Review

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DISSECTING THE INTERPLAY BETWEEN INTESTINAL MICROBIOTA AND HOST IMMUNITY IN HEALTH AND DISEASE: LESSONS LEARNED FROM GERMFREE AND GNOTOBIOTIC ANIMAL MODELS

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This review elaborates the development of germfree and gnotobiotic animal models and their application in the scientific field to unravel mechanisms underlying host–microbe interactions and distinct diseases. Strictly germfree animals are raised in isolators and not colonized by any organism at all. The germfree state is continuously maintained by birth, raising, housing and breeding under strict sterile conditions. However, isolator raised germfree mice are exposed to a stressful environment and exert an underdeveloped immune system. To circumvent these physiological disadvantages depletion of the bacterial microbiota in conventionally raised and housed mice by antibiotic treatment has become an alternative approach. While fungi and parasites are not affected by antibiosis, the bacterial microbiota in these "secondary abiotic mice" have been shown to be virtually eradicated. Recolonization of isolator raised germfree animals or secondary abiotic mice results in a gnotobiotic state. Both, germfree and gnotobiotic mice have been successfully used to investigate biological functions of the conventional microbiota in health and disease. Particularly for the development of novel clinical applications germfree mice are widely used tools, as summarized in this review further focusing on the modulation of bacterial microbiota in laboratory mice to better mimic conditions in the human host.

Keywords: isolator-raised germfree animals, gnotobiotic animals, secondary abiotic mice, commensal gut microbiota, *in vivo* model, bacteria/pathogen-host interaction, inflammatory bowel diseases, gut–brain axis

Introduction

In the natural environment macroorganisms including plants and animals are colonized by microorganisms forming a specific ecological community of commensal, symbiotic and pathogenic microorganisms referred to as 'microbiota' herein [1, 2]. The indigenous physiological microbiota of mammals consist of bacteria, fungi and protozoa colonizing inner and outer surfaces. In addition to permanently colonizing species, a transient microbiota is present which is characterized by a huge number of environmental microorganisms only temporarily colonizing the host. The composition of the constant and transient microbiota depends on the respective body region. The microbiota composition on the inner and outer surfaces of the host is extremely variable. The dominating species on the skin, in the oral cavity or in the gastrointestinal tract differ fundamentally [3]. Altogether the normal bacterial microbiota of vertebrates may involve several hundreds of different species and genera. It is of note that many of them are still undescribed or poorly characterized. This particularly concerns the microbial composition of the microbiota in the intestinal tract [4, 5]. In healthy humans the esophagus, the stomach and the upper duodenum are not continuously colonized by microorgamisms due to antibacterial effects exerted by gastric juice, digestive enzymes, bile, mucins and defensins. Only the extremely host-adapted *Helicobacter pylori* bacteria are able to permanently colonize the human stomach, and this may result in chronic inflammation subsequent leading to serious sequelae in many cases [3–5]. However, in healthy individuals the lower parts of the gastrointestinal tract are

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colonized by lactobacilli, enterobacteria and enterococci dominating the microbiota in the duodenum, jejunum and ileum. These facultative aerobic bacteria metabolize sugars and increase in concentrations to distal locations in the terminal ileum. Deeper in the intestines the facultative aerobic species are replaced by obligate anaerobes metabolizing complex polysugars and amino acids. With a total of 96% of the bacterial species *Bacteroidetes* (mainly *Bacteroides* spp.) and *Firmicutes* (mainly clostridia) dominate the microbiota composition in the terminal ileum and the colon. The remaining species belong to facultative anaerobic genera such as *Escherichia, Proteus, Klebsiella, Enterobacter, Enterococcus* among others. In other body regions the bacterial microbiota is less complex and bacterial loads are significantly lower [3–5].

Preconditions for experimental application of germfree animal models

The broad variety and high load of the intestinal microbiota in vertebrates impact the reproducibility and scientific significance of experimental results obtained from animal models in general. Moreover, it is well-established that the constant conventional microbiota promote endogenous infections particularly in immune-compromised hosts. In order to ensure the reproducibility of scientific results in animal facilities worldwide, the specific pathogen free (SPF) status of experimental animal models plays an essential role not only in animal welfare and hygiene, but also in good scientific practice [6]. Given that breeding of germfree animals is elaborated and laborious, the vast majority of animal experiments are performed with SPF mice worldwide. These animals harbor a complex autochthonous microbiota defined as being free of particular pathogens. This ensures that experimental results are not falsified by specific metabolic characteristics of different microbiota compositions or by infectious diseases. The SPF hygiene status of animals is preferentially realized by a barrier concept. Rooms for animal husbandry are equipped with high-efficiency particulate air filters. All cage materials including food are sterilized. The access to the animal facility is restricted to authorized personnel using disinfection shower and protective clothing. An additional validation of the SPF hygiene level can be achieved by applying special micro-isolator cages such as filter top cages and individual ventilated cages (IVCs). Compared to conventional animals all these measures lead to an increased life expectation and an enhanced rearing power [7, 8].

However, research focusing on biological microbiota functions creates the essential need for animals that are free of either autochthonous or pathogenic bacteria. The animal facilities focusing on breeding and handling of animals under sterile conditions have therefore to be equipped in minimum with sterilization of cage materials in autoclaves and sterile filtration of the air supplies [7, 8].

History of germfree animals

Biological consequences of the molecular interactions between the microbiota and vertebrates can be useful, harmful, indifferent, beneficial or even essential for the host. In 1885, Louis Pasteur formulated the hypothesis, that interspecies relationships with microorganisms are essential for maintaining homeostasis in higher plants and vertebrates. He propagated that essential life functions of macroorganisms depend on the symbiosis with microorganisms meaning that life in a sterile environment is *"per se"* impossible [9]. However, during the turn of the 19th century and in the following years other researchers tried to address this fundamental hypothesis experimentally. In these times, it was already possible to breed and rear gnotobiotic guinea pigs and chicken in sterile environments until the age of several weeks [10–17]. These experiments were performed in a technically correct way, and during the World War I researchers used sterile environments realized in isolators as technical tools to substantiate that life in a germfree environment is, in fact, possible. Most remarkably, germfree goats survived in sterile isolators until the age of 35 days [18]. Loss of feed-derived essential vitamins following heat sterilization, however, was limiting experimental success. In the following years, research with germfree and gnotobiotic animals was continued in Sweden, Japan and in the United States of America [19–22]. In 1946, Gustafsson was the first investigator, who achieved the successful rearing of germfree rats delivered by cesarean section [23]. The isolator techniques had substantially improved and animal nutrition was supplemented with synthetic vitamins until then [24]. Production of the first germfree mice started in 1959 and it was subsequently possible to breed rats, monkeys, goats, cats and other vertebrates in sterile isolators [24, 25]. Today, most of the professional animal facilities are equipped with sterile isolators in minimum to generate a pool of germfree rodents for hygiene purposes. Huge animal producing companies such as Charles River Laboratories, Inc. (an U.S. corporation specialized in a variety of pre-clinical and clinical laboratory services for the pharmaceutical, medical device and biotechnology industries) still use the germfree techniques developed in the early 20th century [26]. In the sterile environments of the isolators offsprings generated by embryo transfers or implanting are born by germfree foster mothers.

The Severe Combined Immuno Deficiency (SCID) mouse model is a good example for the use of sterile techniques in animal breeding. These mice were commonly used with great success during the 20th century in tumor biology, xenograft research, transplantation and immunobiology [27]. Remarkably, SCID mice suffer from a severe combined immunodeficiency affecting both T and B lymphocytes resulting in susceptibility to infection and other diseases including an altered social behavior and autism spectrum disorder [28, 29]. For successful rearing, it is essential to continuously rederive the original breed stock by aseptic standard caesarean hysterectomy followed by propagation in a germfree environment. The germfree sta-

tus of these animals has been maintained since that time and is verified serologically on a daily basis [27]. Apart from breeding, maintenance and shipping of SCID mice is extremely laborious. The exposure of these immune-compromized animals to potentially pathogenic organisms must be strictly prevented because of the underlying severe immunodeficiency. All housing systems that can be used for rearing and maintaining SCID mice require sterilization of feed, bedding, water and all cage components. The use of filter-top or individually ventilated caging systems, coupled with laminar flow work and change stations, and strict adherence to aseptic techniques for handling these animals is a common method of ethical animal husbandry of SCID mice in the research environment. SCID mice produced by commercial companies grow up in flexible film and semi-rigid isolators using certified aseptic handling protocols. For shipping, animals are transferred from isolators into sterile laminar flow workstations by the use of aseptic transfer techniques and placed into small isolator-like containers that have been pre-sterilized (Gnotosafe™ shippers) [27]. Taken together, all these technical measures and efforts make breeding, handling and shipping of germfree mice extravagantly expensive.

Characteristic features of germfree mice reared in isolators

Laboratory mice propagated permanently in isolators under strict germfree conditions develop specific anatomical, physiological and behavioral abnormities. The cecum, for instance, is heavily enlarged and the intestinal wall hypotrophic [24, 25]. It is well-established that the differentiation of Peyer's patches and enterocytes within the gut lining is triggered by bacterial molecules including short chain fatty acids (SCFA) such as butyrate. The fecal consistence is soft and shows an altered redox potential as compared to conventional animals [24, 25]. It is thus not surprising that the lifestyle in the sterile isolator environment leads to serious health impairments in animals, as mentioned at the early beginnings by Pasteur (see above). The enlarged cecum may favor the development of mechanical ileus and hence, decreases live expectancy of germfree animals in general. The specific husbandry in isolators with a continuous airflow render animals anxious and destressed. Besides an underdeveloped immune system, mice have a high risk to suffer from vitamin deficiencies caused by the absence of vitamin K and vitamin B12 producing bacteria inhabiting the microbiota. Deficiencies in both vitamins are known to cause severe health problems such as anemia and bleeding [30–33].

The commensal microbiota prevents mice from pathogenic colonization by competing for attachment sites or for essential nutrients within ecological niches. The commensal bacteria produce a variety of substances ranging from relatively non-specific fatty acids and peroxides to highly specific bacteriocins, which inhibit or even kill invading pathogens [30]. The poorly developed lymphatic organs in germfree mice indicate that molecules produced by the normal microbiota are essential for the proper development of the immune system. Upon experimental recolonization, germfree mice display an increased susceptibility to infections caused by bacteria, fungi, viruses and parasites. Finally, due to the low differentiation and stimulation of B cell populations germfree mice lack "natural antibodies", and innate as well as adaptive immune responses to bacterial infection are severely compromized.

As mentioned above, the caecum of germfree animals is enlarged, thin-walled, and fluid-filled if compared to conventional counterparts. Also, based on the absence of immunological differentiation and stimulation, the intestinal lymphatic tissues of germfree animals are only poorly developed. Taken together, these specific features of germfree animals are of vital importance for the scientific significance and reproducibility of experimental results. Based on this knowledge it becomes obvious that the comparison of results from germfree and conventionally colonized (SPF) mice is problematic *"per se"*. This point of view is further supported by studies reporting essential differences of disease severities and outcomes in conventional versus germfree mice [30–35]. Thus, valid scientific results from studies focusing on differences between germfree and conventional mice essentially depend on the definition and use of correct and scientifically valid controls.

Simplified gnotobiotic mice models

In order to compensate for the specific physiological and anatomical characteristics observed in isolator-raised germfree animals and to protect animals from morphological and functional abnormities and resulting diseases mentioned above, germfree animals are associated with defined microorganisms by secondary recolonization. The resulting gnotobiotic ("gnotos bios", a Greek expression for "defined colonized") mice combine the efforts of a developed immune system, a normal gut anatomy, a cecum of regular size, together with the experimental benefit of the well-defined intestinal bacterial species composition. The need for simplified models to investigate host–microbe interactions has previously been recognized and is already established. Gnotobiotic mice co-associated with *Bacteroides thetaiotaomicron* and *Eubacterium rectale,* for instance, were successfully used to investigate the niche specialization of both species as well as their interactions with each other and the host [36]. Other studies investigated the responses of a simplified microbiota consisting of *Lactobacillus johnsonii, Bifidobacterium longum* and *Escherichia coli,* in gnotobiotic mice to the addition of probiotic strains [37]. Another simplified microbiota mimicking conditions in human infants was successfully established in gnotobiotic mice [38]. Furthermore, gnotobiotic rats were associated with a distinct mixture of bacterial species dominant in the gut of humans in order to investigate metabolic properties exerted by the microbiota in

obesity [38]. The bacterial mixtures consisted of *Anaerostipes caccae, Bacteroides thetaiotaomicron, Bifidobacterium longum, Blautia producta, Clostridium ramosum, Escherichia coli* and *Lactobacillus plantarum* without (SIHUMI) or with *Clostridium butyricum* (SIHUMIx) [39]. The functionally most important biochemical pathways of the human gut microbiota were established by reassociation of germfree rats with those bacteria. Thus, this "artificial" bacterial community is somehow representative for a complex conventional microbiota. Most importantly, the total bacterial counts and numbers of single strains within the feces of the simplified human microbiota (SIHUMI)-associated rats did not differ from human feces, whereas the SCFA content was, however, 90% lower than in human feces. Interestingly, the corresponding SCFA concentrations were similar to those reported for human infants, whose microbial diversity is rather low as compared to adults. Furthermore, the response of the SIHUMI microbiota upon selected dietary interventions was investigated. The results revealed that feeding a fiber-free diet to SIHUMI rats caused a significant decline in bacterial numbers as compared to a fiber-rich standard chow [39]. This is in agreement with conditions reported for the human microbiota. The microbial community established in gnotobiotic rodents can easily be extended by adding strains with distinct metabolic features not covered by the SIHUMI microbiota. Importantly, one striking advantage of the SIHUMI model is its stability and reproducibility.

In conclusion, gnotobiotic rodents harboring a defined intestinal microbiota can serve as useful experimental model systems for investigating host–microbiota and bacteria– (including pathogen-) microbiota interactions.

Generation of secondary abiotic mice by broad-spectrum antibiotic treatment

To circumvent technical difficulties of rearing germfree mice in isolators and to avoid the high expenses associated with germfree technology, scientists tried to find more convenient and flexible alternatives to eradicate the commensal microbiota in laboratory mice. The method of choice that is now used worldwide is the oral or intravenous administration of antibiotic compounds [40]. It is well-documented that antibiotic treatment decreases the gut bacterial loads in the gastrointestinal tract and induces changes in the microbiota composition termed dysbiosis [41–43]. Decreased loads of aerobic and anaerobic bacteria were reported in obese mice after treatment with norfloxacin and ampicillin, for instance [44]. Treatment of SPF mice with vancomycin increased the prevalences of *Proteobacteria, Tenericutes* and *lactobacilli* and decreased the bacteria loads of the *Lachnospiraceae* family [45].

In order to virtually completely eradicate the intestinal microbiota in conventionally raised, housed and colonized mice we and others administer a cocktail of five different antibiotic compounds perorally to SPF mice of different age (depending on the disease model of interest). To achieve this, mice are transferred to sterile cages and treated by adding a mix of ampicillin plus sulbactam (1 g/L), vancomycin (500 mg/L), ciprofloxacin (200 mg/L), imipenem (250 mg/L) and metronidazol (1 g/L) to the drinking water *ad libitum* for 6–8 weeks [46]. The intestinal colonization status of the mice is controlled once a week by highly sensitive cultural analysis of fecal samples (i.e. incubating in brain heart infusion and thioglycollate enrichment broths for at least 7 days). As early as three weeks of broad-spectrum antibiotic treatment quality controls indicate complete eradication of the intestinal microbiota, as demonstrated by negative results from both, culture and molecular detection of bacteria using real-time PCR targeting of bacterial 16S rRNA genes. This quality control is relatively cheap and easy to perform. Most strikingly, numbers of 16S rRNA gene copies detected in fecal samples do not differ from those detected in autoclaved food pellets. To avoid contaminations, mice are continuously kept in a sterile environment (autoclaved food and drinking water, sterile filtered antibiotic cocktail) and handled under strict aseptic conditions. Given that eradication or modulation of the microbiota by antibiotic treatment results in a loss of colonization resistance against exogenous microorganisms, these secondary abiotic (gnotobiotic) mice can subsequently be mono-associated with bacterial commensals, pathogens, fungi, parasites or a complex microbiota derived from humans or mice (or a selected combination of respective components) in order to elucidate the mechanisms underlying bacterial (pathogenic) / microbiota / host interactions.

Applications of germfree rodent models in biomedical research

The role of the microbiota in human diseases

Results from a huge number of scientific investigations revealed a general impact of the commensal microbiota in host metabolism and in metabolic diseases including obesity, diabetes mellitus, cardiovascular disorders, kidney stones, or cancer [47–49]. Even the dysbiosis in the gut or the respiratory tract has been linked to alterations in immune responses and to disease development [50]. Also the severity of malaria seems to be modulated by the composition of the gut microbiota [51], and in patients with human immunodeficiency virus 1 (HIV-1) infection a linkage between alterations in the gut microbiota and the increase in microbial translocation and systemic inflammation could be confirmed [52]. In addition, researchers were able to determine a link between the intestinal microbiome and hepatitis B virus infection given that the gut microbiota composition was shown in a hydrodynamic transfection mouse model to contribute to the age dependence of hepatitis B virus clearance and immunity [53].

Germfree animal models were thus successfully used in the last decades to investigate the association of various

diseases with the microbiota composition and metabolism of the host [54]. In order to unravel the underlying molecular mechanisms it is essential to gain a deeper understanding of the distinct structures of the respective microbiota in health and disease. The comparisons of very recently sequenced fecal metagenomes of human individuals from four countries with previously published data sets revealed the individual dominance of three bacterial genera, *Bacteroides, Prevotella* and *Ruminococcus* in humans that were used to define three distinct enterotypes [55]. The phylogenetic composition of the recently sequenced samples confirmed that the *Firmicutes* and *Bacteroidetes* phyla constitute the vast majority of the dominant human gut microbiota [56]. *Bacteroides* was the most abundant, but also most variable genus across samples, which is well in line with previous observations [57, 58]. In general, enterotypes do not seem to differ in functional richness, and virtually none of the ascertained host characteristics including gender, age, body mass index or nationality correlated with respective enterotypes. Notably enterotypes are not restricted to humans, but can also be defined for animals [55].

Xenobiotic metabolism

The xenobiotic metabolism is defined as a biochemical modification of pharmaceutical substances or xenobiotics by living organisms, usually by specialized enzymatic systems. Through this metabolism lipophilic chemical compounds are converted into more readily excreted hydrophilic products, for instance. The gut microbiota plays an important role in xenobiotic metabolism [59–61] and the intestinal microbiota has been further shown to modulate the xenobiotic metabolism in the liver [62, 63]. A very recent study investigated the effect of the gut microbiota on the ontogeny of drug processing genes (DPGs) in the liver [64]. The researchers collected the livers of germfree and conventional mice between 1 day and 90 days of age. By performing RNA sequencing, quantitative real-time PCR, western blot and liquid chromatography-tandem mass spectrometry (LC-MS/MS) four specific gene patterns could be identified. One of them is specific for conventionally raised mice, whereas another is enriched predominantly in livers of 15-day-old mice, among which a special sterol-efflux transporter is down-regulated in germfree animals. The application of germfree mice in this study for the first time unraveled the effect of intestinal microbiota on the ontogeny of DPGs during development, and thus, did greatly improve our understanding of "bug-drug interactions" [64].

Obesity

Different studies in both, mice and men demonstrated that obesity is associated with an altered gut microbiota composition and microbial ecology, characterized by a lowered microbial diversity, decreased levels of *Bacteroidetes* and increased abundances of *Firmicutes* [65–69]. Distinct shifts in the microbial composition are associated with alterations in the gut microbial metagenome. Notably, bacterial genes involved in energy harvest are enriched in obesity [70]. Investigations on the influence of the nutrition on obesity in germfree and conventional mice revealed that germfree mice display reduced food intake and are resistant to diet-induced obesity [71, 72]. Furthermore, germfree mice consume fewer calories, excrete more fecal lipids, and gain significantly less weight as compared to conventional counterparts. The analysis of associations between obesity and human enterotypes did, however, not reveal any correlation between body mass index and the *Firmicutes/Bacteroidetes* ratio. Thus, the relationships between distinct microbiota compositions and obesity are still under debate and await further investigation [55].

Cancer

Several lines of evidence exist that intestinal dysbiosis observed in patients or animal models is associated with cancer, and that the microbiota is causatively involved in cancerogenesis [73–75]. For several forms of cancer disease promoting effects of the bacterial microbiota have been reported in mice and men [73]. Detailed studies revealed the de-/increase of distinct bacterial species in different forms of cancer like colorectal carcinoma [76–84]. For various organs including skin, colon, liver, breast and lungs, studies in germfree animals have revealed tumor-promoting effects of the microbiota in spontaneous, genetically induced, and carcinogen induced cancer types [85]. Populations of the phyla *Prevotella* as well as *Ruminococcus* spp. and *Pseudobutyrivibrio ruminis* were found to be decreased in patients with colorectal cancer, whereas *Acidaminobacter, Phascolarctobacterium, Citrobacter farmeri* and *Akkermansia mucinophila* abundances were increased [83]. Significant shifts in the microbiota were associated with colon tumorigenesis in an inflammation-based murine model of tumorigenesis as shown in both, SPF and germfree mice [84]. Cancer was induced by intraperitoneal injection of the chemical carcinogen azoxymethane followed by a 2% dextran sodium sulphate (DSS) treatment. Results revealed dramatic shifts in the relative abundances of bacterial populations, including those related to the genus *Bacteroides* that were associated with tumorigenesis. Moreover unclassified genera within the family *Porphyromonadaceae* declined during cancer development. The authors hypothesized that these bacteria may protect from cancerogenesis and are thus of basic importance for intestinal health [84]. Other studies indicate that different species of the genus *Helicobacter* play major roles in development of distinct gastrointestinal carcinoma types [86].

It could be further demonstrated that tumor-bearing mice that were either germfree or had been subjected to antibiotic compounds directed against Gram-positive bacteria displayed a decrease in T helper cell (Th)-17 responses

and tumors that were resistant to cyclophosphamide [87]. In addition cyclophosphamide treatment altered the composition of the gut microbiota in the small intestine of mice and induced the translocation of distinct Gram-positive bacterial species into secondary lymphoid organs, where these bacteria stimulated the generation of a specific subset of "pathogenic" T helper 17 (pTh17) cells and memory Th1 immune responses [87]. These results provide strong evidence that distinct gut bacterial species may contribute to anti-cancer immune responses [88]. Also probiotic compounds that modulate the composition of the gut microbiota are thought to suppress cancer growth in mice. In a hepatocellular carcinoma model, for instance, probiotic feeding of mice resulted in a 40% reduction of tumor size and weight [89].

Inflammatory bowel diseases

Results from many scientific reports suggest the involvement of a perturbed intestinal microbiota (i.e. dysbiosis) and dysregulated immune responses in the multifactorial etiology of inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis, representing chronic inflammatory processes within the gastrointestinal tract with acute episodes (relapses) and remission phases [90, 91]. Whereas Crohn's disease can virtually affect the entire gastrointestinal tract with the terminal ileum as predilection site, the inflammatory process involves exclusively the colon in ulcerative colitis [92–94]. In healthy individuals, the colonic mucosal surface is thought to be kept virtually sterile by the presence of a continuous adherent mucus layer and the abundance of antimicrobial peptides including defensins and glycoproteins such as mucins (e.g. MUC2) [95]. In the small intestine, however, the mucus layer is discontinuous facilitating nutrient absorption [96]. In the case of active ulcerative colitis the adherent mucus layer almost completely disappears, allowing for direct contact and interaction between intestinal bacteria and the surface epithelium [96]. The loss of intestinal barrier integrity and function associated with ulcerative colitis subsequently results in an increased uptake of bacterial antigens across the gut mucosa. These antigens can then interact with Toll-like receptors (TLRs) on epithelial cells or NOD-like receptors on dendritic cells, thus triggering the activation of innate and adaptive immune responses [97]. Both, quantitative and qualitative changes in the fecal microbiota have been reported in IBD, particularly a reduced biodiversity in comparison to healthy controls [98]. The gastrointestinal microbiome of healthy humans is predominated by members of the phyla *Firmicutes* and *Bacteroidetes,* whereas IBD is associated with an imbalance mainly characterized by a lower proportion of *Firmicutes,* as well as by increased bacteria from the *Proteobacteria* phylum [99, 100]. In search of the microbiota driven mechanisms underlying IBD, several animal models of intestinal inflammation have been developed including chemically induced models, adoptive transfer models and genetically modified models [101]. The two most widely used chemicals to induce IBD in animal models are 2,4,6-trinitrobenzene sulphonic acid (TNBS) and dextran sodium sulphate (DSS) [102]. Both agents lead to an acute destruction of the intestinal barrier, whereas the immunopathological mechanisms induced by TNBS and DSS more closely mimic Crohn's disease and ulcerative colitis, respectively [103]. Another way of investigating the IBD underlying mechanisms is the application of genetically modified models such as gene knockouts and transgenic animals including IL-10 gene-deficient mice (see below), multi-drug resistant gene-deficient mice, and $Nod2^{2939iC}$ mice [104]. In most of the cases mice with gene-knockouts are integrated into other animal models like the T cell transfer model, for instance, among others. Some of the gene-knockout mice develop spontaneous colitis as a result of the genetic modification, whereas others can require additional intervention to trigger the onset of inflammation [105].

Large intestinal inflammation models

The most intriguing advances in the understanding of bacterial contributions to IBD pathophysiology have been developed by the use of germfree mice [106–109]. Seminal studies unraveled that germfree IL-$10^{-/-}$ mice do not develop enterocolitis, in contrast to counterparts kept under SPF conditions or germfree mice inoculated with an SPF microbiota after birth [110]. Furthermore, extent of the immunopathological process differed depending on the age at which the mice had been inoculated with the respective microbiota. This finding highlighted for the first time that the intestinal microbiota is essentially involved in the initiation and propagation of intestinal inflammation [111]. The impact of individual bacterial species or distinct T cell populations in the course of IBD development was further successfully investigated in murine adoptive transfer models. Colitis can be induced by the transfer of T cells or immune tissue from donors into histocompatible adoptive hosts and is characterized by transmural inflammation, epithelial cell hyperplasia, polymorphonuclear and mononuclear leukocyte infiltration, crypt abscesses, and epithelial cell erosions [102]. In the adoptive transfer model the onset and severity of intestinal disease are precisely "synchronized", and this condition is furthermore ideal to study the role of T regulatory (Treg) cells in suppressing or limiting the onset and/or perpetuation of intestinal inflammation [112]. An elegant study evaluated the clinical course and the histology after transfer of $CD4^+CD62L^+$ lymphocytes from germfree and conventionally housed donor mice into SCID recipients [113]. Results of this study revealed that animals that had received cells from germfree donors developed an earlier onset of colitis as compared to mice reconstituted with lymphocytes from conventionally housed animals. Additionally, $CD4^+CD62L^-$ cells from germfree mice were not able to abrogate colitis

induced by co-transfer of $CD4^+CD62L^+$ lymphocytes, whereas $CD4^+CD62L^-$ T cells from normal mice ameliorated disease. IL-10 production after priming by dendritic cells suggests the presence of Treg cells within the $CD4^+CD62L^+$ lymphocyte subset derived from conventional housed mice and assumes a lack of Treg cells within germfree mice. These results thus indicate that bacterial antigens are crucial for the generation and/or expansion of Treg cells in a healthy individual. Hence, bacterial colonization was shown to be crucial for maintaining the immunological balance within the vertebrate host [113]. Whether the role of T cell spontaneous proliferation driven by microbiota is due to microbiota antigenic stimulation or its interaction with host innate cells, and how microbiota-driven T cell spontaneous proliferation contributes to the induction of colitis is still unclear and warrants further investigation [114].

Small intestinal inflammation models

To date only very few animal models for ileal inflammation exist [115]. Oral infection of susceptible mouse strains with a high dose (i.e. 100 cysts) of the protozoan *Toxoplasma gondii* triggers a Th1-type dependent acute inflammation in the small intestines resembling key features of acute epidoses in ileal Crohn's disease [46, 116]. Remarkably, secondary abiotic mice were protected from disease development, whereas reassociation of gnotobiotic mice with a complex SPF microbiota resulted in *T. gondii* induced ileitis comparable to infected SPF controls [46]. Ileitis development was accompanied by a marked overgrowth of the inflamed ileal lumen with small intestinal commensal Gram-negative species such as *E. coli* and *Bacteroides / Prevotella* spp. [46, 117]. Recolonization studies with respective Gram-negative strains in secondary abiotic mice further revealed that initiation and perpetuation of *T. gondii* induced ileitis was depending on TLR-4 dependent signaling of lipopolysaccharide (LPS) derived from the overgrowing microbiota components [117]. Notably, similar shifts in the microbiota composition towards Gram-negative species have been reported in human IBD [118–121]. We could further show that upon peroral recolonization of secondary abiotic mice and subsequent ileitis induction, viable *E. coli* and *Bacteroides/ Prevotella* spp. were able to translocate to subepithelial tissue sites and to mesenteric lymph nodes subsequently further exacerbating the inflammatory scenario [46, 117].

These intriguing results further underlines the pivotal role of the intestinal gut microbiota in initiation and perpetuation of acute inflammatory processes in the intestinal tract.

Another ileitis model makes use of a subline of the senescence accelerated mouse (SAM) P1/Yit strain which develops spontaneous enteric inflammation even if propagated under SPF conditions [122]. Most importantly, germfree (SAM) P1/Yit mice showed no signs of intestinal inflammatory disease that was seen under SPF conditions. This again further supports the impact of the intestinal microbiota in the pathogenesis of ileitis.

Another elegant study assessed the impact of intestinal bacterial communities in a spontaneous model of chronic Crohn's disease-like ileitis [123]. The $TNF^{deltaARE}$ mice used in this disease model carry a deletion in the tumor necrosis factor (TNF) AU-rich (adenosin-uracil) elements (ARE) leading to transmural inflammation in the distal ileum after colonization with caecal microbiota from SPF mice [123]. By the use of antibiotics treated mice, mice housed at different hygienic conditions and germfree mice the authors dissected the relationship between microbiota changes and ileitis development in the $TNF^{deltaARE}$ model. Germfree $TNF^{deltaARE}$ mice were protected from intestinal inflammation, thereby demonstrating the essential role of microbial triggers in a model that shares some clinical features with human Crohn's disease. In contrast, severe ileitis developing under SPF conditions was characterized by a loss of antimicrobial peptides and dysbiosis. Analysis of the microbiota composition by high-throughput 16S rRNA gene sequencing unraveled that *Clostridiales* and *Porphyromonadacaeae* (order of *Bacteroidales*) increased and decreased, respectively, during ileitis. The authors point out that members of *Clostridiales* were found in lower abundance in patients with Crohn's disease, while they were increased in $TNF^{deltaARE}$ ileitis [123]. Hence, in accordance with results obtained from the murine *T. gondii* ileitis model this study provided further evidence for disease-related causality of microbiota, bacterial dysbiosis and experimental ileitis.

Bacterial enteritis

C. difficile

During the last decades gastrointestinal pathogens were extensively investigated in germfree or gnotobiotic animal models [124, 125]. The rod-shaped Gram-positive bacterium *Clostridium difficile* for instance, induces serious diarrheal disorders in vertebrate hosts including humans [126–128], given that its enterotoxins cause enterocyte apoptosis and inflammation in experimental models and infected (and intoxicated) patients [129], and may also have the ability to reactivate IBD [130]. Different gnotobiotic mouse models were successfully evaluated for the study of interactions between *C. difficile* and the indigenous cecal microbiota [131]. Results revealed that *C. difficile* infection was strongly suppressed in gnotobiotic mice that were recolonized by a complex microbiota derived from hamsters [131]. In another *C. difficile* infection study could be shown that a single component of the murine gut microbiota, a member of the family *Lachnospiraceae,* is able to partially restore colonization resistance against *C. difficile* in germfree mice [132].

Candida albicans

Also the fungus *Candida albicans* has been shown to trigger the immunopathogenesis of IBD [133, 134]. Oral administration of *C. albicans* resulted in crop infections in germfree chicks, whereas conventional chicks were not stably infected [135], which was underlined by results in germfree and SPF mice [136]. In another murine study one susceptible strain of ex-germfree mice showed considerable loss of body weight, preponderance of hyphal colonization of the intestine, and evidence of infection in the stomach, which is in line with the crop infection of *C. albicans* challenged ex-germfree chickens [137]. Conventional mice, however, were not clinically affected and displayed colonization of their gut mainly by the yeast form of *C. albicans*. The findings suggest that the normal microbiota in susceptible hosts, maintained on adequate diets, will prevent the morphogenesis of *C. albicans* to hyphal forms and thereby allow only mild infections with the organism. In this protective action only certain components of the microbiota, such as *E. coli,* might be involved. In nutritionally deficient conventional animals, the picture is considerably changed by the inability of the host to prevent the systemic invasion by yeast cells and perhaps other pathogens [124].

Listeria monocytogenes

Very similar results were obtained by infection studies with the genus *Listeria*. The major human enteric pathogen, *L. monocytogenes,* causes listeriosis, a serious infection caused by contaminated food. Recent studies point towards *L. monocytogenes* as an infectious factor in IBD etiopathology [138]. Remarkably, germfree mice were shown to be more susceptibility to *Listeria* infections than conventionally colonized controls [139].

Bacteroides fragilis

Bacteroides fragilis comprise other bacterial species associated with IBD development [140, 141]. In the majority of adults, these obligate anaerobic bacteria form part of the colonic commensal microbiota [142]. A subset of *B. fragilis* strains, termed enterotoxigenic *B. fragilis* (ETBF), however, secretes a proinflammatory zinc-dependent metalloprotease toxin that is suspected to cause diarrhea in children and adults. ETBF has been further identified in up to 19% of patients suffering from clinically active IBD [143]. In animal studies, inoculation with ETBF was associated with colitis of severe inflammation and with overproduction of IL-17, a central regulator of inflammation and autoimmunity [144]. Further studies explored the administration of DSS to germfree IL-10$^{-/-}$ mice after monocolonization with ETBF negative *B. fragilis* [145]. The clinical observations confirmed that *B. fragilis* did not cause any clinical signs of intestinal inflammation such as rectal bleeding or diarrhea. This provided first evidence of the protective effects of *B. fragilis* in the germfree DSS mouse model and might provide a novel therapeutic approach for IBD [145].

Mycobacteria

For some other species such as *Helicobacter* spp. [146–148] and *Mycobacterium avium* subspecies *paratuberculosis* a direct linkage to IBD is not clear so far [149–151]. Mycobacteria can cause chronic granulomatous enterocolitis in ruminants with features similar to IBD. In Crohn's disease patients, mycobacteria have been isolated in higher frequencies, although the evidence for the presence of this pathogen in these patients is inconsistent at best, with studies reporting detection rates ranging from 0 to 100% [152, 153]. Clinical studies of triple antimycobacterial therapy, which would be expected to clear *Mycobacterium avium* subspecies *paratuberculosis* infections, have failed to show sustained responses, further suggesting that this pathogen might rather not be involved in the initiation or progression of Crohn's disease [154].

Enterobacteria (Salmonella, Klebsiella)

Germfree mouse models can further be used to elucidate immunopathological properties of colitis caused by the common intestinal pathogen *Salmonella enterica* serovar Typhimurium, *Salmonella* belong to the genus of the rod-shaped Gram-negative bacteria of the *Enterobacteriaceae* family and are suspected to contribute to IBD induction [155]. Conventional mice are inherently protected from *Salmonella* serovar Typhimurium colonization and enterocolitis by physiological colonization resistance that is mediated by the resident intestinal microbiota [156]. Upon infection, SPF mice pre-treated with antibiotics such as streptomycin or germfree mice, however, developed distinct immunopathological features of enteritis. Providing comparable results, the two different applied murine infection models for *Salmonella* serovar Typhimurium induced colitis have their particular advantages. For instance, SPF mice allow for the use of a wide variety of knockout and transgenic strains obtained from SPF certified facilities, whereas germfree mice guarantee that a bacterium/pathogen of interest (*Salmonella* serovar Typhimurium in this case) is the only bacterium present in the intestine and that there is no contamination by remains of the indigenous microbiota [156].

The major advantages in using germfree models together with SPF mice were also demonstrated by the study of non-motile rod-shaped enterobacteria such as *Klebsiella. Klebsiella* species may cause a wide range of disease states including pneumonia, urinary tract infections, septicemia, meningitis, diarrhea, and soft tissue infections [157]. Germfree mouse models could be used in this context to compare bacterial and viral infections (for example with cytomegalovirus) [158].

There are additional bacterial pathogens causing gastrointestinal infections (see [159]). For example *Yersinia* spp. [160], *Fusobacterium* spp. [161], *Methanosphaera stadtmanae* [162], Norovirus [163] and *Campylobacter* spp., such as *Campylobacter jejuni* and *Campylobacter*

concisus [155, 164, 165] were successfully analyzed in germfree and gnotobiotic mouse models [166–169].

Campylobacteriosis

Campylobacter jejuni are microaerophilic rod-shaped Gram-negative bacteria [170, 171]. Already for more than a century the awareness of the public health implications of *Campylobacter* infections has evolved [172]. *Campylobacter* was identified as a human diarrheal pathogen in 1973 by the development of different selective growth media [173]. Over the decades *Campylobacter* infections became a leading cause of bacterial gastroenteritis reported in the United States with more than 2.4 million infections per year [174]. The bacteria are widely distributed and found in most warm-blooded domestic and wild animals. Many of those infected hosts do not display any symptoms, whereas manifested infections are self-limiting in the vast majority of cases. Rare complications include post-infectious sequelae such as Guillain–Barré syndrome, Reiter's syndrome, reactive polyarthritis or irritable bowel syndrome [172, 173, 175]. In the last years different studies proved that different strains of the same species often display significant diversity within their repertoire of virulence factors [176, 177] and metabolic properties [178–182]. In addition, *C. jejuni* isolates are equipped with different sets of chemoreceptor genes that respond to a variety of potential nutrients [183, 184].

For quite many years, appropriate *C. jejuni* infection models were missing [185–190]. First trials were undertaken with pigs [191] and piglets [192], ferrets [193] and primates [194], but all these models showed several disadvantages in convenience, cost and disease reproducibility. Also the chicken model was insufficient concerning the robustness for *C. jejuni* colonization and immunopathogenesis studies [190]. Mice with a defined, limited gut microbiota led to efficient establishment and reproducible colonization at high levels resulting in mild inflammation of the large intestines followed by pathogenic clearance after several weeks, whereas SCID mice with limited gut microbiota remained persistently colonized at high levels and exhibited severe intestinal inflammation [195]. Another promising murine model for colonization applies mice deficient in the myeloid differentiation factor 88 (MyD88), an adaptor protein essentially required for signaling of most TLRs. Finally, a lot experimental effort was undertaken to determine whether the observed colonization resistance of mice against *C. jejuni* is caused by the conventional murine microbiota. To address this, it was inalienable to find a suitable novel murine *C. jejuni* infection model with standardized results. The gnotobiotic mouse model seems to fit in here for the use in the research field of molecular mechanisms underlying human campylobacteriosis. With the possibility to modify or even deplete the intestinal microbiota from mice *C. jejuni* has been shown to be able to colonize at high loads alongside the entire gastrointestinal tract and to induce pro-inflammatory immune responses in the colon upon murine antibiotic treatment [169, 196]. Our research group proved stable colonization of three different *C. jejuni* strains (namely reference strains ATCC 43431, 81–176 and B2) in gnotobiotic, but not SPF mice [169]. The results confirmed that conventional mice bred and maintained under conventional SPF conditions display a strong colonization resistance against the pathogen, as within 48 hours after infection *C. jejuni* was already expelled from the gastrointestinal tract. Strikingly, colonization resistance was completely abrogated in gnotobiotic (i.e. secondary abiotic) mice generated by antibiotic treatment, and all three *C. jejuni* strains could stably colonize the intestines at high loads [169]. Remarkably, following peroral fecal transplantation, secondary abiotic mice harboring an intestinal human, but not murine microbiota could be stably infected with the pathogen upon peroral *C. jejuni* infection [169]. With these experimental studies we were able to validate the fact that the colonization resistance against *C. jejuni* is caused by the murine intestinal microbiota, whereas susceptibility to *C. jejuni* infection pivotally depends of the host dependent (i.e. human) composition of the inherent intestinal microbiota or its pertubations (i.e. dysbiosis). The induced pro-inflammatory responses upon *C. jejuni* infection of gnotobiotic as well as with respect to the microbiota "humanized" mice were mimicking key features of human campylobacteriosis, thus further underlining the feasibility of the applied infection model [196].

The facts above let researchers hypothesize that rapid mutation in contingency genes generates genetic diversity in *C. jejuni* subpopulations that enhances the ability to colonize poultry and to subsequently colonize and cause disease in humans. To address this hypothesis, Mansfield et al. analyzed the ability of three *C. jejuni* human disease isolates (namely strains 11168, 33292 and 81-176) and their derivatives to colonize broiler chickens (Ross 308) and C57Bl/6J IL10-deficient (IL-$10^{-/-}$) mice as models for human disease [197] by comparing microarray DNA/DNA hybridization analysis and DNA sequence analysis in culture, in the poultry reservoir, and after passage through mice [198]. The authors concluded that the host immune system generates pathogenic subpopulations with specific patterns of mutations within distinct contingency genes predicting the potential virulence of specific genotypes [198].

Germfree and secondary abiotic IL-$10^{-/-}$ mice for the investigation of bacterial pathogenesis

In order to better define the potential of distinct bacterial/pathogenic species in triggering enterocolitis scientists developed different experimental models. One prominent approach is the application of mice that are gene deficient for IL-10, an anti-inflammatory cytokine and essential immune modulator in the intestinal tract. Due to IL-10 gene

deletion, these mice develop a spontaneous form of large intestinal inflammation when kept under conventional conditions, whereas isolator-raised germfree IL-10$^{-/-}$ mice do not. The intestinal inflammatory phenotype can, however, be rescued upon reassociation of germfree IL-10$^{-/-}$ mice with a complex microbiota indicative for the pivotal role of bacterial ligands derived from the commensal intestinal microbiota in chronic IL-10$^{-/-}$ colitis. Histologically the segmental and transmural enterocolitis is characterized by inflammatory cell infiltrates in the lamina propria and the submucosa, erosions and ulcerations of the mucosa and hyperplasia of the mucosal layer, an abnormal architecture of the crypts, a depletion of the globlet cells as well as crypt abscesses [199–201]. Notably, no other organ systems beside the intestinal tract are so far known to be affected by the inflammatory process. The fact that germfree IL-10$^{-/-}$ mice develop significant intestinal disease and pathological lesions similar to those observed in humans when infected with enteropathogens such as *C. jejuni* offers the possibility to unravel molecular mechanisms underlying bacterial enteritis [197].

In an own study we addressed whether peroral *C. jejuni* infection of secondary abiotic IL-10$^{-/-}$ mice would induce intestinal inflammation. In order to virtually eradicate the intestinal microbiota and thus delete potential colitogenic stimuli derived from the inherent intestinal bacteria, conventionally raised IL-10$^{-/-}$ were subjected to quintuple antibiotic treatment for approximately 10 weeks. Remarkably, secondary abiotic IL-10$^{-/-}$ mice developed acute and non-self-limiting enterocolitis within one week following peroral *C. jejuni* infection, whereas abiotic IL-10$^{-/-}$ mice infected with a commensal intestinal murine *E. coli* strain were as uncompromized as were uninfected abiotic IL-10$^{-/-}$ controls [202]. The immunopathological responses upon infection were highly dependent on TLR-4 mediated signaling of *C. jejuni* derived lipooligosaccharides (LOS). Our study further underlines the feasibility of the IL-10$^{-/-}$ mouse model for dissecting the triangle relationship between pathogens, intestinal microbiota and host immune responses.

Due to substantial research progress that had been made possible by novel innovative techniques to delete or overexpress genes encoding cytokines or other genetic components of the immune system, the genetic basis of inflammatory conditions, irrespective of its etiology, can be nowadays unraveled in more detail by using distinct genetically modified animals, besides IL-10$^{-/-}$ mice [203]. For instance, rodents deficient in IL-2 [204], different T cell receptor chains ((TCR)-α, TCR-β) [205] or transforming growth factor (TGF)-β, as well as transgenic rodents overexpressing IL-7 and tumor necrosis factor (TNF) [206] are available, and all of them can even be used under germfree/gnotobiotic conditions. Although many of these mouse mutants might share many symptoms of intestinal inflammation, they also exhibit distinct pathological changes and unique cytokine profiles that have been associated with either Crohn's disease or ulcerative colitis in humans [203, 207, 208].

Respective inflammation models might be considered to be applied in unraveling host–pathogen interactions upon infection with norovirus [167], the intestinal helminth *Heligmosomoides polygyrus* [209] or *Helicobacter hepaticus* [210]. Furthermore, also the carcinogenic impact of defined bacterial species might be elucidated, given that colonic inflammation in IL-10$^{-/-}$ mice was associated with distinct changes in the gut microbiome that was characterized by an accumulation of tumor-promoting *E. coli* strains [211].

Behavior

Handling and maintenance conditions of experimental animals have a direct influence on their behavior. Germfree mice are generally housed in isolators. Due to the permanent audibly airflow germfree mice are more anxious than conventional mice. Results from recent studies point towards interactions between commensal microbiota in the gut and the brain [212]. In these investigations the influence of commensal microbiota on host behaviors was analyzed in a contamination-free environment. Open-field and marble-burying tests were used to analyze anxiety-like behaviors and locomotor activity. The monoamine levels in several brain regions were measured in germfree mice and gnotobiotic mice re-colonized with a commensal fecal microbiota. Even after 24 hours exposure to the environment outside the sterile isolators, germfree mice were shown to be less anxious as compared to not contaminated counterparts, whereas no differences in locomotion could be observed. In most regions of the brain the norepinephrine, dopamine, and serotonin turnover rates were higher in the recolonized mice as compared to the germfree mice. These results strongly support the current view that gut microorganisms modulate brain development and behavior [212]. Other investigations focused on the impact of the host microbiota on maturation and activation of microglia, the resident macrophages of the central nervous system (CNS) [213, 214]. These experiments revealed that germfree mice display global defects in microglia with altered cell proportions and an immature phenotype, leading to impaired innate immune responses. Temporal eradication of host microbiota severely changed microglia properties. Limited microbiota complexity also resulted in defective microglia function. In contrast, recolonization with a complex microbiota partially restored microglial features. Short-chain fatty acids, products derived from commensal bacterial fermentation, regulated microglia homeostasis [213]. In support of these observations, Möhle and colleagues demonstrated very recently that broad-spectrum antibiotic treatment negatively affected hippocampal neurogenesis and memory function [214]. Following reconstitution of mice with complex commensal intestinal microbiota, treatment with a probiotic compound (i.e. VSL#3), or running exercises, however, the antibiotics-induced effects could be reversed resulting in higher intracerebral numbers of Ly6C monocytes [214].

These findings uniquely suggest that host bacteria vitally regulate maturation and function of distinct neuronal cell populations. We were further addressing the clinically absolutely relevant question as to whether microbiotal colonization or a distinct intestinal microbiota composition were crucial for stroke outcome. Strikingly, depletion of the intestinal microbiota upon antibiotic treatment did, in fact, worsen the outcome after stroke in mice [215]. Furthermore, alterations of the intestinal microbiota affect intracerebral biochemical pathways and, in turn, behavioral responses in mice [216]. Of note, depression and anxiety are also commonly observed in IBD patients and are associated with a more active course of disease [217–219]. This further points towards regulatory features of the intestinal bacteria within the gut–brain axis. Investigations in this field are mostly based on the use of germfree mice/rats compared to SPF counterparts or on colonization of a distinct germfree mouse strain with intestinal microbiota derived from another mouse strain. Since evidences from experiments uniquely indicate that behavioral responses in rodents are affected upon manipulation of their intestinal microbiota composition, it is important to note that genetic differences across strains influence behavior and, therefore, that studies addressing the role of microbiota in behavior needs to be reproduced in several strains [220]. Also administration of pathogens such as *C. jejuni* can induce anxiety-like behaviors in healthy mice. Within few hours post infection the altered behavior is clearly visible suggesting that modifications in gut microbiota can relatively rapidly induce biochemical changes within the CNS [221, 222]. Moreover, alterations in gut microbiota have been described in autism [87, 223–225]. These studies provide at least indirect evidence for a crosstalk between the intestinal microbiota and the CNS that needs to be further characterized [226].

The impact of the intestinal microbiota in extra-intestinal diseases besides the neurological/psychiatric field opens a novel research focus [228–230]. Other immunopathological conditions including dental diseases (e.g. caries, periodontal inflammation), uremia, mycoplasmosis, tuberculosis, yeast or protozoal infections among many others could be experimentally addressed by using germfree or gnotobiotic animal models [124]. In light of microbiota dependent variations in the major histocompatibility complex (MHC) germfree mouse models might also present suitable tools germfree mouse model in transplantation medicine [231].

Summary and outlook

Both, germfree and gnotobiotic mice have been extensively and successfully used to investigate functional properties of the conventional microbiota in health and disease. Many physiological and pathological functions of the intestinal microbiota in humans and other vertebrates have been unraveled by the use of these important scientific tools, further substantiating our understanding of bacterial/pathogenic host interactions. In particular roles of distinct commensal bacteria in intestinal infections, cancer, obesity, cardiovascular and neurological diseases render germfree and gnotobiotic experimental models essential tools for both, basic research and the development of novel clinical applications in many clinical disciplines.

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