

Mucosal Immunology and Probiotics

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Abstract The cross-talk between the mucosa-associated immune system and microbiota is critical in mucosal tissue homeostasis as well as in protection against infectious and inflammatory diseases occurring at mucosal sites. This recent evidence has paved the way to therapeutic approaches aimed at modulating the mucosa-associated immune system using probiotics. Different strains of probiotics possess the ability to finely regulate dendritic cell (DC) activation, polarizing the subsequent T cell activity toward Th1 (e.g. *Lactobacillus (Lb) acidophilus*), Th2 (*Lb.reuteri* and *Bifidobacterium bifidum*) or, as more recently demonstrated, Th17 responses induced by specific strains such as *Lb.rhamnosus* GG and Lac23a, the latter isolated in our laboratory. Here, we review some recent advances in our understanding of probiotics effects on mucosal immunology, particularly on cells of the innate immunity such as DCs. We also highlight our own experiences in modulating DC functions by commensal bacteria and discuss the relevance of probiotics administration in the treatment of human immunopathologies.

Keywords Probiotics · Commensal bacteria · LAB · Microbiota · Lactobacilli · Bifidobacteria · Dendritic cells · T-cell polarization · Th1 · Th2 · Th17 · Inflammatory bowel diseases · Allergy · Crohn's disease · Ulcerative colitis · Immune-mediated diseases · Interleukins · TLRs · MALT · GALT · IEC · Immunotherapy · Immunopathology · Autoimmunity · PRR · PAMP · Mucosal immunology

Introduction

Probiotics have been described as “live microbial supplements that beneficially affect the consumer by improving intestinal microbial balance” (FAO/WHO 2002) [1]. Most probiotics in use today belong to the genera *Lactobacillus* and *Bifidobacterium*. In particular, lactobacilli are characterized by their production of lactic acid and are predominant participants in many industrial and artisanal plants, meat, and dairy fermentations. Currently, there is a certain

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consensus that probiotic bacteria can help in the relief of symptoms of human immunopathologies, including inflammatory bowel diseases (IBD) and, in general, atopic diseases [2]. The mechanisms of action of the probiotics can be quite disparate. It has been shown that they modulate the permeability of epithelial barriers, alter the inflammatory potential of epithelial cells, compete with pathogens for mucosal colonisation, or directly modify the activity of immune cells [3]. The interplay between the mucosa-associated immune system and microbiota certainly plays a pivotal role in mucosal tissue homeostasis as well as in protection against infectious and inflammatory diseases occurring at mucosal sites, which remain the main gateway for pathogens invading our body. Therefore, therapeutic approaches aimed at modulating the mucosa-associated immune system using probiotics might be considered as an interesting tool to treat several human immunopathologies. Here, we review some recent advances in our understanding of the effects of probiotics on mucosa immunology, particularly on cells of the innate immunity such as dendritic cells (DCs), and highlight the potential applications of probiotics in the treatment of human diseases.

Commensal Bacteria and Probiotics

Bacteria can be used to improve human health. A ‘live microorganism’, that when administered in adequate amounts confer a health benefit to the host, would be called a probiotic. A consensus definition of the term states that oral probiotics, upon ingestion in specific numbers, exert benefits beyond those of inherent basic nutrition and do not necessarily colonise the human intestine [1].

Most of the bacteria or microbiota of our body reside in the gastrointestinal tract from the mouth to the anus. They represent a large and diverse population of bacteria and some of them are beneficial to the host, while others are not. The intestinal habitat of an individual contains 300–500 different species of bacteria. The stomach and small intestine contain only a few species adhering to the epithelia and some other bacteria in transit and it seems this is because of the composition of the luminal medium (acid, bile, pancreatic secretion), which kills most ingested microorganisms; on the other hand, the colon is a reservoir of numerous species of microorganisms with concentrations of 10^{11} or 10^{12} cells/g of luminal contents. Several bacteria living in the gut affect host homeostasis. Some of these are potential pathogens and can be a source of infection and sepsis under some circumstances, for instance when the integrity of the bowel barrier is physically or functionally breached [3].

Lactic acid bacteria (LAB) are normal members of the human gut microflora and most are regarded as safe when administered as probiotics. They are predominantly non-

pathogenic, Gram-positive, catalase-negative, non-spore-forming, facultative anaerobe and obligate fermentative; LAB are characterised by their main metabolite, and comprise bacteria belonging to several genera (*Lactococcus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, and *Lactobacillus*) [4•].

“Probiotics” are also the genus *Bifidobacterium* and other microorganisms, for instance certain strains of *Escherichia coli* and *Saccharomyces cerevisiae* [5]. The effect of a bacterium is strain-specific and cannot be extrapolated even to other strains of the same species.

If the immune system is not stimulated by gut bacteria after birth, it does not develop completely, comprises fewer T cells (including Tregs) [6, 7] and dendritic cells [8], and generates weaker antibody responses [9]. Furthermore, specific recognition of commensal microorganisms occurs only in the mesenteric lymph nodes [10], and this stimulation might skew the immune system away from the neonatally dominating Th2 response towards a balanced Th1 immune profile [11]. These data emphasise the relevance of the interactions occurring between microbiota and the cells of the immune system, particularly at the mucosal level.

Mucosal Immune System in Protection and Tolerance

Mucosal immune system has a key role in the defence against pathogens. Indeed, most antigens or infectious agents penetrate the body through the mucosa, which include the surface lining of the gastrointestinal, respiratory and genitourinary tracts. In these mucosal surfaces, mucus represents the first barrier against the entry of microorganisms, whereas mucosa-associated lymphoid tissue (MALT), such as intestinal Peyer’s patches or tonsils and adenoids, are critical organs for exerting an efficient protective immune response [12–14].

In particular, for their importance in blocking the entry of commensal and pathogens, gastrointestinal mucosal surfaces have been widely investigated. It has been described that there are two mucus layers in the stomach and colon, an outer loose layer and an inner thick layer close to the epithelium, while in the small intestine, there is a discontinuous mucus layer. Mucus is secreted at the top of the crypts, where Goblet cells have the function of secreting the mucin, and then moves between the villi. Other important epithelial cells are Paneth cells for their ability to produce antimicrobial peptides [12]. In addition to mucus, intestinal epithelial cells (IEC), closely linked through tight junctions, represent a physical barrier to pathogens and commensal antigens. Under this epithelial cell layer there is the lamina propria, which contains the gut-associated lymphoid tissue (GALT) providing immune responses to the intestinal mucosa. The importance of protecting the gut from pathogens is evident

in the abundance of lymphoid tissue and immune cells. The GALT consists of isolated and aggregated lymphoid follicles, including Peyer's patches in the small intestine, and the mesenteric lymph nodes (MLN) [14]. T and B cells, DCs, macrophages and neutrophils cells are harboured in GALT and protect the mucosa against harmful pathogens inducing inflammatory responses to bacteria and antigens and scavenging dead cells. Peyer's patches are responsible for the defence against pathogens and the immune tolerance (oral tolerance) to not-harmful antigens. In fact, one of the most difficult challenges that the gastrointestinal immune system has to face is to eliminate pathogens while maintaining tolerance towards food antigens and commensal microflora.

Intestinal antigens are acquired through enterocytes, able to transport antigens from the intestinal lumen to the lamina propria. Antigen sampling also occurs in Peyer's patches (PPs). PPs are surrounded by a particular epithelium, the follicle-associated epithelium (FAE), which contains enterocytes and specialised cells named M cells, differing from enterocytes by the absence of microvilli and mucus production. M cells transport luminal antigens and bacteria to the basolateral side by transcytosis. An enormous variety of pathogens take advantage of the properties of M cells to penetrate the intestinal mucosa and invade the host [14]. At their basal surface, M cells are surrounded by lymphocytes and DCs that activate or inhibit the immune response leading to either tolerance or systemic immune responses. Finally, lamina propria DCs can send their dendrites between intestinal epithelial cells opening tight junctions and capturing antigens from either commensal or pathogenic gut bacteria [14, 15•, 16] (Fig. 1).

It is known that DCs sense microbes through pattern recognition receptors (PRR) and migrate to secondary lymphoid organs to prime naive T cells. In the GALT, there are two predominant subsets of DCs with several specialisations, and oral tolerance is mediated in part by a subset promoting the conversion of naive T cells in FOXP3⁺ Treg cells. CX3CR1⁺ monocyte-derived DCs have the ability to extend dendrites through epithelial tight junctions to sample the luminal contents, while CD103⁺ DCs are considered tolerogenic and induce Treg cells by producing high level of retinoic acid and TGF- β [17•, 18••, 19]. IEC express tolerogenic factors, such as thymic stromal lymphopoietin (TSLP), and promote the differentiation of these CD103⁺ DCs [20]. TSLP is an IEC-derived factor produced in response to signals from microbiota, inducing IL-10 production from DCs and promoting Th2 responses. In addition, CD103⁺ DCs transport commensal bacteria to MLN, where they help to generate commensal-specific IgA responses. The abundance of intestinal IgA-secreting plasma cells depends on the presence of commensal bacteria, and it has been demonstrated that the absence of commensal bacteria

in germ-free mice and antibiotic-treated mice leads to the downregulation of IgA secretion [17•, 21].

The most important IgA production in the body occurs in the mucosa-associated immune system. In fact, the gastrointestinal, genitourinary and respiratory tracts are important entrance doors for microorganisms. Defence of these regions is ensured by antibodies, mainly IgA produced by MALT, which bind to microorganisms and toxins, neutralising and preventing access to the host cells.

In the gut, B cell isotype switching to IgA occurs especially into PPs and MLN. IgA translocates across the intestinal epithelial cell layer toward the lumen and is deposited in the intestinal mucus layer. The mucosal immune system is highly adapted to the presence of the commensals because the presence of high IgA concentrations in the mucus layer restricts microbes from gaining entry to the lamina propria [17•, 21]. It has also been described that commensals can penetrate the lamina propria and survive within DCs for only a few hours, conversely to intestinal pathogens that could be resistant to phagocytosis. It has been proposed that these commensal-loaded DCs induce IgA but, because they are confined in the MLN, they do not induce systemic immune responses [21].

Therefore, the influence of microbiota on mucosal immunity, gut homeostasis and immunological tolerance plays a relevant role. Because DCs are key players in the development of tolerance, probiotics could also modulate the immune response by influencing DC activation [22, 23].

In the respiratory tract, under physiological conditions, a large number of bacterial species colonise the nasopharynx (predominantly *Staphylococcus epidermidis* and *Staphylococcus aureus*); conversely, the lower respiratory tract is free of microorganisms. But, if the epithelium is damaged, pathogens such as *H. influenzae* or *Streptococcus pneumoniae* can gain access.

In the genitourinary tract, the normal flora of the anterior urethra consists of *Staphylococcus epidermidis*, *Enterococcus faecalis* and streptococci. In addition, *E. coli* and other enteric bacteria may be found, and they probably represent contaminants from the skin, vulva or rectum. The vagina is colonised by staphylococci, streptococci and lactobacilli.

In the gut, the proximal small intestine has a Gram-positive flora, consisting mainly of lactobacilli and *Enterococcus faecalis*, while the distal part contains additional species, including *E. coli*, coliforms and *Bacteroides*. The flora of colon is rich in coliforms, enterococci, clostridia and lactobacilli, but the predominant species are *Bacteroides* and *Bifidobacteria*.

Commensals in the gut have the ability to protect their host from pathogens and to stimulate the activity of the mucosal immune system. In fact, alterations in the intestinal microbiota contribute to gastrointestinal diseases, and probiotics, primarily strains of the *Lactobacillus* or *Bifidobacterium* genera, have beneficial effects in a variety of

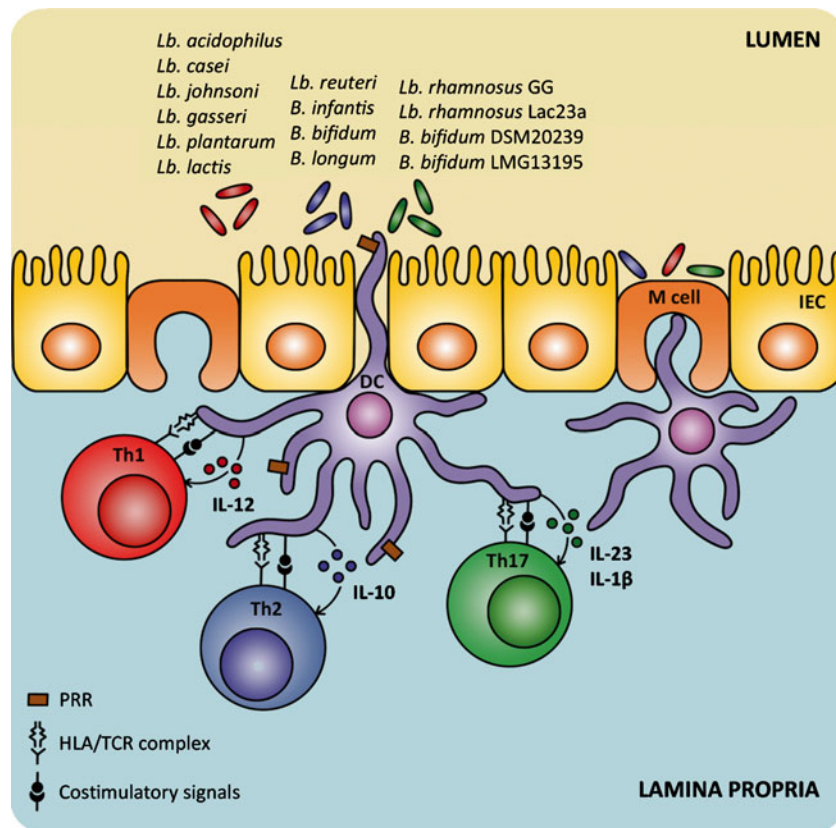


Fig. 1 Distinct commensal bacteria can trigger different polarisations of the adaptive immune response. Dendritic cells (DC) can encounter commensal bacteria either sampling directly the lumen by sending their dendrites between epithelial cells or via M cell transcytosis. The different combinations of pathogen associated molecular patterns expressed by the distinct classes of probiotics will be recognised by the related pattern recognition receptors (PRR) on DCs and could induce a divergent cytokine release. The resulting bacteria-dependent

cytokine milieu will represent the “third” signal for T cells after recognition of antigen by T cell receptor (TCR) (first signal) and costimulation (second signal). This third signal will eventually determine the polarisation of the subsequent T cell response occurring in mucosa-associated lymphoid tissue. The indicated bacteria are grouped by their ability to induce respectively type 1, 2 or 17 T cell responses. *Lb* = *Lactobacillus*; *B* = *Bifidobacterium*

immunopathologies, such as inflammatory bowel diseases and atopic diseases [22], as further discussed below.

Immunomodulatory Properties of Probiotics

The action of probiotics involves different effector signals, cell types and receptors, and distinct strains may differ in their respective ability to trigger specific signals in both immunocompetent and intestinal epithelial cells [24].

In recent years, the ability of LAB to influence innate and adaptive immune responses has been demonstrated. They can act in the host via binding to PRR expressed on immune cells and many other tissues, including the intestinal epithelium [25•].

PRR recognise conserved molecular structures known as pathogen-associated molecular patterns (PAMPs), and signal to induce the production of cytokines and chemokines and expression of markers on antigen-presenting cells, such as DCs [26••]. PRR can be divided into three families: Toll-

like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors that recognise viral RNAs (RLRs), and nucleotide oligomerisation domain-like (NOD) receptors (NLRs). The most studied PRRs are the TLRs. For probiotic signalling, one of the best investigated is TLR2 which, in conjunction with TLR1 or TLR6, recognises cell wall peptidoglycan, lipoteichoic acid and lipopeptides, and TLR9, which binds CpG DNA [27].

TLR signalling pathways have been recently reviewed in detail and, except for TLR3, involve recruitment of MyD88, which in turn activates the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor KB (NF-KB) pathway signalling cascades [28, 29••, 30••].

In an inactivated state, NF-KB is located in the cytosol as a protein complex with the inhibitory protein IκBα, but TLR and NLR signalling leads to the phosphorylation of IκBα, its ubiquitination and degradation by the cell proteasome. Liberated NF-KB then translocates into the nucleus and induces the transcription of specific genes and thus the production of large range of chemokines and cytokines

including interleukins (ILs) and tumour necrosis factors (TNFs) [31, 32].

Amongst the many benefits associated with the administration of probiotics, modulation of the immune system has received the most attention. Several studies have provided unequivocal evidence that specific strains of probiotics are able to induce the polarisation of the immune responses by stimulating the release of selected cytokines by activated DCs [33]. These cytokines would act as the “third” signal for naive T cells, which consequently should differentiate into Th1, Th2, or Th17 (Fig. 1).

Alteration of the gut microbiota has been suggested as a pathologic etiology in IBD, including ulcerative colitis (UC) and Crohn's disease (CD), which are chronic inflammations of the gastrointestinal tract. Dysfunction of the mucosal immune system evokes intestinal inflammation through activation of both innate and acquired immunity. There is evidence that in CD there is a predominately Th1-mediated process, while UC seems to be a Th2-like disorder [34•].

A potential role for probiotics in modulating DC functions suggest that certain species or strains could be exploited in the different clinical setting requiring modifications of the magnitude and quality of the adaptive T cell response [35]. For instance, the property to inhibit IL-12 release exerted by some LABs (such as *Lb.reuteri* and *B.bifidum*) may be of importance in Th1-mediated disease in restoring gut immune system homeostasis [4•, 26••, 36].

Recently, IL-17-producing T-helper cells (Th17 cells) have been regarded as crucial for the control of several chronic inflammatory diseases such as multiple sclerosis and IBD. Th17 cells are abundant in the intestine, and the development of these cells seems to be regulated by DCs and macrophages in the lamina propria [37]. Thus, orally administered probiotics (such as *Lb.rhamnosus* GG, a strong inducer of TGF- β , IL1- β and IL-23) could affect the development of Th17 cells and ameliorate clinical symptoms. Indeed, Th17-inducing properties seem to be associated with several *Lb.rhamnosus* strains. *Lb.rhamnosus* Lac23a, a strain recently isolated in our laboratory, leads strongly to a Th17 adaptive immune response (V. Rizzello, unpublished data).

Most likely, differences in PAMPs, such as LTA, peptidoglycan structures and nonmethylated CpG motifs, could explain the different cytokine release by human DCs in response to specific probiotic species or even strains. However, the molecular mechanisms of these different DC responses to distinct probiotics largely remain to be elucidated.

Administration of Probiotics for Human Health

Generally, in adults, the microbial environment of the gut and, in general, of the mucosae is relatively stable,

particularly at the genus and species level. However, dietary components can influence the numbers of particular bacteria in the gut [38].

The addition of probiotics to the intestine may re-establish the colonic and intestinal microbial balance. Probiotics may restore balance to the intestinal microbiota population by changing the intestinal pH and producing antimicrobial substances such as bacteriocins, organic acids, and hydrogen peroxide [39, 40]. The increase in beneficial bacteria results in competition with pathogenic bacteria for nutrients and, thus, survival. Experimental animal and human studies have shown that probiotics may normalise intestinal permeability, provide nutrition to the colonocytes by forming short-chain fatty acids and some amino acids, stimulate proliferation of colonocytes, and participate in the regulation of intestinal functions [38]. Probiotics have also been shown to positively affect immunoglobulin production, antibody response, and other cellular immune responses that may contribute to good health and disease resistance [41].

There is also evidence that intake of probiotics is effective in the prevention and/or management of acute gastroenteritis, rotavirus diarrhoea, antibiotic-associated diarrhoea, intestinal inflammatory disorders such as CD and paediatric atopic disorders [41]. These studies have indicated that probiotics might exert an effective modulation of both normal and pathological immune response.

Finally, from published data, the use of probiotics can also be considered as nonspecific adjuvants of innate immune response to increase the body's defences, e.g. by promoting potent type-1 immune responses, which is desirable in infections and cancers [36, 42].

Probiotics in Immune-mediated Diseases

According to what we have described above, it can be envisaged that probiotics have a variety of beneficial effects on the health of the host, and at least some of them occur by their interactions with the immune system. These effects are induced through different mechanisms, including the inhibition of the pathogens to the epithelium, the production of specific antibodies and the modulation of the immune system through the DCs [43].

Dendritic cells are known to play a pivotal role in the pathogenesis/protection of autoimmune diseases because they are capable of either priming effector T cells or activating Treg cells, depending on the maturation stimuli and/or DC subsets [44, 45]. The exact role of DCs in the aetiology of IBD is unknown, but several observations in models of IBD suggest that DCs may play a pathogenic role. DCs accumulate at sites of inflammation in patients with IBD, mainly as a consequence of upregulated mucosal

expression of chemokines or of addressins for which DCs possess specific receptors [46].

It has been shown that the interplay between the commensal bacteria and TLRs on cells of the innate immunity, in particular DCs, is necessary for the intestinal homeostasis and the prevention of epithelial injury. If this recognition is dysregulated, chronic inflammation and tissue damage may occur [16, 47]. The pathogenesis of IBDs, such as UC, CD, etc., as well as other autoimmune diseases such as celiac disease and Type 1 diabetes (T1D), appears to be associated with a pathological reactivity towards intestinal microflora [48]. At the same time, the break of tolerance to self-antigens in autoimmune diseases, including T1D, is due to deficient immune regulation [49, 50].

Regular intake of probiotic bacteria might contribute to immune homeostasis by altering microbial balance or by interacting with the gut immune system, explaining their potential effect in IBD [51].

The management of allergic diseases by means of probiotics and their effects on gut microflora has also been an object of clinical interest. The 'hygiene hypothesis' postulates that the recent drastic increase of allergic diseases in industrialised countries is caused by decreased levels of infections during childhood. Such infections would otherwise 'fine-tune' the immune system into an allergy-protective response. A related extrapolation of the hygiene hypothesis has speculated that changes in the gut microflora (e.g. caused by antibiotic use or components of the modern diet) could also be associated with the increase of allergic diseases, that are IgE-mediated and associated with a polarisation of the immune-system toward a Th2 response [52•]. Several studies have been shown that children with atopic dermatitis were less often colonised with lactobacilli and bifidobacteria compared with healthy children, suggesting that dysregulation of gut microflora might be involved in the allergic disease [53, 54•].

Conclusions

In recent years, it has become clear that probiotics could potentially play a role in the control of the immune network by affecting diverse sets of immune-regulatory cells at mucosal sites.

Selected probiotics can stimulate DC regulatory functions by targeting specific PRR, thus indicating a role of DCs in the immunological effects induced by probiotics and providing a strong rationale for their use in immune-intervention therapies during mucosal tissue injury. Indeed, the use of probiotics seems to positively influence the course of immune-mediated diseases and disorders related to the malfunction of the intestinal barrier.

Probiotics might have the ability to differently polarise T cell polarisation via specific stimulation of DCs, and this could justify their use in some diseases that require modifications of the adaptive T cell response. The presence of LAB early in life can skew the immune system towards a Th1 response, possibly through the intermediate action of NK cells [4•, 36, 55–57], and this may aid in the prevention of Th2-mediated allergy. This putative modulation of T cell polarisation is a very attractive application for probiotics, which, however, requires further in vivo confirmations.

Although several studies have clearly shown that commensal bacteria have the potential to influence both normal and pathological immune response, the efficacy of probiotic administration against infections, cancer and immunological disorders such as IBDs, allergy, asthma, diabetes, and arthritis still remains to be proven in humans. Important gaps exist about the mechanisms by which probiotics modulate immune functions. Optimum dose, frequency and duration of treatment required for different conditions in different population groups also remain to be determined. Lately, we learned that different probiotic strains might vary widely in their ability to modulate the immune system, since two different strains can sometime induce opposite outcomes. For this reason, the effects and the efficacy of each probiotic strain now needs to be carefully demonstrated through rigorously designed studies.

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