects (Hugot *et al.*, 2001; Ogura *et al.*, 2001). Thus, indigenous bacterial strains, including probiotic bacteria, selected for their properties to activate an epithelial inhibitory response may be a rational nutritional intervention in IBD patients.

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