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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.

MAIN TEXT

Defined Communities Mimicking the Normal Intestinal Microbiota *In Vivo*

A number of recent studies addressed host-microbe interactions *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).

(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,

143 blunt-ended rods were eliminated, which represented the majority of isolator contaminants. Also,
144 the amount of facultative anaerobes was limited, as they outgrew aerobic isolator contaminants and
145 thus, impeded the ability to detect the latter (13). The ASF consists of six Firmicutes (*Clostridium*
146 species (ASF356), *Lactobacillus intestinalis* or *acidophilus* (ASF360), *Lactobacillus murinus* or
147 *salivarius* (ASF361), *Eubacterium plexicaudatum* (ASF492), *Pseudoflavonifractor* sp. (ASF500)
148 and *Clostridium* sp. (ASF502)), one Bacteroidetes (*Parabacteroides distasonis* (ASF519)*)* and one
149 Deferribacteres (*Mucispirillum schaedleri* (ASF457)).
150 The ASF has been used multiple times as a reference or minimal defined microbiota, and its
151 applications were extensively reviewed elsewhere (14). Several studies involving ASF in mice (or
152 other animals) reported its effect on host parameters (Table 1a-c). The list is probably not
153 exhaustive, given the wide application of ASF mice as control or minor population in studies,
154 which makes these studies harder to identify.
155 The applications of ASF in rodents varied from wild-type strains (mostly C57BL/6, but also
156 C3H/HeN and Swiss-Webster mice) to models prone to diseases including IBD (15-17), type I
157 diabetes (18) or colorectal cancer (19). The ASF lacks Proteobacteria, a phylum shared by mice
158 and humans, whereas some researchers did introduce Proteobacteria to ASF mice, such as
159 *Oxalobacter formigenes* (20) and *Escherichia coli* (21). Other studies included only selected
160 members of the ASF, because not all were found to successfully colonize the murine caecum (18)
161 or to test the level of colonization resistance of different combinations of ASF members (22).
162 Overall, the application of ASF to study host-microbe interactions has been quite diverse, regarding
163 host strain, gut region of interest and host parameters studied.
164 Although the ASF has been used multiple times as a reference microbiota and has aided in the
165 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^{12}, was constructed in an attempt to provide full colonization resistance against *Salmonella enterica* serovar Typhimurium (*S.* Tm) (22). Twelve strains were selected to represent the five most prevalent and abundant phyla of the laboratory mouse intestine, i.e. Firmicutes: 'Acutalibacter muris', *Flavonifractor plautii*, *Clostridium clostridioforme*, *Blautia coccoides*, *Clostridium innocuum*, *Lactobacillus reuteri*, *Enterococcus faecalis*; Bacteroidetes: 'Bacteroides caecimuris', 'Muribaculum intestinale'; Actinobacteria: *Bifidobacterium longum* subsp. *animalis*; Proteobacteria: 'Turicimonas muris' and Verrucomicrobia: *Akkermansia muciniphila*. Colonization resistance of ASF mice or mice colonized with Oligo-MM^{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^{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 (*E. coli*, *Streptococcus danieliae* and *Staphylococcus xylosus*), underrepresented in Oligo-MM^{12}, increased coverage and furthermore, conferred full colonization resistance (22). C57Bl/6 mice stably colonized with Oligo-MM^{12} have been designated stable Defined Moderately Diverse Microbiota mice (sDMDMm2). The designers of Oligo-MM^{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^{12} found to lack

189 the enzymatic pathway to carry out 7α-dehydroxylation, an important bile acid transformation, the
190 addition of *Clostridium scindens* (a 7α-dehydroxylating bacterium) was tested in another study.
191 This modification normalized large intestinal bile acid composition in mice, which was
192 accompanied by colonization resistance against *Clostridium difficile* and decreased intestinal
193 pathology (25). Finally, Oligo-MM^{12} served as a defined reference microbiota to verify the
194 significant difference between the bacterial composition in the large intestinal outer mucus layer
195 and the lumen (26), but host parameters were not assessed. Note that the latter two studies that
196 applied of Oligo-MM^{12} left out the three additional facultative anaerobes that were found to be
197 crucial for full colonization resistance.

198 **SIHUMI(x)**

199 Because ASF was found to poorly represent the dominant intestinal bacteria and ASF mice hardly
200 differed from GF mice in a key set of microbial biochemical activities (23) (Midtvedt criteria, see
201 below), a simplified human intestinal microbiota (SIHUMI) was established in rats to provide a
202 highly standardized animal model to study host-microbe interactions. Species were selected
203 according to their prevalence in humans, their fermentative capacity, the availability of their
204 genomic sequence and their ability to stably colonize the rodent gut. SIHUMI(x) includes four
205 Firmicutes (*Anaerostipes caccae*), *Lactobacillus plantarum*, *Blautia producta* and *Clostridium*
206 *ramosum*), one Bacteroidetes (*Bacteroides thetaiotaomicron*), one Actinobacterium (*B. longum*)
207 and one proteobacterium (*E. coli*). All seven members successfully colonized the rat intestinal tract
208 and total bacterial numbers in faecal samples did not differ from those in human faeces. The amount
209 of short-chain fatty acids (SCFAs) produced, however, was dramatically lower compared to
210 humans, probably owing to the smaller number of species. An eighth species was added to the
211 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 microbiotas 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 microbiotas 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-MM[12], 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

283 samples of all colonized mice, independent of dietary intervention (51). No host parameters,
284 however, were assessed in this study.

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

292 **Defined communities in non-rodents**

293 Previously discussed defined microbiota were either isolated from rodents or applied to them.
294 Laycock *et al.* stressed the need for a well-established intestinal colonization microbiota for pigs,
295 given the higher representability of these animal models in early immune development studies (52):
296 in pigs, there is no transfer of maternal immunoglobulin G *in utero* (53, 54), and a poorly developed
297 mucosal system in neonates (55). Furthermore, pigs are genetically more similar to humans than
298 mice (56), and their digestive physiology is comparable to ours (57). Colonization of germ-free
299 piglets with ASF members turned out to be largely unsuccessful and only the most consistently
300 colonizing ASF member (*Parabacteroides* sp.) was incorporated in the novel 'Bristol' microbiota.
301 Additional strains were selected based on their representation of the major phylogenetic groups in
302 gut sections of 12-18 week-old pigs, and either their ability to grow on a wide range of metabolic
303 carbohydrate structures (*Roseburia intestinalis*) or their presence in unweaned pigs (*Clostridium*
304 *glycolicum* and *Lactobacillus amylovorus*). Except for *R. intestinalis*, the novel microbiota
305 successfully colonized the GIT after administration to germ-free piglets, with high clinical safety

306 and an expected increase in immunoglobulin serum levels (52). The Bristol microbiota was
307 exploited by other researchers as a simplified starter microbiota to study additional effects of a
308 complex microbiota on early life microbiota development (58), the intestinal expression of a
309 butyrate-sensing olfactory receptor (59) and on the gastric transcriptome (60). Note that in the latter
310 three studies, the piglets were not maintained in a sterile environment, hampering comparison of
311 the effect of the Bristol microbiota on host parameters between studies. A different ten-membered
312 porcine gut microbiota, originally designed as a competitive exclusion culture for pigs, was used
313 to investigate antibody repertoire development in ex-germ-free newborn piglets (61). Another
314 'defined commensal microflora' (DMF) included seven porcine bacterial species and was similar
315 in composition to ASF. Species were originally isolated from the caecal contents of six week-old
316 healthy pigs and administered to germ-free pigs to evaluate the interactions between intestinal
317 commensals, antibiotics, probiotics and human rotavirus. This model was primarily applied as a
318 model commensal gut microbiota of neonates (62, 63).

319 **Other Defined Communities *In Vivo***

320 Apart from the defined communities as model for the normal (human) gut microbiome to study
321 host-microbe interactions, other kinds of communities have been composed for application in
322 gnotobiotic animals. These communities, however, are not listed in Table 1a-c and their application
323 goes beyond the scope of this review, as they did not aim to represent the 'normal' microbiota. For
324 instance, these include disease-specific consortia, e.g. IBD-related (15, 64-67). Others are age-
325 specific, such as the Human Baby Microbiota (68-70), DMF (62, 63) and a recently developed
326 *Bifidum*-dominated model consortium (71). Lastly, some communities were developed for
327 therapeutic or probiotic purposes. A well-studied and globally marketed multispecies probiotic is
328 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, *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 *E. rectale*'s ability to generate butyrate as a major end product of fermentation, a 'marriage was arranged' between *E. rectale* and *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 (*B. thetaiotaomicron* and *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 *in vivo* models to study host-microbe interactions. The next section discusses the representability of these communities, focussing 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

398 phyla and various metabolic capacities have been frequently put forward. A meta-analysis was
399 performed comparing the composition of the core mouse gut microbiome (based on five different
400 mouse models, i.e. varying in age, phenotype and sampling site) with the human gut microbiome
401 (based on 16 individuals) (87). Apart from the differences within the mouse microbiota,
402 Bacteroidetes and Firmicutes were clearly the most dominant phyla in all samples (together 87-
403 97%). (87) The same is true for the composition of well-established defined communities ASF,
404 SIHUMI(x) and Oligo-MM^{12} (75-87.5%). Similar to most murine microbiota included in the meta-
405 analysis, however, ASF and SIHUMI(x) lack Verrucomicrobia, which was found among the five
406 most abundant phyla in human and some murine samples (87). In that sense, Oligo-MM^{12},
407 originally designed to represent the murine microbiota, is compositionally more complete than
408 SIHUMI(x), which was meant to represent the human microbiota. The frequently used ASF also
409 lacks Actinobacteria and Proteobacteria, which are abundant in both murine and human samples
410 (87-89). Similarly, a large part of the other defined communities discussed here (Table 1a-c) did
411 not include representatives of all five major phyla of the human microbiota, some not even one of
412 the two most prominent phyla. Note that species selection has been mostly based on microbiota
413 composition of Western individuals.
414 Further, community design has been limited by availability of genomes and cultivability of strains.
415 In the case of ASF, the number of species was limited for financial reasons, i.e. taking into account
416 the monitoring costs. Nevertheless, this community has been frequently used in gnotobiotic animal
417 models. The assumption that ASF mice can be regarded as conventional mice with respect to their
418 gut microbiota, has been criticized (23). Several functional activities in faecal materials from ASF
419 mice were analysed and compared to samples from GF and conventional rodents and other
420 mammalian species, including humans. The five biomarkers investigated, the so-called Midtvedt
421 criteria (i.e. conversion of cholesterol to coprostanol, conversion of bilirubin to urobilinogens,

422 degradation of β-aspartylglycine, degradation of mucin, and the absence of fecal tryptic activity
423 (23)) are claimed to reflect host-bacterial interactions, independent of the intestinal localization of
424 the bacteria involved and the kind of species. With regard to these criteria, faecal samples from
425 ASF mice showed patterns more resembling GF rather than conventional mice (23), which
426 complemented previous results demonstrating an abnormal microbiota in SPF mice (90). Although
427 this could be due to one of the limitations of ASF, i.e. its low diversity, ASF mice were shown to
428 be immunologically, reproductively and metabolically similar to conventional mice (23). The
429 Midtvedt criteria were also used to assess the suitability of SIHUMI(x) as a model microbiome.
430 SIHUMI(x)-associated rats shared four criteria with conventional rats, of which three were,
431 however, less pronounced (27).

432 A major difference between ASF and a consortium such as SIHUMI(x), is the fact that the
433 latter involves human-derived bacterial strains. Most members of recently developed communities,
434 except for Oligo-MM, are of human origin as well. This may be obvious, given the fact that,
435 although their microbiota is similar at the division (superkingdom) level, 85% of the microbial
436 genera and species detected in mice are not found in humans (91). Although qualitatively, humans
437 and mice share a largely similar core, their intestinal microbiota is quantitatively very different
438 (87). On the other hand, the development of small intestinal immune maturation was found to be
439 host-specific, with humanized mice resembling more closely GF mice than mice associated with a
440 murine microbiota (92). This host-specificity might also, at least partially, explain the unsuccessful
441 colonization of piglets with ASF (52). Additionally, humanized rodent models were claimed to
442 have been utilized mainly for short-term biomedical research studies (14). The question remains
443 how human-derived bacteria would adapt during long-term colonization and vertical transmission
444 in murine hosts (14, 93, 94), and thus, which kind of microbiota would be most reliable to study
445 host-microbe interactions when using murine hosts. The maximum colonization time reported in

446 the studies discussed here (Table 1a-c) was less than one year. With respect to vertical transmission,
447 stability after transfer to offspring has been addressed mainly for murine microbiota only (ASF
448 (95) and Oligo-MM (22)). Within the humanized defined communities, SIHUMI(x) is an
449 exception, of which bacterial concentrations in caecum were verified between founder rats as well
450 as their offspring. At the age of eight weeks, SIHUMIx-rats harboured similar bacterial levels as
451 their founders, but not at two weeks (except for *E. coli*) (27).

452 **Simplified versus complex communities**

453 The distinction between minimal communities, with two or three members, and larger defined
454 communities is not black-and-white. For instance, ASF, initially used as a microbiota to standardize
455 mouse models, slowly adopted the role of a minimal community, instead of one representing the
456 normal microbiota of mice. Nonetheless, the simplicity of a defined community also has some
457 advantages over more complex communities. The limited nature of ASF should, as proposed by
458 Brand *et al.*, allow investigators to evaluate the *in vivo* effect of the removal or addition of bacterial
459 species on mucosal homeostasis and colonization dynamics, or potentially, factorial interactions of
460 the community (14). Indeed, some of the studies discussed here (Table 1a-c) used only a subset of
461 the ASF species or added species to already established defined communities, including ASF and
462 SIHUMI(x). Additionally, one- and two-member communities could be applied to model aspects
463 of a more complete microbiota, such as depletion of certain dietary compounds or metabolites (86).
464 Finally, as already discussed, a simplified consortium makes each species traceable, as opposed to
465 a very complex community (28, 40).

466 On the other hand, complex communities might more closely resemble the normal human
467 gut microbiota and are more likely to confer colonization resistance to opportunistic pathogens,
468 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^{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,

493 the proximal colon should be viewed as a partially mixed bioreactor rather than a clearly
494 compartmentalized gut section with spatially segregated communities. A next step would be to
495 quantify the distribution of nutrients and metabolites and the role of host factors such as diet, gut
496 motility and mucus composition (48). Vice versa, it would be interesting to study the effect of
497 spatial organization on relevant host parameters, which were unfortunately not addressed in the
498 latter study. The authors did admit that the 15-membered community used may not be complex
499 enough to demonstrate stronger spatial associations with food particles, host cells and mucus (48),
500 reinforcing, all in all, the need for more complex communities.

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

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

506 One way to obtain a more complex model community is to start with a complex sample, e.g. human
507 stool, and narrowing the amount of species down via one or more enrichment steps, e.g. by
508 culturing on selective media (top-down approach (100)) or using fermentation models. Table 1a-c
509 includes only a few examples with regard to normal microbiota (Oligo-MM^{12} (22), (40)). The
510 majority of the studies listed in Table 1a-c used a bottom-up approach, in which single, previously
511 cultured and characterized strains are combined into a synthetic bacterial community, e.g. based
512 on selection criteria previously mentioned, and administered to germ-free animals. An advantage
513 of the latter method is the known composition of the microbiota, as previously emphasized. A
514 drawback, however, is formed by the risk that the desired phenotype (in this case a normalized
515 host) cannot be entirely recapitulated (100).

Future design

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 (*Methanobrevibacter smithii*) and a bacterium (*B. 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

562 to be mentioned. The high value of using gnotobiotic animals as models of humans, i.e. their known
563 composition and controllability, seem to be weakened by poor control of host parameters known
564 to influence the human gut microbiome, such as diet, genotype, sex, part of the gut studied, age
565 and the immune system.

566 **Host parameters influencing the microbiota**

567 Diet is a complex and strong determinant of gut microbiota composition (reviewed in (111,
568 112)). The individual species levels were assessed of a ten-membered defined community in mice
569 fed with diets systematically varying in protein, fat, polysaccharides and simple sugars, in order to
570 develop a model to predict the variation in species abundance. Next, the model was validated with
571 48 random combinations and concentrations of four ingredients selected from a set of eight human
572 baby foods. Approximately 60% of the variation in species abundance could be explained by the
573 known concentrations of pureed foods (41). This study exemplified the application of defined
574 communities to systematically assess the response of individual gut members to various food
575 components, which are, moreover, typical for the human diet. Clearly, a standardized diet of a
576 laboratory animal is different from that of humans, which varies per region, season, individual taste
577 and even per day. Some studies listed in Table 1a-c incorporated a previously developed prototypic
578 "Western style" diet (27, 32, 39, 42, 46, 85), containing high amounts of saturated and unsaturated
579 fats and carbohydrates commonly used as human food additives (i.e. sucrose, maltodextrin and/or
580 corn starch). A lack of standardization in lab animal feeding protocols, however, has been
581 emphasized previously for instance with respect to diet composition and texture (113) and indeed,
582 diets used by studies discussed here are highly variable (Table 1a-c). Moreover, in ~40% of the
583 studies, the diet was not clearly defined or not even reported, which is alarming given the large
584 impact of diet on the gut microbiome.

585 The choice for mouse genotype also varied per study (Table 1a-c), although an effect of
586 host genotype on microbiota composition was established within species (114-118). These results
587 were corroborated by studies with defined communities such as ASF (119) and SIHUMI(x) (64).
588 Additionally, colonization of different mouse strains with SIHUMI(x) demonstrated host-specific
589 caecal levels of polyamines and SCFAs (31). In mice associated with *B. longum* and *B.*
590 *thetaiotaomicron*, host genetic background was found to affect the overall transcriptome of the
591 latter bacterium, but not the expansion of the bacterial substrate range of this bacterium (84).
592 Obviously, defined communities allow the careful investigation of such host-dependent effects, but
593 validation of host-microbe interactions in a wide range of host strains seems crucial before drawing
594 conclusions and extrapolation to humans.
595 Although reports on the effect of gender have been contradictive (106, 117, 120-124) it
596 might be a crucial determinant in gut microbiota composition and/or behaviour. In turn, commensal
597 microbiota was shown to affect sex hormone levels (125, 126). Sex differences in gut microbiota
598 composition were, recently, comprehensively investigated in 89 common inbred mouse strains.
599 After excluding confounding by host genetics, diet, age or cage effects, the researchers detected
600 gender-specific differences in taxa abundances and diet responses. These differences could be
601 partially explained by sex hormones (127). Among the studies discussed here (Table 1a-c), one
602 reported differences in metabolic profiles in urine and plasma between both sexes, but no
603 explanation was put forward (39). In an older study, male mice were found more susceptible to
604 death after *C. botulinum* infection, which could be explained by their coprophagic behaviour or a
605 more general higher susceptibility to disease (36). In contrast, other studies reported an absence of
606 gender-specific effects on, for instance, levels of *Oxalobacter formigenes* colonized in ASF mice
607 (20) or assembly of a synthetic microbiota (43). Whereas some studies discussed here (Table 1)
608 reported to have used a gender-mixed population, others included only one gender (n = 12 of 53

609 studies), in which male more often than female (nine vs. three) animals were used. Remarkably,
610 the establishment of SIHUMI(x) was verified in both genders, whereas the effect of dietary fibre
611 was tested in male and the effect of high-fat diet was investigated in female rats (27). A similar
612 discrepancy was found in a study that assessed the effect of five fermented milk product strains in
613 human female twins, but male gnotobiotic animals. Although microbiota responses were more or
614 less similar in both species (40), such a gender-mismatch may complicate translation. Lastly, not
615 all studies clearly reported the gender used per experiment, and approximately half of the studies
616 did not report animal gender at all. This too, may hinder data reproduction and, more importantly,
617 translation.

618 Defined communities allow the quantitative comparison of microbial compositions along
619 the GIT, within and between models. ASF-associated mice were used to quantitatively demonstrate
620 that the microbiota of the colon is poorly reflected in faecal samples (95). Relative abundance of
621 species were also different between faeces (rectal swabs) and colon in pigs colonized with a defined
622 microbiota (63). In rats colonized with SIHUMI(x), however, bacterial concentrations of caecum,
623 colon and faeces were similar (27). Additionally, increases in relative abundances of mucin-
624 degrading bacteria in caecum and colon upon switching to a fibre-free diet, were reflected in faeces
625 (43). In a mouse model associated with a 12-membered community, individual bacterial levels
626 were also similar between faeces and caecum (46). These conflicting results could be explained by
627 various factors, including host, community composition and sampling time. Irrespective of the
628 actual difference between GIT sites, it is disappointing that some other studies relied solely on
629 faecal bacterial content. In a study applying a 92-membered community, for instance, not even half
630 of the members could be detected in faeces. Other species may have established themselves in
631 different regions of the gut, but this was beyond the scope of the paper (51). Nevertheless, due the
632 invasiveness of sampling, systematic studies comparing colonic and faecal bacterial content are

633 lacking in humans as well (99, 112). The variation in GIT sites looked at by the studies included
634 in Table1a-c, makes it hard to compare the colonization pattern of the defined communities to
635 natural colonization. Apart from differences along the GIT, capturing the transversal heterogeneity
636 within one compartment may be crucial for properly modelling and understanding host-microbe
637 interactions, as discussed above.

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

649 A last factor determining gut microbiota composition and behavior is the immune system,
650 which in turn is influenced by, amongst others, aforementioned factors and the gut microbiota
651 itself. Looking at the studies discussed here (Table 1a-c), several researchers investigated
652 immunological parameters such as serum immunoglobulin levels and the presence of (subsets of)
653 immunological cells in the gut. Nevertheless, due to the complexity of the immune system, it is
654 hard to quantify and compare the model hosts used with respect to immunological parameters. The
655 key findings on the interactions of gut microbiota members and their products with the immune
656 system have been recently reviewed elsewhere (100). The authors emphasized the value of minimal

657 microbiomes and subsequent standardized (animal) models. Determining the effects of specific gut
658 microbiota on the host, could help to identify host-microbe interactions that shape the immune
659 system (100). Most studies discussed in this review did not make a distinction between the
660 contributions of each specific microbe to immunological effects observed.

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

663 **Validation of *in vivo* models**

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

677 A better example was recently described in a study in which the effect was tested of a
678 fermented milk product in both humans and gnotobiotic mice humanized with a 15-membered
679 microbiota. The proportional representation of the intestinal bacterial species and genes and

680 metabolic changes upon introduction of the probiotic strains, were hardly different between mice
681 and men, but the researchers also acknowledged the limitations of their gnotobiotic animal model
682 with respect to translatability (40). In most other studies (Table 1), control groups were limited to
683 conventionally raised and GF animals or animals with a control treatment, for which translatability
684 of the results to the human situation remains speculation.

685 **Defined Communities *In Vitro***

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

695 **Modelling the intestinal microbiota *in vitro***

696 The use of fermentation models has proven successful in modelling the intestinal microbiota *in*
697 *vitro*, ranging from short-term batch incubations to multi-compartmental continuous systems. As
698 discussed already, most defined communities applied in *vivo* (Table 1a-c) were constructed bottom-
699 up, by selecting species based on their function, prevalence or other criteria. Alternatively,
700 communities can be composed top-down by inoculating GIT-mimicking chemostats with human
701 faeces. Well-known examples of these chemostats, such as the MacFarlane/Gibson three-stage
702 continuous culture system, (M-)SHIME, EnteroMix, Lacroix Model and TIM-2, have been

703 extensively reviewed elsewhere (132-134). The high reproducibility, stability, and complexity of
704 bacterial communities cultured in chemostats (135, 136) has allowed the development and
705 application of representative communities of the human intestinal microbiota *in vitro*. Most of these
706 models, however, did not include a host component. The HMI^{TM} module comprised a promising
707 exception in which first, faeces from a healthy volunteer was fed into an adapted SHIME system,
708 with fluid compartments mimicking the stomach, small intestine and ascending colon.
709 Subsequently, the SHIME-effluent was exposed to an artificial mucus layer, separated by a semi-
710 permeable membrane from a compartment containing Caco-2 cells. This module allowed the co-
711 culture of bacteria with enterocytes up to 48 hours (137), which is discussed in more detail below.

712 **Modelling the host *in vitro***

713 With respect to well-established defined communities, the probiotic cocktail VSL#3 and the faecal
714 transplant substitute MET-1 have been tested on various human or animal intestinal cell lines
715 (Caco-2, T84 and HT-29) (e.g. (138-140)). In most studies, however, the use of bacterial lysates or
716 conditioned media was preferred over live bacteria (e.g. (72, 141-144)), because the – mainly
717 anaerobic – gut bacteria cannot survive under the aerobic conditions needed for intestinal cell
718 culture. In these 2D models, the interaction with the immune system or other tissues, cannot be
719 studied. Although the direct effect of VSL#3 was tested on spleen and dendritic cells (145, 146),
720 tissue-tissue interactions were lacking in these models. This problem can be (partially) solved in
721 Transwell co-culture models, in which bacteria, mucosal immune cells and intestinal epithelial
722 cells can be studied together (147). A Transwell model with an apical anaerobic compartment
723 enabled the co-culture of an anaerobe bacterium with an intestinal cell line to study host-microbe
724 interactions (148). Still, these cell lines lacked their tissue-specific context, including all major
725 types of epithelial cells (e.g. Goblet cells, enterocytes, enter endocrine and Paneth cells) organized

726 in crypts and villi. Moreover, as cell lines are tumor-derived, their epithelial characteristics are
727 affected. These issues have been overcome by the development of gut organoids, self-organizing
728 3D epithelial structures derived from intestinal stem cells (149) or human pluripotent stem cells
729 (150). The use of organoids to study host-microbe interactions was reviewed elsewhere (151). The
730 closed structure of organoids, in which the lumen is sealed with epithelial cells and a mucus layer,
731 may facilitate the establishment of hypoxia in the core lumen (151). The anaerobic pathogen *C.*
732 *difficile* survived up to 12 hours within organoids, but luminal oxygen levels still ranged from 5-
733 15%, which may be tolerated by specific strains of *C. difficile* only (152). More recently,
734 researchers developed an organ culture system for the mouse intestine, in which the stromal and
735 hematopoietic components of the normal intestine were preserved *ex vivo*. The device supported
736 the survival and growth of both anaerobic and aerobic microbiota, allowing the investigation of
737 their effects on neuronal parameters (153).
738 The co-culture of defined microbial communities with human cells in Transwells, organoids or
739 organ culture systems has been limited, probably owing to the static nature of these models. More
740 advanced *in vitro* models to study host-microbe interactions have been developed (as recently
741 reviewed in (133)) of which only a few have hitherto allowed the co-culture of multiple bacteria
742 with intestinal cells or cell lines.
743 Organ-on-a-chip technology is an emerging concept within biomedical research, to replace
744 conventional cell culture and animal testing. Organ-on-chips are microfluidic devices in which
745 cells are cultured with organ-relevant spatiotemporal chemical gradients and dynamical
746 mechanical cues, thereby aiming to reconstitute the structural tissue arrangements and functional
747 complexity of living organs *in vitro* (154). Several gut-on-chips have already been developed (155-
748 158), only one in which multiple intestinal bacteria were successfully cultured (158). In this device,
749 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^{TM} 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^{TM} 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,

774 mechanical cues and tissue-tissue interaction). To date, the cultivation of a defined intestinal
775 microbiota in this device, has not yet been reported.

776 **Validation of *in vitro* models**

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

791 **Conclusions and Future Outlook**

792 Our understanding of the human gut microbiome has rapidly grown over the past decades, which
793 has definitely supported the design of defined communities representative of the human gut
794 microbiome. Whereas defined communities were initially aimed to normalize germ-free hosts to
795 conventionalized mice, they could be a valuable tool to study host-microbe interactions, because
796 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

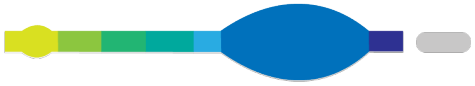
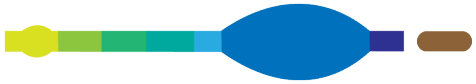
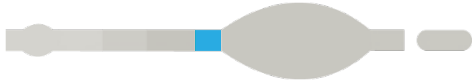
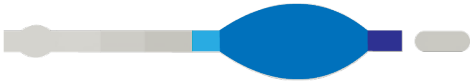
Hauke Smidt studied Biotechnology at the Technical University of Braunschweig, Germany, and obtained his PhD from Wageningen University. Following a postdoc position at the University of Washington, Seattle, he rejoined the Laboratory of Microbiology at Wageningen University to head the Molecular Microbial 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.

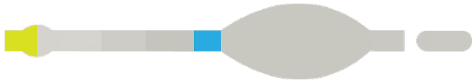
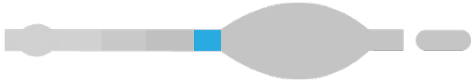
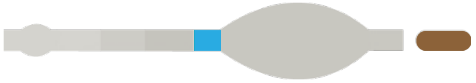
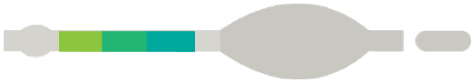
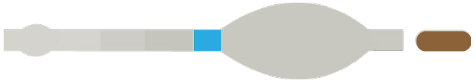
FIGURE LEGENDS

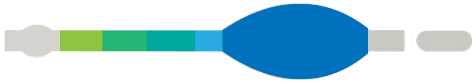
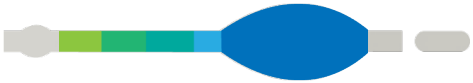
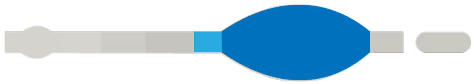
Figure 1. *In vitro* models of the human gut and gut microbiota. Models are organized from bottom to top, with the most representative and complex at the top and the most controllable and traceable - with respect to host parameters or microbial species - at the bottom.

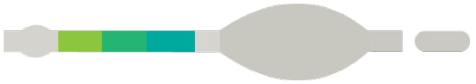
1428 **TABLES**

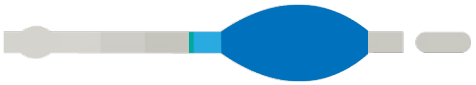
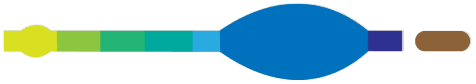
Table 1a. **Studies using defined communities to study host-microbe interactions *in vivo*: Specifically named communities** (n = 30)
The following study characteristics are listed: microbial consortium name (if applicable), taxonomic affiliation, strain source, host species and strain, part of the gut 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

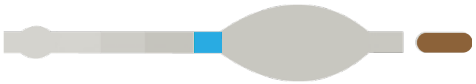
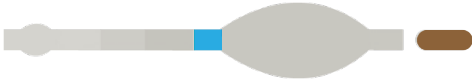
Ref.	Name consortium (no. of species*)	Phylum division Firmicutes Bacteroidetes Actinobacteria Proteobacteria Verrucomicrobia Other	Strain source	Host species (strain)	Part of the gut studied Stomach Duodenum Jejunum Ileum Caecum Colon Rectum Faeces	No. of animals per group	Chow	Sex (M/F, both or NR)	Age (col. time**)	Study outcomes***
(9)	**Schaedler flora (5)** 2 *Lactobacillus* sp., anaerobic *streptococcus* sp. (group N), *Bacteroides* strain, *Enteroccocus sp.*, coliform strain		Mouse	Mouse (NR)		20	NR	NR	4 wk (3 wk - 4 mo)	Colonization pattern; caecal size
	Altered Schaedler Flora (ASF)									
(36)	**ASF (8)** ASF356: *Clostridium species* ASF360: *Lactobacillus intestinalis* or *acidophilus* ASF361: *Lactobacillus murinus* or *salivarius* ASF457: *Mucispirillum schaedleri* ASF492: *Eubacterium plexicaudatum* ASF500: *Pseudoflavonifractor* sp. ASF502: *Clostridium* sp. ASF519: *Parabacteroides distasonis*		Mouse	Mouse (HA/ICR)		30	NR	Both	Adult (14-56 d)	Death after *C. botulinum* infection; faecal *C. botulinum* toxin excretion; colonization pattern of *C. botulinum*
(170)			Mouse	Rat (F344)		1-5	Sterile food (Charles River) ad libitum	M	NR (2 wk)	Hepatic genotoxicity of mononitrotoluene isomers; metabolic activation of 2NT by intestinal bacteria; caecal bacterial content
(16)			Mouse	Mouse (scid C.B-17)		4-6	Autoclaved pelleted diet ad libitum	NR	NR (8-12 wk post reconstitution	(After *H. hepaticus* infection) Rectal prolapse; clinically severe disease; grossly thickened colon, cecum and rectum on necropsy; colonic

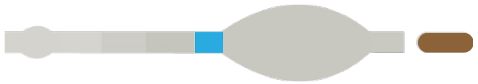
									CD4+ T-cells)	inflammation score; colonic epithelial cell proliferation; histopathology
(15)			Mouse	Rat (HLA-B27 on 33-3/F344)		7-11	NR	at least M	2 mo (1 mo)	Gross gut score, levels of MPO and IL-1B in caecal tissue; histologic inflammatory score of caecum and antrum
(171)			Mouse	Mouse (C3H/HeN)		4-8	Irradiated diet (Harlan Teklad)	NR	6-8 wk (9-14 wk)	After colonization with *H. bilis* or *B. hyodysenteriae*: Caecal pathological gross and histological scores; serum IgG1 + IgG2a ab response
(172)			Mouse	Mouse (C3H/HeN)		7-10	Irradiated diet (Harlan-Teklad)	NR	6-8 wk (10 wk)	Faecal bacterial contents; (after *H. bilis* infection:) caecal pathological scores; caecal histological changes; serum immunoglobulin
(173)			Mouse	Mouse (SW)		2-5	NR	NR	6-9 wk (NR)	Presence of Th17 cells and Foxp3+ regulatory cells in LP of small intestine
(174)			Mouse	Mouse (C57BL/6)		NR	NR	NR	NR	Total intestinal IgA and intestinal IgA anti-CBir1; proliferation of splenic CBir1 TgT-cells after CBir1 gavage
(175)			Mouse	Mouse (B6.Rag-/-)		NR	NR	F	8-10 wk (10 d)	Homeostatic and spontaneous proliferation of TCR Tg T-cells in LP
(97)			Mouse	Mouse (C57BL/6)		5-8	Autoclaved chow	NR	8 wk (at least 3 dpi)	After infection: *S. Typhimurium* levels in mesenteric lymph nodes, spleen, caecum and feces; caecal pathology score;

										caecal microbiota density; bacterial content and microbiota complexity in feces
(21)	**ASF (8,9)** 8: ASF 9: ASF + *Escherichia coli* HA108 or HA107	**ASF (9)** 	Mouse	Mouse (C57BL/6)		3	NR	NR	NR (119 d)	No. of IgA plasma cells per intestinal villus in duodenum, jejunum, ileum and colon; IgA-bacterial binding in intestine; anti-*E.coli* IgA titre
(176)	**ASF (8)**		Mouse	Mouse (NMRI, C57BL/6, BALB/c, NIH Swiss, SW, NMRI, MyD88-/-Ticam1-/-, SMARTA, C57BL/6.CD45.1+)		3-10	NR	NR	NR (up to 28 d)	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
(17)			Mouse	Mouse (Nod1 -/- and Nod2-/- on C57BL/6)		NR	NR	NR	6-9 wk (NR)	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
(177)			Mouse	Mouse (C57BL/6)		NR	Autoclaved food	Both	8-12 wk (8-12 wk)	REGIII-gamma RNA and protein expression in ileum and colon

(178)			Mouse	Mouse (C57BL/6 and C57BL/6 TSLPR-/-)		3-5	NR	NR	NR (28 d)	Expression of thymic stromal lymphopoietin mRNA in intestinal epithelial cell or colonic LP (LP); percentage of CD4+ T cells secreting IL-17A and IFN gamma in the colonic LP and MLN; expansion of colonic Treg cells in colonic LP and MLN; expression of receptor for TSLP by CD4+ and regulatory T-cells
(179)			Mouse	Mouse (NIH Swiss)		4	NR	NR	3 d (3 d)	Structure of myenteric plexus, nerve density, average no. of HuC/D-positive myenteric neurons per ganglion, cell body size and average no. of nNOS-positive neurons per myenteric ganglion in duodenum, jejunum and ileum; small intestinal motility (frequency and amplitude of muscle contractions) in duodenum, jejunum and ileum before and after general neural or specific nitrergic blockade
(81)			Mouse	Mouse (C57BL/6)		5-14 per group	Autoclaved mouse breeder's diet (Harlan), unlimited access	Both	6-12 wk (3 wk)	Colonic histology, inflammatory (MPO) activity, enteropathy (presence of faecal albumin) and cytokine expression; faecal microbiota profiles; colonic gene expression; proportion of T-cell subtypes in colonic LP and

										other mucosal and systemic immune compartments
(26)	**ASF (8)** Oligo-MM[12] was also used, but no host parameters were assessed		Mouse	Mouse (C57BL/6)		3 (ASF), 5-23 (Oligo-MM)	NR	Both	NR	Thickness of colon total and colon inner mucus (ASF); mucus turnover time (ASF); alpha diversity in colon and cecum (Oligo-MM)
(20)	**ASF (8,9)** 8: ASF 9: ASF + *Oxalobacter formigenes*	**ASF (9)** 	Mouse	Mouse (SW)		4-7 per group	LM-485 autoclavable rodent diet, free access	M (no gender-effect observed)	3-9 mo (3-9 mo + 6 wk)	Bacterial levels in stomach, caecum, proximal colon and caecal mucosa; body weight; dietary oxalate intake; caecal and faecal oxalate levels; urine volume; urinary metabolite levels; caecal wet weight; caecal water metabolites
Partial ASF										
(18)	**Partial ASF (6)** ASF 356, 361, 492, 502, 519 and 500 ASF 360 and 457 not colonized		Mouse	Mouse (NOD.MyD88KO)	None	9-23	NR	Both	NR (up to 30 wk)	Incidence of diabetes; histological scores of pancreatic islet destruction
(19)	**Partial ASF (4,5)** 4: ASF360, ASF361, ASF457, ASF519 5: 4 + *Butyrivibrio fibrisolvens* (type I, ATCC 19171 and type II, ATCC 51255)	(4)  (5) 	Mouse and bovine	Mouse (BALB/c)		4-5	Autoclaved low-fiber diet (5SRZ, 1813680) or high-fiber diet (5SVL, 1813901) or tributyrin diet (5AVC 1814961)	NR	NR (2.5-5 mo after colorectal cancer induction)	Colorectal tumor multiplicity, tumor size and tumor grade; levels of LDHA, lactate, butyrate, H3ac and total H3 in colonic tissue and tumors; luminal SCFA levels; H3ac and expression levels of Fas, p21 and p27 genes in colonic tissue and tumors; apoptosis and cell proliferation levels in colonic tissue and tumors

(22)	**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')	(4), (5)  (7), (7) 	Mouse	Mouse (C57BL/6)		4-6	NR	Both	0 or 8-12 wk (8-12 wk or 40 d)	Faecal bacterial content; bacterial load of *S. typhimurium* in feces, caecum and MLN; relative caecal weight; functional genomic analysis of bacteria
Oligo-MM										
(22)	**Oligo-MM (12, 15, 17)** 12, Oligo-MM: 'Acutalibacter muris' KB18, *Flavonifractor plautii* YL31, *Clostridium clostridioforme* YL32, *Blautia coccoides* YL58, *Clostridium innocuum* I46, *Lactobacillus reuteri* I49, *Enterococcus faecalis* KB1, 'Bacteroides caecimuris' I48, 'Muribaculum intestinale' YL27, *Bifidobacterium longum* subsp. *animalis* YL2, 'Turicimonas muris' YL45 *Akkermansia muciniphila* YL44 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  with 5 ASF 	Mouse	Mouse (C57BL/6)		4-6	NR	Both	0 (8-12 wk)	Faecal bacterial content; bacterial load of *S. typhimurium* in feces, caecum and MLN; relative caecal weight; functional genomic analysis of bacteria

(25)	**Oligo-MM (12,13)** 12: Oligo-MM 13: 12 + *Clostridium scindens* ATCC35704	Oligo-MM  + *C. scindens* 	Mouse	Mouse (C57BL/6)		5-8	NR	NR	0 (6-12 wk)	Faecal and caecal bacterial contents; caecal levels of lipocalin-2; calprotectin expression in caecal tissue; histopathology of caecum; caecal bile acid metabolome

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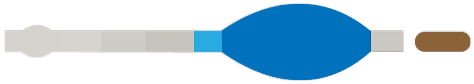
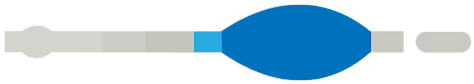
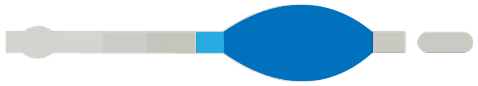
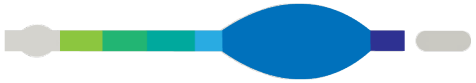
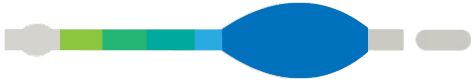
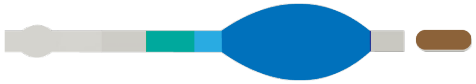
SIHUMI (x)										
(27)	**SIHUMI(x) (7,8)** *Anaerostipes caccae* DSM(Z) 14662 or 14667 *Bacteroides thetaiotaomicron* DSM(Z) 2079 *B. longum* NCC 2705 *Blautia producta* DSM(Z) 2950 *Clostridium ramosum* DSM(Z) 1402 *E. coli* K-12 MG1655 *Lactobacillus plantarum* DSM(Z) 20174 (x) *Clostridium butyricum* DSM(Z) 10702	SIHUMI  SIHUMI(x) 	Human	Rat (Spraque-Dawley)		3-21	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	Both	0-3 mo (2-38 wk)	Stability of microbiota in offspring; SCFA concentrations and pH in caecum, colon and feces; bacterial counts in caecum, colon and feces; Midtvedt criteria
(28)	**SIHUMI(x) (8,9)** 8: SIHUMI(x) 9: 8 + *A. muciniphila* ATCC BAA-835	(9) 	Human	Mouse (C3H)		5-10	NR	NR	12 wk (5-15 d)	Bacterial cell numbers and proportions in caecum and colon; caecal and colonic histopathology score; expression of pro-inflammatory cytokines in caecal and colonic mucosa; serum protein levels of pro-inflammatory cytokines; cell number of *S. Typhimurium* in MLN and spleen; size of MLN; macrophage infiltration in caecal tissue; localization of *A. muciniphila* and *S. Typhimurium*; mucin formation, mucus

										thickness, mucus composition and number of mucin-filled cells
(30)	**SIHUMI(x) (8,9)** 8: SIHUMI(x) 9: 8 + *Fusobacterium varium* ATCC 8501	(9) 	Human	Mouse (C3H/HeOuJ)		12	Irradiated standard chow R03-40	F	0 (8 wk)	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
(31)			Human	Mouse (Prm/Alf, C3H/He)		12-13	Sterilized pelleted standard chow R03-40	F	0 (56 ± 1 d)	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
(32)	**SIHUMI(x) (7,8)** 7: SIHUMI(x) without *C. ramosum* 8: SIHUMI(x)	SIHUMI 	Human	Mouse (C3H/HeOuJ)		3-9	Irradiated low-fat or high-fat diet ad libitum	M	0 (16 wk)	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
(29)	**SIHUMI(x) (8,9)** 8: SIHUMI(x) 9: 8 + *A. muciniphila* ATCC BAA-835	(9) 	Human	Mouse (C57BL/6.129P2-Il10^{tm1Cgn})		5-6	Irradiated standard chow (Altromin fortified type 1310; Altromin, Lage, Germany) ad libitum	M	0 or 8 wk (3 wk)	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

										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)

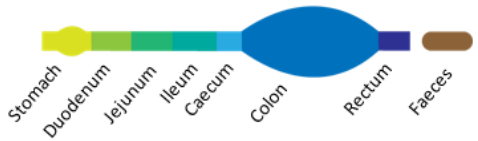
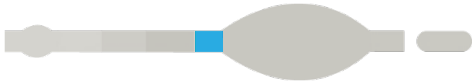
The following study characteristics are listed: microbial consortium name (if applicable), taxonomic affiliation, strain source, host species and strain, part of the gut studied, no. of animals per experimental group, diet, sex, age and study outcomes reported.

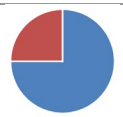
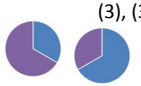
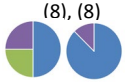
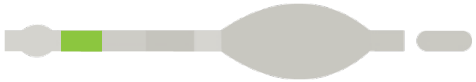
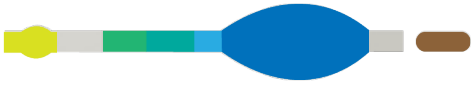
* Two different strains tested are counted as one species. Strains were not always reported. Pathogenic species, in case of an infection model, are not included.

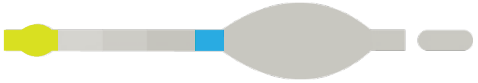
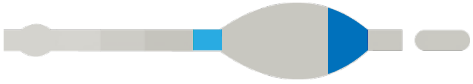
**The colonization time includes the time from colonization (0 in case of transfer of microbiota to offspring) till and including the time of sacrifice or end of experimental (e.g. dietary) manipulations, in case this is clearly stated in the paper. If age is given and animals are colonized at birth,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

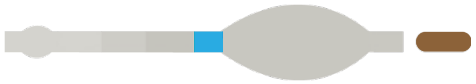
*** Study outcomes are only reported for the animals colonized with the defined community of interest

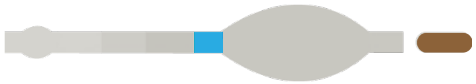
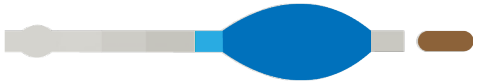
Abbreviations: LP = lamina propria; MLN = mesenteric lymph nodes; MPO = myeloperoxidase; NR = not reported; SCFA = short-chain fatty acids; Treg = regulatory T-cell

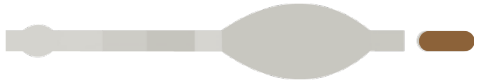
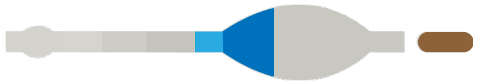
Ref.	Name consortium (no. of species*)	Phylum division Firmicutes Bacteroidetes Actinobacteria Proteobacteria Verrucomicrobia Other	Strain source	Host species (strain)	Part of the gut studied  	No. of animals per group	Chow	Sex (M/F, both or NR)	Age (col. time**)	Study outcomes***
(33)	**NA, F- and N-strains (2,9,11,41, 130)** 2: *E. coli* C25 + *Lactobacillus* 9: 2 + enterococcus + *Lactobacillus* + Candida + 4 morphologically different strains of gram-negative anaerobes 11: 9 + 2 strains of gram-negative anaerobes with fusiform morphology 41: 11 + 30 additional strains of gram-negative anaerobes 130: 50 strains of gram-negative strict anaerobes (N) + 80 facultative anaerobes (F)	*E. coli* C25 +  *Lactobacillus* Others not specified	Mouse	Mouse (CD-1)		4-57	Autoclaved Lobund diet L-356 or pelleted sterile diet from Charles River Mouse Farms	NR	NR (1-60 d)	Caecal number of E. coli C25; caecal size; histopathology of stomach, small intestine, caecum and colon
(34)	**N- and F-strains (60,96,96,97)** 60: N-strains + 14 facultative anaerobes + *E. coli* C25 96: F-strains + *E. coli* C25 or *E.coli* 40T or *Shigella* 97: F-strains + *E. coli* C25 + *Shigella* or *E. coli* 40T	Not specified	Mouse	Mouse (CD-1)		5-75	Sterilized Lobund diet L-356 , Charles River Formula 7RF, Lobound diet L-485 or Purina Breeder Chow	NR	NR (4 wk)	Caecal size; caecal levels of fatty acids; caecal levels of E. coli; pH of caecal contents

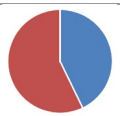
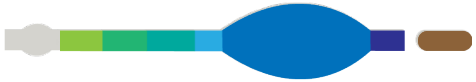
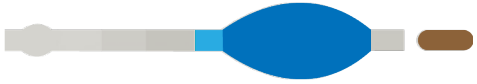
(180)	**na (4)** *Lactobacillus* sp. 1 and 2, *Bacteroides* sp., *Streptococcus* group N		Rat?	Rat (Sprague-Dawley)	None	2	Autoclaved standard diet (Ref 7) supplemented with caffeic acid	NR	NR	Urinary metabolites of caffeic acids
(38)	**na(2,2,2,2,3,3,4,5,6,6,6,8,8,9,13,15,17)** 2: *Actinobacillus* s3 + *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 8: 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	(2,2), (2), (2)  (3), (3)  (6), (6), (6)  (8), (8)  (4,9,13,15), (17) 	Human and mouse	Mouse (CD-1)		≥ 2	Sterilized commercial diet (Usine d'Alimentation Rationnelle) ad libitum	Both	2-5 mo (4 wk after last inoculation)	Number of IgA plasmocytes in duodenum
(181)	**na (2,2,2,2,2,2,2,2,3)** 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*	(2,2,2,2,2), (2)  (2,2), (3) 	Mouse, rat, human	Mouse (C3H)		2-6	Autoclaved commercial diet	NR	Adult (up to 51 d)	Faecal bacterial counts; (mucosal) histology of stomach, jejunum, ileum, caecum, colon

	3: *Clostridium* E and P + *E. coli* K-12									
(37)	**UW-GL (9)** Genera *Lactobillus, Bacillus, Clostridium* and *Corynebacterium*. Species not defined.	UW-GL 	NR	Mouse (Balb/c)		Total of 3	Sterilized Ralson Purina 5010C	Both	0 (60-90 d)	Caecal levels of bacteria and *Candida albicans*; histology of tongue and stomach
(108)	**na (6)** *Streptococcus (Enterococcus) faecalis, Lactobacillus brevis, Aerobacter aerogenes, Staphylococcus epidermidis, Bacteroides spurius* (?), a yeast fungus		NR	Mouse (Balb/c/ABOMf)	None	3-6	Sterilized food (two different procedures)	NR	0 (14 wk)	Serum levels of IgG1, IgG2, IgM and IgA
(36)	**Partial or complete UW-GL (2,3,9)** 2: *Lactobacillus* + *Clostridium* 3: 2 + *Bacillus* 9: UW-GL	Partial UW-GL 	NR and mouse	Mouse (HA/ICR)		10-48	NR	Both	Adult (14-56 d)	Death after *C. botulinum* infection; faecal *C. botulinum toxin* excretion; colonization pattern of *C. botulinum*
(109)	**na (2)** *B. thetaiotaomicron* VPI-5482 + *Desulfovibrio piger* ATCC 29098		Human	Mouse (NMRI/KI)		4-5	Autoclaved polysaccharide-rich (B&K) *ad libitum*	M (subset)	Adult or 12 wk (14-28 d)	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

(182)	**na (2, 6, 10)** 2: *Staphylococcus epidermidis* + *Veillonella parvula* 6, 10: anaerobic strains isolated from a conventional male mouse (not specified)	(2) 	Mouse	Mouse (B10.BR)		45-73	Sterilized ST1 (Institute of Physiology AS CR)	M	21 d (12 mo)	Occurrence of ankylosing enthesopathy of the ankle; colon histology; bacterial content in ileum and colon
(85)	**na (2)** *B. thetaiotaomicron* + *Eubacterium rectale*		Human	Mouse (NMRI-KI)		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" (Harland Teklad 03317)	M	11 wk (14 d)	Bacterial gene expression; caecal colonization levels; fermentation efficiency in caecum; colonic gene expression; protein expression in caecum
(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)	(3)  (8), (9), (10) 	Human	Mouse (C3H/HeN)		15 in total	Sterile standard chow diet or switch to high-fat diet ad libitum	Both	7 wk (70 d after 1st inoculation)	Faecal and caecal bacterial cell counts; body weight; metabolites in urine and plasma

(40)	**na (15, 19)** 15: *Bacteroides caccae*, *Bacteroides ovatus, B. thetaiotaomicron*, *B. uniformis*, *B. vulgatus*, *Bacteroides WH2*, *C. scindens*, *Clostridium spiroforme*, *C. aerofaciens*, *Dorea longicatena*, *E. rectale*, *F. prausnitzii*, *Parabacteroides distasonis*, *Ruminococcus obeum*, *R. torques* (strain info not accessible) 19: 15 + *Bifidobacterium animalis* subsp. *lactis* CNCM I-2494, *Lactobacillus delbrueckii* subsp. *bulgaricus* CNCM I-1632+CNCM I-1519, *Lactococcus lactis* subsp. *cremoris* CNCM I-1631, *Streptococcus thermophilus* CNCM I-1630	(15)  (19) 	Human	Mouse (C57Bl/6J)		5	Autoclaved low fat, plant polysaccharide-rich diet (B&K rat and mouse autoclavable chow #7378000)	M	6-8 wk (42 d)	Faecal and caecal bacterial content; bacterial gene expression; urinary metabolites
(183)	**na (2)** *B. thetaiotaomicron* VPI-5482 (ATCC 29148) + *F. prausnitzii* A2-165 (DSM 17677)		Human	Rat (F344)		6-16	Irradiated polysaccharide-rich diet (R03, SAFE)	M	< 3 months (30 d after inoculation *F. prausnitzii*)	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

(86)	**na (2)** *B. thetaiotaomicron* VPI-5482 + *B. longum* NCC2705		Human	Mouse (SW)		3	Standard diet (Purine LabDiet 5K67)	NR	NR (10 d)	Faecal bacterial content; metabolites in feces and urine
(42)	**na (2,8,9)** 2: *B. thetaiotaomicron* + *D. piger* 8: *B. thetaiotaomicron, B. caccae*, *B. ovatus*, *E. rectale*, *Marvinbryanthia formatexigens*, *C. aerofaciens, E. coli, Clostridium symbiosum* 9: 8 + D. *piger*	(2)  (8), (9) 	Human	Mouse (NMRI)		4-20	Irradiated low-fat/high-plant polysaccharides or high-fat/high-simple sugars ad libitum or the HF/HS diet with modified sulfate concentrations (600-fold range) or HF/HS diet supplemented with chondroitin sulfate	M	7-8 wk (2 wk)	Faecal bacterial relative abundance; faecal metatranscriptome; gene expression of *D. piger*; gene expression of mouse proximal colon; caecal metabolites

(45)	**na (14) + virus-like particles** *C. aerofaciens* ATCC 25986, *B. caccae* 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		Human	Mouse (C57BL/6J)		5	Autoclaved low-fat/high-plant polysaccharide diet (B&K) ad libitum	NR	8 wk (46 d)	Gut barrier and immune function; overall health status; body weight and adiposity; number of CD4+ and CD8+ T-cells in spleens and MLN; faecal bacterial content and viral abundance; genetic changes upon viral attack (phage resistance); bacterial content of proximal and distal small intestine, caecum, colon; prophage activation
(43)	**No name (14)** *B. ovatus* DSM 1896, *Bacteroides uniformis* DSM 8492, *B. thetaiotaomicron* DSM 2079, *B. caccae* DSM 19024, *Barnesiella intestinihominis* YIT11860, *Roseburia intestinalis* 14610, L1-82, *E. rectale* DSM 17629, A1-86, *F. prausnitzii* DSM 17677, A2-165, *Marvinbryanthia formatigenes* DSM 14469, I-52, *C. symbiosum* DSM 934, *C. aerofaciens* DSM 3979, *E. coli* HS, *A. muciniphila* DSM 22959 Muc, *D. piger* ATC29098		Human	Mouse (SW)		Total of 51	Autoclaved standard fiber-rich (15% dietary fiber), fiber-free or prebiotic (addition of purified soluble glycans) ad libitum	Both	8-9 wk (54 d)	Microbial composition in feces, caecum, colonic lumen and mucus layer; bacterial CAZyme expression in caecum; mucin specific transcript in *B. caccae, A. muciniphila* and *B.thetaiotaomicron* caecal microbial enzyme activity; levels of SCFA and organic acids; colonic mucus layer thickness; colonic expression of

										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

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Table 1c. Studies using defined communities to study host-microbe interactions in vivo: Communities in non-rodents (n = 6)

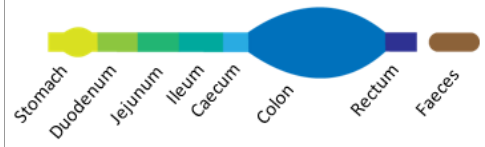
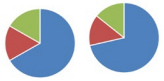
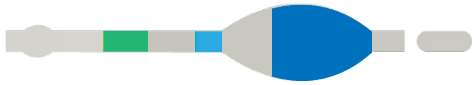
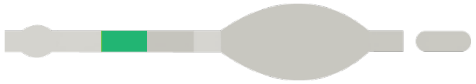
The following study characteristics are listed: microbial consortium name (if applicable), taxonomic affiliation, strain source, host species and strain, part of the gut studied, no. of animals per experimental group, diet, sex, age and study outcomes reported.

* 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

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

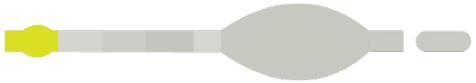
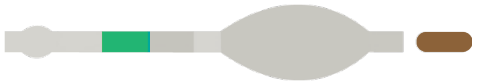
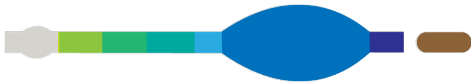
(60)	**Bristol (3)**		Pig	Pig ((Great York x Pietrain) x 'Dalland' cross		6	Sow serum or pasteurized sow colostrum, followed by ad libitum milk replacer diet (day 0-4), followed by a control diet or medium chain fatty acid diet	NR	1 d (2-3 wk)	Oxyntic mucosa transcriptome
(62)	**DMF (7,8)** *Bifidobacterium adolescentis, B. longum, B. thetaiotaomicron, E. faecalis, L. brevis, S. bovis* and *C. clostridioforme* 8: DMF + *E. coli* Nissle	DMF  (8) 	Pig	Pig (Landrace × Yorkshire × Duroc cross-bred)		3-6	NR	NR	7 d (35 d)	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 intestient epithelial cells
(63)			Pig	Pig (NR)		3-5	NR	NR	5 d (14-35 d)	Bacterial content in rectum, duodenum, jejunum, ileum, colon and feces/rectal swabs; diarrhoea and virus shedding after virulent human rotavirus challenge

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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.

Factor	Advantage (vs. human research)	Pitfalls in practice
Inoculum (defined community)	• Controllable composition *Healthy vs. diseased microbiota (e.g. missing key stone species), human- vs. animal-derived*	• Animal microbiome ≠ human microbiome • Difficulties in defining a healthy or normal microbiota • Host-specific selection of microbiota
Diet	• Controllable composition, timing, amount *Tailored to human diet (region, age, season, etc.)*	• Lack of standardization in lab animal feeding protocols • Not always reported (Table 1a-c)
Host genotype	• Controllable – genetic changes possible *Ability to introduce disease*	• Validation of HMIs in multiple strains needed before extrapolation to humans • Animal genotype ≠ human genotype
Sex	• Controllable	• Only one gender investigated (Table 1a-c) • Not always reported (Table 1a-c)
Part of the gut	• Ability to measure bacterial levels in virtually all intestinal parts • Ability to capture transversal heterogeneity	• Anatomy and physiology different from humans • Variations in relative abundance per gut region different per model (Table 1a-c) • Focus on specific gut regions or faeces only (Table 1a-c)

Colonization time	• Controllable	• Long-term effects not studied (Table 1a-c) • 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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FIGURES

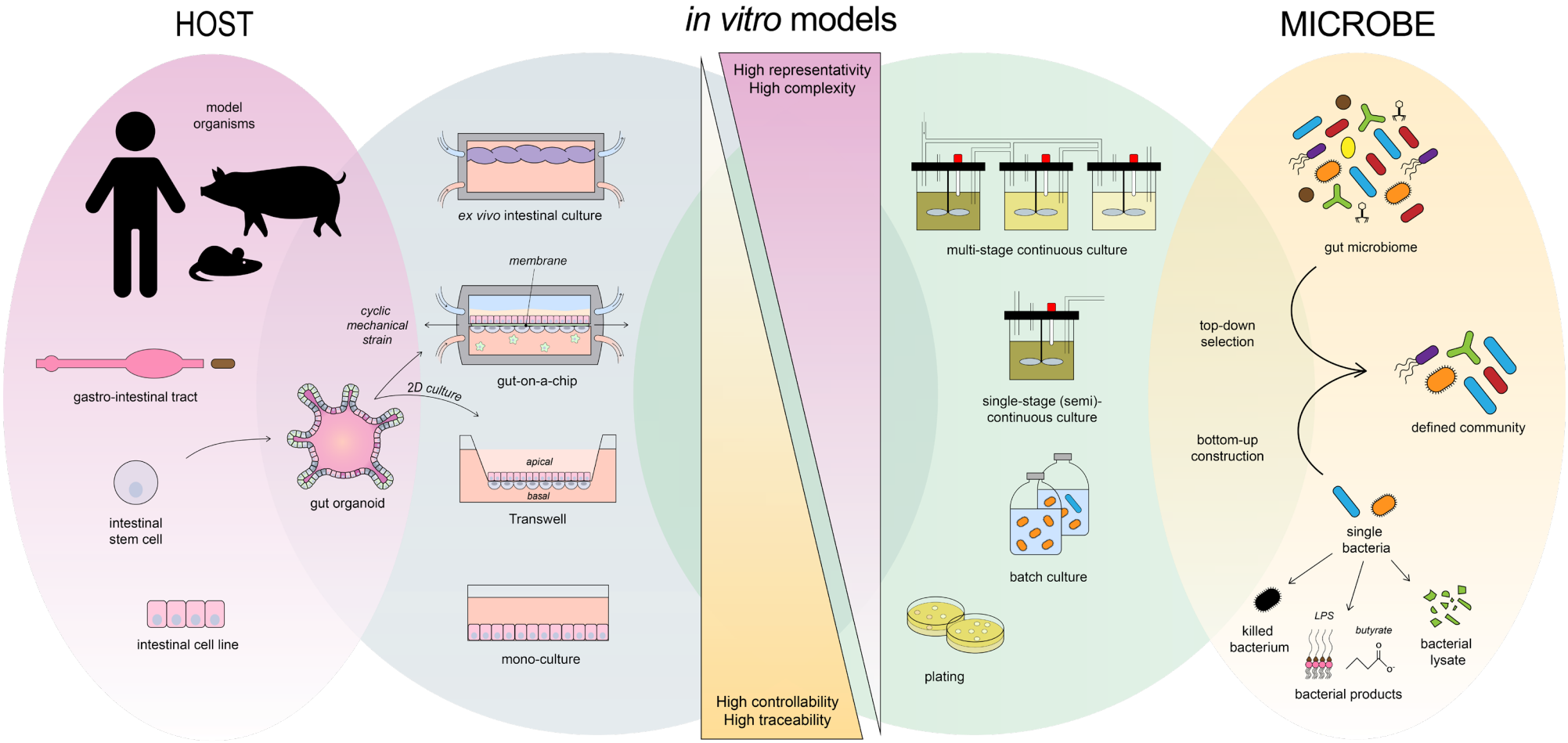
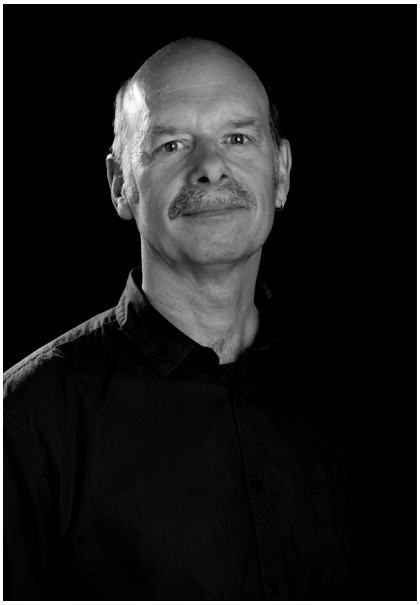


Figure 1. *In vitro* models of the human gut and gut microbiota. Models are organized from bottom to top, with the most representative and complex at the top and the most controllable and traceable - with respect to host parameters or microbial species - at the bottom.

AUTHOR PHOTOGRAPHS

Janneke Elzinga



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