Chapter 16
Germ-free Mice Technology: Opportunity for Future Research

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Abstract The most popular approach to measure key functions of any living entity is to remove it and then study the consequences of its removal. Microorganisms influence their host in several manners and their role can be studied by eliminating them from their host and observe the host's response, in their absence. Numerous studies have justified the vital role of microbiota in human health and disease development. Germ-free (GF) animal models are useful tools to improve our understanding of the host–microbiota relationship in vivo. Although different animal models, lacking microbiota (partially or completely) have been extensively used in research but germ-free (GF) mice are the most widely used rodent model in human research due to its close proximity to humans. In modern research, GF technology is one of the most attractive and informative tools for getting insights into host's microbial community. Each body part harbors unique microorganisms with unique functions. Because of the advancement of microbial characterization techniques, the human microbiota community is expanding day by day. GF mice model can efficiently reveal the role of these valuable partners of humans. In spite of its high cost and obligation of skilled experts, GF research is a hot field for investigators and has a huge possibility for future applications. The present book chapter is a summary of the basics of GF technology and its main applications with future prospects.

Keywords Germ-free · Gnotobiotics · Microbiota · Microbiome · Mice

16.1 Introduction

All multicellular organisms including humans live in close association with the microbial communities (Mendes and Raaijmakers 2015). All the microorganisms that live in or on the human body are collectively known as microbiota (Human Microbiome Project Consortium 2012). The members of this microbial community

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have been shown to interact with each other and also with their host in different manners. It is a huge task to define the function of each individual microorganism of this complex community and their relation with the host. Germ-free animal research has been done extensively on three different animal models: Germ-free (GF) animals, gnotobiotic (GN) animals, and specific pathogen-free (SPF) animals. Germ-free or axenic animals are completely devoid of any kind of living microorganisms. Gnotobiotic animals are initially germ-free and subsequently inoculated with either one strain (monoxenics) or two different strains (dixenics) of microorganisms and so on. These animals thus have a defined and known microbiota. Specific pathogen-free (SPF) animals are those from which a defined set of microorganisms, usually pathogenic organisms are excluded (Festing and Blackmore 1971). Since the use of humans in such studies is not ethically possible, use of an animal model having several similarities with humans is the only left option. Mouse is the most common germ-free model in human studies (Luczynski et al. 2016a). However, rats, guinea pigs, chickens, piglets, and calves have also been used (Luczynski et al. 2016a, b). Experimental germ-free (GF) mice models are valuable tools for establishing the impact of microbiota on host metabolism, physiology and to explore interactions between microbiota and with their host (Fiebiger et al. 2016; Bhattarai et al. 2017). Recent studies performed on germ-free mice have proved the specific role of specific microbial communities for their host (Braniste et al. 2014; Jourová et al. 2017; Kaden-Volynets et al. 2019). Although germ-free mice technology has broadened our knowledge of microbe–microbe and host–microbe interactions but, due to the huge cost and expertise associated with the GF facilities, this field has not been explored as per the expectations and possibilities. This book chapter covers the basics of GF mice technology, its applications and future prospects.

Axenic: free of all detectable microorganisms
Monoxenic: a culture in which one organism is grown with only one other organism
Dixenic: a mixed culture of one organism together with two other organisms
Germ Free (GF): historical term same as axenic but continues to remain the more popular than axenic
Gnotobiotic (GN) animal: an animal with known and defined microorganisms
Pathogen-free (PF) animal: an animal free of all known pathogens
Specific pathogen-free (SPF) animal: an animal from which a defined set of microorganisms, usually pathogenic are excluded
Conventional (CV) animal: an animal maintained under accepted husbandry practices
Altered Schaedler flora (ASF): a model community of eight cultivable microorganisms derived from mice and used for establishing stable GI colonization in the GF mouse

16.2 Germ-Free Mice Technology

16.2.1 History

Louis Pasteur (1885) conceptualized the idea of a germ-free animal more than a century ago with the remark that bacteria-free existence is impossible (Pasteur 1885). Ten years later in 1895, Nuttall and Thierfelder produced the first GF guinea pigs, which survived for two weeks (Nuttall and Thierfelder 1895). Due to the technology developed by James Arthur Reyniers and Philip C. Trexler, the hypothesis of germ-free life came into reality in the 1950s (Reyniers 1957). By the late 1950s, researchers successfully developed GF mice, rats, guinea pigs, and chicks inside sterile stainless steel and plastic housings (Reyniers 1959a). The first GF mice were successfully developed by Pleasants in 1959 (Pleasants 1959).

16.2.2 GF Technology

Today's methodology for keeping GF animals has not changed much since 1959. To start a germ-free colony, pups must be delivered from the mother's womb through a careful cesarean section to protect them from exposer of microorganisms that inhabit on the mother's vagina and skin (Gustafsson 1959a; Reyniers 1959b).Then, the newly born pups are introduced to the GF foster mother and raised in an aseptic isolator and only exposed to food, water, and other equipment that has also been sterilized. GF animals can also be produced via embryo transfer, in an isolator by the implantation of cleaned embryos into GF female in well-controlled conditions. A recipient female normally delivers and caress the pups assuming them her own offsprings, hence enhancing the survival rate of pups. These mice regularly monitored in order to guarantee GF status by analyzing the presence of any kind of microorganism in their feces using cultural and sequencing techniques (Smith et al. 2007). Once GF animals are produced next lineage can be generated by crossing GF individuals (Gustafsson 1959a; Reyniers 1959b). Then GF mice colonies can be shipped in a sterile container for different purposes including GF research. Isolators maintain a sterile environment for GF animals. A typical isolator has an air supply, air inlet and outlet, transfer port, and arm-length gloves, as well as a special tank filled with disinfectant and used for the transfer of mice in and out. Bedding, food, water, and equipment, including cages, must first be sterilized before putting them into the isolator through the sterile lock. Sterilization of entire steel isolators is accomplished by autoclaving the whole isolator, as well as with portable vacuum and steam equipment. Plastic isolators are sterilized by steam accomplished with germicidal vapor (2% peracetic acid and chlorine dioxide). Air sterilization is ensured upon entry and exhaust by mechanical air filtration under positive pressure.

Autoclave jars are used for transferring of animals in and out of the isolator. Maintenance of the GF status of rodents during the execution of the entire experiment is technically challenging. The probability of their contamination is always high. The experimentation cost of the GF facility is extremely high since multiple mouse strains and multiple inoculation groups are housed in separate isolators. Recently the use of positive-pressure isocages has been increased in short duration experiments since they offer low cost and space effective (Hecht et al. 2014). Future research is needed to optimize these isocages to apply them in long-term experiments.

16.2.3 Customized Flora and Control Group for Experiments

In 1965, Schaedler and Dubos characterized the bacterial population of the gastrointestinal (GI) tract of conventional mice (Schaedler et al. 1965). One of the most pronounced phenotypes observed in almost all GF rodents is unusually enlarged cecum that is of normal length in conventional rodents (Wostmann and Bruckner-Kardoss 1959). Surprisingly a reduction in cecum size was observed upon colonization of normal gut microbiota. On the basis of these findings, Schaedler colonized GF animals with a mixed bacterial population of Bacteroides, lactobacilli, an anaerobic Streptococcus, and a slow lactose-fermenting coliform (Schaedler et al. 1965). This flora is subsequently known as the "Schaedler flora" and has been used globally as an essential tool for the standardization of experimental animals' microbiota. In 2015, extremely oxygen-sensitive (EOS) bacteria were included in Schaedler's flora. This altered flora is now known as Altered Schaedler Flora (ASF) (Orcutt et al. 1987). Recently a synthetic bacterial community is created for experimental GF mice known as "Oligo-Mouse-Microbiota" (OMM^{12}) which consists of 12 sequenced and easily available bacterial strains isolated from mice (Brugiroux et al. 2016; Lagkouvardos et al. 2016). The specific pathogen-free (SPF) mice which are free from particular pathogens are treated as a control group for GF mice research (Smith et al. 2007). SPF mice are generally exposed to the defined colonization of modified Schaedler flora or other customized flora (Wymore Brand et al. 2015; Brugiroux et al. 2016). Broader categories of mice models are summarized in Fig. 16.1.

16.3 Why Mice Model?

Although many GF animals have been used in investigations, GF mice is the most acceptable GF model in human studies for long period (Haldane 1928). Over 95% of animal studies have been conducted on the *Mus musculus* model because of almost

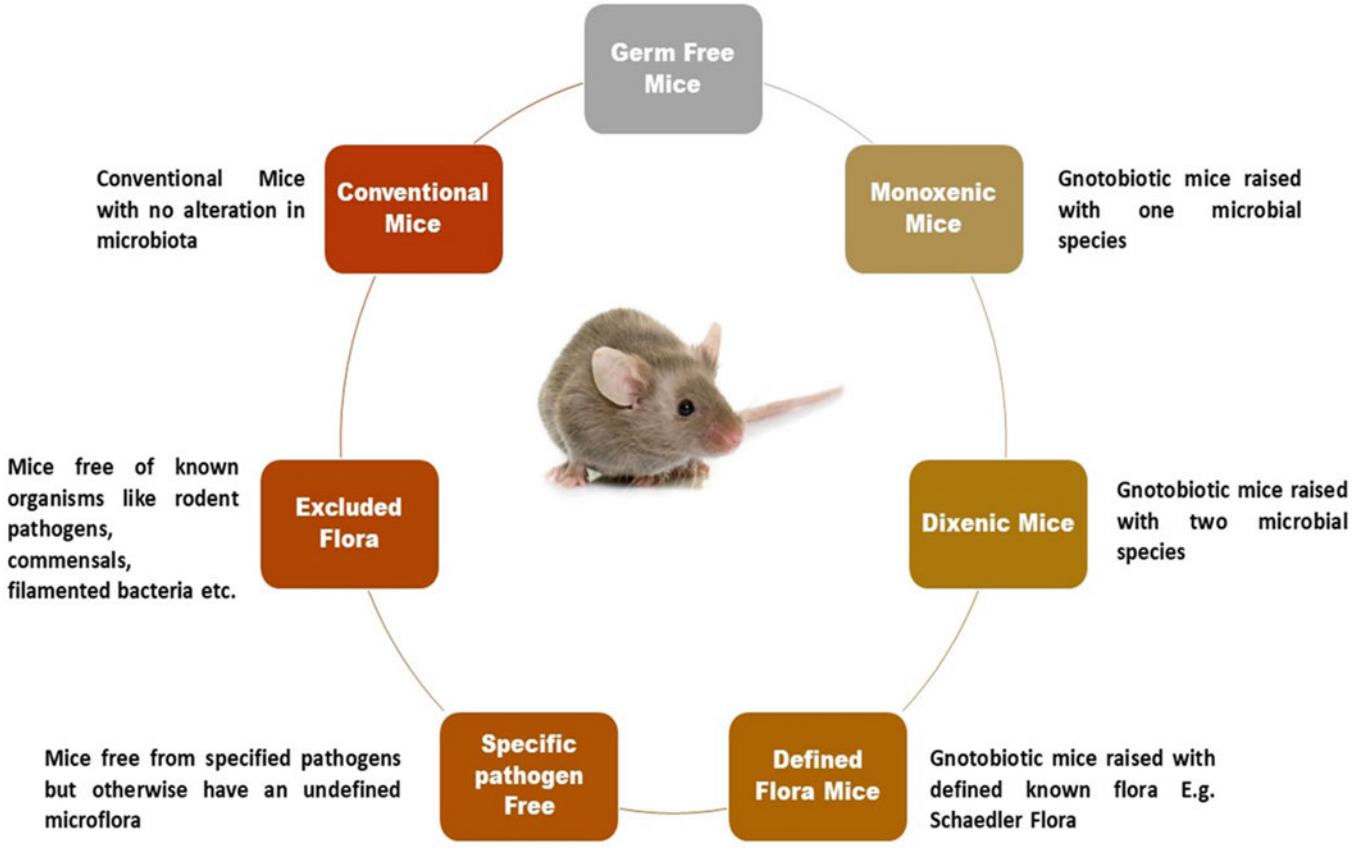

Fig. 16.1 Different types of mice models used in gnotobiotic technology

99% genetic homology with the human genome, availability of good genetic/molecular technologies for mice research, and replicability of many human conditions in mice (Gregory et al. 2002; Mouse Genome Sequencing Consortium et al. 2002). Investigators prefer mice model for various other reasons including their small size, easy to maintain, and adaptability in altered conditions. Because of quick reproduction and a short life span of two to three years, many generations can be observed in a short period of time. They are relatively inexpensive and can be bought in large quantities from commercial producers. Their mild-tempered and docile nature makes them easy for researchers to handle (Canales and Walz 2019). Due to constrain of working space inside the GF isolator and extremely higher maintenance cost mice offer more experimental units in a relatively smaller area in a cost-effective manner.

16.3.1 Differences Between Germ-free and Conventionally Raised Animals

Germ-free animals exhibited several physiological and functional alterations, not associated with conventionally raised animals. Some crucial differences are summed up in Table 16.1. These dissimilarities have created the research-ground for gnotobiotics, to assess the effect of microbiota in postnatal development and metabolism of host.

16.3.2 GF mice Technology: Applications and Future Guideline

Germ-free animal models are essential tools of investigators to explore the complexity and functions of host's microbiota. GF mice provide researchers with a better way to get insights of host–microbe and microbe–microbe interactions. GF mice have been widely used in the field of nutrition, metabolism, drug response, neuroscience, immune response, cancer biology, and infectious diseases and some of the important fields are also highlighted in Fig. 16.2. Germ-free mice technology has revealed the crucial role of commensal microbiota in normal aging, and normal functioning and development of immune system, GI system, and nervous system (Grenham et al. 2011).

Table 16.1 Key differences between GF animals and conventionally raised animals

	GF Animal (Comparison with conventional animals)
Gastrointestinal tract physiology	
Weight of the small intestine	Lighter (Gordon and Bruckner-Kardoss 1961)
Mucosa of the small intestine	Thinner (Gordon and Bruckner-Kardoss 1961)
Mucosal surface area, Lamina propria	Reduced by approximately 30% (Gordon and Bruckner-Kardoss 1961)
Digestion and absorption	More efficient (Phillips and Smith 1959; Heneghan 1963)
Passage time through the small intestine	Increased (Abrams and Bishop 1967)
Cecal	Excessively enlarged (Wostmann and Bruckner-Kardoss 1959)
Mitosis indexes of epithelial cells	Lower
Renewal rates of intestinal epithelium	Reduced (Abrams et al. 1963)
Cells, tissues, and organs of immune system	
Expression of certain TLRs	Decreased or absent (Shanahan 2002; Grenham et al. 2011)
IgA secretion	Decreased (Abrams and Bishop 1961; Wostmann et al. 1970)
Peyer's patches, lymphoid follicles in the intestine	Fewer and smaller (Abrams and Bishop 1961; Wostmann et al. 1970)
Lymphoid tissue and lymph nodes	Undeveloped and smaller (Abrams and Bishop 1961)
γ-globulin-bearing plasma cells in mesenteric lymph nodes	Absent (Hobby et al. 1968)
Antibody-producing cells of lymph-node	Reduced (one-twelfth) (Olson and Wostmann 1966)
Antibody-producing cells after Ag challenge	Increased (Olson and Wostmann 1966)
Total white blood cell count	Lower (Reyniers et al. 1960)
B- and γ-globulins in the serum	Reduced (Gustafsson and Laurell 1958)
Plasma cells synthesizing IgA	Less (10%) (Crabbé et al. 1968)
Lysozyme in saliva	Absent (Makulu and Wagner 1967)
Thymus	Smaller in size (Wilson et al. 1965)
Nutrition, digestion, and metabolism	
Nutrient requirements	Consume more food (Wostmann et al. 1983)
Diet-induced obesity	Not observed (Bäckhed et al. 2007)
Water intake	Higher (Coates 1973)
Lipid: requirement for essential fatty acids	Lower (Coates 1973)
Cholesterol absorption	Absorb up to 50% more
Protein:	
Fecal nitrogen	More excreted (Levenson and Tenivant 1963)
Urinary nitrogen	Less excretion (Reddy et al. 1969)
Starving conditions	Lesser survival rate (Loesche 1969)
Minerals: urinary calcium excretion	Higher (5X higher) (Gustafsson and Norman 1962)

(continued)

Table 16.1 (continued)

	GF Animal (Comparison with conventional animals)
Vitamin B & K in diet	Required (Gustafsson 1959b; Sumi et al. 1977)
Trypsin and chymotrypsin in feces	Higher (Borgstrom et al. 1959)
Serum cholesterol levels	Higher (Danielsson and Gustafsson 1959)
Cardiovascular system	
Heart weight, total blood volume, and cardiac output	Reduced (Gordon et al. 1963)
RBC count, Hematocrit values	Higher (Gordon et al. 1963)
Age	Live significantly longer (Gordon et al. 1966; Tazume et al. 1991)

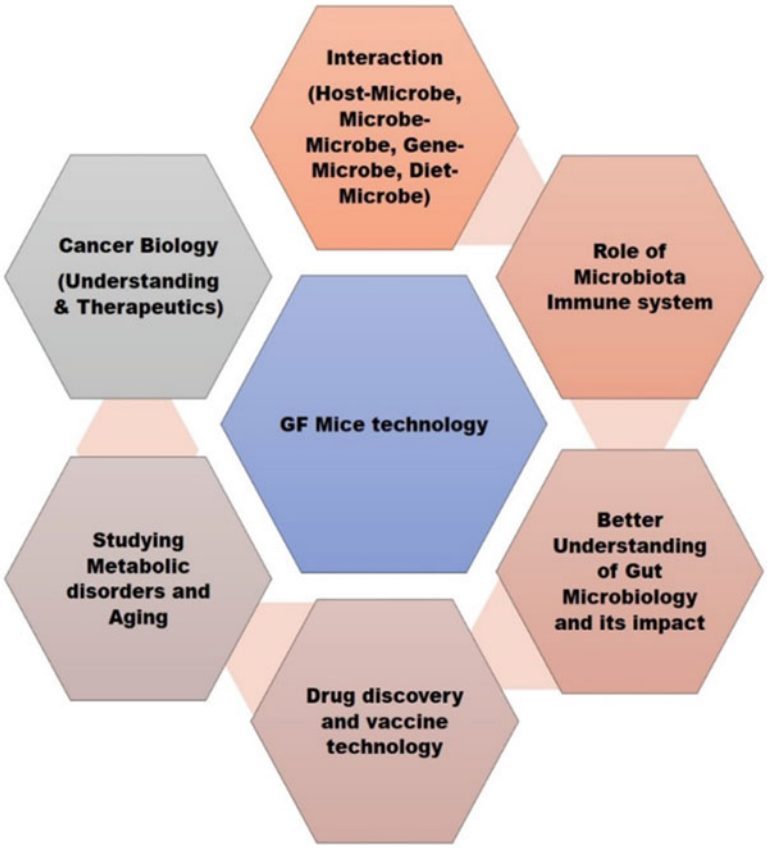

Fig. 16.2 Key applications of germ-free mice technology

16.4 Metabolic Disorders

Our generation is facing many metabolic disorders including obesity, heart disease, stroke, type 2 diabetes, hyperglycemia and hyperlipidemia, phenyl-ketonuria (PKU), and a number of other hepatic disorders. Studies performed on GF mice have established the very fact that the presence or absence of specific gut microbiota is correlated with certain metabolic disorders (Karlsson et al. 2013). It is found that the composition of gut microbiota is unstable and far sensitive for alteration but surprisingly found relatively stable during obesity, which suggests the possible role of gut microbiota in obesity. Jeff Gordon et al. showed that the transfer of gut microbiota of obese humans or mice to GF mice with no change to mouse diet, resulted in weight gain relative to GF mice that had received microbiota transplants from lean donors (Turnbaugh et al. 2006; Ridaura et al. 2013). Transfer of microbiota from third-trimester pregnant mothers to GF mice promoted low-grade inflammation, increased adiposity, and insulin resistance relative to GF mice receiving microbiota from first trimester pregnancies (Koren et al. 2012). As gut microbiota synthesizes additional essential nutrients, its absence affects the process of absorption and digestion (Sekirov et al. 2010; Grenham et al. 2011). GF mice have reduced production of short-chain fatty acids, which are beneficial to host metabolism and are produced when dietary fiber is fermented by gut bacteria (Høverstad and Midtvedt 1986; denBesten et al. 2013). GF rats have deficient thiamine absorption: when these animals are fed radio-labelled thiamine, large quantities of the nutrient are found within the feces but little is found within the tissue (Wostmann et al. 1962). A recent study proposed that fecal microbiota transplantation is the recent technology used for treating various neurological diseases (Tripathi et al. 2022). This suggests that the gut microbiome plays an important role in influencing metabolism and adiposity. Phenylketonuria (PKU) is a genetic disorder related to an inability to metabolize phenylalanine (Phe), which may end in neurotoxicity. Recently a genetically engineered *Escherichia coli* strain administration to PKU mouse model showed significantly reduced blood phenylalanine concentration independent of dietary protein intake (Isabella et al. 2018). Collectively, such studies certainly prove that not only the commensal microbiota but genetically modified microorganisms can also be used to target genetic disorders along with altered metabolism. Gut microbiota is the most abundant microbiota and also termed as "neglected endocrine organ" (Clarke et al. 2014). Gut microbiome contains hundred-times more genes than the human genome (Qin et al. 2010). This microbiome comprises an enormous possibility of future investigation because every member of this massive microbial community may have a unique function for its host that can be assessed efficiently with the assistance of GF technology. Once the individual role of every member of microbiota revealed, specific microorganisms can be selectively administered or removed from the host to treat several metabolic disorders. Initial findings with microbiota research might be milestones for future investigations during which GF animals are going to be considered as an indispensable tool for exploring the role of

microorganisms to study the complexity of microbiota-gut-brain axis and metabolic disorders.

16.5 Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Disease (IBD) is a set of chronic inflammatory conditions of the gastrointestinal tract (Belkaid and Hand 2014). IBD features a complex etiology and is influenced by the genetic factors, host immune system, and external factors like the microbiota (Maloy and Powrie 2011). IL-17-producing Th17 cells are correlated with IBD while RORγt$^+$ Treg cells adversely affect IBD by maintaining homeostasis at the mucosal barrier. Studies performed on mice showed the elevated number of IL-17-producing Th17 cells upon colonization of mice with anaerobic, uncultivatable segmented filamentous bacteria while the introduction of commensals showed an adverse effect (Nakanishi and Tamai 2015). In another study transfer of microbiotas from IBD donors into germ-free mice increased numbers of intestinal Th17 cells and Th2 cells and decreased numbers of RORγt$^+$ Treg cells while microbiota from healthy donors exhibited an adverse effect (Britton et al. 2019). Administration of fecal microbiota transplants from patients with IBS to germ-free mice induced alterations in GI motility, also as hypersensitivity to colonic distension (Crouzet et al. 2013; De Palma et al. 2014). The absence of IBD in germ-free animals is that the classical evidence that microorganisms are crucial for the development of IBD. *We can't neglect the colonic microbiota and a heavier metagenome and its functions in several physiological conditions including IBD. Future attempts should be focused on manipulating the amount and composition of commensal or altered microbiota to alleviate the severity of IBS either by oral administration of probiotic formulations, fecal microbiota transplants from healthy donors, and/or diet modifications. Germ-free models might be a milestone in the collaborative attempts to cure IBD.*

16.5.1 Host Immune Response

The role of microbiota in the development and regulation of the immune system has been extensively studied with the assistance of GF mice. Factually the exposure to microbes early in life is essential for the proper development and performance of the immune system (Blümer et al. 2005; Douwes et al. 2008; Kaplan et al. 2011). The host's immune system and gut microbiota have a mutualistic relationship. The microbiota helps in shaping our immune system, and the later shape the composition of host microbiota (Nicholson et al. 2012). These hypotheses further get strength by the fact that about 80% of the host's immune cells are located in or around the gut (Abbas et al. 2017). Commensal microbiota of host is crucial for proper intestinal immune response, protection from pathogens, and suppression of detrimental

inflammatory reactions. GF mice show many abnormal features generally absent in conventionally raised mice, including the presence of fewer and smaller Peyer's patches, a reduced number of $CD4^{+}$T cells, low level of IgA producing plasma cells, under-developed gut-associated lymphoid tissues (GALT), fewer intraepithelial lymphocytes as well as reduced production of antimicrobial peptides (Wostmann and Bruckner-Kardoss 1959; Gordon and Bruckner-Kardoss 1961; Shanahan 2002; Round and Mazmanian 2009). Upon administration of normal microbiota, most of those altered structures and immunological functions of GF mice are corrected and restored. These inducible structures normally develop in conventional animals exposed naturally to diverse populations of microorganism, suggesting a complex relationship between the host's immune response and its commensal microbiota. *Microbes produce a variety of known and unidentified metabolites that can modulate the host's metabolic pathways in a complex manner. Class, quantity, and roles of metabolites are influenced by the composition of the host's microbiota. Despite advanced molecular characterization technology most of the commensal microorganisms, their different metabolites and function of every metabolite are yet to be defined. There is an enormous challenge and also an opportunity for the investigators to address this issue and define the functionality of each single metabolite of this complex immune response regulated by the host and its microbiota.*

16.5.2 Vaccine Response

More recently, mice treated with a cocktail of antibiotics, exhibited impaired IgG responses upon systemic Ag ova challenge, the same could be restored after the colonization with a mixed bacterial population (Lamousé-Smith et al. 2011). Pre-antibiotic-treated mice showed enhanced antibody response upon oral administration of Rota-virus (Uchiyama et al. 2014). These findings suggest that gut flora can enhance systemic vaccine responses but can suppress oral vaccine responses. A study on seasonal influenza vaccine showed that after vaccination, in germ-free or antibiotic-treated mice, IgG and IgM antibody responses were significantly impaired (Oh et al. 2014). It is also reported that microbial metabolites can modulate various immune cell types including Mfs, DCs, T cells, and B-cells (Dorrestein et al. 2014). *However, further investigations are desirable to conclude whether these effects occur in all situations or only observed in some special circumstances. Currently, the knowledge of various microbial metabolites and their role in vaccine response is in its infancy. Future research can reveal the potential mechanisms by which the gut microbiome modulate vaccine response in various populations. The study of gut microbiota for a successful vaccination strategy may open a new area for investigators with unlimited possibilities and GF mice may serve as a valuable tool in this cause.*

16.5.3 Host–microbe and Microbe–Microbe Interaction

Germ-free mouse facilitates to introduce microbes individually or sequentially in its different body parts to assess the role of a single bacterium or known consortia of bacteria on host function in vivo (Reigstad et al. 2015). Germ-free mice offer to study microbe–microbe interaction, to better understand how the introduction of a new microbial member affects the whole microbial community and host functions. Germ-free models provide insights into the host processes regulated by the presence and/or composition of the microbiota in health and disease (Reyniers 1959b; Smith et al. 2007). Based on their interaction with the host, members of the microbiota can be classified as beneficial species (Commensals) including probiotic bacteria, like *Bifidobacterium* and benign organisms such as members of the defined "altered Schaedler flora," or pathogenic species, including pathobionts such as *Helicobacter pylori* and opportunistic pathogens (Fanning et al. 2012; Biggs et al. 2017). The development of gnotobiotic animal models provides an opportunity to compare them with conventionally raised animals but also the ability to introduce one or few bacterial species at a time to understand host–microbe interactions in a simplified environment (Williams 2014).

16.5.4 Host–pathogen Interaction

Very soon after the invention of germ-free technology, gnotobiotic animals became a key tool to study the host–microbiota interaction and later used to investigate the host immune responses to pathogens. Initial studies performed on the mono-associated animal to assess the resisting power of host towards infections demonstrated that lack of an intestinal microbiota impairs early innate immunity. Mono-associated animals showed higher sensitivity towards *Listeria monocytogenes* infection while di-associated mice having commensal flora, remained unaffected from the pathogen (Zachar and Savage 1979; Czuprynski and Balish 1981). A similar conclusion concerning the significance of native flora on the host's immunity was made in other studies conducted with *Salmonella typhimurium* and *Vibrio cholera* infections (Nardi et al. 1991; Butterton et al. 1996). A commensal microbiota competes for space, nutrients and mediates the production of antimicrobial metabolites, which subsequently prevent growth and colonization of numerous pathogenic bacteria (Mack et al. 1999, 2003; Srikanth and McCormick 2008). GF mice models in combination with mono-associated and di-associated gnotobionts will be obligatory tools in future investigations, aimed to recognize the pathogenesis and treatment of newly emerging infections.

16.5.5 Reproductive Health

Germ-free mice are considered reproductively inferior to their conventional counterparts. Surprisingly, when germ-free female mice di-associated with *B. distasonis* and *C. perfringens* displayed a normalized estrous cycle, and increase rates of copulation and implantation (Shimizu et al. 1998). The lower reproductive tract of the female mouse is anatomically almost like that of humans (Leppi 1964). During a recent study, GF mice vaginally inoculated with *Prevotella bivia* displayed increased numbers of mucosal activated $CCR5^+$ $CD4^+$ T cells (HIV target cells) within the female genital tract compared to mice inoculated with *Lactobacillus crispatus*. Hence colonization of altered bacteria is often associated with increased HIV risk and other STIs (Gosmann et al. 2017). In future investigations, germ-free models can be exploited to address different aspects of reproductive health correlation with host microbiota.

16.5.6 Cancer Biology

Cancer development in GF rodents can partially be associated with the absence of commensal flora (Pollard and Teah 1963; Walburg Jr 1973; Pollard et al. 1985). Experimental cancer yields were found to be lower in GF rodents when the carcinogens tested required enzymatic metabolic activation (Weisburger et al. 1975). Generally, the oncogenic potential is that the same as in conventional rats, but tumor-related changes are more clearly defined in GF animals (Pollard et al. 1968). GF rodents with either spontaneous or induced tumors have higher numbers of plasma cells but haven't any germinal zones in their lymph nodes (Pollard et al. 1968). Gnotobiotic animals are particularly suitable for testing candidate viral carcinogens, since derivation by hysterectomy and gnotobiotic maintenance has been found to eliminate all known viruses from GF rodents (Luckey 1963; Pleasants 1974). Cycasin from cycad bean flour is carcinogenic for conventional rats because the microbiome present in them converts it into a carcinogen, whereas it doesn't induce tumors in GF rats (Laqueur et al. 1967; Luckey 1968). Spontaneous colon adenomas are twice as prevalent in GF rats (Weisburger et al. 1975). The foremost frequent spontaneous tumors in aged GF rats involve the mammary and pituitary glands (Pittermann and Deerberg 1975).

16.5.7 Aging

GF mice tend to live longer than their conventionally colonized counterpart animals (Reyniers and Sacksteder 1958; Gordon et al. 1966; Tazume et al. 1991). There is growing evidence indicating that gut microbiota influences the aging process. As GF

mice are raised in sterile conditions, their longer life span is likely due to the absence of pathological infections. Premature mortality of GF mice is mainly due to infection or by environmental factors. Delayed morbidity in 2- to 3-year-old GF rodents is a common observation, which shows them to be virtually free of the age-related kidney, heart, and lung changes (Pollard and Kajima 1970; Pollard 1971). In humans, microbial diversity and stability decrease with age and are accompanied by a cognitive decline (O'Toole and Claesson 2010; Borre et al. 2014). These findings have prompted the thought that restoring microbial diversity within the elderly could improve general and mental health.

16.5.8 Drug Response and Xenobiotics

It is often assumed that gastrointestinal tract microbiota is probably the first which interacts with ingested xenobiotics. The gut microbiome can activate or deactivate pharmaceuticals and may alter their metabolic consequences. An altered microbiota also influences the outcome of various therapies, this proves the importance of intact microbiota in host immune responses (Pope et al. 2017). In experimental germ-free mice with induced tumors, immune cells poorly respond to immunotherapy that slows cancer growth and prolongs survival. These germ-free rodents hardly exhibited any response toward anticancer drugs like oxaliplatin and cisplatin (Viaud et al. 2015). Clinical use of anticancer drug cyclophosphamide (CTX) on tumor-bearing mice caused the translocation of some bacterial species into mesenteric lymph nodes and the spleen, where they stimulate a Th1 and Th17 immune response. Germ-free mice failed to generate the same response and were found immune to the CTX (Viaud et al. 2013). A study performed in National Cancer Institute (NCI) reported that in germ-free mice having subcutaneous tumors exhibited lower cytokine production and tumor necrosis after CpG-oligonucleotide treatment and deficient production of reactive oxygen species and cytotoxicity upon chemotherapy. This finding advocates the necessity of an intact microbiota for proper response to anticancer therapy (Iida et al. 2013). Recently in two parallel studies, the microbial population of fecal samples of melanoma patients was characterized prior to treatment with the anticancer drugs which block a T cell receptor PD-1. In both studies, certain bacterial species were reported in greater numbers in those patients who responded properly to the drug. When the same microbes were administered into the germ-free mice model, an anti-tumor immune response was observed (Gopalakrishnan et al. 2018; Gong et al. 2019). While some drugs get activated through bacterial metabolism, others can be inactivated due to microbial action. A single bacterium *Eggerthella lenta* inactivates the drug Digoxin, a treatment for heart failure by converting it into inactive form dihydrodigoxin (Lindenbaum et al. 1981). The microbiome also inactivates Parkinson's disease drug L-DOPA, initially by Enterococcus faecalis mediated decarboxylation and later *Eggerthella lenta* A2 mediated dihydroxylation. Treatment with broad-spectrum antibiotics can reverse this activity (Rekdal et al. 2019). Recently Klatt

et al. reported that vaginal bacteria *Gardnerella vaginalis* could rapidly metabolize and breakdown the active form of "Tenofovir Microbicide" the drug for HIV treatment and thus results in a high HIV acquisition in those women. These findings highlight the contribution of intact microbiota and its poorly known factors in the therapy of cancer and other diseases (Klatt et al. 2017).

16.5.9 Gastrointestinal System and Enteric Nervous System

It is reported that gastrointestinal (GI) transit time was significantly faster in conventionally raised mice as compared to GF mice (Abrams and Bishop 1967). Subsequently, several investigators have shown the introduction of mouse-derived or human gut-derived bacteria into GF mice alters GI motility and transit time (Husebye et al. 2001; Kashyap et al. 2013). Various studies highlight the utility of GF mice as a model to understand host–microbe interaction and how microbes modulate GI motility and secretions (Husebye et al. 2001; Kashyap et al. 2013; Kaji et al. 2015; Reigstad et al. 2015; Yano et al. 2015). The microbiota was found essential for the postnatal development of the enteric sensory and motor neurons (Luczynski et al. 2016a, b).

The changes that have been reported in central nervous system development in GF mice are reflected during the maturation of the enteric nervous system (ENS) (Collins et al. 2014; Luczynski et al. 2016a). At postnatal day 3, the structure, neurochemical composition, and function of enteric neurons in the jejunum and ileum of GF mice were significantly altered, also in the small intestine, GF mice have decreased overall nerve density (Collins et al. 2014). The ganglia of intrinsic sensory neurons of the ENS are embedded in the gut wall and it has been established that the electrophysiological properties of afterhyperpolarization (AH) neurons are altered in the absence of colonizing bacteria (Forsythe and Kunze 2013; McVey Neufeld et al. 2013, 2015). As mentioned earlier, GF mice have altered intestinal motility and these sensory neurons synapse on enteric motor neurons controlling gut motility, so this may provide a possible explanation for the dysfunction. The AH sensory neurons also synapse, both anatomically and functionally, with vagal nerve endings in the gut and thus could represent a direct neural route whereby the intestinal bacterial status is transmitted to the brain (Powley et al. 2008; Perez-Burgos et al. 2014). All these studies carried out with help of GF mice provide us a crucial link between microbiota and development of the GI system and also how it alters the functioning of ENS when compared with conventional models.

16.6 Future Potentials of Germ-Free Technology

16.6.1 Technological Aspects

Introduction of automation, sterile room facilities and robotics could prove to be boon in the technology of in vivo mice models. Major challenges for this technology are scarcity of proficient technicians, and the time and space required to accommodate the bulky isolators along with huge cost associated with maintaining GF facilities is the major challenge for this technology (Mallapaty 2017). Innovative ideas such as the use of positive-pressure isocages for short-term experiments can be helpful to reduce the overall cost (Hecht et al. 2014). Availability of highly skilled technical staff will be another breakthrough in GF technology. Colonies of GF mice of specified genetic strains and novel minimal bacterial consortia should be established for the common laboratory species for target-based studies. Long-term rearing, development of breeding colonies of GF mice, and development of devices for shipping GF animals require extensive operations. In future, a centralized or region-based laboratory exclusively for GF mice technology can be established which not only will be center for resources but also skilled manpower.

16.6.2 Future Bio-therapeutic Agents and Pharmaceuticals Products

Due to increased incidences of antibiotic-resistance and therefore the side-effects of those drugs on host and off-target flora, alternate strategies should be developed to target pathogens (Meropol et al. 2008). Manipulation of the commensal microbiota and hence enabling its over-growth and competition with pathogens thus ultimately replace drug-resistance flora could also be a potent solution in the form of probiotics (Imperial and Ibana 2016). Germ-free mice colonies are often used as an experimental model to develop probiotics against antimicrobial-resistance pathogens. However, consistent monitoring of microbial load in germ-free models is critical for researchers to determine the load of contaminants or antibiotic-resistant microbes. These gnotobiotic models can also be potentially utilized in vaccine development program. Since it's evident that host microbiota can modulate vaccine response, hence it can be a decisive factor for a successful vaccination strategy (Wang et al. 2010; Cram et al. 2018). Microbiota features a capacity to alter the efficacy of any pharmaceutical formulation applied on its host. Different pharmaceutical products can be activated or inactivated by selective microorganisms (Iida et al. 2013; Klatt et al. 2017; Rekdal et al. 2019). Hence germ-free mice associated with such bacteria can be utilized in preclinical trials to develop stable and effective drugs (Fig. 16.3).

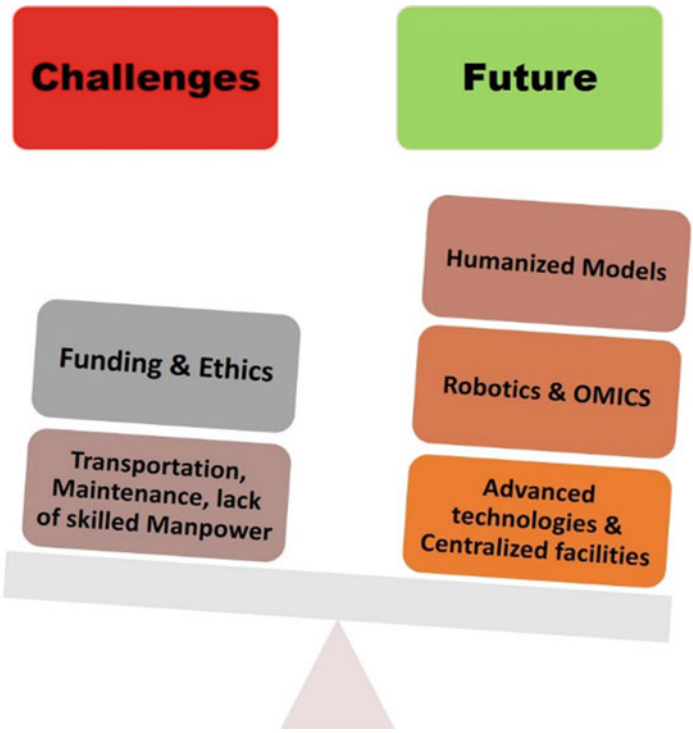

Fig. 16.3 Current challenges vs proposed future advancements in germ-free mice technology

16.7 Future Models

Humanized mouse model: GF mice can be colonized with human gut microbiota by using either a reductionist or holistic approach. In a reductionist approach, investigators seek the effect of known organisms, which affect the host, affected by the host or interact with one another. In the holistic approach, complex gut microbiota from diseased or healthy human donors is transferred in GF mice and their role in humans is predicted by profiling the alterations in recipient mice. The generation of "humanized mouse models" can support translational aspects of future research by creating human-like conditions within the mouse gut (Basic and Bleich 2019).

Knockout-gnotobiotic mouse model: Recently knockout-gnotobiotic animal models have been successfully developed and exploited to review the immune response modulated by a pathogen, in absence of crucial immune-modulatory genes (Balish et al. 1998; Yugo et al. 2018). Although the development of the knockout-gnotobiotic animal model is not an easy task, it will immensely help to understand the role of a specific bacterium in special circumstances.

1. In a genetic disease due to defective gene/gene product: to explore the role of various bacterial species or their metabolites that can perform an identical role to cure the disorder
2. To understand the infection dynamics of a pathogen in immune-compromised individuals

Model recombinant microorganisms: Engineering Human Microbiome is a novel concept (Kali 2015). Recombinant DNA technology can be employed to modify the genome of resident microflora and genetically modified microbes can be assessed to achieve unprecedented goals when associated with GF animals (Kayser et al. 2019).

Fecal Microbiota Transplantation (FMT): Administration of stool sample in solution from healthy donor to intestinal tract of recipient in order to change the gut health.

Cohousing: *Cohousing* recommendations for individual species are based, in part, on behavioral characteristics such as the desire to nest near a cage mate. In a study of male mice, *animals* given the choice to nest in an inhabited or empty cage preferred the proximity of another *animal*.

16.7.1 Combination of OMICS and GF Technology

The bioinformatics approaches in GF mice technology can prove to be indispensable in terms of applications in the future. When combined with approaches such as genomics, transcriptomics, metabolomics, and proteomics, GF mice technology can lead to the discovery of the exact functions and mechanisms of host colonization. It can also lead to a better understanding of the interaction and communication of specific microbiota representatives amongst each other and also with their respective hosts.

16.8 Conclusion

Here, we have summarized the foremost important outcomes of germ-free mice technology within the fields of health and allied sciences. These animal models offer immense advantages over other existing approaches for studying the role of varied microbial species and to know pathogenesis through host–microbe interaction, microbe–microbe interaction, gene–microbe interaction, diet–microbe interactions and senescence. GF animals are going to be the most important tool to investigate, how certain microorganisms are ready to colonize and survive within the host, while others can't. Germ-free mice have been extensively used for deciphering some mechanisms linked to metabolic diseases like Type II diabetes mellitus, behavioral functions at the gut-brain axis and autism, cardiovascular diseases, and cancer. Like every technology developed in the past few decades, germ-free mice technology is

additionally into its evolving phase. The fact that technologies for detecting and characterizing microorganisms is continuously evolving, GF mice technology also needs to go in with pace.

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