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Review

Small intestine vs. colon ecology and physiology: Why it matters in probiotic administration

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SUMMARY

Research on gut microbiota has generally focused on fecal samples, representing luminal content of the large intestine. However, nutrient uptake is restricted to the small intestine. Abundant immune cell populations at this anatomical site combined with diminished mucus secretion and looser junctions (partly to allow for more efficient fluid and nutrient absorption) also results in intimate host-microbe interactions despite more rapid transit. It is thus crucial to dissect key differences in both ecology and physiology between small and large intestine to better leverage the immense potential of human gut microbiota imprinting, including probiotic engraftment at biological sensible niches. Here, we provide a detailed review unfolding how the physiological and anatomical differences between the small and large intestine affect gut microbiota composition, function, and plasticity. This information is key to understanding how gut microbiota manipulation, including probiotic administration, may strain-dependently transform host-microbe interactions at defined locations.

INTRODUCTION

The gut microbiota—defined as the trillions of microbes living in the gastrointestinal (GI) tract—orchestrates human health.[1] While the term "gut microbiota" typically refers to the multitude of bacterial species residing in the GI tract, non-bacterial microbes, including fungi and bacteriophages, have emerged as equally important modulators of host-microbe interactions.[2,3] Unwarranted fluctuations in gut microbiota composition, as well as alterations in the crosstalk between the host and its microbiome—comprising the microbiota, its components and products, and the environment[4]—tend to influence disease

development. The spectrum ranges from metabolic disorders[5] and inflammatory bowel disease (IBD)[6–8] to cancer[9] and major depressive disorders.[10,11]

In addition, the gut microbiota can enzymatically transform drug structures, altering their bioavailability, bioactivity, and toxicity.[12–14] Molecules secreted by human gut bacteria can further metabolize a range of host-mediated products, including bile acids, and ultimately coordinate GI immune homeostasis.[15]

To this end, the administration of natural or designer probiotics that carry relevant enzymes may help to prevent unwarranted immunity toward the gut microbiota and facilitate physiological processes such as lactose degradation in otherwise intolerant

subjects. Indeed, emerging research points toward advanced microbial therapeutics[16] (AMT) as a promising strategy to alleviate human disease traits.[17,18] A key concept of AMT is microbial engineering.[19] Although bacteria are attractive tools for genetic modulation, the eucaryotic nature of probiotic yeasts offers additional opportunities for complex expression systems, including post-translational modifications, resistance to antibiotics,[20] and advanced containment systems.[21] Despite the promising potential of (1) the gut mycobiome,[22] (2) engineered yeasts as delivery vehicles for requested products, as well as (3) microbiomes beyond the GI tract, this review focusses its narrative on the intimate relationship between the host and its bacterial constituents of the small and large intestine.

NUTRIENT DIGESTION AND ABSORPTION: EXPLORING THE ANATOMY OF THE SMALL INTESTINE AND COLON

The physiology and biochemical environment, including oxygen concentrations, pH, and redox potential, vary significantly throughout the intestine.[23] pH varies not only along the GI tract (Figure 1) but also horizontally, where, e.g., the ventricle is protected by a diffusion barrier enabling luminal proton concentrations to exceed the concentrations at the epithelial surface by >1 million-fold. This, together with oxygen fluctuations shaping the epithelial barrier along intestine,[24] is paramount for compartmentalized microbial colonization, nutrient digestion, and host metabolism. Considering the separate locations, the duodenum represents the most dynamic part of the gut, receiving acidic chyme from the stomach, neutralizing it with bicarbonate, and adding pancreatic secretions and bile. In addition to the acidity (defined by proton concentrations, cf. above), the bile secreted into the duodenum can reach concentrations between 2.6 mM (fasted) and 11.2 mM (fed),[25] which is toxic to many microbial species. The pancreatic secretions further contain a cocktail of proteases, lipases, and glycosidases that may generate antimicrobial compounds from foods.[26]

The mucosal environment in the proximal small intestine (SI) also diverges from the more distal parts. As it is the main site of digestion and absorption, the mucosal surface is covered in long villi (1–2 mm),[27] maximizing the absorptive surface area (Figure 1). The cells in the intestinal epithelial layer develop from epithelial stem cells in the base of the crypts of Lieberkühn and gradually differentiate as they migrate up the villi, where they are shed and undergo apoptosis at the tip.[28] In humans, this process takes 3–5 days and creates significant amounts of cellular debris, such as high-viscosity DNA, in the region around the villi tips.[29]

The mucus layer covering the intestinal epithelium is secreted by goblet cells. Its thickness gradually increases from ~120 μm in the duodenum to 850 μm in the colon[30] (Figure 1). In addition, the colonic mucus forms a bilayer, where the dense inner adherent layer of the healthy colon is largely devoid of bacteria, while the loose outer layer nourishes the resident microbiota.[31] In the duodenum, the loosely adherent mucus is broken up by the movement of the intestinal content, allowing chyme to penetrate between the villi.[32]

The limited discontinuous mucus barrier in the SI gains immunological support from host defense peptides (HDPs), such as

defensins, lysozyme, and regenerating islet-derived protein III-gamma (REG3g) released by crypt-resident Paneth cells. In this way, the crypts remain largely sterile,[31,33] a process that is further substantiated by goblet cell mass-secretion essentially purging the crypts upon acetylcholine-mediated neural stimulation.[34] Trypsin, a key protease in pancreatic secretions, processes human alpha defensins, such as HD5, from the pro-peptide to mature forms. The reducing environment near the epithelial barrier renders the peptides sensitive to proteolytic cleavage,[35] which, instead of peptide degradation, may generate novel bioactive fragments[36] exceeding the bactericidal repertoire of the full-length peptide.[37] This, along with the high concentration of other toxic compounds such as bile acids, antimicrobial peptides and bioactive substrates of food origin, and secretory immunoglobulin A (IgA), limits microbial growth.

More than 90% of lipids, proteins, absorbable sugars, and lipid-soluble micronutrients have been removed by the time the digesta leaves the SI, while the concentration of non-absorbable components, including dietary fiber, has increased. This becomes important when considering region-specific engraftments of substrate-specific probiotics. A balance must be found between overgrowth and failure to establish due to the presence of either nutrients or antimicrobial components.

MICROBIOTA DISTRIBUTION AND FUNCTION: IMPLICATIONS FOR PROBIOTICS

Along the various regions of the gut, microbes colonize the intestinal content (lumen) and the gut mucosa.[38] Microbial colonization of these environments has rarely been studied in humans as sampling in the intestine has traditionally required invasive procedures. Among the limited number of studies that have analyzed the microbial composition of the human gut in vivo, samples were taken from widely different regions of the small and large intestine. Figure 2 provides a detailed overview of the characteristics of the studies considered for the current review along with the microbial taxa identified in specific regions. Overall, despite the variations in sampling sites, and sampling and analysis techniques, these studies consistently demonstrate the existence of different microbial communities along the human GI tract. That is, one community colonizes the duodenum down to the proximal ileum and one colonizes the distal ileum down to the rectum (Figure 2). These findings are beautifully mirrored by two recent studies that developed an ingestible capsule device to sample multiple regions of the GI tract in 15 healthy individuals during normal digestion.[39,40] It is important to note, however, that mentioned studies primarily focus on healthy individuals and that differences may also depend on disease status. Probably this is where the most important implications for the use of probiotics lies. Not only are there substantial differences in the individual microbiota composition, hence posing different levels and types of competition with the probiotic administered, there is also compositional variation along and across the GI tract, which may determine where a probiotic exhibits its main activity.

From a functional perspective, these studies indicate that the small-intestinal microbiota—ranging from 10^4–10^5 microbes/mL in the duodenum to 10^7–10^8 microbes/mL in the ileum—consists

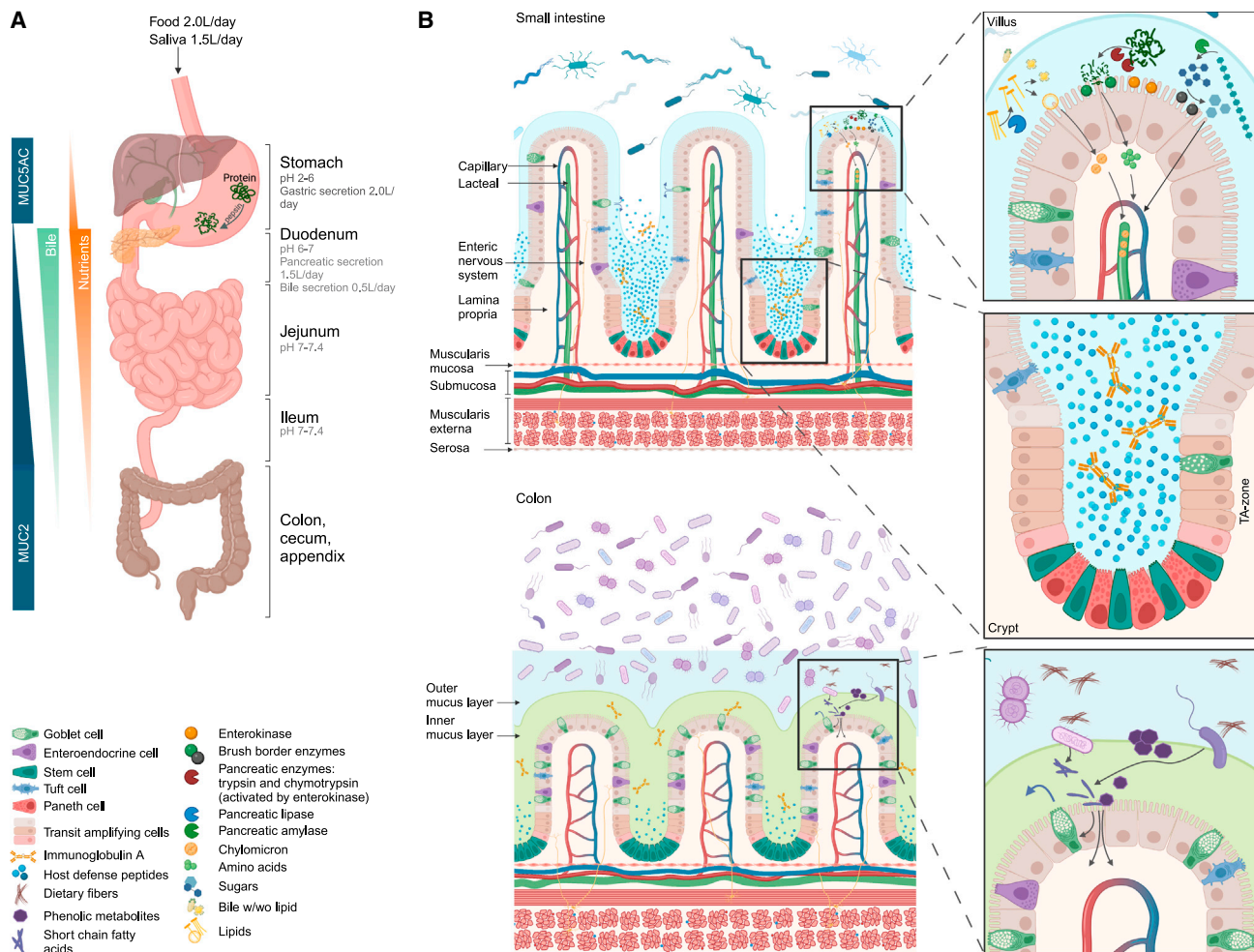# Cell Reports Medicine
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Figure 1. Anatomy and physiology of the small intestine (SI) versus colon

(A) Overview of the gastrointestinal tract displaying spatial distribution of key processes influencing microbial colonization. In the healthy individual, liquid volume presented to the lumen of the SI is 8.5 L per day, for which pancreatic and bile secretion accounts for 1.5 and 0.5 L, respectively; ∼6.5 L are reabsorbed in the SI, while ∼2 L enters the colon. Here ∼1.9 L are reabsorbed and ∼0.1 L is lost in fecal secretions. Both secretion and absorption of liquids are affected by diet and diseases. Acidic ventricle secretion is neutralized by natrium hydrogen carbonate-buffered pancreatic juice in the upper duodenum. Proximal duodenum is also the site of bile acid (BA) secretion from the gall bladder. Nominal amounts of bile are passively absorbed through the entire length of the intestine, while the majority is recirculated by active transport in the distal ileum. Simple carbohydrates and protein are absorbed in the SI with a gradually decreasing absorption from proximal duodenum to distal ileum. Complex carbohydrates and proteins escaping digestion in the SI are converted into short-chain fatty acids (SCFAs) by microbial fermentation in the large intestine.

(B) Inner mucus layer (colon): green. Loosely attached mucus layer (outer layer in colon and only layer in SI): blue. Villus: in healthy individuals, most dietary lipids are absorbed in the first 60 cm of the SI, where BA concentrations are highest. As chylomicrons are too large to pass through the fenestrae of villus blood capillaries, they instead enter the lymph through larger inter-endothelial channels of the lacteals. Dietary proteins and carbohydrates are digested by pancreatic and brush border enzymes, respectively. Crypt: crypt-residing Paneth cells secreting abundant amounts of HDPs further protect and nurture the neighboring stem cells. In the transit-amplifying zone, lineage-committed progenitor cells swiftly divide to fuel the rapid intestinal cell turnover. Colon: the outer mucus layer provides a niche for mucolytic bacteria, many of which can ferment complex carbohydrates/dietary fibers, thereby generating SCFAs fortifying barrier function, including goblet cell-mediated mucus secretion. IgA, immunoglobulin A; TA-zone, transit-amplifying zone.

of fast-growing, primary fermenting bacteria, such as lactic acid bacteria and enterobacteria, and secondary fermenting bacteria, such as *Veillonella*, which grow on the lactate produced by primary fermenters.[41]

Metagenomic and metatranscriptomic analyses of ileostomy microbiota have indeed indicated that the rapid uptake and conversion of simple carbohydrates drives the microbial ecosystem in the SI.[42] This contrasts with the colonic ecosystem, which is driven by the conversion of complex carbohydrates. A likely explanation this dichotomy is the enormous difference in transit time between the small and large intestine combined with the continuous removal of nutrients by the small-intestinal epithelium during transit. An *in vitro* modeling study has, in fact, shown that an ileum-like community can be obtained from a fecal inoculum when mimicking small-intestinal characteristics, including transit time.[43] When developing novel probiotics that target the
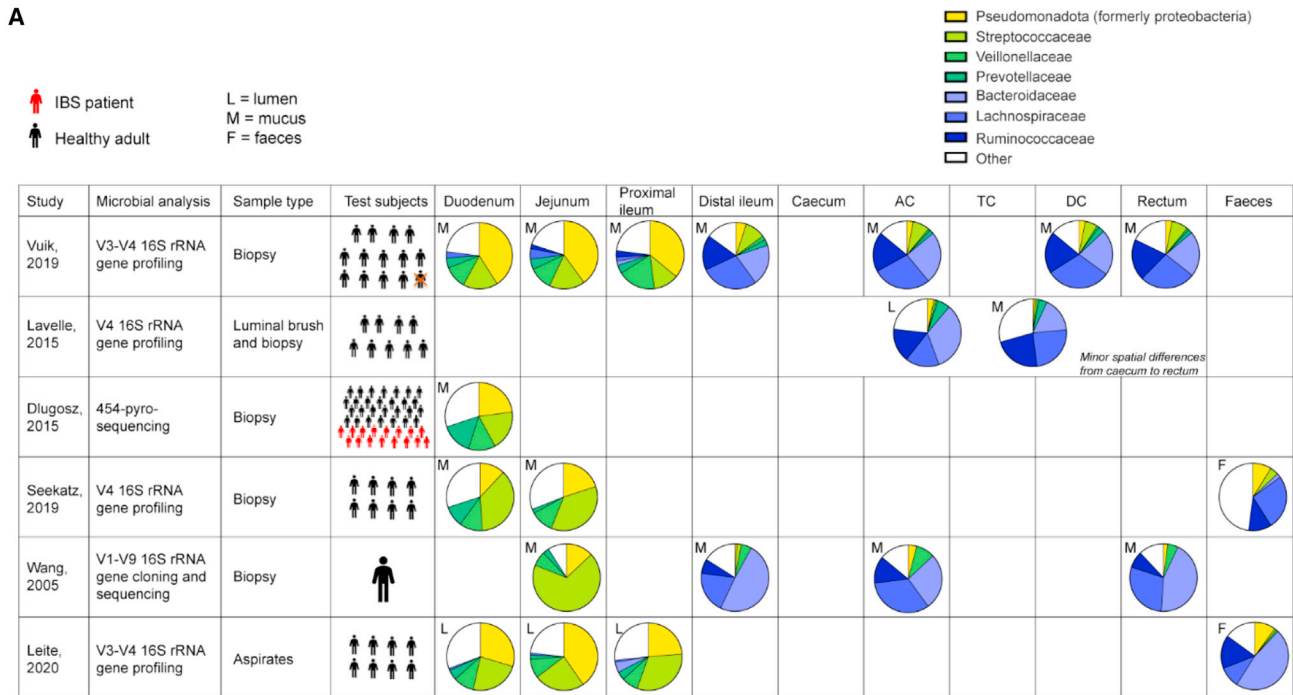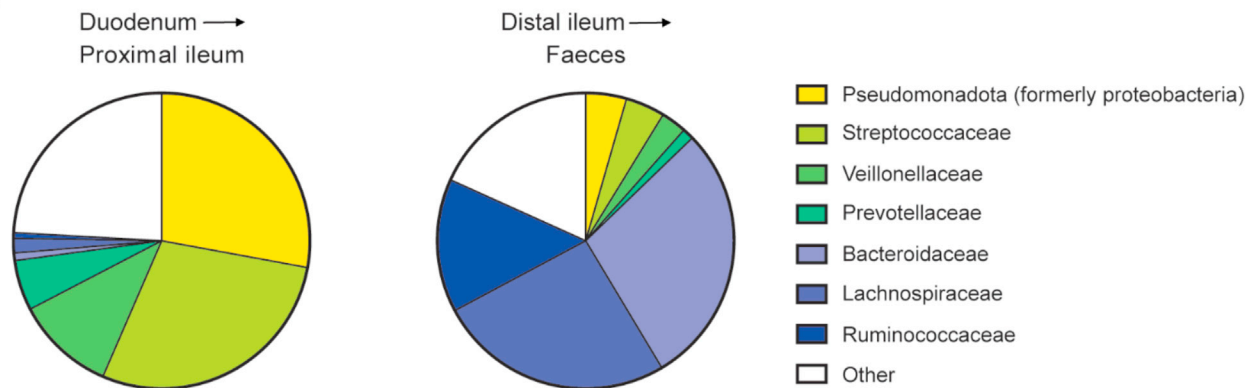
Figure 2. Longitudinal differences along human gastrointestinal (GI) tract

(A) The abundance of key taxa in a specific gut area, as averaged across all individuals tested in a given study. Six studies are included based on the requirement that they investigated different gut regions within specific individuals. Despite the differences in sampling and microbial analysis techniques, this revealed the consistent difference between microbial communities of the upper and lower GI tract. One community colonizes the duodenum down to the proximal ileum (generally dominated by Pseudomonadota, Streptococcaceae, and Veillonellaceae) and one colonizes the distal ileum down to the rectum (generally dominated by Bacteroidaceae, Lachnospiraceae, and Ruminococcaceae).

(B) Weighted average of all the studies included in (A).

SI, it will thus be relevant to consider their ability to thrive in an environment fundamentally different from the colon.

In addition to easily fermentable carbohydrates, numerous other potential energy sources enter the SI that can be used not only by the host but also by the SI microbes. Human data on how, e.g., fats and proteins interact with the SI microbiota are limited. Fats are generally processed and absorbed in the duodenum and jejunum[44] but, as energy-rich sub-

strates, they can also be used by intestinal microbes. Ingestion of fats may exhibit a further indirect effect on the microbiota via the release of bile acids, which may inhibit microbes depending on their ability to hydrolyze bile salts[45] and thus influence the survival of consumed probiotics. Proteins are another potential energy source for proteolytic microbes. While they are mainly metabolized in the SI, any remaining proteins may undergo colonic fermentation,

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resulting in the production of hazardous components, such as amines, sulfide, and phenolic compounds. The physiological impact of intestinal protein fermentation by *Prevotella copri* and other intestinal microbes can also increase the occurrence and cross-epithelial transport of by-products, such as branched-chained amino acids,[5] exhibiting negative impact on insulin sensitivity in humans and mice.[5]

MICROBIAL CROSSTALK AND ITS IMPACT ON PROBIOTIC ENGRAFTMENT POTENTIAL

One of the main implications of the intestinal microbiota is its resistance to incoming microbes. This effect, commonly known as competitive exclusion or colonization resistance, protects the host from infections with pathogens but also affects the ability of probiotic organisms to persist in the GI tract. Fast-growing organisms or organisms with a high affinity for available nutrients have an advantage to outcompete other, less well-adapted counterparts. Still, growth rate is not the only competitive edge observed in microbial communities. Speedy microbes (high motility), even when growing slow, may colonize new microhabitats faster than their immediate competitors,[46] presenting speed, in either growth or motility, as a key evolutionary trait to facilitate efficient colonization.

In addition to competition, there is also collaboration between intestinal microbes. One of the most common examples of this is cross-feeding. As briefly alluded to above, metabolites from primary fermenters can be consumed by secondary fermenters as energy sources while simultaneously relieving primary fermenters from metabolite inhibition. For example, lactate is commonly formed by many primary fermenter species, among others lactobacilli and bifidobacteria, and is metabolized further by secondary fermenters to, e.g., propionate and butyrate. Besides carbon and nitrogen cross-feeding, there is also cross-feeding of micronutrients, including vitamins.[47] To what extent this affects local probiotic persistence remains to be determined. A general observation is, however, that probiotics cannot be detected in feces 1–2 weeks after consumption has ceased,[48] although exceptions exist.[49]

THE CHIT-CHATTING BEHAVIOR BETWEEN THE MICROBIAL COMMUNITY AND HOST IMMUNITY

Apart from an apparent vertical variation in GI physiology and microbial colonization as described above, the anatomy of the intestinal immune system also exhibits profound variation from the SI to the colon. Specifically, the SI harbors visible Peyer's patches (PPs), which mature in response to the microbiota. PPs are characterized by a follicle-associated epithelium interspersed with microfold cells (M cells), which are important for antigen uptake from the lumen into the underlying lymphoid tissue. As their presence reflects microbial loads, PPs are more abundant in the ileum than in the proximal SI.[23]

The large intestine contains colonic patches and the cecal patch. Isolated lymphoid follicles are also present, covering all stages of maturity from immature cryptopatches to mature isolated lymphoid follicles.[23,50] These lymphoid structures are collectively known as gut-associated lymphoid tissue (GALT).

The GALT comprises the immune-inductive site of adaptive immunity and moderates a tolerogenic or pathogen-protective immune response toward microorganisms and dietary antigens.[51] The constant balancing act between tolerance and pathogen suppression may explain why the intestinal immune system is the largest and most diverse in the body.

The intestinal lamina propria, located between epithelium and underlying smooth muscle cells (muscularis mucosae), consists of loose connective tissue made of extracellular matrix protein produced by mesenchymal stromal cells. This nurtures the epithelial layer and provides a structure for the diverse immune cell populations to migrate along and become regionalized along the length of the intestine.[52] Certain immune cell subsets then become dominant in different parts. For example, interleukin (IL)-17-producing CD4$^+$ T helper (Th17) cells are more abundant in the SI, while the number of peripherally induced IL-17-secreting FoxP3$^+$RORyt$^+$ regulatory T cells (iTregs) is higher in the colon.[23] Notably, this Treg subset is induced by the gut microbiota.[53,54] Exhibiting increased immunosuppressive capabilities compared to thymus-derived counterparts,[55] it is an eminent example of host-microbe interactions.

The bidirectional interplay between gut microbes and host immunity was further corroborated by a recent investigation of the immunomodulatory potential of microbial antigens used as a source of dietary protein.[56] This dietary intervention facilitated iTreg blooms in the small and large intestine and corrected gut dysbiosis in obese mice, restoring it to a state resembling that of lean counterparts. The latter was observed as a substantial, >5-fold increase in *Parabacteroides*, known for their immune-regulating capabilities.[57] The increase was dependent on a functional adaptive immune system,[56] suggesting that, while mutualistic microbes can orchestrate mucosal immunity, adaptive immune cells can dictate microbial composition.

In addition to the regionalization of mucosal immune subsets, intraepithelial lymphocytes also exhibit spatially separated phenotypic characteristics. Similarly, the gut epithelium comprises distinct compositions of epithelial cell types from the proximal to the distal intestine. In general, the crypts harbor the intestinal epithelial stem cell (IESC) niche, which provides all types of mature epithelial cells. These cells develop as they migrate along the crypt and through the transit-amplifying zone, away from the stem cell-maintaining factors in the crypt area.[28]

The only mature epithelial cell interspersed with the IESCs in the crypt is the small-intestinal-specific Paneth cell.[28] Paneth cells provide IESC niche factors and secrete HDPs into the crypts, protecting this crucial area and potentially making up for the thin and discontinuous mucus layer of the SI. During IBD, ectopic Paneth cells have been reported in the colon, probably a response to the dysbiotic nature of such diseases.[58] This observation is of critical importance to probiotic usage as bacteria, such as *Ruminococcus gnavus*, otherwise thriving in the healthy colon and even exhibit tolerogenic capabilities,[59] can promote inflammation when exposed to Paneth cell derived products. In other words, disease activity is aggravated when the inflammation-driven occurrence of Paneth cells in the large intestine release lysozyme into the
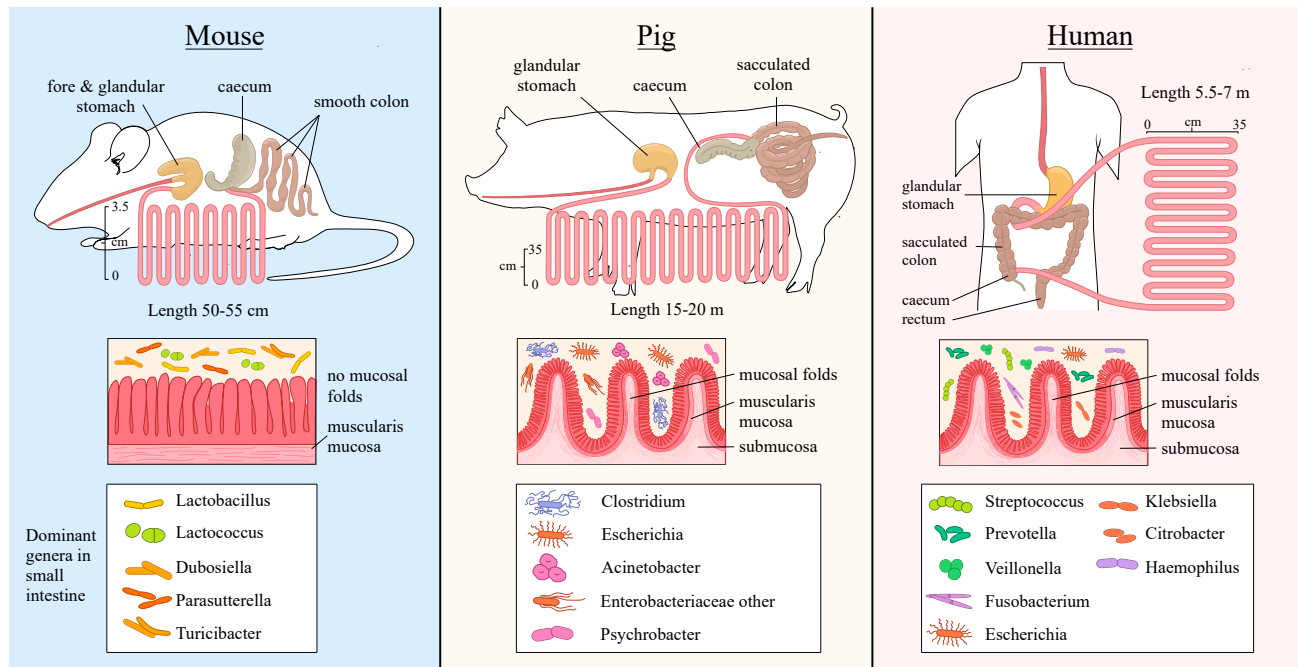
Figure 3. Differences in anatomy and microbiota (on genus level) of the small intestine (SI) between two animal models (mouse and pig) vs. humans
Upper part: the length of the SI in comparison with the other gut compartments is shown. Mice have a forestomach and a glandular stomach, while pigs and humans have a glandular stomach, but the pig's stomach is two to three times larger than the human stomach. Middle part: the anatomical structure of the luminal small-intestinal wall is shown, highlighting the absence of plicae circulares or mucosal folds in mice in contrast to pigs and humans. In mice, the finger-shaped villi are directly oriented on the small-intestinal muscle layer (muscularis mucosa), making the mucosal surface smooth. In pigs and humans, there are plicae circulares in distal duodenum, jejunum, and proximal ileum. Lower part: dominant bacterial genera in the SI.

R. gnavus habitat, disrupting the membrane of the bacterium and releasing intracellular proinflammatory components.[60]

HOW TO COLLECT MICROBIOTA SAMPLES FROM THE SI?

The properties of the microbiota in the human SI are not well characterized, primarily because of the challenges involved in obtaining samples from this part of the digestive tract. To enhance resolution of engraftment potential and interrogate *in situ* dynamics in the SI, we are in desperate need of better sampling techniques, allowing us to, e.g., investigate how the microbiome is assembled in the SI from early life to aging in the healthy population. Such information would aid in characterizing disruptions caused by GI conditions and help elucidate how probiotic administration may counter or even reverse these effects.

A recent study demonstrated that probiotic supplementation altered the antibiotic-resistance gene reservoir along the human GI tract, including in the SI, in a person-specific and antibiotic-dependent manner.[61] However, as this research is still in its infancy, standardized sampling techniques with spatial and temporal resolution are urgently needed. Specifically, rather than the biopsy-related techniques frequently used in the clinic, non-invasive access to the SI microbiota will ultimately improve our understanding of SI community dynamics and enable more precise studies on the efficacy of selected probiotics at this location.

An appealing option is the use of swallowable smart capsule systems that allow samples to be taken directly on site without need of endoscopy.[62] The newest development of such capsules enables multi-omics analyses of the intestinal environment under physiological conditions at spatially separated locations.[40] Selected sampling devices for the SI are shown in Table S1.

Future research should address the development of affordable sampling tools to provide a detailed picture of probiotic activity in the SI under physiological conditions, both among different sex and age groups of the healthy population and among those with a disease. These tools could initially be used in probiotic clinical trials and ultimately made available to practitioners and consumers for monitoring probiotic survival, engraftment, and activity.

IN VIVO MODELS FOR STUDYING THE SMALL-INTESTINAL AND COLONIC MICROBIOTA

As an alternative to human samples, animals can be used to obtain valuable insights about the human microbiota. Figure 3 and Table S2 summarize specific characteristics of the human, mouse, and pig GI tract.

While mice generally surpass most other preclinical models in experimental flexibility, due to their easy accessibility, modest housing requirements, short breeding cycles, and a multitude of available genetic models for delicate mode-of-action (MoA)

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studies, their GI tract does not resemble human physiology in adulthood. The developing intestine does, however, share marked similarities.[63] The most notable differences in the adult mouse intestine relate to the lack of a transverse segment, a substantially enlarged caecum used as a fermentation sack, and a smooth outer mucosa in the SI, unlike the circular folds in the human mucosa.[64] Therefore, it is reasonable to speculate whether host-microbe interactions in mouse models are relevant for studying the engraftment potential of probiotics selected for human use.

A catalog of the mouse gut metagenome was produced in 2016[65] and expanded in 2021.[66] Although the mouse gut shares only 4% of its microbial genes (95% identity, 90% coverage) with those of the human gut microbiome, a 95% similarity was found between functional pathways. It is important to note that the mouse provider and, to some extent, housing conditions seem to have a pronounced effect on the composition of the gut microbiota,[67,68] similar to household[69] and/or enterotype[70–72] differences in human studies. This potential drawback introduces heterogeneity to an otherwise homogeneous setup. On the upside, however, it can be used to (1) corroborate scientific relevance and rigor, if the data are reproduced in mice from various vendors and preferably also different strains or genotypes,[56,73] and (2) tease out critical information about specific communities and, potentially, identify driver species of relevant phenotypes.[74]

Despite the limited overlap between the mouse and human microbiota, mice have repeatedly been used as translators in microbiome research where seminal discoveries of host-microbe interactions have been established.[75–79] This has confirmed the relevance of mice as a notable tool for, at least, colonic microbiota research.

When evaluating the applicability of mouse models to probiotic engraftment, a key point to consider relates to the dietary composition. As gut microbiota community structures fluctuate depending on the available substrates, it is no wonder that the microbial composition in mice fed regular mouse feed differs from the microbial communities in human subjects with substantially different dietary habits. This point is evident from the few mouse studies where experimental animals were fed human diets. An experimental strategy to improve probiotic engraftment could, therefore, be to feed experimental mice a humanized diet.[80] Such an approach was indeed recently employed to tease out how experimental diet affects the metabolic benefits of probiotics in obesity,[81] a concept that was further corroborated in a randomized controlled human trial,[82] thus advocating clinical translatability.[83]

Notable limitations to the above are the lack of small-intestinal samples and the fact that most samples included in the gene catalogs originate from C57BL/6J mice, which could affect their applicability in studies of other mouse strains. A recent study of the regional diversity of the gut microbiota in mice showed a high abundance of Lactobacillaceae and Muribaculaceae in the SI; this is quite different from humans.[84] Another drawback of using mice is their coprophagic behavior. It has been shown that continuous self-exposure to the fecal microbiota has substantial quantitative and qualitative effects on the SI microbiota.[85] By contrast, the SI of non-coprophagic mice had a lower

microbial load, reduced abundance of anaerobic microbes, and bile acids predominantly in the conjugated form, resembling the human SI more closely.

As it is both difficult and ethically challenging to ensure non-coprophagic behavior in mice, other, more appropriate models may be used to study the intricate relationship between SI microbes, probiotic engraftment, and host phenomics. To this end, pigs appear as a suitable model.

Like humans, pigs are monogastric, omnivorous animals. They have a simple stomach and a relatively long SI (Figure 3).[86] Although the large intestinal morphology and physiology of pigs resembles the human large intestine more closely than that of mice, conclusions about the effects of orally dosed probiotics should be made with caution. Pigs lack an appendix, but their caecum is more developed compared to humans, and the colon has a spiral arrangement.[87] Moreover, their large intestinal fermentation may yield up to 30% of their maintenance energy requirement, compared to an estimated 7% in humans.[88]

A reference gene catalog of the pig gut microbiome was collated in 2016[89] and recently expanded to include samples from a wide range of sources.[90] The pig metagenome exhibited a higher alpha diversity than both the human and mouse microbiomes. The relative abundances of the same bacterial species were seen to differ significantly in different gut locations. A modest 9.5% overlap between the human and pig microbiome was found, while 96% of the functional pathways identified in the human catalog were also present in the pig catalog and hence were close to the mouse-human overlap described above.[65] However, compared to mice, the use of pigs has notable limitations, including the higher cost of animal maintenance and husbandry, longer reproductive cycles, and retentive time to maturity. Their genetic background is also more difficult to control.[87] Still, the use of cannulated pigs makes it possible to study nutrient digestibility and the dynamics of intestinal microbiota composition through repeated sampling. In addition to the collection of chyme, this model also allows easy endoscopic examination, enabling the collection of ileal effluent and fecal samples from the same individual, which is not possible in human ileostomy patients. Besides animal welfare concerns, a disadvantage could be that the cannulation itself affects GI functioning.[91]

This said, accurate and representative models are crucial to the study of the intestine and its interaction with the host. For this reason, *in vivo* models remain indispensable. By comparison, *in vitro* models cannot adequately simulate the full spectrum of host-microbe interactions, both within and beyond the GI tract, as discussed below.

SIMULATING THE GUT *IN VITRO*

Various *in vitro* systems have been developed to simulate different parts of the intestine. The systems range from batch-fermentation models for parts of the SI or colon to continuous-flow models for the upper gut and/or colon.[92–95] While the SI models typically mimic host physiology (digestive enzymes, bile, pH), allowing studies of digestion and absorption, the lower-gut models simulate the colonic microbiota, enabling studies of microbial fermentation processes. Although these

models do not generally simulate the host response or the mucosal layer, they can simulate the *in vivo* situation reasonably well, thus providing an opportunity to investigate the influence of different environments on, for example, probiotic survival, fiber fermentation, and microbiota composition and activity.

An improvement to these models would be to include a simulated SI microbiota, which has only been attempted in a limited number of studies. Cieplak et al.[96] developed "the smallest ntestine" (TSI) stand-alone model where seven strains from six genera (*Escherichia*, *Streptococcus*, *Enterococcus*, *Bacteroides*, *Veillonella*, *Flavonifractor*) were incubated for 8 h to simulate the duodenum, jejunum, and ileum. The growth and activity of the various species were not investigated, however, which is necessary to understand how closely the TSI model represents the colonization of SI microbiota. Stolaki et al.[43] developed a simulation model of the ileal microbiota using TNO gastro-intestinal model (TIM)-1 and TIM-2 technology.[97,98] Here, human ileostomy effluent and fecal inocula were incubated in environmental conditions representative of the ileum (3.5-h transit time, pH 7.2) over a period of 14 days, resulting in the establishment of an *in vitro* microbiota with a degree of similarity to *in vivo* ileal samples. However, the microbiota was dominated by phyla such as Bacteroidota that typically enrich colonic *in vitro* models, possibly due to the high content of fibers in the simulation medium.[99] Finally, Roussel et al.[100] developed an extension of the mucosal simulator of the human intestinal microbial ecosystem (M-SHIME) model[101] in which an ileum reactor was seeded with a suspension from the simulated ascending colon reactor. Following a 20-day incubation period, during which a fill-and-draw principle was applied to simulate short transit, the simulated ileal microbiota was found to be strongly enriched with Pseudomonadota. This was potentially due to the addition of simple sugars to the nutritional medium.

Compared to *in vitro* models for the human colon microbiota, models for the small-intestinal microbiota are at an early stage of development. While the studies described above have conducted essential pioneering work, a method to preserve the composition and functionality of an *in vivo*-derived small-intestinal microbial community has yet to be developed. Biorelevance for the *in vivo* situation must be demonstrated before *in vitro* models may be used to broaden our understanding of how probiotics thrive in the SI environment.

Intestinal epithelial *in vitro* and *ex vivo* models can be valuable to study host-microbe interactions, as summarized elsewhere.[102,103] Although widely used, immortalized epithelial cell lines, such as Caco-2 and HT29, possess many non-physiological characteristics. This can be overcome in part using 3D stem cell-based organoids or organ-on-a-chip approaches. Comprising different cell types and embedment in an extracellular matrix, these can enhance the reflection of *in vivo* microarchitecture.[102,104] Use of primary intestinal cells has the additional advantage that host genetic features, including disease specific traits, can be captured. The models mentioned facilitate studies of the direct interactions between probiotic strains or their metabolites and epithelial cells under normal and stressed conditions. They have been used to study the preventive effects of probiotics on bacterial adhesion or invasion and barrier dysfunction, including underlying mechanisms.[103] Still, major

drawbacks relate to their limited robustness for high-throughput analyses, poor accessibility to human specimens, and, especially, the oxygen requirements that hinder exposure to strict anaerobic bacteria.

HOST-DIET-MICROBE-DISEASE INTERACTIONS IN THE SI

Diet is the most obvious factor affecting the composition and function of the GI microbiota. In addition to dietary factors, a multitude of intestinal diseases influence microbial composition both directly (disease-microbe interactions) and indirectly (drug-microbe interactions). To this end, celiac disease (CeD) is an inflammatory disorder that primarily affects the SI, triggered by dietary gluten exposure in genetically predisposed individuals. While recognized that gluten is the trigger in CeD, evidence suggests that genetics only explain 55% of disease susceptibility, pointing toward a strong environmental influence, with intestinal microbiota involved in the pathogenesis, progression, and clinical symptoms.[105] Consequently, probiotics has been proposed to relieve the condition. This includes the possibility to rebalance the altered microbial composition in the SI, thereby improving intestinal barrier function, or to detoxify gliadin peptides to reduce the load of peptides with immunogenic properties.[106] Clinical trials that have studied the effect of various probiotics on the prevention or treatment of CeD, however, lack homogeneity. Despite the demonstration of good safety profiles, symptom management appears to be a greater opportunity than disease prevention.[107,108]

Another well-defined disease affecting the SI is food allergy (FA), starting in early childhood and representing a major public health concern.[109] It is well established that gut leakiness enhances unwarranted immunity toward dietary antigens in susceptible individuals.[110] However, while a perturbed barrier may increase the likelihood of an allergic response, it does not seem to be the primary cause of disease onset.[110] A recent study has shown that a gradually evolving dysbiosis drives FA in infants.[111] Gut microbial dysbiosis—characterized by diminished abundances of a consortium of species belonging to the Clostridiales order—inhibits the formation of iTregs in the infant GI tract. Fecal transplants from FA infants to predisposed recipient mice replicated the diminished iTreg phenotype observed in human donors. Conversely, bacteriotherapy with a consortium of species belonging to the Clostridiales order has been seen to suppress FA in experimental mice while promoting enhanced retinoid orphan receptor (ROR)-γt⁺ iTreg abundances in their mesenteric lymph nodes.[111]

ROR-γt is required to antagonize effector programs in GI Tregs,[112] and Treg-specific deletion of ROR-γt removed Clostridiales-mediated protection against FA.[111] This suggests that ROR-γt⁺ iTregs are imperative to protect against FA, a finding that lends further credence to the hypothesis of probiotic treatment as an effective strategy in FA treatment. Further support comes from conceptual findings that cell surface polysaccharides from *Bifidobacterium bifidum* engage with lamina propria-residing dendritic cells to promote a 2-fold upregulation of colonic iTregs.[113] As one study has shown, a French-press-lysed non-intestinal bacterium, *Methylococccus capsulatus*

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Bath, facilitated a 3- to 5-fold increase in ROR-γt$^+$ iTregs in both small and large intestine.[56] The suggestion is that exogenously administered, relevant bacteria strains can not only nourish intestinal immunity but also stimulate the induction of the immune subset required to suppress FA at the preferred anatomical niche.[77]

PROBIOTIC TRIALS IN IBD: LESSONS LEARNED AND THE POTENTIAL FOR OTHER GI TRACT DISEASES

IBD is the umbrella name for Crohn's disease (CD), ulcerative colitis (UC), and IBD unclassified (IBDU), which are chronic disorders characterized by relapsing inflammation of temporal intensity in the GI tract. The multifactorial nature of IBD, including its complex underlying genetics, represents a challenge to diagnosis and therapy. A defective and leaky gut lining, contributing to chronic inflammation associated with defects in the innate and adaptive immune responses, is a hallmark of this disease. Apart from the varying symptoms, clinical diagnosis mainly distinguishes CD from UC by the fact that CD can precipitate transmural inflammation in the entire GI tract, whereas UC is restricted to mucosal inflammation in the colon.

The steep increase in the number of IBD cases over the last 50 years clearly indicates that the environment and microbiota are contributing factors alongside genetic predisposition. The microbial impact on IBD has been suspected for over a century,[114] supported by animal models where disease severity is microbiota dependent,[115] just as IBD-associated communities can transfer the donor phenotype to recipients of fecal microbiota transplants.[116] The latter was further corroborated in recent work using multi-omics analyses of the UC fecal microbiota to identify bacterially shed proteases that promote disease severity. *Bacteroides vulgatus* proteases disturbed colon barrier function both *in vitro* and in mono-colonized, IL-10-deficient, colitis-prone mice. Disease activity was curbed by simultaneous administration of broad-spectrum protease inhibitors. The importance of host-microbe interactions in disease etiology is further rooted in the empiric observation that antibiotic use, especially at early life stages, is associated with IBD prevalence.[117,118] Apart from changes in bacterial[119] and viral[120] populations, reduced diversity in the fungal[121] community has also been observed in IBD patients.

The molecular mechanism of conventional probiotics administered in clinical trials to IBD patients has rarely been investigated, although some potential MoAs are occasionally supported by animal models. It is, however, worth noting that it is inherently difficult to translate murine findings to human IBD based on the vastly diverging disease etiology between species. Still, there is general consensus that both strain and dose are paramount for treatment efficacy. Perhaps more importantly, disease severity seems equally imperative.[122,123] To gauge this disparity, it is important to consider the underlaying cause and multifactorial consequences of mentioned diseases. Both UC and CD are characterized by mucosal barrier defects that imbalance host-microbe interactions,[124] and while altered community structures by probiotic administration may curb host immunity, it does not treat the underlying cause of disease, namely the barrier defect. Thus, if disease activity is

too pronounced, probiotic administration may not only fail to dampen excessive immunity but on the contrary may succeed to penetrate the compromised mucosa, hence further fueling the inflammatory process, which—although installed to avoid bacterial translocation—may further compromise barrier function. Moreover, as both the molecular etiology and intestinal manifestation of the disease differs between UC and CD,[125,126] both strain (predictive for its MoA) and location (small versus large intestine) are essential parameters to consider. Based on the evidence available from clinical trials to date, it seems that patients with active UC may benefit mainly from treatment with *Escherichia coli* Nissle 1917 and the probiotic mixture VSL#3 (now marketed as "De Simone Formulation"). The latter is a probiotic cocktail of eight live freeze-dried bacterial species comprising different lactobacilli and bifidobacteria mainly provided in high doses. The most recent Cochrane Review performed on this topic concluded that "low-certainty evidence suggests that probiotics may induce clinical remission in active UC when compared to placebo."[127] However, the same review also noted that "no evidence exists to assess whether probiotics are effective in people with severe and more extensive disease, or if specific preparations are superior to others." Interestingly, and further corroborating the importance of location, the route of probiotic administration significantly alters its efficacy. Rectally administered *Lacticaseibacillus casei* DG-modified colonic microbiota, while significantly elevating mucosal IL-10 levels in UC patients.[128] However, these effects were not replicated by oral administration of this strain at the same dosage, corroborating that the delivery of active strains at biologically sensible niches is crucial to maximize probiotic efficacy. The situation is, however, considerably more complicated in CD than in UC. As CD can involve any part of the GI tract, the microbial community will differ between the sites affected. For this reason, it is challenging—if not impossible—to establish generic mechanistic links to benefit patients. While animal models have supported the use of probiotics to manage CD,[129] the effectiveness of probiotics, prebiotics, and synbiotics in inducing or maintaining remission in CD patients has not been confirmed.[130] A 2020 Cochrane Review on the use of probiotics for the *induction* of CD remission concluded that "The available evidence is very uncertain about the efficacy or safety of probiotics, when compared with placebo, for induction of remission in Crohn's disease."[131] There is a lack of well-designed randomized clinical trials in this area and further research is needed. An earlier Cochrane Review from 2006 suggested there was a lack of clear evidence to support the use of probiotics for the *maintenance* of remission in CD.[132] However, the included studies were small. More adequately powered studies are necessary to determine if probiotics are of benefit for CD patients as a whole or subgroups thereof.

A potential confounder of probiotic trials in CD relates to genetic polymorphisms, which potentially interfere with probiotic MoA. For example, frameshift mutations in genes responsible for innate immune sensing of the intestinal microbiota, such as NOD2, IL-23R, and ATG16L1, increase the risk of IBD in humans.[133–135] NOD2 polymorphism is a particularly interesting case that may provide conceptual learnings applicable to probiotic treatment of other GI diseases. NOD2, the first IBD
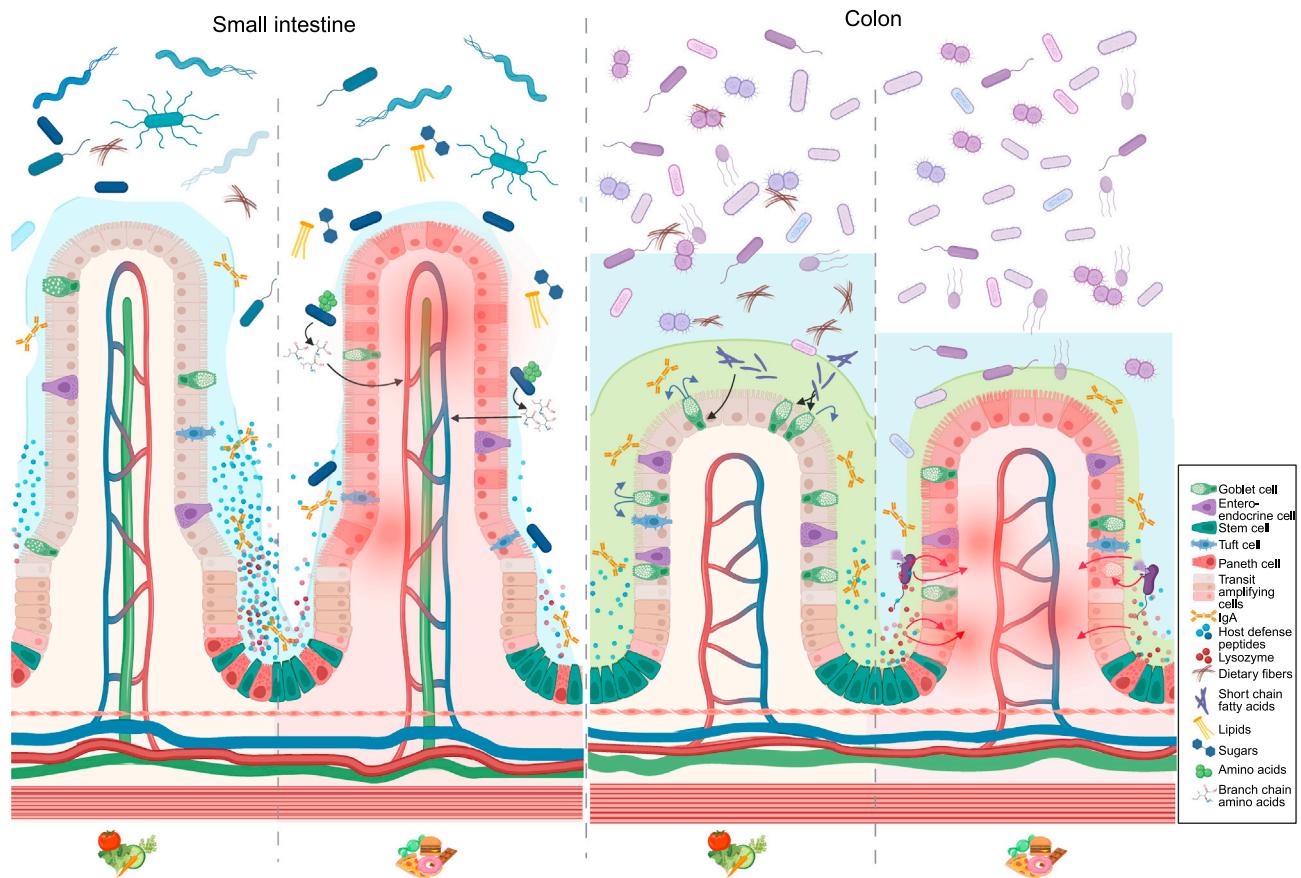
Figure 4. Regional differences in host-diet-microbe interactions

In the healthy SI, conditioned by high-fiber dietary intake, crypt-residing Paneth cells secrete an ample amount of host defense peptides (HDPs). Barrier integrity is further bolstered by the loosely adherent mucus layer ensured by well-functioning goblet cells. Changing dietary habits from a balanced high-fiber diet to a typical westernized diet (low in fiber; high in fat, animal protein, and simple sugars) disrupts immune balance, mucus production, Paneth cell function, and microbiota composition. On a westernized diet, but not a balanced fiber-rich diet, *Prevotella copri* was able to enhance cross-epithelial transport of branched-chain amino acids (BCAAs), potentiating metabolic syndrome. Next, while dietary fibers are passing through the SI, they are metabolized by colonic microbes. The microbial metabolites from this process, SCFAs, stimulate goblet cell function, hence enhancing barrier integrity by increased mucus production. Conversely, in the inflamed colon—often mediated by disease activity potentiated by westernized diets—decreased mucus production enables bacterial encroachment to the otherwise sterile inner mucus layer. As a host-mediated counterresponse, this process fuels Paneth cell hyperplasia, enabling this cell type that is otherwise restricted to the SI to steadily emerge in the inflamed colon. Here, they secrete HDPs and lysozyme to ward of potential introducers. Since the microbial composition in colon diverges from the SI, so will the physiological response to, e.g., host-mediated defense mechanisms. As an example, Paneth cell-secreted lysozyme is one of the most important protectors of bacterial invasion in the SI. However, when these cells emerge in the inflamed colon, their secreted lysozyme promotes bacterial lysis of microbes. This includes the otherwise beneficial *Ruminococcus gnavus*, which is overly abundant in colon but not SI. Lysis of, e.g., *R. gnavus* liberates inflammatory effector molecules potentiating intestinal inflammation.[41]

susceptibility gene to be identified,[134] and its polymorphism have been linked to poor effectiveness of some probiotics in CD.[136] Specifically, the anti-inflammatory effect of *Ligilactobacillus salivarius* Ls-33 peptidoglycan correlated with the release of a specific muropeptide sensed by NOD2.[137] In Paneth cells, NOD2 mediates the activation of nuclear factor κB (NF-κB), leading to the induction of defensins. Therefore, NOD2 polymorphisms in CD may reduce the production of defensins such as HD5, HD6, and hBD-2.[138–140]

Defensins are innate defense molecules produced at a variety of epithelial surfaces to balance protection against pathogens, while maintaining tolerance toward the intestinal microbiota.

Attenuated expression of defensins will compromise host immunity, as seen in CD where reduced alpha defensin levels are observed in patients with ileal disease and reduced hBD-2 levels in those with a colonic form of the disease.[141] In mechanistic studies, recombinant hBD-2 has been shown to curb inflammation in murine and human peripheral blood mononuclear cells via the Toll-like receptor 4 (TLR4)/NF-κB pathway. Consequently, it was possible to mitigate disease activity in three different colitis models.[142] Confirming a niche for probiotic administration, flagellin shed by probiotic *E. coli* Nissle 1917 but not the apathogenic *E. coli* strain, ATCC 25922, promotes hBD-2 expression.[143] As such induction is bound to be compromised in

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patients with frameshift mutation in the NOD2 gene, this patient group may benefit from orally administered hBD-2 but is unlikely to respond to probiotic treatments intended to stimulate endogenous defensin production. However, patients without this mutation may benefit substantially from intake of probiotic strains capable of eliciting an endogenous defensin (or other HDP) response. Such hypothesis is further supported by the above-described observation that rectally administered *E. coli* Nissle 1917 reduce inflammation in UC,[128] which is not characterized by the mentioned frameshift mutation but rather decreased colonic mucus secretion and thus is fully capable of eliciting an appropriate hBD-2 response upon stimulation. Indeed, 3-week oral administration of Symbioflor 2, containing one strain of several viable genotypes of *E. coli*, to 23 healthy individuals stimulated hBD-2 synthesis and fecal excretions.[144] Subsequent *in vitro* analyses revealed that this effect was mediated by a single genotype performing on par with *E. coli* Nissle 1917. Interestingly, the fecal hBD-2 peptide was still increased 9 weeks post treatment cessation, pointing toward sufficient engraftment potential of this probiotic.

MITIGATING RISKS OF POTENTIAL ADVERSE EFFECTS FROM PROBIOTIC USE

Safety is one of the prime characteristics of probiotics. However, being live microorganisms, they may cause harm under unfortunate conditions.[145] For example, a recent report demonstrated that over-the-counter probiotic usage curbed the efficacy of checkpoint inhibitor immunotherapy.[146,147] During clinical trials, it is important to assess such risks and act promptly in the event of adverse effects. As studies of small intestinal bacterial overgrowth (SIBO) show, the endogenous microbiota may also become detrimental to health. While the organisms associated with SIBO are similar to those commonly detected in the SI, their higher numbers cause symptoms to develop,[148] driven by a breakdown in defense mechanisms, such as the gastric acid barrier.[149] Similar breakdowns in microbial control mechanisms due to diet, lifestyle, or disease may increase sensitivity to otherwise benign endogenous microbes.[150] For this reason, it is of crucial importance to monitor and discuss the potential side effects of probiotics. Context-dependent microbial traits observed for *P. copri*, *Akkermansia muciniphila*, and *R. gnavus* (Figure 4), to name a few, may cause intestinal microbes generally regarded as safe to turn into foes.[77] This is especially relevant in susceptible hosts, including those with a leaky intestinal barrier or altered immune function.

CONCLUSIONS

Most of our understanding of the human intestinal microbiota is based on analyses of fecal samples. For this reason, there is a need for tools such as capsule systems to study the intestinal microbiota in the upper GI tract. *In vitro* and *in vivo* models provide substantial opportunities to study the interaction between the intestinal microbiota and various organ systems. However, these models need to be further developed, particularly to increase translatability. Finally, to improve our understanding of the

mechanisms of probiotic action in general and in the SI in particular, our knowledge of the intestinal microbiota and its activity needs to be expanded beyond the colon to also include the upper parts of the GI tract.

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