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Identification of probiotic effector molecules: present state and future perspectives

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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.

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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.*

acidophilus than mentioned above [24]. Yet, also the human transcriptomic responses were highly individual. Comparing the transcriptome signatures induced by the consumption of different probiotic strains and drugs further revealed clues towards the optimal field of application of specific probiotic products, although this is not so easy to pinpoint. The growth phase-dependent capability of *L. plantarum* to modulate NF-κB associated pathways in the mucosa of healthy human volunteers was further explored by adding trypsin-treated *L. plantarum* surface samples to a dedicated Caco-2 intestinal epithelial cell line NF-κB reporter. A subsequent proteomics analysis revealed that one of the surface proteins that was found to be up-regulated in the late stationary phase was StsP, a very large, serine/threonine rich, sortase-dependent protein that was previously shown to be induced in the intestinal tract of mice and humans [25]. Increased StsP expression was achieved by genome editing and was shown to elicit strong NF-κB attenuation in the NF-κB reporter assay, whereas this effect was completely abolished in a StsP-negative derivative strain [26]. These experiments provide evidence for the role of this cell surface protein in host cell signaling. However, trypsination of the proteome did not lead to reduced attenuation capacity, suggesting that specific peptides within StsP are also sufficient to exert the NF-κB attenuation which might enable a more pharmaceutical approach employing synthesized peptides rather than probiotic cells, although acid and bile resistance of these peptides will be an issue.

Probiotic effectors molecules have also been identified in other strains. For example, using knock-out mutants of *Lactobacillus salivarius* UCC118 unable to express the bacteriocin Abp118, as well as genetically modified *Listeria* mutants expressing the bacteriocin immunity genes, Hill and colleagues demonstrated that Abp118 limited *Listeria monocytogenes* infection in mice [27]. In this seminal paper, the key mechanism of action was shown to be direct antagonism and not immunomodulation or competitive exclusion, at least not in this case of this pathogen. Of note, the same probiotic *L. salivarius* UCC118 was also able to protect against *Salmonella*, but this effect was independent of the Abp118 bacteriocin, and thus might be a more generic mechanism such as immunomodulation, competitive exclusion or lactic acid, to which *Salmonella* is quite sensitive *in vitro* [28]. In another *L. salivarius* strain, Ls33, it was shown that the measurable anti-inflammation potential of the strain was correlated with a local IL-10 production and was abolished in Nod2-deficient mice. The muropeptide fraction of peptidoglycan, M-tri-Lys, whether purified from Ls33 or synthesized, could rescue mice from colitis in an IL-10-dependent manner and favored the development of CD103+ DCs and CD4+Foxp3+ regulatory T cells [29].

Many probiotic mechanisms have been linked to metabolites. For example, *L. reuteri* strains have been shown to reduce inflammation via tryptophan-derived indole derivatives that activate the aryl-hydrocarbon receptor and induce regulatory T-cells [30••] or histamine-related metabolites [31••]. Moreover, *L. reuteri* 6475 was recently demonstrated to produce a soluble bacterial enzyme known as diacylglycerol kinase (Dgk) that suppressed intestinal type 1 histamine receptor-mediated proinflammatory responses, via diminished protein kinase C phosphorylation-mediated mammalian cell signaling. This elegant study using a *dgk* mutant showed that this probiotic could act as a 'microbial antihistamine' [31••].

Compared to most lactobacilli, bifidobacteria are more refractory to genetic engineering, but also major advances have been made in the past decade [32]. Using these methods, in the model probiotic strain *Bifidobacterium breve* UCC2003, specific type IVb tight adherence (Tad) pili were identified to be only expressed *in vivo*. Moreover, these Tad pili promote colonization of the gut [33]. In *Bifidobacterium longum* 35624, the surface associated exopolysaccharides were shown to play an essential role in dampening host pro-inflammatory responses and in repressing local helper Th17 responses [34•]. Other factors of bifidobacteria have also been postulated in probiotic effects, such as serine protease inhibitor (or serpins) of *B. longum* [35].

Recent advances in tools and model systems enable further mechanistic research

Genetic engineering and genome editing

The Molecular Koch's postulates encompass a paradigm whereby the products of individual genes are confirmed to be pivotal for establishing molecular cause-effect relationships of microorganisms to human health. Although classically applied to identify virulence factors, this same approach can be applied to identify health-benefiting effector molecules made by probiotic bacteria and is exemplified for the different probiotic model organisms described above. Such molecular studies are of course elegant to pinpoint active molecules of probiotics. This is especially relevant in the context of live microorganisms, because — ideally — a gene deletion mutant contains still all other active molecules, except for the single gene/molecule knocked out. However, such genome editing strategies are extremely challenging and time-consuming for non-model organisms, even for strains within the same species. A major contributing factor to the limited applicability of developed genome editing tools is the extraordinary genetic diversity of the genus *Lactobacillus*, a genus that is more diverse than a family [36••]. Nevertheless, recent developments of genome editing tools that can generate subtle genome edits without the need for antibiotic selection, including single stranded DNA recombineering and CRISPR-Cas genome editing, show great promise [37,38]. These techniques not only enable a better understanding of probiotic gene-function relationships, but they can also be applied

to promote the rational selection and even the development of tailored probiotics with increased stress tolerance, or enhanced metabolic activity, for example, to enhance probiotic function. Of note, these emerging genome editing tools can be used to make user-defined single-base changes and could therefore be considered as non-GMO, although this is still under debate [39].

In vivo models and new insights in microbial ecology

The development of the human gut microbiota commences at birth, with bifidobacteria being among the first colonizers of the sterile newborn gastrointestinal tract. Bifidobacteria have thus also a large potential as probiotics, especially in children [40]. *Lactobacillus* strains form the largest part of commercial probiotic products on the market, but some authors have questioned in the past whether these microbes are ideal probiotics, because they are often s allochthonous members or transient passengers of the gut microbiota [41]. Yet, recent microbiome studies underscore that lactobacilli form a pivotal part of the human microbiota, in the gut up to 1–5% [42] and more dominant in the vaginal microbiota up to 99% relative abundance based on 16S rDNA amplicon sequencing [43]. In addition, lactobacilli are dominant members of the fermented foods we consume [44]. Moreover, colonization is not a requirement for a probiotic and several modes of action such as immune stimulation might actually be promoted by more allochthonous strains.

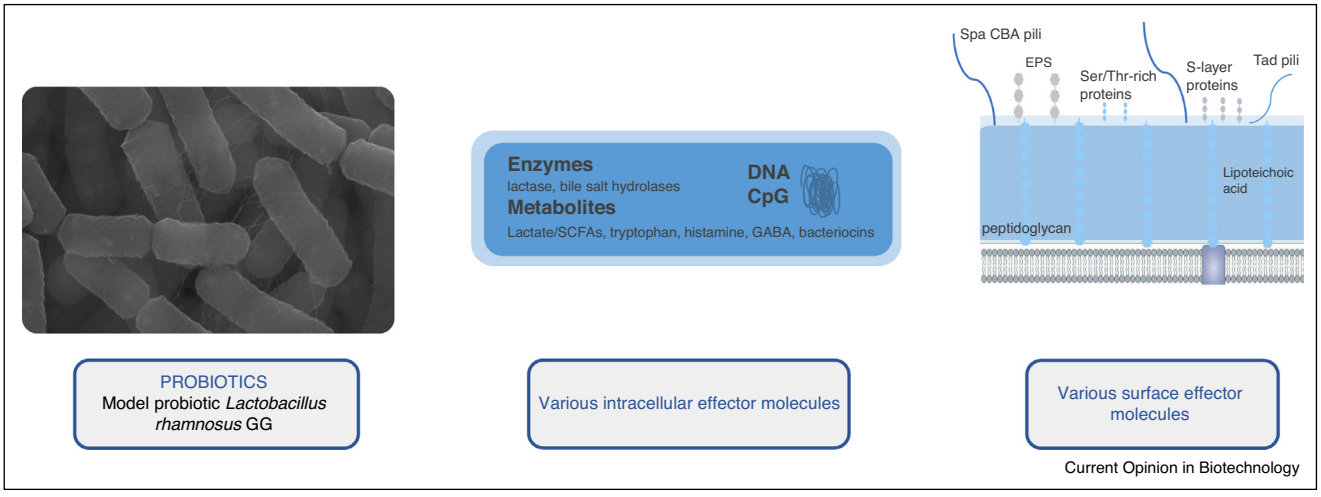
Niche-adaptation, niche-flexibility and biogeography of lactobacilli [41] and bifidobacteria [45••], are receiving renewed interest, stimulated by the recent advances in comparative genomics approaches (e.g. [36••]) and improved detection in human microbiomes, for example, with tools such as DADA2 exemplified for *Lactobacillus crispatus* detection in vaginal samples [46••]. In addition to DNA-based high-throughput approaches, RNA, protein and metabolic approaches reveal many new insights. Metabolically, lactobacilli and bifidobacteria probably play a larger role than one could estimate based on relative abundance data. For example, as yet mentioned, *L. reuteri* was recently shown to be among the microbiota members that produce tryptophan-derived ligands inducing regulatory T-cells, and thus balancing mucosal reactivity [30••]. Although this study, like many other mechanistic microbiome and immunological studies, was carried out in murine animal models, the metabolic activity of lactobacilli, in this case *L. plantarum* WCFS1 colonic transcriptomes, is rather well conserved between humans and mice [25]. Recently, the *L. plantarum* transcriptome in the ileum of rhesus macaques, who are more closely related to humans than mice, was also explored [47•]. Genes required for tolerating oxidative stress, modifying cell surface composition, and consumption of host glycans were clearly expressed in the small intestine of these macaques, again pointing to similar adaptation mechanisms and metabolic pathways active in the different mammalian gut ecosystems. Moreover, in SIV-infected macaques, *L. plantarum* was also shown to prevent gut epithelium damage [48], in agreement with previous *in vitro* and human clinical trials showing that *L. plantarum* can have a positive effect on epithelial barrier function [49]. Thus, macaques might form a non-human primate, alternative animal model for mechanistic probiotic studies and might be used to validate cause-effect relationships in studies that cannot be carried out in humans.

Conclusions

In this review, we focused on the few established microbial-produced effector molecules, responsible for specific probiotic effects and emerging methods and findings in microbial genetics and ecology that are being used to accelerate our interpretation of probiotic mechanisms. However, these advances are balanced by caveats that are well-recognized in the probiotic field. One caveat is that molecular mechanisms of action might be strain-specific or they might be shared among most members of a larger taxonomic group [1]. Several of the molecules described above are not merely strain-specific, but also have homologs in other strains and species or even beyond, such as the Tad pili which appear to be a genus-wide property of *Bifidobacterium* [33]. Secondly, the presence or absence of a specific mechanism in a probiotic might not be able to predict the translation of that mechanism into a net health benefit. e Each probiotic has a complex cell wall, DNA/RNA, proteins, amino acids, sugar precursors, primary and secondary metabolites, and produces enzymes such as lactase and bile salt hydrolases (Figure 1). Totaled together, all these molecules will result in the overall health benefit expressed. The expression of those cell products also depends on the environmental context to which probiotics are exposed in prior to [50] or after [51] application. Nevertheless, this complexity of live microorganisms with their hundreds or thousands of probably bioactive molecules could be embraced to elucidate multi-factorial interactions between probiotics and their hosts. Probiotic efficacy could be viewed as a continuum of complexity, with some examples being quite complex, requiring the interaction of many different aspects of the probiotic, but others being simpler, such as lactase expression to promote lactose digestion in the large intestine [1]. Such complexity is already appreciated on a macroscale for the gut microbiome and its enormous taxonomic and functional diversity.

Presently, in most cases it is thus not yet confirmed whether the known probiotic effector molecules are the actual drivers of the clinical effects observed in human trials. Hereto, properly designed clinical trials with dedicated isogenic knock-out or knock-in mutants of the probiotic microorganisms or with proper formulated isolated bio-active compounds in human subjects are

Figure 1



Embrace the complexity of probiotics as live microorganisms applied in adequate amounts to confer a health benefit to the host. Although it is a paradigm in molecular microbiology that key microbial effector molecules can be elegantly identified based on phenotypic comparisons of wild-type and knock-out micro-organisms lacking a specific effector molecule or comparisons with the isolated microbial molecules, it is crucial for probiotic applications that whole microbial cells, their cellular content and cell surface are taken into account when exploring modes of action. This picture represents a progressive zooming in on probiotic structures. The left panel shows a dense culture of the model probiotic *L. rhamnosus* GG — as visualized by scanning electron microscopy (SEM). The thin structures between the bacterial cell are the pili, key for mucus adhesion and some immunomodulatory interactions, but they are not the only factors involved. In the middle panel, we aim to highlight that all metabolites, enzymes, DNA/RNA and cell wall molecules could impact on host physiology and indigenous microbiome (Box 1). The last panel is a more detailed overview of the cell wall architecture of typical Gram-positive probiotic bacteria, because many of the key probiotic effector molecules so far are cell surface associated molecules.

needed. Such trials are very complex to perform, for a variety of reasons, ethically, regulatory, technically, financially, logistically, among others. To succeed, evidently the potency of the probiotic strain itself matters, but also dose, viability, formulation, targeted pathogen, targeted host response, targeted host site, prevention or treatment set-up, difficulties of measuring certain biomarkers, combination effects, time frames for the probiotic activities (seconds, minutes, hours, weeks), are all aspects that need to be taken into account, especially if more than one effector molecule is involved.

Although detailed mechanistic trials could ultimately extend the field from food to pharma, the benefits of this reductionist approach provides a way to address lingering questions such as predicting which probiotic microorganism to administer and explaining inter-individual variation in responses to probiotics. Just because such identification of mechanisms is highly complex does not mean that we should not try. Remember the famous phrase of John F. Kennedy. 'We choose to go to the Moon in this decade and do the other things, not because they are easy, but because they are hard'. This is the 'moonshot' for the probiotics field.

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- •• of outstanding interest

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