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Review

Use of Germ-Free Animal Models in Microbiota-Related Research

Maha Al-Asmakh[1,2*] and Fahad Zadjali[3]

[1]*Department of Health Sciences, College of Arts and Sciences, Qatar University, P.O. Box 2713, Doha, Qatar*
[2]*Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, 171 77 Stockholm, Sweden*
[3]*College of Medicine and Health Sciences, Sultan Qaboos University, 123 Alkoudh, Oman*

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*Corresponding author
Phone: +974-44034782;
Fax: +974-44931351;
E-mail: maha.alasmakh@qu.edu.qa

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The large intestine is a home for trillions of microbiota, which confer many benefits on the host, including production of vitamins, absorption of nutrients, pathogen displacement, and development of the immune system. For several decades, germ-free animals have been used to study the interaction between the host and its microbiota. This minireview describes the technical aspects for establishing and maintaining germ-free animals and highlights the advantages and disadvantages for germ-free animals as experimental models.

Keywords: Germ-free animals, germ-free mice, microbiota, gnotobiotic, isolator technology

Introduction

Accumulating evidence reveals that the gut microbiota play a major role in promoting health, as a result of which it is often referred to as the "forgotten organ" [20]. These microbiota are key factors in maintaining homeostasis, with functions affecting virtually every organ in the body, such as the regulation of bone mass [24], brain development and behavior [1, 5, 7], hepatic function [4], and aspects of adipose tissues [18] and the cardiovascular system [27]. In mammals, microbial colonization starts *in utero* by the maternal microbiota and is influenced thereafter by the mode of birth and type of infant feeding and exposure to antibiotics [21, 22]. Consequently, the microbiota are heterogeneous and unstable until approximately 2–4 years of age, when it becomes more stable and begins to resemble the adult microbiota [6].

Germ-free (GF) animals provide an invaluable experimental tool for examining interactions between a host and its microbiota. The term germ-free (axenic) refers to an animal demonstrably free from microbes, including bacteria, viruses, fungi, protozoa, and parasites, throughout its lifetime [29, 30]. GF animals selectively colonized with one or more bacterial species are referred to as gnotobiotic [8, 25] (a term sometimes used synonymously with GF). This term is derived from the Greek "gnotos," meaning known, and "bios" which means life [3, 29].

Historical Aspects of GF Experimentation

The concept of a germ-free animal was recognized more than a century ago by Louis Pasteur (1885), although he concluded that bacteria-free existence is impossible. Ten years later in 1895, Nuttle and Thierfelder at Berlin University produced the first GF animals (guinea pigs), which survived for as long as 13 days. However, owing to the lack of knowledge concerning nutrition, it took 50 more years until the first GF rat colonies were established in the late 1940s. Subsequently, the first GF mice were successfully developed by Pleasants in 1959 [28−30].

At first, GF animals were housed in stainless steel isolators (Fig. 1) designed by Professor Bengt Erik Gustafsson (1959), a pioneer in the design of equipment and procedures for producing GF rats [12]. However, these stainless steel isolators are very heavy, expensive, limit the field of view and are not flexible. Therefore, flexible plastic isolators are more commonly used now to house GF animals [3] (Fig. 2).

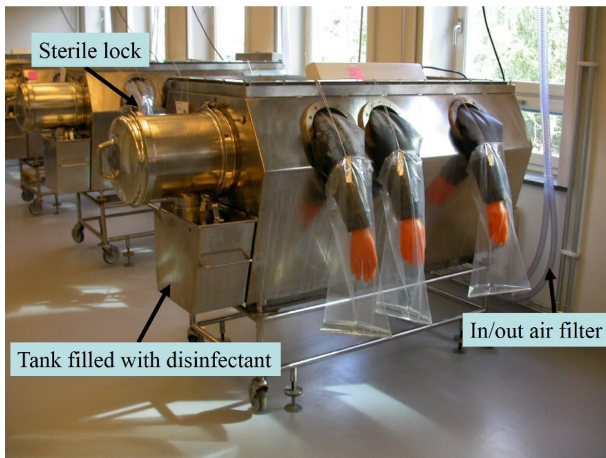

Fig. 1. Gustafsson steel isolator.

Isolator Technology

Isolators provide physical barriers that allow creation of a sterile environment. These devices have 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 (Figs. 1 and 2). Maintaining an isolator is very laborious work and requires special training. All manipulation of mice and supplies occurs inside the isolator through gloves and sleeves attached to the isolator walls. In terms of potential contamination, the gloves are most vulnerable, and the most common cause of contaminations are due to holes in the gloves.

Bedding, food, water, and equipment, including cages, must first be sterilized (autoclaved) and are then put into the isolator through the so-called sterile lock. Sterilization of entire steel isolators is accomplished by autoclaving the whole isolator, as well as with portable vacuum and steam equipment. In the case of plastic isolators, which cannot tolerate the heat of steam sterilization, sterilization is accomplished with germicidal vapor (2% peracetic acid and chlorine dioxide). Air is sterilized upon entry and exhaust by mechanical filtration under positive pressure. The transfer of animals in and out of the isolator is usually carried out *via* autoclave jars (Fig. 3).

Colony maintenance and experimentation using a GF

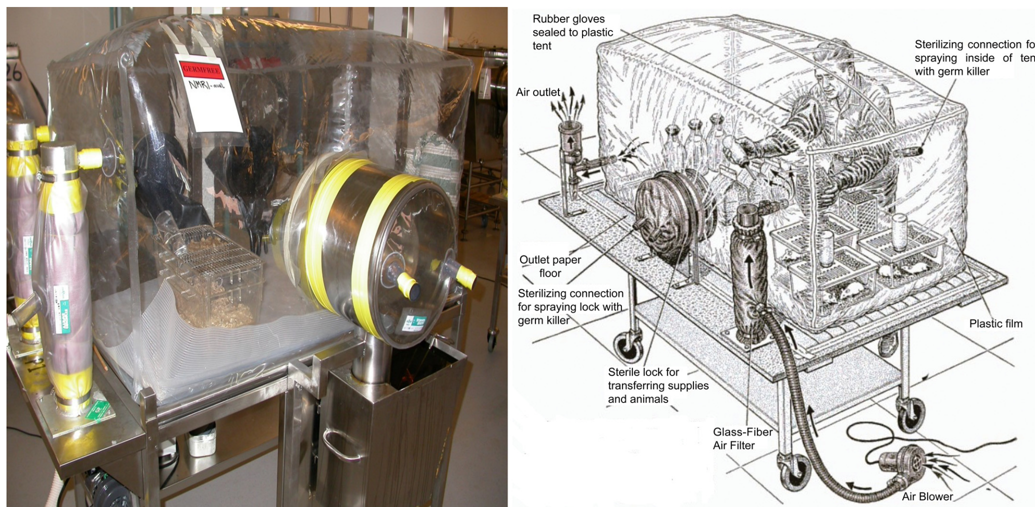

Fig. 2. Composition of the plastic isolator.

Fig. 3. Transfer of mice from inside the isolator.

The mouse is placed in an autoclaved glass jar and transferred through a sterilized lock into the tank filled with disinfectant.

environment is technically challenging. Germ-free animals can be contaminated very easily. The common practice is to separate multiple mouse strains and multiple inoculation experimental groups in separate isolators [3], altogether increasing the cost and space for such experimentation. Transfer of GF mice from isolators to positive-pressure isocages has recently been shown to be cost and space-effective for short-term experiments [13]. The use of a positive-pressure isocage for long-term maintenance of GF mice needs further optimization.

Establishment of GF mice

Establishment of new strains of GF mice requires that the fetus remains sterile in the uterus. The pups are most commonly delivered by sterile Caesarean section and then transferred while still in the uterine sac to a GF foster mother (Fig. 4). Thereafter, it is relatively straightforward to maintain and breed colonies of GF mice in isolators with free access to autoclaved food and water [14, 25]. It is not advisable to use the first generation of GF mice for experiments, since their mother was not GF, and virus, bacteria, and bacterial metabolites can be transmitted transplacentally from the mother to the fetus. The GF status of the mice should be monitored regularly by culturing fecal samples for aerobic/anaerobic bacteria and fungi and by 16S PCR testing for bacteria that cannot be cultured [25].

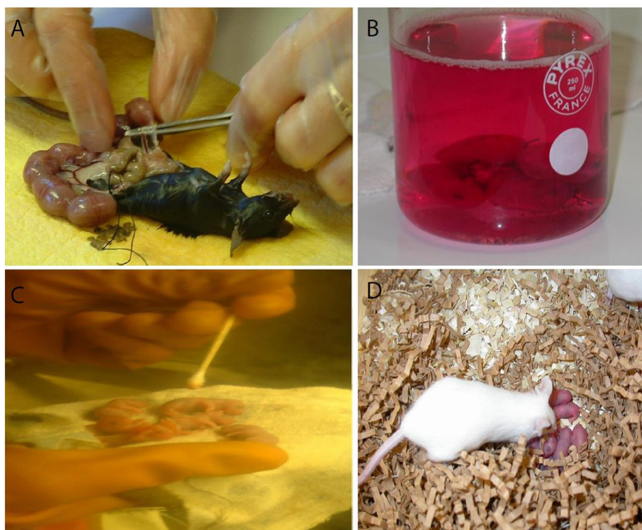

Fig. 4. Establishment of GF mice by Caesarian section.
(**A**) The uterine sac is removed and clamped together at the top of each horn and at the base close to the cervix. (**B**) The uterine sac is placed in a glass jar containing desinfectant. (**C**) The uterine sac is transfered to the isolator, where it is opened, and the pups are removed, cleaned, and stimulated to breath. (**D**) The pups are introduced to the GF foster mother.

Establishment of the Control Group for GF Mice

GF and gnotobiotic mice are compared to the specific pathogen-free (SPF) animals, which are free from known pathogens that cause clinical or subclinical infections that can bias research findings [25]. Although SPF mice are usually housed in special rooms, for reliable comparison they should be housed in the same environment as the GF mice (*i.e.*, also in isolators), but this is seldom done because isolators are too expensive.

SPF mice should be screened and tested for pathogens, as recommended by the Federation of Laboratory Animal Science Associations [19]. It is important to note that SPF animals are normally colonized with commensal bacteria, but the diversity and type of colonization are rarely known with any accuracy. To achieve balanced and identified colonization, commercial breeders and animal facilities tend to expose SPF mice to the modified Schaedler flora, containing eight species of bacteria, five belonging to the genera *Clostridium*, *Eubacterium*, and *Bacteroides*; one a spirochete from the *Flexistipes* group (*Mucispirillum schaederli*); and two *Lactobacillus* species [25].

Anatomical and Physiological Characteristics of GF mice

If their diet is supplemented with vitamins, including K and B, GF animals are viable and healthy. However, these animals show a number of important developmental and physiological differences in comparison with SPF animals. For example, the cecum is enlarged by 4–8-fold, due to the accumulation of mucus and undigested fibers. This is in contrast to other GF animals, including dogs, pigs, sheep, goats, and chickens, which due to the anatomy of the junction between their small and large intestines show little or no such enlargement. When body weight is corrected for cecal weight, adult GF rodents weigh less than their SPF counterparts [29].

Moreover, the small intestine of GF rodents is less developed, with a considerably smaller surface area, slower peristalsis, irregular villi, and reduced renewal of epithelial cells. Consequently, the ability of GF animals to utilize nutrients is compromised. Interestingly, GF rats live longer and develop spontaneous cancers less frequently than do SPF rats [29]. GF animals are also more prone to infections and have altered immune systems. Additional

Table 1. Anatomical and physiological features of germ-free mice that differ from those of specific-pathogen-free and wild-type mice [15].

Characteristic	Difference
Nutrition	Requirement for vitamins K and B in diet
	Decreased percentage body fat
	Normal or increased food intake
Fluid balance	Increased intake of water
Metabolism	Decreased basal metabolic rate
	Increased secretion of free amino acids and urea and little excretion of acetic acid
	More urea and little ammonia in intestinal contents
	More nitrogen in the cecal contents and feces
	Elevated oxidation-reduction potential of the cecal contents
	Altered response to anesthetics
Circulation	Reduced total volume of blood
	Decreased cardiac output
	Decreased blood flow to skin, liver, lungs, and digestive tract
	Increased cholesterol level, numbers of red blood cells, and hematocrit in blood
Liver	Reduced size
	Increased levels of ferritin and cholesterol
Lungs	Thinner alveolar and capsular walls
	Fewer reticuloendothelial elements
Intestinal morphology	Reduction in total intestinal mass
	Decrease in the total surface area of the small intestine
	Slender and uniform villi of the small intestine
	Shorter ileal villi and longer duodenal villi
	Shorter crypts of the small intestine
	Lamina propria of the small intestine thinner, with fewer cells and slower cell renewal
	Larger cecum with a thinner wall
Intestinal motility	Increased muscle tissue, with elongated and hypertrophied muscle cells in the cecum
	Longer transit time
Intestinal physiology	Reduced osmolarity in the small intestine
	Elevated oxygen tension and electropotential in the small intestine
Intestinal function	Enhanced absorption of vitamins and minerals, alterations in the absorption of other ingested materials
	Altered enzyme content, elevated levels of typsin, chymotrypsin and invertase in the feces
	High levels of mucin (mucoproteins and mucopolysaccharides) in the feces
	Less fatty acids and no cyclic or branched-chain fatty acids in the intestinal content, excretion of primarily unsaturated fatty acids
Endocrine function	Less uptake of iodine by the thyroid
	Decreased motor activity and hyperresponsiveness to epinephrine, norephinephrine and vasopressin
Electrolyte status	More alkaline cecal contents
	High levels of calcium and citrate and little phosphate in the urine
	Somewhat less sodium and low levels of chloride in the intestinal content

differences between SPF and GF mice are presented in Table 1.

Conclusions: Advantages and Disadvantages for GF Mice as Experimental Models

Murine models provide excellent tools to study microbiota-associated human diseases. Germ-free animal models have been used to explore host-microbiota interactions in entire fields, including neurogastroenterology [1, 7, 16, 17], cardiology [27], reproductive biology [2, 23], lipid metabolism [18], and bone homeostasis [24]. GF and gnotobiotic mice are valuable experimental tools for examining host-microbe interactions, since monocolonization of single bacteria is achievable. Furthermore, genetically modified mice can be made germ-free in order to study

interactions between any particular gene and the microbiota. Inoculation of human gut microbiota into GF mice, humanized gnotobiotic models, allows recapitalization of the human microbiota phylogenetic composition [9]. These models provide powerful tool in understanding the cause and effect of gut microbiota in the human-like system.

The major questions concerning host-microbe interactions include how colonies of microbiota are established and maintained, how these affect their host, how the host shapes the populations of microbiota, and how the microbiota influence the development of diseases. However, information obtained by comparing GF and SPF mice cannot be directly applied to humans, and it often remains uncertain whether a disruption in the microbiota associated with a disease in humans is a cause, contributing factor, or merely a consequence of the disease state. Although such comparisons provide hints concerning the pathogenesis of diseases such as cancer, cardiovascular disease, diabetes, and multiple sclerosis, the underlying mechanisms remain unknown and, as a result, GF findings can seldom be readily translated into treatments and/or prevention.

Several factors could contribute to this failure. One caveat is that several bacterial species that colonize the murine gut are not found in humans. Furthermore, the distinct physiology and anatomy (including skin, fur, orapharyneal structures, and compartmentalization of the GIT) and behavior (*e.g.*, coprophagia) of mice will undoubtedly influence microbial communities [8, 10, 11, 15, 26].

Despite these pitfalls, the GF mouse remains the most powerful model system for studying host-microbe interactions.

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