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Identification of probiotic effector molecules: present state and future perspectives

Sarah Lebeer[1], Peter A Bron[2], Maria L Marco[3], Jan-Peter Van Pijkeren[4], Mary O'Connell Motherway[5], Colin Hill[5], Bruno Pot[6,7], Stefan Roos[8] and Todd Klaenhammer[9]

Comprehension of underlying mechanisms of probiotic action will support rationale selection of probiotic strains and targeted clinical study design with a higher likelihood of success. This will consequently contribute to better substantiation of health claims. Here, we aim to provide a perspective from a microbiology point of view that such comprehensive understanding is not straightforward. We show examples of well-documented probiotic effector molecules in *Lactobacillus* and *Bifidobacterium* strains, including surface-located molecules such as specific pili, S-layer proteins, exopolysaccharides, muropeptides, as well as more widely produced metabolites such as tryptophan-related and histamine-related metabolites, CpG-rich DNA, and various enzymes such as lactase and bile salt hydrolases. We also present recent advances in genetic tool development, microbiome analyses and model systems, as well as perspectives on how the field could further progress. This opinion is based on a discussion group organized at the annual meeting of the International Scientific Association on Probiotics and Prebiotics (ISAPP) in June 2017.

Addresses
[1] University of Antwerp, Department of Bioscience Engineering, Groenenborgerlaan 171, 2020 Antwerp, Belgium
[2] NIZO Food Research, Ede, Netherlands
[3] Department of Food Science & Technology, University of California, Davis, USA
[4] Department of Food Science, University of Wisconsin-Madison, Madison, WI 53706, USA
[5] School of Microbiology and APC Microbiome Institute, National University of Ireland, Western Road, Cork, Ireland
[6] Yakult R&D, Europe, Almere, The Netherlands
[7] Vrije Universiteit Brussels, Belgium
[8] Swedish University of Agricultural Sciences & BioGaia AB, Sweden
[9] Department of Food, Bioprocessing & Nutrition Sciences, North Carolina State University, Raleigh, USA

Corresponding author: Lebeer, Sarah (sarah.lebeer@uantwerpen.be)

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Introduction

Recently, the International Scientific Association on Probiotics and Prebiotics (ISAPP) reinforced the FAO/WHO definition of probiotics, with minor changes: 'live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host' [1]. Documentation of health benefits is essential, but not a trivial task, because the monitoring of targeted health benefits of the applied probiotics is difficult to establish. Moreover, a plethora of modes of action has been postulated behind these health benefits from a host perspective (Box 1). Furthermore, because of the limited knowledge of the underlying mechanisms by which probiotics elicit their effects, reproducibility and rational strain selection is challenging. Here, we aim to provide a microbiological perspective that comprehensive understanding of probiotic mechanisms is not yet in our grasp, because the path there requires rigorous and laborious scientific investigation. We can however show examples of well-documented probiotic molecules of action — also termed probiotic effector molecules — in *Lactobacillus* and *Bifidobacterium* strains. We also highlight recent advances in the genetic tool development, microbiome analyses and model systems to unravel the molecular mechanisms that drive probiotic effects. These examples are also relevant for the increasing exploration of next-generation probiotics based on the recent advances in gut microbiome research [2].

Selected examples show that impactful probiotic effector molecules have been identified

Probiotic bacteria exert a variety of beneficial effects, such as alteration of the microbiota composition, regulation of the epithelial barrier function, modulation of immune responses or interaction with the gut-brain barrier (Box 1). *Lactobacillus rhamnosus* GG is one of the best clinically documented and most commercialized probiotic micro-organisms, with documented health benefits ranging from gastro-intestinal health [3] towards immune modulatory effects such as prevention of upper respiratory tract diseases [4] and atopic eczema in children [5]. The knowledge on its mode of action has long been lagging behind because genome editing technologies were not readily available in this organism [6]. We now know that transformation of this bacterium is difficult at least partially due to the presence of long pili structures at

Box 1 Probiotic mechanisms of action from a host perspective.

While the major part of the manuscript is focused on probiotic mechanisms of action from a microbiological perspective, possible molecular mechanisms of action of probiotics from a host perspective can be broadly divided into the following categories:

(1) Modulation of the composition and activity of the indigenous microbiota — at least temporarily
Most probiotics applied to day are lactic acid bacteria, which all have a broad antimicrobial activity, for example, against *Salmonella* through production of lactic acid [28]. More specific microbiota-targeting mechanisms include pathogen inhibition by bacteriocin production (e.g. [27]), competition for nutrients such as between the probiotic *E. coli* Nissle 1917 and the pathogen *Salmonella* [52] and alteration of the intestinal metabolome (e.g. [53]). Also effects on digestive capacity (e.g. lactose digestion), stool consistency and frequency could be classified here because:

(2) Enhancement of epithelial barrier function
These mechanisms include decreasing permeability by promoting tight junction functionality such as shown by [49], and improving cell proliferation/inhibiting apoptosis of the epithelial cells [14].

(3) Modulation of the immune system
All probiotics interact with pattern recognition receptors of the immune system such as Toll-like receptors. They have effects on cells of the innate and adaptive arm of the immune system, mainly through interactions with monocytes, macrophages and dendritic cells, which further modulate the balance of T-helper and T-regulatory cells or antibody production by B-cells. However, the exact immunological outcome of each specific probiotic strain applied is different because the sum of the interactions is strain-specific (such as reviewed in [54]).

(4) Modulation of systemic metabolic responses
In addition to direct metabolic responses in the gut, systemic metabolic responses can also be induced by probiotics, for example, by bile salt hydrolase activity, impacting on satiety hormones (e.g. [55]) and endocrine modulations (e.g. [56]). These effects can be quite general, such as the bile salt hydrolase [57] or more strain-specific.

(5) Signaling via the central nervous system
Various direct and indirect mechanisms of probiotic signaling with the central nervous system have also been shown during the past years, such as via tryptophan-derived products, γ-aminobutyric acid (GABA) [58], oxytocin production [59•]. Also antinociceptive effects such as by *L. reuteri* DSM 17938 through the TRPV1 channel [60] could be classified here. Effects on gut motility could also be classified here.

its surface [7]. These SpaCBA pili were identified through comparative genome analysis [7] and can best be observed when the outer layer of surface exopolysaccharides is removed [8]. Comparative analysis of isolated pili (subunits) and *L. rhamnosus* GG wild-type and isogenic pili mutants have subsequently shown that SpaCBA pili are key for adhesion to human mucus and intestinal epithelium, modulate immunoregulatory interactions with monocytes and dendritic cells [9,10], and even promote pathogen exclusion such as of pilliated *Enterococcus faecium* [11]. In a human fetal ileal organ culture model, *L. rhamnosus* GG also attenuated inflammatory cytokine production in response to *Salmonella*, at least partially through the SpaC subunit of the pili [12]. Moreover, by comparison of wild-type and a SpaCBA pilus mutant in mice, the pili were also demonstrated to be involved in specific signaling mechanisms promoting cell proliferation in intestinal crypts, as well as protection against radiological insults [13]. Pili in LGG thus serve as an example of the complexity of mechanisms of action mediated by a single structure. Besides pili, various other effector molecules have been identified and confirmed to play a key role in some mechanisms of *L. rhamnosus* GG supporting health, such as the major secreted proteins p40 and p75 (enzymes degrading peptidoglycan) that prevent cytokine-induced apoptosis and colitis and protect against TNF-induced epithelial damage [14], lipoteichoic acid that negatively modulates colitis [15,16], CpG-rich DNA that suppresses allergen-specific IgE [17] and exopolysaccharides that alleviate adipogenesis in high-fat-diet fed mice [18].

The *L. acidophilus* species encompasses several strains that are commercially employed as probiotics, with *L. acidophilus* NCFM being the model probiotic strain. One of the most prominent cell surface features of *L. acidophilus* NCFM are its surface (S)-layer proteins. The S-layer of *L. acidophilus* NCFM is encoded by three Slp-encoding genes: *slpA* (LBA0169), *slpB* (LBA0175), and *slpX* (LBA0512). For this species, a versatile genetic and biochemical toolbox has been developed over the years. This was employed to identify diverse functional roles for Slps (and other surface molecules) of *L. acidophilus* NCFM, including cell shape determinants, molecular sieves, protective layers against viral infection, anchoring sites for surface-associated enzymes, and facilitators of cellular adhesion through immune receptors [19]. Recently, by comparing a purified SlpA subunit and a mutant only expressing the major SlpA, SlpA was shown to be a probiotic factor able to bind to the C-type lectin, host immune receptor SIGNR3. This modulated regulatory signals, which resulted in mitigation of colitis, maintenance of healthy gastrointestinal microbiota, and protection of gut mucosal barrier function in mice [20••]. Similarly, a mutant deficient in lipoteichoic acid of *L. acidophilus* NCFM was also able to mitigate colitis through a mechanism that involved interleukin-10 and CD4(+)FoxP3(+) T regulatory cells to dampen exaggerated mucosal inflammation [21].

Lactobacillus plantarum WCFS1 is another well-documented model strain of which the genome sequence was the first published whole genome sequence of *Lactobacillus* [22]. By host transcriptomics studies, *L. plantarum* WCFS1 was shown to modulate various NF-κB-dependent pathways in duodenal biopsy samples from healthy human volunteers only when stationary phase-harvested cells were employed [23]. These expression patterns were distinct from the transcriptome responses observed in humans that consumed other probiotics such as *L. rhamnosus* GG and another — but related strain of *L.*