VERSITA

Biologia, Bratislava, **61**/6: 735—740, 2006
Section Cellular and Molecular Biology
DOI: 10.2478/s11756-006-0150-5

Review

Regulation of immune response at intestinal and peripheral sites by probiotics*

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Abstract: The gut associated lymphoid tissue (GALT) should protect intestinal mucosa against pathogens, but also avoid hypersensitivity reactions to food proteins, normal bacterial flora and other environmental macromolecules. The interaction between epithelial cells and microflora is fundamental to establish gut mucosal barrier and GALT development. The normal colonization of intestine by commensal bacteria is thus crucial for a correct development of mucosal immune system. Probiotic bacteria are normal inhabitants of microflora and may confer health benefits to the host. The modification of the intestinal microflora towards a healthier probiotics enriched microflora may generate beneficial mucosal immunomodulatory effects and may represent a new strategy to cure intestinal and allergic diseases. The health benefits may be specific for different probiotic strains. Ongoing research is providing new insights into the probiotic beneficial effects and related mechanisms. This review represents an update of immunomodulatory activity of different probiotics and of the more accredited mechanisms underlying such activities.

Key words: probiotics; health effect; intestinal inflammation; allergy.

Abbreviations: GALT, gut associated lymphoid tissue; IBD, inflammatory bowel disease; *L.* GG, *Lactobacillus rhamnosus* GG; MAMP, microbial associated molecular patterns; MLN, mesenteric lymph-nodes; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells; TLR, Toll like receptor; UC, ulcerative colitis.

The intestinal epithelium as defence against pathogens and luminal antigens

The intestine is continuously exposed to a myriad of antigens and thus, other than exerting the primary role of nutrients absorption, should protect the mucosa against pathogens and dangerous substances. The intestinal epithelium represents the first line of defence against pathogens and dangerous environmental agents by different means. Among them, the mucus layer produced by goblet cells may create a physical barrier against pathogens by entrapping them and allowing their elimination by intestinal peristalsis (Mayer, 2003). A further protection against pathogens is provided by the secretion of antimicrobial defensins by the Paneth cells (Keshav, 2006). In addition, the intestinal mucosa ensures a relevant immune defence in virtue of the gut associated lymphoid tissue (GALT), which is one of the largest immunological tissues of the body. The GALT comprises specialized inductive sites of the intestinal immune response, the Peyer's patches, that are numerous lymphoid follicles surrounded by the specialized M cells, responsible for the transport in the patches of most antigens and bacteria coming from intestinal lumen. Antigen-primed B and T lymphocytes migrate from inductive sites through lymph and reach peripheral blood for subsequent homing to the mucosal effector sites, lamina propria and epithelial compartments, where effective immune responses against pathogens are mounted (Simecka, 1998; Brandtzaeg et al., 1999).

The gut immune system has to be able to protect the mucosa against pathogens, but also to avoid hypersensitivity reactions to food proteins, normal bacterial flora and other environmental macromolecules. To achieve such purposes, the GALT may act through two different immune mechanisms. The first is an active immune response mediated by both B and T lymphocytes. B lymphocytes produce antibodies IgA that recognize and remove the antigens from the mucosa without activating the inflammatory response. When the antigens escape from this first defence mechanism and reach the

* Presented at the Second Probiotic Conference, Košice, 15–19 September 2004, Slovakia.
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lamina propria, an inflammatory response occurs by IgG mediated complement activation. T lymphocytes are the effector cells regulating the immune response through cytokines and chemokines secretion, and comprise T helper (Th) cells that can develop to Th1 and Th2 classes, and cytotoxic T cells. The second immune mechanism is the "oral tolerance", induced to avoid hypersensitivity reactions against innocuous substances, like food and enteric flora antigens (Brandtzaeg, 1996; Strobel & Mowat, 1998). Oral tolerance has recently been defined as "any mechanism by which a potentially injurious immune response is prevented, suppressed or shifted to a non injurious class of immune responses" (Weiner, 2001a). Oral tolerance is achieved through three different mechanisms: anergy, clonal deletion and active suppression of immune response mediated by cytokines (Wittig & Zeitz, 2003). Anergic lymphocytes are not able to recognize a specific antigen with the consequent inactivation of the antigen specific cellular clone. The anergy is induced essentially when the antigen is presented by "non professional" antigen presenting cells, such as enterocytes or endothelial cells. Clonal deletion occurs in peripheral lymphoid tissues by apoptotic death of cells reactive to a specific antigen. Active suppression of immune response is mediated by regulatory cytokines. Upon antigen presentation, these cytokines induce the generation of antigen-driven regulatory T cells (Treg; Allez & Mayer, 2004), that are T regulatory 1 (Tr1) cells, characterized by high levels of IL-10, low levels of IFN-γ and no IL-4 secretion (Groux et al., 1997); Th1–like Treg cells producing IFN-γ and IL-10 (Stock et al., 2004); Th2–like Treg cells producing IL-10, some IL-4 but no IFN-γ (Akbari et al., 2002). These Treg cells are adaptive Treg, opposed to "natural" thymus-derived $CD4^+$ $CD25^+$ Foxp3 Treg cells (Faria & Weiner, 2005). Other important Treg cells involved in oral tolerance induction are the Th3 cells producing high levels of TGF-β (Chen et al., 1994). The adaptive Treg cells migrate to different lymphoid tissues and organs and inhibit both antigen specific and not specific response, secreting regulatory cytokines and reducing inflammatory processes (Weiner, 2001b).

Intestinal microflora and probiotics

The interaction between epithelial cells and microflora is fundamental to establish gut mucosal barrier and GALT development (Kalliomaki et al., 2001), to promote intestinal integrity and prevent cell dysfunction (Bouhnik et al., 1992; Donnet-Hughes et al., 1992; Goldin et al., 1992; Ouwehand et al., 2002). The normal colonization of intestine by commensal bacteria is thus fundamental for a correct development of mucosal immune system, and starts during the delivery when the newborn encounters the maternal enteric bacteria and environmental microbes. During weaning and early childhood intestinal microflora develops to a composition that remains almost stable throughout the life, but can transiently change in response to different factors, such as alterations in diet, health status and external conditions (Chin et al., 2000). Normal inhabitants of microflora comprise probiotic bacteria, such as *Bifidobacterium* and *Lactobacillus* strains, that are live microrganisms which may confer health benefits to the host (Ouwehand et al., 2002). The probiotics must be able to survive the gastric transit and may transiently colonize intestinal mucosa by integrating with enteric bacteria, and in such way maintain or induce a healthy intestinal microflora. Several benefits have been ascribed to the ingestion of probiotics, although they still need to be confirmed in humans. The beneficial effects may be specific for the different probiotic strains and ongoing research is providing new insights into their benefits and related mechanisms.

Immune modulation by probiotics

Among the various beneficial effects exerted by probiotics, a large field of interest has recently been focused on their interaction with immune system, which may lead to improvement in inflammatory intestinal disease, oral tolerance and food allergies (Salminen et al., 1998; Chin et al., 2000; Roselli et al., 2005). However, the mechanisms underlying these activities are still unclear. Immune modulation by probiotics includes effects on innate and adaptive immunity, lymphocyte function, antibody production and cytokine expression (Perdigon et al., 1999; Gill et al., 2000; Pessi et al., 2000). Several *in vitro* and animal studies have shown that certain strains of probiotics, such as *Lactobacillus rhamnosus*, *L. acidophilus* and *Bifidobacterium lactis*, were able to stimulate macrophage and neutrophil populations, and to enhance natural killer cells activity (Gill et al., 2000; Matsuzaki & Chin, 2000). *L. casei* was found to increase the size of T helper lymphocyte population in the GALT of mice (Perdigon et al., 1999). In addition, an enhanced IgA secretion was observed in mice challenged with cholera toxin and orally treated with *L. acidophilus*, *B. infantis* or *B. bifidum* (Tejada-Simon et al., 1999), and in serum of volunteers challenged with *Salmonella typhimurium* and fed with yogurt containing *L. acidophilus* La1 and bifidobacteria (Link-Amster et al., 1994). An increase in innate immune response has also been shown in humans after supplementation with dairy products containing probiotics (Schiffrin et al., 1995; 1997), suggesting that lactic acid bacteria could be used as non-specific adjuvants in innate immune response to accelerate the early defensive mechanisms against intestinal infections.

One of the most studied activity on adaptive immunity by probiotics is their regulation of anti- and pro-inflammatory cytokine production. However, available data are quite conflicting and the activity of probiotics on cytokine expression is not completely understood. Several *in vitro* studies indicate an increase of the pro-

inflammatory IL-12, IFN-γ and IL-18 cytokines induced by *L. rhamnosus*, *L. bulgaricus*, *L. paracasei* and *L. casei* strain *Shirota*, in both human peripheral blood mononuclear cells (PBMC) and mice splenic lymphocytes (Miettinen et al., 1998; Hessle et al., 1999; Kato et al., 1999). An increased expression of IL-12, associated with a reduction of IL-10, was also found in human PBMC after stimulation with three lactobacilli isolated from human intestinal mucosa, namely the *L. plantarum*, *L. rhamnosus* and *L. paracasei* (Hessle et al., 2000). Other studies confirmed that lactobacilli may induce the expression of pro-inflammatory cytokines, showing an increase of IFN-γ and IL-12 in human PBMC treated with *L. johnsonii* and *L. sakei* (Haller et al., 2000), or an increase in plasmatic values of IFN-α and IFN-β in mouse injected with yogurt bacteria (Solis-Pereyra et al., 1997). Despite these studies, an ability of probiotics to induce anti-inflammatory cytokine expression has been shown. An *in vitro* study on intestinal Caco-2 cells and PBMC treated with different probiotics has shown that *L. sakei* induced an increase of IL-10 mRNA level in human PBMC, but also of IL-1β and TNF-α mRNA when the cells were co-cultured with PBMC (Haller et al., 2000). In addition, *L. johnsonii* was able to increase TGF-β mRNA level in Caco-2 cells when co-cultured with PBMC. This study suggests an important role for epithelial cells in regulation of mucosal immune response, by modulating cytokine expression after stimulation with non pathogenic bacteria. A hypothesis was delivered by the authors of this study (Haller et al., 2000), that bacterial signal may reach enterocytes through PBMC produced soluble mediators. A recent study has clarified that probiotics may differently regulate cytokine expression of intestinal cells, depending on the presence or absence of a pathogenic stimulus. Indeed, an increase of IL-1β was found when intestinal Caco-2 cells were treated with *L. rhamnosus* GG (*L.* GG), whereas treatment of the cells with *B. animalis* and *L.* GG simultaneously with *Escherichia coli* counteracted the pathogen induced increase of IL-1β, TNF-α and decrease of TGF-β (Roselli et al., 2006). An explanation on whether probiotics may regulate cytokine mediated anti-inflammatory response, comes from a study of Von Der Weid et al. (2001). These authors have shown that *L. paracasei* induced the development of a population of $CD4^+$ T lymphocytes with low proliferative capacity and high TGF-β and IL-10 production, reminiscent of Tr1 cells implicated in oral tolerance and gut homeostasis. Since it is known that probiotics may stimulate dendritic cells to enhance IL-10 production (Drakes et al., 2004; Hart et al., 2004) and that IL-10 is essential for Tr1 expansion (Wakkach et al., 2002), it is possible that probiotics induce the development of Tr1 through dendritic cells stimulation.

Recent advances in understanding the mechanism of immunomodulatory activity of probiotics have been achieved by the discovery of the Toll like receptors (TLRs) pathways. The TLRs are a family of transmembrane proteins highly expressed on the surface of immunocompetent cells, monocytes, macrophages and dendritic cells, as well as epithelial cells (Cario et al., 2002). They recognise highly conserved structural motifs expressed by microorganisms, the microbial associated molecular patterns (MAMPs; Medzhitov & Janeway, 2000). Different MAMPs selectively activate different TLRs. For example, TLR2 recognises peptidoglycans and lipoteichoic acids that are wall components of Gram positive bacteria including lactobacilli and bifidobacteria (Yoshimura et al., 1999; Neuhaus & Baddiley, 2003), and TLR4 is the major receptor for lipopolysaccharide, the main component of Gram negative bacteria wall (Chow et al., 1999). The binding of TLRs with MAMPs may lead to inflammatory cascade through activation of nuclear factor kB (NF-kB), with subsequent generation and release of Th1 pro-inflammatory cytokines, chemokines, lipid mediators and reactive oxygen and nitrogen species (Watson & McKay, 2006). It is known that TLRs cannot distinguish between pathogens and commensal bacteria. However, under physiological conditions the pro-inflammatory cascade does not occur, suggesting a fine regulation between immune defence and immune tolerance toward commensal bacteria (Otte et al., 2004). At this regard, it has been shown that continuous exposure of intestinal cells to bacterial components results in a state of hyporesponsiveness mediated by increased expression of Toll-interacting protein (Otte et al., 2004). In addition, probiotics have been shown to reduce NF-kB activation in intestinal inflammation (Petrof et al., 2004; Fig. 1). Some authors provided evidence that different *Bifidobacterium* strains led to a significant attenuation of DNA binding activity of NF-kB complex in biopsy specimens of probiotic-treated Crohn's disease patients, thus inhibiting the expression of pro-inflammatory cytokines (Cui et al., 2004). A subsequent study confirms the role of probiotics in inhibition of NF-kB by showing a down-regulation of the nuclear translocation of NF-kB by *L. casei* strain *Shirota* in intestinal lamina propria mononuclear cells isolated from mice with chronic inflammatory bowel disease (Matsumoto et al., 2005). A possible mechanism by which probiotics interfer with NF-kB pathway was provided by a recent study reporting that DNA from a mixture of probiotic bacteria (VSL#3) elicits immunosuppressive activity by stabilizing IkB levels and inhibiting NF-kB activation and IL-8 secretion (Jijon et al., 2004). Since unmethylated CpG dinucleotides in bacterial DNA are known to have immunostimulatory properties through recognition by TRL9 (Krieg, 2002), the authors of the above-mentioned study suggested that the immunosuppressive activity of VSL#3 DNA may be due to alterations in CpG content, degree of methylation or presence of novel immunosuppressive motifs.

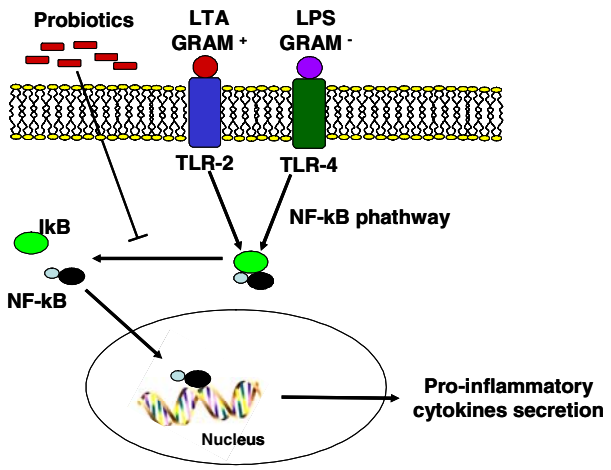

Fig. 1. Toll like receptors (TLRs) binding with lipoteichoic acids (LTA) wall components of Gram positive bacteria and with lipopolysaccharide (LPS), the main component of Gram negative bacteria wall, cause activation of nucler factor kB (NF-kB) pathway, leading to pro-inflammatory cytokines release. Probiotic bacteria may inhibit NF-kB activation and traslocation into the nucleus.

Health effects of probiotics on intestinal inflammation, allergy and oral tolerance

The incidence of inflammatory bowel disease (IBD), including ulcerative colitis (UC), Crohn's disease (CD) and pouchitis, as well as of food allergies and atopic diseases, in the form of dermatitis, allergic rhinitis and asthma, appears to be increasing in more developed countries. Such increase may be caused by an incomplete development of intestinal immune system, due to an insufficient bacterial exposure (hygiene hypothesis; Shanahan, 2004), leading to development of adverse reactions against intestinal flora, that plays a critical role in the pathogenesis of IBD and other immune-based disorders. In favour of this hypothesis, some studies reported an increased number of *Bacterioides* spp. and a decreased number of lactobacilli in faecal samples and colonic mucosa from UC patients (Fabia et al., 1993; Swidsinski et al., 2002). A recent accredited theory to explain the increases of IBD and allergic disease, is that a reduced consumption of fermented foods results in defective maturation of Treg cells and thus in imbalance between them and the Th cells, leading to the development of IBD characterized by a Th1 response, or to allergy and intolerance caused by a Th2 response, depending on individual immunological history (Rook & Burnet, 2005). Based on this assumption, the modification of the intestinal microflora towards a healthier probiotics enriched microflora appears a good strategy against IBD and allergic diseases. Several experimental and preliminary human studies have shown promising results (Kalliomaki & Isolauri, 2003; Bischoff & Crowe, 2004; Fedorak & Madsen, 2004). Indeed, studies on animal models of IBD reported that *L. reuteri* diminished the severity of acetic acid induced colitis in rats (Fabia et al., 1993), *L. plantarum* prevented the intestinal permeability increase and the bacterial traslocation in rats affected by enterocolitis (Mao et al., 1996), and *L. salivarius* and *B. infantis* resulted in attenuation of colitis in IL-10 deficient mice (McCarthy et al., 2003). Clinical studies have shown that administration of VSL#3 to patients affected by active mild to moderate UC alleviated disease severity (Bibiloni et al., 2005). Moreover, treatment of mucosal explants from CD patients with *L. casei*, *L. bulgaricus* or *L. crispatus* counteracted the symptoms of CD, by down-regulating TNF-α expression which plays a key role in the pathogenesis of CD (Burruel et al., 2002). All these results support a role for probiotics in preventing or treating IBD and have encouraged the scientific community to further explore the role of these bacteria in preventing human inflammatory diseases.

Relating to allergic diseases, symptoms of atopic dermatitis were diminished in infants receiving *L.* GG and atopic disease development was reduced in newborns from mothers, which received the same probiotic during pregnancy and breastfeeding (Majamaa & Isolauri, 1997; Kalliomaki et al., 2001; 2003). Moreover, studies on the effect of probiotics during the development of autoimmune diseases have shown that mice treated with *Lactobacillus casei* strain *Shirota* during the development of arthritis induced by type II collagen, which is similar to human rheumatoid arthritis, were less susceptible to the induction of the disease (Kato et al., 1998).

Recently, it has been found that long-term feeding of *B. animalis* and *L.* GG to rats immunized with ovalbumin (OVA) injection, may modulate intestinal immune response towards a tolerogenic status. Indeed, the proliferation of mesenteric lymph-nodes (MLN) lymphocytes was lower in probiotic treated than untreated rats, whereas it was similar in splenic lymphocytes, indicating that probiotics may induce a different OVA-specific immune response at intestinal and peripheral sites. This hyporesponsiveness was associated with an increased IL-10 expression in MLN lymphocytes suggesting that *B. animalis* and *L.* GG may induce the development of IL-10 secreting Tr1–like population (Britti et al., 2003). These results are in agreement with the findings of several studies showing that probiotic treatment prevents or eliminates inflammatory reactions against food antigens in intolerant and allergic subjects (Pelto et al., 1998; Pessi et al., 1999) and that probiotics may induce and modulate Treg cell populations (Christensen et al., 2002; McGuirk & Millis, 2002). However, the mechanisms used by probiotics in the management of tolerance and allergic disease are not fully understood.

Conclusions

There is a growing body of evidence that probiotics induced beneficial effects in inflammatory and allergic diseases. The numerous health probiotics benefits observed in animal studies need to be confirmed in humans. However, available data are in favour of a potential application of probiotics as a therapeutic or preventing strategy to cure certain pathologies, such as IBD and allergies, that have been considerably growing in recent years. New insights into the mechanisms of probiotic anti-inflammatory activities have been recently achieved, but further studies are necessary to better define the mode of action of probiotics as well to characterize the specificity of the activities of the different probiotic strains.

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Received September 14, 2005
Accepted August 10, 2006