Systematic Review

Annals of Nutrition & Metabolism

Ann Nutr Metab 2012;61:160–174
DOI: 10.1159/000342079

Received: July 19, 2012
Accepted: July 20, 2012
Published online: October 2, 2012

Probiotic Mechanisms of Action

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Key Words
Antimicrobial responses · Bifidobacteria · Lactic acid bacteria · Lactobacilli · Probiotic mechanism of action · Probiotics

Abstract
Probiotics are live microorganisms that provide health benefits to the host when ingested in adequate amounts. The strains most frequently used as probiotics include lactic acid bacteria and bifidobacteria. Probiotics have demonstrated significant potential as therapeutic options for a variety of diseases, but the mechanisms responsible for these effects have not been fully elucidated yet. Several important mechanisms underlying the antagonistic effects of probiotics on various microorganisms include the following: modification of the gut microbiota, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune system to convey an advantage to the host. Accumulating evidence demonstrates that probiotics communicate with the host by pattern recognition receptors, such as toll-like receptors and nucleotide-binding oligomerization domain-containing protein-like receptors, which modulate key signaling pathways, such as nuclear factor-κB and mitogen-activated protein kinase, to enhance or suppress activation and influence downstream pathways. This recognition is crucial for eliciting measured antimicrobial responses with minimal inflammatory tissue damage. A clear understanding of these mechanisms will allow for appropriate probiotic strain selection for specific applications and may uncover novel probiotic functions. The goal of this systematic review was to explore probiotic modes of action focusing on how gut microbes influence the host.

Introduction

According to the Food and Agriculture Organization of the United Nations and the World Health Organization [1], probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts. In particular, strains belonging to *Bifidobacterium* and *Lactobacillus*, which are the predominant and subdominant groups of the gastrointestinal microbiota, respectively [2], are the most widely used probiotic bacteria and are included in many functional foods and dietary supplements [3–5]. *Saccharomyces boulardii* yeast has also been shown to have health benefits [6]. After a long history of safe use of probiotics in fermented dairy products and an increased recognition of their beneficial effects on human health [7], the food industry has become increasingly interested in these types of microorganisms. Often the criteria for the selection of probiotics include the tolerance to gastrointestinal conditions (gas-

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tric acid and bile), ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens [8, 9]. The mechanisms underlying the beneficial effects of probiotics are largely unknown but are likely to be multifactorial. Several mechanisms related to the antagonistic effects of probiotics on various microorganisms include the following mechanisms: secretion of antimicrobial substances, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune system [10].

The results of evidence-based analyses from human studies and animal models have shown the clinical potential of probiotics against many diseases [11]. Probiotics have been reported to suppress diarrhea [12], alleviate lactose intolerance [13] and postoperative complications [14], exhibit antimicrobial [15] and anti-colorectal cancer activities [16, 17], reduce irritable bowel symptoms [18] and prevent inflammatory bowel disease [19]. However, generalizations concerning the potential health benefits of probiotics should not be made because probiotic effects tend to be strain specific. Thus, the health benefit attributed to one strain is not necessarily applicable to another strain even within one species [20].

In the present study, we sought to conduct a systematic review on the mechanisms of action of probiotic strains. Using the following equation: 'epithelial barrier' [All Fields] OR 'antimicrobial substances'[All Fields] OR 'bacteriocins'[All Fields] OR 'BIF'[All Fields] OR 'adhesion'[All Fields] OR 'competitive exclusion'[All Fields] OR 'defensins'[All Fields] OR 'mucins'[All Fields] OR 'bacterial adhesins' [All Fields] OR 'antifungals'[All Fields] OR 'intestinal microbiota'[All Fields] OR 'fatty acids'[All Fields] OR 'mechanisms'[All Fields] OR 'TLR2'[All Fields] OR 'TLR4'[All Fields] OR 'TLR9'[All Fields] OR 'toll-like receptor'[All Fields] OR 'NOD1'[All Fields] OR 'NOD2' [All Fields] OR 'inflammasome'[All Fields] OR 'NLRP3' [All Fields] AND 'probiotics'[MeSH], we have selected 165 relevant articles of 1,731 articles published until June 25, 2012, from the PubMed and SCOPUS databases.

Mechanisms of Action of Probiotics

Major probiotic mechanisms of action include enhancement of the epithelial barrier, increased adhesion to intestinal mucosa, and concomitant inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of anti-microorganism substances and modulation of the immune system (fig. 1).

Enhancement of the Epithelial Barrier

The intestinal epithelium is in permanent contact with luminal contents and the variable, dynamic enteric flora. The intestinal barrier is a major defense mechanism used to maintain epithelial integrity and to protect the organism from the environment. Defenses of the intestinal barrier consist of the mucous layer, antimicrobial peptides, secretory IgA and the epithelial junction adhesion complex [21]. Once this barrier function is disrupted, bacterial and food antigens can reach the submucosa and can induce inflammatory responses, which may result in intestinal disorders, such as inflammatory bowel disease [22–24]. Consumption of non-pathogenic bacteria can contribute to intestinal barrier function, and probiotic bacteria have been extensively studied for their involvement in the maintenance of this barrier. However, the mechanisms by which probiotics enhance intestinal barrier function are not fully understood.

Several studies have indicated that enhancing the expression of genes involved in tight junction signaling is a possible mechanism to reinforce intestinal barrier integrity [25]. For instance, lactobacilli modulate the regulation of several genes encoding adherence junction proteins, such as E-cadherin and β-catenin, in a T84 cell barrier model. Moreover, incubation of intestinal cells with lactobacilli differentially influences the phosphorylation of adherence junction proteins and the abundance of protein kinase C (PKC) isoforms, such as PKCδ, thereby positively modulating epithelial barrier function [26].

Recent data have indicated that probiotics may initiate repair of the barrier function after damage. *Escherichia coli* Nissle 1917 (EcN1917) not only prevents the disruption of the mucosal barrier by enteropathogenic *E. coli*, but it even restores mucosal integrity in T84 and Caco-2 cells. This effect is mediated by the enhanced expression and redistribution of tight junction proteins of the zonula occludens (ZO-2) and PKC resulting in the reconstruction of the tight junction complex [27, 28]. Similarly, *Lactobacillus casei* DN-114001 [29] and VSL3 (a mixture of pre- and probiotics) [30] are capable of sustaining the intestinal barrier function by similar mechanisms. A recent paper has reported that VSL3 protects the epithelial barrier and increases tight junction protein expression in vivo and in vitro by activating the p38 and extracellular regulated kinase signaling pathways [31].

A link between altered levels of pro-inflammatory cytokines and intestinal permeability has been described in a number of intestinal diseases [32]. Using probiotics, the prevention of cytokine-induced epithelial damage, which is characteristic of inflammatory bowel disease [24], may

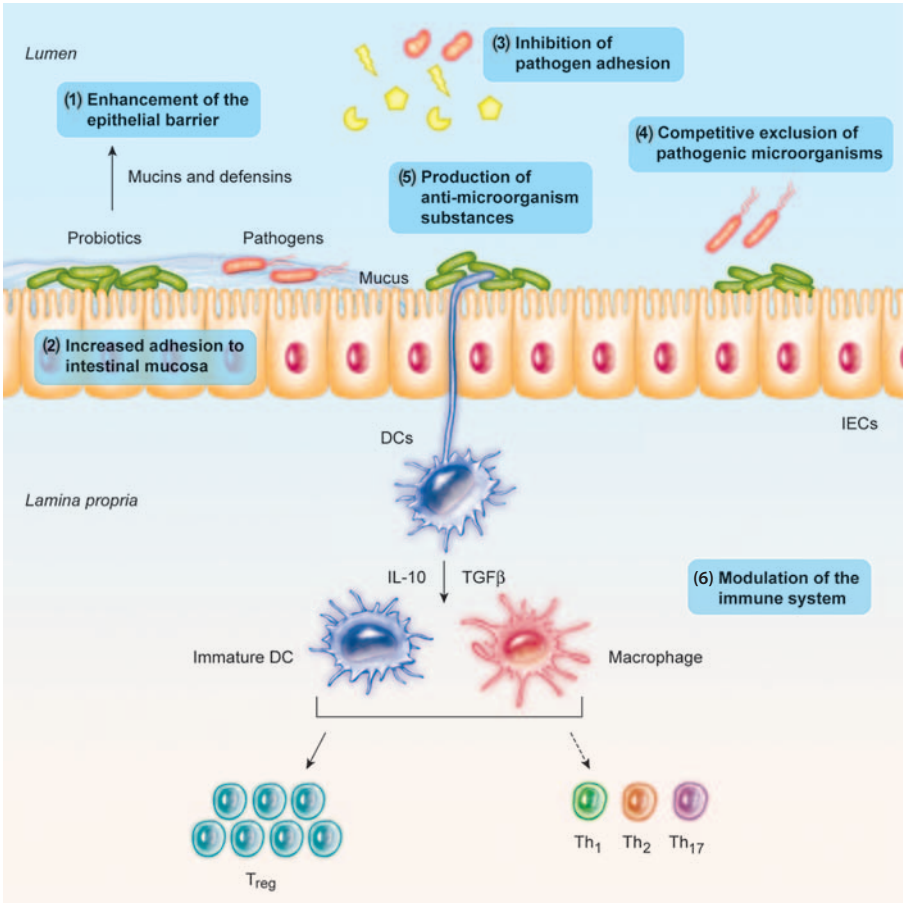

Fig. 1. Major mechanisms of action of probiotics.

also contribute to the reinforcement of the mucosal barrier. Two isolated and purified peptides secreted by *Lactobacillus rhamnosus* GG (LGG), which are designated p40 and p75, have recently been demonstrated to prevent cytokine-induced cell apoptosis by activating the anti-apoptotic protein kinase B (PKB/Akt) in a phosphatidyl inositol-3′-kinase-dependent pathway and by inhibiting the pro-apoptotic p38/mitogen-activated protein kinase (MAPK) [33, 34]. The evidence that p40 and p75 are responsible for the observed effects is derived from the observation that the anti-apoptotic function is abolished when p40- and p75-specific antibodies are added in vitro to murine and human epithelial cells or to colon explants derived from mice [34]. Other low-molecular-weight (LMW) peptides secreted from LGG induce expression of heat shock proteins and activate MAPKs [35].

Mucin glycoproteins (mucins) are major macromolecular constituents of epithelial mucus and have long been implicated in health and disease. Probiotics may promote mucous secretion as one mechanism to improve barrier function and the exclusion of pathogens. Several *Lactobacillus* species increase mucin expression in human intestinal cell lines. However, this protective effect is dependent on *Lactobacillus* adhesion to the cell monolayer, which likely does not occur in vivo [36, 37]. Conversely, another group has shown that *Lactobacillus acidophilus* A4 cell extract is sufficient to increase *MUC2* expression in HT29 cells independent of attachment [38]. Additionally, VSL3, which contains some *Lactobacillus* species, increases the expression of *MUC2*, *MUC3* and *MUC5AC* in HT29 cells [30]. In vivo studies are less consistent because only a few have been performed. Mice given VSL3 daily for 14 days do not exhibit altered mucin expression or mucous layer thickness [39]. Conversely, rats given VSL3 at a similar daily dose for 7 days have a 60-fold increase in *MUC2* expression and a concomitant increase in mucin secretion [40]. Therefore, mucous production may be increased by probiotics in vivo, but further studies are needed to make a conclusive statement.

Increased Adhesion to Intestinal Mucosa

Adhesion to intestinal mucosa is regarded as a prerequisite for colonization and is important for the interaction between probiotic strains and the host [41–43]. Adhesion of probiotics to the intestinal mucosa is also important for modulation of the immune system [43, 44] and antagonism against pathogens [45].

Thus, adhesion has been one of the main selection criteria for new probiotic strains [41, 46–48] and has been related to certain beneficial effects of probiotics [49]. Lactic acid bacteria (LABs) display various surface determinants that are involved in their interaction with intestinal epithelial cells (IECs) and mucus. IECs secrete mucin, which is a complex glycoprotein mixture that is the principal component of mucous, thereby preventing the adhesion of pathogenic bacteria [47, 50]. Additionally, lipids, free proteins, immunoglobulins and salts are present in mucous gel [51]. This specific interaction has indicated a possible association between the surface proteins of probiotic bacteria and the competitive exclusion of pathogens from the mucus [52–54]. As mentioned above, several *Lactobacillus* proteins have been shown to promote mucous adhesion [54], and bacteria display surface adhesins that mediate attachment to the mucous layer [55]. This process is mainly mediated by proteins, although saccharide moieties and lipoteichoic acids have also been implicated [56]. The most studied example of mucus-targeting bacterial adhesins is MUB (mucus-binding protein) produced by *Lactobacillus reuteri* [55, 57]. The proteins playing a role in the mucous adhesion phenotype of lactobacilli are mainly secreted and surface-associated proteins, which are either anchored to the membrane through a lipid moiety or embedded in the cell wall [58–61]. The involvement of surface proteins in the interaction with human plasminogen or enterocytes has been reported in *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum*, respectively. Under certain circumstances, these proteins may play a role in facilitating the colonization of the human gut through degradation of the extracellular matrix of cells or by facilitating close contact with the epithelium [62–66]. MapA (mucous adhesion-promoting protein) has been reported to mediate the binding of *L. reuteri* and *L. fermentum* to mucus [52]. Probiotics, such as *L. plantarum*, have been reported to induce MUC2 and MUC3 mucins and to inhibit the adherence of enteropathogenic *E. coli*. These observations indicate that enhanced mucous layers and glycocalyx overlying the intestinal epithelium as well as the occupation of microbial binding sites by *Lactobacillus* spp. provide protection against invasion by pathogens [45, 67, 68]. Collado et al. [69] evaluated the adhesion of *Bifidobacterium longum* and *Bifidobacterium catenulatum* strains to human intestinal mucus and compared the results to those of control experiments that were run with the original acid-sensitive strains. They reported that in half of the 4 studied cases, the acid-resistant derivative shows a greater ability to adhere to human intestinal mucus than the original strain. The ability of bifidobacteria to inhibit pathogen adhesion to mucus is not generally improved by the acquisition of acid resistance. Overall, the induction of acid resistance in bifidobacteria may be a strategy for selecting strains with enhanced stability and improved surface properties that favor their potential functionality as probiotics against specific pathogens.

The mixture of probiotics and VSL3 has been reported to increase the synthesis of cell surface mucins and to modulate mucin gene expression in a manner dependent on the adhesion of bacterial cells to the intestinal epithelium [40].

Probiotics also cause qualitative alterations in intestinal mucins that prevent pathogen binding [68]. The bacterial component involved in the adhesion of the LB and BG2FO4 *L. acidophilus* strains is protease resistant and is associated with the bacterial surface [70–72]. Interestingly, the bacterial component is also degraded into an antimicrobial peptide, which lends anti-pathogenic properties to the host and provides an example of how large surface proteins may exhibit evolutionarily beneficial pleiotropic effects [73].

Probiotic strains can also induce the release of defensins from epithelial cells. These small peptides/proteins are active against bacteria, fungi and viruses. Moreover, these small peptides/proteins stabilize the gut barrier function [74]. Observations have indicated that in response to attack by pathogenic bacteria, the host engages its first line of chemical defense by increasing the production of antimicrobial proteins (AMPs), such as α- and β-defensins, cathelicidins, C-type lectins and ribonucleases [75–80]. Many AMPs are enzymes that kill bacteria by carrying out an enzymatic attack on cell wall structures and/or non-enzymatic disruption of the bacterial membrane. Enzymes expressed by Paneth cells attack the bacterial membranes. Lysozyme hydrolyzes the glycosidic linkage of wall peptidoglycan [81] and phospholipase A_2 bacterial membrane phospholipids [82]. Defensins comprise a major family of membrane-disrupting peptides in vertebrates. The interaction is non-specific and mainly by binding to anionic phospholipid groups of the membrane surface through electrostatic interactions. This interaction creates defensin pores in the bacterial mem-

brane that disrupt membrane integrity and promote lysis of microorganisms [83]. Cathelicidins are usually cationic, α-helical peptides that bind to bacterial membranes through electrostatic interactions and, like the defensins, induce membrane disruption [84].

The microbial adhesion process of LAB also includes passive forces, electrostatic interactions, hydrophobic interactions, steric forces, lipoteichoic acids and specific structures, such as external appendages covered by lectins. A wide variety of molecules mediating the adhesion of pathogenic bacteria has been characterized. However, the understanding of the factors that mediate adhesion for *Lactobacillus* is extremely limited [85–87]. Further studies are needed for the identification and analysis of the functional significance of various components of mucous layers as well as the complex interactions of mucous layers, microbiota (including probiotics) and epithelial cells with underlying innate and adaptive immune systems [68].

Competitive Exclusion of Pathogenic Microorganisms

In a report addressing the total exclusion of *Salmonella typhimurium* from maggots of blowflies published in 1969, Greenberg [88] first used the 'competitive exclusion' term for the scenario in which one species of bacteria more vigorously competes for receptor sites in the intestinal tract than another species. The mechanisms used by one species of bacteria to exclude or reduce the growth of another species are varied, including the following mechanisms: creation of a hostile microecology, elimination of available bacterial receptor sites, production and secretion of antimicrobial substances and selective metabolites, and competitive depletion of essential nutrients [89].

Specific adhesiveness properties due to the interaction between surface proteins and mucins may inhibit the colonization of pathogenic bacteria and are a result of antagonistic activity by some strains of probiotics against adhesion of gastrointestinal pathogens [90]. Lactobacilli and bifidobacteria have been shown to inhibit a broad range of pathogens, including *E. coli*, *Salmonella*, *Helicobacter pylori, Listeria monocytogenes* and *Rotavirus* [91–97]. Exclusion is the result of different mechanisms and properties of probiotics to inhibit pathogen adhesion, including the production of substances and the stimulation of IECs. Competitive exclusion by intestinal bacteria is based on a bacterium-to-bacterium interaction mediated by competition for available nutrients and for mucosal adhesion sites. To gain a competitive advantage, bacteria can also modify their environment to make it less suitable for their competitors. The production of antimicrobial substances, such as lactic and acetic acid, is one example of this type of environmental modification [98]. Some lactobacilli and bifidobacteria share carbohydrate-binding specificities with some enteropathogens [99, 100], which makes it possible for the strains to compete with specific pathogens for the receptor sites on host cells [101]. In general, probiotic strains are able to inhibit the attachment of pathogenic bacteria by means of steric hindrance at enterocyte pathogen receptors [102].

The effect of probiotic bacteria on the competitive exclusion of pathogens has been demonstrated using human mucosal material in vitro [45, 103] as well as chicken [104] and pig mucosal material in vivo [105]. Hirano et al. [45] showed that *L. rhamnosus,* a strongly adhering strain, is capable of inhibiting the internalization of EHEC (enterohemorrhagic *E. coli*) in a human intestinal cell line.

Production of Antimicrobial Substances

One of the proposed mechanisms involved in the health benefits afforded by probiotics includes the formation of LMW compounds (<1,000 Da), such as organic acids, and the production of antibacterial substances termed bacteriocins (>1,000 Da).

Organic acids, in particular acetic acid and lactic acid, have a strong inhibitory effect against Gram-negative bacteria, and they have been considered the main antimicrobial compounds responsible for the inhibitory activity of probiotics against pathogens [106–108]. The undissociated form of the organic acid enters the bacterial cell and dissociates inside its cytoplasm. The eventual lowering of the intracellular pH or the intracellular accumulation of the ionized form of the organic acid can lead to the death of the pathogen [109, 110].

Many LAB produce antibacterial peptides, including bacteriocins and small AMPs. Bacteriocins produced by Gram-positive bacteria (usually LAB, including lactacin B from *L. acidophilus*, plantaricin from *L. plantarum* and nisin from *Lactococcus lactis*) have a narrow activity spectrum and act only against closely related bacteria, but some bacteriocins are also active against food-borne pathogens [111]. The common mechanisms of bacteriocin-mediated killing include the destruction of target cells by pore formation and/or inhibition of cell wall synthesis [112]. For example, nisin forms a complex with the ultimate cell wall precursor, lipid II, thereby inhibiting cell wall biosynthesis of mainly spore-forming bacilli. Subsequently, the complex aggregates and incorporates peptides to form a pore in the bacterial membrane [113]. Several studies have revealed that bacteriocin production

confers producing strains with a competitive advantage within complex microbial environments as a consequence of their associated antimicrobial activity. Bacteriocin production may enable the establishment and increase the prevalence of producing strains as well as enable the direct inhibition of pathogen growth within the gastrointestinal tract [114].

Some specific antibacterial compounds have been described for several *Bifidobacterium* strains, and a unique bacteriocin, bifidocin B, which is produced by *B. bifidum* NCFB 1454 and is active towards Gram-positive bacteria, has been described as well [108, 115]. Liévin et al. [116] described a strong killing activity of two *Bifidobacterium* strains against several pathogenic bacteria, including *Salmonella enterica* ser. *typhimurium* SL1344 and *E. coli* C1845. This activity has been attributed to the production of a potential LMW lipophilic molecule [117]. In addition, an LMW protein termed BIF, which is produced by *B. longum* BL1928, is the only compound characterized thus far that is active against Gram-negative bacteria [100, 118, 119]. This protein has no direct inhibitory or killing effect, but it inhibits the binding of *E. coli* to human epithelial cell lines.

Intestinal bacteria also produce a diverse array of health-promoting fatty acids. Indeed, certain strains of intestinal bifidobacteria and lactobacilli have been shown to produce conjugated linoleic acid (CLA), a potent anticarcinogenic agent [114, 120]. An anti-obesity effect of CLA-producing *L. plantarum* has been observed in diet-induced obesity in mice [121]. Recently, the ability to modulate the fatty acid composition of the liver and adipose tissue of the host upon oral administration of CLA-producing bifidobacteria and lactobacilli has been demonstrated in a murine model [114].

Finally, probiotic bacteria are able to produce so-called de-conjugated bile acids, which are derivatives of bile salts. De-conjugated bile acids show a stronger antimicrobial activity compared to that of the bile salts synthesized by the host organism. It remains to be elucidated how probiotics protect themselves from their own bactericidal metabolites or if they are resistant to de-conjugated bile acids at all [122].

It is well known that some strains of probiotics produce metabolites that inhibit the growth of fungi and other species of bacteria [123, 124]. Some researchers have reported that *Lactobacillus* can produce antifungal substances, such as benzoic acid, methylhydantoin, mevalonolactone [125, 126] and short-chain fatty acids [127]. Magnusson and Schnürer [128] discovered that *Lactobacillus coryniformis* can produce proteinaceous compounds exhibiting antifungal properties, and Rouse et al. [129] characterized the antifungal peptides produced by LAB. These reports showed that the antifungal culture has the ability to prevent the growth of molds found in apple spoilage. Dal Bello et al. [130] reported the identification and chemical characterization of four antifungal substances produced by *L. plantarum* FST 1.7, including lactic acid, phenyllactic acid and two cyclic dipeptides [cyclo(L-Leu-L-Pro) and cyclo(L-Phe-L-Pro)]. A study described the antifungal culture as having the ability to retard growth of *Fusarium culmorum* and *Fusarium graminearum* found on breads. Another such study has reported the production of the antifungal cyclic dipeptides, cyclo (L-Phe-L-Pro) and cyclo(L-Phe-traps-4-OH-L-Pro), by LAB, which inhibit the growth of food- and feed-borne filamentous fungi and yeasts in a dual-culture agar plate assay [131].

Probiotics and the Immune System

It is well known that probiotic bacteria can exert an immunomodulatory effect. These bacteria have the ability to interact with epithelial and dendritic cells (DCs) and with monocytes/macrophages and lymphocytes. The immune system can be divided between the innate and adaptive systems. The adaptive immune response depends on B and T lymphocytes, which are specific for particular antigens. In contrast, the innate immune system responds to common structures called pathogen-associated molecular patterns (PAMPs) shared by the vast majority of pathogens [132]. The primary response to pathogens is triggered by pattern recognition receptors (PPRs), which bind PAMPs. The best-studied PPRs are toll-like receptors (TLRs). In addition, extracellular C-type lectin receptors (CLRs) and intracellular nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLRs) are known to transmit signals upon interaction with bacteria [133].

It is well established that the host cells that interact most extensively with probiotics are IECs. In addition, probiotics can encounter DCs, which have an important role in innate and adaptive immunity. Both IECs and DCs can interact with and respond to gut microorganisms through their PPRs [132, 133]. Figure 2 shows a summary of how probiotics may interact and modulate the immune system

TLRs and Probiotics

TLRs are transmembrane proteins expressed on various immune and non-immune cells, such as B cells, natural killer cells, DCs, macrophages, fibroblasts, epithelial

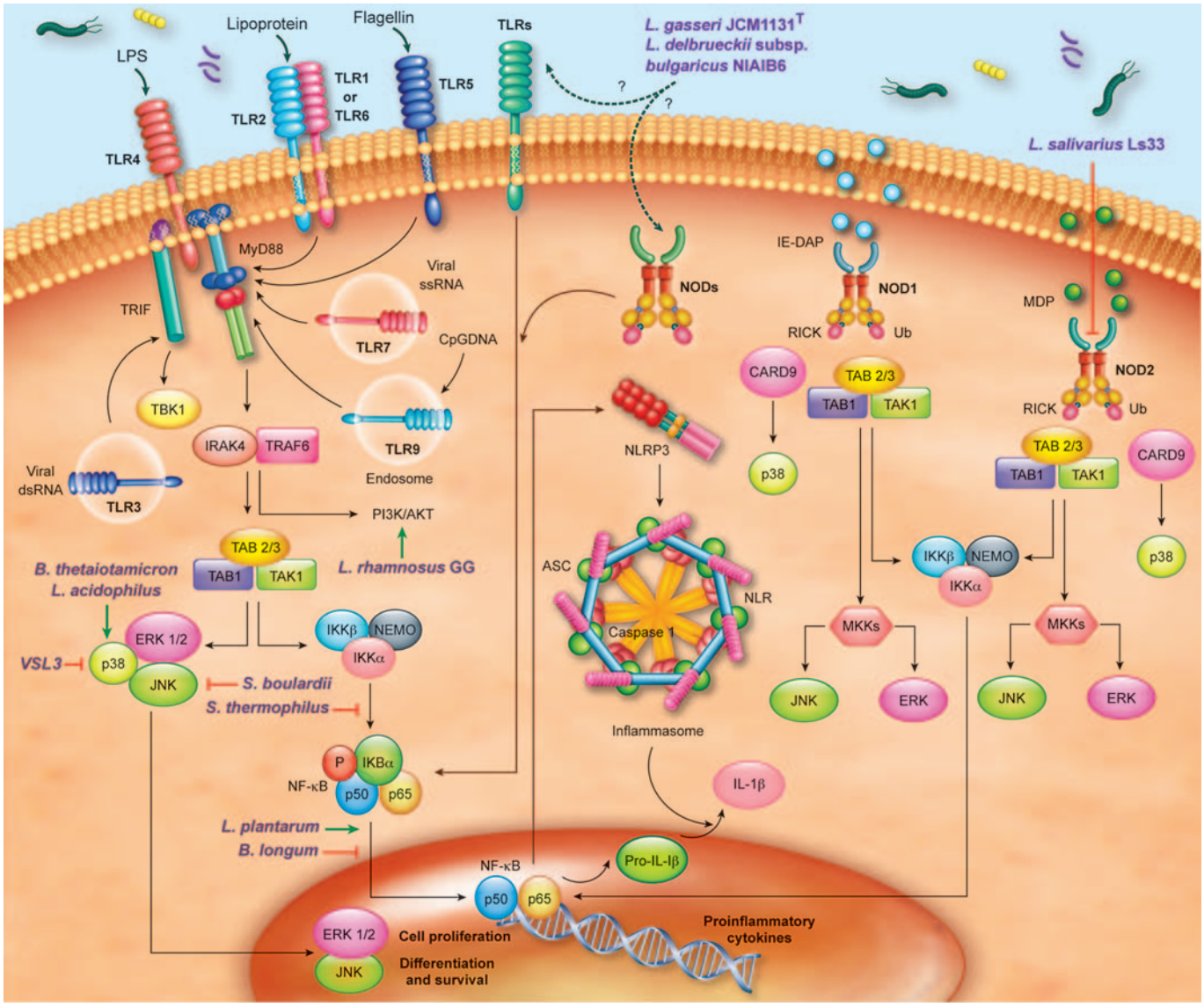

Fig. 2. Interaction of probiotics with the gut-associated immune system. ASC = Apoptosis-associated speck-like protein containing a CARD; *B. thetaiotamicron* = *Bacteroides thetaiotamicron*; CARD9 = caspase recruitment domain-containing protein 9; ERK = extracellular regulated kinase; IE-DAP = D-gamma-glutamyl-meso-DAP; IKK = IκB kinase; IRAK4 = IL-1 receptor-associated kinase 4; JNK = Jun N-terminal kinase; MDP = muramyl dipeptide; MKK = mitogen-activated kinase kinase; NEMO = NF-κB essential modulator; TAB1/2/3 = TAK binding proteins; TAK1 = ubiquitin-dependent kinase of MKK and IKK; TBK1 = serine/threonine-protein kinase 1; TRAF6 = TNF receptor-associated factor 6; Ub = ubiquitin.

cells and endothelial cells. In mammals, the TLR family includes eleven proteins (TLR1–TLR11). However, there is a stop codon in the human TLR11 gene that results in a lack of production of human TLR11. Activation of TLRs occurs after binding of the ligand to extracellular leucine-rich repeats. In humans, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are outer membrane associated and primarily respond to bacterial surface-associated PAMPs. TLR3, TLR7, TLR8 and TLR9 are found on the surface of endosomes where they respond primarily to nucleic acid-based PAMPs from viruses and bacteria [132]. Dimerization of TLRs and the highly conserved toll-interleukin-1 (IL-1) receptor (TIR) domains leads to the recruitment of adaptor molecules, such as myeloid differentiation primary response protein (MyD88), TIR domain-containing adaptor protein and TIR domain-containing adapter-

inducing interferon (IFN)-β (TRIF), to initiate signaling activation. The TLR signaling pathway, except for TLR3, involves the recruitment of MyD88, which activates the MAPK and nuclear factor (NF)-κB signaling pathways [133–135]. TLR3 utilizes the adaptor protein TRIF, leading to the expression of type 1 IFNs [135]. Furthermore, TLR-mediated signaling has been shown to control DC maturation inducing the upregulation of various maturation markers, such as CD80, CD83 and CD86, as well as the CCR7 chemokine receptor. Moreover, commensal and probiotic microorganisms can create an overall tolerant state mediated by the action of TLRs on DCs. It is clear that TLR9 signaling is essential to mediate the anti-inflammatory effect of probiotics. However, different studies have implicated other TLRs, such as TLR3 and TLR7, in the tolerance induced by commensal and probiotic bacteria. After activation by commensal and probiotic microorganisms, DCs initiate an appropriate response, such as the differentiation of Th_0 to T_{reg}, which has an inhibitory effect on Th_1, Th_2 and Th_{17} inflammatory responses.

It is well established that probiotics can suppress intestinal inflammation via the downregulation of TLR expression, secretion of metabolites that may inhibit TNF-α from entering blood mononuclear cells and inhibition of NF-κB signaling in enterocytes [132].

In this regard, cell wall components of lactobacilli can potentially signal through binding TLR2 in combination with TLR6. The diacylated membrane anchors of lipoproteins and lipoteichoic acids bind to TLR2 and TLR6, thereby promoting dimerization and MyD88-mediated activation of the canonical pathway of NF-κB [135]. Stimulation of TLR2 increases the production of cytokines, and TLR2 activation has an important role in enhancing transepithelial resistance to invading bacteria [136].

TLR2 recognizes peptidoglycan, which is the main component of Gram-positive bacteria, including the *Lactobacillus* genus. Several studies have demonstrated that TLR2 is required for some *Lactobacillus* strains to exert their immunomodulatory effects. Vinderola et al. [137] demonstrated that *L. casei* CRL 431 interacts with epithelial cells through TLR2 and that the interaction between *L. casei* and gut-associated immune cells induces an increase in the number of CD-206 and TLR2 receptors, mainly in the cells involved in the innate immune response.

In addition, Shida et al. [138] showed that *L. casei* induces a high level of IL-12 production in both wild-type and TLR2-deficient macrophages, and that peptidoglycan induces low levels of IL-12 production in wild-type macrophages and even lower levels in TLR2-deficient macrophages. They also suggested that the intact peptidoglycan of lactobacilli actually signals via TLR2 to inhibit IL-12 production. Although the recognition by TLR2 is essential, 12–48% of IL-12 production in TLR2-deficient macrophages is inhibited by peptidoglycan, thus suggesting that other TLR2-independent mechanisms may also be involved. Furthermore, it has been demonstrated that *Lactobacillus* strains, such as *L. rhamnosus* GG (LGG) and *L. plantarum* BFE 1685, enhance TLR2 in vitro in experiments using human intestinal cells, and more recently, *L. casei* CRL 431 has been shown to exert a similar effect on healthy mice and mice infected with *S. enterica* serovar *typhimurium* [139, 140]. For instance, probiotic administration to healthy mice increases expression of TLR2, TLR4 and TLR9, and it improves the secretion of TNF-α, IFN-γ and IL-10 in Peyer's patches [140].

Similarly, when porcine IECs encounter *Lactobacillus jensenii* TL2937, TLR2 may act synergistically and cooperatively with one or more PRRs, which may result in a coordinated sum of signals that induce the upregulation of several negative regulators of TLRs, including A20, Bcl-3 and MKP-1 [141].

TLR2 also has an important role in the recognition of bifidobacteria. Hoarau et al. [142] reported that a fermentation product from *Bifidobacterium breve* C50 can induce maturation, high IL-10 production and prolonged survival of DCs via the TLR2 pathway.

Similarly, Zeuthen et al. [143] showed that TLR2–/– DCs produce more IL-2 and less IL-10 in response to bifidobacteria, and they concluded that the immuno-inhibitory effect of bifidobacteria is dependent on TLR2.

Recently, Kailova et al. [144] reported that oral administration of *B. bifidum* OLB 6378 to rats with necrotizing enterocolitis (NEC) stimulates TLR2 expression in the ileal epithelium, enhances epithelial expression of COX-2 and increases intestinal production of prostaglandin E_2. Indeed, pretreatment of IEC-6 cells with the probiotic strain stimulates TLR2 and COX-2 expression and blocks cytokine-induced apoptosis. However, there is no evidence of a clear link between TLR2 activation and the upregulation of COX-2.

In contrast, it has been shown that the *L. reuteri* strains DSM 17938 and ATCC PTA 4659 have a beneficial effect on preventing NEC in rats. In response to the probiotic, mRNA expression of IL-6, and expression levels of TNF-α, TLR4 and NF-κB are significantly downregulated, and mRNA levels of IL-10 are significantly upregulated. Moreover, *L. reuteri* treatment leads to de-

creases in intestinal protein levels of TLR4, IL-1β and TNF-α in newborn rats with NEC. Furthermore, *L. reuteri* significantly increases survival rate, reduces both the incidence and severity of NEC and decreases pro-inflammatory cytokine levels in parallel with inhibition of TLR4 signaling via the NF-κB pathway.

Moreover, TLR4 has a significant role in the host defense against *Salmonella* infection in vivo. In healthy mice, *L. casei* CRL 431 activates this receptor and can be used as a surveillance mechanism against pathogenic bacteria [140]. Activation of TLR4 leads to the induction of pro-inflammatory mediators, an increase in TLR2 expression, and a reduction in its own expression, which leads to the recruitment of inflammatory cells and the initiation of the appropriate responses in the spleen. Collectively, these events allow for the control of bacterial replication [140, 146, 147].

Similarly, heat-inactivated LGG and *Lactobacillus delbrueckii* subsp. *bulgaricus* can decrease TLR4 expression similar to lipopolysaccharide (LPS) after 12 h in human monocyte-derived DCs. Moreover, LGG downregulates p38 expression, and *L. delbrueckii* subsp. *bulgaricus* reduces inhibitor protein κB (IκB) expression. In addition, these probiotic strains can modify the immune response at the post-transcriptional level by modifying miRNA expression [148].

Another relevant TLR is TLR9, which recognizes bacterial CpG DNA and synthetic unmethylated CpG oligonucleotide mimics (CpG-ODN). Unmethylated DNA fragments containing CpG motifs that are released from probiotics in vivo have the potential to mediate anti-inflammatory effects through TLR9 signaling at the epithelial surface. It is known that *Lactobacillus* species differ in their C+G composition. Thus, the ability of different species to stimulate TLR9 is likely to be different [135, 149]. TLR9 activation through apical and basolateral surfaces activates different intracellular signaling pathways in polarized epithelial cells. Whereas basolateral TLR9 triggers IκBα degradation and NF-κB pathway activation, apical TLR9 induces cytoplasmic accumulation of ubiquitinated IκB and inhibition of NF-κB activation [150].

Using polarized HT29 and T84 cell monolayers, Ghadimi et al. [151] showed that binding of natural commensal-origin DNA to the apical TLR9 initiates an intracellular signaling cascade in a specific manner that is associated with the attenuation of TNF-α-induced NF-κB activation and NF-κB-mediated IL-8 expression. When LGG DNA was apically applied, they showed a detracted TNF-α-induced NF-κB activation by reduced IκBα degradation and p38 MAPK phosphorylation, thereby indicating that intracellular chemical signals may coordinately regulate multiple properties of TLR9 expression that are relevant in multicellular functional responses of TLR9 to bacterial DNA. They also showed that TLR9 silencing abolishes the inhibitory effect of natural commensal-origin DNA on TNF-α-induced IL-8 secretion.

Similarly, *B. breve* (NumRes 204), *L. rhamnosus* (NumRes 1) and *L. casei* (DN-114 001) strains induce different cytokine production levels by human and mouse primary immune cells. It has been demonstrated that the *B. breve* strain induces lower levels of the pro-inflammatory cytokine IFN-γ than *L. rhamnosus* and *L. casei*. Moreover, *B. breve* and lactobacilli induce cytokines in a TLR9-dependent manner, and the lower inflammatory profile of *B. breve* is due to inhibitory effects of TLR2 [152].

In addition, it has been shown that purified genomic DNA from *L. plantarum* (p-gDNA) does not substantially stimulate pro-inflammatory cytokines. However, p-gDNA inhibits LPS-induced TNF-α production by THP-1 cells. Furthermore, p-gDNA reduces the expression of TLR2, TLR4 and TLR9, which induces the activation of NF-κB through the LPS signaling pathway, leading to the upregulation of inflammatory cytokines [153, 154]. Pretreatment of p-gDNA inhibited the phosphorylation of MAPKs and NF-κB, and also inhibited LPS-induced TNF-α production in subsequent LPS stimulation. In this regard, *L. plantarum* genomic DNA-mediated inhibition of signaling and TNF-α was accompanied by the suppression of TLR2, TLR4 and TLR9, as well as the induction of IL-1 receptor-associated kinase M (a negative regulator of TLR) [154].

NLRs and Probiotics

As mentioned before, there is another family of membrane-bound receptors: NLRs. They are located in the cytoplasm and are important in tissues where TLRs are expressed at low levels. The most thoroughly characterized members are NOD1 and NOD2, but currently more than 20 different NLRs have been identified [155]. Unlike NOD1, which is ubiquitously expressed, the expression of NOD2 is restricted to DCs, macrophages, Paneth cells, intestinal cells, lung cells and oral epithelial cells, and it is expressed at low levels in T cells. NOD1 can sense peptidoglycan moieties containing meso-diaminopimelic acid, which are associated with Gram-negative bacteria, but NOD2 senses muramyl dipeptide motifs, which can be found in a wide range of bacteria [156]. Upon recogni-

tion of their agonist, both NOD1 and NOD2 self-oligomerize to recruit and activate the adaptor protein RICK, a protein kinase that regulates CD95-mediated apoptosis, which is essential for the activation of NF-κB and MAPKs, resulting in the upregulation of transcription and production of inflammatory mediators (e.g. cytokines, chemoattractants, COX-2 and inducible nitric oxide synthase) [157].

There are a few studies showing the effect of probiotics on NLR. However, Fernandez et al. [158] recently demonstrated that the protective capacity of *L. salivarius* Ls33 correlates with local IL-10 production, which is abolished in NOD2-deficient mice. Indeed, these authors showed that the anti-inflammatory effect of Ls33 is mediated via NOD2.

Another important pathway activated by NLRs involves apoptosis-associated speck-like protein with caspase recruitment to activated caspase 1, an adaptor protein which is necessary for the cleavage of pro-IL-1β and pro-IL-18 into their mature and biologically active forms. NLRs participate in the formation of inflammasomes, which leads to the activation of caspase-1. There are three principal inflammasomes named after the NLR involved as follows: NOD-like receptor family, pyrin domain containing protein (NLRP) 1, NLRP3 and NLRC4. NLRP3 detects LPS, muramyl dipeptide, bacterial RNA and viral RNA [157].

The following two steps are required for the complete activation of the NLRP3 inflammasome: a priming step to induce transcription of NLRP3 mRNA and a sequential step to recognize various PAMPs and danger-associated molecular patterns by fully expressed NLRP3 itself [159, 160]. With regard to probiotic mechanisms associated with NLRP3, Tohno et al. [161] found that *L. delbrueckii* subsp. *bulgaricus* NIAI B6 and *L. gasseri* JCM1131^T are able to enhance NLRP3 expression in the GALT of adult and newborn swine. Their results suggested that immunobiotic *Lactobacillus* strains directly promote NLRP3 expression via TLR and NOD-mediated signaling, resulting in the induction of appropriate NLRP3 activation in porcine GALT. Furthermore, their results indicated that NLRP3 expression is upregulated by TLR2, TLR9, NOD1 and NOD2 agonists in adult and newborn porcine GALT. It has been suggested that NLRP3 has an important role in the regulation of human intestinal inflammation, such as in Crohn's disease [162], and that dysregulated NLRP3 expression results in the disruption of immune homeostasis associated with autoinflammatory disease in humans [163]. Because the potential expression level of NLRP3 is low in immune cells, induction of cellular NLRP3 expression itself is a first step to evoke the appropriate activation of the NLRP3-mediating signaling pathway in order to respond to danger-associated molecular patterns and PAMP stimuli [159, 160, 164, 165].

Conclusions

Probiotics have considerable potential for preventive or therapeutic applications in various gastrointestinal disorders. However, it is important to note that many probiotic health claims have not yet been substantiated by experimental evidence. In addition, the efficacy demonstrated for one given bacterial strain cannot necessarily be transferred to other probiotic organisms. Moreover, the mechanisms underlying probiotic action have not yet been fully elucidated.

This study reviewed the mechanisms of action of probiotics. Several important mechanisms underlying the antagonistic effects of probiotics on various microorganisms include the following: modification of the gut microbiota, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune system to convey an advantage to the host. The recent characterization of the host families of pattern-recognition molecules, such as TLR and NOD-like receptors, as well as modulating key signaling pathways, such as NF-κB and MAPK, with respect to their ability to enhance or suppress activation and influence downstream pathways will shed light onto the complex interplay of host-microbe interactions. Stimulation of these receptors by commensal bacteria has a crucial role to elicit measured antimicrobial responses with minimal inflammatory tissue damage.

Future Perspectives

In the present review, we provided an overview of the mechanisms of action of probiotics. It must be noted that many reported mechanisms of probiotic action are the results of in vitro experiments. Considerable effort has been invested in the development of methods enabling the in-depth analysis of the molecular mechanisms of probiotics. The complex and dynamic interactions that exist between the intestinal epithelium and bacteria on the luminal side as well as between the epithelium and the underlying immune system on the basolateral side must be reconciled in co-culture experiments with probiotics,

DCs and IECs as well as in 3D models. Other models include tissue explants, bioreactors and organoids. In vitro models have improved our current knowledge regarding specific probiotic modes of action. However, a number of limitations have to be taken into account. For example, results obtained with different IECs have to be carefully interpreted because not all cell lines share the same characteristics. It should also be noted that culture conditions may influence the expression of certain molecular characteristics.

The molecular elucidation of probiotic action in vivo will help to identify true probiotics and to select the most suitable ones for the prevention and/or treatment of particular diseases. It is important to note that results obtained in animal models cannot be directly transferred to humans. The physiology of animals differs considerably from that of humans, but this disadvantage is outweighed by the possibility of using animals with virtually identical genetic backgrounds, such as human microbiota-associated animals.

The quest for a better understanding of how probiotics operate has catalyzed an enormous interest in the molecular processes underlying host-microbe interactions. Gaining insight into the mechanisms of probiotic action may not only help to improve the credibility of the probiotic concept but also to foster the development of novel strategies for the treatment or prevention of gastrointestinal and autoimmune diseases.

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