Structural Bacterial Molecules as Potential Candidates for an Evolution of the Classical Concept of Probiotics[1,2]

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ABSTRACT

A large number of experimental and clinical studies published in recent years have demonstrated the beneficial role of probiotic bacteria in the health of the host. However, because the different receptors of the innate immune system can recognize only specific bacterial molecular patterns, knowledge of the role played by individual probiotic molecular patterns is essential to move from the current confused era of live probiotic bacteria to the era of the pharmacobiotic strategies. This article reviews the current knowledge on the probiotic activities of bacterial structural molecules (nucleic acids and surface molecules), which represent the fundamental basis to set up experimental and clinical studies in this emerging field with very promising and potentially invaluable future prospects. *Adv. Nutr. 2: 372–376, 2011.*

Introduction

Probiotic bacteria have shown therapeutic effects in infectious, inflammatory, and allergic disorders. Although probiotics are commonly defined as live microorganisms preferentially of human origin that upon ingestion in specific and sufficient numbers exert health benefits, the signaling pathways engaged by these bacteria are poorly understood and the molecular details underpinning these pathways remain largely unknown. Understanding how probiotics exert their beneficial effects is critical for the establishment of definite selection criteria for pharmacobiotic strategies in specific clinical conditions.

In recent years, there have been tremendous advances in our understanding of the structure and function of signal receptors, and the pivotal role of PRR[7] and cells of innate immunity in processing bacterial and food components is now well established (1–4). PRR include trans-membrane TLR and Dectin-I, endosomal PRR (TLR 3, 7/8, and 9), and cytosolic nucleotide oligomerization domain-like receptor: (NOD1 and NOD2), RLH (retinoic acid-inducible gene-1) and iron-regulated surface determinant sensors. The involved cells are DC, intraepithelial lymphocyte, macrophages, neutrophils, and enterocytes. MAMP are first recognized by a PRR, and activation of the receptor by binding of the MAMP sequentially activates intracellular molecules such as the cytoplasmic adapter molecule MyD88, leading to the activation of transcription factors, including NF-κB and activator protein-1 (AP-1), which are required for gene transcription and cytokine synthesis. The different receptors of the innate immune system are obviously only able to process specific molecular components of microorganisms and foods, whereas the recognition of a whole bacterium or food does not appear possible, although simultaneous activation of several PRR may be characteristic of a specific organism or food and lead to a different outcome than activation by single PRR. For example, studies on host mucosal gene expression following exposure to different whole bacteria have demonstrated upregulation of different gene networks for each organism. Networks stimulated by these probiotic bacteria included cell proliferation, Th1/Th2 balance, control of blood pressure, tissue development, water and ion regulation, and wound healing. Major host differences were noted in the stimulated transcriptome. The pathways stimulated by the whole organism corresponded

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[7] Abbreviations used: CD4, cluster of differentiation-4; DC, dendritic cell; IBD, inflammatory bowel disease; LTA, lipoteichoic acid; MAMP, microorganism-associated molecular pattern; NOD, nucleotide oligomerization domain; PRR, pattern recognition receptor; PSA, polysaccharide A; SFB, segmented filamentous bacteria; TLR, Toll-like receptor.

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to pathways stimulated by known pharmacological preparations. However, the specific molecules of the bacteria that caused these effects are currently unknown (5). Further, whether the bacterium is alive or dead does not seem relevant for the recognition of a molecular pattern by specific PRR. The accessibility of MAMP for PRR and the presence of other microbial effector molecules, such as toxins produced by pathogens, have a pivotal role in the modulation of host immune response. Other important factors determining the host response are host-derived direct or indirect negative regulators of PRR signaling.

To date pathogenic, probiotic and commensal bacteria are considered to induce different levels of immune response: a strong host response stimulated by pathogens, an intermediate response induced by probiotics, and finally a homeostatic control of the response is triggered by commensal bacteria. An important exception to this concerns a restricted number of commensal bacteria, the prototype of which is the SFB, which could largely recapitulate and orchestrate a broad spectrum of B and T cell responses (6,7). SFB-colonized mice had low levels of ATP, suggesting that host sensing of SFB does not involve TLR or NOD receptors (8). We recently showed that the progressive penetration of the holdfast segments of these bacteria within the specialized epithelial cells of the terminal ileum could permit an impressive presentation of bacterial antigens directly to the lymphocytes contained in the lymphoid packets characteristic of the M cells and to antigen-presenting cells (9).

It should also be remembered that interactions between PRR and ligands are not as specific as those between antigens and antibodies, and ligands for PRR such as TLR are generally present in repetitive structures to increase avidity. Therefore, some very important and specific questions concerning immune-mediated probiotic activity are as follows:

- Are whole live bacteria essential to promote biological effects on the immune system?
- Can the concept of probiotics be extended to include bacterial-derived molecular bioactive components?
- Moreover, can probiotic molecules be also produced by nonprobiotic bacteria?

Based on the outcomes of clinical trials, on the one hand there is firm evidence concerning the utility of probiotic therapy for clinical conditions, such as acute gastroenteritis and antibiotic-associated diarrhea (10,11), in which mechanisms of colonization and competition by live bacteria surely play a pivotal role (12,13). However, on the other hand, in clinical situations that present a more complex physiopathology, such as IBD, irritable bowel syndrome, and allergic conditions, the outcomes of studies carried out so far do not permit any final conclusion on the usefulness of probiotics. On the basis of the finding that cell wall components and DNA sequences can modulate immune responses (13,14), in these more complex disease states, a pharmacobiotic approach using specific MAMP may have a better theoretical potential to improve the therapeutic success in the future.

On this basis, we decided to review the present knowledge about molecular bacterial components presenting a potential role as probiotic MAMP suitable for a pharmacobiotic approach during experimental and clinical studies confining our evaluation to structural molecules. Any other non structural bacterial-derived molecular products have not been taken into consideration for the aim of this article.

Search strategy

To our knowledge, this is the first article to review the few published experimental paper on the probiotic activities of structural bacterial molecules.

Primary published papers used for compiling this review were searched with a Pub Med search strategy for the terms "probiotic molecules," "probiotic DNA," and "probiotic surface molecules."

At the moment, there is only one other review article, also published this year in March (15), which takes into account only the bacterial surface molecules but fails to consider the role of probiotic bacterial DNA.

Bacterial DNA

Bacterial genomic DNA of probiotics in VSL-3 induced a remarkable strain-specific immune response in humans as evaluated by the release of IL-1β, IL-6, and IL-10. Total bacterial DNA from feces increased the Th-1 cytokine IL-1β more than IL-10 compared to DNA from the probiotic bacteria, which had the reverse effect. However, total DNA from feces after being given a course of the probiotic bacteria had a greater stimulation of IL-10 compared to IL-1β (16). Notably, the respective roles of IL-1β and IL-6 in the beginning and maintenance of a Th17 response is well known (17,18). An important and provocative study (19) showed that in a mouse IBD model, the protective effects of probiotics contained in VSL-3 are mediated by their DNA rather than by their ability to colonize the gut mucosa. TLR 9 signaling is essential in mediating the antiinflammatory effects of probiotics. TLR-9 is an endosomal TLR that is known to interact with bacterial DNA upon bacterial lysis. The authors suggested that DNA-TLR 9 signaling resulted in the differentiation of naive CD4 T lymphocytes into regulatory T cells, mediating the protective action. Another example of the immunomodulatory capacity of probiotic DNA is represented by DNA of *Lactobacillus rhamnosus GG* that induces B cell proliferation and activates DC (20). More recently, the effects on the Th1/Th2 balance by genomic DNA of different probiotic bacteria (*L. rhamnosus GG*, *Lactobacillus gasseri*, *Bifidobacterium bifidum*, and *Bifidobacterium longum*) were compared with the effects of live bacteria by using peripheral blood mononuclear cells from healthy individuals and from patients with allergy against house dust mite (21). Compared with live *Lactobacilli*, bacterial DNA inhibited IL-4 and IL-5 secretion in a similar way, and based on the maximal effects achieved with *Lactobacilli* and their DNA, >50% of these effects seem to be due to their DNA.

The immunomodulatory activity of DNA is characterized by unmethylated CpG motifs, which can activate innate

immune responses through binding to TLR9 and triggers the translocation of NF-κB and AP-1 from the cytoplasm to the nucleus, thereby upregulating gene expression pathways. Stimulatory oligodeoxynucleosides contain the CpG within a flanking region to give a motif of Pur-p-Pur-p-CpG-p-Pyr-p-Pyr . Typically, more than one CpG is present in the immunostimulatory oligodeoxynucleoside and maximal effect occurs if they are separated by 1–2 bp. A 5′ TpC- and pyrimidine-rich 3′ end also increases the immunostimulatory effects. In terms of a potential therapeutic, the in vivo degradation can be decreased by synthesizing a phosporthiorate backbone that increases the stimulatory activity of the motif (22). A very recent study based on entire genome sequences from 5 bifidobacterial strains (23) showed that *Bifidobacterium* genomes contained several CpG motifs and biologically active sequences previously identified in *Lactobacilli*. These bioactive sequences induced the production of monocyte chemotactic protein-1 (MCP-1) and TNFα through a pattern of TLR-9 stimulation of macrophages. An inter- and intraspecies investigation of 71 strains of *Bifidobacteria* of various origins showed that these bioactive DNA sequences were highly conserved in the genus. The results of these studies clearly suggest the necessity of further investigation.

Molecules present at the bacterial surface

Bacterial cell wall molecules are potential probiotic ligands that can interact with PRR and induce signaling pathways resulting in probiotic effects.

The immune system is capable of recognizing virtually any biological polymer found in the bacterial cell wall and presenting it to T cells. Most probiotics are typically Gram-positive bacteria, in which the cell wall is composed of a thick peptidoglycan layer with proteins, teichoic acids, and polysaccharides (24). However, a few Gram-negative probiotics exist, such as *Escherichia coli* strain Nissle 1917; in this case, the cell wall is composed of a thin peptidoglycan layer and an outer membrane that contains LPS, which is further decorated with proteins and polysaccharides (25).

Although adaptive immune responses have been considered for some time to be caused by antigenic proteins or glycoproteins whereas carbohydrates were considered not to be recognized by the adaptive immune system, recent studies have questioned this assumption (26,27). Bacterial wall polysaccharides and glycolipids are now considered to be perhaps the more likely targets in the search for immunomodulatory molecules. Interestingly, bacterial capsular PSA, the most immunodominant among the zwitterionic polysaccharides elaborated by *Bacteroides fragilis*, a commensal Gram-negative anaerobe that colonizes the mammalian lower gastrointestinal tract, has been demonstrated to be the archetypal bacterial molecule capable of mediating the development of the host immune system (28). PSA, presented by intestinal DC, activates CD4+ T cells and elicits appropriate cytokine production. *Bacteroides* are among the earliest gastrointestinal colonizers and the most abundant microorganisms of the gut microbiota (29) but are not considered probiotic species. More recently, Mazmanian et al. (30) showed that the *B. fragilis*-produced PSA protects mice from experimental colitis induced by *Helicobacter hepaticus*; purified polysaccharides are required to suppress proinflammatory IL-17 production by intestinal T cells, and it also protects from inflammatory diseases by induction of IL-10–producing CD4+ T cells. Therefore, although bacteria may have developed polysaccharide capsules to avoid recognition by the immune system, it may be that the host immune system not only tolerated but also coevolved with commensal bacteria. Strikingly, PSA from the nonprobiotic *B. fragilis* is a natural antiinflammatory molecule that promotes health, and so clearly performs important probiotic activities. This provides a fundamental platform for the discovery of new biomolecules having important probiotic effects independently of their bacterial derivation.

Polysaccharides synthesized extracellularly (EPSO) also represent attractive candidates as probiotic effector molecules interacting with PRR. Exopolysaccharides are produced by probiotic, symbiont, and also potentially pathogenic bacteria but have not been studied in detail yet.

On the other hand, LTA is considered the major immunostimulating component of the cell wall of Gram-positive bacteria via TLR 2 (most of the known probiotics, *Lactobacilli* and *Bifidobacteria*, are Gram-positive bacteria) in the same way that LPS is the major immunostimulating component in the cell wall of Gram-negative bacteria via TLR 4. Two important concepts concerning LTA have emerged in recent years: the first concerns the much lower potency to stimulate the TLR 2 pathway to induce proinflammatory molecules by using purified LTA from a probiotic strain of *Lactobacillus plantarum* compared with a pathogenic strain of *Staphylococcus aureus* (31); the second very important concept is related to the possible modification of LTA molecules to induce a substantial reduction in D-alanine content with a marked increase in glucose substitutions (32). These modified LTA may be candidates as probiotic effector molecules able to induce secretion of antiinflammatory IL-10.

Furthermore, LPS synthesized by Gram-negative bacteria of the gut microbiota have been recently involved in the development of inflammation, obesity, and type 2 diabetes induced by a high-fat diet (33). If confirmed, these findings open up a new possible role in this field not only for a direct bacterial competition by live probiotics but also for the research of nonimmunostimulating molecules competing with LPS for the TLR 4 pathway.

Lipoproteins and glycoproteins present at the cell surface are also attractive candidates as probiotic molecules because of their interactions with TLR 2 receptors. For instance, flagellins of the *E. coli* Nissle 1917 have been shown to induce the expression of human β-defensin 2, an inducible antimicrobial peptide (34). However, up to now, the role of lipoproteins and glycoproteins has been poorly investigated even in pathogenic bacteria (35). Thus, further studies in this field are desirable.

Discussion

This review article focuses only on structural bacterial molecules having a potential role as probiotic MAMP, whereas other nonstructural molecules, including biopeptides, polysaccharides, and other molecules naturally synthesized by bacteria (bacteriocins, bioactive molecules found in fermented substrates, supernatant, or biofilms), are not considered here. For example, 2 secreted proteins of the probiotic *L. rhamnosus* GG, designated p40 and p75, were recently found to promote the homeostasis of the intestinal epithelium and could be important in the prevention of IBD (36), but they are not considered in this article, because they are secreted and not structural molecules. Important bioactive peptides produced by epithelial cells of the host such as goblet cells-derived mucins, defensins produced by Paneth cells, and epithelial-derived bactericidal lectins are not taken into consideration in this review. All these aspects, being potential downstream effectors of probiotics rather than probiotics per se, deserve an attentive and separate evaluation and review.

Current data concerning potential molecular candidates for a pharmacobiotic approach among structural bacterial molecules seem to prove that the time has arrived for a substantial evolution in the concept of a probiotic agent. In fact, in the near future it will be possible to select, depending on the host response, structural bacterial molecules for probiotic therapy. For instance, one may anticipate that the choice of bacterial molecules will be different for patients with IBD than for patients with allergic conditions. Allergic diseases result from exaggerated Th2 immune response; by contrast, for IBD the Th1 and Th17 immune responses are predominant. Therefore, for the prevention of allergic diseases, probiotic agents need to be able to beneficially modulate T cell response into an increase in Th1 and CD4+ CD25+ regulatory T cell responses. By contrast, for IBD treatment, the molecules present at the probiotic bacterial surface that can counterbalance the proinflammatory factors might need to be selected, taking into account the disturbed epithelial barrier. Therefore, IBD patients show a polymorphism in PRR that results in modified signaling pathways, e.g., NOD2 in many Crohn's disease patients (37). The selection of optimal probiotic molecules for specific disease conditions requires further molecular studies dealing with both the cell surface molecules and DNA of bacteria and the interacting host cells and their receptors. As highlighted in this short review, an important concept is that host-microorganism interactions are not univocal but involve the complex interactions of various MAMP with different PRR; the final host response is determined by the coordinated action of the signals induced by the different receptors in multiple cell types. The identification and characterization of the bacterial molecules as ligands of these specific receptors of the host are mandatory for understanding the function of probiotics and the resident microbiota. If it is true that probiotics are increasingly popular as possible alternatives for antibiotics and/or antiinflammatory drugs, surely the philosophical concept about what has to be considered as a probiotic agent has to change. The medical community cannot continue to consider probiotics only as live *Lactobacilli* and *Bifidobacteria* having generic beneficial effects, as often demonstrated in a few clinical trials, without any definite pharmacological rationale or precise knowledge of their intimate mechanism of action. Variability is probably the keyword in the probiotic world: it surely represents the major confounding factor and includes the various definitions of the term probiotic, the high number of genera, the very high number of species and even larger number of strains used, the doses investigated, the different formulations (capsules, solutions, yogurts, etc.), the clinical trial methodology, and the end points and outcomes (38). The extreme variability regarding each separate factor makes it, in most cases, absolutely impossible to perform formal meta-analyses only allowing data collection and analysis. In only a few cases of pediatric interest has it been possible to carry out a formal meta-analysis (39,40).

A live whole microorganism carries out many functions by synthesizing different molecules; thus, it can be expected that these various molecules interacting with host sensors may produce both positive and negative effects.

On these bases, even pathogenic bacteria may produce MAMP that could be investigated for their probiotic effects. The trillions of bacteria in our gut microbiota (probiotics, symbionts, and potentially pathogens) have coevolved with us and it should not be surprising if our symbionts or even pathogenic bacteria produce native or adaptable molecules very useful for their probiotic action. Efficient probiotic *Lactobacillus* and *Bifidobacterium* strains, in fact, resemble pathogens in many aspects, such as in survival and adherence, and one can imagine that for efficient competition with pathogens, efficient probiotics must utilize similar molecular mechanisms. For example, it is interesting in this respect to look at the molecular mechanisms that some pathogens or symbionts use for immune evasion and downregulation of inflammatory responses.

A detailed molecular understanding should lead to a more rational use of probiotics as efficient therapy for specific disorders such as IBD, allergic disease, and gastroenteritis. Fundamental studies involving different disciplines and focusing on potential candidates as probiotic effector molecules may contribute to change and improve the actual concept of probiotic therapy and the laws regulating this important emerging field.

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