



The pros, cons, and many unknowns of probiotics

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1 The pros, the cons, and the many unknowns of probiotics

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44 Abstract

Consumption of over-the-counter probiotics has been globalized in recent years. Emerging health trends, extensive commercial endorsement, and conflicting clinical results have led to a highly polarized state, in which, on the one hand, probiotics use has been greatly popularized by the general public, but on the other hand many proposed probiotics health indications remain non-sufficiently substantiated, and are accompanied by a highly debated medical literature. Emerging insights from the microbiome field now enable a re-assessment of probiotics gut colonization, strain-level activity, interactions with the indigenous microbiome, safety and impacts on the eukaryotic host, in reaching more comprehensive conclusions on physiological effects and potentially useful medical indications. In this perspective, we will highlight key advances, challenges, and limitations in striving towards an unbiased interpretation of the large, but often debatable data regarding over-the-counter probiotics, and propose avenues to improve the quality of evidence, transparency, public awareness, and regulation of their use.

81 Introduction

82 The concept of oral consumption of microorganisms as means of inducing health benefits has 83 intrigued humans for centuries. The term 'probiotics' first appeared in this context in 1974 and 84 conceptually evolved to the current common definition suggested by the FAO/WHO in 2002: 85 "live microorganisms, which, when administered in adequate amounts, confer a health benefit 86 on the host"¹. Nowadays, over-the-counter microbial therapy constitutes a constantly growing 87 multi-billion-dollar industry² and is one of the most commonly consumed forms of food 88 supplements worldwide³. Probiotics are supplemented to foods such as yogurt, cheese, ice 89 cream, snacks and nutritional bars, breakfast cereals, and infant formulas, as well as cosmetic 90 products, and are also commercialized in the form of lyophilized pills⁴. Probiotics consumption 91 is widely supported by physicians⁵, and specifically gastroenterologists⁶. This popularity 92 notwithstanding, data from decades of research on the efficacy of probiotics in treating or 93 preventing disease often points towards opposing conclusions, and remains conflicting, debated 94 and confusing in many cases. Moreover, the major medical regulatory authorities, such as the 95 European Food Safety Authority⁷ or the US Food and Drug Administration⁸, have yet to approve 96 any probiotic formulation as a medical intervention modality. As a result, probiotics marketing 97 as dietary supplements is often driven by properties such as safety, viability in the GI tract and 98 lack of impact on food taste, rather than by unequivocal health-promoting effects⁹. This 99 confusing state merits seeking better evidence-based proofs of probiotics impacts on humans 100 and their adverse effects¹⁰. In this perspective, we will highlight and discuss some of the major 101 prospects and limitations of the current approach to probiotics, present challenges in 102 interpretation of available data, and suggest possible strategies to clarify these issues and 103 transform probiotics into a more reproducible and universally accepted measurement-based 104 approach.

105 In providing this critical perspective, we would like to emphasize that the reviewed over-the-106 counter microbial interventions will be termed 'probiotics' regardless of their benefit, efficacy 107 or lack thereof. Importantly, while aiming to offer a critical overview of the state of probiotics, 108 we do not wish to 'throw the baby out with the bathwater'. The uncertainty created by some of 109 the opposing evidence with regards to probiotics notwithstanding, we conceptually believe that 110 rigorous research and regulation has a promising potential of materializing into an effective 111 medical intervention in selected indications, some of which are exemplified below. Of note, this 112 perspective is not aimed at reviewing investigational, non-commercially available "next 113 generation" microbial therapy approaches that are being proposed as interventions in various 114 medical indications. These are discussed elsewhere^{11,12}.

115 It is unrealistic to include all probiotics studies and their suggested indications in one 116 perspective. Therefore, we will highlight notable examples to discuss **A**. The 'knowns' and 117 challenges with respect to strength of evidence and clinical interpretation of studies assessing health benefits of probiotics. B. Suggested mechanisms of probiotics, touching upon the gut
colonization debate C. Interactions of probiotic strains with the gut microbiome. D. Safety, and
E. Future directions.

121

122 Clinical efficacy

123 The effects of probiotics on humans have been extensively studied both by scientists and the 124 food and drug industry for decades, leading to multiple suggested prophylactic and therapeutic 125 health indications and claims, including prevention or treatment of acute, antibiotic-associated, 126 and *Clostridium difficile*-associated diarrhea, amelioration of inflammatory bowel disease and 127 irritable bowel syndrome and risk reduction for neonatal late-onset sepsis and necrotizing 128 enterocolitis. Other claims include, among many others, eradication of Helicobacter pylori, 129 reduction in incidence and severity of respiratory infections, alleviation of depression, 130 prevention or treatment of atopic dermatitis and reduction of cardiovascular risk factors 131 associated with cardiometabolic syndrome¹³. Regretfully, despite the fact that some clinical 132 trials related to the above health claims are of high methodological quality and validity¹⁴⁻¹⁸, 133 careful examination of the large body of evidence reveals that, for most of the above indications, 134 there are also studies of similarly high methodological quality featuring negative or opposing 135 results, collectively leading to conflicting, ambiguous and debatable overall conclusions.

136 This confusing situation may stem from a number of reasons, including the fact that many of the 137 probiotics trial readouts are based on empiric clinical data that varies in its collection 138 methodology, clinical end-points, and analytical rigor. Many reports use of qualitative, self-139 reported parameters of "well-being"^{19,20}, others provide quantification of markers that do not 140 necessarily have clinical significance, for example reduction of C-reactive protein (CRP) in 141 healthy individuals²¹, or elevation of glucose-stimulated glucagon-like peptide 1 (GLP-1) in 142 glucose-tolerant individuals²². Likewise, a great variability exists as to the systems analyzed, 143 ranging from cell cultures, in vitro studies, animal models and human studies spanning 144 observational or randomized, placebo-controlled trials. At times, even within high quality 145 placebo-controlled studies, probiotics putative benefits are conflicting between trials^{23,24}.

Another contributor to the variability between probiotics studies is the profusion of studied 146 147 microorganisms. With observations made over a century ago^{25,26}, the dominant microorganisms 148 used in the probiotics industry even nowadays belong to two genera: Lactobacillus and 149 *Bifidobacterium.* Each of these genera includes multiple species, subspecies and strains that 150 feature with both class effects and, in some cases, distinct strain-specific traits. Additional 151 common microorganisms used in the probiotics industry include *Lactococcus spp., Streptococcus* 152 thermophilus, E. coli Nissle 1917, and the yeast Saccharomyces boulardii²⁷. Importantly, some 153 health benefits may require interaction between different strains, contrasting with the current 154 approach of considering probiotics as a homogenous therapeutic entity.

155 To counteract the above methodological and analytical limitations and to overcome 156 underpowered findings researchers and clinicians frequently integrate results from multiple 157 studies in the form of systematic reviews and meta-analyses. The use of such tools may be 158 highly useful in revealing general trends, however it may also be susceptible to biases that can 159 be introduced in each analytical step²⁸, such as obscuring actual effects or their lack thereof by 160 outlier studies that dominate the collective results and artificially resolving contradictory 161 trends. In particular, meta-analyses concerning probiotics tend, at times, to group studies 162 testing various unrelated supplemented bacteria under the same umbrella, thereby risking 163 over- or misinterpretation of results^{29,30}. Consequently, even meta-analyses addressing similar 164 topics may conflict each other^{31,32}. Thus, in our view, meta-analyses can complement, but not 165 replace high-quality, large-scale, multi-center, randomized controlled clinical trials.

Moreover, unlike animal models, humans are highly heterogeneous in terms of diet, age range, genetic background and their gut microbiome configuration, and may therefore respond differently to the same intervention (**Fig. 1**). Nevertheless, these readily measurable personalization issues have not been sufficiently addressed in the probiotics literature. As described in the 'Gut colonization' section below, humans feature a differential and highly personalized gut colonization capacity for probiotics, which may drive differential probiotics effects on the host and/or on its indigenous gut microbiome.

Finally, many of the probiotics studies are linked, funded, initiated and endorsed by commercial entities of the probiotic industry or by professional lobby groups heavily associated and funded by the same industry³³. While this reality by itself does not necessarily compromise the validity of such studies, there is a need and interest in independent corroboration of efficacy claims to be reproduced through non-affiliated research by scientific and medical entities. Examples of some of the most notable suggested probiotics indications include:

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180 Acute gastroenteritis. Probiotics have been suggested to be effective as preventive or 181 therapeutic means in various pediatric and adult etiologies manifesting as acute diarrhea. 182 Several meta-analyses and systematic reviews indicate that some preparations³⁴, especially 183 those containing S. boulardii³⁵, Lactobacillus rhamnosus GG (LGG)³⁶ and other strains within the 184 Lactobacillus genus³⁷ may ameliorate acute diarrhea in children and shorten its duration by 185 approximately one day. Likewise, probiotics have been shown effective in the prevention and 186 treatment of acute diarrhea in adults, and various preparations, in particular S. boulardii and L. 187 rhamnosus, have been suggested to improve antibiotic-associated diarrhea both in healthy 188 children^{38,39}, adults^{40,41}, and in hospitalized patients⁴². In contrast, other studies and meta-189 analyses have shown contradictory results as for diarrhea prevention in children⁴³, adults²³, and 190 in the elderly^{41,44}. Notably, the results of two recent high-quality, large-scale, multi-center, 191 randomized placebo-controlled trials assessing treatment with L. rhamnosus (LGG or R0011)

192 with or without *L. helveticus* R0052 in over 1800 children presenting to the emergency 193 department with acute gastroenteritis demonstrated no clinical benefits^{45,46}. One meta-analysis 194 in children has noted that the quality of evidence with regard to this indication was low to very 195 low⁴⁷, leading to the omission of probiotics from one clinical management guidelines⁴⁸, whereas 196 another still advocates the use of *LGG* and *S. boulardii* while stating that the evidence upon 197 which these recommendations are based is of low quality⁴⁹. Notwithstanding the dispute, many parents "self-treat" their children, when contracted with gastroenteritis, with "functional foods" 198 199 containing probiotics⁵⁰.

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201 *Clostridium difficile-associated diarrhea (CDAD)*. *Clostridium difficile* thrives in the gut when 202 microbiome-conferred colonization resistance is compromised, such as upon antibiotics 203 treatment in hospitalized patients, thereby causing a disease that can range in severity from 204 mild diarrhea to a life-threatening condition termed pseudo-membranous colitis. Several meta-205 analyses have shown a cumulative beneficial outcome for probiotics in preventing C. difficile 206 infection or its associated morbidity⁵¹, especially when administered close to antibiotics 207 exposure⁵². A follow up 2017 meta-analysis further supported moderate beneficial evidence, 208 but indicated a considerable heterogeneity between trials, and utilized a post-hoc analysis that 209 suggested no significant effect to probiotics on CDAD in trials featuring low and moderate 210 baseline CDAD risk⁵³. Another meta-analysis concluded that of the various probiotic strains, 211 only S. boulardii was effective against C. difficile⁵⁴, though a different meta-analysis relating 212 specifically to *S. boulardii* found that it reduced CDAD risk in children, but not in adults⁵⁵, with 213 low quality of evidence noted⁵⁶.

214 In taking a closer look on the individual studies forming the basis of these meta-analyses, C. 215 *difficile* incidence was non-existent (8 trials, **Table 1**) or low in the majority of the trials 216 regardless of treatment group, while the vast majority of trials included in meta-analyses (34 217 trials, **Table 1**) did not demonstrate a significant effect for probiotics of different strains on 218 CDAD or *C. difficile* infection. While this may be related to insufficient power of these studies to 219 demonstrate an effect in the context of low incidence of C. difficile, two RCTs featuring 220 populations with a high incidence of *C. difficile,* including the largest trial of probiotics for this 221 indication to date, did not find a difference between the treatment and placebo groups^{44,57}. Thus, 222 the effects observed in meta-analyses are mostly contributed by a minority of works 223 demonstrating a significant effect^{18,42,58-61}, of which two are non-peer-reviewed conference 224 abstracts^{62,63}. While *C. difficile* incidence in the placebo group was very high^{18,42,62} in most works 225 showing a beneficial effect^{18,42,58,59,61}, other works, in which CDAD was uncommon pointed 226 towards a lower level of evidence with respect to probiotics efficacy in preventing CDAD^{33,64}. 227 Together, variable baseline risk of CDAD among cohorts may potentially explain the differences

in outcomes between studies, as well as the fact that the majority of meta-analyses aggregated
 studies testing a variety of probiotic strains, both fungal and bacterial⁶⁵.

230

231 Irritable Bowel Syndrome (IBS) and digestive complains. IBS is a common and clinically 232 variable disorder of unclear etiology. Trials assessing interventions to alleviate IBS are often 233 limited by the fact that this condition is defined by subjective criteria. As such, it is of paramount 234 importance to ensure that symptoms alleviation by probiotics is not equal or inferior to that of a 235 placebo effect⁶⁶. One recent meta-analysis has suggested that probiotics may be efficacious in 236 treating symptoms of IBS⁶⁷, although it should be noted that none of the single strain 237 preparations was proven effective for abdominal pain alleviation or for treatment of bloating, 238 flatulence and urgency. Even within probiotic combinations some were found effective in 239 reducing symptom persistence and abdominal pain scores, while others were not, emphasizing 240 the importance of informed strain selection on disease outcome. Correspondingly, a systematic 241 review of 9 systematic reviews and 35 RCTs did not find evidence for various probiotic strains 242 efficacy in IBS68.

243

244 Neonatal sepsis. A promising indication for the efficacy of probiotics is the prevention of 245 neonatal late-onset sepsis and/or necrotizing enterocolitis (NEC), a gastrointestinal disease 246 typically affecting premature newborns^{69,70}. The protective mechanism against NEC may involve 247 anti-pathogen mucosal protection, coupled with induction of maturation of innate immunity and 248 intestinal epithelial cells by some probiotic strains (such as *LGG*), which prompt an attenuated 249 inflammatory response^{71,72}. Furthermore, a recent large-scale RCT strengthened these findings 250 by showing that rural Indian infants who received a combination of oral preparation of L. 251 plantarum PP 11-217 and fructooligosaccharide were protected from neonatal sepsis and 252 death¹⁴. It still remains debated whether probiotics reduces the risk for late-onset sepsis in 253 extremely low birth weight neonates⁷³⁻⁷⁵, and whether milk-fed preterm infants feature a better 254 response to this intervention as compared to formula-fed or infants kept on mixed feeding⁷⁶. 255 Importantly, the long-term consequences of probiotics on the development of the indigenous 256 gut microbiome and their effect on gut immune, metabolic, and anatomical development⁷⁷ 257 warrants further studies.

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Acute respiratory infection. Some systematic reviews and meta-analyses of studies empirically testing probiotic strains, suggest that they may be effective in reducing the severity, duration or incidence of common cold, respiratory infections and influenza-like symptoms in children, adults, the elderly, and even in athletes^{78,79}, however quality of evidence was stated as low to very-low and heterogeneity between studies was deemed significant. A meta-analysis encompassing both children and adult studies proposed that probiotics might reduce the

265 severity and duration of respiratory tract infections, but not their incidence⁸⁰. These 266 discrepancies may stem, at times, from reliance on subjective or indirect measures to assess infection, such as self-reporting⁸¹⁻⁸⁴, or antibiotic treatment and days of absence from 267 268 work/daycare^{78,85}. Discrepancies may also result from unadjusted results when treatment groups were different at baseline⁸⁵, subsampling with no clear clinical or biological 269 270 justification^{86,87}, unexplained exclusion of trials from meta-analyses⁷⁸, and attributing an effect 271 to treatment despite a counter-intuitive dose-response relationship⁸⁷. On a causal level, there is 272 a great need of a data-driven explanation of mechanisms by which gastrointestinal-localized 273 probiotics would impact a disease involving a remote organ.

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275 Gut colonization

276 An unresolved issue associated with probiotics mechanisms of action relates to the 277 administered microorganisms capacity to stably or even transiently colonize the host 278 gastrointestinal mucosal surface, and whether their colonization is necessary to exert beneficial 279 impacts on the host. The proximity of probiotic strains to the host lining epithelial layer may be 280 mechanistically crucial, as mucosal adhesion or even presence at low titers, may provide the 281 micrometer distance of probiotics strains to the host gastrointestinal epithelium, which is a 282 prerequisite for many activities including contact-dependent immune modulation^{88,89}, 283 metabolite secretion in effective concentrations⁹⁰, and mucus layer modification⁹¹. This 284 decades-long debate is comprised of two inherently distinct colonization-related questions, 285 which have often been confusingly intermingled with each other, in the absence of concrete 286 experimental data:

287 Question 1: Do probiotics colonize the gut mucosa during consumption? Surprisingly, this 288 critically important topic has not been directly explored in a comprehensive manner in humans 289 until recently. Most probiotics colonization claims have been extrapolated from assessment of 290 their abundance in stool, without directly examining whether this actually reflects their 291 colonization capacity, or merely a passage of non-engaging microbes across the GI tract and 292 their excretion into stool⁹². Like stool assessment, probiotics adherence to human 293 gastrointestinal cells *in vitro*^{93,94} may be a poor indicator of *in vivo* colonization due to a myriad 294 of host and microbiome factors that are absent in the *in vitro* setting.

Direct quantification of mucosal probiotics colonization was determined by endoscopies in a handful of trials, with some studies in humans⁹⁵⁻⁹⁸ and pigs^{99,100} suggesting that probiotic bacteria could be universally isolated from various gastrointestinal organs during or even after supplementation, while others showing a highly limited and variable colonization patterns, observed in only a minority of tested individuals¹⁰¹⁻¹⁰⁴. Noteworthy, the universal utilization of culturing or 16S rDNA techniques in these studies considerably limits the ability to distinguish between the administered probiotic strain and endogenous closely related commensals of the

302 same species/genus (see **Box 1**). A species- and strain-sensitive metagenomic assessment of 303 human participants evaluated by colonoscopy and gastroscopy before and after consumption of 304 11 probiotic strains belonging to the four most widely used probiotic genera (or placebo)⁹² 305 featured a significant expansion of the mucosa-associated probiotics in 60% of the 306 supplemented individuals, and a near-total colonization resistance in the other 40%, even when 307 measured by ultra-sensitive quantitative PCR. The degree of mucosal association was unrelated 308 to the bloom of probiotic strains in stool, and could be predicted by a combination of baseline 309 host and microbiome factors, highlighting a potential future prospect of probiotics tailoring to 310 the individual. Interestingly, transplantation of fecal microbiome from 'resistant' or 'permissive' 311 individuals into germ-free (GF) mice recapitulated the donor susceptibility to probiotics 312 colonization, indicating a dominant microbiome-mediated colonization resistance mechanism⁹². 313 Other postulated non-colonization-dependent probiotics effects on the host, such as impacts on 314 food digestion merit evidence-based experimental proof. With this respect, in the above study⁹² 315 probiotic strains in 'resistant' individuals were not detected even in the gut lumen during active 316 consumption (Gut Microbes, in press), suggesting that temporarily/persistently colonizing

- 317 mucosa-associated probiotics may serve as an important reservoir for luminal bacteria.
- 318

319 Question 2: Do probiotics persistently colonize the gut mucosa, even after cessation of 320 consumption? Even in 'permissive' individuals, it remains unclear whether probiotic 321 colonization is maintained after supplementation ceases. In rats fed a fermented milk product 322 (FMP) containing 5 probiotic strains, all strains were shed during feeding, but only a subset of 323 rats continued to shed one of the five probiotics strains (L. lactis CNCM I-1631) two days 324 following supplementation. Transferring the distinct microbiomes of 'permissive' or 'resistant' 325 rats to GF rats replicated colonization permissiveness of the donors¹⁰⁵. In humans, detectable 326 shedding of probiotics in stool samples during supplementation that diminishes following 327 cessation has been described for *Bifidobacterium* strains *infantis* 35624¹⁰⁶, *animalis* sbsp. *lactis* Bb-12¹⁰⁷, Lactobacillus strains acidophilus R52¹⁰⁸, casei DN-114 001¹⁰⁹, johnsonii La1^{104,110}, 328 329 plantarum 299v¹¹¹, reuteri DSM17938^{112,113}, rhamnosus (LGG, R11, 19070-2)^{103,108,113}, and 330 *salivarius* CECT5713¹¹⁴ among others¹¹⁵. However, follow-up periods were limited to 1-2 weeks 331 after cessation of consumption in most studies. Patterns emerging from longer follow-ups 332 suggest both strain- and person-specific persistence variability. Two months following 333 supplementation cessation, L. rhamnosus was detected only in 1/10 individuals¹¹⁶, whereas one-334 third of *B. longum* AH1206 consumers continued to shed the probiotic species in stool up to 6 335 months after discontinuation¹¹⁷. Subject- and strain-specific post-cessation shedding were also 336 noted in humans supplemented with the aforementioned 5-strains mix FMP, in which only L. 337 *lactis* CNCM I-1631 was shed in stool samples five weeks following cessation, and only by a 338 subset of individuals characterized by a distinct microbiome composition¹⁰⁵.

339

340 Mechanism of activity

341 Beneficial effects of probiotics have been postulated to occur through diverse mechanisms, 342 including induction of immunomodulation, protection against physiological stress, suppression 343 of pathogens, microbiome modulation and improvement of gut epithelium barrier function (Fig. 344 **2)**. These mechanistic probiotics studies often suffer from several major limitations, including 345 heavy reliance on utilization of cell culture systems that do not account for the myriad of crucial 346 physiological cues that dictate microbe-microbe and microbe-host interactions within the 347 complex GI mucosa microenvironment, and are thus often not replicated in *in vivo* trials. Other 348 limitations stem from the poor colonization capacity of exogenous 'human compatible' 349 probiotics in the murine GI mucosa, compared to that noted in humans^{92,118}. Host discordance 350 may be functionally significant, as administration of human commensals to mice can result in a 351 markedly distinct effect on the immune system^{119,120} or host metabolome¹²¹ compared to mice 352 harboring a murine microbiome. Importantly, some probiotic traits may represent class effects 353 and be uniformly present between different members of the species or even the genus, for 354 example both Bifidobacterium spp. and Lactobacillus spp. produce the enzyme beta-355 galactosidase, which may compensate in lactase insufficiency^{122,123}, while other traits may be species-¹²⁴ or even strain-specific¹²⁵, or require interaction between probiotic strains¹²⁶, as 356 357 further discussed. Several major mechanisms have been suggested to be involved in probiotics 358 effector functions:

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360 *Immunomodulation.* Many probiotics studies suggested *in vitro* effects on expression of 361 immune-related genes, inflammatory pathways activity and immune marker levels, including 362 modulation of intestinal epithelial cell (IEC) NF κ B, mitogen-activated protein kinase (MAPK), 363 Akt / phosphoinositide 3-kinase (PI3K), peroxisome proliferator-activated receptor γ activity, 364 CRP, IL-6, IL-8, tumor necrosis factor (TNF)- α , IL-1 β , and interferon γ , through multiple, mostly 365 contact-dependent mechanisms (reviewed in¹²⁷). Interestingly in some studies, live and dead 366 bacteria featured a differential effect on gene expression, suggesting that both cell surface and 367 actively secreted molecules may affect intestinal transcriptome¹²⁸. Additional examples of 368 suggested immune impacts include Lactobacillus-mediated TLR2-dependent stimulation of TNF-369 α secretion through lipoteichoic acid (LTA)¹²⁹, *B. longum-mediated* contact-dependent IL-10 370 secretion¹³⁰, sortase-dependent pili in *Bifidobacterium* evoking a TNF- α response⁹³, cell surface 371 exopolysaccharide (sEPS) in *B. longum* 36524 modulating proinflammatory cytokines and Th17 372 responses in the gut and the lung¹³¹, and immuno-stimulatory cell surface appendages termed 373 SpaCBA in LGG, mediating (in vitro) both binding to human intestinal mucus and TLR2-374 dependant modulation of TNF- α , IL-6, IL-10, and IL-12¹³².

375 Additional examples of suggested in vivo mechanisms include LGG inducing the generation of 376 reactive oxygen species and consequently inhibiting TNF- α -induced intestinal NF κ B activation 377 through SpaC-mediated adhesion to intestinal epithelium¹³³; Peptidoglycan from *L. salivarius* 378 Ls33, but not L. acidophilus NCFM, protecting mice from chemically induced colitis in a 379 nucleotide-binding oligomerization domain-containing protein 2 (NOD2)-IL-10-dependent 380 manner¹²⁴; *L. acidophilus* L-92 binding to microfold ('M') cells mediated immune modulation by 381 its surface layer protein A (SlpA)¹³⁴; *B. infantis* 35624 inducing TLR2-depended T regulatory 382 cells in humans¹³⁵; and *B. animalis* sbsp. *lactis* Bb-12 inducing IgA secretion^{136,137}. Collectively, 383 most of the above examples point to a requirement of physical contact or proximity between 384 host cells and probiotics to potentially induce both pro- and anti-inflammatory responses, 385 highlighting the importance of the context in which they are administrated. The clinical outcome 386 of such changes observed in colonized individuals, whether beneficial or not, merits further 387 human studies.

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389 *Protection against pathogens.* Probiotics have been suggested to inhibit pathogen colonization 390 by attaching to epithelial cells and physically blocking the pathogen ability to adhere. This has 391 been shown in culture¹³⁸ and indirectly in mice for Salmonella and L. acidophilus LAP5 or L. 392 fermentum LF33¹³⁹. L. acidophilus A4 can also antagonize adhesion of E. coli O157:H7 to IEC 393 through up-regulation of MUC2, IL-8, IL-1 β , and TNF- α^{140} . Several *Bifidobacterium spp.* have 394 been shown to produce acetate in vivo, consequently inhibiting Shiga toxin-producing E. coli 395 (STEC) 0157:H7 through acidity-related mechanisms^{141,142}. Several lactic acid bacteria can 396 produce bacteriocins, compounds that demonstrate antimicrobial activity¹⁴³. For example, 397 production of Abp118 bacteriocin by *L. salivarius* UCC118 protects mice from infection with *L.* 398 *monocytogenes*¹⁴⁴. Other mechanisms may involve the disruption of quorum sensing (QS), for 399 instance L. acidophilus La-5 inhibited autoinducer-2 (AI-2) and reduced the expression of some 400 virulence factors of *E. coli* 0157:H7 in vitro¹⁴⁵; *L. acidophilus* GP1B inhibited AI-2 activity for *C.* 401 difficile in vitro and its administration to mice with C. difficile infection improved their 402 survival¹⁴⁶; and *L. reuteri* RC-14 produced mediators to interfere with *S. aureus* QS and thus 403 repressed its virulence, including the expression of toxic shock syndrome toxin-1147. 404 Importantly, production and response to QS signals is a trait shared between pathogens and 405 commensals¹⁴⁸, thus the complexity of QS signals and abundance of responders *in vivo* may 406 differ from that of *in vitro* experiments¹⁴⁹, and QS manipulation *in vivo* can even result in 407 inhibition of commensal bacteria¹⁵⁰.

408

Improved barrier function. Several underlying mechanisms have been suggested for probiotics
 stabilization of gut barrier function, and are reviewed elsewhere¹⁵¹, including up-regulation of
 tight-junction (TJ) proteins (Claudin-1, Occludin, and ZO-1) and improved transepithelial

412 resistance, promotion of mucus secretion (by up-regulating MUC2, MUC3 and MUC1), elevation 413 of butyrate levels, as well as microbiome modulation. These effects may be mediated by locally 414 secreted metabolites, for example L. plantarum produces hydroxy-cis-12-octadecenoic acid 415 (HYA), which has been demonstrated to suppress TJ permeability and the down-regulation of 416 occludin, ZO-1, and claudin-1 induced by IFN- γ and TNF- α in culture, by regulating TNFR2 417 expression via the G protein-coupled receptor (GPR) 40/mitogen-activated protein kinase 418 (MEK)/extracellular-signal-regulated kinase (ERK) pathway¹⁵². In mice, HYA decreased skin 419 TNF- α and increased claudin-1 in a model of atopic dermatitis¹⁵³, and ameliorated pathogen-420 induced gingival epithelial barrier disruption in a GPR40-dependent manner¹⁵⁴. Two secreted 421 proteins purified from *LGG* (termed p40 and p75) have been suggested to promote intestinal 422 epithelial homeostasis by inhibiting cytokine-induced epithelial cell apoptosis¹⁵⁵. Other effects 423 may require direct mucosal adherence, as demonstrated for MUC3 mucin expression induced by 424 Lactobacillus strains in HT29 cells¹⁵⁶, as well as MUC2 and L. casei GG in Caco-2 cells⁹¹. The 425 requirement for adhesion may explain why VSL#3 supplementation in vivo results in conflicting 426 findings regarding the ability to increase mucin secretion^{157,158}. Importantly, when attempting to 427 validate these findings in clinical trials the results were inconclusive, with probiotics-associated 428 improvement observed in some trials¹⁵⁹⁻¹⁶¹, but not in others¹⁶²⁻¹⁶⁵, across multiple underlying 429 conditions. Whether these discrepancies represent the result of variable probiotics colonization 430 not appreciated by early studies remains to be established.

431

432 *Additional suggested mechanisms.* Resistance to bile inhibition is one of the prerequisites for 433 commercial probiotics. For example, Lactobacillus and Bifidobacterium spp. feature bile 434 resistance by the production of bile salt hydrolases (BSH), which deconjugate glycine or taurine 435 from the steroid core¹⁶⁶. BSH activity has been associated with systemic beneficial metabolic 436 effects, including reduction in mouse weight gain, plasma cholesterol, and liver triglycerides¹⁶⁷, 437 as well as cholesterol lowering in humans¹⁶⁸. Nonetheless, deconjugation of bile acids may lead 438 to impaired digestion of dietary lipids and the formation of gallstones¹⁶⁶, as well as impaired 439 glucose tolerance¹⁶⁹.

440 Probiotics were also suggested to affect signaling to the enteric and central nervous systems, 441 and conduce anxiolytic, antidepressant, and ant nociceptive effects on the host¹⁷⁰. Mice fed with 442 L. rhamnosus JB-1 experience specific regional changes in mRNA for γ -aminobutyric acid 443 (GABA)-A and -B receptor in the brain, associated with attenuation of the corticosterone 444 response to stress and an anxiolytic phenotype, which was not observed in vagotomized 445 animals¹⁷¹. Nonetheless, the same strain failed to modulate stress or cognitive performance in 446 humans¹⁷². In mice, maternal high-fat diet results in dysbiosis of both the dam and the offspring, 447 which has a causal role (as demonstrated by transplantations to GF mice) in impairing social 448 behavior in the offspring. Treatment with L. reuteri ATCC PTA 6475, but not L. johnsonii ATCC

449 33200, restored oxytocin levels in the paraventricular nuclei that were reduced by maternal 450 HFD, and improved social behavior¹⁷³. L. reuteri DSM 17938 may also present an ant nociceptive 451 effect in rats in a transient receptor potential vanilloid 1 (TRPV1)-dependent manner¹⁷⁴. L. 452 *acidophilus* NCFM induced expression of μ -opioid and cannabinoid receptors in intestinal 453 epithelial cells, and had an analgesic effect in rats¹⁷⁵. With the potential of beneficially 454 influencing the gut-brain axis by probiotics notwithstanding, key molecular players are still 455 unknown and will be critical for proper translation of findings in animal models to human-456 relevant therapies.

457

458 Interactions with the indigenous microbiome

459 While probiotics impact on the host may not necessarily relate to their interactions with the 460 indigenous microbiome, their use is often associated with claims related to 'beneficial 461 modulation of the microbiota' and 'normalization of perturbed microbiota', either as favorable 462 outcomes on their own or as a mechanism by which probiotics protect the host against disease¹. 463 Nonetheless, the extent, if any, by which probiotics modulate the intestinal microbiota in 464 healthy individuals remains highly debated, as highlighted by a 2015 systematic review that 465 reported lack of evidence for probiotics effect on the microbiota in 6/7 analyzed studies¹⁷⁶, as 466 well as an earlier systematic review analyzing different trials, of which only 21% resulted in 467 microbiome alterations¹⁷⁷. Presumable effects on the microbiome may stem from analytical 468 biases (Box 1). In all, the majority of studies on probiotics in healthy adults, children, and 469 elderly individuals reported no effect of probiotics on the fecal microbiota composition, 470 regardless of the supplemented strains, dose, duration or microbiome analysis method (Table 471 **2)**. Importantly, there is a paucity of trials characterizing the effect of probiotics on the 472 gastrointestinal microbiome in situ (Box 2).

473 One important determinant that may affect the ability of probiotics to modulate the microbiome 474 is the pre-exposure assembly, which may differ between individuals. Antibiotics significantly 475 perturb the microbiome¹⁷⁸, thus relieving colonization resistance to probiotics¹¹⁸, but also to 476 pathogens¹⁷⁹. In this context, probiotics are postulated to serve as placeholders in the cleared 477 niche, preventing pathogen colonization and antibiotic-associated diarrhea³⁸, or as means of 478 correcting antibiotic-associated dysbiosis¹, but evidence to support an ability of probiotics to 479 facilitate reconstitution of the gut microbiome following antibiotics perturbation is often based 480 on bacterial cultures or specific FISH or qPCR probes, which represent only a minimal fraction 481 of the perturbed microbiome, and even using this methodology, the restoration reported may be 482 partial^{180,181} or minimal¹⁸² and is highly debated¹⁷⁷. Overall, the majority of studies do not 483 support a role for probiotics in compositional or functional microbiome modulation, other than 484 transient presence of the probiotic strains themselves during the consumption period (Table 485 2)^{176,177}. Among the studies that report probiotics-associated microbiome alterations, it is

difficult to point towards commonly altered microbial patterns of change (Box 1). While some
works reported microbiome alterations to co-occur with health promoting effects, none
demonstrated causality, and it is thus far impossible to *a priori* claim that such microbiome
alterations are beneficial.

490

491 Safety

492 While the efficacy of probiotics in treating or preventing disease constitutes a decades-long 493 ongoing debate, human supplementation with probiotic microorganisms is generally considered 494 safe, and is recognized as such for most probiotic strains by regulatory authorities¹⁸³. This safety 495 profile is mainly based on history of safe use in foods, and on observations noted in clinical 496 trials assessing probiotics efficacy, rather than safety as the major readout⁴. While probiotics 497 may be safe in healthy adults, their use has been associated with higher risk for infections 498 and/or morbidity in young infants¹⁸⁴ and very low birth weight neonates¹⁸⁵, critically ill adult 499 and infant patients in intensive care units, and postoperative, hospitalized or immuno-500 compromised patients, in part due to bacteremia and fungemia^{38,186-188}. Of note, two large-scale 501 systematic reviews of hundreds of probiotics trials concluded that adverse events and safety 502 issues are poorly reported^{189,190}, calling for the performance of non-industry sponsored 503 independent, high quality, multi-centered controlled trials assessing both efficacy and adverse 504 effects in the above at-risk populations, preferentially coupled with regulatory body 505 assessment¹⁹¹.

506 Interestingly, following antibiotics treatment, enhanced probiotics colonization was 507 associated with a persistent long-term probiotics-induced dysbiosis¹¹⁸, which significantly 508 delayed the reconstitution of both the fecal and the GI mucosal microbiome compared to no 509 post-antibiotics intervention. Soluble factors secreted from the administered Lactobacillus 510 species were suggested (at least *ex-vivo*) to directly inhibit human microbiome growth¹¹⁸. In 511 agreement, two additional trials demonstrated post-antibiotics probiotics administration to be 512 associated with a lower number of observed species compared to no probiotic treatment^{192,193}. 513 Importantly, inhibiting reconstitution of the microbiome quantity and diversity towards its pre-514 antibiotic configuration may result in significant long-term health effects. Such persistent 515 dysbiosis hampers the colonization resistance to pathogens conferred by the microbiome, 516 which may potentially explain several associations made between probiotics use after 517 antibiotics and increased risk of communicable^{38,185,187,194,195,196}, and non-communicable disease 518 such as type 1 and type 2 diabetes, obesity, idiopathic arthritis, asthma and allergies, and IBD¹⁷⁹. 519 Given these observations, it is crucial, in our view, to better assess probiotics long-term safety in 520 this context in future clinical trials, and in particular in children, immunosuppressed 521 individuals, and the critically ill.

523 Future directions

524 The probiotics field is one of the most opinionated and polarized disciplines in biomedical 525 sciences. Data, personal beliefs, solid proof, intuition and commercial interests, coupled with 526 lack of medical regulation, are often intermingled in ways making objective interpretation close 527 to impossible. With this unfortunate situation notwithstanding, we envision that recent 528 discoveries in the microbiome field and the introduction of novel high-throughput sequencing 529 and experimental techniques may allow to revisit some elementary notions about probiotics 530 and focus on biologically relevant questions to facilitate the transition from empirical into 531 target-, disease- and patient-oriented therapeutics (Fig. 3). Instead of a 'black-box' modus 532 operandi, that is, haphazardly administering one member or more of a limited array of bacteria 533 with the intent to elicit health-promoting effects, a mechanism-oriented approach should be 534 adopted, in which probiotic preparations are devised *ad hoc*, following a set of meticulously 535 established criteria. These may include careful consideration of the population to be treated and 536 the medical indication to be targeted. The aim of microbial therapy should be similarly carefully 537 determined: is the effect on the host mediated remotely or indirectly through secretion of 538 molecules by allochthonous bacteria, by modulation of the indigenous microbiome, or by other 539 putative contact-dependent mechanisms inter-linking these bacteria to the intestinal 540 epithelium? Are the intended probiotic effects strain-specific or represent a class effect? Could a 541 nonfood-grade strain be suited to address a particular medical indication? For example, A. 542 *muciniphila* supplementation in mice prevents diet-induced metabolic syndrome and protects 543 against chemically induced colitis¹¹. Faecalibaterium prausnitzii is inversely correlated with 544 Crohn's disease activity, IBS, and colorectal cancer, and suggested to protect mice from 545 chemically-induced colitis¹¹. As with currently available commercial probiotics, it would be 546 important to deepen our understanding of the interactions between these novel potential 547 microorganisms, the host and its resident microbiome, when administered exogenously.

548 Development of means of tackling colonization resistance may be necessary in many instances, 549 and should require careful patient-subset selection¹⁹⁷, development of predictive algorithms 550 assessing colonization potential based on baseline host and microbiome features^{92,105,117,118}, 551 rational co-administration of "prebiotics"¹⁴, colonization modifying agents¹⁹⁸, or those tailored 552 to support an administered strain¹⁹⁹, generation of defined consortia fitting individualized 553 patterns, and counteracting commensal-generated inhibitory mechanisms. The adverse effects 554 of probiotics on post-antibiotic host and indigenous microbiome reconstitution need to be 555 comprehensively assessed with more antibiotic regimens, probiotic strain combinations, and 556 modeled using human microbiome transfers into GF mice, allowing for the assessment of the 557 potential long-term clinical consequences of probiotics-induced dysbiosis. However, the very 558 same potentially negative impact of probiotics-associated dysbiosis noted in the post-antibiotic 559 setting, may be harnessed as positive therapeutic means in other clinical contexts. As such, the

560 apparent improved colonization of probiotics following 'niche freeing' induced by antibiotics 561 may be utilized as means of potentiating probiotics function, by allowing their colonization in a 562 variety of microbiome-associated multi-factorial disorders. Such shift from the empiric "onesize-fits-all" scheme into a person- and condition-tailored approach would inherently 563 564 necessitate a better understanding of the forces shaping exogenous bacterial colonization and 565 resistance to colonization along the human gut interface. However, it may hold promise in 566 generating more robust and reproducible results in relation to specific strains utilization, in 567 specific human subpopulations, in specific clinical contexts, while accounting for consumer 568 safety.

569 Finally, diligently planned large-scale randomized and blinded clinical trials, preferentially 570 devoid of commercial interests, should be the mainstay of evidence-based policy formulation. 571 Endpoints should be objectively assessed and stratified to account for inter-individual 572 differences that might mask effect sizes or confound desirable or undesirable outcome. Adverse 573 reactions should be better studied, reported, and published. Unbiased risk and benefit 574 assessment by treating physicians and consumers alike should be encouraged, in improving 575 accurate data-driven decision-making at various clinical settings. Data should be made readily 576 accessible and shared to allow for a global collaborative effort to reproduce positive results 577 before guidelines are drafted or modified. In contrast to the unfortunate historical lack of 578 sufficient medical regulation for currently available probiotics, one cannot underscore the 579 critical importance of a formal regulatory approval process to be utilized with 'next generation' 580 probiotics, similarly to any other human medical intervention.

581

582 Figure legends

583 Figure 1. Precision aspects of probiotics. Distinct initial host and microbiome conditions and 584 environmental exposures can result in different outcomes when supplemented with the same 585 probiotic preparation. Probiotic bacteria isolated from distinct host populations may present 586 with differential properties, such as adhesion, hydrophobicity and autoaggregation^{197,200}. 587 Underlying medical conditions, such as atopic dermatitis²⁰¹ or milk hypersensitivity²⁰², modified 588 the effects probiotics exerted on host immune cells. Features of the indigenous microbiome can 589 also account for different impacts of probiotics on the host, as microbiomes that allow 590 colonization were associated with ameliorated clinical responses in women with IBS²⁰³ and 591 murine models of colitis²⁰⁴ and depression²⁰⁵. These 'permissive' microbiomes were also more 592 prone to compositional and functional alterations in response to probiotics, and their hosts' gut 593 epithelium exhibited enrichment in distinct pathways compared to 'resistant' microbiomes⁹². 594 Pre-supplementation butyrate levels were associated with a differential effect of probiotics on 595 the microbiome and butyrate²⁰⁶. Diet may also affect properties of probiotics, as dietary 596 polyunsaturated fatty acids (PUFA) modulated probiotics adhesion in vitro. Similarly, it may affect clinical outcome, as preterm infants fed with human milk showed a reduced risk of lateonset sepsis and a shorter time to achieve full enteral feeding, while formula-fed infants did
not⁷⁶.

600

601 Figure 2. Mechanistic interactions of probiotics with the host and its microbiome. 602 Probiotic may have several effects on the host, including metabolism of nutrients to improve 603 digestion (lactose) or produce systemic effects (bile salts), direct and indirect pathogen 604 antagonism (but potentially also promoting virulence), improved barrier function, altering the 605 microbiome, affecting signaling to the nervous system, and immunomodulation. These may be 606 contact-dependent and/or mediated by surface molecules (such as LTA, sEPS, SpaCBA, and 607 sortase-dependent pili), or by secreted molecules (such as SCFA, bacteriocins, p40 and p75). 608 Dashed lines represent putative mechanisms. BSH, bile salt hydrolase; B-gal, beta-galactosidase; 609 QS, quorum sensing; SlpA, S-layer protein A; sIgA, secreted immunoglobulin A; M-Cell, microfold 610 cell; DC, dendritic cell; MOR, mu-opioid receptor; GABA, Gamma-Aminobutyric Acid; PVN, 611 paraventricular nucleus of the hypothalamus; TGF-b, Transforming growth factor beta; LTA, 612 lipoteichoic acid; TLR, toll-like receptor; IFNg, interferon gamma; TNFa, tumor necrosis factor 613 alpha; HYA, 10-Hydroxy-cis-12-octadecenoic acid; GPR40, G-protein-coupled receptor 40; Akt, 614 Protein kinase B; LPS, lipopolysaccharide; ROS, reactive oxygen species.

615

616 Figure 3. Common limitations of the current approach to probiotic research and 617 **proposed strategies to overcome them.** Translating the large body of probiotic research into 618 clinical guidelines can sometimes be challenging due to inconclusive or conflicting evidence 619 deriving from suboptimal study conduct and data analysis methodology. A novel perspective to 620 probiotics may include expanding the variety of administered strains and examining them 621 separately per-strain and per-individual according to personalized considerations, such as 622 baseline host and microbiome parameters, the medical condition to treat and the specific aim of 623 treatment. This will require a mechanism-based approach, implemented through meticulously 624 planned high-quality studies in humans, preferably regulated by health authorities, which 625 directly assess the organ of interest and do not overlook long-term safety.

626

Table 1. Individual trials included in meta-analyses addressing a role for probiotics in *C. difficile* diarrhea, infection or recurrence. Trials with more than one intervention arm appear as separate rows with the difference indicated in the "probiotics intervention" column. Eight trials had a significant effect, and 34 trials did not. P-values and confidence intervals (CI) are taken from the published works, NA indicates that these values were not calculated as part of the work. CDAD, *Clostridium difficile*-associated diarrhea.

634 Table 2. Probiotics supplementation effect on fecal microbiome composition of healthy

individuals. *, Significant taxonomic differences relative to the control group / baseline,
excluding the administered strains; N.D., not determined / no data; MZ, monozygotic; CFU,
colony forming units. S, Streptococcus; L, Lactobacillus; B, Bifidobacterium.

638

639 Box 1: microbiome analysis strategies in probiotics research

640 Advances in the field of microbiome research now offer implementing a finer resolution when 641 studying the interaction between probiotics and the resident microbial community, while 642 addressing previous methodological limitations and biases to potentially resolve contrasting 643 reports. A major contributor to this confusion is the lenient definition of "microbiome 644 alterations". The majority of reports assessing probiotics-induced microbiota modulation utilize 645 16S rDNA relative abundances (RA) in stool samples. As supplemented probiotic bacteria are 646 excreted in stool, increase in their RA concomitantly leads to a spurious reduction in RA of other 647 community members, sometimes misleadingly interpreted as microbiota modification²⁰⁷. Thus, 648 an increase in the RA of the administered probiotic strain should not be interpreted as a *bone*fide effect on the microbiome²⁰⁸. Interestingly, even introduction of heat-killed bacteria was 649 650 suggested to result in supposed microbiome alterations²⁰⁹. Utilization of culture-based methods 651 or species-specific probes can overcome this caveat by describing probiotics-associated changes 652 in their absolute abundances²¹⁰, while accounting for viability²¹¹, but cannot describe global 653 shifts in microbiome configuration compared to pre-supplementation or placebo (beta 654 diversity) or alterations in species richness (alpha diversity). While shotgun metagenomic 655 sequencing may also result in conflicting reports^{212,213}, it offers the advantage of strain-level 656 resolution and characterizing potential probiotics effect on microbiome function. Interestingly, 657 several studies have reported probiotics-related effects on microbiota-encoded function or its 658 associated metabolites, despite no apparent effect on global composition, although these 659 functional microbiome alterations may represent genes contributed by the supplemented 660 probiotic strain, rather than global modulation^{117,214,215}. An additional limitation concerns the 661 definition of the sought "healthy microbiome" that probiotics presumably contribute to. Even when assessing the studies that do suggest probiotics-associated microbiome modulation, no 662 663 consensus signature of such impacts can be reached (Table 2), and reports of microbiome 664 changes induced by probiotics are in many times conflicting, for example in the case of 665 *Clostridium perfringens*^{209,210,216} or *Escherichia*^{211,216,217}, and in various clinical contexts¹⁷⁷. For 666 example, a probiotics-associated fecal bloom of butyrate-producing bacteria (belonging mainly 667 to Clostridiales), and a reduction in *Bilophila wadsworthia* and *Parabacteroides distasonis*, was 668 noted in individuals with IBS (n=28)²¹², and mirrored (for *B. wadsworthia*) in a separate cohort of individuals (n=107) in a subset of "responders", which experienced alleviation of symptoms 669 670 following the intervention²⁰³ but was not reproduced by a third RCT (n=55)²¹⁸. Importantly,

even in cases in which probiotics administration was associated with microbiome changes,
these changes could be stemming from disease modulation rather than directly from exposure
to probiotics. To the best of our knowledge, no study to date has demonstrated a direct causal
role for probiotics-related microbiome modulations in improving a disease phenotype.

675

676 Box 2: quantifying probiotics effect on the gastrointestinal microbiome *in situ*.

677 While stool samples may not accurately represent the GI mucosa-adherent microbiome²¹⁹, only 678 a handful of studies have characterized the effect of probiotics on the intestinal microbiome *in* 679 *situ*. A culture-based study of *L. plantarum* 299v-supplemented individuals (n=29) 680 demonstrated an enrichment of Clostridia in fecal samples, but not in the rectal or ascending 681 colon mucosa¹⁰². Likewise, no significant alterations at the lower GI luminal or mucosal 682 microbiome was noted in probiotics-supplemented humans, compared either to their own 683 baseline or to placebo-administered individuals⁹². In rats, VSL#3 exacerbated the reduction in 684 luminal species diversity associated with the induction of chemically-induced colitis, but had no 685 effect on the mucosa-associated microbiome²²⁰. In contrast, in a mouse model of colitis-686 associated colorectal cancer (azoxymethane-treated Il10-/- mice), VSL#3 supplementation 687 resulted in mucosal expansion of Proteobacteria, and reduction in Verrucomicrobiaceae, 688 Porphyromondaceae, and Clostridium, changes that were associated with enhanced 689 tumorigenesis²²¹. Conflicting results regarding probiotics-related microbiome modulation were 690 also observed in patients with pouchitis^{159,222}, although the reported alterations may be merely 691 stemming from the introduction of the VSL#3 bacteria into the niche²²².

- 692
- 693

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723		
724		References
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