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Shared mechanisms among probiotic taxa: implications for general probiotic claims

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Shared mechanisms among probiotic taxa: implications for general probiotic claims

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Strain-specificity of probiotic effects has been a cornerstone principle of probiotic science for decades. Certainly, some important mechanisms are present in only a few probiotic strains. But scientific advances now reveal commonalities among members of certain taxonomic groups of probiotic microbes. Some clinical benefits likely derive from these shared mechanisms, suggesting that sub-species-specific, species-specific or genus-specific probiotic effects exist. Human trials are necessary to confirm specific health benefits. However, a strain that has not been tested in human efficacy trials may meet the minimum definition of the term 'probiotic' if it is a member of a well-studied probiotic species expressing underlying core mechanisms and it is delivered at an effective dose.

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Introduction

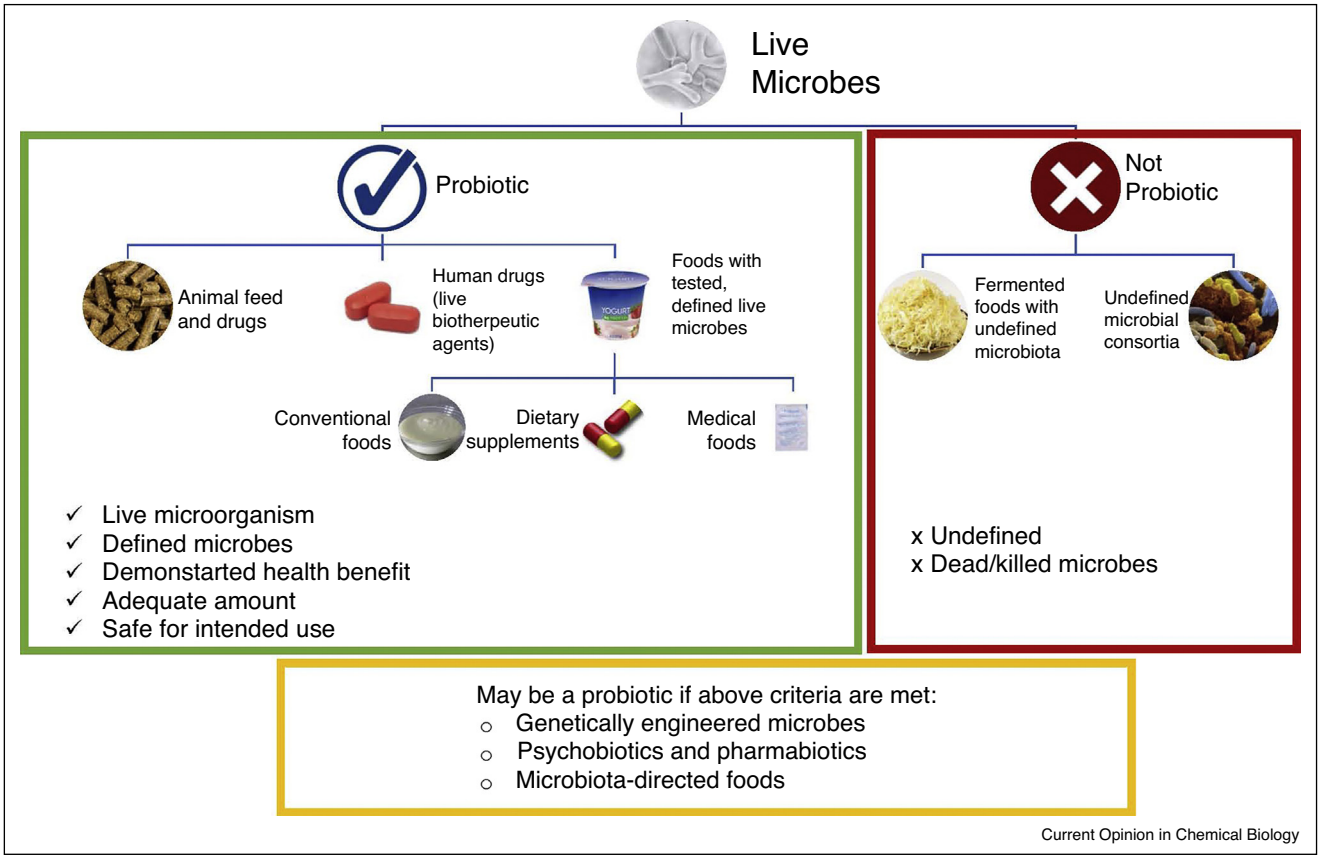
Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [1••]. Probiotics span a wide range of uses, including different regulatory categories, different target host species, and different routes of administration (oral, intravaginal, topical) (Figure 1). The strain-specificity of probiotic effects has been accepted for decades as a fundamental principle based on mechanistic research by scientists in the field. Today, it is rare to see commercial products that do not list strain designations or to read a clinical trial that does not identify the probiotic to the strain level. Globally recognized guidelines have reinforced this concept [2].

Here we examine the concept that shared mechanisms exist among taxonomic groups that include many different strains (Figure 2). This concept was introduced previously [1••,3•], but here we expand on the mechanistic evidence to support this concept. Studies have shown, and continue to show, that certain types of live microorganisms are beneficial to human health as assessed through a range of digestive health endpoints. The number of tested strains is large and the range of health benefits demonstrated is wide. We explore the significance of these observations in the context of science-based, responsible communication of health benefits of probiotics to consumers and healthcare providers. We focus on examples of *Lactobacillus* and *Bifidobacterium*, because these are the traditionally used probiotics, but the concepts are also valid for the next generation of probiotics [4].

Shared mechanisms, shared benefits: mechanistic rationale

In the probiotic field, it is clear that not all probiotics function in the same manner. For example, the specific bacteriocin produced by *Lactobacillus salivarius* UCC118, which conferred resistance to *Listeria monocytogenes* infection when expressed in a mouse model [5], has not been found in other strains of *L. salivarius* [6]. However, it is equally clear that not all probiotic strains function in a purely unique manner. As we discuss more completely below, the ability to produce short chain fatty acids (SCFAS) is a feature shared by many different probiotic taxa, and surely plays a significant role in probiotic-mediated health benefits. Consider the body of research conducted on clinical effects of probiotics for prevention of necrotizing enterocolitis: many taxa have been tested, most of which resulted in similar clinical outcomes [7], supporting the conclusion that probiotics from different taxa have benefits in necrotizing enterocolitis. Here we provide examples of mechanisms, which likely play important roles in directing probiotic health benefits, that have been identified among strains within probiotic taxa. In some cases the traits may be shared broadly, among most strains in a genus. In other cases, the distribution of the mechanism may be much narrower. When

Figure 1



Probiotics: what is encompassed under this term (updated from Hill *et al.* [1••]). By definition, a health benefit must be demonstrated for a probiotic, at either a strain-specific or, as discussed in this paper, at a taxonomic level where mechanisms are shared. Probiotics can be administered via different routes (oral, intravaginal, topical, etc.) and are not limited to human use (companion animals, livestock, fish). The probiotic definition is not restricted by regulatory category, as clarified here. Dead microbes, microbial endproducts, microbial components and undefined microbial mixes do not come under the probiotic classification [4,65–68].

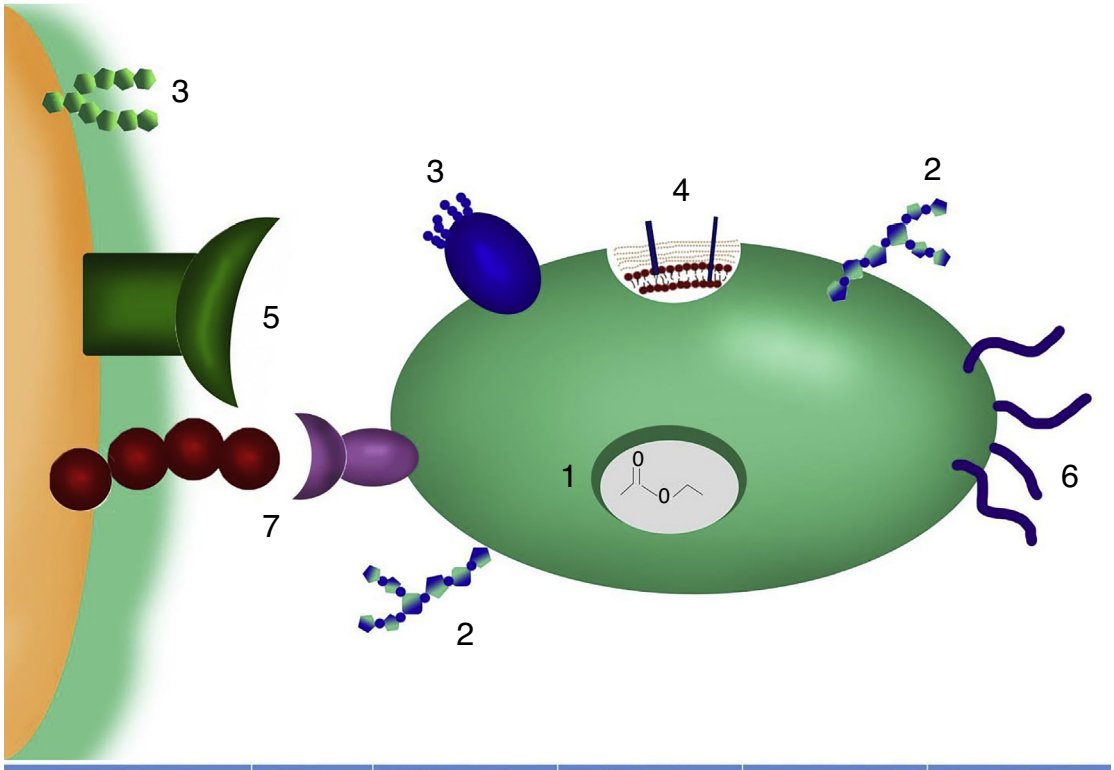
mechanisms important to the expression of a health benefit are shared, shared health benefits may result.

Bifidobacterium shunt and short chain fatty acid production

The genus *Bifidobacterium* contains species that are generally known for their ability to degrade complex carbohydrates and metabolize the derived monomers through a shared central metabolic pathway known as the *Bifidobacterium* shunt (Bif Shunt). This pathway depends on key enzymatic steps catalyzed by a phosphoketolase enzyme that degrades hexose and pentose phosphates. A unique feature of the Bif Shunt pathway is its increased capacity for energy harvest from five- and six-carbon sugars, which generally yields one additional ATP per every two molecules of glucose compared to pathways in other organisms [8]. Reliance of *Bifidobacterium* species on the Bif Shunt pathway as a central metabolic resource likely reflects an evolutionary adaptation in *Bifidobacterium* that confers a fitness advantage during natural colonization.

In addition to energy generation, the Bif Shunt pathway yields SCFA as end products, specifically, lactate and acetate (two moles of lactate and three moles acetate per two moles of glucose) [8]. Increases in fecal SCFAs have been observed in studies that administer probiotic species of *Bifidobacterium* as well as studies where prebiotics are used to stimulate abundances of native colonizing *Bifidobacterium* species [9–12]. These SCFAs have a broad array of positive effects on the human GI tract, either directly or by conversion to other SCFA such as butyrate by other members of the microbiota [13•,14,15,16]. These positive effects are mediated through multiple SCFA receptors on colonocytes that directly control energy and non-energy dependent motility and electrolyte transport [17–21]. Beyond the transport and contractile functions, SCFAs can also influence local inflammation by affecting synthesis of inflammatory cytokines, inhibiting

Figure 2



Mechanism	Gram+	*Bifidobacterium* genus wide	*Bifidobacterium* - some species	*Lactobacillus* genus wide	*Lactobacillus* – some species
1. *Bifidobacterium* shunt pathway,leading to acetate and lactate		•			
1. Lactate (not via *Bifidobacterium* shunt)				•	
2. Surface-layer-associated proteins					•
3. Mucus binding protein		•		•	
4. Lipoteichoic acid	•				
5. TLR receptors	•				
6. Sortase-dependent pilin proteins			•		•
7. Fibronectin-binding proteins					•
Lactocepin					•
CpG DNA			•		•
Vitamins			•		•

Current Opinion in Chemical Biology

Shared probiotic mechanisms and their taxonomic distribution.

synthesis of TNF-alpha and IL-6 and stimulating synthesis of the anti-inflammatory cytokine IL-10 in macrophage and neutrophils [22–25]. These effects improve inflammatory tone and have added to earlier interest in incorporating SCFAs into anti-inflammatory enemas and dietary SCFA-stimulating prebiotics as treatments in patients with inflammatory bowel disease [26–30].

Comparative genomic analysis of publically available *Bifidobacterium* genomes reveals that all thirteen enzymes that comprise the Bif Shunt pathway are among the core set of ~480 genes shared across all species of *Bifidobacterium*. This finding implies that the pathway is ancestral to the common ancestor of extant *Bifidobacterium* species and strains. If this pathway indeed provides a fitness advantage for growth of *Bifidobacterium* species in the GI tract, it is reasonable to suspect that signatures of such an evolutionary adaptation would be observable in phylogeny of the Bif Shunt enzymes, particularly the key phosphoketolase Xfp, which encodes a bifunctional fructose-6-phosphate/xylulose-5-phosphate phosphoketolase. Phylogenetic analysis of the Xfp phosphoketolase from several species and strains of *Bifidobacterium*, along with phosphoketolases from other members of the Phylum *Actinobacteria*, as well as members of the *Firmicutes*, *Proteobacteria*, and *Cyanobacteria* supports this hypothesis. As illustrated from the unrooted dendrogram in Figure 3, the Xfp proteins from the family Bifidobacteraceae (*Bifidobacterium*, *Scardovia*, *Gardenella*) form a very distinct cluster, with long branch length (greater genetic distance), distinctively separating this cluster from other members of the Actinobacteria, a result that would be expected if Xfp has undergone evolutionary adaptation.

The shared ancestry of the Bif Shunt pathway in *Bifidobacterium* species, the potential fitness advantage for colonization, and the shared benefits of SFCAs produced from this pathway provide support for a core benefit that is shared across species. Although not addressed in detail here, in a similar vein *Lactobacillus* species share key metabolic traits since they predominately derive energy through fermentation of sugars into lactic acid, via either homofermentative or heterofermentative pathways, which also cluster in separate phylogenetic clades [31].

Probiotic cell surface architecture

Flooding the upper small intestine with ingested Gram-positive bacteria ($\sim 10^{8-9}$/gm) temporarily overwhelms the resident population ($\sim 10^{4-7}$/cm^2) during the transient passage of microbes through the GI-tract. Because of the dynamic changes constantly occurring in the mucosal surface of the small intestine, such as variable mucin production and accumulation, orally delivered microbes are more likely to have greater access to the intestinal mucosa, microvilli, Peyer's Patches, and dendritic cells signaling the immune system.

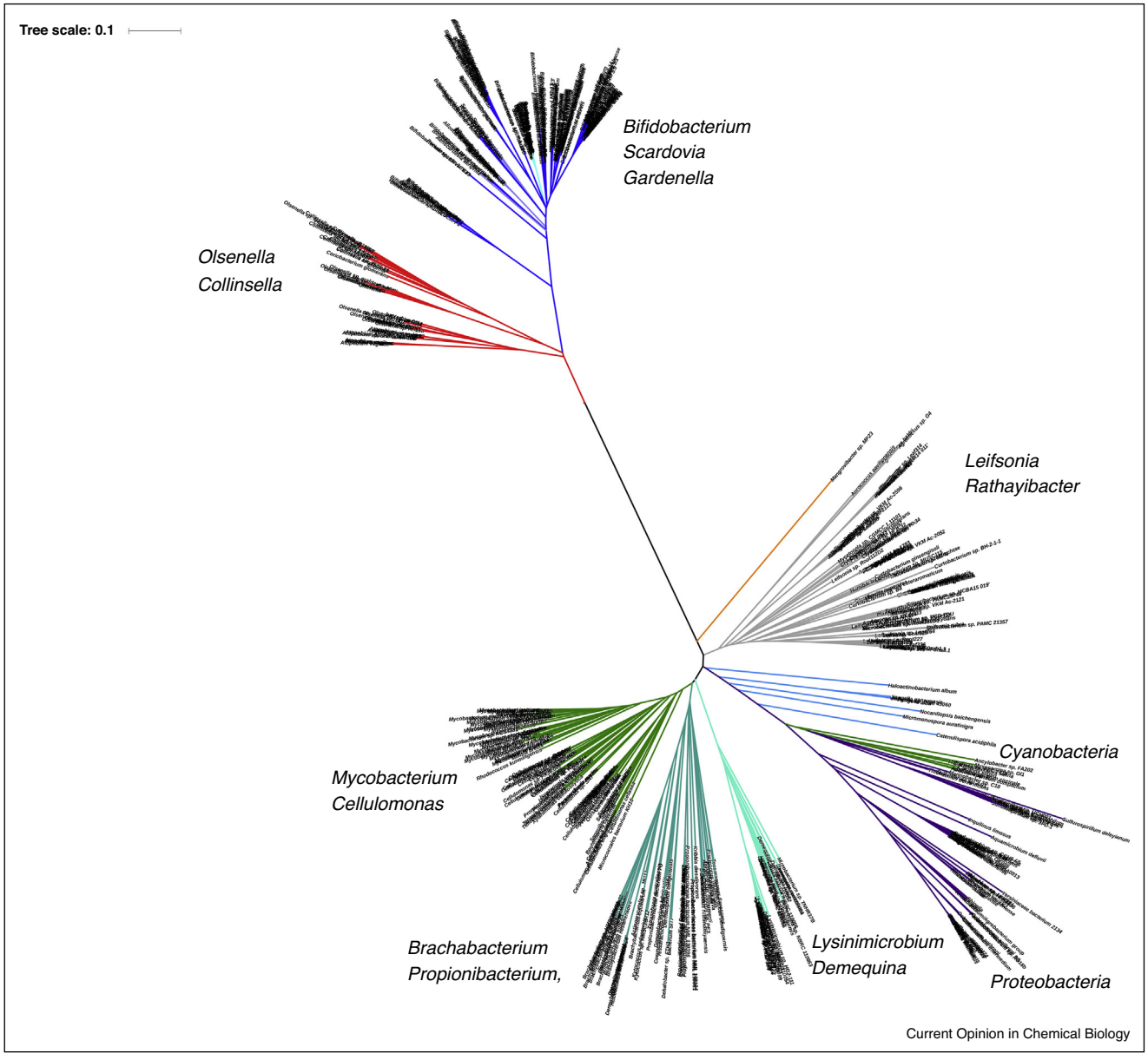
Important core mechanisms that underlie common probiotic functions are found in the shared architecture of the cell surface structures of Gram-positive microbes. Clearly, not all Gram-positive microbes expressing these traits are necessarily probiotics or beneficial. But when these mechanisms are distributed among certain taxa known to be probiotics, they may contribute to certain health benefits in a somewhat predictable manner. These shared architectures vary considerably among species and likely impact the host's physiological responses to beneficial microbes that are either delivered orally or applied spatially. These core cell surface architectures found among species of Gram-positive bacteria are: peptidoglycan; cell wall teichoic and lipoteichoic acid (LTA); and common but varying components including exopolysaccharides, surface layer associated proteins (SLAPS), mucin-binding proteins (MUBs), fibronectin binding proteins, and pili. These bacterial cell surface macromolecules are key factors in this beneficial microorganism-host crosstalk, as they can interact directly with the intestinal epithelium, mucus, and host pattern recognition receptors of the gastrointestinal mucosa.

Mucus-binding, fibronectin-binding and pilin proteins

The sortase pathway is the most common mechanism to transport, deliver and link extracellular proteins to the surface of Gram-positive bacteria [32]. This mechanism underlies the presentation of MUBs, with their repeated mucus-binding domains (reviewed by Etzold *et al.* [33]). Linkage of MUBs to the peptidoglycan cell wall occurs via a conserved Leu-Pro-any-Thr-Gly motif. First recognized and characterized in *Lactobacillus reuteri*, MUBs are widely distributed among commensal and probiotic lactobacilli and bifidobacteria [33]. Genetic knockouts of MUB proteins result in loss of mucin binding ability and shorter retention times during transit through the mammalian GI-tract [34,35].

Sortase-dependent, cell surface pili have also been defined in both lactobacilli (*rhamnosus* and *ruminus* [36]) and bifidobacteria (*bifidum*, *longum*, *dentium*, *adolescentis* and *lactis* [37]). Close interaction with the intestinal mucosa and mucin binding are key attributes commonly exhibited by these pilin-like appendages. Mucin binding via MUB's and pili are considered key mechanisms of 'core' probiotic activity that impacts retention in the GI-tract, competitive exclusion of pathogens [38], immunomodulation and structural integrity of the intestinal mucosa. For instance, the sortase-dependent SpaCBA pili of the model probiotic *L. rhamnosus* GG promote human intestinal retention [36], competitive exclusion with the pathogen *Enterococcus faecium* having similar mucus-binding pili [38] and immunomodulation in macrophages [39]. In addition, certain pili such as these glycosylated SpaCBA pili of *L. rhamnosus* GG, also more specifically modulate immune responses via special PRRs such as the DC-SIGN receptor of dendritic cells [40]. Although improved

Figure 3



Phylogenetic analysis of the Xfp phosphoketolase from *Bifidobacterium* and representative species from multiple bacterial phyla. A total of 500 phosphoketolase proteins from the RefSeq database were aligned by pairwise BLAST with Kimura distance metrics. The unrooted tree was resolved with minimum evolution and plotted using the interactive Tree of Life (https://itol.embl.de/). Major groups of taxa are colored by clade and dominant genera within these clades are indicated in large letters.

persistence of a probiotic does not necessarily lead to a health benefit, it provides an increased opportunity for a probiotic to interact with its host.

Fibronectin-binding proteins

Fibronectin is a multi-domain glycoprotein found ubiquitously in human body fluids and extracellular matrices of a variety of mammalian tissues, including intestinal epithelial cells. Fibronectin-binding proteins (FnBPs) have been identified and characterized in a wide variety of host-associated bacteria, including both pathogens and commensals [41]. Colonization or retention in the GI-tract highlights the role of multiple adhesions, including FnBP's. FnBP's are core adhesions found among Gram-positive commensals and probiotics and found in *L. acidophilus* (S-layer associated — see below),

and the non-S-layer producing species of *L. casei*, *L. plantarum*, *L. brevis* and *L. rhamnosus*.

Surface-layer proteins

Another widely used commercial probiotic species, *L. acidophilus*, produces an extracellular crystalline protein (SlpA) that creates a surface layer. These types of proteins coat the cell surface of lactobacilli taxonomically assigned to the *Lactobacillus acidophilus* group A; including *L. acidophilus*, *L. helveticus*, *L. crispatus*, *L. amylophilus*, *L. gallinarum*, *L. jensenii*, *L. kefiranofaciens* and *L. amylovorus*. In recent studies with *L. acidophilus*, it has been discovered that multiple SLAPs exist (~20–40) and are loosely embedded within the major crystalline surface layer protein, SlpA [42]. They likely act as extracellular cell surface proteins able to interact with mucosal tissues, and exert significant physiological, enzymatic and immunological consequences. One notable example is that the fibronectin binding protein of *L. acidophilus* is a SLAP [41]. SLAP proteins vary among *Lactobacilllus* species, and are absent from species lacking an S-layer; notably the probiotic species of *L. gasseri*, *L. johnsonii*, *L. reuteri*, and *L. (para)casei* [43]. Yet certain probiotic *Lactobacillus* species clearly produce the major surface layer proteins (which alone have physiological and immunological responses), and these S-layers also embed loosely associated proteins that may intimately interact with the intestinal mucosa. Thus, both S-layer proteins and SLAPs represent a collection of proteins that likely elicit common core mechanism for this bacterial group.

Peptidoglycan and lipoteichoic acid

During probiotic administration, a high dose of microbial-associated molecular patterns (MAMPs) is consumed, which can all interact with host PRRs resulting in various immunomodulatory effects. Toll-like receptors (TLRs) are the best documented PRRs being able to detect various probiotic MAMPs. Since the most common probiotic taxa are Gram-positive bacteria, TLR2 is a key PRR because peptidoglycan and LTA are documented MAMPs (reviewed in [44••]). The core molecular architecture of peptidoglycan and LTA is well conserved among lactobacilli — and bifidobacteria (e.g. [45]). TLR2 has an important barrier-protective function in intestinal epithelial cells [46]. Positively impacting intestinal barrier function is a major probiotic mechanism [47]. However, it should be noted that — although *Lactobacillus* LTA is a well-documented MAMP interacting with TLR2/6 heterodimers [48], immunostimulation by LTA of lactobacilli should be carefully taken into account, for instance for applications in inflammatory bowel disease. Modification of LTA [49] or removal of LTA [50] have elicited significant anti-inflammatory consequences observed in mouse models of both colitis and colon cancer. In this case, the core mechanism is that probiotic Gram-positive microbes with reduced expression of LTA are more likely to modulate the anti-inflammatory immunological consequences that affect inflammatory bowel diseases (as also reviewed in [51]).

Multiple other MAMP-PRR interactions between probiotic bacteria and host cells can also occur and impact on the final host responses. Inside the probiotic bacterial cells, unmethylated cytosine-guanine (CpG)-containing DNA is an important ligand for TLR9. TLR9 is expressed by many cell types located in the intestine, including epithelial cells and classical immune cells. TLR9 signaling is also important for gut epithelial homeostasis. Recently, a bioinformatics analysis was performed on the frequency of potentially immunostimulatory CpG motifs in the genomes of gut commensal bacteria across major bacterial phyla [52•]. The frequency of these motifs (all hexamers) was linearly dependent on the genomic G + C content: species belonging to Proteobacteria, Bacteroidetes and Actinobacteria (including bifidobacteria) carried high counts of GTCGTT, the optimal motif stimulating human TLR9. However, despite having an A + T rich genome content, *Lactobacillus casei*, *Lactobacillus plantarum and Lactobacillus rhamnosus* strains that have been marketed as probiotics were found to have high counts of GTCGTT motifs. Indeed, CpG-rich DNA from the widely used probiotic strain *L. rhamnosus* GG has been shown to interact with TLR9 on endosomes to stimulate Th1 responses [53,54]. Not all probiotic immunomodulatory molecules function through PRRs. For example, some widely excreted enzymes seem to actively impact cytokine activity, such as the conserved cell envelop associated PrtP-proteases (also named lactocepin), which has been shown to selectively degrade proinflammatory cytokines [55••]. In their recent large genome comparison of lactobacilli, Sun *et al.* [56•] also highlight these proteases as potential probiotic factors, which show clear clade association, notably with the *L. delbrueckii*, *L. casei* and *L. buchneri* clades, part of the *L. salivarius* clade, and the *Carnobacterium* clade. The presence of this probiotic feature is thus a nice example of a property existing beyond the strain-specific dogma. Furthermore, several bacterial metabolites such as the SCFAs mentioned above and various aminoacids (e.g. trypthophan-derived products), all can impact directly on the host immune status (e.g. [57]). In line with these more 'metabolic modes of action', the only 'probiotic' mechanism of action currently approved in Europe by EFSA is improved lactose digestion by live yoghurt cultures containing at least 10^8 CFU/g *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, because these bacteria are known to express the necessary enzymes (lactase or beta-galactosidase) to degrade lactose. Similarly, strains producing high levels of vitamins (e.g. vitamin B12 by certain lactobacilli [58] and folate by certain bifidobacteria [59]) are being selected that produce enough to meet minimum daily requirements.

Category-based evidence

Systematic reviews (SR) and meta-analyses (MAs) are important tools for assessing the totality of evidence for a given intervention. An essential component of the SR/MA process is clear definition of the intervention being researched. In the case of probiotics, over 200 meta-analyses have been published and most of these have not been conducted on individual strains. The implied rationale for this is that the outcomes being studied are expected to be expressed by many different strains. Although the essence of this assumption may not be clearly considered or even understood by many of the authors of these SR/MAs, the rationale may be sound [60]. To the extent that a common mechanism can be described among a group of different strains, it is scientifically sound to pool results on clinical outcomes on those strains.

The process of assigning health benefits to a category, rather than a defined entity, is not unique to the probiotic field. Fecal microbial transplant to prevent recurrent *Clostridium difficile* disease, an intervention widely accepted in the medical community, is a case-in-point. The fecal preparations are wholly undefined, vary by donating host, are delivered in different manners, and are undoubtedly immensely impacted by handling practices, which surely take a toll on strict anaerobe populations.

Other examples come from dietary recommendations. The broad category of 'dietary fiber' is recommended and fully endorsed, yet dietary fibers are a broad category, differing in their structure and their physiological impact. Perhaps resulting from the explosion of human microbiome research revealing the importance of live microbes in human health, fermented foods have surged in popularity. Fermented foods that retain their live microbial constituents (i.e. have not been processed to kill or remove the fermentation microbes) have been around for millennia, but are being recognized anew for their potential health benefits [61]. Fermented foods often have a rich, undefined microbial content. The health benefits due to the microbial components of these foods are examples of expression of health promoting mechanisms likely shared among many different taxa.

It is widely accepted that there are class effects of medications, yet different medications comprise a given class. For example, as a class all beta-adrenergic blocking agents (beta blockers) work by blocking receptor sites epinephrine and norepinephrine resulting in a lower pulse rate and reduced blood pressure. However, there are many differences among the various beta blocking medications and they have a wide range of indications. The same type of overall class benefit is seen with 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors, proton pump inhibitors, selective serotonin reuptake inhibitors and many other drug classes.

Overall implications

Probiotic claims

Assuring that consumers are not misled is a common objective among global agencies responsible for oversight of health benefit claims on probiotic products. Preventing unsubstantiated claims is a top priority. Unfortunately, at times this can put a limit on consumer access to legitimate information, with negative implications for the health of adults and children around the world. One example is the situation in the EU, which currently prohibits the use of the word 'probiotic' on foods. As previously asserted [1••], consumers are not misled by products being labeled as 'probiotic' if the products contain adequate amounts of a well-studied probiotic species documented in numerous studies to confer a beneficial effect. This idea is reinforced through the common mechanistic properties of probiotic taxa presented here. We provide the scientific rationale and well-documented examples of mechanisms for defining commonalities that exist for groupings of individual strains. It is noteworthy that this approach has been accepted by regulatory approaches in Canada and Italy, and is consistent with the claim EFSA allowed for the species *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [62].

Probiotic product labels

It should be emphasized that the evidence presented herein does not eliminate the responsibility for researchers and commercial entities to continue to fully define and disclose the content of probiotic products to the strain level. Fermented foods, as indicated in Figure 1, to the extent their microbial content is undefined, are not probiotic products. But any product claiming to be a probiotic must accurately disclose the levels of the microbes present and identify them to the genus, species and strain level, ideally including complete genome sequences. Such disclosure is needed to assure product consistency, transparency in marketing and the ability to repeat research. Additionally, human studies with defined strains and doses are necessary to support specific health benefit claims.

Conclusion

In 2014, a consensus group of experts recommended to 'Include in the framework for definition of probiotics microbial species that have been shown in properly controlled studies to confer benefits to health' [1••].

Although laboratory assessments document many ways in which strains may differ [63], we lack direct evidence on how different strains may utilize similar mechanisms to exert similar clinical outcomes. Most clinical studies do not compare multiple strains of the same species.

However numerous meta-analyses show that similar clinical benefits are achieved by many different strains [64].

It is not possible to assert with 100% confidence that the presence of certain metabolic pathways or molecular mechanisms will confer a given clinical benefit. A strain may be deficient in overall physiological fitness or may otherwise possess traits that override adequate expression of encoded properties, which precludes detection of an overall health benefit. However, as research continues to evolve, associations between the presence of specific mechanisms and clinical benefits will continue to strengthen confidence. The net clinical effect of a live bacterium will be dictated by the complex array of genetic expression and technological production factors for probiotic cultures that impact viability and shelf-life. Human trials to confirm that a given mechanism drives the observed clinical effects will improve our understanding. The probiotic field would benefit from agreement among experts regarding what constitutes adequate evidence of assignment of a clinical benefit to a larger taxonomic group.

We present here evidence for mechanisms that are shared among probiotic taxonomic groups at higher levels than strain. Although further research is needed to confirm the link between a given mechanism and clinical benefit, we propose that the distribution of such mechanisms among all members of a taxon provides a rationale that some general probiotic benefits can be expected in a non-strain-specific manner.

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- • of special interest
- •• of outstanding interest

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