Chapter 8

Germ-Free Mice Model for Studying Host–Microbial Interactions

Yogesh Bhattarai and Purna C. Kashyap

Abstract

Germ-free (GF) mice are a relevant model system to study host–microbial interactions in health and disease. In this chapter, we underscore the importance of using GF mice model to study host–microbial interactions in obesity, immune development and gastrointestinal physiology by reviewing current literature. Furthermore, we also provide a brief protocol on how to setup a gnotobiotic facility in order to properly maintain and assess GF status in mice colonies.

Key words Germ-free, Gnotobiotic, Gut microbiome

1 Introduction

The use of modern techniques such as genomics and metabolomics has recently started to unravel the enormous genetic diversity and the metabolic complexity of the microbiota in the gastrointestinal (GI) tract. It is now known that the human GI tract consist of more than 100 trillion commensal bacteria and a large number of other microorganisms including viruses, fungi, protozoa and archaea [1–3]. Among these microorganisms, commensal bacteria form the predominant part of the gut microbiota and interacts intricately with the host to regulate the development and function of the GI tract. Study of microbial communities in humans poses a challenge because targeted microbial manipulation in humans is challenging, and it is difficult to uncouple the effect of microbes from host genetics and environment. Therefore, to better understand how microbiota affect GI function, gnotobiotic animal models are often used. The term *gnotobiotic* is derived from the Greek word "*gnotos*" and "*biota*" meaning known flora and fauna [4]. Gnotobiotic animal models are essential to translational medicine as they help deconstruct complex interactions and allow the study of the effects of specific bacteria on the host in a highly

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controlled experimental environment, as well as provide a "frame-of-reference" to understand the role of microbes in regulating host function.

1.1 Historical Perspective: Establishment of Gnotobiotics as a Tool for Studying Host–Microbial Interactions

Gnotobiotic experimentation for studying host–microbial interactions began with Louis Pasteur in 1855, when he postulated that life was dependent on microbial colonization [5]. To test whether germ-free (GF) life of an animal host is possible, Nuttall and Thierfelder raised guinea pigs under GF conditions for the first time at the University of Berlin [6]. The cesarean delivered GF guinea pigs appeared to be healthy but few striking differences such as decreased body weight and enlarged cecum (five to ten times the cecal volume relative to the conventional counterpart) were observed in GF guinea pigs [6]. The caecum in these animals was filled with "a brown liquid which contained cheese like coagula" [6]. Further study of the cecal contents in GF animals showed that it contains bioactive substances that are toxic to the animal and causes altered smooth muscle contractility [7]. These early observations led researchers to hypothesize that although GF life is possible, bacteria are essential for regulating host physiology including "normal" digestion in order to inactivate or reduce toxic substances produced by the host [6]. Subsequent studies suggest that bacteria not only help eradicate/neutralize toxic substances but also interact closely with the host to affect the development and function of the GI tract.

Rearing and maintenance of healthy GF animals was a challenging task due to technological constraints until mid-1900s. Studies with GF animals started systematically when Reyniers and his colleagues established academic organizations in mid-1940s and early 1950s devoted to understand host–microbial interactions [8]. By late 1950s, researchers were successfully rearing GF guinea pigs, mice, and chickens [9]. The availability of gnotobiotic animal model gave researchers not only the ability to compare GF animals with conventionally raised animals with a microbiota but also the ability to introduce one or few bacterial species at a time to understand host–microbial interactions in a simplified environment [10]. Utilizing the advantages these gnotobiotic animal models had to offer, scientists have examined various microbial species in GF mice to understand microbe–microbe interaction, gene–microbe interaction, diet–microbe interactions and factors affecting microbial colonization of the GI tract.

The development of tools such as specific culture media for different bacteria and incubation techniques were enormously helpful to enumerate the bacterial species present in the gut. However, given the fact that the vast majority of gut bacteria are unculturable it was insufficient to study the microbial ecology of the GI tract in detail [11]. Now with recent advancement in next generation sequencing technology, it is possible to investigate the gut microbial ecology in much greater detail. 16s rRNA based

microbial community sequencing [12] and metagenomic sequencing [13–15], has now revealed that there are about ~1000 different species of bacteria in the human and the mouse intestine [16] among which those belonging to the phylum Bacteroidetes and the Firmicutes are the most abundant [1, 16]. With the advent of next generation sequencing and advances in microbial ecology, the use of gnotobiotic models that started as an underutilized tool in the last century is now an invaluable resource to understand host–microbial interactions in health and disease.

1.2 GF Mice Model as a Tool for Studying Host–Microbial Interactions in Human Health and Disease

GF mice are gnotobiotic mice that are free from all forms of microbial life including bacteria, viruses, protozoa, archaea, and parasites. Different strains of laboratory mice serve as an important genetic tool to study host–microbial interactions for three main reasons. First, the genotypes of laboratory mice have been well characterized [17]. Second, mice and human genomes are 99% similar in gene function [18]. Third, transgenic mice unlike any other lab mammalian species, allows us to introduce precise genetic modifications (genetic addition, ablation and modulation) and examine of the effects of these modifications in a living organism [19]. The genotypic characterization and similarity with humans enables us to use mice as a model organism to understand how gene interacts within an organism, and help extrapolate these data to human biology [17], while ability to perform genetic modification facilitates the identification of genes participating in normal and disease pathways in order to help understand host–microbial interaction in health and disease [20]. The ability to rederive these genetically modified mice as GF further allows us to understand the effect of environment and the host genes on the microbial community, as well as the effects of microbial community on host gene expression, epigenetic changes and host physiology.

1.3 GF Mice as a Tool to Understand the Role of Gut Microbiome in Obesity

16s rRNA based microbial community profiling and metagenomic sequencing from humans and genetic mice models have revealed that the phyla Firmicutes and Bacteroidetes are the predominant part of gut microbiota [21, 22]. Interestingly, obesity has been associated with a change in Firmicutes to Bacteroidetes ratio [21]. In particular, obese mice have been shown to have 50% reduction in the abundance of Bacteroidetes and a significant concomitant increase in Firmicutes compared to lean mice [21, 23]. However, the studies that correlate disease phenotype with gut microbiota composition cannot determine if gut microbiota is driving the phenotype or an innocent bystander that changes in response to a disease. Gnotobiotic mice provide an ideal tool to address hypotheses generated from such studies to help investigate the role of gut microbiota in driving a disease phenotype.

As an example, Turnbaugh et al. colonized GF mice with microbiota from obese mice and found the microbiota transfer from obese mice led to greater adiposity in gnotobiotic mice implicating gut microbiota as one of the factors driving the obese

phenotype [22]. Turnbaugh et al. subsequently used a humanized mouse model (ex-GF mice colonized with human fecal microbial communities) and showed that humanized mice when fed a high-fat western diet shifts the structure of gut microbiota with increased representation of Firmicutes. Furthermore, transplantation of microbiome from these high-fat fed humanized mice to GF mice leads to increased adiposity in the recipient mice [24]. Together these studies suggest that the differences in gut microbial ecology in lean and obese individuals may in part be responsible for the metabolic disturbance. An area of interest in this regard is the metabolic potential (capacity to harvest energy from diet) of different microbial communities. Although the physiological contributions of increased Firmicutes to the intestinal ecosystem and to fuel partitioning are unclear in obesity, few studies have reported that certain members in Firmicutes could affect the metabolic potential of the host because they are highly enriched for glycoside hydrolases and polysaccharide lysases and help in efficient extraction of calories from otherwise indigestible common polysaccharides in the diet [22, 25]. Thus, the increased Firmicutes–Bacteroidetes ratio potentially creates a microbial mix that is highly efficient in extracting energy from diets and could potentially promote adiposity [22, 25]. In follow-up studies, Riduara et al. showed that gnotobiotic mice colonized with microbiota from an obese co-twin gain weight as compared to those colonized with microbiota from the lean co-twin [26]. Interestingly, microbes from mice associated with lean microbiome can invade the mice colonized with obese microbiome and prevent weight gain as long as mice were fed a healthy diet [26].

Bariatric surgery is one of the most effective therapies for medically complicated obesity [27]. Tremaroli et al. compared gut microbiota of patients who underwent Roux-en-Y gastric bypass to obese subjects who did not undergo surgery [28]. They found that the surgical procedures causes long term durable changes on the gut microbiota including a decrease in Firmicutes compared to control obese subjects [28]. The variation in microbial ecology corresponds to lower respiratory quotient and decreased utilization of carbohydrates in subjects who underwent Roux-en-Y bypass [28]. While this finding suggested a potential role of gut microbiota in mediating beneficial effects of bariatric surgery on the metabolic phenotype, the authors used a gnotobiotic mouse model to further investigate the relevance of this finding. GF mice were colonized with gut microbiota from patients that either underwent surgery or had no surgical intervention and interestingly microbiota from patients following bariatric surgery led to reduced fat deposition in recipient mice compared to microbiota from control obese subjects [28]. This suggests that the gut microbiome plays an important role in influencing metabolism and adiposity after a bariatric surgery and highlights the utility of using GF mice to investigate the role of gut microbiome in driving obesity.

1.4 GF Mice as a Tool to Understand the Effect of Microbiota on the Development and Function of the Immune System

The role of microbiota in development and regulation of the immune system has been extensively studied. Recent studies suggest that exposure to microbes early in life is essential for the proper development and function of the immune system [29–31]. This is especially true in the GI tract and gnotobiotic mouse model has been extensively utilized to show that commensal gut microbiota interacts with the host to enhance host immunity and defend against enteric pathogens.

Multiple studies have demonstrated that surface antigen and metabolic-end products of gut microbiota modulate immune system activation and production of cytokines [32–37]. Franchi et al. using a GF and conventionally raised mouse model showed that commensal bacteria modulate immune system and cytokine production by priming intestinal macrophages for pathogenic infection via upregulation in pro-IL-1β activity [33, 38]. This in turn leads to an increase in "mature" enzymatically active IL-1β production and ultimately causes neutrophil recruitment for pathogen eradication [33]. This observation is supported by the fact that neutrophil count and macrophage function such as phagocytosis and microbicidal activities including phagocytic superoxide anion production is lower in GF mice [39, 40].

Autoimmune disorders such as inflammatory bowel disease (IBD) and rheumatoid arthritis have been associated with alteration in gut microbial ecology [41]. In IBD, a reduction in Firmicutes and Bacteroidetes and a concomitant overgrowth of Proteobacteria has been observed [42, 43]. Although it is not well understood how the alteration in the gut microbiota composition (dysbiosis) results in inflammation in IBD, it is hypothesized that systemic $CD4^+$ T cell might play a role. In this regard, Mazmanian et al. showed that colonization of GF mice with *Bacteroides fragilis* directs the cellular and physical maturation of the developing immune system via a bacterial polysaccharide (PSA) mediated pathway and corrects systemic $CD4^+$ T cell deficiencies by restoring T helper 1 (T_H1; crucial for the host defense against microbial infection) and T helper 2 (T_H2; crucial for eliminating parasitic infections) balance as the GF immune response is biased towards T_H2 response [32]. Microbial fermentation end products such as short chain fatty acids have also been shown to regulate colonic regulatory T cells and protect against colitis in gnotobiotic mice [44].

Besides impacting T cell lineages, changes in microbiota also differentially impact microbial recognition by affecting pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors to cause disease phenotypes [33, 45]. Since, genetic knockdown of TLR receptors and NOD2 gene can contribute to development of inflammatory disorders such as IBD [46], and absence of gut microbiota impairs the development and production of TLR receptors [46, 47], it is possible that commensal microbiota play an important role in protecting against

inflammation by impacting the development of PRRs. To this end, GF mice have been used to show that recognition of commensal microbiota by TLR is required for maintenance of intestinal homeostasis [48], while recognition of NOD by intestinal microbiota is responsible for regulation of innate immunity [45, 47]. Overall, these studies highlight the utility of GF mice as a model to understand how microbiota can regulate development and function of different aspects of the immune system.

1.5 GF Mice as a Tool to Understand the Effects of Microbes on GI Physiology Including Motility and Secretion

The GI tract harbors a more diverse microbial ecosystem than any other part of the body. These bacteria can modulate the development of enteric nerves to influence colonic motility and secretion [49, 50].

1.5.1 GI Motility

GF mouse model has played an important role in evaluating the effect of gut microbiota on GI motility. Abrahams and Bishop in 1967, using a non-absorbable radioactive tracer found that GI transit time is significantly faster in conventionally raised mice as compared to GF mice (conventional mice passed >90% of radioactivity in feces within 16 h while it was less than 30% in GF) [51]. Subsequently, several investigators have shown introduction of mouse-derived or human gut-derived bacteria into GF mice alters GI motility and transit time [52, 53]. Recent work in this area has increased our understanding of the effect of gut microbes on the neuromuscular apparatus. Anitha et al. showed that gut microbiome interacts with enteric neurons via LPS mediated activation of TLR-4 in order to increase neuronal survivability and intestinal motility using gnotobiotic mouse model [50]. Furthermore, human or mouse-derived complex microbial communities introduced in GF mice have been shown to accelerate GI transit by increasing serotonin (5-HT) biosynthesis and release in the gut, an effect which can in part be blocked by systemic 5-HT antagonism [52, 54]. These studies highlight the utility of GF mice as a model to elucidate host–microbial interaction and how microbes modulate GI motility.

1.5.2 GI Secretion

Epithelial ionic and water secretion is an important physiological function of the GI tract. Gut microbiota regulates GI secretion possibly via 5-HT production [54, 55]. Since, 5-HT is a neurotransmitter that stimulates ion (bicarbonates and chloride) secretion in the colon to balance luminal fluidity [56–58], imbalance in 5-HT secretion caused by alterations in gut microbiome can potentially disrupt luminal fluidity and lead to dehydration of the feces and disruption of bowel movements, a hallmark of GI motility disorders.

Lomansey et al. reported similar response of colonic mucosa–submucosa preparations from both GF and conventionally raised

mice, to neural, epithelial and bacterial stimulation using Ussing chamber [49]. However, GF mice exhibit a heightened response to forskolin/cAMP response, suggesting that commensal gut microbes may influence colonic ion transport, via cAMP-mediated responses. Similarly bacterial toxins from pathogenic bacteria such as Cholera toxin, have been shown to irreversibly activate adenylate cyclase producing copious amounts of cAMP which ultimately results in continuous salt and water secretion [59]. This is yet another example of how GF mice are important to advance our understanding of effect of microbes on host physiology.

1.6 Limitations

GF animal model is very useful tool to study microbe–microbe and microbe–host interaction in health and disease; however, a few limitations have been noted. Previous studies show that GF mice have biochemical and physiological abnormalities which causes altered immune systems [32], mild chronic diarrhea [60], disrupted metabolism [61], and reduced reproductive abilities [62]. Although these observations raise some concerns, these changes likely represent normal physiology needed to survive in the GF state. The introduction of complex microbial communities in GF mice leads to changes in physiological parameters such that they resemble conventionally raised mice. This ability of GF mice to respond to introduction of bacteria suggests they are indeed a good model system to study the effects of microbes on host development and function. In fact, we know that babies start in a GF state and acquire a microbiota right before or at birth from the environment. This primary succession is somewhat similar to introducing microbial communities in GF mice. Thus, even though there are a few concerns as with any animal model, gnotobiotic mice serve as an important tool to understand host–microbe interactions in health and disease as well as a preclinical model to test microbiota directed therapies.

2 Materials

2.1 Setting Up a Gnotobiotic Facility

Flexible film isolators with isolator port, transfer sleeves, and HEPA filtered air inlets.

Autoclavable diets.

Sterilized tap water.

Shelves.

Autoclavable cotton bags for feed and bedding.

Sterilizing cylinder for autoclaving supplies.

Clidox®.

Tweezers.

2.2 Monitoring of GF Status

Fecal pellets.

Brain heart infusion (BHI) broth, Sabouraud dextrose, and nutrient broth (All prepared using manufacturers' recommendation).

1.5 ml sterile eppendorf tube.

Culture tubes.

Anaerobic chamber.

37 °C incubator.

Bunsen burner.

Sterile microscope slide.

Sterile swabs.

Reagents: Crystal violet, Gram's iodine solution, acetone–ethanol (50:50 v/v; decolorizing agent), 0.1 % basic fuchsin solution.

2.3 PCR

Fecal pellets.

Phenol–chloroform DNA isolation kit.

Go Taq.

dNTPs.

Taq Buffer.

PCR cleanup kit.

Nanopure water.

PCR machine.

Primers.

Universal bacterial 16S rRNA primers:

8F-5′-AGAGTTTGATCCTGGCTCAG-3′.

1391R-5′-GACGGGCGGTGWGTRCA-3′.

3 Methods

3.1 Setting Up a Gnotobiotic Facility

The advancement and standardization of gnotobiotic methods has led to rapid expansion of gnotobiotic facilities across the country given the relative ease of setting up a new facility. The facility however requires dedicated infrastructure in terms of space and personnel. A gnotobiotic facility can be set up in small spaces as long as it fulfills certain criteria such as but not limited to, restricted personnel access, adequate sound barrier, emergency power back up and central alarms, HEPA filtered air inlets and option for a positive pressure space. Within the facility mice are housed in flexible film isolators wherein factors such as temperature, humidity, pressure, air flow must be precisely controlled [63]. Food, water and other supplies must be appropriately sterilized using autoclave, irradiation or treatment with

disinfectants such as chlorine dioxide based sterilant Clidox®. Supplies can be introduced into isolators from autoclavable cylinders carrying dry or wet loads using transfer sleeves, which have to sterilized with a disinfectant prior to the transfer. Alternately non-autoclavable supplies can be placed in the entry port and disinfected prior to transfer inside the isolator. Similarly supplies or samples can be removed from the isolator using a similar protocol. In order to optimize functionality of a facility, mice are bred in larger isolators with shelving system to accommodate 18–20 cages whereas for experimental purposes, mice are transferred to smaller isolators, which can typically accommodate 4–5 cages. This reduces risk of contamination in a large colony due to specific experimental procedures such as special order diets, specialized equipment such as exercise wheels or introduction of specific bacteria (*see* **Note 1**). The facility should have a dedicated technologist who is responsible the day-to-day activities of the facility as well as periodic maintenance and appropriate tests on the mice.

3.2 Monitoring of GF Status

To access GF status three screening assays are typically used. These include anaerobic/aerobic liquid culture, Gram stain, and PCR using universal and specific 16S rRNA bacterial primers. Recent studies however suggests that in practice bacterial culture and Gram stain are adequate for screening GF status as they both offer high sensitivity and specificity as opposed to PCR which although offers high specificity but has lower sensitivity [64].

3.2.1 Liquid Culture

Liquid culture is a routinely used method to detect GF conditions. However, since cultures can easily be contaminated, precautions must be taken to avoid potential loss of time and animals [64].

Pellets are collected from GF mouse in a sterile eppendorf tubes and transferred into the culture tubes containing nutrient broth, Sabourad dextrose, or BHI broth. One set of tubes is stored in anaerobic chamber while the other set is incubated in an aerobic incubator maintained at 37 °C to detect anaerobic and aerotolerant bacteria respectively. The culture tubes are checked every day for 7 days. Clear culture tube is indication of GF conditions while cloudy tube is an indication of bacterial contamination. Although bacterial culture is a sensitive measure, it might still miss some unculturable and or species that show poor growth.

3.2.2 Gram Stain

Besides culture, Gram stain is also routinely used as an inexpensive tool to detect contamination in GF mice. However, unlike culture, Gram stain can used to screen unculturable bacterial species contamination, provided they are present in large quantities in the intestine [64].

For Gram stain, fecal material is thinly spread over a surface of a sterile glass slide, air-dried, and heat fixed. Subsequently crystal violet stain is added over the fixed slide and allowed to stand for 30 s.

The stain is then rinsed with a stream of sterile water, followed by iodine solution, decolorizer, and basic fuchsin solution. Basic fuchsin is finally washed with water and the slides are later air dried and examined under a microscope. Presence of purple or pink staining is an indication of gram-positive and gram-negative bacteria respectively.

One of the major concerns at present is that positive Gram stain could be due to dead bacteria present in autoclaved/sterilized diet. A recent study however suggests that only very few to no dead bacteria are detected in feces of GF mice [64] (*see* **Note 2**).

3.2.3 PCR

GF status can also be verified by checking the presence of 16S rRNA bacterial gene in mice fecal pellet using universal bacterial primers.

A typical PCR cycle used is:

Initial denaturation at 94 °C for 2:00 min.

35 cycles of denaturation, annealing, and extension at

94 °C for 1:00 min,

55 °C for 0:45 min,

72 °C for 2:00 min, respectively.

Final extension at 72 °C for 20:00 min.

3.3 Rederivation

GF mice can be rederived either via embryo transfer or via hysterectomy and fostering to an axenic mother [65]. To perform embryo transfer, embryos are collected from ovulated females and washed to prevent pathogen contamination. These embryos are then transferred surgically into the uterus of surrogate axenic mother kept in a GF isolator. To perform hysterectomy, uterus from donor strain is removed by a sterile surgical technique and passed through a tank containing germicide. The fetuses are then removed from the uterus in GF isolators and placed on heating pads. These pups are adopted by foster mother whose pups have recently been removed.

4 Notes

1. In order to prevent loss of a colony of valuable mouse strains, which were rederived as GF, it is helpful to keep mice in two separate isolators so that the mouse strain can be expanded again in an event of a contamination.
2. In some instances where few bacteria or "bacteria-like particles" were present they were below the detection limit and did not interfere with the specificity of the assay [64].

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