



ADDENDUM

## Probiotics in the next-generation sequencing era

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### ABSTRACT

Technological developments, including massively parallel DNA sequencing, gnotobiotics, metabolomics, RNA sequencing and culturomics, have markedly propelled the field of microbiome research in recent years. These methodologies can be harnessed to improve our in-depth mechanistic understanding of basic concepts related to consumption of probiotics, including their rules of engagement with the indigenous microbiome and impacts on the human host. We have recently demonstrated that even during probiotic supplementation, resident gut bacteria in a subset of individuals resist the mucosal presence of probiotic strains, limiting their modulatory effect on the microbiome and on the host gut transcriptional landscape. Resistance is partly alleviated by antibiotics treatment, which enables probiotics to interact with the host at the gut mucosal interface, although rather than promoting reconstitution of the indigenous microbiome and of the host transcriptional profile, they inhibit these components from returning to their naïve pre-antibiotic configurations. In this commentary, we discuss our findings in the context of previous and recent works, and suggest that incorporating the state-of-the-art methods currently utilized in microbiome research into the field of probiotics may lead to improved understanding of their mechanisms of activity, as well as their efficacy and long-term safety.

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### Probiotics: decades of conflicting reports

The concept of promoting human health through consumption of beneficial microorganisms has evolved during the last century, starting with Metchnikoff's observation at the beginning of the twentieth century that fermented dairy products are associated with longevity,<sup>1</sup> through Fuller's initial definition of probiotic therapy,<sup>2</sup> to its current common definition as coined by the FAO/WHO in 2001: "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host".<sup>3</sup> Thousands of studies have been published under this broad definition, attempting to demonstrate a beneficial effect for probiotic microorganisms (mostly within the *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus*, and *Saccharomyces* genera) in a long list of clinical conditions, with highly mixed results. For example, several studies suggested that probiotics may prevent or limit acute gastroenteritis,<sup>4-7</sup> while other studies did not find such an effect,<sup>8-13</sup> including two recent multi-center

prospective trials encompassing nearly 2,000 children.<sup>14,15</sup> Conflicting reports also question the efficacy of probiotics in antibiotics-associated diarrhea.<sup>16-21</sup> More specifically, several meta-analyses have reported a beneficial role for probiotics in prevention of *Clostridium difficile* infection or its associated comorbidities,<sup>22-26</sup> although some also indicated considerable heterogeneity between the analyzed trials,<sup>27</sup> and low<sup>28</sup> to intermediate<sup>29</sup> quality of evidence. Of note, when each of the analyzed trials is considered independently, the majority of works did not find a significant effect of probiotics on *C. difficile*-associated diarrhea (CDAD) or *C. difficile* infection.<sup>30-60</sup> Studies that did find a significant positive effect of probiotics in these conditions were associated with a rarely encountered *C. difficile* infection rate of over 5%,<sup>61-63</sup> and one trial even associated probiotics use with enhanced incidence of *C. difficile* infections.<sup>64</sup> These conflicting results greatly highlight a need for high-quality, sufficiently powered prospective trials to resolve the usefulness of probiotics in this and other indications. Conflicting findings were also

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reported in the context of irritable bowel syndrome (IBS),<sup>65-67</sup> respiratory infections,<sup>12,68-84</sup> *Helicobacter pylori* infections,<sup>85-88</sup> inflammatory bowel disease,<sup>89</sup> improving antiretroviral treatment,<sup>90,91</sup> and the metabolic syndrome.<sup>92,93</sup> A more convincing indication of probiotics has been recently documented in a neonatal population at high risk of sepsis, in which synbiotic supplementation (*Lactobacillus plantarum* plus prebiotic fructooligosaccharide) significantly prevented morbidity and mortality.<sup>94</sup> In contrast, a 2014 Cochrane meta-analysis reported a beneficial effect for various probiotic regimens in the prevention of necrotizing enterocolitis (NEC) and all-cause mortality in preterm infants, but not sepsis,<sup>95</sup> suggesting that efficacy might depend on additional factors, such as baseline risk or the addition of prebiotics. Evidence regarding probiotics efficacy in preventing NEC are equally equivocal, as a later multi-center trial with >1300 infants found no effect for *B. breve* BBG-001 in this indication.<sup>96</sup>

This confusing reality resulted in a peculiar state, in which, on the one hand, probiotics are not approved as medical interventions by regulatory authorities such as the US Food and Drug Administration<sup>97</sup> and the European Food Safety Authority,<sup>98</sup> and on the other, they are globalized, popularized and integrated into foods, cosmetics, and supplements.<sup>99</sup> This discrepancy highlights the great interest of the public in probiotics, and the concurrent need to improve the state of evidence with regard to this therapeutic modality.

### **Circumventing old obstacles with new technology**

The considerable variation in outcome between trials may be attributed to multiple factors, including the wide array of studied microorganisms in each context, heterogeneity between participants in response to probiotics, and technical differences between studies. Discrepancies associated with the treatment may be related to species<sup>100</sup>- and strain<sup>101</sup>-specific probiotic traits, resulting in differential clinical efficacy between trials comparing various probiotic microorganisms;<sup>102</sup> in the case of commercially available products, the batch-to-batch variation might result in different outcomes.<sup>103</sup> Host factors that might affect probiotics colonization

and/or efficacy include diet,<sup>104</sup> age,<sup>105,106</sup> antibiotics exposure,<sup>107,108</sup> underlying medical conditions,<sup>109,110</sup> and baseline microbiome composition<sup>111-113</sup> and function.<sup>114,115</sup> Methodological advances in next-generation sequencing, metabolomics, and gnotobiotics, now enable a better understanding of concepts such as colonization resistance or permissiveness to exogenous microorganisms, biogeographical diversity, inter-individual variability in microbiome configuration, and how these may affect the response to therapies.

In our recent studies<sup>107,111</sup> we aimed at tackling elementary questions regarding the current commercially available probiotics by employing multi-omic analyses complemented by *in vivo* and *in vitro* experimentation, while disentangling potential confounding factors that may arise from next-generation sequencing data. Our focus and design were not purposed to show clinical benefit or harm to the host, but rather to invasively and directly characterize the effects of exogenous probiotic microorganisms administration on the gut mucosa and the indigenous microbiome at various sites along the gastrointestinal tract. To that end, we subjected human volunteers to probiotics and other treatments and collected sequential stool samples before, during and after the interventions. Additionally, we performed endoscopic examinations of their gastrointestinal tract to obtain mucosal and luminal contents before and during the intervention. Stool, mucosal, and luminal samples underwent 16S rRNA and shotgun sequencing to assess the microbiome component, while mucosal biopsies underwent RNA sequencing to directly assess the host. We further utilized probiotics species-specific quantitative PCR to achieve absolute abundances of selected bacteria. The entire study design was replicated in mice.

By this combined approach we aimed to overcome several shortcomings in probiotics research: First, the vast majority of previous studies have inferred mucosal colonization of probiotics from their persistence in stool after discontinuing the treatment with the probiotic preparation, and thus were unable to determine mucosal adherence of these strains during the period of administration. We directly sampled the mucosal microbiome to bypass this limitation. Second, we set out to

sample luminal and mucosal samples throughout the entirety of the human digestive tract, reachable proximally and distally by an endoscope, to account for biogeographical differences. Third, we characterized the human transcriptome in different regions of the gastrointestinal tract and evaluated its alterations in response to various treatments. Furthermore, we delineated gene expression landscapes as harbingers of response to probiotics. Fourth, we examined carefully selected 11 probiotic strains, which on the one hand, shed light on strain-dependent impacts on the host and the microbiome, and on the other hand, featured multiple representative strains of the most commonly used probiotic genera (*Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus*), thereby enabling to assess class effects. Finally, in the most invasive study of its kind, we portrayed the host and the microbiome, both before and during probiotics exposure, thereby allowing for a person-specific evaluation.

### The colonization debate

The question whether allochthonous probiotic strains are capable of colonizing the gut, and whether this colonization is transient or persists after cessation of probiotics consumption, remains highly debated. Based on the constantly expanding body of knowledge regarding interactions between the host and resident commensal and pathogenic bacteria, it is logical to assume that probiotics may require contact with, or approximation to the host epithelium to exert direct, or metabolite-mediated effects. For example, the sortase-dependent pili of *Bifidobacterium bifidum* mediate adherence to the host cells and tumor necrosis factor (TNF)-alpha production;<sup>116</sup> Similarly, the SpaC pili of *Lactobacillus rhamnosus* are required for adherence to the gut mucosa, stimulation of reactive oxygen species production, and promotion of cell proliferation and protection against intestinal injury,<sup>117</sup> and the degree of *Lactobacillus reuteri* strains' adhesion to the mouse ileal mucosa is correlated with their immunomodulatory properties.<sup>118</sup> Competitive exclusion of pathogens from the gut mucosa and their displacement has been suggested to be among the antagonistic mechanisms of probiotics.<sup>112,119-122</sup> A beneficial

effect of probiotic microorganisms on intestinal barrier integrity may also require interaction with the epithelium: Binding of *L. casei* GG to specific receptors on enterocytes is required to upregulate MUC2,<sup>123</sup> and adhesion of *Lactobacillus* strains to epithelial cells enhances production of MUC3.<sup>124</sup>

However, whether such probiotics mucosal colonization universally exists remained debated. A handful of studies directly sampling the host mucosa in humans<sup>125-128</sup> and porcine models<sup>129,130</sup> suggested that probiotics universally inhabit the gut mucosa during administration or shortly thereafter, while others have shown inconsistencies across participants and sites along the gastrointestinal tract,<sup>131-134</sup> however due to relatively small sample sizes, host and microbiome factors that promote or resist colonization have not been fully elucidated in these studies. Other non-invasive works, which examined probiotic strains presence in stool samples as a proxy for their persistence in the gut mucosa following cessation of their administration, have yielded similarly confusing findings, as probiotics were detected only in subsets of examined human individuals<sup>112,133-145</sup> and rats.<sup>113</sup> Several other recent works associated the extent to which supplemented probiotics colonized the guts of animals or humans to their ability to ameliorate colitis<sup>146</sup> (in mice), depression<sup>147</sup> (in rats) or IBS (in humans).<sup>148</sup>

Some researchers would still argue that probiotics might benefit the host without even transiently being detected at the proximity of the host-microbiome mucosal interface.<sup>149,150</sup> As colonization of the human intestinal mucosa with probiotics has been quantified *in situ* only in a limited number of trials, and none have demonstrated an effect on the host in the absence of mucosal colonization, this hypothesis remains to be experimentally validated. Current indirect evidence for an effect exerted by probiotic microorganisms that do not actively engage the mucosa stem from trials with killed bacteria,<sup>151-153</sup> or those conducted with their secretomes.<sup>112,154-156</sup> Most probably, even mechanisms of activity involving signaling to the host through metabolite secretion or passive sensing of surface molecules<sup>149</sup> necessitate microbial mucosal approximation to enable effective metabolite concentrations to reach their epithelial/immune cellular targets through the mucus layer. In future trials,

next-generation sequencing can be utilized to determine the physiological state of probiotic bacteria in mucosal samples, and how it affects their ability to exert an effect on the host.<sup>157</sup> Importantly, our recent studies directly assessing the presence of probiotics along the entirety of the human gastrointestinal tract<sup>107,111</sup> failed to detect exogenously consumed strains among participants resistant to probiotics mucosal colonization during their consumption even in **luminal** samples (Figure 1), rendering even putative luminal activities of probiotics, such as degradation of dietary lactose<sup>158</sup> or deconjugation of bile salts<sup>159</sup> unlikely in those individuals. Collectively, these results suggest that in the absence of mucosal colonization, a consistent luminal presence of probiotics is debated and limited at best, with consumed probiotics rapidly finding their way into stool.<sup>111</sup>

The introduction of next-generation techniques has considerably improved our ability to address the colonization question even at strain resolution to differentiate between endogenous and exogenous bacteria.<sup>111,114</sup> In our recent studies we showed that regardless of global shedding in stool, even during supplementation to healthy treatment-naïve

individuals, probiotic strains did not colonize the gut mucosa equally among participants, and we categorized them as probiotics ‘resisters’ or ‘permissives’ according to their level of colonization. This personalized colonization capacity may stem from host factors, as we showed that ‘resister’ and ‘permissive’ individuals differed in mucosal immune-related gene expression in some gastrointestinal organs. Additionally, it may also result from autochthonous bacteria-mediated colonization resistance, which we could demonstrate through several observations: First, a reduction or a complete lack of gut microbiome seen in antibiotics-treated or germ-free mice, respectively, exhibited improved probiotics colonization; Second, ‘humanized’ mice, transplanted with feces derived from ‘resister’ or ‘permissive’ humans, which were treated with oral probiotics, recapitulated the degree of colonization resistance to probiotics of the donor. Our data support the notion that colonization resistance patterns follow the phylogenetic exclusion principle. Of note, in many of the aforementioned studies colonization was assessed by enumeration of the probiotic bacteria in feces, and not directly by mucosal sampling,



**Figure 1.** Luminal presence of supplement-specific probiotics strains in human individuals.

Probiotics strain quantification based on mapping of metagenomic sequences to unique genes, which correspond to the strains found in the supplemented probiotics pill, in the descending colon lumen of healthy individuals treated with an 11-strain probiotic mix (N = 10) or placebo (N = 5). Permissive individuals are those, who were significantly colonized by probiotics in the lower GI mucosa, compared to resistant individuals, in whom no significant colonization of the mucosa was determined. Red, the sample contains the same probiotic strain present in the supplement; Dark gray, the sample contains a different strain of the same species. Strain identification was performed as previously described.<sup>111</sup> LAC, *Lactobacillus acidophilus*; LCA, *Lactobacillus casei*; LPA, *Lactobacillus casei* sbsp. *paracasei*; LPL, *Lactobacillus plantarum*; LRH, *Lactobacillus rhamnosus*; BLO, *Bifidobacterium longum*; BBI, *Bifidobacterium bifidum*; BBR, *Bifidobacterium breve*; LLA, *Lactococcus lactis*; STH, *Streptococcus thermophilus*.

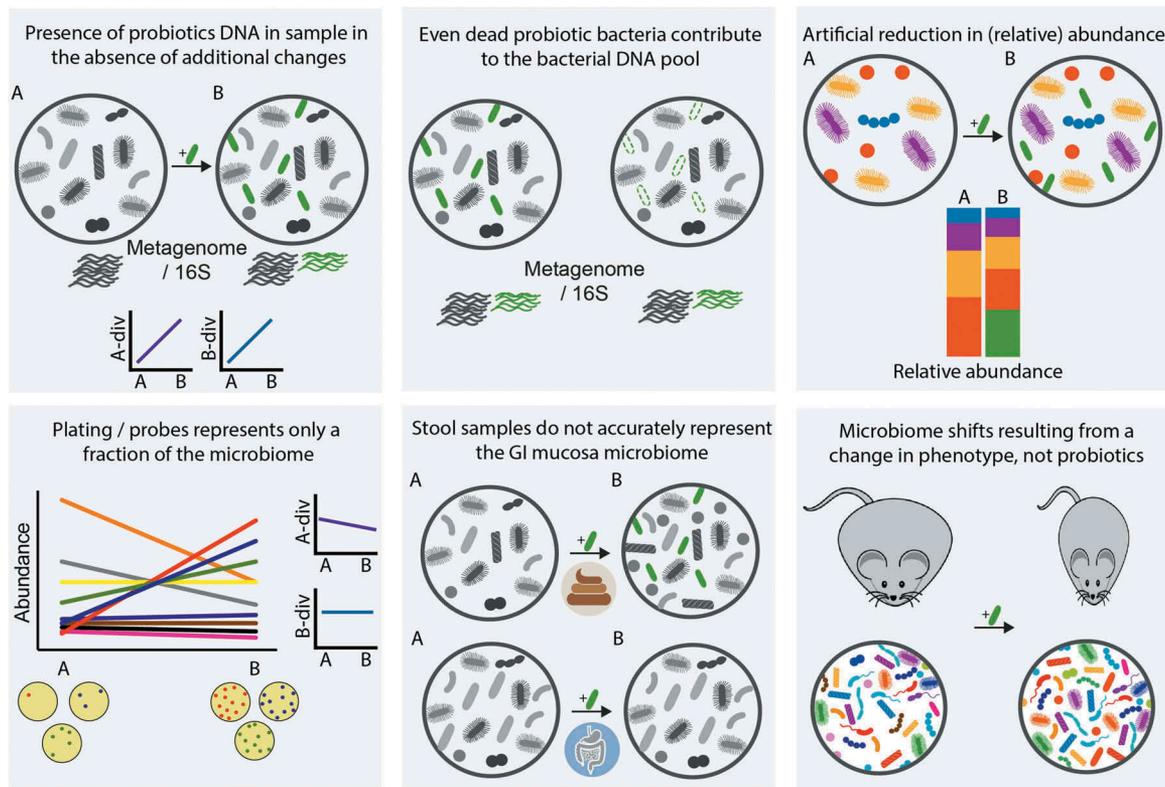
and no causative colonization-modulating effect was demonstrated for the indigenous microbiome or host gut function. We showed that probiotics affected ‘permissive’ individuals differently than ‘resisters’ in terms of their cecal and colonic transcriptional landscape. Collectively, there is sufficient evidence to conclude that probiotics adherence to the gut mucosa is adjusted by a host- and microbiome-driven colonization resistance, and that mucosal colonization may be a prerequisite for mediating some, if not most, microbiome alterations and clinical effects on the host.

### Probiotics effect on the microbiome

In addition to an effect on the host exerted through direct probiotic-host cell interactions, probiotics may indirectly act through modulation of the indigenous commensal microbial community. In their 2014 consensus statement regarding the definition of probiotics, the International Scientific Association for Probiotics and Prebiotics states “modulation of perturbed microbiota” as a widespread activity, shared across probiotics genera. An even more generalized statement regarding probiotics ability to beneficially modulate the microbiota is often promoted by other lobbying and commercial entities. Nonetheless, due to the complex nature of host-microbiome interactions, in which the same bacteria or metabolite can produce context-dependent opposing effects (reviewed in<sup>160</sup>), it is often difficult to attribute definitions such as “beneficial” or “detrimental” to a microbial assemblage. To achieve such definitions, a microbiome altered by probiotics should produce a beneficial effect upon transplantation to gnotobiotic animals, however, such experiments are so far surprisingly lacking. Regardless of the physiological effect, even the ability of probiotics to modulate the microbiome is contested. The majority of trials do not support the claim that probiotics can modulate the microbiome in the absence of prior perturbation, as reflected by systematic reviews that found such evidence only in 14%,<sup>161</sup> 20%,<sup>162</sup> and 21%<sup>108</sup> of trials included in the analyses. Even this limited number of supportive trials should be considered with care, as in some cases, the mere presence of administered probiotics species in a stool sample was reported as microbiome alterations, and in others, a reduction in relative

abundance of taxa might be a concomitant result of adding the exogenous probiotics species, rather than an actual effect.<sup>163,164</sup> An additional limitation in some of the trials is the use of culture- or probe-based methods of limited scope to characterize the effect of probiotics on the microbiome, thus giving excessive weight to specific taxonomic units of choice, yet without the ability to report on global community measurements such as alpha and beta diversity. Nonetheless, even global community measurements such as 16S rDNA analysis, may be confounded by the presence of the administered probiotics, due to their limited resolution beyond the genus level. Finally, as mentioned above, stool samples may not accurately mirror an effect (or the lack of it) in the gut mucosal niche. These common limitations in studies assessing probiotics effect on the microbiome are summarized in [Figure 2](#).

In our work, we sought to address these limitations by employing a multi-omic, invasive bio-geographical analysis of two homeostatic hosts: mice and humans. Indeed, the microbiome of mice supplemented with an 11-strain probiotic mixture displayed a bio-geographical discrepancy, as probiotics significantly elevated the alpha and beta diversity of the lower gastrointestinal (LGI) tract mucosal, but not the fecal, microbiome. In humans, probiotics affected both the composition and the function of the fecal microbiome, as well as the total bacterial load, compared to baseline or placebo, with several species, genes and pathways altered even one month following cessation of probiotics supplementation. In contrast, no significant effect was observed in the LGI lumen and mucosa. Functional alterations to the fecal microbiome were also reported in several trials, though these may be attributed to the probiotics species themselves.<sup>114,165-167</sup> Importantly, the extent to which probiotics affected the microbiome was associated with their colonization capacity. In our study, changes to the fecal microbiome composition, function, diversity, and bacterial load were significantly more pronounced in ‘permissive’ individuals, which displayed significant probiotics colonization in their gut mucosa. Similarly, both the mucosal microbiome composition and bacterial load were more affected in ‘permissive’ individuals.<sup>111</sup> This observation is in line with a report by Zhang



**Figure 2.** Common biases and challenges observed in studies assessing probiotics effect on microbiome configuration. Supplemented probiotic bacteria are illustrated in green. A-div, alpha diversity; B-div, beta diversity. A, before supplementation; B, during or after supplementation.

*et al.*, in which individuals colonized with *Lactococcus lactis* given as a component of a probiotic food supplement had a greater effect on fecal microbiome beta diversity, compared to colonization-resistant individuals.<sup>113</sup> To conclude, probiotics may have a limited effect on the microbiome composition and potentially function, which may depend on the ability of the administered microorganisms to colonize the host, at least transiently.

Further support to the importance of probiotics colonization in inducing microbiome alterations stems from trials in antibiotics-perturbed subjects. In our study,<sup>107</sup> we administered probiotics after the cessation of antibiotics and an invasive assessment of antibiotics-associated dysbiosis, thereby enabling to accurately quantify the impact of probiotics on the host and the microbiome without having the probiotics strains present and confounding during baseline measurements. Surprisingly, while antibiotics-induced depletion of the indigenous microbiome enhanced the colonization of probiotics in the LGI

mucosa of humans (but only marginally in mice), they persistently inhibited the restoration of the pre-antibiotics microbial community composition, function, diversity and bacterial load in both mice and humans, as well as the human naïve gut transcriptional landscape, compared to both autologous fecal microbiota transplantation (FMT) and spontaneous recovery. Our study's design suggests that this probiotics-induced inhibition of microbiome and host reversion to their naïve state would probably be even more pronounced, if probiotics were allowed to colonize the host also during the administration of antibiotics.

*In vitro*, we demonstrated that this inhibition might be mediated by soluble factors secreted by *Lactobacillus spp.*<sup>107</sup> Surprisingly, while many probiotic species were reported to possess anti-bacterial properties through the production of acid<sup>168,169</sup> or bacteriocins,<sup>130,170,171</sup> these have been so far described solely in the context of pathogens, despite no mechanistic explanation to support a pathogen-specific, commensal-tolerant activity. In line with this notion,

several studies provided data that do not support a beneficial effect for probiotics in the recovery of the microbiome from antibiotics or even its inhibition. In mice, a four-strain probiotic mixture did not result in an improved alpha-diversity when given either during or following antibiotics, and was in fact associated with lower diversity compared to no treatment.<sup>172</sup> In humans, a combination of *L. acidophilus* and *B. bifidum* given during antibiotics resulted in a lower restoration to baseline of anaerobes, facultative anaerobes and specifically *Bacteroides* compared to placebo or probiotics given following antibiotics,<sup>173</sup> and administration of *S. boulardii* CNCM I-745 during antibiotics did not restore the microbiome to an antibiotics-naïve configuration, and reduced bacterial diversity compared to no intervention.<sup>174</sup> Importantly, prolonged antibiotics-associated dysbiosis and reduced diversity may expose the host to long-term complications due to infectious and other non-communicable diseases.<sup>175-192</sup>

To conclude, while antibiotics may alleviate colonization resistance to probiotics and enable their supposedly beneficial ‘place-holder’ effect against pathogen colonization (an effect that remains to be thoroughly validated in humans), this may result in inhibition, rather than restoration of the indigenous gut microbiome and host gut function. As prolonged dysbiosis may have considerable health implications, this previously underappreciated trade-off merits further clinical exploration. We suggest a framework that combines longitudinal whole community composition and function characterization, utilizing both relative and absolute quantifications and prospective long-term and sufficiently powered clinical assessment.

## Perspective

The reports discussed in this commentary point to several aspects in which methodological advances might improve our understanding of probiotics-host-microbiome interactions. First, a strain-level resolution achieved with shotgun sequencing enables to distinguish the supplemented organism from closely related strains already present in the sample. Synthesizing studies that utilized this approach lead to the conclusion that intestinal colonization with probiotics was observed only in

some individuals, both in the long term and even during the supplementation itself. Strain-level resolution and functional microbiome analysis also point to the prior presence of phylogenetically related species, or functional redundancy of the resident microbiome with that contributed by the supplemented probiotic, as factors inversely correlated with colonization. While global shedding in stool masks heterogeneity in gut mucosal colonization, fecal samples may still be used to predict permissiveness to colonization. Transplantations of these fecal samples into germ-free mice enable to validate a causative role for the resident microbiome in dictating colonization resistance, and may be further utilized to identify specific crucial factors required for allochthonous colonization. Importantly, due to the complexity of sampling the GI mucosa, it would be useful to develop computational algorithms that predict mucosal colonization based on sequencing of fecal samples.

Advancing from limited culture- and probe-based techniques, the combined knowledge from 16S rDNA, metagenomics, and metabolomics now enables better elucidation of probiotics colonization, and direct or microbiome-mediated impacts (or their lack thereof) on the human host, paving the way for improved efficacy and safety of probiotics.

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## Author contributions

All authors have researched data for the article, made substantial contribution to the discussion of content, and wrote, reviewed and edited the manuscript before submission.

## Disclosure of potential conflicts of interest

EE is a paid consultant at DayTwo and BiomX. None of his work on probiotics is related to, funded or endorsed by, shared or discussed with or licensed to any commercial entity.

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