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CHAPTER

21

Gnotobiotics and the Microbiome

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PROLOGUE

This chapter is dedicated to the memory of two giants in the modern era of gnotobiotics who were lost to us since the last edition of this chapter: Philip C. "Trex" Trexler, creator of the eponymous plastic isolator, which brought gnotobiotic technology into broad use, and Henry "Hank" Foster, founder of Charles River Laboratories and a past author and coauthor of this chapter.

In the more than a decade since the publication of the second edition of *The Laboratory Rat*, the explosion of interest in the field of the host animal's microbiome[1] has fueled expansion of work in the field of gnotobiology. This current growth of research interest has been almost exclusively through studies using laboratory mice. To a great extent the work of Jeffrey Gordon, his colleagues and students at Washington University-St. Louis used the techniques created through the Human Genome Project to move the study of the host microbiome from classical bacteriological characterizations to the gene level (Gordon, 2012). This approach has permitted the study of noncultivable microbes. The Gordon laboratory used a variety of mammals, and even germfree zebrafish (*Danio rerio*) (Rawls et al., 2004), but the great part of their studies has been with gnotobiotic mice. The use of laboratory rats in gnotobiology has diminished significantly over the same time period and decades-old laboratories using and maintaining germfree rats have closed. This trend may reverse itself in the not too distant future, so a new section at the end of the chapter, titled "Resources," has been added to provide current information to researchers choosing to use gnotobiotic rats at some future point.

Special attention should be given to the form of the references provided for the 19th century publications. Through decades of authors who were unable to access the original publications and thus reference them through secondary sources, the information over time often became terribly corrupted. Through the resources made available by the Karolinska Institutet, Stockholm, the original German papers were accessed for the previous edition of this chapter. Readers should be confident that the reference information, primarily to the work of Nuttall and Thierfelder, is accurately presented.

I. INTRODUCTION

A. History

The earliest description of research involving gnotobiotic or germfree[2] animals, *Thierisches Leben ohne Bakterien im Verdauungskanal* ("Animal life without bacteria in the digestive tract"), by George H. F. Nuttall and H. Thierfelder working in Berlin, dates back to the late 19th century. These animals, maintained in the most rudimentary devices (Fig. 21.1), were first guinea pigs (Nuttall and Thierfelder, 1895, 1896), then chickens

[1] The term "microbiome" is used in replacement of the term "microflora" of older editions of this chapter since the former term is the more correct, encompassing all viable organisms, and also reflects currently accepted usage.

[2] Germfree or germ-free (the latter is the more grammatically correct but most authors use the former, some using both in different papers as can be observed in the References), gnotobiotic, and axenic will be discussed in Section 1.B.

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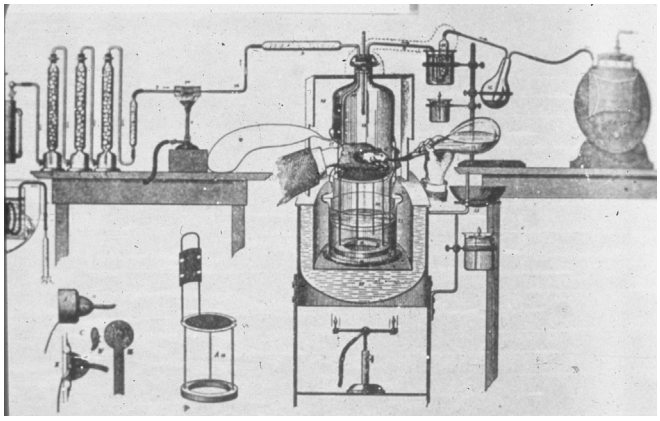

FIGURE 21.1 Early (c. 1897) isolator of the type used by Nuttall and Thierfelder. *Courtesy University of Notre Dame attributed to Nuttall and Thierfelder, 1895.*

(Nuttall and Thierfelder, 1895) (they had considered using the latter first but were concerned about reports of in ovo infections), and then other mammals by later investigators. Significant advances in the production, use, and characterization of germfree animals did not occur until the 1930s, and was virtually simultaneous at the University of Notre Dame in Indiana by James A. "Art" Reyniers and coworkers (Fig. 21.2) and by Bengt Gustafsson (Fig. 21.3), his professor, E. Gösta Glimstedt, and colleagues at the University of Lund, Sweden (later moving to the Karolinska Institutet in Stockholm). These groups later reported the establishment of the first germfree rat colonies (see Gustafsson, 1948; Carter, 1971 for a review of the early work). Interest in gnotobiotic science and technology appeared later in Asia with the work of Masazumi Miyakawa (Fig. 21.4) and colleagues at Nagoya University, Japan.

FIGURE 21.3 Bengt Gustafsson. *Courtesy Gustafsson Family.*

Since that time there have been major advances in methodology, which have facilitated the breeding and utilization of gnotobiotic rats in a continually expanding spectrum of biomedical research. A literature survey for recent decades would yield thousands of references on germfree rats and their gnotobiotic and disease-free descendants. The vast majority of these research reports are concerned with other experimental uses of these animals rather than their derivation, rearing, and establishment. The scientific literature contains reviews specific for the germfree laboratory rat by Pollard (1971a),

FIGURE 21.2 J. Arthur Reyniers, Jr. (*left*) with colleagues Philip Trexler (*center*) and Robert Ervin (*right*). *Courtesy University of Notre Dame.*

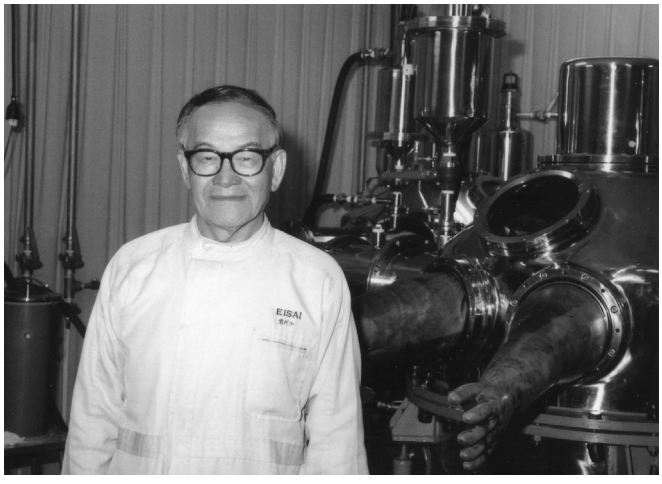

FIGURE 21.4 Masazumi Miyakawa. *Courtesy of Professor Sakakibara.*

Maejima et al. (1974), and Miyakawa (1968), while more current publications by Coates and Gustafsson (1984) and Wostmann (1996) provide a general overview. The Website of the Association for Gnotobiotics (2020) provides regularly updated information on resources available as well as information on scientific and technical conferences (see Section IV). This useful site also provides links to the Websites of allied organizations. A listserv for direct information exchange is maintained at the University of Alabama-Birmingham (see Section IV).

The quantum jump in recent decades in the use of these animals was facilitated in large part by the introduction of the Trexler flexible plastic film isolator system in 1957 (Trexler and Reynold, 1957). This innovation greatly reduced costs while simultaneously increasing design flexibility. Prior to the late 1950s, germfree research was conducted in rigid isolators made of stainless steel or steel and glass (Fig. 21.5). The most commonly used type consisted of a stainless-steel cylinder bolted together and gasketed at the joints. Many of these systems contained a steam autoclave as a pass-through lock. These isolator systems were heavy, cumbersome, very costly, and occupied a large amount of floor space, since their weight prohibited placing them in tiers. The advent of the plastic isolator not only reduced the cost to one-tenth to one-fifteenth of the cost of steel isolator systems, but allowed units to be stacked in tiers of two and three in a multitude of sizes and configurations to conserve floor space (Fig. 21.6). This marked the birth of a new era permitting vast expansion in the production and availability of gnotobiotic animals. It also paved the way for the development of lightweight, disposable shipping units that made the transport of gnotobiotic animals to distant locations a practical reality.

Finally, of some historical interest in this past year (2019) of the 50th anniversary of the landing of the first humans on the surface of the moon is the fact that germfree LOBUND (Laboratories of Bacteriology, University of Notre Dame) rats were used a few years earlier by a NASA laboratory in Houston, Texas, to examine "moon dust" for infectious agents (M. Pollard, director of LOBUND, personal communication, c. 1967). It was considered the most reasonable approach in attempting to assess possible threats to astronauts scheduled to land on the moon as well as to people on earth working with the specimens. It was only after the live animal and other tests proved negative for infectious agents that the moon specimens (popularly referred to as "moon rocks") were released to museums for view by the public. The germfree laboratory rat was chosen as the preferred test animal because it is not infected by any known infectious agent transmitted either horizontally or vertically (unlike the laboratory mouse, which carries a vertically transmitted leukemia virus). Rats from LOBUND were used because of the high confidence that the animals were of a pedigree known to be free of foreign agents, without accidental contamination, over a period of years.

FIGURE 21.6 Gnotobiotic facility utilizing flexible film isolators. *Courtesy Charles River Labs.*

FIGURE 21.5 Stainless-steel isolators. *Courtesy University of Notre Dame.*

B. Terminology

The word gnotobiotic is derived from the Greek words *gnotos* and *biota* meaning known flora or fauna. Therefore when referring to gnotobiotes, one refers to an animal with a known flora or fauna. This term is also applicable when a microbial flora does not exist or is not detectable. In other words, gnotobiotic is the broad term encompassing axenic, germfree, and defined flora/fauna-associated animals (Luckey, 1963).

The general review of gnotobiotics by Pleasants (1974) defines a gnotobiotic animal as follows:

> One of an animal stock or strain derived by aseptic caesarian section or sterile hatching of eggs that is reared and continuously maintained with germfree technics under isolator conditions and in which the composition of an associated fauna and flora, if present, is fully defined by accepted and current methodology.

Axenic animals are gnotobiotes known to be free of all detectable microorganisms. This term is often used synonymously with germfree, although the latter is more commonly utilized. The detection of leukemia virus particles in mice by Pollard (1972) raises the question of whether axenic animals exist at all, although these endogenously transmitted viruses have not yet been detected in cesarean-derived rats. The ability to achieve this state may be limited by the inability to exclude endogenous viruses integrated within the host genome. At present, animals are accepted as axenic when they are free of bacteria, fungi, and metazoan organisms by routine sampling. These animals are also expected to be free of detectable exogenous viral pathogens using standard diagnostic tests for rodent infectious agents.

Germfree is the historical term utilized over the longest period of time and is part of the colloquial scientific language, especially in North America. Its definition is the same as axenic, and even though those working within the field prefer axenic as being more accurate, germfree continues to remain the more popular term.

Specific pathogen-free (SPF) animals are those from which a defined set of microorganisms, usually pathogenic bacteria, fungi, parasites, and exogenous viruses, are excluded (Festing and Blackmore, 1971; Treuting et al., 2012). Designation of animals as SPF carries the connotation that they are housed in a facility where entry of new animals is restricted through quarantine and testing or rederivation, husbandry practices are used to combat pathogen entry, and pathogen monitoring is conducted to ensure the maintenance of SPF status.

This term causes confusion because some facilities exclude microbes that others do not. Furthermore, large research institutions may have multiple facilities or sections of facilities where exclusion lists differ. Efforts to standardize testing and reporting of rodent infectious agents have been under way (Mähler et al., 2014). To add to this confusion, the term SPF is often used in the scientific literature to refer to animals housed under undefined standard husbandry conditions or those with an undefined, complex animal facility microbiota. This is done despite known differences between the husbandry practices of animal facilities and the microbiota of research animals obtained from different sources (Ericsson et al., 2015; 2018; Ivanov et al., 2009).

Cesarean-derived, cesarean-derived and barrier-maintained, and cesarean-originated and barrier-maintained or sustained are terms that imply an initial derivation of axenic animals and their subsequent association with a defined microflora (i.e., defined flora, DF) followed by the continuing maintenance within a controlled barrier where all materials entering the barrier are subjected to a procedure that removes or destroys pathogenic microorganisms. While these terms carry important historical relevance, embryo transfer-based methods are now most commonly utilized for derivation of breeding stock by vendors. These animals may be reconstituted with defined microbial communities (i.e., altered Schaedler flora) at birth. Whether derived by cesarean section or embryo transfer, progeny so generated are often marketed as harboring restricted or defined microbiota. It is noteworthy that rodents produced in this manner may be free of microbes associated with opportunistic infections, such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Proteus* spp., and beta-hemolytic *Streptococcus* spp.

Pathogen-free is incorrectly used loosely and interchangeably with SPF, since both categories are implied to be free of pathogens. Some argue that it may be theoretically possible to maintain animals free of pathogens through testing and eradication as well as through the use of broad-spectrum antibiotics (van der Waaij et al., 1971).

Conventional animals are all other animals maintained under accepted husbandry practices but which do not fall within any of the previously described definitions. To some working in the field of gnotobiotics, animals are either gnotobiotes or conventional. Today, the largest category of research animals falls in between gnotobiotic and noncontainment, conventionally raised animals.

For the purposes of this chapter the following abbreviations will be used and reflect the terminology most commonly referred to by the respective authors: GF, germfree, a gnotobiote without any introduced microbes; DF, defined flora/fauna gnotobiotes, those having introduced, defined organisms; GN, gnotobiote (either GF or DF); SPF, specific pathogen-free; and CV, conventional. This is summarized in Table 21.1.

TABLE 21.1 Summary of Terminology.

Axenic	"Without strangers" (preferred)
Germ-free or germfree (GF)	Common usage for axenic animals
Gnotobiotic (GN)	"Known life"
Defined flora (DF)	Gnotobiotes colonized with known microbes
Pathogen-free	Animals lacking all known pathogens
Specific pathogen-free (SPF)	Animals lacking specific pathogens
Conventional (CV)	Animals raised in open environments

II. THE GERMFREE AND DEFINED FLORA LABORATORY RAT

One of the main advantages of using GF and DF laboratory rats in biomedical research is that the nutrition and physiology of many such colonies and strains have been well established. They have been used extensively, for example, in metabolic experiments. These animals are quite prolific in the isolator environment, notwithstanding the greatly enlarged cecum, which is thought to impair reproduction in GF guinea pigs.

There are many research areas where the investigator utilizing microbiologically sterile animals can elicit information that cannot be obtained using animals with normal flora. These research areas have included nutrition, immunology, infectious diseases, and dental caries studies. It is probably a lack of training and confidence in gnotobiotic technology on the part of investigators that limits more extensive use of GN animals, though this can be addressed through the establishment of core facilities. Technicians in the field of gnotobiotics typically receive specialty training from colleagues or those already knowledgeable and successful in maintaining these animals. Staff are selected for their strong understanding of rodent husbandry and attention to detail. While formal education is not required, it is important that employees understand the rationale behind procedures performed and commit to repeating them thoughtfully and without deviation.

The other major uses and importance of GN rats are as nucleus seed stocks for the production of disease-free animals and as diagnostic tools for infectious disease studies, particularly in situations where routinely used culture media are inadequate. In the middle 1950s major laboratory animal breeders reported the use of GN rats as foster nursing stock for the rederivation of breeding colonies (Foster, 1959b). It became apparent to the laboratory animal breeding industry that testing, eradication, and selection techniques for the elimination of infectious diseases and parasites were often inconclusive as well as tedious and not totally reliable. Therefore as a natural evolution of the technology developed for the production of GN animals, the latter became the building blocks and nucleus stock for the production of microbially associated DF and disease-free animals. This was accomplished by introducing a known microbiome to GF animals, placing them in a barrier, and maintaining them in an environment that precluded the entry of pathogenic organisms.

When a clinical syndrome or a set of pathological findings fail to elicit an etiologic agent by routine microbiologic techniques, the GF rat provides an excellent model for transmission and diagnostic studies. It provides the almost perfect model to establish Koch's postulates, since the use of this definable animal model frequently assures valid results in the determination of specific etiologic agents, i.e., the effect of a single organism can be evaluated, in the absence of other microbial forms.

A. Derivation Philosophy

Although embryo transplantation technology (Smithies, 2007) is used by some commercial suppliers and others (Inzunza et al., 2005) to quickly derive new strains of GF rodents or those that have been genetically manipulated, the primary method of deriving GF rats for noncommercial operations is through surgical intervention of pregnancy at term. Another technique has been reported whereby the gut tract has been rendered sterile through the successive use of a variety of broad-spectrum antibiotics. Van der Waaij et al. (1971) reported that mice have been rendered GF within sterile isolator systems. The presumed advantages are the rapidity with which this regime can be accomplished as opposed to the traditional and proven method of cesarean derivation. For certain types of studies of short or medium duration, animals can be freed from viable microorganisms and maintained within an isolator utilizing gnotobiotic techniques. Understandably, such a decontamination approach would have limited use since it raises questions about the continued presence of microorganisms undetectable by current technologies.

In spite of the previously mentioned success using embryo transfer to obtain GF mouse lines (Inzunza et al., 2005), those researchers found the traditional cesarean delivery, the classic method of deriving axenic animals, to be preferred (Norin, 2019). Since this approach is still widely used in research institutions (Carter, 1976), academic or private, and by small and large commercial lab animal suppliers, it is described in detail below. Furthermore, embryo transfer is predicated on a recipient dam of transferred embryos to be, itself, GF.

Early workers delivered GF rats in stainless-steel GF tanks where visibility was possible only through small viewing ports. The surgical technician's movements were restricted by the rigid steel isolator, even though there was sufficient mobility to perform the cesarean section. Since the weakest member in any GF system is probably the rubber sleeves and gloves, it was established in the 1950s that flexible film polyethylene or polyvinyl chloride isolators afforded at least the same degree of microbiological security with the same type of neoprene sleeves and gloves (Trexler and Reynold, 1957; Trexler, 1959) used in the earlier rigid systems.

Therefore since the late 1950s the most common procedure for cesarean delivery of GF rats is utilization of a 1.5×0.6-m flexible film isolator fitted with at least two pairs of neoprene sleeves fitted to 22.9-cm glove ports

attached to the isolator wall. In addition, standard surgical gloves are affixed to the wrists of the rubber sleeves to permit maximum tactile sensitivity. There is a 30.5- or 46-cm transfer port in the isolator for the introduction of supplies and instruments. An additional port is installed at one end, which is attached to a long tapered sleeve or a rigid clear plastic tube approximately 7.6 cm in diameter. This sleeve or tube terminates beneath the surface of a liquid germicide trap filled with a warm, 38°C, chlorine solution (a 0.525% solution of sodium hypochlorite, which can be prepared by a 1:9 dilution of classic bleach solution that most often comes as a 5.25% solution of sodium hypochlorite). A thermostatically controlled electric heating pad is placed between the exterior floor of the isolator and the rigid surface supporting the isolator. This provides warmth to the neonates after delivery. Within the sterile system, in addition to the required surgical instruments, sponges, and water, a plastic cage 35.5 cm long, 30.5 cm wide, and 14.5 cm deep is fitted with a taut Mylar membrane (a product of Dupont Co., East Orange, New Jersey), which provides a work area for the surgeons and which can be replaced after each procedure with a new membrane. This arrangement permits the uterus and fetal membranes to drop to the floor of the plastic cage.

B. Cesarean Methods

Delivery of GF rats can be accomplished by a two-stage hysterectomy technique or by a single-stage hysterotomy procedure (Foster et al., 1967b; Wostmann, 1970; Pollard, 1971a). In the latter procedure a plastic Mylar membrane in the floor of the isolator is sealed to the shaved and surgically prepared abdominal wall of the pregnant rat. The surgical technician performs the hysterotomy through the Mylar membrane window in the isolator floor. This method is more tedious than the preferred and more rapidly performed hysterectomy. In addition to speed, the hysterectomy method permits an almost mass production routine, since the two stages can be performed simultaneously by separate surgical teams. One team is responsible for the extra isolator phase, which consists of preparation, euthanasia, hysterectomy, and introduction of the uterus into the sterile surgical isolator. Another team of usually two technicians performs the actual cesarean, removing the fetuses from the uterus and its membranes. With proper planning and coordination, the two surgical teams can perform six to eight cesareans in one hour.

C. Derivation Procedure

A vital key to successful cesarean delivery of GF rats is the assurance that the pregnant rat has completed the normal gestation period of 20–21 days (Foster, 1959a,b; Pollard, 1971a). This is best accomplished by observed or timed matings that are confirmed by the presence of the spermatic plug in the vaginal opening. If the plug is not seen, confirmation can be made by vaginal smear for the presence of spermatozoa.

The timed, gravid female is shaved along the ventral portion of the abdomen from the xiphoid cartilage to the genital opening. The use of a depilatory assures complete removal of hair and a clean incision without the contamination of animal fur. The female is euthanized outside of, but close to, the surgical isolator. The surgical site is washed and disinfected. The abdomen is draped with a surgical drape containing an elliptical opening through which a midline incision is made. Good surgical technique is required to prevent the accidental incision of the intestines with its abundance of microorganisms. The uterus, with its cervix and cornuae clamped, is lifted from the abdomen onto sterile drapes. After severance from the maternal body, the uterus is lifted and removed to a 38°C primary germicide of 0.525% sodium hypochlorite solution where it remains for 5 s. It is then placed into a perforated container, which is lowered via the sleeve or rigid tube by a nylon cord from inside the sterile isolator to beneath the surface of a 5% iodide germicide. After an additional 15–30 s, the container still beneath the surface of the germicide is guided into the mouth of a 10.4-cm wide rigid clear plastic tube connected to the isolator wall. A surgical technician, with arms inside the isolator via the surgical sleeves and gloves, raises the uterus within the perforated container to the interior of the sterile isolator allowing the germicide to drain down to the germicidal trap. Two technicians on opposite sides of the isolator rapidly remove the fetuses from the uterus and separate them from their fetal membranes utilizing a taut Mylar membrane secured to a cage top as a surgical table. They are quickly washed with surgical sponges and rendered clean from amniotic fluid and blood. This is essential since a foster mother might cannibalize them if body fluids and remnants of the fetal membranes remain. These procedures must be accomplished rapidly, since maternal support is lost upon separation of the placenta from the uterus.

The neonates are dried and massaged to stimulate breathing and the umbilical cord is separated by clamping and cutting or electric cautery (Pollard, 1971a). If additional procedures are to be performed, the neonates are loosely wrapped in a small surgical towel and placed on the isolator floor above the warmth of the heating pad resting beneath the isolator floor. The Mylar membrane attached to the plastic cage is punctured, permitting the uterus and membranes to fall below to the floor of the cage. A new membrane is placed across the mouth of the cage and once again held in place by rubber

bands. This procedure can now be repeated many times without breaching the integrity of the sterile system.

It is good practice to transfer the neonates to a rearing isolator where they are foster nursed on lactating GF mothers until weaning age (Pollard, 1971a). This method replaced the more cumbersome hand rearing and feeding (Gustafsson, 1948; Griffiths and Barrow, 1972), principally because of the more readily available lactating GF mothers and the increased survival rate as compared to hand-feeding methods. One would only consider hand rearing where minimal antigenic stimulation is desirable, and in these instances highly purified synthetic formulas are utilized. The surgical procedures, equipment, etc., are reviewed by Pleasants (1974). Figs. 21.7–21.10 show an adaptation of the procedure just described using tandem laminar flow hoods. Numerous references exist on the establishment and maintenance of breeding colonies of GF rats (Reyniers et al., 1946; Gustafsson, 1948; Foster and Pfau, 1963; Lev, 1963; Gordon et al., 1966; Reid and Gates, 1966; Miyakawa, 1968; Kappel et al., 1969; Kellogg and Wostmann, 1969; Yale and Linsley, 1970; Coates, 1973; Maejima et al., 1974).

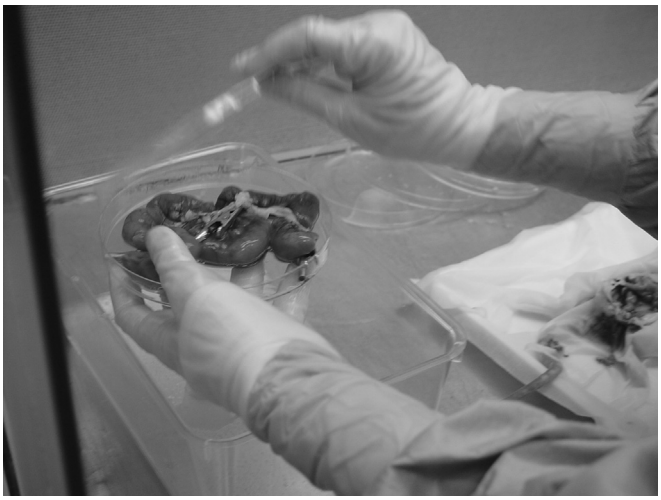

FIGURE 21.7 The clamped, gravid uterus, after being taken from the donor dam following euthanasia, is placed into a plastic dish, immersed in disinfectant, and moved to a laminar flow hood. *Courtesy Charles River Labs.*

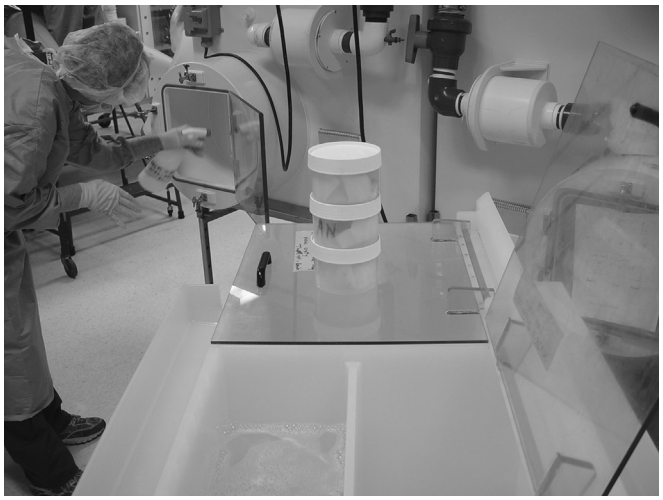

FIGURE 21.9 The clean, active pups are placed into a sealed container, passed through disinfectant and sprayed into an isolator. *Courtesy Charles River Labs.*

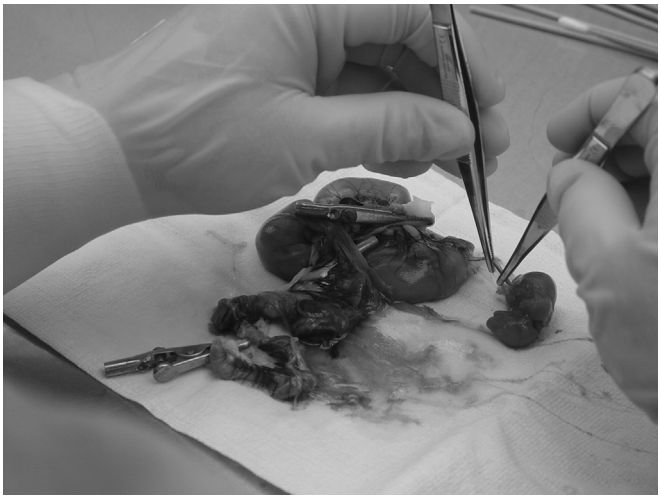

FIGURE 21.8 The pups are removed from the uterus, each placenta detached, and the pups cleansed prior to being taken into an isolator. *Courtesy Charles River Labs.*

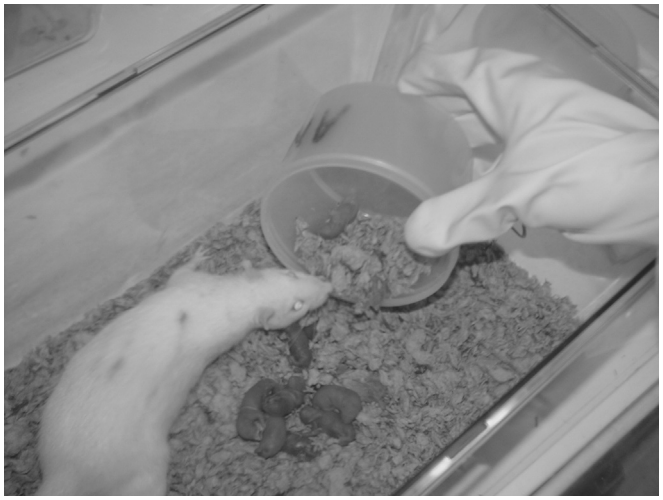

FIGURE 21.10 The container of pups is introduced into the isolator and placed with a receptive foster mother. *Courtesy Charles River Labs.*

D. Production of Defined Microbiome Rats

The elimination of the rat's normal gut microbiome by cesarean derivation results in dramatic changes in the host's physiology, nutrition, tissue morphology, and defense against infectious agents. The most pronounced anomaly of the GF state in rats, as well as other species, is the enlargement of the cecum that can lead to volvulus at the ileocecal–colonic junction and eventual death (Wostmann and Bruckner-Kardoss, 1959). The content of the cecum and intestines are fluid, and the animal is said to have a chronic mild diarrhea. In addition, aside from low levels of antigenic material in the feed and bedding, the immune system of the GF rat is unstimulated (Gordon and Pesti, 1971). The lamina propria is thin and almost devoid of antibody-producing plasma cells, and lymph nodes are smaller (Gordon and Wostmann, 1960; Gordon et al., 1966). Also, due to the absence of its vitamin K synthesizing gut flora, the GF rat must have this vitamin added to its food, or it rapidly develops prolonged prothrombin times and hemorrhages (Gustafsson, 1959). GF rats are also much more susceptible to infections than their CV counterparts, which is why they may die soon after being introduced into a CV colony (Luckey, 1963). This can be prevented if they are first colonized by at least several members of their normal gut microbiome.

Gordon and Wostmann demonstrated that GF rats could be normalized by feeding them cecal contents of CV rats (Gordon and Wostmann, 1959). However, no attempt was made to determine which member(s) of the microbiome was responsible for this phenomenon until Schaedler's and Dubos' classic work describing the bacterial colonization of the gastrointestinal tract of mice, which subsequently became the cornerstone for much of the work that followed (Dubos et al., 1965; Schaedler et al., 1965a, 1965b). They reported that soon after birth the entire gastrointestinal tract was populated by *Lactobacillus* spp. and a group N *Streptococcus*. During the second week of life, high concentrations of aerobic bacteria, such as enterococci and slow lactose-fermenting coliforms, were observed in the large intestine. Their numbers abruptly dropped during the third week of life when obligately anaerobic bacteria, such as *Bacteroides* species, colonized this organ. Throughout the adult lives of mice, the obligately anaerobic bacteria remained at very high levels, and the aerobic component of the microbiome remained suppressed at very low levels. The microbiome of the rat has been found to closely resemble that of the mouse (Smith, 1965; Savage, 1969).

Schaedler then proceeded to colonize GF animals with a flora consisting of *Bacteroides*, lactobacilli, an anaerobic *Streptococcus*, and a slow lactose-fermenting coliform (Schaedler et al., 1965b). This mix of microflora was able to drastically reduce the size of the cecum and therefore almost normalize the animals. Consequently, this flora, and variations of it, has been used extensively to colonize both GF mice and rats prior to their removal from an isolator into a new colony. A process for colonizing GF animals, commonly referred to as "associating animals," merely consists of colonizing an initial isolator of GF rats with pure cultures of each of the individual members of the flora. Additional associations are then achieved by simply introducing an associated animal into a GF isolator and placing fecal pellets from the associated animal into the water bottles of the GF animals on 2 consecutive days. During the first day the aerobic bacteria colonize the GF animals and lower the oxidation–reduction potential, so that on the second day the extremely oxygen-sensitive fusiform-shaped anaerobes are able to colonize the animals. It should be noted that these few bacteria represent a very small fraction of the gut microbiome, and many additional members are necessary to normalize a GF animal completely (Syed et al., 1970).

An excellent review and perspective by Meghan Wymore Brand and coauthors (2015) on what is now known as the altered Schaedler flora was published on the 50th anniversary of the publication of the initial reports from the laboratory of René Dubos at The Rockefeller Institute in 1965 (that same year The Rockefeller Institute became The Rockefeller University). Brand and her coauthors finished their 2015 perspective with the following statement: "… the availability of defined microbiota rodent models offers unique opportunities to study host–microbiota interactions well beyond what may have been envisioned by Schaedler in 1965." In fact, the current use of fecal transplantation in the treatment of primary *Clostridium difficile* infection as a cause of pseudomembranous colitis in humans is just one example (Gustafsson et al., 1999; Juul et al., 2018).

E. Microbiological Testing

It is good practice to perform certain examinations on the euthanized dam. Historical data of the health status of the donor female provide excellent reference material should subsequent contaminations occur in the GF or SPF colony. Therefore prior to losing the identification of a cesarean-delivered litter, examination for *Mycoplasma* and intestinal parasites and serological examination for murine viruses are recommended. Certainly, at the very least, careful culturing of the ovaries and uterus for *Mycoplasma* should be performed (Ganaway et al., 1973; Schultz et al., 1974), since there have been occasional reports of *Mycoplasma pulmonis* isolation from GF rats, which may have resulted from in utero contamination of the dam (Kappel et al., 1969; Schultz et al.,

1974). Careful workers discard neonates as a precaution if the donor female exhibits positive *Mycoplasma* in the reproductive system.

Subclinical *Pasteurella pneumotropica* infections have been reported in GF rats (Ganaway et al., 1973; Pleasants, 1974), and these might be transmitted to GF progeny. Microbiological testing by fecal swabs of the neonates 24–48 h after delivery assures the asepsis of the surgical procedure as well as the sterility of the rearing isolator. Detailed methods are described elsewhere (Wostmann, 1970). The methods for gross observation and the detection of bacteria, fungi, and parasites are relatively simple and well standardized. The methods for the presence of exposure to murine viruses are usually accomplished through serological tests for the specific antibodies such as the enzyme-linked immunosorbent assay or the indirect immunofluorescence assay. Wagner (1959) worked out detailed sterility testing procedures, which are still utilized as standard procedures in many laboratories, albeit with updated analytics. Usually, when an isolator becomes contaminated with bacteria, the exhaust air loses its nonanimal-like almost sweet odor to the more familiar odor of laboratory rats. In nearly all instances, contaminants can be observed in fecal wet mounts prior to routine culturing. Culturing 24–48 h on appropriate media readily reveals typical contaminants at 37°C. Since 21, 37, and 55°C are standard incubation temperatures, molds and thermophilic organisms are also detected in the less common contaminations. Molecular methods, such as polymerase chain reaction (PCR) and quantitative (q)PCR, may be utilized alongside culture-based methods to monitor isolator sterility (Nicklas et al., 2015; Fontaine et al., 2015; Packey et al., 2013). Because these methods utilize primers that recognize conserved regions of the 16S rRNA gene, they readily detect bacteria that are not easily cultured. When compared to bacterial culture, 16S qPCR is more specific and less sensitive (Fontaine et al., 2015).

Intrauterine infection represents a potential hazard to the axenic integrity of GF rats (Asano, 1969; Altura et al., 1975), since vertical transplacental transmission of Kilham rat virus (rodent Protoparvovirus 1) (Kajiwara et al., 1996; Jacoby et al., 2001) has been noted but, unlike mice (Kajima and Pollard, 1965), leukemia and mammary tumor viruses have not been observed in GF Fischer, Wistar, and Sprague-Dawley rat strains examined (Pollard and Kajima, 1966). The confirmation of encephalitozoan in GF rabbits (Hunt et al., 1972) suggests the likelihood of such vertical transmission possibilities in rats and other species. With these limited reports as background, the examination of donor stock would be the only means currently available to help reduce the vertical transmission potential in newly derived colonies. Unfortunately, searching histologically for viruses is tedious and negative results would not be conclusive. With regard to encephalitozoan, immunofluorescent and India dye tests are fairly straightforward procedures and provide a high degree of accuracy (Wosu et al., 1977a,b). In summary, the previously referenced reports should be cautionary for investigators attempting gnotobiotic derivation of new strains or species. Established commercial breeders, using the most current testing methods for microbiological analysis, have long-standing pedigrees yielding a high degree of assurance in their gnotobiotic status.

F. Anatomy, Reproduction, and Lifespan

While strain, sex, age, and organ must be taken into account in making comparisons (Banasaz et al., 2000), the GF rat is an experimental animal that differs significantly from the CV rat in a number of characteristics. GF rats provide a uniform and relatively stable baseline of morphologic and physiologic activities, which, in turn, facilitate studies of superimposed changes. The earliest noted and most conspicuous effect of the GF status in rodents, including rats, is enlargement of the cecum. It becomes voluminous, usually five times larger than its CV counterpart on the same diet, and may approach 25% of body weight (Coates, 1973). This enlargement sometimes interferes with normal reproduction but can be significantly reduced with dietary manipulation of inorganic ions (Coates, 1973). The cecal wall is much thinner in GF rats and the cecal contents more liquid than in CV animals. This is due to an excess of water and anionic-soluble mucins, the latter being degraded in the CV rat (Asano, 1967; Pleasants, 1974; Carlstedt-Duke et al., 1986). Cecectomy of GF rats restores most functional and metabolic parameters within the CV range (Wostmann, 1975).

The enlarged cecum is associated with altered metabolic functions, particularly slower cholesterol and bile conversion (Einarsson et al., 1973), depressed reducing capacity of the cecal contents, and reduced cecal concentrations of chloride and carbonate ions (Thompson and Trexler, 1971). These animals also require less exogenous choline (Nagler et al., 1969), and there is a total absence of metabolism of flavonoid compounds in the gut (Griffiths and Barrow, 1972). If the GF rat's cecal contents are replaced with saline, then the water absorption capacity of the cecum becomes normal or greater than in CV rats (Gordon, 1974). GF rats have been reported to accumulate a compound or compounds in their cecum, which changes the tone and reactivity of mesenteric vascular smooth muscle to adrenaline (Baez and Gordon, 1971) and which is normally destroyed by the CV normal flora (Carlstedt-Duke et al., 1986). Current understanding suggests that alpha-pigment, prostaglandins, eicosanoids, fatty acids, and kallikrein all contribute to change in cecal muscle tone (Bruckner-Kardoss and Wostmann, 1974).

The weight and surface area of the small intestine and the associated lymphoid cells and tissues of GF rats are generally decreased (Gordon et al., 1966). Depending on the diet, the rate of peristalsis may be the same (Gustafsson and Norman, 1969) or slower (Saquet et al., 1973) in GF than in CV rats. The rate of mucosal sloughing is generally half that of CV controls (Gordon et al., 1966; Reddy and Wostmann, 1966), and digestive enzymes, such as proteases, lipase, and amylase, persist longer and farther down the gut under GF conditions (Lepkovsky et al., 1966; Reddy et al., 1969a; Norin et al., 1986). Urease appears to be absent under GF status (Delluva et al., 1968). The GF rat gut is more efficient in digestion and absorption, in part because the villi are longer and more even (Coates, 1973).

Nutrient requirements in the diet of GF rats is usually higher than CV requirements but vary with experimental conditions (Pleasants, 1974). In general, there is a higher need for total food and water, for vitamin K, the B vitamins, and for choline to prevent liver cirrhosis. GF rats maintained on a diet without supplemental vitamin K rapidly develop a hemorrhagic condition, while CV rats on the same diet do not (Coates, 1973). Antagonism between vitamins A and K occurs only when vitamin A intake is 10 times above normal (Wostmann and Knight, 1965; Reddy and Wostmann, 1966). On the other hand, GF rat nutrient requirements are less than CV requirements for vitamin A (Rogers et al., 1971; Coates, 1973), lysine, cysteine, and vitamin E to prevent liver necrosis, protein (Pleasants, 1974), calcium, and magnesium and zinc (Smith et al., 1973). GF rats given vitamin A-deficient diets survived much longer than CV rats (Coates, 1973). Assessment of the rat's nutritional requirements is often difficult because of coprophagy.

GF rat studies show unequivocally that the CV rat's microbial flora has a significant effect on the basal metabolism and on the response to adrenaline, cardiac output, and vascular distribution (Pleasants, 1974). The overall metabolic rate of GF rats has been reported to be one-fourth that of CV rats of the same strain (Wostmann et al., 1968). This undoubtedly results from reduced (one-third normal) cardiac output and oxygen consumption (Wostmann et al., 1968), reduced regional blood flow and distribution (Gordon et al., 1966), decreased heart weight (Gordon et al., 1966; Albrecht and Souhrada, 1971), decreased total blood volume (Bruckner, 1997), and decreased pulmonary partial pressure of carbon dioxide values (Schwartz, 1975). Aortas and portal veins of GF rats have an attenuated reactivity to angiotensin, vasopressin, and epinephrine (Altura et al., 1975). Production of short-chain fatty acids are related to microbial activity (Håverstad and Midtvedt, 1986) and reduction of total body fats has been reported (Reina-Guerra et al., 1969). In GF rats the lymph nodes and Peyer's patches are small, lack germinal zones, and contain few, if any, plasma cells (Miyakawa et al., 1969; Carter, 1971; Balish et al., 1972). Serum globulin values are one-third those of CV rats, and GF rats have less total serum proteins (Balish et al., 1972). The decreased immunological stimulation of GF animals leads to very low titers of agglutinating antibodies for *Streptococcus fecalis*, *Proteus vulgaris*, *P. aeruginosa*, *Lactobacillus acidiphilus*, and *Bacillus fragilis* (Balish et al., 1972). This is accompanied by decreased severity of conjunctival inflammation when infected by bacteria (McMaster et al., 1967). In relation to the foregoing, it has also been reported that the mucosa of the nasal cavity and middle ear have few lymphocytes and no inflammatory infiltrates (Giddens et al., 1971).

Tissue enzyme levels usually tend to be lower in GF rats. There is less mitochondrial succinate oxidase and glycerophosphate dehydrogenase activity in the liver (Sewell et al., 1975). Lower muramidase levels have also been reported (Ikari and Donaldson, 1970). However, some tissue enzyme activities are higher in GF rats, namely, peroxidase-mediated antibacterial activity of the salivary glands (Morioka et al., 1969), liver microsomal hydroxylation of steroid hormones (Einarsson et al., 1974), and fatty acid synthetase and citrate lyase activity in the liver (Reddy et al., 1973; Wostmann, 1975).

In general, GF rats eat more and grow better. They absorb saturated and unsaturated fats better, particularly palmitic and stearic acid (Demarne et al., 1973); have greater serum and liver cholesterol concentrations (Reina-Guerra et al., 1969); use more total fat in the diet (Nolen and Alexander, 1965); and have a higher cholesterol conversion rate (Reina-Guerra et al., 1969). A report on experimental cholesterol synthesis in GF and CV rats (Ukai et al., 1976) indicates that there is an inverse proportionality between the log phase rate of hepatic cholesterol synthesis and liver cholesterol levels in GF rats. Therefore the endogenous cholesterol synthesis in GF rats may not be responsible for the high cholesterol levels in plasma or in the liver. Liver cholesterol may play a major role in the regulation of hepatic cholesterogenesis in the GF rat by a mechanism similar to that in the CV rat.

There is total conjugation of bile acids in GF rats compared with almost total lack of conjugation in the cecum of CV rats (Madsen et al., 1976), which is dependent upon clostridial species (Midtvedt and Gustafsson, 1981). The bile turnover rates are higher (Reina-Guerra et al., 1969) as is the pH of the cecal contents (Thompson and Trexler, 1971). In GF cecal contents, the colloid osmotic pressures are approximately 100 mm Hg. This results in a pressure gradient of 60–70 mm Hg between the gut lumen and the blood plasma, in contrast to a smaller gradient in CV rats (Gordon, 1974). GF rats have greater reabsorption of bile acids from the gastrointestinal tract and therefore have greater recirculation of bile (Einarsson et al., 1973). Use of the GF rat in studies

of cholesterol metabolism are particularly concerned with the factors that influence the absorption of cholesterol from the gut and its elimination from the body as bile acids via the feces (Wostmann, 1973, 1975). The GF rat appears to be unable to decrease the reabsorption of bile acids in the lower gut, a function of normal microbial flora. Work indicates that differences in the histochemical nature of mucosaccharides are dependent on whether they are located in areas of normal bacterial flora in CV rats or in areas relatively free of intestinal flora (Yamada and Ukai, 1976).

Other metabolic parameters that tend to be higher or greater in GF animals as compared to CV animals include pH of cecal contents (Thompson and Trexler, 1971), mean intracolonic oxygen pressure (Bornside et al., 1976), pulmonary arteriovenous oxygen values (Schwartz, 1975), plasma levels of some steroids (Einarsson et al., 1973), urinary citrate excretion (Gustafsson, 1948), and fasting blood glucose (Pleasants, 1974). In addition, aortas and portal veins have a higher total calcium content in GF animals (Altura et al., 1975), and the microvasculature is refractory to catecholamines (Gordon et al., 1966; Baez and Gordon, 1971).

The GF state has little influence on the functional respiration or oxidative phosphorylation of mitochondria isolated from the liver of adult rats (Sewell and Wostmann, 1975). Serum chemistry and hematological values are within the normal range except for the depressed leukocyte level (Burns et al., 1971). Minimal differences have also been reported in serum β-lysin (Ikari and Donaldson, 1970), fasting blood glucose and glucose tolerance (Sewell et al., 1976), metabolism of nicotinamide and nicotinic acid (Lee et al., 1972), carbon dioxide production in the gut (Rodkey et al., 1972), mean pulmonary arterial oxygen partial pressure (Schwartz, 1975), and activities of hepatic enzymes of urea synthesis (Nuzum, 1975; Norin et al., 1986). No differences were found in the histology of the eye of GF rats (McMaster et al., 1967).

It is evident that there are many basic physiological and morphological parameters of GF rats that have not yet been studied. Furthermore, one must keep in mind that often reports cannot be reliably compared because of variables of age, sex, and strain of rat as well as environmental conditions, diet, and unknown interactions among these factors. For example, it has been reported that differences in thyroid function and related hepatic enzymes tend to lessen with age of the animals (Sewell et al., 1975) and that the qualitative and quantitative composition of the bile acids varies considerably between male and female GF rats (Gustafsson et al., 1975).

G. Nutrition

It can be generally stated that the nutritional requirements of animals are inversely proportional to their biosynthetic capacity (Luckey, 1963). The need for special diets for GN animals has been reviewed by Wostmann (1975). Special diets are necessary principally because food sterilization methods usually require compensation for the loss of vitamins and the reduction of nutrient value of proteins resulting from heat sterilization (Weisburger et al., 1975). The dietary requirements for microbiologically synthesized vitamins are higher (Coates, 1973; Wostmann, 1975), because the lack of normal microbial flora affects the absorption, which is greatly enhanced in the GF rat and which leads to the formation of urinary calculi unless dietary levels of calcium are reduced (Gustafsson and Norman, 1962; Smith et al., 1973).

Diets tend to vary according to the specific GF research objectives (Luckey, 1963), i.e., antigen-free diets for immune system studies or high sugar content diets in dental caries studies. Some diets have been found nutritionally adequate for short-term experiments even if autoclaved, as long as the diets are supplemented with filter-sterilized heat-labile vitamins (Oace, 1972). A canned, moist, presterilized (autoclaved) diet of known composition can be provided by spraying it into the isolator system (Foster and Pfau, 1963). Autoclavable diets are also available (Kellogg and Wostmann, 1969; Oace, 1972; Pleasants, 1974) as are chemically and water-soluble ones. The latter can be filter sterilized (Pleasants, 1974) and used as special purified diets for nutritional research (Wostmann and Kellogg, 1967). Growth of GF rats on these diets is comparable to that of CV animals. Reddy et al. (1969b) grew GF rats from birth to maturity using membrane-filtered, chemically defined, water-soluble diets based on amino acids and glucose. Diets sterilized by gamma irradiation have also been used in rearing GF rats (Paterson and Cook, 1971). Radiation sterilization using ^{60}Co irradiation is recommended for studies of cholesterol and bile acid metabolism in GF rats (Wostmann et al., 1975). Current diets suitable for studies in nutrition and metabolism of GF rats are listed in Wostmann's review (1975). However, besides the nutritional adequacy, moisture content of autoclavable diets must also be considered. The diet cannot be so hard that the animal is unable to feed normally, and if the diet is too soft, a rat's teeth will continue to grow and may need to be trimmed manually (Norin, 2018).

H. Strains and Stocks

1. *LOBUND*

LOBUND maintained GF colonies of the Fischer strain and the Wistar and Sprague-Dawley rat stocks (Maejima et al., 1974) and Pleasants (1959) reported experiments with the Holtzmann and LOBUND stocks of

rats bred in closed colony. LOBUND had a distinguished history as a site for training, supplies, and animal stock resource, in addition to research, into the 1990s; this laboratory is now much reduced in size and scope and does not currently maintain GF animals.

2. *Karolinska Institutet*

Like LOBUND, the gnotobiotic facilities in Sweden trace its origins to the 1930s. Begun at the University of Lund and subsequently moved to Stockholm, Gustafsson (1948) used the Long-Evans hooded stock and AGUS strain, which are not currently used in Sweden. Tore Midtvedt and colleagues expanded the use of GF rats but the facilities are now much reduced in size and scope with only GF mice currently maintained.

3. *University of Wisconsin*

An internationally known laboratory was established in the 1960s by Balish and colleagues in Madison, Wisconsin, that created and maintained large colonies of GF rats, mice, and, uniquely, beagle dogs for three decades. This facility has recently been reestablished within the Biomedical Research Model Services department (formerly Laboratory Animal Resources), currently maintaining GF mice, but availability of GF rats is anticipated. As before, the facility will be a resource for the university campus and beyond.

4. *Commercial Suppliers*

Charles River Laboratories, Envigo (formerly Harlan, Inc.; Harlan Sprague Dawley, Inc.), Taconic Biosciences, IFFA-Credo (Charles River France), and a few others are commercial entities that usually maintain various gnotobiotic stocks and strains, generally not catalog items, as seed stocks for their commercially available pathogen-free animals.

5. *Others*

The Gifu hybrid has been produced in the GF state by Miyakawa in Japan (1968) and Dajani and colleagues utilized the GF AGUS strain (Dajani et al., 1975).

While there are many laboratories active in the use of gnotobiotic rats, such as those supported by the National Institutes of Health gastrointestinal disease center at North Carolina State University, the University of North Carolina at Chapel Hill, at Tokai University in Japan, and the University of Minas Gerais in Brazil, none is large enough to maintain stocks equivalent to those mentioned previously that would provide more than a few breeding pairs of animals. These organizations, like others, have maintained GF rats intermittently based upon the needs of their researchers. There is a profound need for a central supplier of GF rats for use by the world's research laboratories.

III. RESEARCH APPLICATIONS OF GNOTOBIOTIC RATS

A. Infectious Diseases

The infectious and chronic diseases of CV rats are described by Tuffery and Innes (1963) and the more recent ACLAM series (Schoeb and Eaton, 2017), and may serve as a basis for comparison of monoassociation and experimental infection studies. *M. pulmonis* is the primary pathogen in chronic respiratory disease of CV rats (reviewed in Sugiyama and Bruckner, 1975) as demonstrated by researchers at the University of Alabama-Birmingham (Lindsey et al., 1971), but is now effectively controlled, if not eradicated from research animal stocks, through wide use of the cesarean derivation of breeding populations. Schoeb et al. (1985) and Schoeb and Lindsey (1987) also used GF rats to show that Sendai virus and rat coronavirus each exacerbates murine respiratory mycoplasmosis. Rats infected with *M. pulmonis* alone displayed mild disease, while those subsequently administered viruses developed advanced respiratory mycoplasmosis as well as more mycoplasmal colony-forming units in their respiratory tracts. Luckey (1968) provided an extensive bibliography on the effects of bacterial species on the monoassociated rat. Cassell and colleagues made effective use of monoassociated mice and rats to study the pathogenesis of *Mycoplasma* diseases and the host response (Cassell et al., 1974; Cassell and Davis, 1978). No differences were found in the susceptibility of GF rats to *Plasmodium berghei* primary infections via mosquito-borne sporozoites, nor were there any differences in the resulting pathology (Martin et al., 1966). Work in Balish's laboratory (Rogers and Balish, 1976) indicates that the GF rat can serve as an animal model of nephritis due to *Candida* infections, since the yeasts multiply in the monoassociated rats' kidneys.

B. Cancer

Cancer development in GF rats can be related in part to the absence of microbial flora (Pollard and Teah, 1963; Walburg, 1973; Pollard et al. 1985). Experimental cancer yields are lower in GF rats when the carcinogens tested are of the type necessitating enzymatic metabolic activation (Weisburger et al., 1975). In general, the oncogenic potential is the same as in CV rats, but tumor-related changes are more clearly defined in GF animals (Pollard et al., 1968). GF rats with either spontaneous or induced tumors have higher numbers of plasma cells but have no 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 rats (Luckey, 1963; Pleasants, 1974). Nevertheless, GF rats have a very low rate of spontaneous neoplasm development as compared to GF mice (Walburg, 1973). The most frequent spontaneous tumors in aged GF rats involve the mammary and pituitary glands (Pittermann and Deerberg, 1975).

1. Colon Cancer

Cycasin from cycad bean flour is carcinogenic for CV rats whose microbiome converts it to a carcinogen, whereas it does not induce tumors in GF rats (Laqueur et al., 1967; Luckey, 1968). If cycasin is first hydrolyzed to the aglycone methylazoxymethanol, it is then carcinogenic to GF rats (Laqueur et al., 1967). Spontaneous colon adenomas are twice as prevalent in GF rats (Weisburger et al., 1975). No differences in the incidence of adenocarcinoma have been reported following intracolonic exposure to nitrosoguanadine carcinogens (Weisburger et al., 1975), whereas others report greater susceptibility of GF rats to these same direct-acting carcinogens (Shih et al., 1975). Results are significantly dependent on route of administration, since oral administration of *N*-methyl-*N*-nitro-*N*-nitrosoguanadine produced very few adenocarcinomas in GF rats as compared to CV animals (Miyakawa et al., 1975). It appears that there is a lower incidence of cancer induction in GF animals when the carcinogens being tested are of the type necessitating enzymatic metabolic activation.

2. Breast and Prostate Cancers

GF rats are as susceptible to dimethylbenzanthracene-induced breast cancer as CV rats (Pollard, 1972). Primarily through the efforts of Morris Pollard (Pollard, 1973, 1977, 1992; Pollard and Luckert, 1987; Snyder et al., 1990; Pollard and Wolter, 2000), a spontaneous prostate tumor, which appeared in GF LOBUND-Wistar rats, has been developed into an exquisite animal model for human prostate cancer. This has been further characterized and extended in conventionalized rats of the same line (Fig. 21.11). Although the Wistar rat is not inbred, the LOBUND line has been bred in a closed colony for so many generations that skin grafts are accepted among rats of that line. Pollard and colleagues (Fig. 21.12) received many accolades for their work on prostate carcinoma, perhaps none greater than being the cover photograph for *Cancer Research*, November 1, 1999.

3. Leukemia

A spontaneous, transplantable, lymphatic leukemia called "Nova rat leukemia" has been reported in aged GF Fischer rats (Sacksteder et al., 1973). Leukemia could not be induced by whole-body irradiation, but GF rats were found as susceptible to passage of Gross A leukemia virus as CV rats (Pollard, 1972).

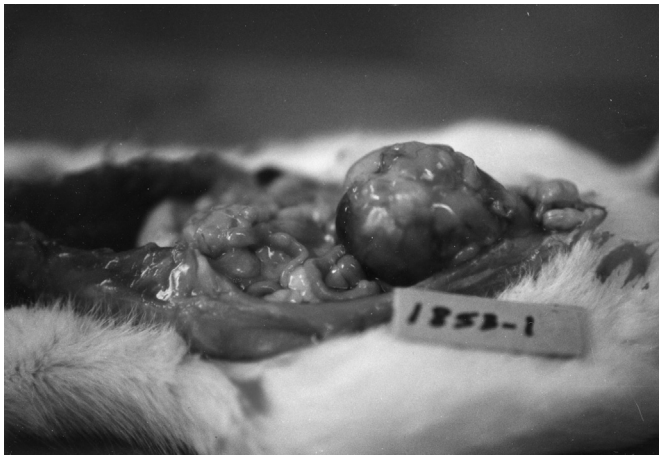

FIGURE 21.11 Spontaneous prostate carcinoma in an aged LOBUND-Wistar rat. *Courtesy M. Pollard, University of Notre Dame.*

FIGURE 21.12 Morris Pollard, DVM, PhD (1916–2011), on the left, an experimental pathologist and noted virologist, was honored by *Cancer Research* as the cover photo in November 1999 (*inset*) for advancing research on prostate cancer through the development of the rat model of prostate adenocarcinoma. Pollard became director of LOBUND in 1961 and is credited with moving the former technology-focused institute to that with a medical research focus while continuing to serve as an international center for training in gnotobiotic technology. Shown with Pollard is research colleague, Mark A. Suckow, DVM, DACLAM (*right*), and their undergraduate research assistant (and future Olympian in 2012, 2016), Molly A. Huddle (*center*). April 2005, Notre Dame, Indiana. *Photo courtesy of the author [PBC]; inset courtesy American Association for Cancer Research.*

4. Urethral Cancer

Urethral cancer is rare in CV rats but is relatively frequent in some older GF rat strains (Pollard, 1973).

5. Endocrine Cancer

Endocrine-related cancers of the nonleukemic type such as thymomas and mammary neoplasms occur in GF rats (Pollard, 1977).

C. Oral Pathology

Mechanisms of oral pathology have been and continue to be clarified through GF rat studies begun at LOBUND (Orland et al., 1954) and continued in recent years by Michalek and coworkers (Crowley et al., 1999; Lynch et al., 2013). This is particularly true in caries research where *Streptococcus* sp. in association with GN rats fed a carcinogenic diet produce carious lesions (Orland et al., 1954; Green et al., 1973), and in periodontal disease studies where a number of streptococci, actinomycetes, and Gram-positive bacilli cause typical periodontal disease under conditions of monoassociation (Green et al., 1974).

D. Senescence and Wound Repair

In general, GF animals tend to live longer than their CV counterparts. Premature death of GF animals may be caused by infection or by environmental factors. Delayed morbidity in 2- to 3-year-old GF rats is a common observation, which shows them to be virtually free of age-related kidney, heart, and lung changes (Pollard and Kajima, 1970; Pollard, 1971b). Postmortem differences include a minimum of odoriferous putrefactive changes and autolysis of the intestinal area by digestive enzymes; dead GF animals undergo drying and mummification if in a dry atmosphere (Luckey, 1963). GF rats are less sensitive by half to X-irradiation because it affects the rate of wound closure (Donati et al., 1973).

E. Immunology

Immunological studies with GF or DF animals enable distinguishing primary mediation lesions from those mediated by possible microbial infections. From work on the biological effects of radiation, it has been determined that GF rats survive larger doses of total-body X irradiation for a longer time (Reyniers et al., 1956). In basic immunological studies, GF or DF rats provide information on the role of the microbial flora in stimulating humoral and cell-mediated immune responses. Immunity, as measured by opsonic activity, is depressed in GF rats infected with *P. aeruginosa*, whereas the GF rat's responsiveness to H and O antigens of *Escherichia coli* and to sheep erythrocytes is increased (McClellan et al., 1974). Differences in phagocytosis depend on differences in opsonic activity rather than on functional differences at the cellular level. In vitro studies with ^{32}P-labeled *E. coli* opsonized with sera of GF rats indicated that cells from GF rats were slightly more active in ingesting capacity than cells from CV rats. Thus the opsonic activity of CV rat sera is higher than that of GF rat sera when tested in vitro (Trippestad and Midvedt, 1971). GF rats have been reported to reject skin allografts more rapidly than CV rats (Lev, 1963; McDonald et al., 1971; Carter and Bazin, 1980), whereas autografts of skin transplants on rat tails heal quickly (Ashman, 1975). The latter is postulated to be due to genetic uniformity of histocompatibility factors in GF animals (Ashman, 1975). The GF allogenic radiation-induced chimera has a greatly reduced or absent T-cell response, whereas the B-cell response is almost normal (Bealmear et al., 1973).

F. Xenotransplantation

Xenotransplantation, focusing on the use of porcine organs in humans, is a topic of great current interest and actively being pursued in swine (M. Rothblatt, personal communications, 2015, 2016). The first successful xenotransplant was actually performed using bone marrow transplantation in GF rats and mice (Pollard et al., 1985; Wade et al., 1987) but porcine studies hold promise for application to humans and funding by private enterprise. The immunological basis for the success of xenotransplantation in this system remains to be defined but gives hope that organ transplants between species may someday be successful.

G. Metabolic Studies and Inflammatory Bowel Diseases

Understanding the etiology and pathogenesis of human inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, has been thwarted by the lack of a suitable animal model. Toward the end of the 20th century, several potential mouse models were investigated (Elson et al., 1995) but none appears to show the overall promise of a transgenic rat model (Sartor, 2000) that has allowed critical evaluation of the host response and the host's associated intestinal flora in the pathogenesis of chronic intestinal disease. The breakthrough was provided by the successful introduction of a human gene into Fischer rats creating a transgenic rat line that reproduced and was cesarean derived into the GF state by Balish (Taurog et al., 1994; Warner et al., 1996). Sartor and coworkers then used this transgenic line to show that spontaneous colitis did not develop in the GF host but did develop in both CV and monoassociated rats (Rath et al., 1996, 1999; 2001; Dielemann et al., 2003). The possibility still exists that many intestinal disturbances could result from the lack of functionally active microbes. Current ongoing studies of the mammalian microbiome have begun to focus thinking on this possibility instead of the presence of disease-causing microorganisms.

Professor Tore Midtvedt and colleagues at the Karolinska Institutet in Sweden pioneered a multidecade study on the impact of the intestinal microbial flora on

host metabolism, which has been titled "MAC/GAC." Midtvedt is credited with introducing the new term in gnotobiology, with the aim of making it easier to discuss the microbiota's function in rats. The concepts are useful tools, especially when studying intestinal functions in other animals and in humans, with some variations depending on anatomical differences and the dietary regimens.

MAC (microbiome-associated characteristic) is defined as the recording of any anatomical structure, biochemical, immunological, or physiological functions in the macroorganism that have been influenced by microbes. When microbes actually influencing the parameter under study are absent, as in GF animals, in healthy newborns, or in relation to antimicrobial therapy, the corresponding structures and functions under study are defined as GAC (germfree animal characteristic). The normal, established microbiota in the host, or dysbiosis in the microbiome, can be followed in fecal samples studying several MACs (Table 21.2). These are suggested as excellent tools for following the microbiota establishment in newborns as well, since they can be used to identify microbiota disturbances in many intestinal diseases.

The earlier work on rat metabolic homeostasis, cecal size, and disease has been reviewed by Midtvedt, Saxerholt, Gustafsson, and others (Gustafsson et al., 1966, 1970; Midtvedt 1989; Saxerholt et al., 1985), as well as the in more recent work by Norin (Norin and Midtvedt, 2006).

TABLE 21.2 Some Anatomical, Physiological, and Biochemical Microflora-Associated Characteristics (MACs) and Corresponding Germfree Animal Characteristics (GACs) in Rats and Mice.

Anatomical/Physiological	MAC	GAC
Intestinal wall	Thicker	Thinner
Cell kinetics	Faster	Slower
Migration motor complexes	Normal	Fewer
Production of peptides	Normal	Altered
Sensitivity to peptides	Normal	Reduced
Cecum size	Normal	Enlarged
Osmolality	Normal	Reduced
Colloid osmotic pressure	Normal	Increased
Oxygen tension	Low	High (as in tissue)
Electropotential Eh, mV	Low (<100)	High (>100)
BIOCHEMICAL		
β-Aspartylglycine	Absent	Present
Bile acid metabolism	Deconjugation	No deconjugation
	Dehydrogenation	No dehydrogenation
	Dehydroxylation	No dihydroxylation
Bilirubin metabolism	Deconjugation	Little deconjugation
	Urobilin	No urobilin
Cholesterol	Coprostanol	No coprostanol
Intestinal gases	Carbon dioxide	Some carbon dioxide
	Hydrogen	No hydrogen
	Methane	No methane
Mucin	Degraded	No degradation
Short chain fatty acids	Large amounts	Far less
Tryptic activity	Little or absent	High activity

Adapted from: Midtvedt, T., 1985. Influence of antibiotics on biochemical intestinal microflora-associated characteristics in man and mammals. In: Wostman, B.S., Pleasants, J.R, Pollard, M, Teah, B.A, Wagner, M. (Eds.), Germfree Research, Microflora Control and its Application to the Biomedical Sciences. Alan R. Liss, New York, pp. 241–245.

H. Irritable Bowel Syndrome

Gnotobiotic rats have been used as a model of irritable bowel syndrome (IBS) (Crouzet et al., 2013). GF rats reconstituted with the fecal microbiota of IBS patients developed hypersensitivity upon colorectal distension, while those reconstituted with fecal microbiota from healthy controls did not. These rats recapitulated the microbial community profile characteristic of IBS patients, which included increases in sulfate-reducing bacteria and Enterobacteriaceae and reduced bifidobacteria relative to healthy controls. This was accompanied by altered gut fermentation, specifically increased hydrogen production, and cecal sulfides production. This work established a direct link between the microbial alterations in IBS patients and their colon hypersensitivity.

IV. RESOURCES

The best resource for current information on the availability and use of gnotobiotic rats in research and testing would be national and international organizations as well as research centers that have had a history of use of such animals. The Association for Gnotobiotics (AG), founded in 1960 and based in the United States, has an international membership and remains an excellent starting point for information. The Japan Association for Germfree Life and Gnotobiology (JAGG) has a membership that is more focused in East Asia and services that region through an annual meeting held in Japan. An international symposium, under the sponsorship of AG and JAGG combined memberships as the International Association for Gnotobiology, is held every

third year with a history of rotating the site among the United States, Japan, and Europe. Since Website addresses may change over the life of this volume, readers should search the Web for current contact information on these organizations.

In addition to the scientific organizations mentioned, practical tutelage in gnotobiotic technologies can be gained on an informal basis at centers in the United States and Japan. Among these are the Center for Gastrointestinal Biology and Disease in North Carolina, founded in 1983, and supported as a resource by funding from the National Institutes of Health, and also the Gnotobiotics Core of the Baylor College of Medicine, Houston, Texas. The Karolinska Institutet in Stockholm, INRA in France, the University of Hannover in Germany, and the Kennedy Institute at the University of Oxford, UK, are resources convenient to European researchers. An international listserv for the purpose of direct exchange of information within the community of workers in the field of gnotobiology is currently maintained at the University of Alabama in Birmingham. Subscription is by email message to LISTSERV@LISTSERV.UAB.EDU with the command "SUBSCRIBE GNOTOBIOTICS Firstname Lastname" in the body of the message.

Some of the larger commercial breeders of laboratory animals maintain stocks using gnotobiotic technologies and are a source for advice on maintenance and derivation of new stocks.

V. CONCLUSIONS

At the New York Academy of Sciences Conference in 1959, "Germfree Vertebrates: Present Status," there were discussions on the state of the art, contemporary research uses, and some practical applications of the GF animal in the development of disease-free breeding colonies. Much of these discussions have come to fruition through further study and expanded implementation of the technology.

Prior to the late 1950s, equipment for GF animals was fabricated principally of stainless steel with various sizes and shapes of viewing areas. The early pioneers were convinced that the security of GF systems could be best achieved when rigid components were used that could withstand impact and be less vulnerable to breakage. Stainless-steel cylinder-shaped GF isolators bolted and gasketed together were the most commonly used system in the United States at the LOBUND Laboratory, the National Institutes of Health, and Walter Reed Army Institute for Research. In Sweden, at the Karolinska Institutet, Gustafsson (1948) developed rectangular rigid steel isolators with glass tops maximizing the visibility inside. These units could be totally introduced into a steam autoclave for sterilization. In both the Reyniers cylindrical tank and the Gustafsson isolator, neoprene sleeves and gloves were used for manipulation constituting a potential weakness, since they were subject to tear or puncture. Miyakawa (1968) in Japan operated remote mechanical arms and hands from outside a small sterile room similar to the equipment sometimes seen in radiation research laboratories. Even though there are very fine research reports from this period, the cost of equipment and its inefficient utilization of space limited the number of workers in the field. Also, the complexity of the fabrication and construction added further to discourage interested scientists. It was not until Trexler developed the low-cost, lightweight, flexible film isolator that GF research came within the budgetary and technical reach of the research community in general. In 1957, a standard flexible film $1.5 \times 0.6 \times 0.6$ m isolator complete with filtration, transfer port, exhaust trap, and flexible sleeves and gloves bore a price of \$300–\$400 compared to \$5000 for a typical stainless-steel tank-type isolator. Thus technological advances produced a system that has proved to be equally or more secure, light in weight to permit use in tiers, and made of clear plastic to allow complete visibility. An extension of the flexible film technology brought forth a lightweight, disposable, flexible film shipping unit (Trexler and Reynold, 1957) weighing 5–6 kg compared to earlier units weighing 70–80 kg and which required a battery-operated blower system. Thus both rearing and research units, plus shipping units, were readily obtainable to those interested in using and transporting GF and GN animals.

Diets that early workers developed were either mixed and formulated in their laboratories or prepared at significant cost by organizations that specialized in small batch mixing of complicated formulas. From the late 1950s onward, commercial feed manufacturers offered standard laboratory rodent diets prefortified with sufficient thermolabile nutrients to withstand sterilization and still support reproduction and growth. There have, from time to time, appeared in the marketplace canned sterile water and prepackaged sterilized bedding for those with limited sterilization capacity or capability.

In effect, it is entirely feasible to conduct a single GF research project without setting up a vast facility with expensive support, laboratories, and personnel. Also, when a single project is completed, the inflated flexible film isolator can be stored flattened in its deflated form for subsequent reuse. The initial set-up costs are nominal as are the operating costs.

Perhaps one of the single most important by-product benefits of GF and GN rats is the utilization of these animals as seed stock for new colonies. By deriving a strain of outbred CV stock of rats into the GF state, all

microorganisms and parasites are eliminated except the few that are thought to be transplacental and thus vertically transmitted. Colonies plagued with external and intestinal parasites, chronic murine pneumonia, and one or more other bacterial or viral diseases can be rendered free of these infections by utilization of the gnotobiotic technology and deriving these animals into the GF state. It is then a routine procedure to associate these rats with a defined "bacterial cocktail" of gut flora prior to removal from isolator systems and introduction into some type of clean barrier facility. Pathogen-free is the commonly used terminology for rats derived in this manner and is the accepted practice by industry, government, and academia for providing healthy animals for research. Even though testing and eradication techniques can work, as does induction of a bacterial-free state through broad-spectrum antibiotics, the gnotobiote must be derived within a sterile isolator system.

Valuable genetic strains of rats are assured continuity by maintaining them GF, greatly reducing and almost eliminating the possibility of loss through an epizootic. The National Cancer Institute for many years has maintained Rodent Genetic Centers under contract whereby valuable genetic strains and stocks have been maintained in the GN state. Through the technology herein described, genetically defined CV animals are cesarean delivered into the axenic state, then subsequently associated with a DF and maintained in isolators assuring microbial definition and uniformity. These rats can be sent with genetic and microbiological pedigree to laboratories for research utilization or breeding programs.

Like the pure or refined chemical reagents available to researchers, the laboratory rat can be obtained in the purest microbiological sense (axenic) or with an easily described and DF within the isolator. The barrier-reared animal, which is an extension of the gnotobiote, can be maintained in a controlled environment to preclude contamination by pathogens. Even though isolator systems break down on occasion, usually through human error, their use is a giant step forward toward supplying defined rats as one of the basic tools of biomedical research. Compared to its CV counterpart, the SPF, cesarean-delivered, cesarean-originated, barrier-sustained, barrier-maintained rat has provided the research community with some point of reference in that at one time during the immediate past they were gnotobiotes. The CV animal usually is not reared in a barrier environment where all materials contacting the animals have undergone decontamination, pasteurization, or sterilization. The probability therefore is far greater that microbiological variability does occur in CV animals because of their more loosely controlled environment.

The science of breeding and rearing GF, DF, and SPF rats is an attempt by professionals in laboratory animal science to keep pace with the rapidly evolving technology in the instrumentation field. What is the value of highly sophisticated instrumentation designed to make finite measurements of biological materials if the biological tool is uncontrolled and undefinable? The horizons of gnotobiotic research applications have expanded to include space travel and its potential and unpredictable effects on humans and their biosphere. Other areas of research include oral pathology, cancer, wound repair, infectious diseases, and nutrition. The GF animal therefore offers a multitude of research opportunities.

Because of the technology developed through the years, the cost of such research using GF animals is within the scope of most budgets. However, there is still an inherent resistance to undertake research with GF rats principally as a carryover from other eras when the cost of this type of research was excessive and the technology was too highly specialized for the average laboratory setting. The contents of this chapter, in connection with the literature citations, should provide those desirous of conducting research on GN rats the necessary technical information with regard to methodology, characteristics, and utilization.

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References

Albrecht, I., Souhrada, J., 1971. Defining the laboratory rat for cardiovascular research. In: Schneider, H.A. (Ed.), Defining the Laboratory Animal. National Academies Press, Washington, D.C, pp. 616–625.

Altura, B.T., Altura, B.M., Baez, S., 1975. Reactivity of aorta and portal vein in germfree rats. Blood Vessel. 12, 206–218.

Asano, T., 1967. Inorganic ions in cecal content of gnotobiotic rats. Proc. Soc. Exp. Biol. Med. 124, 424–430.

Asano, T., 1969. Modification of cecal size in germfree rats by long-term feeding of anion exchange resin. Am. J. Physiol. 217, 911–918.

Ashman, R.B., 1975. A rapid method for skin grafting in germfree rats. Lab. Anim. Sci. 25, 502–504.

Association for Gnotobiotics. 2020. https://gnotobiotics.wildapricot.org/.

Baez, S., Gordon, H.A., 1971. Tone and reactivity of vascular smooth muscle in germfree rat mesentery. J. Exp. Med. 134, 846–856.

Balish, E., Yale, C.E., Hong, R., 1972. Serum proteins of gnotobiotic rats. Infect. Immun. 6, 112–118.

Banasaz, M., Alam, M., Norin, E., Midtvedt, T., 2000. Gender, age and microbial status influence upon cell kinetics in a compartmentalised manner. An experimental study in germfree and conventional rats. Microb. Ecol. Health Disease 12, 208–218.

Bealmear, P.M., Loughman, B.E., Nordin, A.A., Wilson, R., 1973. Evidence for graft vs. host reaction in the germfree allogeneic radiation chimera. In: Heneghan, J.B. (Ed.), Germfree Research. Academic Press, New York, pp. 471–475.

Bornside, G.H., Donovan, W.E., Myers, M.B., 1976. Intracolonic tensions of oxygen and carbon dioxide in germfree, conventional, and gnotobiotic rats. Proc. Soc. Exp. Biol. Med. 151, 437–441.

Brand, M.W., Wannemuehler, M.J., Phillips, G.J., Procter, A., Overstreet, A.-M., Jergens, A.E., Orcutt, R.P., Fox, J.G., 2015. ILAR J. 56, 169–178.

Bruckner, G., 1997. How it started - and what is MAS? In: Heidt, P.J., Rusch, V., van der Waij, D. (Eds.), 1995 Old Herborn University Seminar Monograph No. 9 Gastrointestinal Motility. Herborn Litterae, Herborn-Dill, Germany, pp. 24–34. http://www.old-herborn-university.de.

Bruckner-Kardoss, E., Wostmann, B.S., 1974. Blood volume of adult germfree and conventional rats. Lab. Anim. Sci. 24, 633–635.

Burns, K.P., Timmons, E.H., Poiley, S.M., 1971. Serum chemistry and hematological values for axenic (germfree) and environmentally associated inbred rats. Lab. Anim. Sci. 21, 415–419.

Carlstedt-Duke, B., Midtvedt, T., Nord, C.E., Gustafsson, B.E., 1986. Isolation and characterization of a mucin degrading strain of peptostreptococcus from rat intestinal tract. Acta Path. Microbiol. Immunol. Scand. Sec. B 94, 292–300.

Carter, P.B., 1971. Host Responses to Normal Intestinal Microflora. Ph.D. thesis. University of Notre Dame.

Carter, P.B., 1976. Personal Communication. Using the Traditional Cesarean Approach, the Senior Author (PBC) Was Able to Derive, in a Relatively Short Time, over Six Mouse Strains, Three Rat Strains and Two Inbred guinea Pig Lines as New Breeding Stocks at the Trudeau Institute, Saranac Lake, NY, Following the Difficult Decision by the Research Staff to Depopulate the Animal Breeding Facility in the Wake of a Sendai Virus Outbreak.

Carter, P.B., Bazin, H., 1980. Immunology. In: Baker, H., Weisbroth, S.H., Lindsey, J.R. (Eds.), The Laboratory Rat. Academic Press, New York, pp. 181–212.

Cassell, G.H., Lindsey, J.R., Baker, H.J., 1974. Immune response of pathogen-free mice inoculated intranasally with *Mycoplasma pulmonis*. J. Immunol. 112, 124–136.

Cassell, G.H., Davis, J.K., 1978. Protective effect of vaccination against *Mycoplasma pulmonis* respiratory disease in rats. Infect. Immun. 21, 69–75.

Coates, M.E., 1973. Gnotobiotic animals in nutrition research. Proc. Nutr. Soc. 32, 53–58.

Coates, M.E., Gustafsson, B.E., 1984. The Germfree Animal in Biomedical Research. In: Laboratory Animal Handbooks, vol. 9. London Laboratory Animals LTD, 442 pages.

Crowley, P.J., Brady, L.J., Michalek, S.M., Bleiweis, A.S., 1999. Virulence of a spaP mutant of Streptococcus mutans in a gnotobiotic rat model. Infect. Immun. 67, 1201–1206.

Crouzet, L., Gaultier, E., Del'Homme, C., Cartier, C., Delmas, E., Dapoigny, M., Fioramonti, J., Bernalier-Donadille, A., 2013. The hypersensitivity to colonic distension of IBS patients can be transferred to rats through their fecal microbiota. Neuro Gastroenterol. Motil. 25, e272–e282.

Dajani, R.M., Gorrod, J.W., Beckett, A.H., 1975. Reduction in vivo of (minus)-nicotine-l-N-oxide by germfree and conventional rats. Biochem. Pharmacol. 24, 648–650.

Delluva, A.M., Markley, K., Davies, R.E., 1968. The absence of gastric disease in germfree animals. Biochim. Biophys. Acta 151, 646–650.

Demarne, Y., Flanzy, J., Jacquet, E., 1973. The influence of gastrointestinal flora on digestive utilization of fatty acids in rats. In: Heneghan, J.B. (Ed.), Germfree Research. Academic Press, New York, pp. 553–560.

Dieleman, L.A., Goerres, M.S., Arends, A., Sprengers, D., Torrice, C., Hoentjen, F., Grenther, W.B., Sartor, R.B., 2003. Lactobacillus GG prevents recurrence of colitis in HLA-B27 transgenic rats after antibiotic treatment. Gut 52, 370–376.

Donati, R.M., McLaughlin, M.M., Stromberg, L.R., 1973. Combined surgical and radiation injuryùThe effect of the gnotobiotic state on wound closure. Experientia 29, 1388–1390.

Dubos, R., Schaedler, R.W., Costello, R., Hoet, P., 1965. Indigenous, normal, and autochthonous flora of the gastrointestinal tract. J. Exp. Med. 122, 67–76.

Einarsson, K., Gustafsson, J.A., Gustafsson, B.E., 1973. Differences between germfree and conventional rats in liver microsomal metabolism of steroids. Biol. Chem. 248, 3623–3630.

Einarsson, K., Gustafsson, J.A., Gustafsson, B.E., 1974. Liver microsomal hydroxylation of steroid hormones after establishing an indigenous microflora in germfree rats. Proc. Soc. Exp. Biol. Med. 145, 48–52.

Elson, C.O., Sartor, R.B., Tennyson, G.S., Riddell, R.H., 1995. Experimental models of inflammatory bowel disease. Gastroenterology 109, 1344–1367.

Ericsson, A.C., Davis, J.W., Spollen, W., Bivens, N., Givan, S., Hagan, C.E., McIntosh, M., Franklin, C.L., 2015. Effects of vendor and genetic background on the composition of the fecal microbiota of inbred mice. PLoS One 10, e0116704.

Ericsson, A.C., Gagliardi, J., Bouhan, D., Spollen, W.G., Givan, S.A., Franklin, C.L., 2018. The influence of caging, bedding, and diet on the composition of the microbiota in different regions of the mouse gut. Sci. Rep. 8, 4065.

Festing, M.F.W., Blackmore, D.K., 1971. Life span of specified-pathogen-free (MRC category 4) mice and rats. Lab. Anim. 5, 179–192.

Fontaine, C.A., Skorupski, A.M., Vowles, C.J., Anderson, N.E., Poe, S.A., Eaton, K.A., 2015. How free of germs is germ-free? Detection of bacterial contamination in a germ free mouse unit. Gut Microb. 6, 225–233.

Foster, H.L., 1959a. A procedure for obtaining nucleus stock for a pathogen-free animal colony. Proc. Anim. Care 9, 135–142.

Foster, H.L., 1959b. Housing of disease-free vertebrates. Ann. N.Y. Acad. Sci. 78, 80–88.

Foster, H.L., Pfau, E.S., 1963. Gnotobiotic animal production at the Charles River breeding laboratories, Inc. Lab. Anim. Care 13, 629–632.

Foster, H.L., Trexler, P.C., Rumsey, G.L., 1967. A canned sterile shipping diet for small laboratory rodents. Lab. Anim. Care 17, 400–405.

Ganaway, J.R., Allen, A.M., Moore, T.D., Bohner, H.J., 1973. Natural infection of germfree rats with *Mycoplasma pulmonis*. J. Infect. Dis. 127, 529–537.

Giddens Jr., W.E., Whitehair, C.K., Carter, G.R., 1971. Morphological and microbiologic features of nasal cavity and middle ear in germfree, defined-flora, conventional, and chronic respiratory disease-affected rats. Am. J. Vet. Res. 32, 99–114.

Gordon, H.A., 1974. Intestinal water absorption in young and old, germfree and conventional rats. Experientia 30, 214–215.

Gordon, H.A., Bruckner-Kardoss, E., Staley, T.E., Wagner, M., Wostmann, B.S., 1966. Characteristics of the germfree rat. Acta Anat. 64, 367–389.

Gordon, H.A., Pesti, L., 1971. The gnotobiotic animal as a tool in the study of host-microbial relationships. Bacterial. Rev. 35, 390–429.

Gordon, H.A., Wostmann, B.S., 1959. Responses of the animal host to changes in the bacterial environment: transition of the albino rat from germfree to the conventional state. In: Tunevau, G. (Ed.),

Recent Progress in Microbiology. Almqvist & Wiksell, Stockholm, pp. 336–339.
Gordon, H.A., Wostmann, B.S., 1960. Morphological studies on the germfree albino rat. Anat. Rec. 137, 65–70.
Gordon, J.I., 2012. Honor thy gut symbionts redux. Science 336, 1251–1253.
Green, R.M., Blackmore, D.K., Drucker, D.B., 1973. The role of gnotobiotic animals in the study of dental caries. Br. Dent. J. 134, 537–540.
Green, R.M., Drucker, D.B., Blackmore, D.K., 1974. The reproducibility of experimental caries studies within and between two inbred strains of gnotobiotic rat. Arch. Oral Biol. 19, 1049–1054.
Griffiths, L.A., Barrow, A., 1972. Metabolism of flavonoid compounds in germfree rats. Biochem. J. 130, 1161–1162.
Gustafsson, A., Berstad, A., Lund-Tønnesen, S., Midtvedt, T., Norin, E., 1999. The effect of faecal enema on five microflora-associated characteristics in patients with antibiotic-associated diarrhea. Scand. J. Gastroenterol. 34, 580–586.
Gustafsson, B., 1948. Germfree rearing of rats. Acta Pathol. Microbiol. Scand. 73 (Suppl. l), 1–130.
Gustafsson, B.E., 1959. Vitamin K deficiency in germfree rats. Ann. N.Y. Acad. Sci. 78, 166–174.
Gustafsson, B.E., Cronholm, T., Gustafsson, J.A., 1975. Relation of intestinal bile acids in rats to intestianl microflora and sex. In: Fliedner, T.M., Heit, H., Neithammer, D., Pflieger, H. (Eds.), Clinical and Experimental Gnotobiotics. Gustav Fischer Verlag, New York.
Gustafsson, B.E., Midtvedt, T., Norman, A., 1966. Isolated fecal microorganisms capable of 7-alpha-dehydroxylating bile acids. J. Exp. Med. 123, 413–432.
Gustafsson, B.E., Midtvedt, T., Strandberg, K., 1970. Effects of microbial contamination on the cecum enlargement of germfree rats. Scand. J. Gastroenterol. 5, 309–314.
Gustafsson, B.E., Norman, A., 1962. Urinary calculi in germfree rats. J. Exp. Med. 116, 273–284.
Gustafsson, B.E., Norman, A., 1969. Influence of the diet on the turnover of bile acids in germfree and conventional rats. Br. J. Nutr. 23, 429–442.
Håverstad, T., Midtvedt, T., 1986. Short-chain fatty acids in germ-free mice and rats. J. Nutr. 116, 1772–1776.
Hunt, R.D., King, N.W., Foster, H.L., 1972. Encephalitozoonosis: evidence for vertical transmission. J. Infect. Dis. 126, 212–224.
Ikari, N.S., Donaldson, D.M., 1970. Serum beta-lysin and muramidase levels in germfree and conventional rats. Proc. Soc. Exp. Biol. Med. 133, 49–52.
Inzunza, J., Midtvedt, T., Fartoo, M., Norin, E., Osterlund, E., Persson, A.K., Ahrlund-Richter, L., 2005. Germfree status of mice obtained by embryo transfer in an isolator environment. Lab. Anim. 39, 421–427.
Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V., Tanoue, T., Imaoka, A., Itoh, K., Takeda, K., Umesaki, Y., Honda, K., Littman, D.R., 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 139, 485–498.
Jacoby, R.O., Ball-Goodrich, L., Paturzo, F.X., Johnson, E.A., 2001. Prevalence of rat virus infection in progeny of acutely or persistently infected pregnant rats. Comp. Med. 51, 38–42.
Juul, F.E., Garborg, K., Bretthauer, M., Skudal, H., Øines, M.N., Wiig, H., Rose, Ø., Seip, B., Lamont, J.T., Midtvedt, T., Valeur, J., Kalager, M., Holme, Ø., Helsingen, L., Løberg, M., Adami, H.-O., 2018. Fecal microbiota transplantation for primary *Clostridium difficile* infection. N. Engl. J. Med. 378, 2535–2536.
Kajima, M., Pollard, M., 1965. Detection of viruslike particles in germfree mice. J. Bacteriol. 90, 1448–1454.
Kajiwara, N., Ueno, Y., Takahashi, A., Sugiyama, F., Sugiyama, Y., Yagami, K., 1996. Vertical transmission to embryo and fetus in maternal infection with rat virus. Exp. Anim. 45, 239–244.
Kappel, H.K., Kappel, J.P., Weisbroth, S.H., Kozma, C.K., 1969. Establishment of a hysterectomy-derived, pathogen-free breeding nucleus of Blu-(LE) rats. Lab. Anim. Care 19, 738–741.
Kellogg, T.F., Wostmann, B.S., 1969. Stock diet for colony production of germfree rats and mice. Lab. Anim. Care 19, 812–814.
Laqueur, G.L., McDaniel, E.G., Mateumoto, H., 1967. Tumor induction in germfree rats with methylazoxymethanol (MAM) and synthetic MAM acetate. J. Natl. Cancer Inst. 39, 355–371.
Lee, Y.C., McKenzie, R.M., Gholson, R.K., Raica, N., 1972. A comparative study of nicotinamide and nicotinic acid in normal and germfree rats. Biochim. Biophys. Acta 264, 59–64.
Lepkovsky, S., Furuta, F., Ozone, K., Koike, T., 1966. The proteases, amylase, and lipase of the pancreas and intestinal contents of germfree and conventional rats. Br. J. Nutr. 20, 257–261.
Lev, M., 1963. Germfree animals. In: Lane-Petter, W.L. (Ed.), Animals for Research. Academic Press, New York, pp. 139–175.
Lindsey, J.R., Baker, H.J., Overcash, R.G., Cassell, G.H., Hunt, C.E., 1971. Murine chronic respiratory disease. Significance as a research complication and experimental production with *Mycoplasma pulmonis*. Am. J. Pathol. 64, 675–708.
Luckey, T.D., 1963. Germfree Life and Gnotobiology. Academic Press, New York.
Luckey, T.D., 1968. Gnotobiology and aerospace systems. In: Miyakawa, M., Luckey, T.D. (Eds.), Advances in Germfree Research and Gnotobiology. CRC Press, Cleveland, OH, pp. 317–353.
Lynch, D.J., Michalek, S.M., Zhu, M., Drake, D., Qian, F., Banas, J.A., 2013. Cariogenicity of *Streptococcus mutans* glucan-binding protein deletion mutants. Oral Health Dent. Manag. 12, 191–199.
Madsen, D., Beaver, M., Chang, L., Bruckner-Kardoss, E., Wostmann, B., 1976. Analysis of bile acids in conventional and germfree rats. J. Lipid Res. 17, 107–111.
Maejima, K., Mitsuoka, T., Namioka, S., Nomura, T., Tajima, Y., Yoshida, T., 1974. Bibliography of technology for germfree animal research. Exp. Anim. 24, 229–253.
Mähler, M., Berard, M., Feinstein, R., Gallagher, A., Illgen-Wilcke, B., Pritchett-Corning, K., Raspa, M., 2014. FELASA recommendations for the health monitoring of mouse, rat, hamster, Guinea pig and rabbit colonies in breeding and experimental units. Lab. Anim. 48, 178–192.
Martin, L.K., Einheber, A., Porro, R.F., Sadun, E.H., Bauer, H., 1966. Plasmodium berghei infections in gnotobiotic mice and rats: parasitologic, immunologic, and histopathologic observations. Mil. Med. 131 (Suppl. l), 870–889.
McClellan, M.A., Hummel, R.P., Alexander, J.W., 1974. Opsonic activity in germfree and monocontaminated rat sera. Surg. Forum 25, 27–28.
McDonald, J.C., Zimmerman, G., Bollinger, R.R., Pierce Jr., W.A., 1971. Immune competence of germfree rats. I. Increased responsiveness to transplantation and other antigens. Proc. Soc. Exp. Biol. Med. 136, 987–993.
McMaster, P.R., Aronson, S.B., Bedford, M.J., 1967. Mechanisms of the host response in the eye. IV. The anterior eye in germfree animals. Arch. Ophthalmol. 77, 392–399.
Midtvedt, T., Gustafsson, B.E., 1981. Microbial conversion of bilirubin to urobilins in vitro and in vivo. Acta Pathol. Microbiol. Scand. Sect. B 89, 57–60.
Midtvedt, T., 1989. The normal microflora, intestinal motility and influences of antibiotics; an overview. In: Grubb, R., Midtvedt, T., Norin, E. (Eds.), The Regulatory and Protective Role of the Normal Microflora. The MacMillan Press LTD, London, pp. 147–168.
Miyakawa, M., 1968. Studies of rearing germfree rats. In: Miyakawa, M., Luckey, T.D. (Eds.), Advances in Germfree Research and Gnotobiology. CRC Press, Cleveland, OH, pp. 48–62.
Miyakawa, M., Sumi, Y., Kanzaki, M., Imaeda, F., 1975. Tumor induction by oral administration of A'-methyl-A'-nitro-/V-nitrosoguanadine or aflatoxin Bl in germfree and conventional rats. In: Fliedner, T.M.,

Heit, H., Neithammer, D., Pflieger, H. (Eds.), Clinical and Experimental Gnotobiotics. Gustav Fischer Verlag, New York.
Miyakawa, M., Sumi, Y., Sakurai, K., Ukai, M., Hirabayashi, N., Ito, G., 1969. Serum gammaglobulin and lymphoid tissue in the germfree rats. Acta Haematol. Jpn. 32, 501–518.
Morioka, T., Saji, S., Inoue, M., Matsumura, T., 1969. Peroxidase-mediated antibacterial activity in the salivary gland of germfree and conventional rats. Arch. Oral Biol. 14, 549–553.
Nagler, A.L., Seifter, E., Geever, E.F., Dettbarn, W.D., Levenson, S.M., 1969. The nephropathy of acute choline deficiency in germfree conventional, and open animal room rats. In: Mirand, E.A., Back, N. (Eds.), Germfree Biology. Plenum, New York, pp. 317–324.
Nicklas, W., Keubler, L., Bleich, A., 2015. Maintaining and monitoring the defined microbiota status of gnotobiotic rodents. ILAR J. 56, 241–249.
Nolen, G.A., Alexander, J.C., 1965. A comparison of the growth and fat utilization of caesarean-derived and conventional albino rats. Lab. Anim. Care 15, 295–303.
Norin, K.E., Gustafsson, B.E., Midtvedt, T., 1986. Strain differences in fecal tryptic activity of germfree and conventional rats. Lab. Anim. 20, 67–69.
Norin, E., Midtvedt, T., 2006. Born germfree—microbial dependent. In: Ouwehand, A., Vaughan, E.E. (Eds.), Gastrointestinal Microbiology. Taylor & Francis, pp. 273–284.
Norin, E., 2018. Personal Observation.
Norin, E., 2019. Personal Communication.
Nuttall, G.H.F., Thierfelder, H., 1895. Thierisches Leben ohne Bakterien im Verdauungskanal. Hoppe-Seyler's Z. für Physiol. Chem. 21, 109–121.
Nuttall, G.H.F., Thierfelder, H., 1896. Thierisches Leben ohne Bakterien im Verdauungskanal. (II. Mittheilung). Hoppe-Seyler's Z. für Physiol. Chem. 22, 62–73.
Nuttall, G.H.F., Thierfelder, H., 1897. Thierisches Leben ohne Bakterien im Verdauungskanal. (III. Mittheilung) Versuche an H^{n}hnern. Hoppe-Seyler's Z. für Physiol. Chem. 23, 231–235.
Nuzum, C.T., 1975. Activities of hepatic enzymes of urea synthesis in germfree and conventional rats. In: Fliedner, T.M., Heit, H., Neithammer, D., Pflieger, H. (Eds.), Clinical and Experimental Gnotobiotics. Gustav Fischer Verlag, New York.
Oace, S.M., 1972. A purified soy protein diet for nutrition studies with germfree rats. Lab. Anim. Sci. 22, 528–531.
Orland, F.J., Blayney, J.R., Harrison, R.W., Reyniers, J.A., Trexler, P.C., Wagner, M., Gordon, H.A., Luckey, T.D., 1954. Use of germfree animal technic in the study of experimental dental caries. I. Basic observations on rats reared free of all microorganisms. J. Dent. Res. 33, 147–174.
Packey, C.D., Shanahan, M.T., Manick, S., Bower, M.A., Ellermann, M., Tonkonogy, S.L., Carroll, I.M., Sartor, R.B., 2013. Molecular detection of bacterial contamination in gnotobiotic rodent units. Gut Microb. 4, 361–370.
Paterson, J.S., Cook, R., 1971. Utilization of diets sterilized by gamma irradiation for germfree and specific-pathogen-free laboratory animals. In: Schneider, H.A. (Ed.), Defining the Laboratory Animal. National Academies Press, Washington, D.C, pp. 586–596.
Pittermann, W., Deerberg, F., 1975. Spontaneous tumors and lesions of the lung, kidney and gingiva in aged germfree rats. In: Fliedner, T.M., Heit, H., Neithammer, D., Pflieger, H. (Eds.), Clinical and Experimental Gnotobiotics. Gustav Fischer Verlag, New York.
Pleasants, J.R., 1959. Rearing germfree caesarian-born rats, mice, and rabbits through weaning. Ann. N.Y. Acad. Sci. 78, 116–126.
Pleasants, J.R., 1974. Gnotobiotics. In: Melby Jr., E.C., Altman, N.H. (Eds.), Handbook of Laboratory Animal Science, vol. I. CRC Press, Cleveland, Ohio, pp. 119–174.
Pollard, M., 1971a. The germfree rat. Pathobiol. Annu. 1, 83–94.
Pollard, M., 1971b. Senescence in germfree rats. Gerontologia 17, 333–338.
Pollard, M., 1972. Carcinogenesis in germfree animals. Prog. Immunobiol. Stand. 5, 226–230.
Pollard, M., 1973. Spontaneous prostate adenocarcinomas in aged germfree Wistar rats. J. Natl. Cancer Inst. 51, 1235–1241.
Pollard, M., 1977. Animal model of human disease: metastatic adenocarcinoma of the prostate. Am. J. Pathol. 86, 277–280.
Pollard, M., 1992. The Lobund-Wistar rat model of prostate cancer. J. Cell. Biochem. 16H (Suppl. l), 84–88.
Pollard, M., Kajima, M., 1966. Leukemia in germfree rats. Proc. Soc. Exp. Biol. Med. 121, 585–589.
Pollard, M., Kajima, M., 1970. Lesions in aged germfree Wistar rats. Am. J. Pathol. 61, 25–36.
Pollard, M., Kajima, M., Lorans, G., 1968. Tissue changes in germfree rats with primary tumors. RES. J. Reticuloendothel. Soc. 5, 147–160.
Pollard, M., Luckert, P.H., 1987. Autochthonous prostate adenocarcinomas in Lobund-Wistar rats: a model system. Prostate 11, 219–227.
Pollard, M., Luckert, P.H., Meshorer, A., 1985. Xenogeneic bone marrow chimerism in germfree rats. Prog. Clin. Biol. Res. 181, 447–450.
Pollard, M., Teah, B.A., 1963. Spontaneous tumors in germ-free rats. J. Natl. Cancer Inst. 31, 457–465.
Pollard, M., Wolter, W., 2000. Prevention of spontaneous prostate-related cancer in Lobund-Wistar rats by a soy protein isolate/isoflavone diet. Prostate 45, 101–105.
Rath, H.C., Herfarth, H.H., Ikeda, J.S., Grenther, W.B., Hamm Jr., T.E., Balish, E., Taurog, J.D., Hammer, R.E., Wilson, K.H., Sartor, R.B., 1996. Normal luminal bacteria, especially *Bacteroides* species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. J. Clin. Invest. 98, 945–953.
Rath, H.C., Schultz, M., Freitag, R., Dieleman, L.A., Li, F., Linde, H.J., Scholmerich, J., Sartor, R.B., 2001. Different subsets of enteric bacteria induce and perpetuate experimental colitis in rats and mice. Infect. Immun. 69, 2277–2285.
Rath, H.C., Wilson, K.H., Sartor, R.B., 1999. Differential induction of colitis and gastritis in HLA-B27 transgenic rats selectively colonized with *Bacteroides vulgatus* or *Escherichia coli*. Infect. Immun. 67, 2969–2974.
Rawls, J.F., Samuel, B.S., Gordon, J.I., 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc. Natl. Acad. Sci. U. S. A. 101, 4596–4601.
Reddy, B.S., Pleasants, J.R., Wostmann, B.S., 1969a. Pancreatic enzymes in germfree and conventional rats fed chemically defined, water-soluble diet free from natural substrates. J. Nutr. 97, 327–334.
Reddy, B.S., Pleasants, J.R., Wostmann, B.S., 1973. Metabolic enzymes in liver and kidney of the germfree rat. Biochim. Biophys. Acta 320, 1–8.
Reddy, B.S., Wostmann, B.S., 1966. Intestinal disaccharidase activities in the growing germfree and conventional rat. Arch. Biochem. Biophys. 113, 609–616.
Reddy, B.S., Wostmann, B.S., Pleasants, J.R., 1969b. Protein metabolism in germfree rats fed chemically defined, water-soluble diet and semi-synthetic diet. In: Mirand, E.A., Back, N. (Eds.), Germfree Biology. Plenum, New York, pp. 301–305.
Reid Jr., L.C., Gates Jr., A.S., 1966. A method of sterilizing supplies for germfree isolators in plastic bags. Lab. Anim. Care 16, 246–254.
Reina-Guerra, M., Tennant, B., Harrold, D., Goldman, M., 1969. The absorption of fat by germfree and conventional rats. In: Mirand, E.A., Back, N. (Eds.), Germfree Biology. Plenum, New York, pp. 297–300.
Reyniers, J.A., Trexler, P.C., Ervin, R.F., 1946. Rearing germfree albino rats. Lobund. Rep. 1, 1–84.
Reyniers, J.A., Trexler, P.C., Scruggs, W., Wagner, M., Gordon, H.A., 1956. Observations on germfree and conventional albino rats after total-body x-irradiation. Radiat. Res. 5, 591.

Rodkey, F.L., Collison, H.A., O'Neal, J.D., 1972. Carbon monoxide and methane production in rats, Guinea pigs, and germfree rats. J. Appl. Physiol. 33, 256–260.
Rogers, T., Balish, E., 1976. Experimental *Candida* infection in conventional mice and germfree rats. Infect. Immun. 14, 33–38.
Rogers Jr., W.E., Bieri, J.G., McDaniel, E.G., 1971. Vitamin A deficiency in the germfree state. Fed. Proc. 30, 1773–1778.
Sacksteder, M., Kasza, L., Palmer, J., Warren, J., 1973. Cell transformation in germfree Fischer rats. In: Heneghan, J.B. (Ed.), Germfree Research. Academic Press, New York, pp. 153–157.
Sacquet, E., Lachkar, M., Mathis, C., Raibaud, P., 1973. Cecal reduction in "gnotoxenic" rats. In: Heneghan, J.B. (Ed.), Germfree Research. Academic Press, New York, pp. 545–552.
Sartor, R.B., 2000. Colitis in HLA-B27/beta 2 microglobulin transgenic rats. Int. Rev. Immunol. 19, 39–50.
Savage, D.C., 1969. Microbial interference between indigenous yeast and lactobacilli in the rodent stomach. J. Bacteriol. 98, 1278.
Saxerholt, H., Midtvedt, T., Gustafsson, B.E., 1985. Deconjugation of bilirubin conjugates and urobilin formation by conventionalized germ-free rats. Prog. Clin. Biol. Res. 181, 99–101.
Schaedler, R.W., Dubos, R., Costello, R., 1965a. The development of the bacterial flora in the gastrointestinal tract of mice. J. Exp. Med. 122, 59–66.
Schaedler, R.W., Dubos, R., Costello, R., 1965b. Association of germfree mice with bacteria isolated from normal mice. J. Exp. Med. 122, 77–82.
Schoeb, T.R., Eaton, K.A., 2017. Gnotobiology. Academic Press, London.
Schoeb, T.R., Kervin, K.C., Lindsey, J.R., 1985. Exacerbation of murine respiratory Mycoplasmosis in gnotobiotic F344/N rats by Sendai virus infection. Vet. Pathol. 22, 272–282.
Schoeb, T.R., Lindsey, J.R., 1987. Exacerbation of murine respiratory mycoplasmosis by sialodacryoadenitis virus infection in gnotobiotic F344 rats. Vet. Pathol. 24, 392–399.
Schultz, K.D., Appel, K.R., Goeth, H., Wilk, W., 1974. Experiments to establish a rat stock free of mycoplasma. Z. Versuchstierk. 16, 105–112.
Schwartz, B.F., 1975. Pulmonary gas exchange in germfree and conventional rats. In: Fliedner, T.M., Heit, H., Neithammer, D., Pflieger, H. (Eds.), Clinical and Experimental Gnotobiotics. Gustav Fischer Verlag, New York.
Sewell, D.L., Bruckner-Kardoss, E., Lorenz, L.M., Wostmann, B.S., 1976. Glucose tolerance, insulin and catecholamine levels in germfree rats. Proc. Soc. Exp. Biol. Med. 152, 16–19.
Sewell, D.L., Wostmann, B.S., Gairola, C., Aleem, M.I.H., 1975. Oxidative energy metabolism in germfree and conventional rat liver mitochondria. Am. J. Physiol. 228, 526–529.
Sewell, D.L., Wostmann, B.S., 1975. Thyroid function and related hepatic enzymes in the germfree rat. Metab. Clin. Exp. 24, 695–701.
Shih, C.N., Balish, E., Lower Jr., G.M., Yale, C.E., Bryan, G.T., 1975. Induction of colonic tumors in germfree and conventional rats. In: Fliedner, T.M., Heit, H., Neithammer, D., Pflieger, H. (Eds.), Clinical and Experimental Gnotobiotics. Gustav Fischer Verlag, New York.
Smith, H.W., 1965. The development of the flora of the alimentary tract in young animals. J. Pathol. Bacterial. 90, 495–513.
Smith Jr., J.C., McDaniel, E.G., Doft, F.S., 1973. Urinary calculi in germfree rats alleviated by varying the dietary minerals. In: Heneghan, J.B. (Ed.), Germfree Research. Academic Press, New York, pp. 285–290.
Smithies, O., 2007. Turning Pages: Nobel Lecture, 7 December 2007. The Nobel Foundation. https://www.nobelprize.org/prizes/medicine/2007/smithies/lecture/.
Snyder, D.L., Pollard, M., Wostmann, B.S., Luckert, P., 1990. Life span, morphology, and pathology of diet-restricted germ-free and conventional Lobund-Wistar rats. J. Gerontol. 45, B52–B58.
Sugiyama, T., Bruckner, G.G., 1975. Mycoplasma-bacteria relationships in the pathogenesis of chronic respiratory disease in conventional rats. In: Fliedner, T.M., Heit, H., Neithammer, D., Pflieger, H. (Eds.), Clinical and Experimental Gnotobiotics. Gustav Fischer Verlag, New York.
Syed, S.A., Abrams, G.D., Freter, R., 1970. Efficiency of various intestinal bacteria in assuming normal function of enteric flora after association with germfree mice. Infect. Immun. 2, 376–386.
Taurog, J.D., Richardson, J.A., Croft, J.T., Simmons, W.A., Zhou, M., Fernandez-Sueiro, J.L., Balish, E., Hammer, R.E., 1994. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. J. Exp. Med. 180, 2359–2364.
Thompson, G.R., Trexler, P.C., 1971. Gastrointestinal structure and function in germfree or gnotobiotic animals. Gut 12, 230–235.
Trexler, P.C., 1959. The use of plastic in the design of isolator systems. Ann. N.Y. Acad. Sci. 78, 29–36.
Trexler, P.C., Reynolds, L.I., 1957. Flexible film apparatus for the rearing and use of germfree animals. Appl. Microbiol. 5, 6.
Trippestad, A., Midvedt, T., 1971. The phagocytic activity of polymorphonuclear leucocytes from germfree and conventional rats. Acta Pathol. Microbiol. Scand. Sect. B 79, 519–522.
Treuting, P.M., Clifford, C.B., Sellers, R.S., Brayton, C.F., 2012. Of mice and microflora: considerations for genetically engineered mice. Vet. Pathol. 41, 44–63.
Tuffery, A.A., Innes, J.R.M., 1963. Diseases of laboratory mice and rats. In: Lane-Petter, W. (Ed.), Animals for Research. Academic Press, New York, pp. 48–109.
Ukai, M., Tomura, A., Ito, M., 1976. Cholesterol synthesis in germfree and conventional rats. J. Nutr. 106, 1175–1183.
van der Waaij, D., Berghus-de Vries, J.M., Lekkerkerk van der Wees, J.E.C., 1971. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J. Hyg. 69, 405–411.
Wade, A.C., Luckert, P.H., Tazume, S., Niedbalski, J.L., Pollard, M., 1987. Characterization of xenogeneic mouse to rat bone marrow chimeras. Transplantation 44, 88–92.
Wagner, M., 1959. Determination of germfree status. Ann. N.Y. Acad. Sci. 78, 89–102.
Walburg Jr., H.E., 1973. Carcinogenesis in gnotobiotic rodents. In: Heneghan, J.B. (Ed.), Germfree Research. Academic Press, New York, pp. 115–122.
Warner, T.F., Madsen, J., Starling, J., Wagner, R.D., Taurog, J.D., Balish, E., 1996. Human HLA-B27 gene enhances susceptibility of rats to oral infection by *Listeria monocytogenes*. Am. J. Pathol. 149, 1737–1743.
Weisburger, J.H., Reddy, B.S., Narisawa, T., Wynder, E.L., 1975. Germfree status and colon tumor induction by N-methyl-N-nitro-N-nitrosoguanidine. Proc. Soc. Exp. Biol. Med. 148, 1119–1121.
Wostmann, B.S. (Ed.), 1970. Gnotobiotes: Standards and Guidelines for the Breeding, Care and Management of Laboratory Animals. National Academies Press, Washington, D.C.
Wostmann, B.S., 1973. Intestinal bile acids and cholesterol absorption in the germfree rat. J. Nutr. 103, 982–990.
Wostmann, B.S., 1975. Nutrition and metabolism of the germfree mammal. World Rev. Nutr. Diet. 22, 40–92.
Wostmann, B.S., 1996. Germfree and Gnotobiotic Animal Models: Background and Applications. CRC Press, Boca Raton, Florida, 189pp.
Wostmann, B.S., Beaver, M., Modsen, D., 1975. Effect of diet sterilization on cholesterol and bile acid values of germfree rats. In: Fliedner, T.M., Heit, H., Neithammer, D., Pflieger, H. (Eds.), Clinical and Experimental Gnotobiotics. Gustav Fischer Verlag, New York.
Wostmann, B.S., Bruckner-Kardoss, E., 1959. Development of cecal distention in germfree baby rats. Am. J. Physiol. 197, 1345–1346.
Wostmann, B.S., Bruckner-Kardoss, E., Knight Jr., P.L., 1968. Cecal enlargment, cardiac output, and O_2 consumption in germfree rats. Proc. Soc. Exp. Biol. Med. 128, 137–141.

Wostmann, B.S., Kellogg, T.F., 1967. Purified starch-casein diet for nutritional research with germfree rats. Lab. Anim. Care 17, 589–593.

Wostmann, B.S., Knight, P.L., 1965. Antagonism between vitamins A and K in the germfree rat. J. Nutr. 87, 155–160.

Wosu, N.J., Olsen, R., Shadduck, J.A., Koestner, A., Pakes, S.P., 1977a. Diagnosis of experimental encephalitozoonosis in rabbits by complement fixation. J. Infect. Dis. 135, 944–948.

Wosu, N.J., Shadduck, J.A., Pakes, S.P., Frenkel, J.K., Todd Jr., K.S., Conroy, J.D., 1977b. Diagnosis of encephalitozoonosis in experimentally infected rabbits by intradermal and immunofluorescence test. Lab. Anim. Sci. 27, 210–216.

Yale, C.E., Linsley, J.G., 1970. A large efficient isolator holding germfree rats. Lab. Anim. Care 20, 749–755.

Yamada, K., Ukai, M., 1976. The histochemistry of mucosaccharides in some organs of germfree rats. Histochemistry 47, 219–238.