The Use of Defined Microbial Communities To Model Host-Microbe Interactions in the Human Gut

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The Use of Defined Microbial Communities to Model Host-Microbe Interactions in the Human Gut

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Running Head: Defined Intestinal Microbial Communities In Vivo and In Vitro

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SUMMARY

The human intestinal ecosystem is characterized by a complex interplay between different microorganisms and the host. The high variation within the human population further complicates the quest towards adequate understanding of this complex system that is so relevant to human health and well-being. To study host-microbe interactions, defined synthetic bacterial communities have been introduced in gnotobiotic animals or in sophisticated *in vitro* cell models. This review reinforces that our limited understanding has often hampered appropriate design of defined communities that represent the human gut microbiota. On top of this, some communities have been applied to *in vivo* models that differ appreciably from the human host. In this review, the advantages and disadvantages of using defined microbial communities are outlined, and suggestions for future improvement of host-microbe interaction models are provided. With respect to the host, technological advances, such as the development of a gut-on-a-chip and intestinal organoids, may contribute to more accurate *in vitro* models of the human host. With respect to the microbiota, due to increasing availability of representative cultured isolates and their genomic sequences, our understanding and controllability of the human gut ‘core microbiota’ is likely to increase. Taken together, these advancements could further unravel the molecular mechanisms underlying the human-gut microbiota superorganism. Such a gain of insight would provide a solid basis for the improvement of pre-, pro- and synbiotics as well as the development of new therapeutic microbes.
INTRODUCTION

Given its involvement in metabolic, nutritional, physiological and immunological processes, the human intestinal microbiome can be regarded as an essential organ of the human body (1). Further strengthening its clinical relevance, the intestinal microbiome has been linked to numerous disease conditions, including metabolic and immune disorders, cancer and neurodegenerative diseases (2). Apart from a remarkable increase in genome sequence data of the human gut microbiota, however, progress in functional insight has been hampered by its complexity: the existence of more than 1,000 prevalent species (3) combined with the high interpersonal variation within the human population in terms of genetics, environment and habits, results in a complex entity termed the human-microbiome superorganism (4). The number of known host-microbe interactions has grown rapidly over the past decades, yet many aspects still remain obscure.

To solve this complexity, there is need for a reductionist approach in which both host and microbiome are simplified to the extent that experimental variables can be tightly controlled and deliberately manipulated. Regarding the microbiota, synthetic or defined communities have been proposed as useful models to study microbial ecology (5). In recent years, the number of cultivable gastrointestinal microbial species has rapidly expanded (3) by the use of sophisticated or brute force culturomics approaches (6, 7). These strategies have allowed for the design of defined communities that are representative of the normal human intestinal microbiota. With respect to the human host, laboratory animals, notably mice, have proven valuable models for developing human medicine. The colonization of germ-free (GF) animals with defined bacterial communities, resulting in gnotobiotic animals, has already been applied for decades. During the 1960s and 1970s, it was recognized that the intestines of GF animals display aberrant histological, anatomical and physiological characteristics compared to conventional laboratory animals (8). The development
of the Schaedler cocktail for colonization of the murine gut (9) marked one of the first attempts to
normalize GF mice. An altered version has been widely adopted as a standardised gut microbiota
by animal breeders and biomedical researchers ever since. Over time, various other defined
communities have been designed to generate gnotobiotic animals for purposes beyond
standardisation; they have proven a valuable in vivo tool to study microbial ecology (e.g. microbial
invasion, microbe-microbe interactions, and metabolism) and host-microbe interactions. However,
mice and other animal models have various limitations that hamper their use as models for the
human microbiome, as has been recently reviewed (10, 11). Interesting alternatives concern the
development of sophisticated in vitro models, such as organ-on-chip systems and organoids.

This review summarizes existing models of host-microbe interactions in which defined
communities, as models of the (human) gut microbiota, were applied. We aim to present all in vivo
studies that used defined microbial communities representing the intestinal microbiota of healthy
individuals and in which host parameters were considered. The design of these model communities,
as well as the selection of its host, are compared and critically evaluated. The potential use of
defined communities in in vitro (cellular) models, as a surrogate host, are outlined as well. We
conclude by discussing the increased value, opportunities and possible obstacles when applying
defined communities in to-be-developed in vitro host-microbe interaction models.
Defined Communities Mimicking the Normal Intestinal Microbiota \textit{In Vivo}

A number of recent studies addressed host-microbe interactions \textit{in vivo} by using defined communities representative of the healthy human gut microbiota (Table 1a-c). These include various mouse studies with more or less defined intestinal microbiota that are summarized below. Studies in which animals were antibiotic-treated before bacterial colonization are excluded from our analysis as their reproducibility and gnotobiology cannot be reassured (12). The following section first discusses the specifically named defined communities applied in rodents (Table 1a, n = 31), followed by non-specifically named communities in rodents (Table 1b, n = 16). Finally, the defined communities administered to non-rodent models are discussed (Table 1c, n = 6).

\textbf{(Altered) Schaedler flora}

In 1965, Russel W. Schaedler colonized GF mice with a defined microbial community composed of strains isolated from normal mice, to study the fate of the bacteria in the gastrointestinal tract (GIT) and their effect on caecum size. With respect to these parameters, it turned out that the Schaedler flora (SF) was able to, at least partially, normalize the caecum size of the GF size in comparison with animals raised under conventional conditions (9). The defined microbial population was supplied to animal vendors to serve as a community that could limit the infection of ex-GF rodents with opportunistic pathogens. Schaedler developed several different bacterial cocktails over time. In 1978, Roger P. Orcutt set out to standardize and improve the SF flora, but in view of the monitoring costs, the total number of bacterial species was limited to eight. Orcutt made a selection of bacterial species (Altered Schaedler Flora (ASF)) based on their representation and stable colonization in the murine gut, their ease of identification (morphologically) and their presence in or interference with isolator contaminants. For instance, the cocci and spore-forming,
blunt-ended rods were eliminated, which represented the majority of isolator contaminants. Also, the amount of facultative anaerobes was limited, as they outgrew aerobic isolator contaminants and thus, impeded the ability to detect the latter (13). The ASF consists of six Firmicutes (*Clostridium* species (ASF356), *Lactobacillus intestinalis* or *acidophilus* (ASF360), *Lactobacillus murinus* or *salivarius* (ASF361), *Eubacterium plexicaudatum* (ASF492), *Pseudoflavonifractor* sp. (ASF500) and *Clostridium* sp. (ASF502)), one Bacteroidetes (*Parabacteroides distasonis* (ASF519)) and one Deferribacteres (*Mucispirillum schaedleri* (ASF457)).

The ASF has been used multiple times as a reference or minimal defined microbiota, and its applications were extensively reviewed elsewhere (14). Several studies involving ASF in mice (or other animals) reported its effect on host parameters (Table 1a-c). The list is probably not exhaustive, given the wide application of ASF mice as control or minor population in studies, which makes these studies harder to identify.

The applications of ASF in rodents varied from wild-type strains (mostly C57BL/6, but also C3H/HeN and Swiss-Webster mice) to models prone to diseases including IBD (15-17), type I diabetes (18) or colorectal cancer (19). The ASF lacks Proteobacteria, a phylum shared by mice and humans, whereas some researchers did introduce Proteobacteria to ASF mice, such as *Oxalobacter formigenes* (20) and *Escherichia coli* (21). Other studies included only selected members of the ASF, because not all were found to successfully colonize the murine caecum (18) or to test the level of colonization resistance of different combinations of ASF members (22). Overall, the application of ASF to study host-microbe interactions has been quite diverse, regarding host strain, gut region of interest and host parameters studied.

Although the ASF has been used multiple times as a reference microbiota and has aided in the establishment of other defined microbiota, such as Oligo-MM and the Bristol Microbiota, its
representability of the normal gut microbiota has been criticized (23), as discussed later in this review.

Oligo-MM

Another defined community of murine microbiota, Oligo-MM\textsuperscript{12}, was constructed in an attempt to provide full colonization resistance against \textit{Salmonella enterica} serovar Typhimurium (\textit{S. Tm}) (22). Twelve strains were selected to represent the five most prevalent and abundant phyla of the laboratory mouse intestine, i.e. Firmicutes: ‘\textit{Acutalibacter muris}, \textit{Flavonifractor plautii}, \textit{Clostridium clostridioforme}, \textit{Blautia coccoides}, \textit{Clostridium innocuum}, \textit{Lactobacillus reuteri}, \textit{Enterococcus faecalis}; Bacteroidetes: ‘\textit{Bacteroides caecimuris}, ‘\textit{Muribaculum intestinale}; Actinobacteria: \textit{Bifidobacterium longum} subsp. \textit{animalis}; Proteobacteria: ‘\textit{Turicimonas muris}’ and Verrucomicrobia: \textit{Akkermansia muciniphila}. Colonization resistance of ASF mice or mice colonized with Oligo-MM\textsuperscript{12} and/or (a subset of) ASF strains, were compared to conventional mice. ASF was used as a reference, because of its wide usage in gnotobiotic mouse research. Oligo-MM\textsuperscript{12} mice conferred increased, but not full, resistance compared to mice colonized with a subset of ASF strains with and without Oligo-MM. Functional genomic analysis of Oligo-MM and whole ASF revealed that both consortia together cover 66.6\% of the KEGG modules of a conventional mouse microbiota. Addition of three facultative anaerobes (\textit{E. coli}, \textit{Streptococcus danieliae} and \textit{Staphylococcus xylosus}), underrepresented in Oligo-MM\textsuperscript{12}, increased coverage and furthermore, conferred full colonization resistance (22). C57Bl/6 mice stably colonized with Oligo-MM\textsuperscript{12} have been designated stable Defined Moderately Diverse Microbiota mice (sDMDMm2). The designers of Oligo-MM\textsuperscript{12} stressed the importance of expanding the amount of available mouse-derived strains, as initiated recently (24), in favour of the design of functionally defined and simplified microbial consortia for application in gnotobiotic animals (22). Because Oligo-MM\textsuperscript{12} found to lack
the enzymatic pathway to carry out 7α-dehydroxylation, an important bile acid transformation, the
addition of Clostridium scindens (a 7α-dehydroxylating bacterium) was tested in another study.
This modification normalized large intestinal bile acid composition in mice, which was
accompanied by colonization resistance against Clostridium difficile and decreased intestinal
pathology (25). Finally, Oligo-MM12 served as a defined reference microbiota to verify the
significant difference between the bacterial composition in the large intestinal outer mucus layer
and the lumen (26), but host parameters were not assessed. Note that the latter two studies that
applied of Oligo-MM12 left out the three additional facultative anaerobes that were found to be
crucial for full colonization resistance.

SIHUMI(x)

Because ASF was found to poorly represent the dominant intestinal bacteria and ASF mice hardly
differed from GF mice in a key set of microbial biochemical activities (23) (Midtvedt criteria, see
below), a simplified human intestinal microbiota (SIHUMI) was established in rats to provide a
highly standardized animal model to study host-microbe interactions. Species were selected
according to their prevalence in humans, their fermentative capacity, the availability of their
genomic sequence and their ability to stably colonize the rodent gut. SIHUMI(x) includes four
Firmicutes (Anaerostipes caccae), Lactobacillus plantarum, Blautia producta and Clostridium
ramosum), one Bacteroidetes (Bacteroides thetaiotaomicron), one Actinobacterium (B. longum)
and one proteobacterium (E. coli). All seven members successfully colonized the rat intestinal tract
and total bacterial numbers in faecal samples did not differ from those in human faeces. The amount
of short-chain fatty acids (SCFAs) produced, however, was dramatically lower compared to
humans, probably owing to the smaller number of species. An eighth species was added to the
consortium (SIHUMIx), Clostridium butyricum, which led to increased butyrate production. All
members of the SIHUMIx were successfully transferred to offspring. Dietary interventions varying in fibre and fat content resulted in responses (partially) reflecting those observed in mice and humans (27).

In other studies, SIHUMIx served as a resident community to study the effect of the addition or removal of species. For instance, inclusion of *A. muciniphila*, a mucin-degrading commensal, was found to worsen intestinal inflammation induced by *S. typhimurium* Tm in mice (28). The same researchers recently showed, however, that in a colitis-prone mouse model colonized with SIHUMI, *A. muciniphila* did not induce or exacerbate intestinal inflammation (29). In two other studies, the polyamine-producing *Fusobacterium varium* was added to the low polyamine-producing SIHUMIx in mice, which disclosed that gut morphology was neither affected by increased putrescine concentrations (30), nor by higher levels of other polyamines and SCFAs (31). Additionally, the mechanism underlying the obesogenic potential of *C. ramosum* in a SIHUMIx-associated animal model was further investigated by including or excluding this bacterium in SIHUMIx-associated mice fed a high- or a low-fat diet. The increased body fat deposition in the presence of *C. ramosum* was suggested to be due to the upregulation of small intestinal glucose and fat transporters (32). It should be noted that, although SIHUMI was originally established in rats, all other studies applied the community in mice.

**Towards a normal model gut microbiota**

Since the generation of the Schaedler flora in the 1960s, other defined gut microorganisms have been developed in an attempt to normalize GF animals or generate animal models harbouring a bacterial community representative of the human gut microbiome. During the 1970s, Syed *et al.* aimed to normalize GF mice with respect to caecum size, caecal numbers of *E. coli*, histology of the intestinal tract, and the development of a mucosa-associated microbiota in stomach and large
intestine (33). A mixture of 50 strictly anaerobic (later designated ‘N-strains’ (34)) and 70 facultative anaerobes (‘F-strains’) were found to generate a normal mouse phenotype, whereas less complex bacterial communities led to intermediate phenotypes with respect to the parameters studied, including caecum size, caecal *E. coli* levels, GIT histology and development of a mucosa-associated microbiota in stomach and large intestine (33). The exact taxonomic classification of the species within the F- and N-strains was limited by lack of characterization at that time (33). It was considered likely that a number of the isolates used were identical. Based on morphology and fatty acid production, the total of number of different strains was estimated to be rather in the order of 35 (N-strains) and 60 (F-strains) (34). The N-strains alone could not control the *E. coli* population and caecum size when associated with mice fed on a crude instead of refined diet, but this could be restored by additional association with the F-strains (34). The F-strains were exploited as an indigenous gut microbiota to investigate *E. coli* plasmid transfer *in vivo* (35), but other studies using the N- or F-strains could not be identified.

At the end of the 1970s, the use of the UW-GL (University of Wisconsin Gnotobiote Laboratory) flora was reported, which was used as the intestinal microbiota of heterozygous athymic mice (36). This defined bacteriome consisted of nine Gram-positive species from the genera *Lactobacillus, Bacillus, Clostridium* and *Corynebacterium* (37) and additionally, two Gram-negative species that were not further specified (36). It was used to study its colonization resistance against *Candida albicans* (37) and *Clostridium botulinum* (36). The latter study compared UW-GL with other defined microorganisms including ASF and a partial UW-GL. Whereas death rates significantly dropped compared to GF mice, only complete UW-GL fully prevented *C. botulinum* infection (36). The use of the UW-GL microbiota has not been reported since.

Logically, the conception of a healthy or ‘normal’ microbiota is dependent on the available knowledge on conventional animals and/or healthy human subjects, and thus the composition
varied per study. While testing the effect of bacterial species on intestinal IgA immune system
development, Moreau *et al.* paid specific attention to communities of *Clostridium* species, which
was considered a dominant microbiota of the digestive tract of adult conventional mice (38). In
studies using defined communities with human-derived gut bacteria, species were selected based
on their prevalence in (healthy) human faeces (39, 40) and/or their representation of the major three
or four dominant phyla of the human gut microbiota (40-42). Next to the designers of Oligo-MM12,
only few studies acknowledged the presence of five phyla (including *Verrucomicrobia*) of the
human gut microbiota. A recently designed 14-membered synthetic microbiota that collectively
possessed important core metabolic capabilities was applied to study *in vivo* foraging of host-
derived mucus glycoproteins during fibre deprivation (43). Similarly, other studies took into
account the functional capabilities of species. For instance, one study included species that are able
to break down complex dietary polysaccharides not accessible to the host (*B. thetaiotaomicron*,
*Bacteroides ovatus, Bacteroides caccae*), to consume oligosaccharides and simple sugars
(*Eubacterium rectale, Marvinbryantia formatexigens, Collinsella aerofaciens* and *E. coli*), to
ferment amino acids (*Clostridium symbiosum* and *E. coli*) or to remove the end products of
fermentation by reducing sulfate (*Desulfovibrio piger*) or generate acetate (*Blautia
hydrogenotrophica*) (41). This community has been frequently exploited to study host-microbe
interactions or microbe-microbe interactions by the same research group or adopted by others,
albeit in different combinations ranging from eight to 15 species (40, 42, 44-50). Recently, a more
diverse, complex defined community comprising not less than 92 species was developed (51). The
consortium consisted of phylogenetically diverse, human-derived bacterial strains, which had
previously been cultured and sequenced. It also included strains representing species that were
demonstrated to be age- and/or growth-discriminatory in models of microbiota development during
the first years of life. Of all strains, 44 comprised a core group that could be detected in faecal
samples of all colonized mice, independent of dietary intervention (51). No host parameters, however, were assessed in this study.

Remaining inclusion criteria for defined communities are the availability of the genomic sequence and the cultivability of the species. Obviously, both criteria make each individual species more easily traceable. If the entire genetic repertoire of the defined community is known, gene expression of the whole community as well as its individual members can easily be assessed (28, 40) and their function can be more precisely predicted. Interestingly, although ASF has been used for over 50 years, publications on replication of the four extremely-oxygen sensitive ASF members on a defined medium, is still lacking (14).

**Defined communities in non-rodents**

Previously discussed defined microbiota were either isolated from rodents or applied to them. Laycock *et al.* stressed the need for a well-established intestinal colonization microbiota for pigs, given the higher representability of these animal models in early immune development studies (52): in pigs, there is no transfer of maternal immunoglobulin G in utero (53, 54), and a poorly developed mucosal system in neonates (55). Furthermore, pigs are genetically more similar to humans than mice (56), and their digestive physiology is comparable to ours (57). Colonization of germ-free piglets with ASF members turned out to be largely unsuccessful and only the most consistently colonizing ASF member (*Parabacteroides* sp.) was incorporated in the novel ‘Bristol’ microbiota. Additional strains were selected based on their representation of the major phylogenetic groups in gut sections of 12-18 week-old pigs, and either their ability to grow on a wide range of metabolic carbohydrate structures (*Roseburia intestinalis*) or their presence in unweaned pigs (*Clostridium glycolicum* and *Lactobacillus amylovorus*). Except for *R. intestinalis*, the novel microbiota successfully colonized the GIT after administration to germ-free piglets, with high clinical safety.
and an expected increase in immunoglobulin serum levels (52). The Bristol microbiota was exploited by other researchers as a simplified starter microbiota to study additional effects of a complex microbiota on early life microbiota development (58), the intestinal expression of a butyrate-sensing olfactory receptor (59) and on the gastric transcriptome (60). Note that in the latter three studies, the piglets were not maintained in a sterile environment, hampering comparison of the effect of the Bristol microbiota on host parameters between studies. A different ten-membered porcine gut microbiota, originally designed as a competitive exclusion culture for pigs, was used to investigate antibody repertoire development in ex-germ-free newborn piglets (61). Another ‘defined commensal microflora’ (DMF) included seven porcine bacterial species and was similar in composition to ASF. Species were originally isolated from the caecal contents of six week-old healthy pigs and administered to germ-free pigs to evaluate the interactions between intestinal commensals, antibiotics, probiotics and human rotavirus. This model was primarily applied as a model commensal gut microbiota of neonates (62, 63).

**Other Defined Communities In Vivo**

Apart from the defined communities as model for the normal (human) gut microbiome to study host-microbe interactions, other kinds of communities have been composed for application in gnotobiotic animals. These communities, however, are not listed in Table 1a-c and their application goes beyond the scope of this review, as they did not aim to represent the ‘normal’ microbiota. For instance, these include disease-specific consortia, e.g. IBD-related (15, 64-67). Others are age-specific, such as the Human Baby Microbiota (68-70), DMF (62, 63) and a recently developed *Bifidum*-dominated model consortium (71). Lastly, some communities were developed for therapeutic or probiotic purposes. A well-studied and globally marketed multispecies probiotic is the bacterial cocktail VSL#3, which was recently characterized at the genomic level and has been
used to treat various gastro-intestinal disorders (72-74). Other communities were designed to treat infections (amongst others, \textit{C. difficile} infection (CDI) (75-77) and colitis (78)), or to facilitate recovery of cholera (79). Two remarkable applications of defined communities, which were not per se meant to model the normal human gut microbiota, are discussed in more detail below.

**Therapeutic communities**

Although the concept is not new and pioneered already 30 years ago (75), the interest in faecal transplantations has recently increased and the avenue of synthetic microbiotas as stool substitutes has been suggested (80). A particular example of such a stool substitute is Microbial Ecosystem Therapeutic 1 (MET-1), designed as a synthetic stool mixture to treat recurrent CDI. Sixty-two species were recovered from the stool of a healthy 41-year-old female donor, of which 33 species were selected that were sensitive to a range of antimicrobials and were easy to culture. Two CDI patients that were ‘rePOOPulated’ with MET-1 returned to their normal bowel pattern within a few days and remained symptom-free for at least six months. The use of a synthetic stool mixture has several advantages over conventional stool transplants: (i) the bacterial composition is known, controllable and reproducible, (ii) a pure consortium is more stable than stool, (iii) the formulation is safe, owing to the lack of viruses and pathogens, and (iv) the administered organisms can be selected based on their sensitivity to antimicrobials, which further enhances safety (77). Some of these benefits also strengthen the use of defined communities in host-microbe interaction research. Notably, the application of MET-1 as a defined community in GF animals, instead of antibiotic-treated animals, was limited to one study, in which it was used as a healthy, Firmicutes-rich microbiota to study colitis susceptibility and host immune responses (81).

In contrast to the use of a defined synthetic community, the anaerobically cultivated human intestinal microflora (ACHIM) has been derived from a fecal sample from a healthy Western donor
that has been maintained in anaerobic cultured for more than 20 years now and has been applied in faecal microbiota transplantation (82). Although the microbiota is regularly checked for the absence of pathogenic organisms and multiple CDI patients have been treated successfully with this cultured microbiota transplant from a single donor (82), its composition is not controllable.

Instead of starting with a certain disease or phenotype and generating a defined community to treat this condition, as true for MET-1 and ACHIM, researchers recently tested different defined bacterial communities to generate various phenotypes in mice and to identify the strains responsible for the observed phenotypic variation. By administering GF mice with one of 94 different, defined bacterial consortia of species randomly drawn from the culture collection, strains were identified that modulated adiposity, intestinal metabolite composition and the immune system. According to the authors, a similar approach could be applied to identify and characterize next-generation probiotics or combinations of pre- and probiotics (83).

**Minimal communities**

Another category of defined communities is formed by minimal communities. Essentially, all defined microbial communities are minimal in the sense that they are not as complex as microbiota in vivo. Nonetheless, some studies exploited even more simplified defined consortia, i.e. with a limited amount of species or clearly lacking certain functions, to study host-microbe interactions in general. This is exemplified by bi-association studies involving single members of (dominant) phyla. In a recent study GF mice were colonized with *B. thetaiotaomicron*, as a prominent member of the adult human gut microbiota, plus one of three probiotic strains (*B. longum*, *B. animalis* or *Lactobacillus casei*) to study microbe-microbe and host-microbe interactions (84). In the same lab, gnotobiotic mice were colonized with bacteria from the two dominant phyla in the adult human distal gut microbiota – Firmicutes and Bacteroidetes. Based on
their prominence in culture-independent surveys in the distal human gut, the pattern of representation of carbohydrate active enzymes in their glycobiomes and \textit{E. rectale}’s ability to generate butyrate as a major end product of fermentation, a ‘marriage was arranged’ between \textit{E. rectale} and \textit{B. thetaiotaomicron}. This reductionist approach provided information on microbe-microbe interactions, the microbial response to host diet and the microbial effects on host physiology (e.g. the upregulation of production of (mucin) glycans by the host) (85).

Despite the value of minimal communities for studying microbe-microbe and host-microbe interactions, a study into mice colonized with another simplified microbiota (\textit{B. thetaiotaomicron} and \textit{B. longum}) clearly demonstrated that the simple microbiota could not reconstitute the metabolomic complexity of a humanized microbiota, i.e. derived from human donors (86). Nevertheless, Table 1b-c includes some minimal communities, because of their representation of major phyla of the human gut microbiota or relevant application to study host parameters.

**Critical Evaluation of Defined Communities In Vivo: The Microbiota**

In the preceding sections, we provided an objective description of defined microbial communities that have been applied in \textit{in vivo} models to study host-microbe interactions. The next section discusses the representability of these communities, focusing on their design criteria and source (murine vs. human). Additionally, a comparison is made between simple versus complex, and bottom-up versus top-down constructed communities. Suggestions for future design of defined communities representing the normal intestinal microbiota are provided as well.

**How representative are defined microbiota models of a normal microbiota?**

The development of defined communities representative of the human gut microbiota raises the issue: “What defines a normal microbiota?”. Among the included studies that aimed to design a representative gut microbiota, different selection criteria were used. The representation of the major
phyyla and various metabolic capacities have been frequently put forward. A meta-analysis was performed comparing the composition of the core mouse gut microbiome (based on five different mouse models, i.e. varying in age, phenotype and sampling site) with the human gut microbiome (based on 16 individuals) (87). Apart from the differences within the mouse microbiota, Bacteroidetes and Firmicutes were clearly the most dominant phyla in all samples (together 87-97%). (87) The same is true for the composition of well-established defined communities ASF, SIHUMI(x) and Oligo-MM¹² (75-87.5%). Similar to most murine microbiota included in the meta-analysis, however, ASF and SIHUMI(x) lack Verrucomicrobia, which was found among the five most abundant phyla in human and some murine samples (87). In that sense, Oligo-MM¹², originally designed to represent the murine microbiota, is compositionally more complete than SIHUMI(x), which was meant to represent the human microbiota. The frequently used ASF also lacks Actinobacteria and Proteobacteria, which are abundant in both murine and human samples (87-89). Similarly, a large part of the other defined communities discussed here (Table 1a-e) did not include representatives of all five major phyla of the human microbiota, some not even one of the two most prominent phyla. Note that species selection has been mostly based on microbiota composition of Western individuals.

Further, community design has been limited by availability of genomes and cultivability of strains. In the case of ASF, the number of species was limited for financial reasons, i.e. taking into account the monitoring costs. Nevertheless, this community has been frequently used in gnotobiotic animal models. The assumption that ASF mice can be regarded as conventional mice with respect to their gut microbiota, has been criticized (23). Several functional activities in faecal materials from ASF mice were analysed and compared to samples from GF and conventional rodents and other mammalian species, including humans. The five biomarkers investigated, the so-called Midtvedt criteria (i.e. conversion of cholesterol to coprostanol, conversion of bilirubin to urobilinogens,
degradation of β-aspartylglycine, degradation of mucin, and the absence of fecal tryp tic activity (23) are claimed to reflect host-bacterial interactions, independent of the intestinal localization of the bacteria involved and the kind of species. With regard to these criteria, faecal samples from ASF mice showed patterns more resembling GF rather than conventional mice (23), which complemented previous results demonstrating an abnormal microbiota in SPF mice (90). Although this could be due to one of the limitations of ASF, i.e. its low diversity, ASF mice were shown to be immunologically, reproductively and metabolically similar to conventional mice (23). The Midtvedt criteria were also used to assess the suitability of SIHUMI(x) as a model microbiome. SIHUMI(x)-associated rats shared four criteria with conventional rats, of which three were, however, less pronounced (27).

A major difference between ASF and a consortium such as SIHUMI(x), is the fact that the latter involves human-derived bacterial strains. Most members of recently developed communities, except for Oligo-MM, are of human origin as well. This may be obvious, given the fact that, although their microbiota is similar at the division (superkingdom) level, 85% of the microbial genera and species detected in mice are not found in humans (91). Although qualitatively, humans and mice share a largely similar core, their intestinal microbiota is quantitatively very different (87). On the other hand, the development of small intestinal immune maturation was found to be host-specific, with humanized mice resembling more closely GF mice than mice associated with a murine microbiota (92). This host-specificity might also, at least partially, explain the unsuccessful colonization of piglets with ASF (52). Additionally, humanized rodent models were claimed to have been utilized mainly for short-term biomedical research studies (14). The question remains how human-derived bacteria would adapt during long-term colonization and vertical transmission in murine hosts (14, 93, 94), and thus, which kind of microbiota would be most reliable to study host-microbe interactions when using murine hosts. The maximum colonization time reported in
the studies discussed here (Table 1a-c) was less than one year. With respect to vertical transmission, stability after transfer to offspring has been addressed mainly for murine microbiota only (ASF (95) and Oligo-MM (22)). Within the humanized defined communities, SIHUMI(x) is an exception, of which bacterial concentrations in caecum were verified between founder rats as well as their offspring. At the age of eight weeks, SIHUMIx-rats harboured similar bacterial levels as their founders, but not at two weeks (except for \textit{E. coli}) (27).

\textbf{Simplified versus complex communities}

The distinction between minimal communities, with two or three members, and larger defined communities is not black-and-white. For instance, ASF, initially used as a microbiota to standardize mouse models, slowly adopted the role of a minimal community, instead of one representing the normal microbiota of mice. Nonetheless, the simplicity of a defined community also has some advantages over more complex communities. The limited nature of ASF should, as proposed by Brand \textit{et al.}, allow investigators to evaluate the \textit{in vivo} effect of the removal or addition of bacterial species on mucosal homeostasis and colonization dynamics, or potentially, factorial interactions of the community (14). Indeed, some of the studies discussed here (Table 1a-c) used only a subset of the ASF species or added species to already established defined communities, including ASF and SIHUMI(x). Additionally, one- and two-member communities could be applied to model aspects of a more complete microbiota, such as depletion of certain dietary compounds or metabolites (86).

Finally, as already discussed, a simplified consortium makes each species traceable, as opposed to a very complex community (28, 40).

On the other hand, complex communities might more closely resemble the normal human gut microbiota and are more likely to confer colonization resistance to opportunistic pathogens, which has been a frequently mentioned criterion in the studies described above. In the 1980s, Freter
and co-workers formulated the nutrient-niche theory, which states that a certain bacterium can only successfully colonize if it is able to use a specific limiting nutrient more efficiently than its competitors (96). This implies that colonization resistance correlates with community complexity, as supported by several studies (22, 36, 97). Freter’s theory was corroborated in a recent study in which the relative abundance of each species of a ten-membered community was correctly predicted based on the concentration of individual dietary ingredients (41). The theory assumes, however, an environment in which bacterial growth is balanced and nutrients are perfectly mixed, whereas in reality bacteria are metabolically flexible (i.e. they have the ability to switch nutrient source) and nutrient levels in the gut are spatiotemporally heterogeneous (reviewed in (98, 99)).

Metabolic flexibility was hardly addressed in the studies discussed in this review. Some researchers did assure the inclusion of species in a defined community that, as a whole, was able to thrive on a wide range of nutrients. Once established in vivo, however, the behaviour of the community was seldomly addressed or only for a single species. This could be due to the fact that most of the included studies focused primarily on the effects of the whole microbiota or a subset of species on the host (host-microbe), rather than the exact nutrient niche occupation by its separate species (microbe-microbe interactions). Exceptional is a recent study, which quantified the in vivo response of both mucin-specialists (A. muciniphila and Barnesiella intestinihominis) and -generalists (B. caccae and B. thetaiotaomicron) upon fibre deprivation (43). A fibre-deficient diet stimulated the expansion and activity of the mucus-degrading bacteria, promoting epithelial access and pathogen-induced colitis (43).

With respect to spatiotemporal heterogeneity, Oligo-MM\textsuperscript{12} was used to verify that the bacterial compositions in the large intestinal outer mucus layer and the intestinal lumen are significantly different (26). Due to extensive mucus shedding and mixing in the lumen, however, the differences may be relatively small (98). Indeed, it was recently shown that, at microscale level,
the proximal colon should be viewed as a partially mixed bioreactor rather than a clearly compartmentalized gut section with spatially segregated communities. A next step would be to quantify the distribution of nutrients and metabolites and the role of host factors such as diet, gut motility and mucus composition (48). Vice versa, it would be interesting to study the effect of spatial organization on relevant host parameters, which were unfortunately not addressed in the latter study. The authors did admit that the 15-membered community used may not be complex enough to demonstrate stronger spatial associations with food particles, host cells and mucus (48), reinforcing, all in all, the need for more complex communities.

Both metabolic flexibility and spatiotemporal heterogeneity allow for increased community diversity, which is thought to be crucial for ecosystem robustness (98). Defined communities enable the precise investigation of both concepts, but, on the other hand, the question remains whether they can be made sufficiently complex to properly address these issues.

**Bottom-up versus top-down approaches**

One way to obtain a more complex model community is to start with a complex sample, e.g. human stool, and narrowing the amount of species down via one or more enrichment steps, e.g. by culturing on selective media (top-down approach (100)) or using fermentation models. Table 1a-c includes only a few examples with regard to normal microbiota (Oligo-MM\(^2\) (22), (40)). The majority of the studies listed in Table 1a-c used a bottom-up approach, in which single, previously cultured and characterized strains are combined into a synthetic bacterial community, e.g. based on selection criteria previously mentioned, and administered to germ-free animals. An advantage of the latter method is the known composition of the microbiota, as previously emphasized. A drawback, however, is formed by the risk that the desired phenotype (in this case a normalized host) cannot be entirely recapitulated (100).
A probably more important question is whether a normal microbiota actually exists. In the 1970s, Freter et al. concluded that significant fluctuations occur in the normal microbiota and that there is “no such a thing as a reproducible and precisely definable ‘normal enteric flora’”. Instead, they considered the F-strains collection most optimal to use as a microbiota representing a “state which is sometimes found in ‘normal’ individuals” (34). Clearly, the concept of the normal microbiota has changed over time and has evolved with the development of techniques to sequence the human gut microbiome, with increased insight into its composition, dynamics and function. Recently, researchers aimed to draw the compositional functional core of the human gut microbiota, or the core microbiome. They emphasized that the gut microbiome should be considered as a complex landscape, with both common and individual characteristics, and alternative stable states with respect to composition, structure and function (101). They listed a top set of 50 bacterial genus-like taxa that are part of the phylogenetic core, a common core of bacterial taxa shared by the majority of (adult Western) human individuals, based on data from previous studies (101-103). This core may include keystone species, whose role are crucial for ecosystem structure and function, for instance the breakdown carbon sources to support the growth of other core members (104, 105). Mapping this core including its key stone species, and comparing it with diseased microbiota, could increase our understanding of a normal microbiota and facilitate the design of a defined community representative of a healthy human gut microbiota. Next to the phylogenetic core, increased insight into the minimal intestinal metagenome (106) and the active functional core (107) within the human gut ecosystem might provide new criteria for assessing the ‘normality’ of a designed defined community. The paradigm seems to shift from rather black-box-like measures, such as the Midtvedt-criteria, to actually understanding the function of the gut microbiota and the
contribution of its individual species. Subsequently, this approach could allow a more thorough comprehension and more accurate design of age-, region- and disease-specific defined communities.

Although this review primarily focusses on bacterial communities, it should be mentioned that the human (gut) microbiome also includes fungi, archaea, microeukaryotes and many viruses, mainly bacteriophages. A study from 1980 included a ‘yeast fungus’ in a defined hexaflora, but the specific role of this microbe was not addressed (108). One of the few studies in this area addressed the interaction between the murine host, an archaeon \((\text{Methanobrevibacter smithii})\) and a bacterium \((B. \text{thetaiotaomicron})\) (109). In addition, the same research group designed a gnotobiotic animal model with a simplified defined gut community to study phage-bacterial host dynamics (45). In parallel with the healthy gut microbiome, researchers recently mapped the healthy gut phageome (110), but this field is still in its infancy. It is reasonable to assume that, with increasing insight into the role of non-bacterial gut microbes in host-microbe interactions, the design of defined microbial communities becomes more representative of the whole human gut microbiome.

**Critical Evaluation of Defined Communities In Vivo: The Host**

Next to the discussion on the exact composition of the defined microbial community, the selection of the host animal to study host-microbe interactions is critical. Rodents are the most commonly used mammalian models in which defined communities have been applied. The suitability of rodents as model for the human host was extensively reviewed elsewhere (10) and goes beyond the scope of this review. In summary, murine intestines are anatomically, histologically and physiologically very similar to human intestines, but size, metabolic rates and dietary habits differ largely, leading to qualitative and quantitative differences in microbial composition (10). With respect to the gnotobiotic models discussed in this review, there are some additional discrepancies
to be mentioned. The high value of using gnotobiotic animals as models of humans, i.e. their known composition and controllability, seem to be weakened by poor control of host parameters known to influence the human gut microbiome, such as diet, genotype, sex, part of the gut studied, age and the immune system.

**Host parameters influencing the microbiota**

Diet is a complex and strong determinant of gut microbiota composition (reviewed in (111, 112)). The individual species levels were assessed of a ten-membered defined community in mice fed with diets systematically varying in protein, fat, polysaccharides and simple sugars, in order to develop a model to predict the variation in species abundance. Next, the model was validated with 48 random combinations and concentrations of four ingredients selected from a set of eight human baby foods. Approximately 60% of the variation in species abundance could be explained by the known concentrations of pureed foods (41). This study exemplified the application of defined communities to systematically assess the response of individual gut members to various food components, which are, moreover, typical for the human diet. Clearly, a standardized diet of a laboratory animal is different from that of humans, which varies per region, season, individual taste and even per day. Some studies listed in Table 1a-c incorporated a previously developed prototypic “Western style” diet (27, 32, 39, 42, 46, 85), containing high amounts of saturated and unsaturated fats and carbohydrates commonly used as human food additives (i.e. sucrose, maltodextrin and/or corn starch). A lack of standardization in lab animal feeding protocols, however, has been emphasized previously for instance with respect to diet composition and texture (113) and indeed, diets used by studies discussed here are highly variable (Table 1a-c). Moreover, in ~40% of the studies, the diet was not clearly defined or not even reported, which is alarming given the large impact of diet on the gut microbiome.
The choice for mouse genotype also varied per study (Table 1a-c), although an effect of host genotype on microbiota composition was established within species (114-118). These results were corroborated by studies with defined communities such as ASF (119) and SIHUMI(x) (64). Additionally, colonization of different mouse strains with SIHUMI(x) demonstrated host-specific caecal levels of polyamines and SCFAs (31). In mice associated with *B. longum* and *B. thetaiotaomicron*, host genetic background was found to affect the overall transcriptome of the latter bacterium, but not the expansion of the bacterial substrate range of this bacterium (84). Obviously, defined communities allow the careful investigation of such host-dependent effects, but validation of host-microbe interactions in a wide range of host strains seems crucial before drawing conclusions and extrapolation to humans.

Although reports on the effect of gender have been contradictive (106, 117, 120-124) it might be a crucial determinant in gut microbiota composition and/or behaviour. In turn, commensal microbiota was shown to affect sex hormone levels (125, 126). Sex differences in gut microbiota composition were, recently, comprehensively investigated in 89 common inbred mouse strains. After excluding confounding by host genetics, diet, age or cage effects, the researchers detected gender-specific differences in taxa abundances and diet responses. These differences could be partially explained by sex hormones (127). Among the studies discussed here (Table 1a-c), one reported differences in metabolic profiles in urine and plasma between both sexes, but no explanation was put forward (39). In an older study, male mice were found more susceptible to death after *C. botulinum* infection, which could be explained by their coprophagic behaviour or a more general higher susceptibility to disease (36). In contrast, other studies reported an absence of gender-specific effects on, for instance, levels of *Oxalobacter formigenes* colonized in ASF mice (20) or assembly of a synthetic microbiota (43). Whereas some studies discussed here (Table 1) reported to have used a gender-mixed population, others included only one gender (n = 12 of 53
studies), in which male more often than female (nine vs. three) animals were used. Remarkably, the establishment of SIHUMI(x) was verified in both genders, whereas the effect of dietary fibre was tested in male and the effect of high-fat diet was investigated in female rats (27). A similar discrepancy was found in a study that assessed the effect of five fermented milk product strains in human female twins, but male gnotobiotic animals. Although microbiota responses were more or less similar in both species (40), such a gender-mismatch may complicate translation. Lastly, not all studies clearly reported the gender used per experiment, and approximately half of the studies did not report animal gender at all. This too, may hinder data reproduction and, more importantly, translation.

Defined communities allow the quantitative comparison of microbial compositions along the GIT, within and between models. ASF-associated mice were used to quantitatively demonstrate that the microbiota of the colon is poorly reflected in faecal samples (95). Relative abundance of species were also different between faeces (rectal swabs) and colon in pigs colonized with a defined microbiota (63). In rats colonized with SIHUMI(x), however, bacterial concentrations of caecum, colon and faeces were similar (27). Additionally, increases in relative abundances of mucin-degrading bacteria in caecum and colon upon switching to a fibre-free diet, were reflected in faeces (43). In a mouse model associated with a 12-membered community, individual bacterial levels were also similar between faeces and caecum (46). These conflicting results could be explained by various factors, including host, community composition and sampling time. Irrespective of the actual difference between GIT sites, it is disappointing that some other studies relied solely on faecal bacterial content. In a study applying a 92-membered community, for instance, not even half of the members could be detected in faeces. Other species may have established themselves in different regions of the gut, but this was beyond the scope of the paper (51). Nevertheless, due the invasiveness of sampling, systematic studies comparing colonic and faecal bacterial content are
lacking in humans as well (99, 112). The variation in GIT sites looked at by the studies included in Table 1a-c, makes it hard to compare the colonization pattern of the defined communities to natural colonization. Apart from differences along the GIT, capturing the transversal heterogeneity within one compartment may be crucial for properly modelling and understanding host-microbe interactions, as discussed above.

The age at which animals are colonized was quite variable among the studies, including animals bred with the desired defined community as opposed to GF animals colonized with the community of interest to create a gnotobiotic animal model. In the latter case, animals are inoculated at various time points among studies, whereas timing of microbial colonization was demonstrated to impact, amongst others, immune maturation (128, 129), mucosal homeostasis (130) and gut-brain axis communication in mice (131). Moreover, as previously discussed, colonization time of animals in studies discussed here (Table 1a-c) was limited. Nevertheless, some studies confirmed the stability of their defined community of interest over time and even over generations, which should be sufficient to draw conclusions within a specific colonization time window. This does, however, not allow to infer any information on the long-term effects of colonization.

A last factor determining gut microbiota composition and behavior is the immune system, which in turn is influenced by, amongst others, aforementioned factors and the gut microbiota itself. Looking at the studies discussed here (Table 1a-c), several researchers investigated immunological parameters such as serum immunoglobulin levels and the presence of (subsets of) immunological cells in the gut. Nevertheless, due to the complexity of the immune system, it is hard to quantify and compare the model hosts used with respect to immunological parameters. The key findings on the interactions of gut microbiota members and their products with the immune system have been recently reviewed elsewhere (100). The authors emphasized the value of minimal
microbiomes and subsequent standardized (animal) models. Determining the effects of specific gut microbiota on the host, could help to identify host-microbe interactions that shape the immune system (100). Most studies discussed in this review did not make a distinction between the contributions of each specific microbe to immunological effects observed.

The advantages and the levels of controllability of gnotobiotic research, as well as its pitfalls in practice, as outlined above, are summarized in Table 2.

**Validation of *in vivo* models**

As emphasized earlier, differences exist between humans and animals, not only limited to their intestinal microbiota. In line with the question what a normal or healthy intestinal microbiota defines, one could ask: “When is the animal model sufficiently representative of the human situation?” With regard to the studies discussed here (Table 1a-c), diverse host criteria are applied. For the models exploiting a murine microbiota, validation is relatively easy. Most researchers aimed to normalize GF hosts to conventionally raised animals, thereby focusing on host parameters such as caecal size or weight (9, 22, 33, 34). With respect humanized mice, validation is more complicated, but some studies made an effort. For instance, total bacterial numbers in feces and fecal SCFA levels between humans and SIHUMI(x) rats were evaluated, and a previously reported increase of Erysipelotrichaceae upon high-fat diet in humans was mirrored in SIHUMI(x) animals (27). Other host parameters (e.g. immune system or other systemic parameters) were, however, not taken into account. Similarly, validation was lacking in other studies applying SIHUMI(x), in which, moreover, mice were used instead of rats (28, 30-32).

A better example was recently described in a study in which the effect was tested of a fermented milk product in both humans and gnotobiotic mice humanized with a 15-membered microbiota. The proportional representation of the intestinal bacterial species and genes and
metabolic changes upon introduction of the probiotic strains, were hardly different between mice and men, but the researchers also acknowledged the limitations of their gnotobiotic animal model with respect to translatability (40). In most other studies (Table 1), control groups were limited to conventionally raised and GF animals or animals with a control treatment, for which translatability of the results to the human situation remains speculation.

**Defined Communities In Vitro**

As opposed to *in vivo* models, the use of defined communities to study host-microbe interactions *in vitro* has been limited, so far, although the development of sophisticated *in vitro* model systems is advancing rapidly. In this section we discuss *in vitro* models in which defined communities have been applied or could be applied to study host-microbe interactions. A distinction is made between models focused on the microbiota (e.g. composition and characteristics), and those that were designed to realistically represent the human host *in vitro*. Figure 1 summarizes all existing *in vitro* models of the human host and microbiota, illustrating how their interactions can be studied combining advanced *in vitro* cell based systems with defined communities. Ultimately, the goal is to combine best of both worlds.

**Modelling the intestinal microbiota in vitro**

The use of fermentation models has proven successful in modelling the intestinal microbiota *in vitro*, ranging from short-term batch incubations to multi-compartmental continuous systems. As discussed already, most defined communities applied in *vivo* (Table 1a-c) were constructed bottom-up, by selecting species based on their function, prevalence or other criteria. Alternatively, communities can be composed top-down by inoculating GIT-mimicking chemostats with human faeces. Well-known examples of these chemostats, such as the MacFarlane/Gibson three-stage continuous culture system, (M-)SHIME, EnteroMix, Lacroix Model and TIM-2, have been
The high reproducibility, stability, and complexity of bacterial communities cultured in chemostats (135, 136) has allowed the development and application of representative communities of the human intestinal microbiota in vitro. Most of these models, however, did not include a host component. The HMI TM module comprised a promising exception in which first, faeces from a healthy volunteer was fed into an adapted SHIME system, with fluid compartments mimicking the stomach, small intestine and ascending colon. Subsequently, the SHIME-effluent was exposed to an artificial mucus layer, separated by a semi-permeable membrane from a compartment containing Caco-2 cells. This module allowed the co-culture of bacteria with enterocytes up to 48 hours (137), which is discussed in more detail below.

Modelling the host in vitro

With respect to well-established defined communities, the probiotic cocktail VSL#3 and the faecal transplant substitute MET-1 have been tested on various human or animal intestinal cell lines (Caco-2, T84 and HT-29) (e.g. (138-140)). In most studies, however, the use of bacterial lysates or conditioned media was preferred over live bacteria (e.g. (72, 141-144)), because the – mainly anaerobic – gut bacteria cannot survive under the aerobic conditions needed for intestinal cell culture. In these 2D models, the interaction with the immune system or other tissues, cannot be studied. Although the direct effect of VSL#3 was tested on spleen and dendritic cells (145, 146), tissue-tissue interactions were lacking in these models. This problem can be (partially) solved in Transwell co-culture models, in which bacteria, mucosal immune cells and intestinal epithelial cells can be studied together (147). A Transwell model with an apical anaerobic compartment enabled the co-culture of an anaerobe bacterium with an intestinal cell line to study host-microbe interactions (148). Still, these cell lines lacked their tissue-specific context, including all major types of epithelial cells (e.g. Goblet cells, enterocytes, enter endocrine and Paneth cells) organized
in crypts and villi. Moreover, as cell lines are tumor-derived, their epithelial characteristics are affected. These issues have been overcome by the development of gut organoids, self-organizing 3D epithelial structures derived from intestinal stem cells (149) or human pluripotent stem cells (150). The use of organoids to study host-microbe interactions was reviewed elsewhere (151). The closed structure of organoids, in which the lumen is sealed with epithelial cells and a mucus layer, may facilitate the establishment of hypoxia in the core lumen (151). The anaerobic pathogen *C. difficile* survived up to 12 hours within organoids, but luminal oxygen levels still ranged from 5-15%, which may be tolerated by specific strains of *C. difficile* only (152). More recently, researchers developed an organ culture system for the mouse intestine, in which the stromal and hematopoietic components of the normal intestine were preserved *ex vivo*. The device supported the survival and growth of both anaerobic and aerobic microbiota, allowing the investigation of their effects on neuronal parameters (153).

The co-culture of defined microbial communities with human cells in Transwells, organoids or organ culture systems has been limited, probably owing to the static nature of these models. More advanced *in vitro* models to study host-microbe interactions have been developed (as recently reviewed in (133)) of which only a few have hitherto allowed the co-culture of multiple bacteria with intestinal cells or cell lines.

Organ-on-a-chip technology is an emerging concept within biomedical research, to replace conventional cell culture and animal testing. Organ-on-chips are microfluidic devices in which cells are cultured with organ-relevant spatiotemporal chemical gradients and dynamical mechanical cues, thereby aiming to reconstitute the structural tissue arrangements and functional complexity of living organs *in vitro* (154). Several gut-on-chips have already been developed (155-158), only one in which multiple intestinal bacteria were successfully cultured (158). In this device, two channels simulating the gut lumen and a blood vessel are separated by a membrane coated with
extracellular matrix and Caco-2 cells (158). As opposed to cell monolayers and organoids, the gut-on-a-chip is a dynamic model: shear stress and gut peristalsis are mimicked by continuous medium flow and stretching/relaxing of the membrane, respectively. Interestingly, these environmental cues stimulated Caco-2 cells to undergo differentiation into four types of intestinal epithelial cells, organized in 3D villi-like structures (159). Also, the successful incorporation of endothelial cells and peripheral blood mononuclear cells, was demonstrated (160). The authors claimed the successful cultivation of a single bacterium ‘on chip’ (*Lactobacillus rhamnosus*) for more than one week (158) and the eight-membered VSL#3 for at least 96 hours (160). The viability of the probiotic bacteria was, however, solely based on imaging, and which species exactly succeeded in ‘colonizing’ the crypts, was not exactly determined. The growth of anaerobic bacteria in this device has not yet been reported.

In contrast, another recent study reported the successful co-culture of strictly anaerobic bacteria, *B. caccae*, with *L. rhamnosus* and Caco-2 cells. In their microfluidic-based model mimicking the human gut, HuMiX, bacteria were grown in a separate, anoxic compartment (161). Similarly, the HMI™ module allowed the investigation of bacteria for up to 48 hours under microaerophilic conditions. FISH analysis revealed the presence of strict anaerobic bifidobacteria in the upper part of the mucus layer and the positioning of *F. prausnitzii* at the oxic-anoxic interphase (137). In both the HuMiX and HMI™ module, however, a mucin-coated attachment membrane prevented direct or natural contact between host and microbe. Moreover, as opposed to the gut-on-a-chip, gut peristalsis was not mimicked and the formation of the main epithelial cell types or crypts, were not reported in these models (137, 161).

A promising development in gut-on-chip technology is the incorporation of 2D organoids, which grow in a plane rather than in clumps, in the chip device (162), combining the advantages of organoids (tissue differentiation) with those of gut-on-a-chip technology (controllable flow,
mechanical cues and tissue-tissue interaction). To date, the cultivation of a defined intestinal microbiota in this device, has not yet been reported.

**Validation of *in vitro* models**

In comparison with animal models, validation of *in vitro* models is even more challenging. The cellular processes studied in Transwells, organoids or gut-on-a-chips, cannot be readily validated in human subjects. On the other hand, however, such sophisticated *in vitro* models enable the investigation of processes that cannot be readily studied in humans, increasing our understanding of the molecular mechanisms of certain bacterial compounds or products. Furthermore, they allow the elimination of potentially confounding factors present in *in vivo* models, such as the immune system. At the same time, this is also one of the major drawbacks of aforementioned *in vitro* models: as opposed to *in vivo* models, they lack a systemic component, whereas the impact of the gut microbiota on human health extends beyond the GIT. The emergence of organ-on-chip technologies has led to the concept of a ‘human-on-a-chip’ (163), but its implementation in research is still at an early stage. Nevertheless, the road to such a human-on-a-chip may be just as interesting. ‘Rebuilding’ the human body through assembly of its separate parts (lung-on-a-chip, gut-on-a-chip, kidney-on-a-chip, etc.), might increase our understanding of these building blocks and their contribution to the whole.

**Conclusions and Future Outlook**

Our understanding of the human gut microbiome has rapidly grown over the past decades, which has definitely supported the design of defined communities representative of the human gut microbiome. Whereas defined communities were initially aimed to normalize germ-free hosts to conventionalized mice, they could be a valuable tool to study host-microbe interactions, because of their controllability and traceability. For the same reasons, defined communities have a high
potential for therapeutic application. In this review, however, we showed that these rationally
designed consortia have been applied in *in vivo* models that are not entirely representative of the
human host environment. Next to the obvious and frequently discussed differences between mice
and men, we also discussed the power of gnotobiotic animals has been further undermined by poor
control of the host parameters known to affect gut microbiota composition and behaviour.

Simultaneously with the increasing knowledge on the human gut microbiota, the
implementation of more advanced *in vitro* models of the human gut is accelerating, with the
development of stem-cell derived organoids and gut-on-a-chip approaches. Although the research
is still in its infancy, these systems might partially replace the use of animal models. This
development is beneficial not only for ethical and – on the long-term – financial reasons, but also
from a scientific perspective. Human-inspired *in vitro* systems allow us to model and capture host-
microbe interactions at a more fundamental and controlled level.

Both the design of defined communities and *in vitro* models of the gut have not yet reached
their plateau. The former can be improved, via either bottom-up or top-down approaches. Key is
to further expand our knowledge about the intestinal microbiome in health and disease, in which
the NIH Human Microbiome Project and the European MetaHit project have played a crucial role
(106, 164) (bottom-up). The characterization of gut microbiota and genome sequences facilitates
the *in silico* prediction of host-microbe interactions through constraint-based genome-scale
metabolic modelling (165) or other types of mathematical modelling (166) and, subsequently, the
*in silico* design of representative defined communities (bottom-up). Further exploring our whole
microbiome, including phages, fungi and archaea, will revolutionize the design of microbial
communities as well (bottom-up). Lastly, the increased ability to reproducibly culture the
microorganisms in human faeces *in vitro* using well-established fermentation technologies (135,
167) may open the avenue to study human faeces-derived, functionally-enriched defined
communities at a more personalized level (top-down). In this way, both health- and disease-related microbiota can be easily reproduced. The same level of personalization can be obtained on the host side. For instance, the implementation of 2D organoids from patient-derived induced pluripotent stem cells in in vitro systems, such as the gut-on-a-chip with, can lead to highly personalized screening devices.

All in all, these models will provide a basis for the rational development and screening of novel therapies targeting intestinal diseases, ranging from anti-, pre- and probiotics to manipulate existing gut microbiota, to therapeutic microbes (168), faecal microbiota transplantation (169) and stool substitutes (77).

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Janneke Elzinga
Janneke Elzinga is a PhD candidate at the Laboratory of Microbiology in Wageningen, The Netherlands. She obtained a Bachelor’s degree in Biomedical Sciences and a Master’s degree in Molecular Mechanisms of Disease, both at the Radboud University in Nijmegen, The Netherlands. She has been working in the field of microbiology since March 2017, with a particular interest in key stone species of the human intestinal microbiota and in vitro models of the human gut to study host-microbe interactions. The application of these models may facilitate a better understanding of the molecular mechanisms underlying host-microbe interactions, with the potential to develop (personalized) therapeutic strategies.

John van der Oost
John van der Oost (1958) is leader of the Bacterial Genetics group in the Laboratory of Microbiology at Wageningen University, since 1995. Initially research mainly focused on unravelling unique features of central metabolic pathways in bacteria and archaea, revealing many novel enzymes and their regulation. In 2005 John van der Oost was appointed Full Professor, in 2013 he was elected as EMBO member, and in 2017 as member of the Royal Dutch Academy for Arts and Sciences (KNAW). The last decade, he used NWO grants (VICI-2005, TOP-2010/2015 and Gravitation-2017) to establish a successful research line on prokaryotic anti-viral defence systems (CRISPR-Cas and prokaryotic Argonaute). This has provided an excellent basis for development of unprecedented genome editing tools that currently find applications in biotechnology and molecular medicine (gene therapy).
Willem M. De Vos

Willem M. de Vos studied Biochemistry and obtained a PhD at Groningen University NL, partly done at the Max Planck Institute for Molecular Genetics in Berlin DE. Subsequently he spent a post-doc in Reading, UK and became Molecular Genetics Group manager at NIZO Ede NL. He is over 30 years Professor at Wageningen University NL, holds there the Chair of Microbiology, and serves as Professor of Human Microbiomics at the Medical Faculty of the University of Helsinki FI, where he chairs the Research Program Human Microbiome. His research aims to understand and exploit microbes using molecular, (meta)genomics and systems approaches. Since a dozen years his research interest is focused on the human intestinal tract microbiota and its relation with health and disease.

Hauke Smidt

Hauke Smidt studied Biotechnology at the Technical University of Braunschweig, Germany, and obtained his PhD from Wageningen University. Following a postdoc position at the University of Washington, Seattle, he rejoined the Laboratory of Microbiology at Wageningen University to head the Molecular Microbial Ecology group. In 2008, he has been appointed Visiting Professor at Nanjing Agricultural University, and since 2010, he holds a Personal Chair in “Complex Microbial Ecosystems” at Wageningen University. His research focuses on the integrated application of innovative cultivation and functional genomics-based methods to study composition and activity of intestinal tract microbiota in humans, farm and model animals, as well as their interaction with their host, in relation to host nutrition and health. Further interest lies on evolution and spread of antibiotic resistant bacteria and their genes, following an OneHealth philosophy that links environmental, human and animal health.
FIGURE LEGENDS

Figure 1. *In vitro* models of the human gut and gut microbiota. Models are organized from bottom to top, with the most representative and complex at the top and the most controllable and traceable - with respect to host parameters or microbial species - at the bottom.
TABLES
Table 1a. Studies using defined communities to study host-microbe interactions *in vivo*: Specifically named communities (n = 30)
The following study characteristics are listed: microbial consortium name (if applicable), taxonomic affiliation, strain source, host species and strain, part of the gut studied, no. of animals per experimental group, diet, sex, age and study outcomes reported.
* Two different strains tested are counted as one species. Strains were not always reported. Pathogenic species, in case of an infection model, are not included.
** The colonization time includes the time from colonization (0 in case of transfer of microbiota to offspring) till and including the time of sacrifice or end of experimental (e.g. dietary) manipulations, in case this is clearly stated in the paper. If age is given and animals are colonized at birth, the age is included in colonization time.
*** Study outcomes are only reported for the animals colonized with the defined community of interest
Abbreviations: LP = lamina propria; MLN = mesenteric lymph nodes; MPO = myeloperoxidase; NR = not reported; SCFA = short-chain fatty acids; Treg = regulatory T-cell

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Name consortium (no. of species*)</th>
<th>Phylum division</th>
<th>Strain source</th>
<th>Host species (strain)</th>
<th>Part of the gut studied</th>
<th>No. of animals per group</th>
<th>Chow</th>
<th>Sex (M/F, both or NR)</th>
<th>Age (col. time**)</th>
<th>Study outcomes***</th>
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<tbody>
<tr>
<td>(9)</td>
<td>Schaedler flora (5)</td>
<td>Mouse</td>
<td>Mouse (NR)</td>
<td>Mouse (NR)</td>
<td>20</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>4 wk (3 wk - 4 mo)</td>
<td>Colonization pattern; caecal size</td>
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<td>(36)</td>
<td>ASF (8)</td>
<td>Mouse</td>
<td>Mouse (HA/ICR)</td>
<td>Mouse (HA/ICR)</td>
<td>30</td>
<td>NR</td>
<td>Both</td>
<td>Adult (14-56 d)</td>
<td>Death after <em>C. botulinum</em> infection; faecal <em>C. botulinum</em> toxin excretion; colonization pattern of <em>C. botulinum</em></td>
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<td>(170)</td>
<td>Altered Schaedler Flora (ASF)</td>
<td>Mouse</td>
<td>Rat (F344)</td>
<td>Sterile food (Charles River) ad libitum</td>
<td>M</td>
<td>NR (2 wk)</td>
<td>Hepatic genotoxicity of mononitrotoluene isomers; metabolic activation of 2NT by intestinal bacteria; caecal bacterial content</td>
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<td>(16)</td>
<td>Mouse</td>
<td>Mouse (scid C.B-17)</td>
<td>Mouse (scid C.B-17)</td>
<td>Autoclaved pelleted diet ad libitum</td>
<td>4-6</td>
<td>NR</td>
<td>NR</td>
<td>(8-12 wk post reconstitution (After H. hepaticus infection)) Rectal prolapse; clinically severe disease; grossly thickened colon, cecum and rectum on necropsy; colonic</td>
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<td>Study No.</td>
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<td>Mouse Line</td>
<td>Rat Line</td>
<td>Study Duration</td>
<td>CD4+ T-cells</td>
<td>Inflammation Score</td>
<td>Colon Cell Proliferation</td>
<td>Histological Analysis</td>
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<td>(15)</td>
<td>Mouse</td>
<td>Rat (HLA-B27 on 33-3/F344)</td>
<td>NR</td>
<td>7-11</td>
<td>NR</td>
<td>at least M</td>
<td>Gross gut score, levels of MPO and IL-1B in caecal tissue; histologic inflammatory score of caecum and antrum</td>
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<tr>
<td>(171)</td>
<td>Mouse</td>
<td>Mouse (C3H/HeN)</td>
<td>NR</td>
<td>4-8</td>
<td>Irradiated diet (Harlan Teklad)</td>
<td>NR</td>
<td>6-8 wk (9-14 wk)</td>
<td>After colonization with <em>H. bilis</em> or <em>B. hyodysenteriae</em>: Caecal pathological gross and histological scores; serum IgG1 + IgG2a ab response</td>
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<tr>
<td>(172)</td>
<td>Mouse</td>
<td>Mouse (C3H/HeN)</td>
<td>NR</td>
<td>7-10</td>
<td>Irradiated diet (Harlan-Teklad)</td>
<td>NR</td>
<td>6-8 wk (10 wk)</td>
<td>Faecal bacterial contents; (after <em>H. bilis</em> infection:) caecal pathological scores; caecal histological changes; serum immunoglobulin</td>
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<tr>
<td>(173)</td>
<td>Mouse</td>
<td>Mouse (SW)</td>
<td>NR</td>
<td>2-5</td>
<td>NR</td>
<td>NR</td>
<td>Presence of Th17 cells and Foxp3+ regulatory cells in LP of small intestine</td>
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<td>(174)</td>
<td>Mouse</td>
<td>Mouse (C57BL/6)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Total intestinal IgA and intestinal IgA anti-CBir1; proliferation of splenic CBir1 TgT-cells after CBir1 gavage</td>
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<td>(175)</td>
<td>Mouse</td>
<td>Mouse (B6.Rag-/-)</td>
<td>NR</td>
<td>NR</td>
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<td>NR</td>
<td>Homeostatic and spontaneous proliferation of TCR Tg T-cells in LP</td>
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<td>(97)</td>
<td>Mouse</td>
<td>Mouse (C57BL/6)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>After infection: <em>S. Typhimurium</em> levels in mesenteric lymph nodes, spleen, caecum and feces; caecal pathology score;</td>
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<td>Study ID</td>
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<td>(21)</td>
<td>ASF (8,9)</td>
<td>Mouse (C57BL/6)</td>
<td>Mouse (C57BL/6)</td>
<td>3</td>
<td>caecal microbiota density; bacterial content and microbiota complexity in feces</td>
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<td>(21)</td>
<td>ASF (8,9)</td>
<td>Mouse (C57BL/6)</td>
<td>Mouse (C57BL/6)</td>
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<td>caecal microbiota density; bacterial content and microbiota complexity in feces</td>
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<td>(176)</td>
<td>ASF (8)</td>
<td>Mouse (NMRI, C57BL/6, BALB/c, NIH Swiss, SW, NMRI, MyD88/-/-, Ticam1/-/-, SMARTA, C57BL/6.CD 45.1+)</td>
<td>Mouse (NMRI, C57BL/6, BALB/c, NIH Swiss, SW, NMRI, MyD88/-/-, Ticam1/-/-, SMARTA, C57BL/6.CD 45.1+)</td>
<td>3-10</td>
<td>Caecal bacterial contents; colonic Treg cell response and relative IL-10 expression in spleen, MLN, Peyer's patches, colonic and small intestinal LP, thoracic duct lymph; IL-17 production; relative abundance of strains; microscopic localization in colon and small intestine</td>
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<td>(17)</td>
<td>Mouse (Nod1-/- and Nod2-/- on C57BL/6)</td>
<td>Mouse (Nod1-/- and Nod2-/- on C57BL/6)</td>
<td>NR</td>
<td>6-9 wk</td>
<td>Caecal bacterial contents; intestinal tissue conductance and Cr-EDTA-flux; E-cadherin protein expression and RegIII-gamma mRNA expression in colon; survival, colitis disease severity, histology score and myeloperoxidase activity after DSS-induction; colonic IL-6, IL-10, MCP-1, IFN-c, TNF-a, IL-12p70 levels</td>
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<tr>
<td>(177)</td>
<td>Mouse (C57BL/6)</td>
<td>Mouse (C57BL/6)</td>
<td>NR</td>
<td>Autoclaved food</td>
<td>REGIII-gamma RNA and protein expression in ileum and colon</td>
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Notes:
- ASF: African Swine Fever
- IgA: Immunoglobulin A
- E.coli: Escherichia coli
- MLN: Mesenteric Lymph Nodes
- Peyer's patches: Peyer's patches
- IL: Interleukin
- TNF: Tumor Necrosis Factor
- IFN: Interferon
- MCP: Macrophage Migration Inhibitory Protein
- DSS: Dextran Sodium Sulfate
- REGIII-gamma: RegIII-gamma
<table>
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<tr>
<th>Study</th>
<th>Species</th>
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<th>Treatment Duration</th>
<th>Analysis</th>
<th>Notes</th>
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<tr>
<td>(178)</td>
<td>Mouse (C57BL/6 and C57BL/6 TSLPR−/−)</td>
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<td>3-5</td>
<td>NR</td>
<td>NR (28 d)</td>
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<td>(179)</td>
<td>Mouse (NIH Swiss)</td>
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<td>4</td>
<td>NR</td>
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<td>(81)</td>
<td>Mouse (C57BL/6)</td>
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<td>Autoclaved mouse breeder’s diet (Harlan), unlimited access</td>
<td>5-14 per group</td>
<td>Both</td>
<td>6-12 wk (3 wk)</td>
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<tr>
<td>Study</td>
<td>Treatment</td>
<td>Species</td>
<td>Control</td>
<td>P-value</td>
<td>Gender</td>
<td>Duration</td>
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<tr>
<td>(26)</td>
<td>ASF (8)</td>
<td>Mouse</td>
<td>Mouse (C57BL/6)</td>
<td>3 (ASF), 5-23 (Oligo-MM)</td>
<td>NR</td>
<td>Both</td>
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<tr>
<td></td>
<td>Oligo-MM$^{12}$ was also used, but no host parameters were assessed</td>
<td>Mouse (C57BL/6)</td>
<td>Mouse (C57BL/6)</td>
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<tr>
<td>(20)</td>
<td>ASF (8,9)</td>
<td>Mouse</td>
<td>Mouse (SW)</td>
<td>4-7 per group</td>
<td>LM-485 autoclavable rodent diet, free access</td>
<td>M (no gender-effect observed)</td>
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<tr>
<td>8: ASF</td>
<td>Mouse</td>
<td>Mouse</td>
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<tr>
<td>9: ASF + Oxalobacter formigenes</td>
<td>Mouse</td>
<td>Mouse</td>
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<tr>
<td>(20)</td>
<td>ASF (9)</td>
<td>Mouse</td>
<td>Mouse (SW)</td>
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<tr>
<td>(18)</td>
<td>Partial ASF (6)</td>
<td>Mouse</td>
<td>Mouse (NOD.MyD 88KO)</td>
<td>9-23</td>
<td>NR</td>
<td>Both</td>
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<tr>
<td>ASF 356, 361, 492, 502, 519 and 500</td>
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<td>ASF 360 and 457 not colonized</td>
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<td>(19)</td>
<td>Partial ASF (4,5)</td>
<td>Mouse and bovine</td>
<td>Mouse (BALB/c)</td>
<td>4-5</td>
<td>Autoclaved low-fiber diet (SSRZ, 1813680) or high-fiber diet (SSVL, 1813901) or tributyrin diet (SAVC 1814961)</td>
<td>NR</td>
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<tr>
<td>4: ASF360, ASF361, ASF457, ASF519</td>
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<td>5: 4 + Butyrivibrio fibrisolvens (type I, ATCC 19171 and type II, ATCC 51255)</td>
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### Oligo-MM

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<tr>
<th>4-6</th>
<th>NR</th>
<th>Both</th>
<th>0 or 8-12 wk (8-12 wk or 40 d)</th>
<th>Faecal bacterial content; bacterial load of <em>S. typhimurium</em> in feces, caecum and MLN; relative caecal weight; functional genomic analysis of bacteria</th>
</tr>
</thead>
</table>

#### Partial ASF (4,5,7,7)

| 4: ASF356, ASF360, ASF361, ASF519  |
| 5: ASF360, ASF361, ASF457, SB2 [ASF502], ASF519  |
| 7: ASF356, ASF360, ASF361, ASF457, ASF500, SB2 [ASF502] and ASF519  |

7: 4 + *E. coli* Mt1B1, *Streptococcus danieliae* ERD01G, *Staphylococcus xylosus* 33-ERD13C

(more under ‘Oligo-MM’)

---

#### Oligo-MM (12, 15, 17)

| 15: 12 + 3 facultative anaerobes (E. coli Mt1B1, *Streptococcus danieliae* ERD01G, *Staphylococcus xylosus* 33-ERD13C)  |
| 17: 12 + 5 ASF (ASF360, ASF361, ASF457, SB2 [ASF502], ASF519)  |

Oligo-MM with 3 FA

Oligo-MM with 5 ASF
<table>
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<tr>
<th>(25)</th>
<th>Oligo-MM (12,13)</th>
<th>Oligo-MM + C. scindens</th>
<th>Mouse</th>
<th>Mouse (C57BL/6)</th>
<th>5-8</th>
<th>NR</th>
<th>NR</th>
<th>0 (6-12 wk)</th>
<th>Faecal and caecal bacterial contents; caecal levels of lipocalin-2; calprotectin expression in caecal tissue; histopathology of caecum; caecal bile acid metabolome</th>
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<td>SIHUMI(x)</td>
<td>Human</td>
<td>Rat (Sprague-Dawley)</td>
<td>SIHUMI</td>
<td>Human</td>
<td>Mouse (C3H)</td>
<td>SIHUMI(x)</td>
<td>Mouse (C3H)</td>
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<td>Anaerostipes caccae DSM(Z) 14662 or 14667</td>
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<td>8: SIHUMI(x)</td>
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<td>9: 8 + A. muciniphila ATCC BAA-835</td>
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<td>3-21</td>
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<tr>
<td>Sterilized standard chow (g/kg: 225 protein, 50 crude fat, 65 ash, 135 moisture, 480 N-free extract), fermentable-fiber-free diet, inulin diet, pectin diet, high-fat and low-fat diet</td>
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<td>Both</td>
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<td>0-3 mo (2-38 wk)</td>
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<tr>
<td>Stability of microbiota in offspring; SCFA concentrations and pH in caecum, colon and feces; bacterial counts in caecum, colon and feces; Midtvedt criteria</td>
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<tr>
<td>(30)</td>
<td>SIHUMI(x) (8,9)</td>
<td>Human</td>
<td>Mouse (C3H/HeOuJ)</td>
<td>12</td>
<td>Irradiated standard chow R03-40</td>
<td>F</td>
<td>0 (8 wk)</td>
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<tr>
<td>8:</td>
<td>SIHUMI(x)</td>
<td>Human</td>
<td>Mouse (C3H/HeOuJ)</td>
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<tr>
<td>9:</td>
<td>8 + <em>Fusobacterium varium</em> ATCC 8501</td>
<td>Human</td>
<td>Mouse (C3H/HeOuJ)</td>
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<tr>
<td>(31)</td>
<td></td>
<td>Human</td>
<td>Mouse (Prm/Alf, C3H/He)</td>
<td>12-13</td>
<td>Sterilized pelleted standard chow R03-40</td>
<td>F</td>
<td>0 (56 ± 1 d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(32)</td>
<td>SIHUMI(x) (7,8)</td>
<td>Human</td>
<td>Mouse (C3H/HeOuJ)</td>
<td>3-9</td>
<td>Irradiated low-fat or high-fat diet ad libitum</td>
<td>M</td>
<td>0 (16 wk)</td>
<td></td>
<td></td>
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<tr>
<td>7:</td>
<td>SIHUMI(x) without <em>C. ramosum</em></td>
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<tr>
<td>8:</td>
<td>SIHUMI(x)</td>
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</tbody>
</table>

**Note:**
- Thickness, mucus composition and number of mucin-filled cells
- Body weight; dry mass of caecum and colon; bacterial content caecum and colon; polyamine concentrations in caecum and colon; SCFA concentrations in caecum and colon; histology of caecum and distal colon (thickness of crypt depth, epithelial layer, mucosa, submucosa, muscularis externa); mitosis and apoptosis of caecal and distal colonic tissue
- Length of small, large and whole intestine; thickness of muscle, crypt and villi in proximal and small intestine and colon; faecal and caecal microbial content; caecal concentrations of SCFAs and polyamines
- Body weight; body fat percentage; adipose tissue weight (epididymal, mesenteric and subcutaneous); energy intake; food efficiency; digestibility of high-fat diet; digestible energy; caecal and colonic bacterial content
SIHUMI(x) per species; blood glucose; leptin gene expression in epididymal tissue; liver weight; liver triglyceride levels; liver glycogen contents; expression of genes involved in lipid transport, lipid synthesis, cholesterol synthesis and lipid catabolism; gene expression of proteins involved in small intestinal glucose uptake; SCFA formation in caecum, colon and portal vein plasma; gene expression of SCFA-related proteins in colonic mucosa; gene expression of lipid transport and storage proteins in ileum; parameters of intestinal permeability and low-grade inflammation.

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Species</th>
<th>Description</th>
<th>Duration</th>
<th>Outcome Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>(29)</td>
<td>SIHUMI(x)</td>
<td>Human</td>
<td>Mouse (C57BL/6.129P2-Il10tm1Cgn)</td>
<td>5-6 wk</td>
<td>Body weight; histopathology score in submucosa, LP, surface epithelium, lumen; colon length; relative mRNA levels of Tnfa, Ifng, Reg3g; fecal lipocalin-2 concentration; fecal and caecal bacterial levels; caecal histology; number of goblet cells per 100 epithelial cells in caecum.</td>
</tr>
</tbody>
</table>
and colon; mucus layer thickness in colon; relative Muc2 mRNA levels in distal small intestine, caecum and colon
Table 1b. Studies using defined communities to study host-microbe interactions in vivo: Non-specifically named communities in rodents (n = 16)

The following study characteristics are listed: microbial consortium name (if applicable), taxonomic affiliation, strain source, host species and strain, part of the gut studied, no. of animals per experimental group, diet, sex, age and study outcomes reported.

* Two different strains tested are counted as one species. Strains were not always reported. Pathogenic species, in case of an infection model, are not included.

** The colonization time includes the time from colonization (0 in case of transfer of microbiota to offspring) till and including the time of sacrifice or end of experimental (e.g. dietary) manipulations, in case this is clearly stated in the paper. If age is given and animals are colonized at birth, the age is included in colonization time.

*** Study outcomes are only reported for the animals colonized with the defined community of interest

Abbreviations: LP = lamina propria; MLN = mesenteric lymph nodes; MPO = myeloperoxidase; NR = not reported; SCFA = short-chain fatty acids; Treg = regulatory T-cell

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Name consortium (no. of species*)</th>
<th>Phylum division</th>
<th>Strain source</th>
<th>Host species (strain)</th>
<th>Part of the gut studied</th>
<th>No. of animals per group</th>
<th>Chow</th>
<th>Sex (M/F, both or NR)</th>
<th>Age (col. time**)</th>
<th>Study outcomes***</th>
</tr>
</thead>
<tbody>
<tr>
<td>(33)</td>
<td>NA, F- and N-strains (2,9,11,41, 130)</td>
<td>E. coli C25 + Lactobacillus</td>
<td>Mouse</td>
<td>Mouse (CD-1)</td>
<td></td>
<td>4-57</td>
<td></td>
<td>1-60 d</td>
<td>Caecal number of E. coli C25; caecal size; histopathology of stomach, small intestine, caecum and colon</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2: E. coli C25 + Lactobacillus</td>
<td>Others not specified</td>
<td>Mouse (CD-1)</td>
<td>Mouse</td>
<td></td>
<td>4-57</td>
<td></td>
<td></td>
<td>Autoclaved Lobund diet L-356 or pelleted sterile diet from Charles River Mouse Farms</td>
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<tr>
<td></td>
<td>9: 2 + enterococcus + Lactobacillus + Candida + 4 morphologically different strains of gram-negative anaerobes with fusiform morphology</td>
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<td></td>
<td>NR</td>
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<td></td>
<td>11: 9 + 2 strains of gram-negative anaerobes with fusiform morphology</td>
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<td>41: 11 + 30 additional strains of gram-negative anaerobes</td>
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<td>130: 50 strains of gram-negative strict anaerobes (N) + 80 facultative anaerobes (F)</td>
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<tr>
<td>(34)</td>
<td>N- and F-strains (60,96,96,97)</td>
<td>Not specified</td>
<td>Mouse</td>
<td>Mouse (CD-1)</td>
<td></td>
<td>5-75</td>
<td></td>
<td>4 wk</td>
<td>Caecal size; caecal levels of fatty acids; caecal levels of E. coli; pH of caecal contents</td>
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<td></td>
<td>60: N-strains + 14 facultative anaerobes + E. coli C25</td>
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<td></td>
<td>NR</td>
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<td></td>
<td>96: F-strains + E. coli C25 or E.coli 40T or Shigella</td>
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<td></td>
<td>97: F-strains + E. coli C25 + Shigella or E. coli 40T</td>
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<tr>
<td>(180)</td>
<td>na (4)</td>
<td>Lactobacillus sp. 1 and 2, Bacteroides sp., Streptococcus group N</td>
<td>Rat?</td>
<td>Rat (Sprague-Dawley)</td>
<td>None</td>
<td>2</td>
<td>Autoclaved standard diet (Ref 7) supplemented with caffeic acid</td>
<td>NR</td>
<td>NR</td>
<td>Urinary metabolites of caffeic acids</td>
</tr>
<tr>
<td>(38)</td>
<td>na(2,2,2,2,3,3,4,5,6,6,6,8,8,9,13,15,17)</td>
<td>2: Actinobacillus s3 + Streptococcus s1 2: Bacteroides s8 + Actinobacillus s3 2: Eubacterium S10 + Micrococcus s6 2: Clostridium C1 + C2 3: Bacteroides s8 + Actinobacillus s3 + E. coli s7 3: Shigella flexneri + C5 + C6 4: C1-C4 4: S. flexneri + C3-C5 6: C1-C6 6: Actinobacillus s3 + Streptococcus s1 + Lactobacillus s4 + Corynebacterium s5 + Micrococcus s6 + Streptococcus s2 6: S. flexneri + C5-C9 6: (Actinobacillus, etc.) + Bacteroides s8 + E. coli s7 8: S. flexneri + C3-C9 9: C1-C9, 13: C1-C13, 15: C1-15 17: 8 (Actinobacillus, etc.) + C1-C9</td>
<td>Human and mouse</td>
<td>Mouse (CD-1)</td>
<td>≥ 2</td>
<td>Sterilized commercial diet (Usine d’Alimentation Rationnelle) ad libitum</td>
<td>Both</td>
<td>2-5 mo (4 wk after last inoculation)</td>
<td>Number of IgA plasmocytes in duodenum</td>
<td></td>
</tr>
<tr>
<td>(181)</td>
<td>na (2,2,2,2,2,2,3)</td>
<td>2,2: Clostridium E or P with E. coli K-12 2 (x 6): Clostridium E + E. coli S, Proteus mirabilis, Klebsiella pneumonia, Bacteroides (Alistipes) putredinis, Veillonella alcalescens or Clostridium perfringens</td>
<td>Mouse, rat, human</td>
<td>Mouse (C3H)</td>
<td>2-6</td>
<td>Autoclaved commercial diet</td>
<td>NR</td>
<td>Adult (up to 51 d)</td>
<td>Faecal bacterial counts; (mucosal) histology of stomach, jejunum, ileum, caecum, colon</td>
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<tr>
<td>Study</td>
<td>Design</td>
<td>Intervention</td>
<td>Species</td>
<td>Outcome</td>
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<tr>
<td>(37)</td>
<td>UW-GL</td>
<td>Rat</td>
<td>Clostridium E and P + E. coli K-12</td>
<td>Caecal levels of bacteria and <em>Candida albicans</em>; histology of tongue and stomach</td>
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<tr>
<td>(108)</td>
<td>na</td>
<td>Mouse</td>
<td>Streptococcus (Enterococcus) faecalis, Lactobacillus brevis, Aerobacter aerogenes, Staphylococcus epidermidis, Bacteroides sprius (?)</td>
<td>Serum levels of IgG1, IgG2, IgM and IgA</td>
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<tr>
<td>(36)</td>
<td>Partial or complete UW-GL (2,3,9)</td>
<td>Mouse</td>
<td>Partial UW-GL and mouse</td>
<td>Death after <em>C. botulinum</em> infection; faecal <em>C. botulinum</em> toxin excretion; colonization pattern of <em>C. botulinum</em></td>
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<tr>
<td>(109)</td>
<td>na</td>
<td>Human</td>
<td>B. thetaiotaomicron VPI-5482 + Desulfovibrio piger ATCC 29098</td>
<td>Bacterial content in caecum and distal colon; bacterial gene expression; glycan levels in caecum; SCFA production in caecum; serum acetate; liver triglycerides; epididymal fat pad</td>
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</table>
(182) na (2, 6, 10)
2: *Staphylococcus epidermidis* + *Veillonella parvula*
6, 10: anaerobic strains isolated from a conventional male mouse (not specified)

(85) na (2)
*B. thetaiotaomicron* + *Eubacterium rectale*

(39) na (3, 8, 9, 10)
3: *E. coli* HS, *B. vulgatus* DSM1447, *B. thetaiotaomicron* DSM2079
8: 3 + *B. longum* NCC2705, *Blautia hansenii* DSM20583, *C. scindens* DSM5676, *Eubacterium ventriosum* DSM3988, *Lactobacillus rhamnosus* NCC4007
9: 8 + *Collinsella aerofaciens* DSM3979 (colonized most mice)
10: 9 + *Faecalibacterium prausnitzii* DSM17677 (not colonized)

Mouse (B10. BR)
Mouse (NMRI-KI)
Mouse (C3H/HeN)

45-73 Sterilized ST1 (Institute of Physiology AS CR)
4-5 Irradiated standard low-fat, plant polysaccharide-rich diet (diet 2018 from Harland Teklad) or high-fat, "high-sugar" Western-type diet (Harlan Teklad 96132) or low-fat, "high-sugar" (Harlan Teklad 03317)
15 in total Sterile standard chow diet or switch to high-fat diet ad libitum

M 21 d (12 mo) Occurrence of ankylosing enthesopathy of the ankle; colon histology; bacterial content in ileum and colon
M 11 wk (14 d) Bacterial gene expression; caecal colonization levels; fermentation efficiency in caecum; colonic gene expression; protein expression in caecum
Both 7 wk (70 d after 1st inoculation) Faecal and caecal bacterial cell counts; body weight; metabolites in urine and plasma
<table>
<thead>
<tr>
<th>Study ID</th>
<th>Species</th>
<th>Treatment</th>
<th>Time</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>na (15, 19)</td>
<td><em>Bacteroides caccae</em>, <em>Bacteroides ovatus</em>, <em>B. thetaiotaomicron</em>, <em>B. uniformis</em>, <em>B. vulgatus</em>, <em>Bacteroides WH2</em>, <em>C. scindens</em>, <em>Clostridium spiroforme</em>, <em>C. aerofaciens</em>, <em>Dorea longicatena</em>, <em>E. rectale</em>, <em>F. prausnitzii</em>, <em>Parabacteroides distasonis</em>, <em>Ruminococcus obeum</em>, <em>R. torques</em> (strain info not accessible)</td>
<td>Autoclaved low fat, plant polysaccharide-rich diet (B&amp;K rat and mouse autoclavable chow #7378000)</td>
<td>6-8 wk (42 d)</td>
<td>Faecal and caecal bacterial content; bacterial gene expression; urinary metabolites</td>
</tr>
<tr>
<td>na (2)</td>
<td><em>B. thetaiotaomicron</em> VPI-5482 (ATCC 29148) + <em>F. prausnitzii</em> A2-165 (DSM 17677)</td>
<td>Irradiated polysaccharide-rich diet (R03, SAFE)</td>
<td>&lt; 3 months (30 d after inoculation <em>F. prausnitzii</em>)</td>
<td>Host gene expression in colonic epithelium; SCFA caecal concentrations; oxidoreduction potential in caecal contents; colonic crypt depth; total cells/crypt in colon; expression of differentiation proteins of secretory lineage (KLF-4, ChgA); MUC2 production in colonic epithelium; colonic mucin glycosylation</td>
</tr>
<tr>
<td>Study ID</td>
<td>Treatment</td>
<td>Species</td>
<td>Species</td>
<td>Diet</td>
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<tr>
<td>(86) na (2)</td>
<td>B. thetaiotaomicron VPI-5482 + B. longum NCC2705</td>
<td>Human</td>
<td>Mouse (SW)</td>
<td>3</td>
</tr>
<tr>
<td>(42) na (2,8,9)</td>
<td>2: B. thetaiotaomicron + D. piger 8: B. thetaiotaomicron, B. cacaoe, B. ovatus, E. rectale, Marvinbryanthia formatexigens, C. aerofaciens, E. coli, Clostridium symbiosum 9: 8 + D. piger</td>
<td>Human</td>
<td>Mouse (NMRI)</td>
<td>4-20</td>
</tr>
<tr>
<td>Na (14) + virus-like particles</td>
<td>Human</td>
<td>Mouse (C57BL/6J)</td>
<td>5</td>
<td>Autoclaved low-fat/high-plant polysaccharide diet (B&amp;K) ad libitum</td>
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<td>--------------------------------</td>
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<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>C. aerofaciens ATCC 25986, B. cacao ATCC 43185, B. ovatus ATCC 8483, B. thetaiotaomicron VPI-5482+7330, Bacteroides uniformis ATCC 8492, Bacteroides vulgatus ATCC 8482, Bacteroides cellulosilyticus WH2, Parabacteroides distasonis ATCC 8503, C. scindens ATCC 35704, C. symbiosum ATCC 14940, C. spiroforme DSM 1552, D. longicatena DSM 13814, E. rectale ATCC 33656, R. obeum ATCC 29174</td>
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</tr>
<tr>
<td></td>
<td>No name (14)</td>
<td>Human</td>
<td>Mouse (SW)</td>
<td>Total of 51</td>
</tr>
</tbody>
</table>
mucus-production related genes; number of goblet cells in colon; histopathology; body weight; faecal lipocalin; colon length; caecal transcriptome; after infection with C. rodentium: histological score of caecum and colon, area of inflamed tissue in caecum, survival, ascending and descending colon and rectum, adherent C. rodentium in colon
Table 1c. Studies using defined communities to study host-microbe interactions in vivo: Communities in non-rodents (n = 6)

The following study characteristics are listed: microbial consortium name (if applicable), taxonomic affiliation, strain source, host species and strain, part of the gut studied, no. of animals per experimental group, diet, sex, age and study outcomes reported.
* Two different strains tested are counted as one species. Strains were not always reported. Pathogenic species, in case of an infection model, are not included.
**The colonization time includes the time from colonization (0 in case of transfer of microbiota to offspring) till and including the time of sacrifice or end of experimental (e.g. dietary) manipulations, in case this is clearly stated in the paper. If age is given and animals are colonized at birth, the age is included in colonization time.
*** Study outcomes are only reported for the animals colonized with the defined community of interest.

Abbreviations: LP = lamina propria; MLN = mesenteric lymph nodes; MPO = myeloperoxidase; NR = not reported; SCFA = short-chain fatty acids; Treg = regulatory T-cell

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Name consortium (no. of species*)</th>
<th>Phylum division</th>
<th>Strain source</th>
<th>Host species (strain)</th>
<th>Part of the gut studied</th>
<th>No. of animals per group</th>
<th>Chow</th>
<th>Sex (M/F, both or NR)</th>
<th>Age (col. time**)</th>
<th>Study outcomes***</th>
</tr>
</thead>
<tbody>
<tr>
<td>(52)</td>
<td>Bristol (3,4), Modified ASF (6,7)</td>
<td>Phylum division: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia, Other</td>
<td>Bristol (3), (4)</td>
<td>Pig</td>
<td>Pig (commercial hybrid and Babraham)</td>
<td>2-6</td>
<td>Evaporated milk</td>
<td>NR</td>
<td>0-17 d (14-21 d after 1st inoculation)</td>
<td>Presence of bacteria and mean total bacterial content in proximal and distal jejunum, terminal ileum, caecum and colon; serum immunoglobulin concentrations</td>
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<td></td>
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<td></td>
<td>Mod.ASF (6), (7,7)</td>
<td>Pig</td>
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<tr>
<td>(59)</td>
<td>Bristol (3)</td>
<td>Phylum division: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia, Other</td>
<td>Pig</td>
<td>Pig ('Great York x Pie' x 'Dalland' cross)</td>
<td>6</td>
<td>Pasteurized sow colostrum (first hrs), ad libitum milk replacer diet, (day 0-4), a moist diet (remaining)</td>
<td>NR</td>
<td>Neonates (26-37 d)</td>
<td>Relative OR51E1 expression in jejunum</td>
<td></td>
</tr>
</tbody>
</table>

(52) Bristol (3), Modified ASF (6,7)
3: Lactobacillus amylovorus DSM 16698T, Clostridium glycolicum and Parabacteroides sp. (ASF519)
4: 3 + R. intestinalis
6: Clostridium sp. (ASF356), Lactobacillus sp. (ASF360), Lactobacillus animalis (ASF361), E. plexicaudatum (ASF492), Parabacteroides sp. (ASF519) and Propionibacterium sp.
7: 6 + Staphylococcus sp. or Bacillus sp.

(59) Bristol (3)
<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Species</th>
<th>Intervention</th>
<th>Age</th>
<th>Duration</th>
<th>Outcome Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>Bristol (3)</td>
<td>Pig</td>
<td>Pig ((Great York x Pietrain) x 'Dalland' cross)</td>
<td>6</td>
<td>1 d</td>
<td>Sow serum or pasteurized sow colostrum, followed by ad libitum milk replacer diet (day 0-4), followed by a control diet or medium chain fatty acid diet</td>
</tr>
<tr>
<td>62</td>
<td>DMF (7.8)</td>
<td>Pig</td>
<td>Pig ((Landrace x Yorkshire x Duroc cross-bred))</td>
<td>3-6</td>
<td>NR</td>
<td>Faecal virus shedding; mean duration of diarrhea; diarrhea severity and percentage of diarrhea; gene expression levels of CgA, MUC2, PCNA, SOX9 and villin in jejunal intestinal epithelial cells</td>
</tr>
<tr>
<td>63</td>
<td></td>
<td>Pig</td>
<td>Pig (NR)</td>
<td>3-5</td>
<td>NR</td>
<td>Bacterial content in rectum, duodenum, jejunum, ileum, colon and feces/rectal swabs; diarrhoea and virus shedding after virulent human rotavirus challenge</td>
</tr>
</tbody>
</table>

*Bifidobacterium adolescentis, B. longum, B. thetaotaomicron, E. faecalis, L. brevis, S. bovis and C. clostridioforme*  

8: DMF + *E. coli* Nissle
Table 2. Advantages and pitfalls of gnotobiotic animal models in comparison with human research, with respect to the factors influencing intestinal microbiota composition or behaviour. Based on studies listed in Table 1a-c and literature.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Advantage (vs. human research)</th>
<th>Pitfalls in practice</th>
</tr>
</thead>
</table>
| Inoculum (defined community)  | • Controllable composition<br>

*Healthy vs. diseased microbiota<br> (e.g. missing key stone species), human- vs. animal-derived*<br>

| Diet                          | • Controllable composition, timing, amount<br>

*Tailored to human diet (region, age, season, etc.)*<br>

| Host genotype                 | • Controllable – genetic changes possible<br>

*Ability to introduce disease*<br>

| Sex                           | • Controllable<br>

| Part of the gut               | • Ability to measure bacterial levels in virtually all intestinal parts<br>

• Ability to capture transversal heterogeneity<br>

|                                                             | • Animal microbiome ≠ human microbiome<br>

• Difficulties in defining a healthy or normal microbiota<br>

• Host-specific selection of microbiota<br>

|                                                             | • Lack of standardization in lab animal feeding protocols<br>

• Not always reported (Table 1a-c)<br>

|                                                             | • Validation of HMIs in multiple strains needed before extrapolation to humans<br>

• Animal genotype ≠ human genotype<br>

|                                                             | • Only one gender investigated (Table 1a-c)<br>

• Not always reported (Table 1a-c)<br>

|                                                             | • Anatomy and physiology different from humans<br>

• Variations in relative abundance per gut region different per model (Table 1a-c)<br>

• Focus on specific gut regions or faeces only (Table 1a-c)
| Colonization | • Controllable | • Long-term effects not studied (Table 1a-c)  
| time | | • Animals not always colonized starting at birth (Table 1a-c)  
| | | • Stability over generations not always confirmed (Table 1a-c)  
| | |  
| Immune system | • Controllable at start/birth | • Uncontrollable in long-term studies, especially locally  
| | | • Complex, determined by in- and external factors  
| | | • Not quantified or quantifiable (Table 1a-c)  

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Figure 1. *In vitro* models of the human gut and gut microbiota. Models are organized from bottom to top, with the most representative and complex at the top and the most controllable and traceable - with respect to host parameters or microbial species - at the bottom.
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Willem M. De Vos