A guide to germ-free and gnotobiotic mouse technology to study health and disease

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Running title: Germ-free and gnotobiotic mouse research guide

Abbreviations

AhR	aryl-hydrocarbon receptor
AOM	azoxymethane
ASF	altered Schaedler Flora
CFU	colony forming units
CR	colonization resistance
CRC	colorectal cancer
DSS	dextran sulfate sodium
FMT	fecal microbiota transplantation
GALT	gut-associated lymphoid tissue
GAP	goblet cell-associated passage
GEMM	genetically enginereed mouse model
GF	germ-free
GI	gastrointestinal
HEPA	high-efficiency particulate air
HMA	human microbiota-associated
IBD	inflammatory bowel disease
ICI	immune checkpoint inhibition
IEC	intestinal epithelial cells
MAF	mucosa-associated fungi
MDSC	myeloid-derived suppressor cells
MNV	murine norovirus
OC	ovarian cancer
OMM12	oligo-mouse-microbiota 12
SCFA	schort chain fatty acids
OMM12	oligo-mouse-microbiota 12
PVC	polyvinyl chloride
SCFA	short chain fatty acids
SFB	segmented filamentous bacteria
SI	small intestine
SPF	specific-pathogen-free
Th1	T helper cell type 1
Treg	regulatory T cells
UG	urogenital
WT	wild-type

Keywords

Germ-free, gnotobiotic, microbiota, experimental design

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Abstract

The intestinal microbiota has major influence on human physiology and modulates health and disease. Complex host-microbe interactions regulate various homeostatic processes, including metabolism and immune function, while disturbances in microbiota composition (dysbiosis) are associated with a plethora of human diseases and are believed to modulate disease initiation, progression and therapy response. The vast complexity of the human microbiota and its metabolic output represents a great challenge in unravelling the molecular basis of host-microbe interactions in specific physiological contexts. To increase our understanding of these interactions, functional microbiota research using animal models in a reductionistic setting are essential. In the dynamic landscape of gut microbiota research, the use of germ-free and gnotobiotic mouse technology, in which causal disease-driving mechanisms can be dissected, represents a pivotal investigative tool for functional microbiota research in health and disease, in which causal disease-driving mechanisms can be dissected. A better understanding of the health-modulating functions of the microbiota opens perspectives for improved therapies in many diseases. In this review, we discuss practical considerations for the design and execution of germ-free and gnotobiotic experiments, including considerations around germ-free rederivation and housing conditions, route and timing of microbial administration, and dosing protocols. This comprehensive overview aims to provide researchers with valuable insights for improved experimental design in the field of functional microbiota research.

Introduction

The human body is home to a diverse array of microorganisms, including bacteria, archaea, viruses, fungi and protozoans, collectively known as the microbiota. It is estimated that the ratio of microbial to human cells is approximately 1:1 [1]. While the microbiota colonize the skin and all mucosal surfaces, including the gastrointestinal (GI) tract, urogenital (UG) tract and lungs of the human body, the gut microbiota represents the largest microbial reservoir, with approximately 100 trillion bacterial cells [1-4]. While other reviews focused on skin [5, 6], lung [7] and UG tract microbiota [8-10], we here focus mainly on the gut microbiota. The gut microbiota is dominated by bacteria, belonging to six main phyla: Bacillota (Firmicutes), Bacteroidota (Bacteroidetes), Pseudomonadota (Proteobacteria), Verrucomicrobia, Actinomycetota (Actinobacteria) and Fusobacteria, among which Firmicutes and Bacteroidetes constitute over 90% of the gut microbiota [11-13].

The gut microbiota plays a crucial role in various aspects of human health, such as digestion and nutrition [14], metabolism [15, 16], immunity [17, 18] and neurological and behavioral responses [19-21]. In addition, the gut microbiota protect the host from colonization by external pathogens, termed 'colonization resistance' by direct niche competition and by shaping mucosal and systemic immunity. The mechanisms by which specific bacteria modulate host physiology often involve the production of bio-active metabolites. For example, bacterial fermentation of indigestible dietary fibers generate short-chain fatty acids (SCFAs, acetate, propionate and butyrate) [22], which serve as a vital energy source for colonocytes and modulate barrier stability and immune homeostasis in the gut [23-26]. Gut bacteria metabolize the amino acid tryptophan into bioactive compounds like serotonin and indole derivates, which activate the aryl-hydrocarbon receptor (AhR) [27] and affect immune function [28, 29]. The gut microbiota is also producing essential vitamins [30], including B vitamins (e.g. B12, folate, riboflavin) and vitamin K, which are important for various physiological functions [31, 32]. Moreover, the microbiota converts primary bile acids into secondary bile acids which impact metabolic health [33, 34].

While researchers have made significant advances in uncovering the molecular mechanisms through which certain bacteria influence host physiology, the vast complexity of largely uncharacterized microbial species and their metabolites remains a great challenge. Moreover, microbiota profiling studies have shown that microbiota dysbiosis is central to many human diseases [35], including inflammatory bowel diseases (IBD) [36, 37], metabolic diseases (e.g. obesity and type 2 diabetes) [38, 39], autoimmune diseases [40], respiratory disorders, cardiovascular diseases [41] and cancer [42]. However, the precise impact of our microbiome on human health and disease has yet to be fully understood. As a result, there is a great demand for focused microbiota studies conducted in a reductionist framework.

While this review focuses on germ-free and gnotobiotic mouse technology, it is crucial to recognize that these methodologies can also be applied in alternative animal models, including rats [43, 44], pigs [45-47], chickens [48] and zebrafish [49-51]. This cross-species approach broadens the scope of functional microbiota research, providing valuable insights into the influence of microbial communities on health and disease across the animal kingdom.

Germ-free and gnotobiotic mice

Germ-free (GF) or axenic mice are completely devoid of all microorganisms and are born and raised in sterile isolators, which are under continuous positive pressure of 'high-efficiency particulate air' (HEPA)-filtered air. Alternative husbandry systems have been developed to facilitate experimental procedures, including positive pressured HEPA-filtered individually-ventilated cage (IVC) like the Techniplast IsoCage-P and the Allentown Sentry SPP system, which offer easier access to animals

during experimental procedures [52-54], in a level II biosafety cabinet [52, 53]. Secure positive pressure caging systems also enable higher throughput gnotobiotic experiments, or allow more complex experimental procedures, which are not possible in isolators (e.g. mouse colonoscopy).

Axenic mice are highly artificial in nature, but offer a unique opportunity to investigate the impact of microbiota deficiency on host physiology. GF mice can be colonized experimentally with one specific or with a defined community of microbes, to generate gnotobiotic mice, which allow studying the impact of specific microorganisms on various biological processes and models of human disease [55, 56]. The use of a strictly defined microbiota in animal models also improves standardization reproducibility.

Practical considerations for gf mice

Germ-free rederivation is the process of creating a GF mouse line from a conventionally or SPF housed line, either inbred wild-type mouse lines, or genetically engineered mouse models (GEMM). GF rederivation is either performed by hysterectomy or by embryo transfer-based rederivation. Germ-free rederivation is the process of creating a GF mouse line from a conventionally or SPF housed line, either inbred wild-type mouse lines, or genetically engineered mouse models (GEMM). Hysterectomy rederivation is performed through caesarean section and rests on the principle that the fetus in the uterus is sterile [57]. When parturition is imminent, the entire uterus along with the fetus, is carefully removed through aseptic caesarean section, decontaminated externally and placed in a sterile isolator where sterile neonates are housed together with a lactating GF foster mother [58]. However, this method poses various challenges, including timed matings, failure to foster and the risk of microbial contamination through vertical transfer. Therefore, most GF facilities prefer embryo transfer rederivation [59]. In this process, recipient GF females are paired with vasectomized GF males to generate GF pseudo-pregnant dams. Subsequently, embryos are transferred in the uteri of GF pseudo-pregnant dams, either surgically or through cervical injection [60, 61]. Sterile donor embryos are generated from non-GF donors through in vitro fertilization (IVF) [62]. Unlike hysterectomy, embryo transfer has reduced chance of post-implantation vertical transmission of microorganisms, providing more successful GF rederivation [59], but requires trained and dedicated personnel for IVF, surgical vasectomy and embryo transfer procedures.

The notion of the uterus being a sterile environment remains a controversial subject. Several studies, utilizing sequencing-based techniques, have reported the presence of a low-biomass placental microbiota [63-65], as well as presence of microbiota in amniotic fluid [66, 67] and umbilical cord blood [68]. These findings suggest the possibility of microbial seeding of the fetal gut before delivery. However, the fact that GF colonies can be established through aseptic caesarean section does not support the presence of a bona fide resident microbial population *in utero*. Nonetheless, it is conceivable that there may be instances of transient microbial exposure *in utero*, particularly in humans that have a long gestational period. This ongoing debate has been extensively and critically reviewed in recent articles [69, 70].

The complete absence of the microbiota leads to various structural and functional abnormalities, including altered microvilli morphology, reduced rate of intestinal epithelial cell (IEC) turnover [71] and massive enlargement of the cecum [72]. Commensal bacteria play a vital role in supporting the integrity of the epithelial barrier through cross-talk with epithelial cells [73-75]. GF animals exhibit fewer intestinal goblet cells resulting in a thinner mucus layer and also a higher percentage of neutral mucins in the colon [76-79] and have decreased expression of antimicrobial peptides at mucosal barrier sites [80, 81]. Importantly, the immune system is underdeveloped in axenic conditions. GF animals present smaller mesenteric lymph nodes and Peyer's Patches, as maturation of secondary gut-associated lymphoid tissue (GALT) requires postnatal microbial colonization [82, 83]. Absence of

microbial signals significantly affects both innate and adaptive immune responses, as the gut of GF mice is characterized by fewer macrophages [84, 85], mast cells [86], subsets of group-3 innate lymphocyte cells (ILC3) [87, 88], effector and regulatory T cells and disrupted antibody responses [89-91]. Phenotypical abnormalities in GF mice also include metabolic and behavioral changes, which have been extensively documented elsewhere [92-95]. The impact of the absence of microbiota extends beyond the confines of the gut. For example, research indicates that microbiota play a crucial role in influencing the generation of hematopoietic progenitors, as the early stages of myelopoiesis in the bone marrow of GF mice are compromised, leading to diminished levels of neutrophils and monocytes in both the bone marrow and peripheral sites [96-100]. Additionally, microbial metabolites originating from the gut also control systemic antibody responses. Mice experiencing microbial insufficiency exhibit deficiencies in both homeostatic and pathogen-specific antibody responses, highlighting the far-reaching impact of microbiota on immune function [101].

Practical considerations for gnotobiotic mice

Gnotobiotic mice can be generated by delivering microbes to GF mice using various experimental approaches, depending on the research objectives. Timing, microbial delivery method, duration and dosage of colonization are crucial parameters to take into account when setting up gnotobiotic studies (Figure 1).

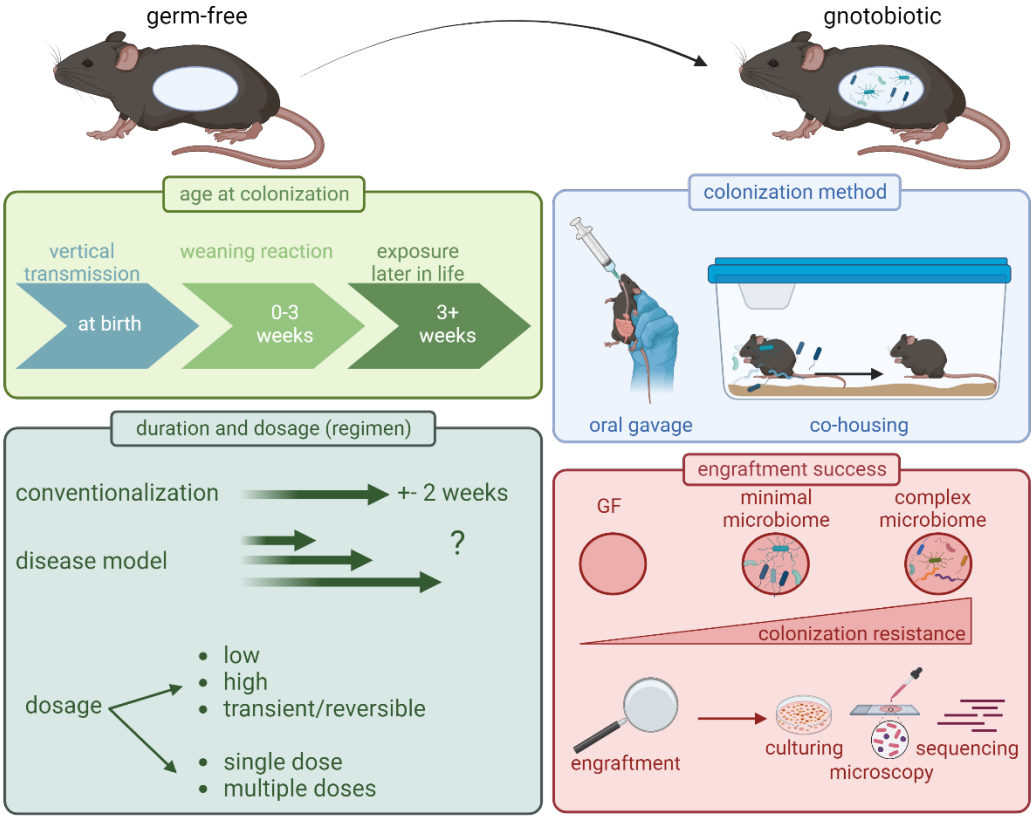

Figure 1. Crucial parameters to take into account when designing gnotobiotic studies. When designing a gnotobiotic study, several experimental factors should be taken into consideration, ranging from the age of the mice, the method of administering the microbes, duration of the colonization experiment as well as dosage. While germ-free mice show minimal colonization resistance, this increases when microbial complexity increases. Engraftment efficiency of microbes of interest should be monitored during the course of the experiment.

Age of colonization

GF mice display various immunological defects, which can largely be restored through colonization at any stage of their lifespan, however, some aspects of immune maturation depend on early-life microbial exposure. For example, neonatal colonization with a diverse microbiota best reverses the hyper-IgE phenotype observed in GF mice [102].

Neonates normally acquire microbiota from the mother through direct vertical transmission during birth [103]. In a gnotobiotic setting, stable vertical transmission of defined minimal microbial consortia has been documented [104-106] and gnotobiotic mice colonized with a complex human microbiota pass down their microbiota to subsequent generations without significant loss of diversity [107]. Vertical transmission of a minimal microbial consortium (such as Altered Schaedler Flora, ASF) supplemented with an *E. coli* LF82 increases susceptibility to dextran sulfate sodium (DSS)-induced colitis, in contrast to mice colonized with ASF+LF82 during adulthood [108]. These findings suggest that the timing of LF82 colonization in gnotobiotic mice impacts the colitis susceptibility later in life and imply that the age at which an individual is colonized by a pathobiont influences the host mucosal immune profile and susceptibility to subsequent inflammatory insults in the future.

Certain phenotypical abnormalities in GF mice can only be reversed by early-life colonization [109]. When colonized mice reach weaning age (3-4 weeks of age), a period when pups gradually transition from maternal milk to solid food, the rapidly changing and expanding intestinal microbiota triggers a robust and broad immune response known as the "weaning reaction". If the weaning reaction is postponed, e.g. by temporal antibiotics treatment, the immune system is imprinted with excessive reactivity, leading to heightened susceptibility to inflammatory conditions like allergic inflammation [109], DSS-induced colitis [109] and azoxymethane (AOM)/DSS-induced colorectal cancer (CRC) [110]. AOM/DSS-treated mice that were born in GF conditions and conventionalized after weaning exhibited increased inflammation and tumorigenesis due to an accumulation of myeloid-derived suppressor cells (MDSCs) [110]. Early-life microbial exposure thus plays a crucial role in establishing intestinal homeostasis and in restraining MDSC-driven CRC in adulthood. Importantly, the critical time window cannot be compensated for by exposure to microbiota later in life, indicating that weaning is a crucial period for microbe-mediated immune education. The protective effect of the weaning reaction is mediated through the generation of RORgt+ regulatory T cells (Tregs), induced by SCFAs [109]. Knoop et al. demonstrated that the critical period for developing tolerance to gut symbionts occurs between 10 to 20 days after birth and coincides with the formation of goblet cell-associated passages (GAPs) in the colon [111]. These GAPs facilitate the delivery of antigens, which in turn promote the generation of Tregs specifically targeting the gut microbiota present during this window, thereby maintaining long-term tolerance. Further, it was described that macrophages capture soluble antigens from the lumen and deliver the antigen to dendritic cells, thereby promoting the development of Tregs and oral tolerance [112].The age at which GF mice are colonized is thus a critical parameter in immune system development and influences disease progression later in life. Microbe-host interactions in early life and its functional consequences have been described extensively elsewhere [113, 114]. The perfect colonization experiment to overcome early life perturbations, is to colonize pregnant dams and use F2 offspring [115, 116], since these mice are born from neonatally colonized dams. For practical considerations however, often F1 offspring is used or mice are colonized around weaning age.

Method of colonization

Delivery of microbial suspensions is preferentially achieved through oral-gastric gavage, in which the bacterial suspension is administered directly into the stomach using a feeding needle [117].

Alternatively, GF mice can be colonized gradually by co-housing with gnotobiotic or SPF donor mice. Bacteria are exchanged through direct physical contact, as well as through coprophagy (ingestion of excreted feces) [118]. This natural approach allows the establishment of colonies with diverse

microbial communities while maintaining genetic uniformity. While this method allows for a diverse microbial community, reproducibility is limited due to the lack of control over dosing and transfer frequency. For specific microbes of interest, it's important to consider their transfer capabilities through this procedure, which favors oxygen-friendly microorganisms. However, conventionalization by co-housing with SPF mice is successful and these ex-GF mice do not exhibit a different microbiota composition compared to the SPF mice [119].

Colonization duration and dosage

Another parameter to take into consideration is the duration of colonization and dosage. These parameters depend strongly on the specific research question and desired read-out. Acute responses (e.g. epithelial expression responses) can rely on short term-colonization experiments (days), while more chronic responses (e.g. disease development, adaptive immune maturation etc.) requires long-term colonization experiments of multiple weeks.

For monocolonization studies (see further), a commonly used dosage is $1*10^8$ colony forming units (CFU) per gavage [120]. However, it is crucial to determine the appropriate dose for each gnotobiotic study. For example, Buschor and colleagues explored the influence of different *Citrobacter rodentium* doses in GF wild-type (WT) mice [121]. Their findings revealed that mice inoculated with a high dose (10^{10} CFU) displayed reduced disease severity compared to those given a low dose (10^4 CFU). The high *C. rodentium* dose more effectively induced an early mucosal innate immune response, whereas the low dose allowed bacteria to remain under the radar of the innate immune response for an extended period and continue their logarithmic growth to cause disease [121]. In case of low-engrafting efficiency, multiple gavages on consecutive days can increase chance of stable engraftment. Moreover, protocols establishing stable microbial consortia in GF mice also describe administering the cocktail twice in 48h to increase colonization success [105, 106]. Dosage may require optimization for specific strains, as too high doses can result in aberrant immune activation and even mouse death.

Reversible colonization protocols have also been established to study host physiology after loss of prior bacterial exposure. For example, auxotrophic mutants of *Escherichia coli* K-12, lacking key enzymes for the biosynthesis of essential amino acids required for its replication (*E. coli* K-12 mutant HA107), have been developed to transiently colonize GF animals. Such reversible colonization models have been successfully used to study the dynamics of microbiota-induced immunity and disease [122, 123].

Typically, conventionalization studies (colonization of GF mice with the total fecal microbial community of SPF or conventionally housed mice) adopt a relatively short timeline, not exceeding 2 weeks. However, a study with conventionalized GF mice demonstrated that the microbiota underwent transient alterations and that approximately 8 weeks are required to reach a similar composition as the conventionally housed counterparts, showing that conventionalization is a slow and complex process [124]. Regarding fecal microbiota transplantation (FMT) studies, dosing can vary from a single dose to multiple administrations per week over a few weeks. For studies utilizing complex fecal samples, a single dosing is not recommended, as it results in cage-dependent shifts of the microbial composition over time [125]. Repeated gavages once a week over an extended period could be a suitable approach to maintain the donor microbiota population for 12 weeks [126, 127]. Multiple rounds of gavage also significantly increased the cumulative number of detected taxa and compositional similarity to the donor, while simultaneously reducing inter-animal variance [127].

In GF conditions, after antibiotic treatment or initial colonization of the neonatal gut, facultative anaerobic bacteria are among the first colonizers, which actively consume oxygen as terminal electron acceptor, thereby effectively lowering the redox potential to facilitate colonization of subsequent

oxygen-sensitive strains [128]. Therefore, engraftment of strict anaerobes in germ-free mice can be facilitated by prior introduction of a facultative anaerobic strain.

Engraftment efficiency and troubleshooting

An intact gut microbiota plays a critical role in mucosal protection from bacterial invasion and disease [129]. The commensal microbiota prevent pathogen colonization by competing for attachment sites or essential nutrients within ecological niches [130, 131]. Eradication of the microbiota by antibiotic treatment also results in a loss of colonization resistance against exogenous microorganisms [132]. Several reports have shown that GF mice, similar to antibiotic-treated mice, are more susceptible to infection than their conventional counterparts [133-135]. Reconstitution or conventionalization of these GF mice can often reverse the phenotype, making them valuable for investigating the role of specific bacteria or host-microbe interactions in colonization resistance.

Successful engraftment can rely on inter-bacterial interactions, eg. cross-feeding interactions [89]. The ability of bacteria to colonize GF mice can also vary among strains of the same species, as different CRC-derived *Fusobacterium nucleatum* subspecies show varying colonization efficiacy in GF mice, with limited mucosal association in gnotobiotic mice [136].

Despite obvious differences between human and mouse, a significant portion (approximately 85%) of the human microbiome can be successfully transferred in mice [107]. Some human-derived strains either fail to engraft or will develop altered microbial functionalities [55, 107, 137-140]. A murine microbiota can simply outcompete an already established human-derived microbiota in the mouse gut, highlighting host-specificity of the intestinal microbiota [141]. Differences in diet between mice and humans also impacts microbiota composition and engraftment efficacy of specific species [107, 142]. Considering these factors, it is unlikely that complex human microbiota is faithfully preserved when transplanted in GF mice. Despite these limitations, human-microbiota-associated mice have proven to be very valuable. As an example, immune-checkpoint-inhibition (ICI) studies using HMA or 'avatar' mice have shown that therapy response is donor-specific (see further) [143-145].

Colonization complexity

The colonization status of a gnotobiotic model can range across a wide spectrum, ranging from low complexity (GF, monocolonization and minimal microbial consortia) to more complex defined microbial consortia and complex undefined microbiota samples from murine or human origin (Figure 2).

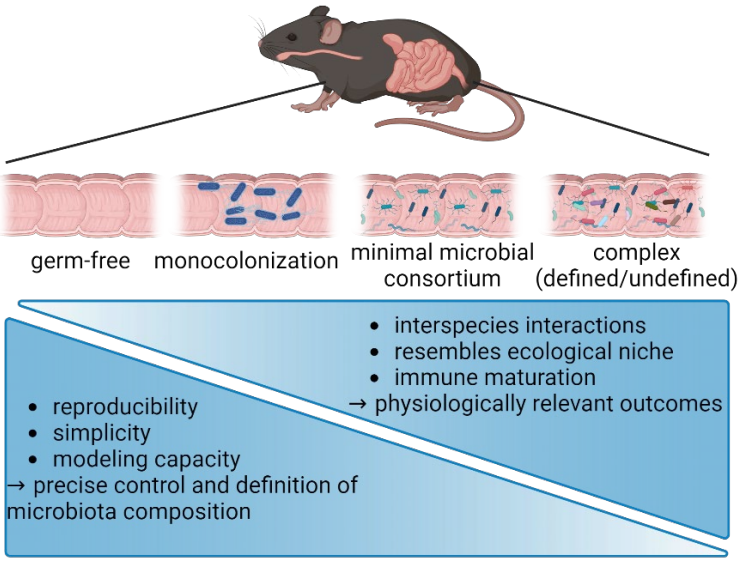

Figure 2. Colonization spectrum. More simple microbial communities allow precise control and definition of the microbiota composition and in-depth investigation into host-microbe interactions. More diverse or complex communities increase physiological relevancy, as it accounts for interspecies interactions, resembles the physiological ecological niches and induces immune maturation.

Monocolonization

In monocolonization experiments, the microbiota is reduced to a single microbe of interest, thus being the most simple form of gnotobiotic studies. These experiments allow in depth investigation of how a single microbial strain affects host physiology and disease development and contributes to immune maturation and activation. Monocolonization experiments allow to study causal relationships between a microbe of interest and host physiology. Although monocolonized mice often largely retain the physiological abnormalities seen in GF mice [146-149], multiple studies have described immune maturation mechanisms in monocolonized mice and identified species that have pronounced T helper cell type 1 (Th1) or Th17 polarizing activity, have anti-inflammatory effects, or influence experimental models of colitis and colorectal cancer [150-157].

Minimal microbial consortia

Gnotobiotic models using minimal microbiota consortia with low and medium diversity offer an expanded antigenic and metabolic microbial capacity and allow studying bacteria of which engraftment depends on specific interspecies interactions [158, 159].

Defined microbial consortia can be designed with specific metabolic requirements or functions in mind (e.g. SCFA producers, mucus degraders etc.) or to generate a minimal representation of the major phyla present in gut microbiota , such as ASF, oligo-mouse-microbiota (OMM12, for mouse) or 14SM (for human microbiota, see further) [104, 160]. For example, a diet-based minimal microbiota was assembled to study interspecies metabolic interactions in the presence of common dietary fibers *in vitro* [161]. Rationally designed bacterial consortia could also be studied in the context of microbiota-based therapeutic interventions. In this aspect, GUT-103 and GUT-108 were designed to independently restore normal function to the inflamed colon in chronic immune-mediated colitis, by complementing missing or underrepresented functions in the dysbiotic microbiome of IBD patients [162].

The defined microbial communities allow researchers to test the effect of a specific microbial strain in a semi-mature immune environment. In addition, minimal complexity consortia reduce limitations

related to variability between animal facilities [55, 163] and contribute to standardization and experimental reproducibility for testing causal host-microbe interactions [56, 164, 165]. These defined microbial consortia offer valuable tools for studying the contribution of specific microbes or examining particular microbial functions. Researchers can supplement these consortia with certain species or remove species and specific functionalities, as needed. For example, elimination of the mucus degrader *Akkermansia muciniphila* from a minimal microbial consortium (GM15, described below) in mice on a fiber rich diet showed the importance of maternal microbiome composition and dietary fiber intake in postnatal microbiome maturation and immune development [166, 167]. However, these minimal microbial consortia have limitations that should be kept in mind when translating gnotobiotic research to more complex settings, as they do not fully recapitulate the functionality of a complex microbiota.

In table 1, we describe some commonly used minimal microbial consortia with bacterial species of both human or mouse origin. It is worth noting that several biopharmaceutical companies, including MRM Health, Vedanta Biosciences and Seres Therapeutics, are actively working on pipelines to develop defined bacterial consortia for use as oral therapeutics. These therapeutics aim to address a wide range of conditions, such as gut inflammation, neurodegenerative and metabolic diseases, autoimmune diseases and as adjuvants in cancer immunotherapy.

Minimal microbial consortia offer the advantage of easy manipulation, allowing to study the contribution of specific microbes or microbial functions by adding or removing specific species. For instance, while OMM12 conferred colonization resistance to *Salmonella enterica Serotype Typhimurium* (S. Tm), it confers only partial colonization resistance to *C. rodentium* infection. The OMM12 was reinforced with an *E. coli* and *Citrobacter amalonaticus* strain, resulting in a clearance of *C. rodentium* infection with kinetics similar to those observed in SPF mice [168]. The addition of these two facultative anaerobes triggered migration of neutrophils required for pathogen clearance and thus further stimulated immunological maturation [168]. Accordingly, several studies have used the OMM12 model to probe for a causal role of individual microbes in providing protection against different pathogens [169-171]. Increasing the bacterial diversity of OMM12 to 19 strains (OMM19.1) further compensates for phenotypical limitations of OMM12, including body composition and changes in T cell subtypes in lamina propria and gut-associated lymphoid tissues. The study also highlights the intermediate phenotype of OMM19.1 between OMM12 and SPF in terms of IgA+ plasma cells in the lamina propria of small intestine and colon [172]. However, whether these effects on the immune system are the result of increased diversity in the microbial community or due to specific functions of added bacteria remains to be elucidated. In addition, since OMM12 cannot carry out 7α-dehydroxylation, addition of *Clostridium scindens* normalized bile acid composition and conferred colonization resistance against *Clostridium difficile* [170]. Minimal microbial consortia with specific functions can also be established from complex human microbiota. For instance, from a healthy human fecal sample, researchers have isolated a consortium of 17 Clostridia species, capable of inducing a CD4+ FOXP3+ regulatory T cell response [173, 174]. This Clostridia consortium attenuated colitis in the TNBS experimental model of colonic inflammation. Furthermore, Faith and colleagues conducted an extensive study where they fractioned an intact, uncultured human gut microbiota in random subsets of various sizes. They subsequently introduced 94 bacterial consortia into GF mice. This comprehensive study discovered a range of bacterial strains that promote the accumulation of colonic Tregs while some strains modulated mouse adiposity and cecal metabolite concentrations [175].

Fungal contributions – the mycobiota

So far, most microbiota-related and gnotobiotic studies have focused on the impact of bacteria. This is not surprising since bacteria constitute the vast majority of the gut microbiota. However, the

mycobiome, which represents only 0.1% of the gut ecosystem [176-178], has long been overlooked, and our understanding of fungal contributions to host development remains limited. Nevertheless, the mycobiome is now increasingly recognized as a regulator of host homeostasis and involved in physiological [179, 180] and pathophysiological processes, including IBD [181-183], obesity [184], alcoholic liver disease [185], allergic airway disease [186] and several cancers [187].

GF and gnotobiotic studies allow us to now explore gut fungi functions, as well as their trans-kingdom interactions with other members of microbial communities (bacteria, archaea, viruses). A recent study established a defined consortium of fungi, representing a subset of fungal genera that are closely associated with the intestinal mucosa (mucosa-associated fungi, MAF). This MAF consortium promotes epithelial barrier function, induces Th17 responses and protects mice from intestinal injury (upon antibiotic treatment) via IL-22 and CD4+ T-cell dependent mechanisms [179].

Gnotobiotic studies exploring inter-kingdom interactions have also yielded intriguing findings. van Tilburg and colleagues used a gnotobiotic approach to demonstrate that fungal colonization alone was insufficient to trigger overt DSS-induced colitis, but when combined with bacterial colonization, it led to shifts in the bacterial microbiome and increased colonic inflammation [188]. This finding highlighted the causal role of fungi in microbial ecology and host immune functionality. Furthermore, quantitative proteome analyses revealed that certain fungal species have a significant impact on the host intestinal proteome compared to a bacterial consortium, particularly through their influence on metabolic proteins [189]. Moving forward, the integration of both bacterial and fungal species in gnotobiotic studies will allow us to further elucidate how the microbiome and mycobiome interact and collectively modulate host physiology and pathology.

Viral contributions – the virome

Viruses represent an important component of the microbiota, mainly bacteriophages which target bacteria, but also host-infecting viruses. Murine norovirus (MNV) infection exacerbates colitis development in SPF Il10-/- mice. These mice are protected in GF conditions and develop little to no colitis upon ASF or OMM12 colonization. However, MNV infection exacerbates colitis only in Il10-/- ASF mice, not in OMM12 Il10-/- mice [190]. In addition, co-colonization with segmented filamentous bacteria (SFB) abolished the MNV-triggered inflammation in ASF colonized Il10-/- mice, while SFB had no effect on OMM12 colonized mice [190], highlighting the importance of microbial context on disease development. In addition, the capacity of *E. coli* Mt1B1 to block *Salmonella Tm* depends on the microbial context. In OMM12 gnotobiotic mice, *E. coli* Mt1B1 depleted galactitol, a substrate that otherwise fuels *Salmonella Tm* colonization and prevented *Salmonella* invasion and mediated colonization resistance. In contrast, *E. coli* Mt1B1 did not provide colonization resistance to *Salmonella Tm* in the background of ASF [169].

Complex microbiota – fecal microbiota transplantation

Defined microbial communities offer a streamlined approach to studying the microbiota, yet subjecting GF mice to complex, undefined microbiota from either mouse or human samples enables the exploration of a microbial community's physiological significance. FMT is a widely used technique to investigate a causal connection between the gut microbiome and diseases [125, 191]. Applying FMT to introduce human feces into GF mice, thereby creating humanized gnotobiotic mouse models, has revolutionized the creation of *in vivo* systems mirroring the human flora [192, 193]. Human microbiota-associated (HMA) mice are exceptionally useful for dissecting host-microbe interactions and have provided new insights in the role of the microbiota in health and disease.

For example, transferring the gut microbiota of ovarian cancer (OC) patients into OC-bearing mice accelerated tumor development, while the addition of *Akkermansia muciniphila* was able to suppress OC progression in mice by restoring gut barrier function and enhancing IFNγ secretion of CD8+ T cells and enhancing its tumor-killing property [194].

Cytotoxic T lymphocyte activation is restricted by specific 'immune-checkpoints', receptors which prevent aberrant T cell activation and autoimmunity upon ligand binding, including cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1). Certain tumors exploit these checkpoints to evade the immune system [195, 196]. Immune checkpoint inhibition therapy (ICI), such as anti-PD1 and anti-CTLA4, has revolutionized anti-cancer treatments in several types of cancer, including melanoma and non-small cell lung cancer [145, 197].

The microbiota composition is a predictive biomarker of ICI therapy response and ICI-therapy response is mirrored in GF mice receiving patient microbiota and ICI therapy [144, 145, 198-201]. The role of specific commensal bacteria in immunotherapy efficacy was highlighted by Vetizou and colleagues, as GF mice failed to respond to CTLA-4 blockade therapy until supplemented with *B. fragilis*, underscoring Bacteroidales' significance in the immunostimulatory effects of anti-CTLA-4 ICI [202].

Numerous studies have leveraged human-to-murine FMT models to explore the connection between gut microbiota and ICI-induced tumor response [117, 203, 204]. Additionally, these models have led to the establishment of defined bacterial consortia that promote anti-tumor immunity and ICI-response. Inosine-producing bacteria have been shown to enhance the effect of ICI therapy in CRC, bladder cancer and melanoma [205]. A consortium of 11 human strains also stimulates IFNγ-producing CD8+ T cells and anti-tumor immunity [206]. This underscores the potential of microbiome modulation as a promising adjuvant therapy for cancer, surpassing the therapeutic scope of FMT.

Although FMTs from humans to mice can provide novel fundamental insights, there are limitations with respect to species specificity, as discussed earlier.

Gnotobiotic mouse technology enables the exploration of precise microbial effects on host physiology, encompassing an in-depth examination of the associated molecular mechanisms. This investigation involves the comprehensive characterization of microbial influences at the transcriptional (RNA), translational (protein), and metabolite levels, employing metatranscriptomics, proteomics and metabolomics approaches, respectively. Through the integration of multi-omics analyses and targeted interventions, researchers can pinpoint the pivotal microbial genes, proteins, and metabolites that significantly influence host physiology [207-210].

Mouse disease models

Various experimental and transgenic mouse models of human disease are very informative to examine microbiota-contributions to disease. Multiple transgenic mouse lines with spontaneous disease phenotype are rescued in germ-free conditions or are influenced by changes in microbiota composition. These genetic and experimental disease models are very interesting to identify microbiota-derived disease mechanisms or therapy-modulating mechanisms. In table 2 we list commonly used genetic and experimental mouse models of IBD, CRC and extraintestinal pathologies that are influenced by microbiota composition. We also incorporated the effect of antibiotic treatment on pathology. Antibiotics treatment and GF rederivation could yield conflicting outcomes, as antibiotic treatment does not entirely deplete the intestinal microbiota Moreover, certain antibiotics may exhibit anti-inflammatory properties [211].

Conclusions and future perspectives

The rapid evolution of high-throughput sequencing, metabolomic profiling and advanced imaging techniques has begun to illuminate the intricacies of host-microbe cross-talk. Understanding the significance of host-microbe interactions in maintaining host homeostasis and contributing to pathology is a profoundly intricate field, riddled with formidable challenges due to its multifaceted nature. Harnessing these technological advances in synergy with gnotobiotic models holds the promise of deciphering the intricate interplay between host and microbial communities. While maximizing standardization is key to capture the multidimensionality of host-microbe interactions, there is no 'one-size-fits-all' approach, as experimental design largely depends on the microbes and the physiological process of interest.

SPF and GF mice are invaluable tools in biomedical research. However, their use has inadvertently created a significant gap with humans, which harbor a more complex microbiota. In an attempt to standardize housing conditions and to limit confounding infections, SPF mice have become abnormally hygienic [212]. Consequently, SPF mice exhibit an immune system that rather resembles neonatal humans, restricting the translational potential of laboratory animal-based studies. Environmental changes result in better recapitulation of features of adult humans, including effector-differentiated and mucosally distributed memory T cells [213]. Co-housing of SPF mice with pet store or feral mice shifts the murine immune system to more closely resemble that of adult humans [213, 214]. Furthermore, exploring the immunological landscape of wild mice emerges as an attractive alternative, offering genetic diversity and exposure to natural environmental factors [215, 216]. Various studies already indicated that wild mice exhibit immunological traits closely resembling their human counterparts [213, 217, 218]. To eliminate genetic effects in wild-mice, wild-mouse microbiota was transferred to C57BL/6 mice through 'reverse germ-free rederivation', which involves C57BL/6 embryo transfer in pseudo pregnant wild mice [219, 220]. The resulting 'wildling' mice had a systemic immune system and microbiota resembling wild mice and phenocopied human outcome in models of T cell activation and TNF-induced septic shock, in contrast to laboratory mice. Surprisingly, wildling mice are also more susceptible to allergic inflammation, which challenges the hygiene hypothesis. It is thus imperative to carefully assess the desired level of microbiota complexity and ideally experiments should be performed at different levels of microbiota complexity. Thoroughly assessing this aspect is crucial to generate comprehensive and clinically relevant insights for biomedical research.

Author contributions

M.J. and L.V. wrote the manuscript.

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Tables:

Table 1. Overview of commonly used minimal microbial consortia in vivo.

	ASF	OMM12	OMM19.1	GM15	14SM
Origin of strains	Mouse	Mouse	Mouse	Mouse	Human
# Bacterial strains	8 strains	12 strains	19 strains	15 strains	14 strains
Phylogeny	Firmicutes, Bacteroidetes, Deferribacteres[221]	Represents 5 most prevalent phyla (Bacteroidetes, Firmicutes, Verrucomicrobia, Proteobacteria and Actinobacteria) naturally abundant in mouse GI [222]	OMM12 + additional phylogenetically and functionally diverse species	Represents 3 different phyla (but encompasses wider range of prevalent intestinal bacterial families compared to OMM12)	Represent dominant phyla of human gut microbiome Possesses core metabolic capabilities
Availability of strains	Not in public collections Near complete genome sequences reported [223]	Fully sequenced + publicly available	Publicly available	Community not publicly available	Fully sequenced + well characterized
Vertical transmission	Stable transmission	Stable transmission	Stable transmission	Stable transmission	Stable transmission
Phenotypical changes	Induce Tregs [156] Induce spontaneous + homeostatic T cell proliferation [224] Normal immune system and GI function [225]	Immune maturation of intestinal blood vessels [168] Activation of immune system to support neutrophil migration [168]	Body composition shifts towards phenotype of conventionally colonized mice Changes in T cell subtypes compared to OMM12: increased RORγt+ CD4+ Th17 cells Increased IgA+ plasma cells compared to OMM12	Increased serum antibodies Increased PP development Phenotypically similar to SPF animals	Immune profile intermediate between GF and SPF [166]
Colonization resistance (CR)	No CR against *Salmonella enterica* serovar Typhimurium (S. Tm)	CR against S. Tm CR against *Clostridioides difficile* [170] Reduced colonization of *C. rodentium* [168]	To be determined	To be determined	No CR to *C. rodentium* [106]
Advantages	Effectively transmitted to offspring Stable consortium Introduced microbes can successfully colonize [190, 226, 227] ASF distribute longitudinally	Long term stability Reproducibly established in different facilities Allows flexible experimental design	Overall intermediate state between OMM12 and SPF colonized mice	Recapitulates functionalities SPF microbiota metagenome Animals phenotypically similar to SPF Less sensitive to deleterious effects of post-weaning malnutrition compared to SPF	Human consortium Allows to validate the impact of fiber supplementation on gut microbiota modulation

	through GI tract and differentially occupy niches similar to complex microbiota [228]			Shares metabolic traits with SOPF (specific and opportunistic pathogen free)	
References	[122, 160, 225, 229-232]	[104, 233, 234]	[172]	[105]	[106, 235]

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1312 *Table 2. Overview GF mouse models of IBD, CRC and extraintestinal pathologies and colonization studies.*

Model	Phenotype SPF	Phenotype/rescue in GF	Colonization studies
Transgenic disease models			
Muc2-/-	Colitis SI adenomas that progress into invasive adenocarcinomas + rectal tumors [236, 237]	N.A.	In SPF: more susceptible to enteric pathogen infections [238-242]
Winnie	Missense mutation in *Muc2* Thinner mucus barrier and increased bacterial translocation Inflammation in distal colon	Partial protection: Colitis substantially reduced but not abolished [243]	N.A.
Il10-/-	Spontaneous chronic colitis [244]	Full rescue [245]	Monocolonization of AOM-treated Il10-/-: *E. coli* NC101 promoted invasive carcinoma in a colibactin-dependent manner [246] Adherent invasive *E. coli* harboring yersiniabactin pathogenicity island promote inflammation-associated fibrosis [247] MNV exacerbates colitis severity in ASF-colonized mice, not in OMM12-colinized mice [190] SIHUMI consortium induced colitis [248] *Klebsiella pneumoniae* isolated from healthy premature infant plays critical role in onset of colonic inflammation [249]
TCRα-/-	Colitis Strong antibody response to self-antigens [250]	Full rescue [251]	No intestinal inflammation when + *Lactobacillus planatarum + E. faecalis + E. faecium + E. coli 09:K36* [251]
Il2-/-	Histological resemblance to ulcerative colitis [252]	Partial protection: delayed and mild focal intestinal inflammation [253]	mono-association with *E. coli* induced colitis but addition of *Bacteroides vulgatus* prevented *E. coli*-induced colitis [254, 255]
Apc$^{Min/+}$	Multiple intestinal neoplasias (mostly small intestinal) [256]	Conflicting data: • Drastic drop in colonic tumor incidence + overall tumor load [257] • no rescue [256]	Mixture of 6 *Fusobacterium nucleatum* CRC clinical isolates failed to enhance intestinal tumorigenesis [258] FMT of CRC patients increased tumor proliferation, impaired gut barrier function and upregulated inflammation

			compared to healthy controls [259]
$Apc^{Min/+}$ $MSH2^{-/-}$	More SI and colon tumors compared to $Apc^{Min/+}$ [260, 261]	On broad-spectrum antibiotics: develop fewer colon tumors [261]	N.A.
$Apc^{Min/+}$; $Il10^{-/-}$	Stronger inflammation induced colon (but not SI) tumorigenesis compared to $Apc^{Min/+}$ [258]	Colon tumorigenesis abolished No effect on SI tumorigenesis [258]	Different bacterial strains affect tumorigenesis: Transfer of GF $Apc^{Min/+}$;$Il10^{-/-}$ mice to SPF: induced colonic inflammation + tumorigenesis [258] Mice are sensitive to microbial status: pks+ *E. coli* enhanced tumorigenesis but mixture of 6 *F. nucleatum* strains did not [258]
AOM-$Il10^{-/-}$ (azoxymethane)	Colitis-associated cancer [262, 263]: • Spontaneous colitis • Colorectal carcinomas	Complete protection [263]	Mono-association with *E. coli* NC101 or *E. faecalis* induced severe colitis, E. coli NC101 aggravated tumorigenesis, *E. faecalis* did not induce tumors [246]
$Zeb2^{IEC\text{-}Tg/+}$	Increased intestinal permeability in SI and colon, colitis and invasive carcinomas in colon [264]	Complete protection from colitis and invasive CRC Intestinal barrier still permeable [264]	SPF: pks+ *E. coli* 11G5 accelerates tumorigenesis in an adhesin-mediated and colibactin-dependent manner [265]
Diabetes type 1: NOD	Diabetes	Diabetes[266, 267]	Delayed onset when colonized with *Bacillus cereus* [267]
Ileitis and arthritis: TNF^{emARE}	TNFR1-mediated Crohn's-like ileitis Peripheral and axial arthritis [268]	Protected from ileitis, not protected from peripheral and axial arthritis [268]	N.A.
Ileitis and arthritis: $TNF^{\Delta ARE/+}$	Crohn's-like ileitis [269, 270]	Protected from ileitis No report on arthritis [269, 270]	Mono-association with E. coli LF82 (CD-related) did not induce colitis, transplantation of disease-associated microbiota induced CD-like ileitis [270]
Experimental disease models			
DSS (dextran sodium sulfate)	Colitis [271]	Aggravated colitis [272, 273] Highly susceptible to epithelial injury [274]	Used to demonstrate probiotic effect of *Lactobacillus kefiranofaciens M1*: ameliorates DSS-induced colitis [275] Protective effects of *Bacteroides ovatus* against DSS injury [276]
TNBS (trinitrobenzene sulfonic acid)	Transmural colitis [277] Disruption of epithelium Invasion of colonic wall by bacteria Colitis partially alleviated by blocking PD-1/PD-L1 [278]	Antibiotics administration: protected from colitis [279]	*Lactobacillus casei*: protective effects against colitis [279]
AOM/DSS	Polyps in distal colon	Contradictory results GF vs broad-spectrum antibiotics: • More and larger tumors in GF	Monocolonization with *Bacteroides fragilis* prevents inflammation and

		compared to SPF (recolonization or LPS administration reduced tumorigenesis), due to delayed tissue repair [280] • SPF + antibiotic cocktail (ampicillin, neomycin, metronizadole, vancomycin): lower tumor burden [281]	tumor formation via TLR4 signaling [152, 282]
Multiple sclerosis: EAE	Inflammation in spinal cord Ascending flaccid paralysis [283]	Resistant to EAE development [284]	Monocolonization with SFB renders mice highly susceptible to EAE symptoms [284]

Figure legends:

Figure 3. Crucial parameters to take into account when designing gnotobiotic studies. When designing a gnotobiotic study, several experimental factors should be taken into consideration, ranging from the age of the mice, the method of administering the microbes, duration of the colonization experiment as well as dosage. While germ-free mice show minimal colonization resistance, this increases when microbial complexity increases. Engraftment efficiency of microbes of interest should be monitored during the course of the experiment. GF: germ-free.

Figure 4. Colonization spectrum. More simple microbial communities allow precise control and definition of the microbiota composition and in-depth investigation into host-microbe interactions. More diverse or complex communities increase physiological relevancy, as it accounts for interspecies interactions, resembles the physiological ecological niches and induces immune maturation.