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From meta-omics to causality: experimental models for human microbiome research

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Abstract

Large-scale ‘meta-omic’ projects are greatly advancing our knowledge of the human microbiome and its specific role in governing health and disease states. A myriad of ongoing studies aim at identifying links between microbial community disequilibria (dysbiosis) and human diseases. However, due to the inherent complexity and heterogeneity of the human microbiome, cross-sectional, case–control and longitudinal studies may not have enough statistical power to allow causation to be deduced from patterns of association between variables in high-resolution omic datasets. Therefore, to move beyond reliance on the empirical method, experiments are critical. For these, robust experimental models are required that allow the systematic manipulation of variables to test the multitude of hypotheses, which arise from high-throughput molecular studies. Particularly promising in this respect are microfluidics-based *in vitro* co-culture systems, which allow high-throughput first-pass experiments aimed at proving cause-and-effect relationships prior to testing of hypotheses in animal models. This review focuses on widely used *in vivo*, *in vitro*, *ex vivo* and *in silico* approaches to study host-microbial community interactions. Such systems, either used in isolation or in a combinatory experimental approach, will allow systematic investigations of the impact of microbes on the health and disease of the human host. All the

currently available models present pros and cons, which are described and discussed. Moreover, suggestions are made on how to develop future experimental models that not only allow the study of host-microbiota interactions but are also amenable to high-throughput experimentation.

Keywords

Causality, diet, human microbiome, hypothesis testing, *in vivo* model, *in vitro* model, *ex vivo* model, *in silico* model, dysbiosis, disease, microfluidics, host-microbe interactions

Review

Introduction

A human individual's microbiota consists of around 100 trillion cells, which represent at least ten times as many cells as human cells constitute the body. These microbiota colonize the surface and deep layers of the skin, are found in saliva and the oral cavity, and in the conjunctiva as well as in the gastrointestinal tract (GIT) [1]. Recent large-scale metagenomic sequencing efforts, including those led by the human microbiome project (HMP; National Institutes of Health initiative) [2,3] and the metagenomics of the human intestinal tract (MetaHIT) [4,5] consortia, have convincingly corroborated the notion that humans should be considered as superorganisms in which the microbial symbionts play essential physiological functions [6,7]. Beneficial effects of the presence of microbial communities on human physiology range from immune cell development and homeostasis [8-10], food digestion *via* the fermentation of non-digestible dietary components in the large intestine [11-14] to balancing the host's metabolism [15-17] and promoting angiogenesis [18,19]. Negative consequences for the host linked to the microbiota include for example chronic inflammation and infection (for recent reviews see [20,21]). Furthermore, shifts in microbial community structure and function (dysbiosis) have been linked to numerous human diseases, including inflammatory bowel disease, diabetes mellitus, obesity, cardiovascular disease and cancer (recently reviewed in [22]).

The largest microbial reservoir of the human body is the GIT and, thus, it is also the most studied and important from a biomedical perspective [23]. Extensive analyses of small subunit (16S) ribosomal RNA gene sequences amplified from fecal samples [24-28], mainly reflecting the luminal microbiota of the distal large intestine, have more recently been supplemented by comprehensive data from large-scale metagenomic sequencing studies to establish a catalogue of microbial organismal and functional diversity in the GIT [4,5,7]. Descriptions at a more detailed taxonomic level reveal many hundreds of species and even more strains in a typical fecal sample [29]. Even though substantial inter-individual variation in microbial community composition has been reported [30], common sets of microbial species include members of the genera *Faecalibacterium*, *Ruminococcus*, *Eubacterium*, *Dorea*, *Bacteroides*, *Alistipes* and *Bifidobacterium* [4,25].

Beyond metagenomics, functional omic approaches, such as metatranscriptomics, metaproteomics and metabolomics (jointly referred to here as 'meta-omic' approaches), are now also rapidly gaining pace [31-33]. Integrated omic approaches provide qualitative and quantitative information on genetic potential, transcripts, proteins and metabolites that are present in microbial communities at specific points in space and time [34]. Moreover, such

approaches have the potential to highlight the systemic impact of microbial communities beyond the vicinity of the GIT and thereby highlight the intricate cross-feeding between both the human and microbial (eco-)systems. For example, Wikoff and coworkers showed using a metabolomic approach that amino acid metabolites found in mammalian blood were particularly affected by the GIT microbiome [35]. In particular, indole-3-propionic acid (derived from tryptophan) could only be detected in conventionally raised mice compared to germ-free mice [35]. Only after colonization of the germ-free mice with *Clostridium sporogenes* could this bioactive amino acid derivative be identified in the plasma of these animals [35]. Another example of metabolic cross-feeding evidenced by metabolomics includes the higher prevalence of tricarboxylic acid cycle (TCA) metabolites in conventionally raised mice *versus* germ-free mice [36].

It is clear that ‘meta-omics’ approaches result in data that is essential for the definition of baseline healthy microbiota and the identification of differences that may be associated with human disease [37]. Recently, integration of different ‘meta-omic’ approaches was successfully used to pinpoint bacterial members of the GIT community, which are active, damaged, or responsive to a given compound [38]. However, to causally link the identified differences in the human microbiota with distinct human phenotypes including diseases, experiments are essential (Figure 1A). Therefore, representative *in vitro* (Figure 1B) and *in vivo* (Figure 1C) experimental models are required, which allow the systematic manipulation of variables and, thus, allow experimental testing and validation of results derived from meta-omics.

Figure 1 From association to causality. (A) Functional co-occurrence networks, established by the analysis of human microbial communities from healthy and diseased cohorts by meta-omic approaches are crucial to define dysbiotic states and to correlate individual microbial community members with disease. (B) In order to gain detailed information about microbial compositional changes and their associated impact on disease, high-throughput *in vitro* experimental systems are essential. *In vitro* co-culture approaches allow the confirmation or the rejection of hypotheses resulting from meta-omic data. (C) In order to causally link changes in microbial community structure or in their associated biomolecular patterns with specific diseases, gnotobiotic animal models are indispensable for *in vivo* validation. In all panels, triangles represent different biomolecules whereas color-coded circles represent different microbial taxa.

***In vivo* models**

Germ-free (GF) animals are reared in sterile isolators to control their exposure to microorganisms, including viruses, bacteria and eukaryotic parasites [39]. If these animals are colonized with specific microorganisms they become gnotobiotic [39]. The colonization of GF animals mimics the birth of an infant from a sterile environment of the womb towards a microbe-dominated environment. A considerable advantage of GF animals is that they can be made gnotobiotic with GIT microbial communities of specific donors (human or other animal species) and therefore allow analysis of the systemic impact of specific microorganisms on the xenograft recipient. Indeed, colonization of GF animals with single individual bacterial species allows one to directly link a putative function or shaping of the GIT to a particular bacterial species or group [40,41]. Being able to associate a specific function to a particular bacterial strain or species is of utmost importance considering the possibility that specific organisms could be used as a treatment for a given disease. However, a major drawback of monocolonization of GF animals with specifically selected bacteria is the lack of scope of

such investigations to uncover the full effects of single bacterial species on the host because of lack of full microbial community context in such approaches. Therefore, the beneficial impacts of a single bacterial species/strain on the host should be validated by taking into account the full microbial community context. In particular, shifts in microbial community structure need to be assessed when adding single strains to communities to avoid potentially triggering a dysbiotic state.

Different GF animal models (zebrafish, mice, rats and even pigs) have already been successfully established (Tables 1 and 2). Undoubtedly, such animal models have proven to be essential for studying host-microbe interactions (in particular immune-microbe feedbacks) and have allowed causal links to be established in a limited number of studies [42,43].

Table 1 Advantages and disadvantages of different animal models commonly used for studying host-microbe interactions

Animal model	Advantages	Disadvantages
Zebrafish	<ul style="list-style-type: none"> • Transparent until adulthood allowing real-time visualization of fluorescently labeled microbes throughout the gut [44]. • Chemical screens and forward genetic tests can be performed to investigate host genetic factors or signaling pathways regulated by microbes [46]. • Relatively short generation time (3 to 4 months) with a progeny size of about 100 to 200 eggs/female [46]. • 3 to 4 cm long as an adult allowing storage of a large number in laboratory facilities [46]. • Genome fully sequenced (http://www.sanger.ac.uk/Projects/D_rerio/). • Well characterized mutant strains [46]. • Gastrointestinal tract (GIT) is homologous to that of mammals, containing a liver, pancreas, gall bladder, and a linearly segmented intestinal tract with absorptive and secretory functions. The intestinal epithelium forms tight junctions and microvilli. Displays absorptive enterocytes, goblet cells, and enteroendocrine cells (reviewed in [45]). 	<ul style="list-style-type: none"> • GIT is homologous to that of mammals but not identical (reviewed in [45]). • Diet and living environment strongly differs from humans • Aging differs strongly from humans • Zebrafish and their natural pathogens exist at a temperature of 28°C, while most human-relevant pathogens are only infectious at 37°C [47]. • Zebrafish do not have distinguishable lymph nodes, Peyer's patches, or splenic germinal centers [48].
Mouse	<ul style="list-style-type: none"> • Numerous mouse specific disease models or genetically altered mice are available [49]. 	<ul style="list-style-type: none"> • Marked differences in the immune system [50]

Rat	<ul style="list-style-type: none"> • Well characterized model; genome fully sequenced (http://www.sanger.ac.uk/resources/mouse/genomes/) and virtually all mouse genes have human homologues. • Relatively small and thus can be easily maintained. • Reproduction rather quick so that several generations can be observed in a relatively short period of time, generally a mouse can live 2 to 3 years. • Mice present the same organs as humans but in different proportions • A lot of rat-specific disease models or genetically altered rats are available (http://rgd.mcw.edu/wg/physiology). • Genome fully sequenced (http://rgd.mcw.edu/). • Relatively small and thus can be easily maintained. • Reproduction rather quick so that several generations can be observed in a relatively short period of time, • Generally a rat can live 2 to 3 years. 	<ul style="list-style-type: none"> • Marked differences in microbiota composition between mice and humans have been noted [51]. • Diet and living environment differs from human. • Diet and living environment differs from human.
Pig	<ul style="list-style-type: none"> • Omnivore. • Physiology of digestion, digestate transit times and associated metabolic processes are very similar between humans and pigs (reviewed in [52]). • Digestive tract shares many anatomical and physiological traits with that of humans. • Immune system similar to humans. • Genome fully sequenced (http://www.sanger.ac.uk/resources/downloads/othervebrates/pig.html). • Conserved homology between human and pig genomes. 	<ul style="list-style-type: none"> • Reproduction rather slow (4 months gestation), generally a pig can live 10 to 15 years. • Important in size and thus expensive and complicated to maintain in laboratory conditions.

Table 2 Examples of successfully conducted microbiota transplantation experiments into germ-free (GF) recipient animals

Animal model	Host Microbiota	^a GF Status <i>versus</i> CONV-R ^b Animals	Donor's Microbiota in the Recipient Animal
Zebrafish	Predominantly <i>Proteobacteria</i> (82% ± 22%) and <i>Fusobacteria</i> (11% ± 15.2%). Minor populations are: <i>Firmicutes</i> , <i>Bacteroidetes</i> , <i>Verrucomicrobia</i> , <i>Actinobacteria</i> , candidate phyla TM6 and TM7, Planctomycetes, <i>Nitrospora</i> and candidate division OP10 [53,54].	Reduced rates of epithelial proliferation [53]. Compromised ability to use nutrients [53].	Mouse to zebrafish: predominantly <i>Firmicutes</i> and <i>Bacteroides</i> [51]; when GF zebrafish are colonized with mouse microbiota the transplanted community resembles its community of origin in terms of the lineages present, but the relative abundance of the lineages changes to resemble the normal gut microbial community composition of the recipient host. Thus, a selective pressure of the host is imposed to the recipient's gut habitat [54].
Mouse	Mouse and human microbiota are similar at the phylum level, but different at the genus level (>99% of the bacterial phylogenetic types from wild-type mice belong to two divisions: <i>Firmicutes</i> and <i>Bacteroidetes</i>) [51].	Most widely used GF animal model and theoretically any mouse strain can be derived to GF status. Numerous immunological differences in GF animals: Peyer's patches are poorly formed; composition of CD4 ⁺ T cells and IgA-producing B cells in the lamina propria is altered [57-63]; impaired development of follicular B- and T-cell areas of the spleen and peripheral lymph nodes [60]. Th17 and Treg CD4 ⁺ T cells are less efficient in GF mice [61-63]. The epithelial cell turnover is decreased by a factor 2 in GF animals compared to CONV-R mice [64,65]. Postnatal gene expression of β1-4-galactosyltransferase stays at low levels in GF mice [69] and in general host gene expression differs between GF and colonized mice [70].	Human to mouse: Transplantation of fresh or frozen adult human fecal microbial communities into GF mice results in stably and heritably colonized mice with a microbiota that reproduces much of the bacterial diversity of the donor's microbiota (all bacterial phyla, 11/12 bacterial classes, and 88% (58/66) of genus-level taxa) [55,56]. Obese mouse to GF mouse: Microbiota transplantation experiments utilizing genetically obese <i>ob/ob</i> mice [66], CONV-R mice fed a Western diet [67], and mice lacking the Toll like receptor 5 [68] have demonstrated that colonization with an obesity-associated gut microbiota results in an increased gain in adiposity relative to colonization with a gut microbiota harvested from lean controls.

Rat	Rat and human microbiota are similar at the phylum level but different at the genus level (wild-type rat microbial communities harbor at least eight different divisions dominated by two major phyla: <i>Firmicutes</i> (74%) and <i>Bacteroidetes</i> (23%)) [74].	<p>Compromised ability to use nutrients in GF animals compared to CONV-R mice [60]. Difference in metabolic signatures in GF mice compared to CONV-R mice and humans [72,73]. Difference in metabolic signatures [75-81]. In CONV rats, the colonic mucus layer is twice as thick as in GF rats [84], and the mucin chemical composition is altered [85,86]. Numerous immunological defects; the proportion of intraepithelial CD4+ and CD8+ T cells is altered [59,87], as well as the composition and T cell receptor repertoire [59]. Decreased enterocyte production [88].</p>	<p>Pig to mouse: when GF mice are colonized with pig microbiota the overall bacterial group distributions are similar, but colony and cell morphologies of bacteria grown on specific media are different between pig and gnotobiotic mice [71]. Human to rat: when GF rats are colonized with human microbiota the transplanted community resembles its community of origin in terms of the group or genus levels but differences at the dominant species level occur. Thus, a selective pressure of the host is imposed on the gut habitat [82]. However, certain metabolic characteristics (high equol-producing and low equol-producing status or cholesterol-to-coprostanol conversion) of human intestinal floras can be transferred to GF rats [82,83]. Human to pig: pigs seem to induce less host specific selection of the donor microbiota [92]. CONV-R pigs to GF pigs: Genes involved in biological processes such as epithelial cell turnover, nutrient transport and metabolism, xenobiotic metabolism, JAK-STAT signaling pathway, and immune responsiveness become upregulated by the colonization of GF pigs [91].</p>
Pig	The microbiome of pigs is dominated by two major phyla: <i>Firmicutes</i> ($\approx 81\%$) and <i>Bacteroidetes</i> ($\approx 11\%$) [89,90].	<p>Host gene expression differs between GF and colonized pigs [91]. Epithelial cell proliferation and differentiation genes are downregulated in GF piglets compared to CONV-R pigs [91].</p>	

^aGF, germ-free

^bConv-R, conventionally raised

From the most fundamental point of view, the study of GF animals demonstrated that life without microbes is possible [39]. Nevertheless, GF animals show a number of important physiological differences when compared to conventionally raised (CONV-R) animals (Table 2). For example, GF animals show a slower epithelial renewal [64,93], an altered immune system [57-63,94] and marked differences in gene expression of mammalian gastrointestinal cells [69,70,91]. Interestingly, GF animals require a higher caloric intake to maintain their body weight, which might be linked to less efficient whole-body metabolism compared to CONV-R animals [6,72,73,75-81,84,95]. Moreover, GF animals, at least GF rats, exhibit a decreased mucus layer in the GIT compared to CONV-R animals, which might be due to the fact that different bacterial strains stimulate the secretion of colonic mucin [85,86,96]. Thus, since a number of essential physiological functions significantly differ between GF and CONV-R animal models, data extrapolated from experiments carried out with GF animal models must be considered with caution (Tables 1 and 2). However, the comparison between an 'all' (CONV-R animals) or 'nothing' (GF animals) situation is still invaluable for the definition of the host's physiological pathways and functions, which are impacted and/or influenced by different microbiota (reviewed in [60]), as well as for the characterization of the biogeography of the GIT [97].

Although different animal models have been developed (Table 1), mice represent the most widely used and best characterized model organisms. Therefore, we chose to illustrate the pros and cons of experimental models by highlighting investigations carried out in GF mice. Several research groups have, for example, investigated how monocolonization by individual bacterial species shape the immune system of the host. In such studies, investigators have demonstrated that colonization of GF mice with particular segmented filamentous bacteria (SFBs) is sufficient to induce Th17 cell populations in the lamina propria of the gut [62,63]. This specific T cell subpopulation, which is important for protecting the host from bacterial and fungal infections, is absent or marginally detectable in GF mice [62,63]. Interestingly, it was recently shown that human colonization with SFBs, in contrast to mice, seems to be age-dependent with the majority of individuals losing SFBs by the age of 3 years [98]. Moreover, colonization of GF mice with human gut microbiota supplemented with SFB only results in a minor increase in the number of intestinal T cells. This indicates that multiple microbial species are involved in the development of intestinal T cells in humans [99] and that most likely different species and strains are important in humans compared to mice. Similarly, colonization of GF mice with indigenous *Clostridium* spp. [61] or *Bacteroides fragilis* [100] by means of a protease-resistant capsular polysaccharide, can increase the frequency or function of colonic CD4⁺ T regulatory cells, which in turn play critical roles in the maintenance of immune homeostasis [8]. However, these findings have yet to be fully validated in humans.

A particularly interesting animal model for investigating microbes-host immune system feedbacks is the reversible microbe colonized GF mouse model that was recently described by Hapfelmeier *et al.* [101]. The reason behind the development of this model is that the continuous presence of commensal intestinal bacteria has made it difficult to study mucosal immune dynamics (for example, kinetics and longevity of IgA's), which in turn does not allow answering fundamental questions such as: Is constant bacterial exposure required to induce antibody production? Is repetitive contact of the same bacterial species with the host immune system required to provide specific antibodies? What is the minimal bacterial concentration required to induce antibody production? This model consists of GF mice, colonized with a triple mutant *Escherichia coli* strain, which cannot divide and persist *in vivo* but which can sustain intestinal colonization up to 48 hours. Using this model the authors

could decipher the dynamics of IgA immune responses and were able to demonstrate that (i) induction of high-titer IgA can be uncoupled from permanent intestinal bacterial colonization, (ii) an intrinsic threshold exists between 10^8 and 10^9 bacteria in the GIT below which the intestinal IgA system shows no response, (iii) the intestinal IgA system lacks classical immune memory characteristics (no observable prime-boost effect), and (iv) the intestinal IgA repertoire represents the most dominant species currently present in the intestine [101].

Using analogous GF approaches, relationships between diet, gut microbial ecology and energy balance have also been investigated (recently reviewed in [21,22,102]). For example, it was demonstrated that transplantation of the cecal microbiota from obese mice fed on high-fat diets into GF recipients increases adiposity, thereby demonstrating a causal relationship between certain microbiota compositions and the host's energy-harvesting capacity [67].

From GF animals experiments it is now well established that microbial communities impact deeply on essential physiological functions of the host. However, it is oftentimes difficult to causally link an apparent dysbiotic state to disease in humans mainly because of marked differences between mouse and man (see also paragraph below). In a few cases, however, causality within the context of dysbiosis-linked diseases has been demonstrated using microbial transplantation experiments. For example, a colitis phenotype was transferable from *Tbx21*^{-/-}/*Rag2*^{-/-} mice, which develop ulcerative colitis in a microbiota-dependent manner, to wild-type mice by adoptive transfer of the implicated microbiota [42]. Similarly, there is also evidence that an altered microbiota associated with a colitogenic phenotype isolated from NLRP6 inflammasome-deficient mice alone is sufficient to drive intestinal inflammation [43].

While all GF as well as disease-specific CONV-R animal models present a number of advantages for studying host-microbe interactions, they often do not yield reliable preclinical results that readily translate into effective human treatments. Two important factors contribute to this failure: i) on the microbial side, bacterial species that colonize the GIT appear to be host-dependent and, thus, a host-specific microbiota is critical for a given host [99]; and ii) on the host side, the immune responses in non-human mammalian species is oftentimes distinct from those seen in human [50,103]. A partial solution to this problem could be the use of animals that show a humanized immune system. These animal models can be generated by grafting immunodeficient animals with suspensions of hematopoietic progenitor cells and/or human peripheral blood cells, and potentially even with supplemental human tissues driving the generation of human immune cells [104-107]. However, in order to causally link dysbiosis with human diseases, these animal models need to be gnotobiotically transplanted further with 'humanized' microbiota. The latter poses additional potential pitfalls, as many microbial species have evolved to fill host-specific niches [55,82,97] and the topology of the mouse GIT is, for example, distinct from that of humans, rendering the mimicking of human-specific niches challenging if not impossible. Consequently, xenograft microbiota may not representatively colonize the GIT of a humanized animal model.

Another conceivable approach to study the interplay between host and microbes is the use of CONV-R animal models treated with antibiotics, leading to a temporary knock-out effect of selected bacterial groups, followed by a repopulation of host GIT with human feces-derived microbial communities. The assumption behind this idea is that these animals do not present significant alterations in essential physiological processes described for GF animals and that the antibiotic intake does significantly enhance the reshaping effect of the transplanted human microbiota. Surprisingly, the combination of antibiotic and transplantation treatments does

not increase the establishment of the donor phylotypes but does interfere with the establishment of the exogenous communities by a yet unknown mechanism [74].

In summary, animal models, especially humanized GF models, can be attractive tools for human microbiome research. However, in addition to some of the pitfalls discussed above, animal models are labor-intensive (there is an immense logistical challenge associated with keeping the animals GF), relatively expensive, tedious, and limited in high-throughput. The establishment of animal models that are widely applicable realistic models of human diseases and that could be used to study the specific interplay between microbiota and their host presents a formidable challenge. Therefore, *in vitro* human-microbial co-culture strategies might offer alternative and complementary strategies, since they have a unique potential to facilitate much needed high-throughput validation of hypotheses that are emerging from state-of-the art molecular data and that link certain microbial community compositions and functions to human disease.

***In vitro* models**

In vitro models mimic microbial processes along the GIT by employing either distinct serially connected bioreactors/microchannels or a single bioreactor/microchannel mimicking specific part(s) of the human GIT (Table 3). Such models represent enticing alternatives over *in vivo* models because they are typically cheaper and offer greatly improved throughput, flexibility and scalability for hypothesis testing. Moreover, downstream high-resolution molecular analyses are more readily carried out on *in vitro* generated samples compared to those derived from *in vivo* experiments. Traditional *in vitro* systems are however usually based around the partitioned cultivation of specific microbiota in dedicated bioreactors connected in series [108]. They typically lack human cells because they have been developed to model the individual steps catalyzed by the microbiota along the human GIT.

Table 3 *In vitro* models used to study host-microbes interactions

Feature	Transwell Inserts	SHIME [109]	M-SHIME [110]	TIM1 [111]	TIM2 [112]	CCS [113]	Gut-on-a-Chip [44]
Human cell culture^a	+ ^b	-	-	-	-	+ ^b	++ ^b
GIT microbiota culture	-	+	+	-	+	-	-
Anaerobic conditions for microbes	-	+	+	+	+	+	-
Mucin	+	-	+	-	-	-	-
pH measurement	-	+	+	+	+	+	-
Throughput	+	-	-	-	-	-	+

^aThe models involving the possibility to incorporate human cells can be subjected to Trans-epithelial Electric Resistance (TEER) measurements (except the model CCS).

^b+, co-culture for 3 to 6 h; ++, co-culture for >1 week.

CCS, continuous culture system; GIT, gastrointestinal tract; M-SHIME, Mucus-Simulator of the Human Intestinal Microbial Ecosystem; SHIME, Simulator of the Human Intestinal Microbial Ecosystem; TIM1, model simulating the stomach and small intestine; TIM2, model simulating the large intestine.

A pioneering example of such an *in vitro* system is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) [109]. Five reactors, harboring mixtures of luminal microbes,

are sequentially connected to mimic acid- and pepsin-mediated digestions in the stomach, metabolism of monosaccharides in the small intestine as well as the distinct microbial fermentative processes that occur in the ascending, transverse and descending colons, respectively. Recently, a mucus layer has been integrated in the SHIME model (M-SHIME) that allows improved simulation of the mucosal and luminal microbiota in the GIT [110]. The SHIME and M-SHIME models allow the study of gut microbiota using either specific isolates or mixed fecal inocula from healthy and diseased (for example, Crohn's disease) donors. Altogether, these models can be used to examine roles of the GIT microbiota in the digestion of specific human food ingredients (for example, fermentation of arabinogalactan, xylan and pectin [114]), to understand the pharmacokinetics of drugs (for example, sulphasalazine [114]) and/or to model the gut microbiota linked to gastrointestinal disorders [115].

Another well-established model is the *in vitro* GI tract system (TIM1 and TIM2) [111,112]. These automated models simulate the actions that occur along the GIT with peristaltic mixing as well as the absorption of water and fermentation products [111,112]. The TIM1 model simulates the stomach and the small intestine [111], whereas TIM2 mimics the large intestine [112]. The unique characteristics of these models are their unique capability to reflect a drug's bioavailability in the intestine. Other unique characteristics include the modeling of the luminal conditions in the GIT of humans and monogastric animals by taking into account the secretion of gastric and small bowel fluids, GI transit times and discharge of microbially metabolized compounds. Therefore, similar to the SHIME model, these models allow investigations of the metabolic capability of the effective microbiota in the GIT. However, the TIM models clearly lack the possibility of partitioning the luminal and mucosal microbiota in artificial niches that reflect the GIT and, thus, cannot be regarded as fully representative models.

A third example of an *in vitro* model is a three-stage continuous culture system (CCS), which was originally designed to study the effect of mucin on microbial sulfate reduction and methanogenesis [113]. The highlight of this model is that it reproduces some of the nutritional features, pH characteristics and fluid retention times of the large intestine with each vessel having a different operating volume and pH. Three different vessels are interconnected and mimic the microbial activities in the cecum, transverse colon and descending colon by taking into account the following characteristics: 1) the cecum is a nutrient-rich environment and has microbial growth at low pH, and 2) the other two subparts of the colon are rather nutrient limited with slow microbial growth at neutral pH. Recently, co-cultures of bacterial communities isolated out of a CCS and human intestinal Caco-2 cells were sustained for approximately 3 h in order to study bacterial adhesion to epithelial cells and to measure bacteria-triggered cytokine release [116,117].

A conceptual colonic fermentation model that is analogous to CCS - three similar sized reactors are connected in a continuous culture system mimicking the proximal, transverse and descending colons each at a distinct pH - has recently been suggested by Payne *et al.* [118]. Human fecal microbiota, either planktonic or immobilized on polysaccharide beads, can be inoculated in the proximal reactor and, thus, the metabolic transformations of different nutrients can be investigated. The results can be compared with the metabolism of the same nutrients in a batch-type reactor mimicking only one compartment and, thus, highlight the relative importance of the different compartments for the digestion of specific nutrients.

Although the above-mentioned *in vitro* models have been successfully used to address specific research questions, they present a number of limitations, which hinder their routine use for the study of versatile questions related to the human GIT microbiome. The major drawback of all the models is the lack of long term co-cultures of human and microbial cells, and the subsequent inability to investigate questions related to host-microbe interactions [102,109]. Furthermore, all of these models are limited in the scope of hypotheses that can be tested. The former shortcoming might be taken care of by using Transwell inserts, in which microbial and human cell cultures can be separated by semipermeable membranes. This arrangement should theoretically allow continuous co-cultures of human cells with microbial consortia from the respective bioreactor compartments. However, the Transwell cell cultures can only be employed as end-point assays [119-122] and bear ample risks for cross-contamination of cultures in human-microbe co-culture experiments. Most importantly, however, the representative inclusion of strictly anaerobic microbiota in Transwell insert setups is not possible.

In contrast, adaptation of microfluidics-based cell culture approaches provide important characteristics for development of improved GIT *in vitro* models. These characteristics include laminar flow profiles, small volumes, continuous diffusion-based perfusion, controlled chemical gradients and the ability to probe cells in spatial confinements mimicking their extracellular matrix *in vivo* [123-125]. Most of these characteristics are unachievable in macro-scale bioreactor setups but they are essential for the ability to directly co-culture human and microbial cells under physiologically relevant conditions [126]. The ability to co-culture human and microbial cells is particularly important to understand the intricate interplay between the human and microbial components that might be driven by direct cell-cell interactions and/or extracellular signaling. Naturally, these interactions have a marked impact on all processes related to the human microbiome and, thus, should be incorporated in any representative *in vitro* model. Consequently, pronounced interest currently exists for the development of microfluidics-based *in vitro* models of the human GIT [127], in particular models that allow human-microbial co-cultures. The enormous potential of such approaches has recently been demonstrated by a study focusing on host-pathogen interactions [128], and by the successful co-cultivation of symbiotic microbial communities in aqueous microdroplets that were probed for synergistic interactions [129]. Conversely, *in vitro* (micro-)fluidics-based systems have so far been mainly used for studying medically relevant biofilm formation using microbial isolate cultures [130-132]. Although several research groups have co-cultured different human cell types [133,134] only a limited number of studies have reported the successful co-culture of human cells with microbial isolates [135-138]. Microfluidic cell co-culture devices typically incorporate semipermeable membranes or porous materials that allow cell feed to diffuse through the permeable barrier to the cells, thereby protecting them from shear stress while simultaneously allowing exchange of nutrients and waste products. Efforts have recently been made to culture multiple cell types across such permeable barriers thereby allowing the mimicking of tissue niches [139]. Incorporation of protective yet permeable barriers is especially pertinent when co-culturing human cells and microbiota due to the large differences in their respective growth rates and possible bacterial virulence to their human counterparts [128].

Microfluidics-based *in vitro* human-microbial co-culture models may offer interesting characteristics for conducting rapid first-pass experiments aimed at proving cause-and-effect relationships (Figure 1B). Moving beyond the traditional lab-scale bioreactors, microfluidic models should allow the co-culture of human and microbial cells for extended periods of time and allow targeted perturbation experiments to be carried out.

Most recently, a promising microfluidics-based Gut-on-a-Chip model has been presented that indeed allows the direct co-culture of epithelial cells with probiotic strains [44]. The model includes many dynamic physical and functional features of the human GIT essential for transport, absorption, and toxicity studies. Therefore, it can be regarded as an essential research tool for drug testing [44]. However, the model still lacks important features, most notably a simulated mucosal barrier and the provision for culturing strict anaerobic microorganisms that dominate the human gastrointestinal microbiota.

Nonetheless the Gut-on-a-Chip model has laid the foundation for the development of novel microfluidics-based devices that allow sustained cultivation of human and representative gut microbial communities (also encompassing anaerobes) to study the links between microbial dysbiosis and disease pathogenesis in a truly systematic and representative manner (Figure 2).

Figure 2 Conceptualization of an idealized *in vitro* gastrointestinal experimental model.

An idealized *in vitro* co-culture model may include three distinct culture chambers, namely microbial, human epithelial and human immune cell culture chambers, each separated by semipermeable membranes allowing molecular cross-talk between the different contingents while preventing microbes from rapidly overtaking human cells due to pronounced differences in their respective growth rates. Furthermore, an idealized gastrointestinal *in vitro* model should reflect the biogeographical distribution of the gastrointestinal microbiota. Such a model should allow the culture of representative microbial communities for the individual sections of the gastrointestinal tract (GIT) including stomach, small intestine, ascending colon, transverse colon and descending colon. All the individual compartments should be connected in series and allow modulation of their respective environmental factors including pH, fluid retention times, growth medium and other physiological factors such as mucin (in green in the microbial chamber) compositions, which actively interact and alter the microbial communities. To represent the GIT in the most realistic way, the microbial growth chamber needs to be depleted of oxygen, which could be achieved by flushing this chamber with anaerobic microbial medium, whereas the human cell chambers need to be flushed with oxygenated medium. Finally, an idealized GIT *in vitro* model suitable for microbiome research must support high-throughput omic analyses and, thus, needs to allow probing of the individual contingents to perform dedicated analyses on the different cell contingents following a particular experimental regime and to relate particular measurements back to the cell populations of origin.

***Ex vivo* models**

Besides *in vivo* and *in vitro* experimental models, *ex vivo* models represent interesting tools to study host-microbiota interactions. Such systems comprise explant cultures (tissue cultures) extracted from the colon or rectum [140]. The advantages of such models, is that the tissue cytoarchitecture, including epithelial, lymphocyte subtypes and follicular-dendritic cell networks, is preserved and, thus, such systems can mimic more closely the *in vivo* situation when compared to traditional *in vitro* systems including isolated human cell types. For example, Tsilingiri *et al.* recently described a human mucosa explant culture model in which an apical to basolateral polarity is preserved during stimulation with bacteria [141]. Using this model, the authors demonstrated that probiotics are not always beneficial for the health of the host but that some postbiotics (metabolic products of probiotics) can protect healthy tissue against the inflammatory properties of invasive *Salmonella* [141].

Major drawbacks of *ex vivo* models are that during surgical resection, the mucus layer is at least partially removed or damaged. Additionally, the tissues are generally treated with a cocktail of antibiotics to avoid microbial contamination and therefore no immediate co-culture of microbial communities and human GI cells is achievable.

Furthermore, explants do not offer the required modularity that would allow analyses to be related to specific cell populations but rather only allows links to whole communities of cells, thereby convoluting any relevant molecular signals.

***In silico* models**

To investigate microbe-microbe and host-microbe metabolic interactions, *in silico* reconstructions of genome-scale metabolic networks combined with constraint-based modeling also exhibit promising attributes. The conversion of a metabolic reconstruction (for example, as derived from the human genome sequences, microbial isolate genome sequences and/or metagenomic sequences) into a condition-specific model (for example, GIT model) requires the transformation of a (hopefully) comprehensive biochemical reaction list into a computable, mathematical matrix format [142]. It also requires the addition of physico-chemical constraints (for example, mass conservation) and system boundaries [142]. Thus, (meta-)genome-scale, manually curated metabolic reconstructions can serve as knowledge-bases to summarize existing knowledge about cellular pathways in target organisms in a well-structured, mathematical manner [142]. However, so far, the knowledge-base for microbe-microbe as well as host-microbe interactions is very sparsely populated and extensive dedicated efforts are required in coming years to establish such a database. Consequently, at present, cross-feeding and/or interaction experiments cannot yet be routinely carried out *in silico*.

Even though, *in silico* constructed models represent powerful tools for modeling and predicting phenotypic characteristics of single organisms living in a community within a particular host [143], they can only be constructed based on existing experimental data. Consequently, *in vitro*, *ex vivo* and *in vivo* experimental data, which allow the unraveling of links between gut microbiota and host metabolism, are crucial for constructing comprehensive host-microbe interaction models as well as for the benchmarking of such models. Once constructed, models can predict what role individual microorganisms have and how their actions influence others within their native community [144]. The resulting hypotheses may drive experiments, which will in turn inform and improve the models. Thus, a combined computational-experimental approach for hypothesis generation and testing has the promise to accelerate new discoveries in the realm of microbe-microbe and microbe-host metabolic interactions. Disadvantages of *in silico* models are that the reconstruction process of such genome-scale high quality metabolic networks requires extensive computational and manual analysis efforts and that any new findings need to be experimentally validated. Furthermore, the resulting findings, especially in the case of *in silico* animal-based models [145], may not always efficiently translate to humans [143,145]. Despite the limitations of current *in silico* reconstructed host-microbe interaction models, such approaches are of utmost importance because they theoretically allow a detailed molecular resolution of the complex relationships within microbial communities and with their host. Therefore, such system approaches could dramatically improve our understanding of individual bacterial taxa within communities and the modes of interactions in which they engage. New links between a host and its microbiota could thus be predicted and perturbation experiments (for example, diet variation for the host or changed medium composition for the microbiota) and their

outcome could easily be simulated. An example of a successful use of such an *in silico* reconstructed model was recently published by Heinken and co-workers [145]. In this study, the authors reconstructed and analyzed the first integrated stoichiometric model of murine and *Bacteroides thetaiotaomicron* metabolism and could demonstrate *in silico* that even at a low growth rate of *Bacteroides thetaiotaomicron* the mouse profited significantly from the presence of this microorganism in the gut lumen [145].

In summary, host-microbe *in silico* models, when combined with experimental data, will greatly strengthen our knowledge on how microbes influence their host and *vice versa*. Consequently, *in silico* models in combination with *in vitro*, *ex vivo* and *in vivo* experimental data will become an invaluable tool to predict metabolic interactions between gut microbes and their host in both diseased and healthy states.

Conceptualization of experimental models

A conceptualized ideal experimental model (Figure 2) for the study of host-microbiota interactions in the GIT and one that would allow testing the myriad of hypotheses linking dysbiosis to disease should allow paired wet- and dry-lab experiments and mimic as closely as possible the GIT. Such a model should in particular include: i) human GIT cells; ii) human microbiota sustainably growing under anaerobic/microaerophilic conditions; iii) a mucus layer simulating the physical separation of human and microbial cell contingents; and iv) the physico-chemical conditions encountered in the GIT including primarily pH, fluid retention times and dissolved O₂ concentrations. Moreover, such a model should reflect simple and controlled experimental settings to allow reproducibility and limit discrepancies in the obtained results due to inter-individual variations occurring in *in vivo* animal models. Finally, in the case of a wet-lab model, it should allow massively parallel screening and validation of results revealed through meta-omic investigations of human subjects.

An *in vitro* living cell-based and microfluidics-based model appears best suited to achieve the highlighted requirements since the gut microenvironment can be simulated by flowing specialized media at defined rates through the respective microchambers seeded with human intestinal cells and microbial communities, respectively. Furthermore, to simulate the physical separation of both human and microbial cells encountered *in vivo* [146], both contingents should be separated either by mucin and/or semipermeable membranes while still guaranteeing molecular cross-talk (metabolites, proteins, nucleic acids, cytokines, chemokines, *etcetera*) between both compartments. The feasibility of such a microfluidics-based *in vitro* model has already been established by the successful co-culture of a single microbial species for over one week on the luminal surface of cultured epithelial cells without compromising epithelial cell viability [44]. However, a future challenge that needs to be overcome in order to be able to reproducibly analyze the interplay between microbes and their host in such an experimental model is the requirement for maintaining aerobic conditions in the human microfluidic chamber while at the same time guaranteeing strictly anaerobic conditions in the microbial chamber, which allow the culture of obligate anaerobic human gut microbes. Finally, an idealized *in vitro* model should include separate compartments mimicking the stomach, small intestine, the ascending colon, the transverse colon and the descending colon, each reflecting the biogeographically distinct characteristics of the GIT.

In summary the conceptualized *in vitro* experimental model could be an elegant supplement to animal *in vivo* and *in silico* models since it would exhibit simple and controlled

experimental settings allowing reproducibility and limit discrepancies resulting from inter-individual variations that occur in *in vivo* animal models. Moreover, by the introduction of human cells the interplay between human and microbial cells should be traceable in real-time. This point seems to be of particular importance since a host-specific microbiota appears to be critical for a given healthy host [99]. Finally, the described conceptualized *in vitro* experimental approach would be well suited for high-throughput experiments in contrast to *in vivo* animal models. However, due to their *in vitro* nature experiments carried out in such a conceptualized experimental model will for the most part still require *in vivo* experimental validation. Combined experimental approaches in animal and *in vitro* models could thus lead to the establishment of causal relationships between microbial community compositions and human diseases.

Conclusions

Understanding of the human gastrointestinal microbiota and its putative role in governing health and disease states has rapidly expanded in recent years. However, the myriads of results, generated by linking ‘meta-omic’ data to disease, still require experimental validation. To reach this goal, we propose a biphasic experimental validation approach: i) rapid first-pass experiments in *in vitro* devices, which allow massively parallel screening and immediate cause-and-effect read-outs (Figure 1B) and ii) *in vivo* experimental validation (Figure 1C).

Microfluidics-based *in vitro* devices appear particularly well suited for high-throughput experiments due to their small footprint, their ability to allow the co-culture of both human and microbial cells for extended periods of time, and their ability to facilitate dynamic perturbation experiments. However, *in vitro* experiments will for the most part still need to be followed up with *in vivo* experiments. Such experimental validation may be achievable using GF animal models. However, owing to specific differences in the living environments of GF and wild-type (CONV-R) animals, the physiologies of both are likely to be distinct, which leads to the somewhat artificial nature of GF animals. Therefore, in order to study the impact of specific microbial strains on the host, gnotobiotic animal models should be directly compared to CONV-R animals. In addition, GF animals should ideally be compared to ex-GF animals colonized by exposing them to the environmental conditions in which CONV-R animals were raised.

We are living through exciting times in human microbiome research. With the advent of high-throughput molecular tools, we are for the first time able to probe the extensive organismal and functional diversity of the human host and identify links between certain microbial community constellations and disease. Beyond this cataloguing effort, experimental validation will become a major component of future studies. Importantly, all the necessary technology for devising representative high-throughput *in vitro* models is available. In the future, such models will become invaluable for large-scale screening efforts prior to *in vivo* experimental and clinical validation.

Abbreviations

CCS, continuous culture system; CONV-R, conventionally raised; GF, germ-free; GIT, gastrointestinal tract; HMP, Human Microbiome Project; MetaHIT, metagenomics of the human intestinal tract; M-SHIME, Mucus-Simulator of the Human Intestinal Microbial Ecosystem; SFB, segmented filamentous bacteria; SHIME, Simulator of the Human Intestinal Microbial Ecosystem; TCA cycle, tricarboxylic acid cycle; TIM 1, model simulating the stomach and small intestine; TIM 2, model simulating the large intestine

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JVF, MSD, PS, JGS, and PW wrote the paper. All authors commented and approved on the final version of the manuscript.

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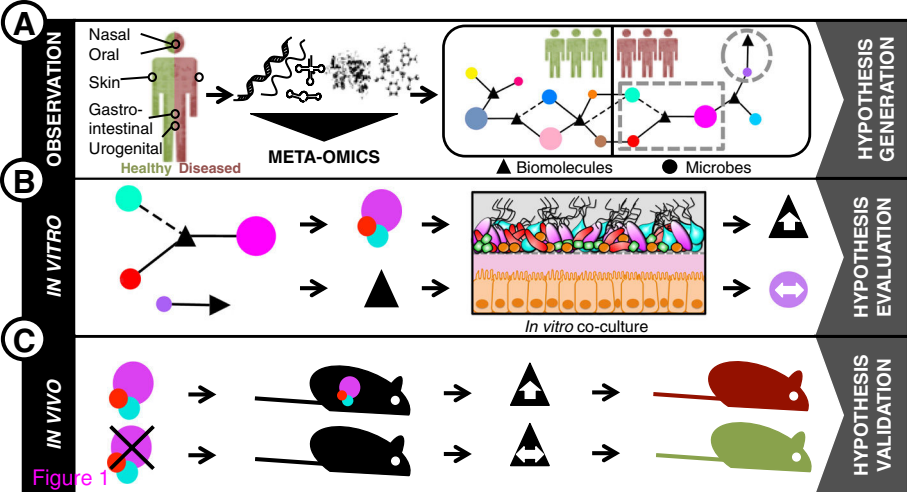


Figure 1

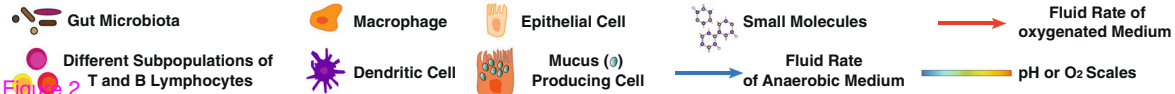
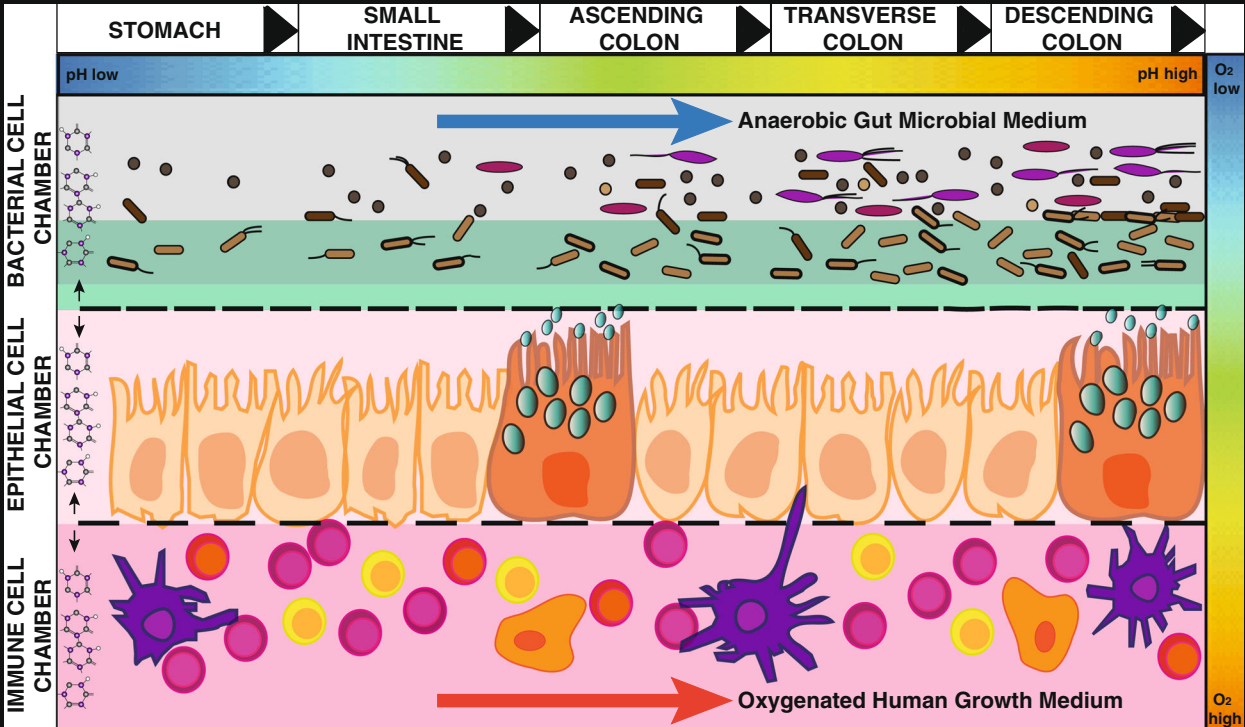


Figure 2