Probiotics as immunomodulators: substances, mechanisms and therapeutic benefits

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The human gut symbionts in whole and probiotics in particular promote intestinal function and health, partially via modulation of gut associated lymphoid tissue. Molecular basis of microbiota-host interaction is pattern recognition receptors (PRRs) and their ligands, *viz.*, microbe-associated molecular patterns (MAMP). Immunomodulatory effects of probiotics are strain-specific, which is important to select specific strains suitable for treatment/prevention of infectious diseases, food allergy and non-infectious inflammatory diseases including autoimmune disorders.

Keywords gut symbionts, modulation of immune system, microbe-associated molecular patterns

1. Probiotics as the part of symbionts and their beneficial functions

The term 'probiotic' was initially used in the 1950s as are opposite of antibiotics [1]. Currently, the most widely used definition of probiotics is 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). Five conditions must be fulfilled for a probiotic to be effective: it must 1) have a proven beneficial effect on the host; 2) not be toxic or pathogenic; 3) contain a sufficiently large number of viable microorganisms per unit; 4) be capable of surviving in the intestine, reproducing, maintaining itself, and having intraluminal metabolic activity; and 5) remain viable during storage and use [2,3]. Controversially to point 3, in some studies was proven that heat-killed and UV-killed probiotics [4,5], bacterial cell fractions [6-8], bacterial substances secreted into growth medium [9,10], pure substances isolated from bacterial cells [11] and their synthetic analogs [12,13] may be used in the form of dietary supplementations for human health improvements, since they have the advantages of allowing a longer shelf-life, easier storage and transportation .

Commonly used bacterial probiotics include *Lactobacillus* and *Bifidobacterium* species. *Escherichia coli*, *Lactococcus lactis*, *Streptococcus* and *Enterococcus* species are used rarely. This list shows that the majority of probiotics are a group of lactic acid bacteria (LAB). Unfortunately, some of them do not possess very good technological features for using as drugs and food additives, because their viability will decrease rapidly under unfavorable conditions, in particular during the passage through the stomach. Therefore, at the present time some spore-forming bacteria (mostly of the genus *Bacillus* currently considered as gut commensals, that have a bimodal life cycle of growth and sporulation in the environment as well as within the gut [14] and yeast cultures of *Saccharomyces boulardii* are recommended as probiotics, although physiological and ecological traits of spore-forming species and yeasts do not qualify them as probiotics of first choice [15]. Taking into account that most probiotics were originally isolated from healthy humans we can supplement the list of probiotics with certain human commensals, *e.g. Bacteroides fragilis* [16] and *Clostridia* [17,18], whose beneficial effects on human health have been proven to date.

Humans contain nearly 100 trillion intestinal bacteria that are essential for health. The concentrations of bacteria in the upper two-thirds of the small intestine (duodenum and jejunum) and more proximal regions (ileum as distal part of the small intestine and large intestine (cecum and colon)) are 10^3 - 10^4 and 10^{10} - 10^{12} colony-forming units per gram of intestinal content, respectively. In fact, 60% of the fecal matter mass in humans is due to bacteria [19]. The human gut microbiota comprises a diverse microbial consortium including upwards of 500 bacterial species closely co-evolved with the human genome and diet. A broad catalog of the human gut microbial genes contains 3.3 million items, 150-fold more than the human genome equivalent [20]. Recent studies using culture-independent techniques of 16S ribosomal RNA gene sequencing and metagenomic sequencing methods estimate the presence of 200–300 species per individual. It was shown that 99% of intestinal resident/commensal microbiota belongs to only four phyla. There are *Firmicutes* (composed mostly of class *Clostridia*, including probiotic genes *Lactobacillus*, *Streptococcus*, *Enterococcus* and *Bacillus* of class *Bacilli*) and *Bacteroidetes* which together constitute more than 90% of the total intestinal microbes; *Proteobacteria* (including *Escherichia coli*, same strains of which are probiotics) and *Actinobacteria* (including genus *Bifidobacterium*) make up the rest [21].

Millions of years of coevolution have molded this human-microbe interaction into a symbiotic relationship in which gut bacteria make essential contributions to human nutrient metabolism and in return occupy a nutrient-rich environment. The importance of the gut microbiota in regulating human health and disease has however been largely overlooked due to the inaccessibility of the intestinal habitat, the complexity of the microbial community, which is difficult to dissect and highly variable between subjects and also the fact that many of commensals resist cultivation.

Currently, it is known that large-scale imbalances in gastrointestinal microbiota, or 'dysbiosis', rather than occurrence of particular microorganisms, are associated with several gastroenterological conditions such as antibiotic-associated diarrhea, Crohn's disease, ulcerative colitis, *etc.* [21].

A commensal microbiota consists of autochthonous species which are able to colonize the mucosal surface of the gastrointestinal tract due to special adhesion factors and from allochthonous species, the presence of which in the intestine has a transient character. Primary function of planktonic intestinal microbiota is metabolic that includes fermentation of indigestible substrates and favoring the growth of beneficial intestinal bacteria. Improvement of lactose digestion, modulation of intestinal gas production, increasing in absorption of Ca, Fe and Mg, synthesis of vitamins (K, folic acid, biotin, B12), genesis of short-chain fatty acids (that serve as source of energy and regulate the growth and differenciation of intestinal epithelial cells, especially in colon) belong to this group. Protective function of planktonic intestinal microbiota is additional and includes detoxification of microbial products like toxins, host metabolites (e.g. bile salts) and food components in the gut, and also synthesis and secretion of lactic acid as end product.

Primary functions of mucosa-associated microbiota are protective and trophic. The trophic function means that mucosa-associated microbiota controls intestinal epithelial cell proliferation/differentiation and maintains new cell growth. The protective function includes prevention of bacterial translocation and systemic infection through (*i*) competitive exclusion of exogenous pathogens, (*ii*) bacteriocin synthesis, (*iii*) induction of non-immune host defense including mucin and defensin synthesis (by goblet cells and Paneth cells, respectively) and (*iv*) regulation and reparation of tight junctions' integrity. The protective function includes also (*v*) immunomodulation; the term means that probiotics/commensals modulate immune homeostasis by affecting the development, differentiation and effector function of different immune cell subsets (mainly at the level of gut associated lymphoid tissue and in other mucosal sites, more rarely at systemic level).

Modulation of non-immune host defense and immunomodulation is most likely important for the prevention and therapy of infectious diseases and for the treatment of chronic inflammation of the digestive tract. In addition, immunomodulation could be important for the eradication of neoplastic host cells and treatment of allergy and non-intestinal autoimmune disorders. Direct effect of probiotics on other microorganisms, commensal and/or pathogenic ones, is important for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut.

The molecular processes underlying host-microbe interactions in general and immunomodulation in particular are far from clarifying. Gaining insight into the mechanisms of probiotic action could not only help to improve the credibility of the probiotic concept but also to develop tailor-made strategies for the prevention or treatment of various diseases. Here we reviewed the known mechanisms of immunomodulation induced by probiotic bacteria and especially bacterial substances and pattern recognition receptors (PRRs) implicated in the mechanisms.

2. Molecular basis of probiotic/symbiont – host interaction

Molecular basis of microbiota-host interaction is PRRs. The current dogma is that PRRs are members of non-adaptive (innate) recognition, which is consistent with their location on the surface and inside intestinal enterocytes and innate immune cells, including macrophages and dendritic cells (DCs). However, growing evidence has demonstrated that TLRs were also able to promote adaptive immune responses, mainly indirectly, via DCs. In addition, it was found that the T and B lymphocytes also have PPR, which suggests that PRR signaling could induce/modulate adaptive immune responses directly [22-26].

Toll-like receptors (TLRs) were the first identified PRR [27-28]. Other PRRs, such as C-type lectin receptors (CLRs) [29], nucleotide-binding oligomerization domain (NOD) leucine-rich-repeat (LRR) containing receptors (NLRs) and retinoic acid inducible gene I protein (RIG-I) helicase receptors (RLRs) [30] continued this list.

Ligands of PPR are microbe-associated molecular patterns (MAMP) [31,32] that are small molecular motifs conserved within a class of microbes. Note, that MAMP are mainly the same molecules as the pathogen-associated molecular patterns (PAMP), the term that was introduced initially, when such molecules were found namely in pathogens and were considered typical for this group of microorganisms. Currently it is known that pathogens have virulence factors that allow adhesion to epithelial cells, entry into cells (for invasive pathogens), evasion and inhibition of the host's immune response. A virulence signal capable of binding to a pathogen receptor, in combination with MAMP has been proposed as one way to constitute PAMP [33]. Thus, both MAMP and PAMP interact with the same PPR.

Generally known MAMP include non-mammalian nucleic acids, *viz.*, viral double-stranded RNA and bacterial unmethylated CpG DNA; proteins that have features unique to bacteria, such as N-formylmethionine initiation; lipids and carbohydrates, *viz.*, Gram-negative bacterial lipopolysaccharides (LPS), Gram-positive bacterial teichoic acids (TA)/ lypoteichoic acids (LTA) and bacterial mannose containing glycopolymers (oligosaccharides and glycoproteins/glycopeptides); peptidoglycan (PGN) from both Gram-negative and Gram-positive bacteria.

2.1 PRRs implicated in the recognition of probiotic/commensals

In the gut, two classes of PRRs play a crucial role in the recognition of commensals, TLRs and NLRs [34]. The TLRs are membrane-anchored proteins either expressed at the cell surface or associated with intracellular organelles. They have a lumen-facing LRR recognition domain and an intracellular toll-interleukin (IL)-1 receptor (TIR) signaling domain [35]. Currently, localization and structure of TLRs, their ligands and adaptor molecules, mechanisms of TLR signaling, negative regulation and cooperation of these mechanisms are well known and reviewed [36-39]. Briefly, the mammalian TLR family investigated in human and in mice comprises 10 and 12 members, respectively, and TLR11, TLR12 and TLR13 are lost in human genome. The ligands for most TLRs were identified through generation of mice deficient for individual TLRs. TLR1, TLR2, TLR4, TLR5, TLR6 and murine TLR10 and TLR11 are expressed exclusively on the cell surface and recognize microbial membrane components such as lipids, lipoproteins and proteins. On the other hand, TLR3, TLR7, TLR8 and TLR9 are localized in intracellular vesicles such as the endosomes or lysosomes and the endoplasmic reticulum and predominantly recognize microbial and viral nucleic acid. TLR2 forms a heterodimer with TLR1 or TLR6, but in other cases TLRs forms homodimers. The ability of TLRs to heterodimerize with one another extends their specificities. For example, dimers of TLR2 and TLR6 are required for responses to diacylated lipoproteins while dimers of TLR2 and TLR1 recognize triacylated lipoproteins [40]. Specificities of the TLRs are also influenced by various adapter molecules (see below) [41] and accessory molecules, such as MD-2 and CD14 that form a complex with TLR4 in response to LPS [42].

Dimerization of TLRs triggers activation of signaling pathways, which originate from a cytoplasmic TIR domain. In the signaling pathways downstream of the TIR domain, 5 adaptors modulate TLR signaling: (*i*) myeloid differentiation primary-response gene 88 (MyD88); (*ii*) TIR domain containing adaptor protein (TIRAP) also called MyD88 adaptor-like (Mal); (*iii*) TIR domain-containing adapter inducing interferon (IFN)-β (TRIF) also known as TIR domain-containing adaptor molecule 1 (TICAM-1); (*iv*) TRIF-related adaptor molecule (TRAM) also known as TICAM-2 and (*v*) sterile alpha- and armadillo-motif-containing protein (SARM). Different TLRs have their own set of adaptor proteins required for signal transduction. Only TLR4 bind all known adaptor proteins.

MyD88 is common adaptor to all TLRs, except TLR3, and MyD88-dependent pathway is the most common signaling pathway [43]. Generally, this pathway leads to mediated activation of nucleic factor-kB (NF-kB), which results in the transcription of proinflammatory cytokines such as tumour necrosis factor (TNF)-α and IL-12. TIRAP is yet another bridging adaptor molecule specifically involved in the MyD88-dependent pathway (downstread MyD88) via TLR1/TLR2, TLR2/TLR6 and TLR4.

In addition, activation of TLR3 and TLR4 signaling pathways and TLR7, TLR8 and TLR9 signaling pathways leads to mediated activation of interferon regulatory factors (IRF), resulting in the transcription of type I IFNs, IFN-α and IFN-β [43]. TLR3 and TLR4 activate IRF3 and IRF7 [44] and triggers the production of type I IFNs through MyD88-independent mechanisms using adaptor TRIF/TICAM-1 [45] and adaptor TRAM/TICAM-2 (only for TLR4) [46]. TLR7, TLR8 and TLR9 signaling leads to activation of IRF5 and IRF7 [47,48] and transcription of type I IFNs through MyD88-dependent pathway [49,50].

TLR2 (TLR4) signaling via MyD88 can induce inflammatory cytokines in a cascade also involving bridging adaptor IL-1 receptor-associated kinase 1 (IRAK-1), that binds to TNF receptor-associated factor 6 (TRAF-6), E3 ubiquitin protein ligase. Downstream TRAF-6, inhibitor of NF-kB kinase complex (IKK complex, including two kinases, IKKα and IKKβ, and a regulatory subunit NEMO/IKKγ) and mitogen-activated protein kinases (MAPKs), including JNKs, p38 kinases and ERK1/2 are activated. They, in tern, activate NF-κB and the activator protein 1 (AP-1), respectively.

NLRs are cytosolic proteins that have N-terminal effector domain and a central nucleotide binding and oligomerization (NOD, also named NACHT) domain, flanked on the C-terminus by a LRR domain. NLRs are composed of several sub-families, depending on the nature of their N-terminal domain [51]. Nucleotide-binding oligomerization domain containing protein 1 (NOD1) and NOD2 have N-terminal caspase recruitment domain (CARD) and are two members of NLR family whose participation in the effects of microbiota is proven to date. Both NOD1 and NOD2 are implicated in the recognition of bacterial PGN. NOD1 senses core peptide chain [52], while NOD2 senses muramyl dipeptide [53]. Interestingly, NOD1 and NOD2 represent the only described NLR family members that turn on the NF-κB pathway upon stimulation, also other NLRs induce the assembly of large caspase-1-activating complexes called inflammasomes that lead to caspase-1 medicated IL-1β activation [54,55].

Another PPR whose participation in the effects of commensals and probiotics is proven to date, is DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) also known as cluster of differentiation (CD) 209. DC-SIGN is a C-type lectin expressed by DCs and macrophages; it recognizes high-mannose-containing biopolymers and initiate specific Raf-1-dependent signaling pathway that modulates TLR-induced NF-κB activation [56].

2.2. Substances of probiotic/commensals implicated in immunomodulation as ligands of PPR

In the gut, three classes of PRRs are implicated in the recognition of probiotic/commensals, TLRs (TLR2, TLR4 and TLR9), NLRs (NOD2) and CLRs (DC-SIGN). Oligodeoxynucleotides with the immunostimulatory CpG motif, high-mannose-containing biopolymers (oligosaccharides and glycoproteins) and bacterial cell wall components are ligands of TLR9, DC-SIGN and either TLR2 or TLR4, respectively.

Briefly, ninety percent of the cell wall of Gram-positive bacteria is composed of PGN, interconnecting glycan strands, cross-linked by short peptides. TA are interwoven with PGN; LTA are inserted into the outer layer of the cytoplasmic membrane via a lipid moiety and extend through the cell wall to the outer surface. Lipopeptides (LP) in Gram-positive bacteria are found in the periplasmic space, where they are involved in nutrient transport [57]. In contrast, Gram-negative bacteria have a PGN monolayer surrounded by an outer lipid bilayer with LPS on the outer surface of the outer layer. Lipoproteins act as a bridge between the inner layer of the lipid bilayer and the PGN monolayer [58]. Among these substances, LPS interact with TLR4; others are ligands of TLR2. In addition, PGN interacts with NOD1 and NOD2. CpG motifs (TLR9 ligands) are numerous in bacterial genomes; in particular, between 18,000 and 27,000 such motifs per DNA strand in *Bifidobacterium* genomes were shown *in silico* [59].

3. Symbionts/probiotics and adaptive immune response

3.1. DCs as innate immune cells controlling adaptive immune response

Among innate immune cells, DCs play a key role in controlling adaptive immune responses, since they are the most effective professional antigen presenting cells for T cell priming and directing T cell differentiation. DCs consist of heterogeneous subsets, as determined by their location, surface markers and cytokine profiles [60]. Two major subsets of DCs have been identified, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). They are present within the gastrointestinal tract in relatively high numbers compared with the peripheral blood. In the mouse, pDCs are numerically dominant within the lamina propria and Peyer's patches, while mDCs dominate in mesenteric lymph nodes [61].

The mDCs express all TLR except TLR7 and TLR9. On the contrary, TLR7 and TLR9 are most characteristic for pDC [62-64]. TLR7 detects single-stranded RNA of single-stranded RNA viruses [65] and short double-stranded RNA with certain sequence motifs (immunostimulatory RNA) [66], while TLR9 recognizes unmethylated CpG motifs within microbial DNA. It leads to production of large amounts of type I IFNs, the most potent antiviral cytokines. The presence of even small numbers of pDCs potently modulates the activity of other immune cell subsets, such as T cells, natural killer (NK) cells, and mDCs [67-69]. Besides, pDCs have been shown to be essential for differentiation of regulatory T cells and maintenance of oral tolerance [70,71]. The mDCs are mainly implicated in T effector/regulatory cell induction and differentiation.

The interaction of microbial products with TLRs leads to DC maturation characterized by upregulation of costimulatory molecules such as CD40, CD80, CD86 and MHC class II, expression of chemokine receptors and production of cytokines.

3.1.1. Th1 and Th2 cells

The functions of T cells in GALT, cytokines that are necessary for their maturation and cytokines that are secreted by mature T cells are reviewed by Delcenserie et al [72]. Briefly, naive T cells proliferate and differentiate into effector cells as a result of their activation by DCs. Mature T cells can be subdivided into T helper cells (CD4+, also called Th) and cytotoxic T cells (CD8+); both types of T cells recognize antigens through T cell receptor (TCR) in the form of an MHC/peptide complexes that are expressed by DCs and macrophages, whose MHC I and MHC II interface with CD8+ and CD4+ T cells, respectively. CD8+ T cells can target infected cells, CD4+ T cells control the immune response by regulating of B cells and macrophages.

In GALT DCs induce differentiation of naïve CD4+ T cells into Th1 and Th2, which generally depend on environmental conditions including cytokines in T cell environment. IL-12 and IFN-γ cytokines induce a Th1 response, whereas the IL-4, IL-5 and IL-13 induce a Th2 response [73]. Th1 cells produce pro-inflammatory cytokines like IFN-γ, TNF-α and IL-2, while Th2 cells produce the cytokines IL-4, IL-5, IL-6 and IL-13. The cytokines produced by Th1 cells stimulate the phagocytosis of microbial pathogens while Th2 cytokine IL-4 generally stimulate B cells for production of antibodies directed toward extracellular infections including large parasites and soluble antigens, specifically secretory bacterial products. IL-5 stimulates eosinophil responses directed against large extracellular parasites. On the negative side, overreactive Th1 pathway seems to be involved in autoimmune diseases, while overreactive Th2 pathway underlay food allergy.

The presence of a further subset of CD4+ T helper cells with pro-inflammatory properties, called Th17, was recently discovered [74]. Induction of Th17 responses includes three distinct steps: transforming growth factor (TGF)-β plus IL-6 induce differentiation of Th17 cells, IL-21 amplifies the frequency of Th17 cells and IL-23 stabilizes the phenotype of previously differentiated Th17 cells [74]. They produce IL-17 and recruit neutrophils, which is important in host protection against Gram-negative bacteria and fungal infections, that are not efficiently cleared by Th1 and Th2-type immunity. On the other hand, Th17 are highly pro-inflammatory and lead to severe autoimmunity in various animal models [75].

The balance between Th1 and Th2 cytokine production can determine the direction and outcome of an immune response. A true balance between Th1 and Th2 profiles can be difficult to maintain, as Th1 and Th2 cells can

antagonize each other's action, either by blocking polarized maturation of the opposite cell type or by blocking its receptor functions. Interestingly, that potent inhibitor of Th17 differentiation are cytokine IL-27 that is important for the differentiation of Th1 cells, and cytokines IL-4 and IFN-γ antagonizing differentiation of Th1 cells and Th2 cells, respectively [74].

Additionally, the gut immune system has diverse mechanisms of tolerance that avoid uncontrolled Th1, Th2 or Th17 effector responses. Particularly relevant among them is the action of regulatory T cells, which act by suppressing effector responses. These include the antigen-induced type 1 Tr cells (Tr1) secreting high levels of IL-10 and from low to moderate levels of TGF-β, type 3 T cells (Th3) [76] secreting TGF-β and also the naturally-occuring, thymic-derived CD4+CD25+Foxp3+ T cells (Treg) producing IL-10 [77]. Tr1 and Th3 are induced from peripheral tissues; they exert suppression via the secretion of soluble factors, IL-10 and TGF-β, in contrast to naturally occurring Treg cells that inhibits responder T cells of unrelated antigen specificity through cell-cell contact [78]. A prominent mechanism by which Treg inhibits T cell responses is cytokine deprivation-induced apoptosis [79]. The frequencies of Treg are considerably elevated in the intestine relative to other tissues [80]. The mucosal Treg cells are continuously generated through the action of specialized DCs subsets via retinoic acid [81,82]; efficient Treg cell activation required the TLR adaptor molecules MyD88 and TICAM-1 [83].

Interestingly, that addition of TGF-β to Foxp3− T cells can lead to Foxp3+ T cells with regulatory activities, whereas activation of Foxp3− T cells in the presence of TGF-β plus IL-6 resulted in the induction of Th17 cells. Thus, there is a reciprocal relationship between Foxp3+ Treg and Th17 cells (known as Treg/Th17 plasticity) [84] and IL-6 plays a pivotal role in Treg/Th17 cell balance.

Microorganisms activate DCs (*i*) directly via their PRRs or indirectly, by the capture of tissue products including (*ii*) apoptotic/necrotic products of other cells dying in response to pathogen exposure and (*iii*) cytokines secreted by epithelial cells, innate immune system cells, *etc*., as result of their interaction with microorganisms [63]. MAMP and PRRs are implicated in DCs control of T-cell polarization in accord with model described in the review of Kapsenberg [85]. Immature DCs can be polarized by type 1, type 2 and regulatory-type MAMP/tissue products to become mature effector DCs that promote the development of naive T cells into Th1, Th2 or Treg cells. An exception is that certain MAMP might inhibit DCs maturation, resulting in regulatory immature DCs (so called semi-mature DCs, a phenotype characterised by increased costimulatory marker expression but low production of proinflammatory cytokines); they promote the development of Treg cells [86]. Interestingly, that T-cell stimulation and polarization require three DCs-derived signals [85]. Signal 1 is the bacterial antigen processed by DC plus MHC class-II-associated peptides; this signal is mediated through TCR. Signal 2 is the co-stimulatory signal; this signal is mediated by triggering of CD28 (expressed by T cell) by CD80 and CD86 (expressed by DCs). Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors; the nature of signal 3 depends on interaction of MAMP with particular PRRs expressed by DCs. Type 1, type 2 and regulatory-type MAMP can be defined as those that selectively prime DCs for the production of high levels of Th1 cells-polarizing factors, Th2 cells-polarizing factors and Treg cells-inducing factors, respectively.

Generally accepted, that balance of IL-12/IL-10 production is crucial for determination of the direction of the immune response. DCs produce IL-12, which contribute to Th1 responses [87], and also produce IL-4 and IL-10, which promote Th2 or Treg responses, respectively [88], though the main producers of IL-10 in intestinal lamina propria are Foxp3+ Treg cells [80].

Certain probiotic strains induce surface markers weakly and suppres IL-12 production though other strains strongly induce IL-12; they could be regulatory type 3 or type 1 DCs-polarizing bacteria, respectively. Cytokine production is regulated by intracellular signal transduction pathways that are activated following recognition of probiotic/commensals via PRRs.

Interestingly, different TLR ligands induce the same responses. For example, unique oligodeoxynucleotides with or without the immunostimulatory CpG motif (ligands of TLR9) have been identified as components of *Lactobacillus rhamnosus* and *L. gasseri* that are active in IL-12 induction [89,90]. Similarly, four 21-mer oligodeoxynucleotides (ODN) and three 25-mer ODNs containing repeated CpG motifs and synthesized on the basis of *Bifidobacterium longum* genomic sequence induced murine macrophage line RAW 264.7 to secrete cytokines IL-6, IL-12p40 and TNF-α [12]. In contrast, the insoluble intact cell wall of *Lactobacillus casei* was required for the induction of IL-12 production by macrophages [91].

Little information is available concerning the components that are active in the induction of IL-10 production. It has been shown that genomic DNA isolated from bifidobacteria or lactobacilli [11], soluble or insoluble cell preparations, obtained from centrifugation of sonicated bifidobacteria [6,7] and TA/LTA from lactobacilli [92] were responsible for induction of IL-10 production.

Other PRR ligands, extracellular proteins of lactobacilli and bifidobacteria, are implicated in regulation of Th1/Th2 balance. Extracellular proteins secreted by *Bifidobacterium breve* C50 were shown to interact with DCs via TLR-2, inducing different functional and physiological changes through different pathways. Prolonged DCs survival was mediated by the phosphatidylinositide 3-kinase (PI-3K) pathway, DC maturation by the p38 and PI-3K pathways, increase in IL-10 production by the MAPKs (p38, ERK) and PI-3K pathways, and finally an increase in IL-12 production was mediated by means of the p38 and glycogen synthase kinase 3 (GSK-3) pathways [93]. S-layer protein

A (SlpA) released from *Lactobacillus acidophilus* NCFM cells has been shown to induce IL-10 production in DCs via a direct interaction of SlpA with DC-SIGN [94]. A SlpA knockout strain, which overproduced another S-layer protein (SlpB), preferentially induced a pro-inflammatory response through an increase in production of IL-12p70, TNF-α and IL-1β. Unfortunately, no information about the components involved in the downstream signal transduction pathway was reported [94].

The cytokine response of antigen-presenting cells to probiotic lactobacilli depends on bacterial susceptibility to intracellular digestion. It was shown [92] that *Lactobacillus plantarum* cells more susceptibility to intracellular digestion than *Lb. casei* that retains its cell morphology following ingestion. In mouse peritoneal macrophages, *Lactobacillus plantarum* potently induced IL-10 but weakly induced IL-12 production, whereas *Lb. casei* potently induced IL-12 but weakly induced IL-10 production. Interestingly, combination of *Lb. plantarum* and *Lb. casei* synergistically induced IL-10 production in macrophages. In this study, cell wall TA and LTA extending from the plasma membrane of *Lb. plantarum* were identified as key factors that trigger IL-10 production, and TLR2-dependent ERK activation was proven as key event that is critical for determination of the IL-10/IL-12 balance [92]. Role of TA was proven by conversion of IL-12 production induced by *Lb. casei* into IL-10 production when combination of *Lb. casei* and TA was used. The data are in accord with study of Shida and colleagues [91], where cell wall structure of lactobacilli that is resistant to intracellular digestion by macrophages induces IL-12 response. Interestingly, that small differences in LAB surface structure result in distinct DCs maturation and cytokine induction patterns, which function as a kind of "strain-fingerprint".

In other study [95] was shown that different structural motifs of lactobacilli and bifidobacteria simultaneously interact with different PRRs in DCs, giving a coordinated sum of signals and determining the DC maturation and Th1/Treg polarization. High and low level of surface markers expression and IL-12 production was detected in DCs after their interaction with lactobacilli (4 species) and bifidobacteria (4 species), respectively. Lactobacilli induce Th1 polarizing DCs by interaction of PGN with NOD2; internalization might depend on binding to DC-SIGN. Simultaneously, LTA of lactobacilli might interact with TLR2 inducing Treg polarization. Bifidobacteria induce Treg polarization by interactions of lipoprotein with TLR1/TLR2 or TLR2/TLR6. When both stimuli are present simultaneously, a competitive interaction of MAMP with NOD2 and TLR2 determines the DC maturation pattern. In sum, commonly known probiotics act as immunoregulators through interaction of lipoprotein with TLR2 and as immunostimulators through interaction of PGN with NOD2.

Note, that the point 'Th1/Th2 balance' is very important taking into account that overreactive Th1 pathway seems to be involved in autoimmune diseases, while overreactive Th2 pathway underlay food allergy [72].

3.1.2. Th17 cells

There are few works reporting probiotic induction of Th17 cells. The comparative analysis of different *Bifidobacterium* strains showed that four strains (*B. bifidum* LMG13195, L22, A8, IF10/10) induce high production of IL-17 and poor secretion of IFN-γ and TNF-α in peripheral blood mononuclear cells, suggesting a Th17 profile, whereas other *Bifidobacterium* strains exhibited mainly a Th1 profile in accordance high L-12/IL-10 ratio. Unexpectedly, any *B. bifidum* strain showed significant capability for Th17 generation, and they were able to generate functional Treg [84]. This study demonstrates great differences between *Bifidobacterium* strains in their ability to activate DCs and supports the fact that commensal bacteria may play a role in Treg/Th17 cell balance in the intestine due to existence of Treg cells with plasticity either to show an effector function, secreting IL-17, or a regulatory action, depending on the environment and the nature of the stimuli.

Interestingly, that DCs matured *in vitro* with specific *Bifidobacterium* strains maintain mucosal tolerance via polarizing of naïve T lymphocytes towards regulatory Foxp3+ cells, whereas in *ex vivo* experiments the same commensal bacteria induce the production of IL-17, probably due to the activation of memory Th17 cells [84]. In addition, under inflammatory stimulation (IL-1β and IL-6) Treg could sustain Foxp3+ expression and begin to produce IL-17, demonstrating transdifferentiation to a Th17 effector subset and a reversible loss of suppressive function [84].

The contribution of the microbiota to intestinal Th17-cell development is highlighted by the virtual absence of this subset in germ-free mice [96]. In addition to IL-6, which is required for Th17 differentiation and its deviation away from induced Treg programming, multiple microbiota-dependent factors favor Th17 development in the intestine, including TGF-β, IL-1β [97], IL-23 [98] and even ATP derived from commensal bacteria [99]. Minor constituents of the microbiota can amplify intestinal Th17 cell numbers. In mice, an unusually potent, but not unique, inducer of Th17 cells is the *Clostridia* sp. *Candidatus arthromitus* [83] known as segmented filamentous bacteria (SFB) [100,101]. Importantly, SFB that are present in a conventional microbiota changed the expression of more genes than commonly known probiotics lactobacilli or bifidobacteria [102]. Though induction of Th17 cells by SFB provides protection against gut pathogens [96], it is not entirely benign since mono-association of mice with SFB induces Th17-mediated inflammatory arthritis [103] and multiple-sclerosis-like symptoms in the experimental autoimmune encephalomyelitis model [104]. At present, it is unclear whether SFB, or related organisms, exert similar effects in humans.

3.1.3. Treg cells

According to the hygiene hypothesis, the increasing incidence of allergy in Westernized societies over the last decades may to some extent be explained by a reduced microbial load early in infancy [105,106] resulting in too little Th1 cell activity and therefore an insufficient level of IFN-γ to cross-regulate optimally Th2 cell responses. Classical hygiene hypothesis was overviewed by Rook and Brunet [107] which took into account simultaneous increase in Th2-mediated allergies, Th1-mediated inflammatory bowel disease and autoimmunity and proposed "old friends" hypothesis. According the view, traditional commensals (lactobacilli) and other transient but harmless organisms (including saprophytic mycobacteria and helmints) induce maturation of DCs that induce Treg specific for allergens, commensals and self antigens, that are target antigens in three groups of chronic disorders. Contact with 'old friends' is greatly diminished in rich countries that lead to insufficient Treg activity and upset effector T cells/Treg balance, which might precipitate immunoregulatory disorders in suspectibile individuals.

Although there are few works reporting bacterial induction of Tr1 cells and Th3 cells [108], there is increasing evidence that some probiotic bacteria might induce naturally-occuring Foxp+ Treg cells from naïve precursors via PRR signaling.

It was shown that *B. breve* but not *Lb. casei* activates intestinal CD103+ DCs to produce IL-10 and IL-27 via the TLR2/MyD88 pathway thereby inducing IL-10-producing Tr1 cells in the large intestine and preventing intestinal inflammation in mice [108]. In other study, *B. longum* AH1206 and *B. breve* AH1205 consumption resulted in increased numbers of Foxp3+ T regulatory cells in mice (in infant, adult and germ-free animals and only in infant mice, respectively) as opposed to *Lactobacillus salivarius* AH102 that did not alter Treg numbers in any animal model tested [109]. *B. longum* AH1206 reduced the Peyer's patch gene expression associated with antigen presentation, TLR signalling and cytokine production while increasing the expression of genes associated with retinoic acid metabolism, which is important for Treg induction [81,82]. In addition, *B. longum* AH1206 protected against airway inflammation in OVA-sensitized animals and blocked the induction of IgE to orally administered OVA. Neither *B. breve* AH1205 nor *Lb. salivarius* AH102 had a protective effect in either model. Thus, bacterial induction of Foxp3+ Treg *in vivo* is strain-specific and associated with prevention of intestinal inflammation and protection from respiratory and oral allergy.

DCs exposed to *B. bifidum* LMG13195 membrane vesicles strongly promoted differentiation of functional CD25(high)Foxp3(high)CD127(-/low) Treg cells as well as induced higher IL-10 levels as compared with proinflammatory cytokines IFN-γ, TNF-α and IL-17 [8]. The results suggest the potential use of *B. bifidum* LMG13195 membrane vesicles as clinically effective adjuvant in allergen-specific immunotherapy to induce antigen-specific Treg cells. In other study [110] was shown that probiotic DNA and the synthetic oligodeoxynucleotides containing CpG motifs were comparable with living probiotics in preventing allergic response in rats orally sensitized with ovalbumin. The Th1/Th2 cytokine balance was shifted away from Th2 side, the percentage of CD4+CD25+Foxp+ Treg cells was increased, and the intestinal barrier function was improved in these three intervention groups compared with the control group. These effects correlated with significant increase in the levels of TLR9 mRNA and NF-κB activity and IκB-α phosphorylation.

Volunteers fed *B. longum* subspecies *infantis* 35624 displayed a selective increase in secretion of IL-10 and enhanced Foxp3 expression in peripheral blood [111]. *In vitro*, mDCs and pDCs expressed indoleamine 2,3-dioxygenase and secreted IL-10, but not IL-12p70, in response to *B. infantis*. The mDC IL-10 secretion was TLR-2/6 dependent, while pDC IL-10 secretion was TLR-9 dependent. Induction of Foxp3+ Tcells by mDCs was TLR-2-, DC-SIGN- and retinoic acid-dependent, whereas induction of Foxp3+ cells by pDCs requires indoleamine 2,3-dioxygenase, a tryptophan-catabolizing enzyme that exert regulatory effects on T cells by tryptophan depletion and is responsible for the self-amplification and maintenance of a stably regulatory phenotype in pDCs [112]. The data suggests that (*i*) human DC subsets use different pathways when exposed to a commensal microbe that enhances Foxp3 expression in autologous CD4 T lymphocytes; (*ii*) cross-talk between TLR-2/6, DC-SIGN, TLR-9 and other PRRs determines the innate and subsequent adaptive immune response to probiotic/commensals; (*iii*) multiple bacterial components including LTA (TLR2 ligand), mannose-rich polysaccharides and glycopeptides (DC-SIGN ligands) and DNA (TLR9 ligands) are all important for optimal induction of the immune regulatory programme *in vivo*.

In whole, bifidobacteria are more efficient inducer of Treg in comparison with lactobacilli, though in other study [113] both *Lb. reuteri* and *Lb. casei*, but not *Lb. plantarum* inducted the Treg cells through monocyte-derived DCs priming, and the induction of the Treg cells was mediated via DC-SIGN.

How probiotics could modulate activated pDCs to produce cytokines and induce Treg remains an interesting field of investigation. It is hypothesized that commensal bacteria may act through TLR9 with the help of humoral immunity or, alternatively, another intriguing scenario may envisage the cooperation of mDCs in LAB-mediated pDC regulation [114]. In support the hypothesis, it was shown [115] that certain spherical LAB (*Lactococcus*, *Leuconostoc*, *Streptococcus* and *Pediococcus*) but not bacillary LAB species induce pDCs activation and IFN-α production in murine bone marrow-derived DCs culture via TLR9 and MyD88-dependent partway, which was correlated with their capacity for uptake by pDC. *Lactococcus lactis* strains also augmented pDC induction of CD4+CD25+Foxp+ Treg compared to the *Lactobacillus* strains. Interestingly, while these responses occurred with purified pDC, IFN-α production was synergistic upon co-culture with mDCs, an interaction that required direct mDC-pDC contact. *In vivo*, oral

administration of *L. lactis* JCM5805 induced significant activation of pDC resident in the intestinal draining mesenteric lymph nodes, but not in a remote lymphoid site (spleen).

Similar to the ability of a limited quorum of commensal bacterial species to induce Th17 cells development, minor constituents of the microbiota can amplify intestinal Treg numbers. The capsular polysaccharide-A moiety of the common commensal *Bacteroides fragilis* mediates interaction of the bacteria with the colonic mucosa, facilitating both colonization and expansion of colonic IL-10-expressing Foxp3+ Treg cells via TLR2–MyD88 pathway [16]. A mixture of 46 *Clostridium* species, mostly comprised of clusters IV and XIVa, drives the efficient expansion of Treg cells in the colons of germ-free mice. In contrast with induction of Treg cells by *B. fragilis*, *Clostridium*-dependent Treg expansion occurs independently of MyD88 through mechanisms yet to be defined [17]. Thus, induction of Treg cell development does not seem to be a unique property of either any bacterial species or specific microbial component.

Since *Clostridia* constitutively induce accumulation and functional activation of Tregs in the colon, the relative abundance of the bacteria may strongly affect the immune status of the host. Loss of clusters IV *Clostridia,* particularly *Faecalibacterium prausnitzii*, and the cluster XIVa *Lachnospiraceae* family [21] was observed in intestinal bowel disease patients [18]. The reduction of *Clostridia* clusters XIVa and IV by neonatal vancomycin treatment promotes airway hypersensitivity in a mouse model [116]. Moreover, abundances of clusters IV and XIVa *Clostridia* have been associated with atopy during childhood [117]. In turn, the crucial role of Treg cells in immune homeostasis to the microbiota is well-documented by the consequences of these cell's absence [118], *viz*., unopposed effector T-cell responses to antigens of the commensals.

3.2. TLR on adaptive immune cells and their role in immunomodulation

Various studies indicate that certain TLR are expressed not only in innate immune cells but also in subsets of T lymphocytes including CD4+ and CD8+ T cells and γδ T cells [22-25] and in B cells [26]. The adaptive immune system (in particular, gut intraepithelial T cells and B cells of Payer patches and isolated lymphoid follicles) is able to respond directly to its immediate environment via TLR, partially negating the requirement for antigen-presenting cells (for T cells) and antigen-presenting cells plus Th2 cells (for B cells).

According to review of Kulkarni et al. [25], human CD4+ T cells express TLR2, 3, 4 and 5; CD8+ T cells – TLR2, 3 and 4; γδT cells – TLR2, 3, 5 and 7/8 (detected as proteins). In addition to effector T cells, Treg also express TLRs, namely, TLR2 and 5 (detected as proteins). Importantly, that activation of T cells with distinct TLR ligands is not possible in the absence of additional TCR stimulation. TLR ligands possess adjuvant properties in inducing antigen-specific T cell responses, enhancing proliferation and effector/regulatory functions.

According to review of Gibson et al. [26], B cells from Payer patches respond strongly to TLR2, 6 and 7, but weakly to TLR4 and TLR9 ligands. Memory B cells, in response to direct TLR stimulation, can potentially differentiate into plasma cells and sustain certain levels of protective antibodies even in the absence of their specific antigen. Since TLR ligation upregulates MHC II molecules and the co-stimulatory molecules in B cells, a type of antigen-presenting cells, they are able potentially to modulate T cell differentiation and regulate T cell responses in a TLR-dependent, but antigen-independent manner.

Mainly pathogenic bacteria and several natural and synthetic TLR ligands were used in the studies that prove direct effects of TLR ligands on T cells expressing the corresponding TLR; a few studies were performed using probiotics/commensals.

In the intestinal mucosa T cells are required to maintain a state of immunological tolerance toward a great number of dietary and bacterial antigens, commonly known as oral tolerance. To accomplish this critical assignment intestinal T cells cycle differently from systemically circulating T cells (peripheral blood T cells, PBT) [119]. About 95% of PBT have TCRs that are composed of α and β polypeptide chains, and 5% of the PBT have a TCR composed of γ and δ chains [120]. On the contrary, γδTCR cells constitute up to 60% of small intestinal intraepithelial lymphocytes [121,122]. TCR of αβ T cells and γδ T cells recognize antigens when they are bound to MHC molecules [123] and directly as intact proteins or nonpeptide compounds [124], respectively.

A direct interaction of probiotics with T cells via TLR was demonstrated for the first time in study of Sturm and colleagues [10]. In this study*, E. coli Nissle* 1917-conditioned medium (CM) inhibited cell cycling and expansion of peripheral blood T cells (PBT) but not mucosal T cells [10]. In addition, *E. coli* Nissle 1917-CM significantly inhibited the expression of IL-2, TNF-α and IFN-γ but increased IL-10 production in PBT. Bacterial lipoproteins mimicked the effect of *E. coli* Nissle 1917-CM; in contrast, heat-inactivated *E. coli* Nissle 1917, lipopolysaccharide, or CpG DNA did not alter PBT cell cycling, which suggest that inhibition of T cells proliferation was TLR-2 dependent. Thus, soluble factors released from *E. coli* Nissle 1917 downregulate the expansion of newly recruited T cells into the mucosa and prevent intestinal inflammation, while already activated tissue-bound T cells continue to eliminate deleterious antigens. The data demonstrated for the first time a direct interaction of probiotics with αβT cells via TLRs. In other study [125], *E. coli* Nissle 1917 ameliorates experimental colitis in mice via TLR2- and TLR4-dependent pathways. The mechanism was proved using coculture of *Escherichia coli* Nissle 1917 and human PBT; increased TLR-2 and TLR-4 protein expression and NF-κB activity were detected.

More recently, the group demonstrates for the first time [126] that a probiotic bacterium can regulate central functions of γδT cells. In contrast to the other probiotic strains tested (*Lb. acidophilus, Lb. casei, L. lactis, B. bifidum, B.*

lactis and *Lb. salivarius*), *E. coli* Nissle 1917 increased activation, cell cycling and expansion of γδT cells and then induced γδ T cell apoptosis, mediated by caspase- and FasLigand-dependent pathways via TLR2.

4. Conclusion

To date, the importance of the gut microbiota in regulating human health is well proven. Besides other benefits, probiotics/commensals provide modulation of immune system by affecting the development, differentiation and effector function of different immune cell subsets (mainly at the level of GALT and in other mucosal sites, more rarely at systemic level). The molecular processes underlying immunomodulation are far from clarifying. Though, it is clear that molecular basis of immunomodulation is PRRs and MAMP, including TA, LTA and LP (ligands of TLR2), PGN (ligand of TLR2 and NOD2), LPS (ligand of TLR4), CpG-containing DNA (ligand of TLR9) and mannose containing glycopolymers (oligosaccharides and glycoproteins/glycopeptides (ligands of DC-SIGN). The current dogma is that PRR are implicated in innate immune response. However, growing evidence has demonstrated that PRR are able to promote adaptive immune responses, mainly indirectly, via DCs that regulate T cells differentiation and effector/regulatory functions.

Commonly known, that immunomodulatory effects of probiotics/commensals are species-specific and strain-specific. It means that thorough selection of applicable bacteria is required for the prevention/treatment of 3 groups of human diseases: infectious diseases, non-infectious inflammation diseases (including systemic and organ specific autoimmune diseases and intestinal bowel disease) and allery. In certain cases, heat-killed and UV-killed bacteria, cell fractions, isolated substances, probiotic growth media containing secretory bacterial substances and syntetic PRR ligands are comparable with living bacteria in their immunomodulatory effects. They have the advantages of allowing a longer product shelf-life, easier storage and transportation and may be used in the form of dietary supplementations for human health improvements along with live probiotics used by tradition.

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