

# The development history of Germfree animal model and its application in the study of microbiota-related diseases

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## *Abstract*

A germ-free animal is an experimental animal with no live microorganisms and parasites in its body. It is a powerful experimental tool for the study of the relationship between microbiota and host health. We reviewed the development history of germ-free animals and provide an overview of their application in medical research, such as intestinal microbiota, immunology and oncology. We provide references for research on microbe-related diseases.

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**Keywords:** Germ-free, microbiota, development history, application

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Received: April 27, 2022

Accepted: February 2, 2023

<https://doi.org/10.14456/tjvm.2023.1>

## Introduction

Germ-free (GF) animal refers to an animal without any living organism or parasite inside and outside its body that can be detected with existing detection technology. A GF animal is free of microorganisms, such as viruses, Rickettsia and bacteria (including spirochetes and Mycoplasma), as well as fungi and protozoa (Al-Asmakh and Zadjali, 2015). The sterility referred to here is a relative concept, which means that the existence of living pathogens cannot be detected using existing scientific knowledge and detection methods. With the development of science, the animals are considered aseptic at present but researchers may be able to detect pathogens rather than GF animals in the future. Therefore, the term "aseptic" is relative (Wostmann *et al.*, 1996; Al-Asmakh and Zadjali, 2015). GF animals are derived from the laparotomy of animals under aseptic conditions and artificial breastfeeding or breast replacement in an aseptic isolator. In rats, mice, guinea pigs, fish, invertebrate models such as drosophila have been successfully cultivated in recent years (Schwarzer *et al.*, 2021).

No uniform microbiological test method exists for GF animals; so, various test methods have been used (Arvidsson *et al.*, 2012; Bhattarai and Kashyap, 2016; Qv *et al.*, 2020). Some of these tests are plagued with issues regarding virus and Rickettsia examination. For example, in the most commonly used GF mice in laboratories, electron microscopy is used to prove that the leukemia virus still exists in thymocytes. Therefore, the so-called GF animals refer to animals in which bacteria, fungi, protozoa, and parasites cannot be detected *in vitro* and *in vivo*.

The biological characteristics of GF animals are significantly different from animals of low microbial level (Reikvam *et al.*, 2011). The differences are not limited to those caused by microorganisms and parasites but also affect host physiology, nutrition, metabolism and immune response. Compared with SPF animals, GF mice show signs of reduced muscle and muscle atrophy (Lahiri *et al.*, 2019), proving that the gut microbiota contributes to the quality and function of mouse skeletal muscle. In addition, the number of intestinal mucosal villi of GF animals is increased and the shape is slender; the turnover rate of intestinal epithelial cells is reduced, the rate of intestinal wall material exchange is decreased and the rate of bile excretion of metabolites is also slowed down. GF animals die due to cecal expansion and a thin intestinal wall, which often causes cecal volvulus and rupture of the intestinal wall. When a GF animal is individually infected by any species of bacteria (Bacteroides, Salmonella, Streptococcus), the cecum will become smaller. Moreover, the lymphatic tissue of GF animals is poorly developed, the ability to produce gamma globulin is weak, the levels of IgM, IgG, and IgA in the serum are low, the number of various immune cells is reduced and the function is defective (Reikvam *et al.*, 2011; Gordon and Pesti, 1971). Therefore, it is extremely sensitive to microbial infection and some microbes that are weak in pathogenicity and must be inoculated through the abdominal cavity or brain to infect ordinary animals can easily infect GF animals.

The establishment of GF animal models has gradually matured after more than a century of development and has been applied to various life science fields, including infectious diseases, metabolic diseases, immune diseases, allergic diseases, respiratory diseases, vaccine development and so on (Al-Asmakh and Zadjali, 2015; Bhattarai and Kashyap, 2016; Gonzalez *et al.*, 2016; Maschirow *et al.*, 2019; Torres *et al.*, 2020) and significant progress has been achieved. Simultaneously, GF animals are important experimental tools for life science research.

## Development history of GF animals

Since ancient times, humans have understood life phenomena through animals and animal experiments have had an irreplaceable role in the study of diseases and the production of medicines and cosmetics (Ulmer *et al.*, 1964; Knudsen *et al.*, 2019). The quality of the experimental animals determines the accuracy and rigor of the experiment, especially because a large number of microorganisms in experimental animals remarkably affect the accuracy of the experiment. At present, experimental animals are divided into conventional (CV) animals, clean (CL) animals, specified pathogen free (SPF), germ free (GF) animals and gnotobiological (GN) animals according to the degree of microbial purification through microbial control, quarantine, disinfection and caesarean section purification. The higher the microbial grade of experimental animals is, the higher the effectiveness of the experimental results. In recent years, with the deepening of disease research, GF animals have become the only tool used to explore the relationship between intestinal bacteria and diseases (Uzbay, 2019). The demand for GF animals among researchers is increasing. Thus, establishing a mature reproductive technology and population of GF animals is very important.

**Establishment process of GF animals:** Within the scope of current sterility testing technology, no detectable microorganisms exist in the intestines of sterile animals. The successful breeding of GF animals depends on a complete sterile barrier system, including isolators, transfer windows, constant temperature heating equipment, lighting, lamps, UV germicidal lamps, air filters and others (Qv *et al.*, 2020). In addition, the caesarean section operation, diet, drinking water, and experimental operation should be conducted under strict aseptic conditions.

Historically, Pasteur first recognized the concept of germ-free life and gnotobiosis. He first put forward the concept of GF animal in 1885 and studied the relationship between bacteria and host. He believed that normal aseptic organisms did not exist and intestinal microbiota promoted the growth and development of animals (Pasteur, 1885). In 1895, Nuttall *et al.*, obtained the first batch of GF animals (GF pigs) in the world through caesarean section. After 8 days of artificial lactation, the guinea pigs were in good health, thereby confirming that the microbiota was not a necessary condition for the survival of animals. Unfortunately, these sterile guinea pigs survived for only 13 days (Nuttall and Thierfelder, 1897). After the

Second World War, with the emergence of antibiotics, life without microorganisms became a popular research topic (Kirk, 2012). Based on previous experiments, scientists developed the first batch of GF rats in 1940. In 1942, Baker *et al.*, reported the study of sterile fish for the first time (Baker *et al.*, 1942). In 1945, the Lobound research team in Notre Dame University successfully bred GF rats and established a simple GF rat breeding system (Reyniers *et al.*, 1946). In 1959, Pleasants *et al.*, successfully established GF mouse and rabbit models (Pleasants, 1959). In the same year, Miyakawa *et al.*, perfected the technical details of aseptic experiment by developing remote control technology (Miyakawa *et al.*, 1959). All the above-mentioned GF animals were obtained by caesarean section and in an aseptic environment. In 2016, Schaeck M *et al.*, prepared sterile sea bass juveniles by continuously adding bacteriostatic drugs. The acquisition of sterile sea bass effectively promoted research into fish juvenile diseases and the development of aquaculture (Schaeck *et al.*, 2016). The establishment of GF animal populations provided an efficient and reliable experimental tool for the study of the causal relationship between microbiota and host health and played an important role in the study of intestinal microbiota, immunity and metabolism.

**Development history of barrier facilities for GF animals breeding:** Three types of positive pressure isolators are used to create a sterile cultivation environment, as well as surgical, transitional and breeding isolators. The surgical isolator is used for the caesarean section of aseptic animals. The experimental materials outside the sterile environment enter the feeding isolator through the transition isolator and the feeding isolator provides a sterile daily feeding environment for GF animals. The development history of cultivation barrier facilities is as follows. In 1895, Kijanizin *et al.*, used aseptic apparatus to cultivate GF rabbits (Wang, 2006). During this period, the diet and drinking water of GF rabbits were kept sterile; however, the weight of the rabbits decreased continuously and they finally died (Nuttall and Thierfelder, 1897). In 1928, Reynier *et al.*, creatively developed the first metal isolator for the cultivation of the first sterile rats in the world (Reyniers, 1932). In 1959, Mr. Miyagawa of Japan took stainless steel as the main part of the isolator and added glass windows and a precision manipulator with remote control to facilitate the experimental operation (Miyakawa *et al.*, 1959). However, the isolator had obvious shortcomings, was bulky and was inconvenient to move; moreover, it was costly to operate (Miyakawa *et al.*, 1959). In the same year, Gustafsson's group used thin-walled stainless steel aseptic tanks. Before the rats were raised to the seventh generation, the bacteria in their feces were sterile and there was no obvious virus (Gustafsson, 1959). In 1957, Lobound *et al.*, in Notre Dame University used non-toxic PVC film as the main part of the isolator and developed a plastic isolator for the first time (Trexler and Reynolds, 1957; Trexler, 1959). Compared with metal and stainless steel isolators, plastic film isolators are lightweight, have a simple manufacturing process, are easy to sterilize and

cost only a few tenths of the price of metal isolators. Thus, plastic film isolators were widely promoted.

In recent years, the isolators used in GF animal laboratories are mostly made of plastic film, thereby ensuring that the isolators are in a sterile state for a long time and guaranteeing the safe production of GF animals. In 2015, Paik J *et al.*, invented a sealed positive pressure box to replace the membrane isolator as the main body of the aseptic environmental barrier system (Paik *et al.*, 2015). This device saves space and further reduces manufacturing costs, thereby achieving ideal results. With the continuous development of isolators (from steel to polyvinyl chloride plastic), caesarean section, high-pressure steam sterilization and the use of artificial galacto nutrition, researchers in many countries have kept GF rats and mice for several generations until an advanced age.

### *Application of GF animal models*

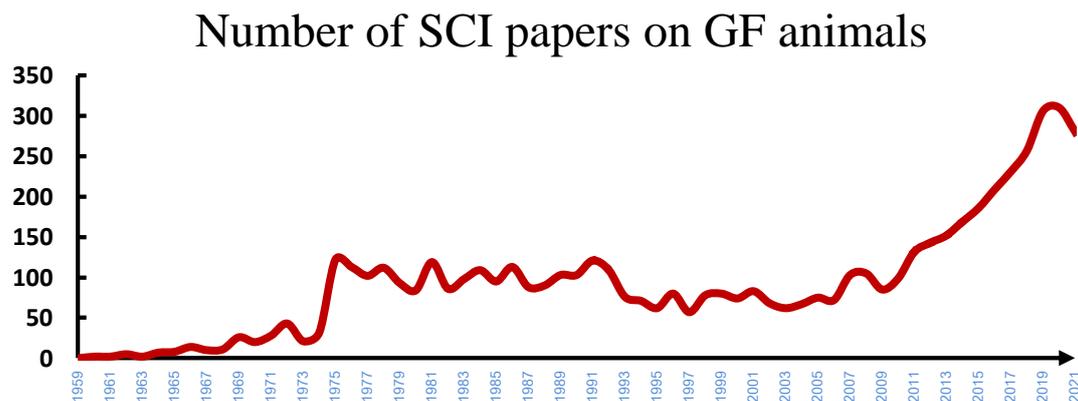
Mammals have 10 times as many symbiotic bacteria in their intestine as there are cells in their bodies (Slack *et al.*, 2009). The dynamic balance of the human internal environment is inextricably linked with a large number of microorganisms. These microorganisms are distributed in multiple parts, including the skin, digestive tract, respiratory tract and endocrine system (Costello *et al.*, 2009). However, microorganisms are mostly in the intestines; thus, research on the interaction between a microorganism and its host is focused on the intestinal microbiota (Frank and Pace, 2008).

The effect of intestinal bacteria on the body is double-sided. On the one hand, intestinal microbiota has a protective effect on the body. The normal microbiota adheres to, colonizes and reproduces in the intestinal mucosa and can synthesize or transform many metabolites, including hormones, essential vitamins and other bioactive substances (Grover and Kashyap, 2014). In addition, intestinal microbiota can participate in host energy metabolism through a variety of ways (Grover and Kashyap, 2014). On the other hand, abnormal intestinal microbiota can cause disease. The changes of intestinal microbiome affect the pathogenesis of many diseases, such as liver disease (Albhaisi *et al.*, 2020) and tumor (Gopalakrishnan *et al.*, 2018). The imbalance between normal intestinal microbiota and pathogens plays a key role in the development of diseases.

These research results are largely derived from the study of GF animals. As the animals with the clearest microbial background, GF animals are indispensable tools for studying the function of the microbiota and the interaction between the intestinal microbiota and the host. At present, GF animal models are widely used in various fields of life sciences, including immunology, oncology, liver disease and the gut-brain axis (Table 1). Fecal microbiota transplantation (FMT) is a method to introduce (transplant) the intestinal microbiota obtained from the feces of healthy donors into the gastrointestinal tract of patients, directly change the intestinal microbiota of recipients, normalize its composition and obtain therapeutic benefits (Wang *et al.*, 2019). This method is an important method to study the role of microorganisms

in sterile animal models and as a new disease correction therapy. The steps of FMT, including the selection of donors, the preparation of fecal bacterial fluid, the way of transplantation and the indications, are constantly updated in the application (Wang *et al.*, 2019; Routy *et al.*, 2018), showing a broad research prospect. In recent years, the study of using FMT to determine the role of microbiome in sterile animal models has gradually increased. Studies have shown that the transplantation of fecal microbiota (FMT) from cancer patients who respond to immune checkpoint inhibitors (ICIs) into sterile mice improves the anti-tumor effect of PD-1 blockade, while FMT from patients who do not respond to ICIs has no such effect. Therefore, it was found that the main resistance to ICIs could be attributed to the abnormal composition of

intestinal microbiome (Routy *et al.*, 2018). In other studies, feces of mice with different diets were transplanted into GF mice, to determine the direct role of cholesterol regulating bacteria in non-alcoholic fatty liver disease (NAFLD) associated hepatic carcinoma (HCC). The feces of GF mice fed with high-fat/high cholesterol mice were characterized by liver fat accumulation, inflammation and cell proliferation. In addition, atorvastatin restored cholesterol induced intestinal flora disorder (Zhang *et al.*, 2020), indicating that cholesterol induced NAFLD-HCC formation was related to intestinal flora disorder. In view of the special application value of GF animals, the number of Science Citation Index (SCI) papers published on GF animals has increased rapidly in recent years (Figure 1).



Note. PubMed database was used to search for documents with the search term of "Germ-free animal." The search value date is 12 February, 2022.

**Figure 1** Number of SCI papers published on GF animal application over the years

**Table 1** Application of GF animal experiment in life science research

DC	RI	Experimental Model	Phenotype of GF Animals Compared with Controls	Ref
Imm	iNKT IgA	oxazolone-induced colitis fecal microbiota, human diet	significantly higher numbers of iNKT cells "intrinsic" properties of bacterial members play a dominant role in dictating IgA responses.	Olszak et al., 2012 Planer et al., 2016
	MTC	Intestinal colonization	expansion of the CD4+CD25+Foxp3+Treg cell population in the colon lamina propria	Geuking et al., 2011
	IGL	FMT	the number of B cells and levels of IgG and IgA are considerably amplified	Li et al., 2020
	IgG1	colonized with the ASF+Akk microbiota	mounted IgG1 and IgA antibody responses specific for <i>Akkermansia muciniphila</i> , which had very consistent titers between mice	Ansaldo et al., 2019
	IgA+	FMT	the IgA+ taxa from the kwashiorkor twin proved lethal in germ-free murine recipients; IgA+ taxa from the healthy twin were well tolerated.	Macpherson et al., 2018
Onc	Mel	FMT from the patients gut microbiome of responders	tumor growth↓, RORγT+ Th17 cells ↑, CD45+ immune and CD8+ T cells↑, responses to anti-PD-L1 therapy↑, and enrichment of innate effector cells↑	Gopalakrishnan et al., 2018
	CRC	transplanted With CRC Stools	proportion of Ki-67-positive cells↑, proportions of Th1 and Th17 cells↑, genes involved in cell proliferation, apoptosis, angiogenesis, invasiveness, and metastasis↑	Wong et al., 2017
	ET	FMT from patients who responded to ICIs, then treated with CTLA-4 and PD-1 mAb	tumor growth delay, PD-L1 in splenic T cells↑, and restored the antitumor activity of simultaneous CTLA-4 and PD-1 blockade	Routy et al., 2018
	LC1	lung cancer induced by Kras mutation and p53 loss	tumor burden↓, tumor numbers↓, percentages of high-grade lesions↓, tumor cell proliferation↓, RORγt expression, and IL-17 production in γδ T cells↓	Jin et al., 2019
	GC	FMT with genotyped <i>Helicobacter pylori</i> strains	Six members of the 15-strain panel from the casecontrol study were recoverable as viable organisms from the stomachs of ex-germ-free 1, 3/4 FT mice.	Singh et al., 2018
	LC2	TLR5-deficient mice were fed with irradiated inulin-containing diet	did not recapitulate either hyperbilirubinemia or hepatocellular carcinoma	Singh et al., 2018
Hep	ALD	single gavage of alcohol	Lack of intestinal microbiota in GF mice increases the susceptibility to alcohol metabolism and alcoholism in the liver	Chen et al., 2015
	NAFLD-HCC	High-fat/high-cholesterol diet	<i>Mucispirillum</i> , <i>Desulfovibrio</i> and <i>Anaerotruncus</i> ↑; <i>Bifidobacterium</i> and <i>Bacteroides</i> ↓	Zhang et al., 2021
	ALI	acetaminophen-induced liver injury; Combination treatment with four antibiotics	improved	Gong et al., 2018
	LC3, HE	Reduction in neuroinflammation by using samples from post-FMT patients to colonize GF mice	a direct effect of fecal microbiota independent of active liver inflammation or injury	Liu et al., 2020
MGBa	PD	OD of specific microbial metabolites	Promoted neuroinflammation and motor symptoms	Sampson et al., 2016
	SCH	FMT from patients with schizophrenia	hyperactivity and reduced anxiety; exaggerated startle response to high-decibel tones (120 db)	Zheng et al., 2019
	ASD	Colonized with human samples from ASD	Bacteroidetes, <i>Bacteroides</i> , and <i>Parabacteroides</i> ↓; <i>Akkermansia</i> , <i>Sutterella</i> , and <i>Lachnospiraceae</i> ↑	Sharon et al., 2019
	AD	mouse models of AD	microglial amyloid-β uptake↑	Mezö et al., 2020
	MDD	FMT from MDD patients	depression-like behavior↑, absence of GM produces decreased immobility time in the forced swimming test.	Zheng et al., 2016

DC: Disease category; RI: Research Indicators; Imm: Immunological/Immunology; iNKT: iNKT Cell Function; IgA: Gut mucosal IgA responses; MTC: Mucosal Treg Cells; IGL: immunoglobulin levels; IgG1: titers of serum IgG1 responses against *Akkermansia muciniphila*; IgA+: proinflammatory relevance of IgA+-enriched taxa; FMT: Fecal microbiota transplants; CTLA-4: cytotoxic T lymphocyte-associated protein 4 ; PD-1: programmed cell death protein 1; Onc: Oncology; Mel: Melanoma; CRC: Colorectal cancer; ET: Epithelial tumors; LC1: Lung cancer; GC: Gastric cancer; LC2: Liver cirrhosis; Ref: Reference; Hep: Hepatology; ALD: Alcoholic liver disease; NAFLD: Nonalcoholic fatty liver disease; HCC: Hepatocellular carcinoma; ALI: Acute (toxic) liver injury; LC3: Liver cirrhosis; TLR5: Toll-like receptor 5; HE: Hepatic encephalopathy; CCl<sub>4</sub>, Carbon tetrachloride; DEN, Diethylnitrosamine; LI: Liver injury; OD: Oral administration; GM: Gut microbiota; MGBa: Microbiota-gut-brain axis; PD: Parkinson's Disease ; SCH: Schizophrenia; ASD: Autism spectrum disorder ; AD: Alzheimer's disease; MDD: Major depressive disorder; GM: Gut microbiota.

**Immunological research:** The unique aseptic state of GF animals means their systemic immune system is always in a dormant state; thus, they can be used as a living test tube to establish a simplified microbial co-system composed of a single microbial species or a

mixture of specific species (Hooper et al., 2012). GF animals are ideal for immunology research.

The microorganisms on the mammalian body have a highly symbiotic relationship with the immune system. The gut microbiome continually interacts with

the immune system and is critical to host physiology. John Rawls summarized the regulatory role of symbiotic flora in the germ-free zebrafish model of innate immunity in vertebrates, and found that the germ-free animal model system is crucial to determine the changes in the function of innate immune cells and intestinal physiological microbial dependence during the development of disease (Murdoch and Rawls, 2019). However, the invasion of pathogenic bacteria still promotes the occurrence of certain diseases and poses a threat to health. Parasitic bacteria and their metabolites profoundly affect the development and regulation of the mammalian immune system. Bacteria decompose carbohydrates into short chain fatty acids by anaerobic fermentation, such as butyrate and acetate (Lu *et al.*, 2016; Nobili *et al.*, 2019; Yao *et al.*, 2021). These metabolites can affect the production of cytokines and the function of macrophages and DC cells; they can promote Treg differentiation (Geva-Zatorsky *et al.*, 2017). The gnotobiotic animal model established by colonizing known bacteria in germ-free animals can be used to explore the relationship between specific bacteria or bacterial metabolites and the immune system. The immune system of GF animals is not fully developed due to the lack of intestinal microorganisms, which greatly increases their susceptibility to bacterial infection (Björkholm *et al.*, 2009). Compared with normal mice, the contents of immunoglobulin IgA, IgG and IgM in sterile mice were significantly lower; the goblet cells producing mucus were fewer and smaller; the mesenteric lymph nodes were fewer; and the immune deficiency of gastrointestinal tract made the sterile mice more vulnerable to infection. However, these defects can be corrected by colonization of symbiotic bacteria (Manolios *et al.*, 1988; Macpherson *et al.*, 2001; Fessler *et al.*, 2019). Hapfelmeier and Macpherson (2010) reported on a reversible sterile colonization system in mice. It was observed that a long-lived, highly specific anti-symbiotic IgA response appeared slowly in GF mice. On the other hand, the intestinal tract of sterile mice was continuously exposed to symbiotic bacteria. The reaction disappears quickly (Hapfelmeier *et al.*, 2010). Thus, the body's various immune functions match the composition of the intestinal commensal bacteria, then applying gnotobiotic technology to commonly used GF animal model has facilitated dissection of the influence of commensal microbes on host physiologies and to clarify the mechanism of autoimmune disease.

**Oncology research:** Intestinal microbiota is an independent organ and plays an important role in human health. It serves as a barrier to prevent bacterial invasion and infection; the maintenance of healthy intestinal microbiota can help patients resist cancer (Routy *et al.*, 2018). In addition, the analysis of metagenomic sequencing technology shows that disorders or imbalances in the normal intestinal microbiota can be used as risk factors for a variety of diseases, including cancer. Abnormal intestinal microbiota may become a driving factor for cancer-promoting properties, because it produces gene toxins, alters immune responses and activates cancer signal pathways, eventually causing tumors, leading to

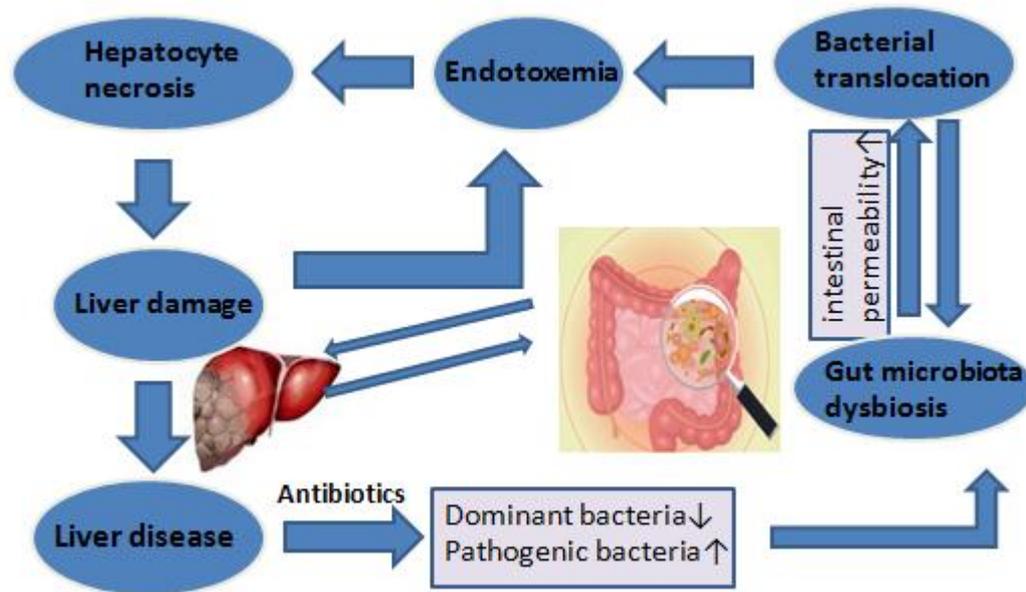
resistance to anti-cancer drugs (such as immune checkpoint inhibitors) and affecting the efficacy of hematopoietic cell transplantation, chemotherapy and immunotherapy (Routy *et al.*, 2018; Temraz *et al.*, 2019). The microbiota has become a key regulator of carcinogenesis and anticancer and the related mechanism of microbes has attracted wide attention in oncology research (Ramírez-Labrada *et al.*, 2020; Hu *et al.*, 2021).

In recent years, GF rodents have been widely used in many fields, such as research on the mechanism of microbial carcinogenesis, the determination of cell and molecular mediators for effective tumor intervention targets and the search for anticancer drugs (Gopalakrishnan *et al.*, 2018; Jin *et al.*, 2019; Xia *et al.*, 2021). The whole body lymphoid tissue of a GF animal is underdeveloped and the level of antibodies is low. A GF animal is more sensitive to danger signals and inflammatory stimuli and is suitable for cancer transplantation experiments. Moreover, the drive of harmful bacteria or the reduction of protective bacteria to produce carcinogens is the inducement of cancer (Wong *et al.*, 2017). In 1975, the incidence of colon adenoma in GF rats was found to be significantly higher than that in conventional rats, thereby confirming the correlation between intestinal microbiota and colorectal cancer (Weisburger *et al.*, 1975). A subsequent preclinical study showed that the adoptive transfer of *Bifidobacterium fragilis* reactive CD4 + T cells could enhance tumor control in GF mice and restore the efficacy of anti-CTLA-4 (Vétizou *et al.*, 2015). Colonizing a single strain or a group of known bacteria into the intestines of sterile animals could systematically and deeply explore the role of specific microbiota in tumor occurrence and development (Wong *et al.*, 2017; Sobhani *et al.*, 2019), which would provide an important reference for people to comprehensively understand tumor pathogenesis, prevention and treatment.

**Hepatology research:** With the increasing incidence rate of infectious and non-infectious liver diseases worldwide, the interest in research on intestinal microbiota has increased rapidly (Chen *et al.*, 2021). Intestinal microbiota is regarded as a "new virtual metabolic organ" (O'Hara and Shanahan, 2006) that connects the gut with extraintestinal organs, such as the kidney, brain and cardiovascular system (Konturek *et al.*, 2018). In recent years, with the continuous deepening of liver disease research, the gut-liver axis has received an increasing amount of attention. The diversity and functional imbalance of intestinal microbiota may be related to the pathogenesis of various liver diseases, such as alcoholic liver disease, nonalcoholic fatty liver disease, fibrosis/cirrhosis, acute (toxic) liver injury, and hepatocellular carcinoma (Hartmann *et al.*, 2019; Chen *et al.*, 2021). Generally, intestinal microbiota affects liver diseases by changing diet metabolism and calories. It increases intestinal permeability, damages the intestinal barrier and leads to bacterial translocation. Bacterial metabolites penetrate the intestinal barrier into the portal blood and reach the liver, thereby damaging the metabolism of bile acid and enterohepatic circulation and promoting intestinal dyskinesia and systemic

inflammation. All of these conditions may lead to intestinal dysfunction, which in turn increases liver damage (Figure 2). However, specific intestinal probiotics can prevent the occurrence of and reduce NAFLD (Yao et al., 2021). In addition, the stage of liver

injury is closely related to the severity of intestinal disorders (Schnabl and Brenner, 2014; Bajaj et al., 2014; Albhaisi et al., 2020). These are the key roles of intestinal microbiota in liver disease, which may have a positive impact on future treatment strategies.



**Figure 2** Gut microbiota and liver disease

GF rodents play a central role in elucidating the function of intestinal microbiota in the pathogenesis of liver diseases. Aseptic state has different effects on various animal strains; even when the same strain is used, opposite results are obtained in various liver disease studies (Canesso et al., 2014; Chen et al., 2015). Compared with the traditional experimental animals, GF rodents have different phenotypes in terms of liver diseases, which may be related to the imperfect immune system and the significant changes of physiological indexes, such as lipid, cholesterol and bile acid metabolism (Hartmann et al., 2012; Albhaisi et al., 2020). GF animals themselves may not produce results that reflect human physiological characteristics. To better reproduce human diseases in rodents, we can combine them with human intestinal microbiota and even “humanized” GF mice (Albhaisi et al., 2020). Many disease studies are difficult to carry out in humans due to ethical considerations. Only by combining human microbiota with GF animals can clinical relevance be established. An example of such a scenario is represented in a study on alcoholic hepatitis. Microbiota from human alcoholic hepatitis patients were transplanted into GF mice, which was followed by transplantation with microbiota from severely ill patients; these mice showed susceptibility to more severe liver disease (Llopis et al., 2016). The gnotobiotic animal models based on GF animals allow researchers to study bacteria that do not exist in mice and to eliminate the influence of microorganisms in mice but not in humans, thereby promoting the early use of microbiome-centric therapy in clinical treatment.

**Microbiota-gut-brain axis research:** In recent years, an increasing number of people have discovered that the resident microbiota can have a considerable impact on the brain and behavior. Thus, the concept of microbe-gut-brain axis has been proposed. Intestinal microorganisms have abundant biotransformation ability and can produce a series of bioactive substances through metabolism. These substances participate in the signal transmission between the gastrointestinal tract, nervous system and immune system and can potentially influence brain and behavior (Tremblay et al., 2021; Foster et al., 2016). This two-way communication occurs through a variety of mechanisms, including binding to receptors in the host brain, tryptophan metabolism, the alteration of central nervous transmission, and the regulation of neuroinflammation, which altogether constitute the microbe-gut-brain axis (Cryan et al., 2019; Tremblay et al., 2021). Research on the potential effects of pathogenic bacteria and probiotics on mental health, neuro developmental diseases and neurodegenerative diseases is based on animal experiments. Changes in the behavior of animals in a carcinogenic environment and immunodeficiency have been observed in studies; such behavior can be rescued by specific microbial colonization or probiotic application (Luczynski et al., 2016; Cryan et al., 2019). Future research will focus on the elucidation of the mechanism of microbiota-gut-brain axis and will attempt to clarify microbe-based intervention and treatment strategies for neuropsychiatric diseases.

Animal models are essential in linking the regulation of basic neural processes, such as neurogenesis and myelin sheath formation, to the activation of microglia microbiomes. A study using aseptic animals without any microorganism can introduce specific bacteria or complete microbial groups in any developmental node, which will provide convincing evidence of the role of bacteria in intestinal brain signals (Bercik *et al.*, 2011; Yano *et al.*, 2015; Zheng *et al.*, 2019). The movement disorder of Parkinson's disease is consistent with the aggregation of  $\alpha$ -synuclein. In a study on Parkinson's disease in SPF mice and humans, significant aggregation of  $\alpha$ -synuclein was observed in the brain area of affected substantia nigra striatum pathway, whereas the aggregation of  $\alpha$ -synuclein in sterile mice was significantly reduced (Sampson *et al.*, 2016). The number of insoluble  $\alpha$ -synuclein in the brain of sterile mice was significantly decreased, indicating that microbiota could regulate the promotion of the accumulation of  $\alpha$ -synuclein or prevent the accumulation and clearance of insoluble protein.

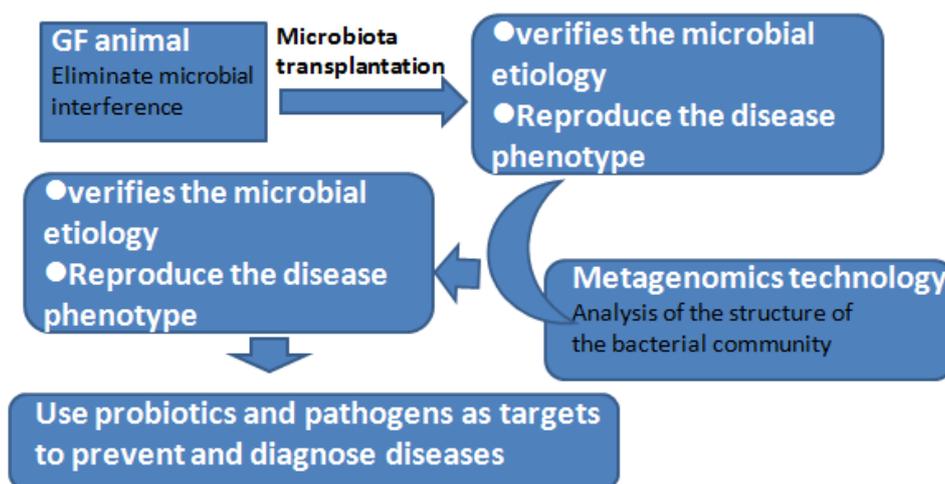
**Stomatology research:** Nearly 90% of the cells in the human body come from the microbiota and only a small number of cells come from the host (Shoemark and Alen, 2015). The oral cavity, as the starting point of the digestive system, contains more than 700 bacteria (Wu *et al.*, 2018). The oral microbiota helps maintain the homeostasis of the oral environment (Schindler *et al.*, 2012) and may also be a risk factor for systemic diseases, including rheumatoid arthritis (Graves *et al.*, 2019), diabetes (Xiao *et al.*, 2017) and cardiovascular disease (Xiao *et al.*, 2015). Altogether, the acquisition of oral bacteria is very important to human health.

The establishment of the GF animal model provides the most critical experimental tool for the study of oral diseases. Relevant results with GF animals as the research object show the following. 1. Bacteria are the key cause of dental caries, among which *Streptococcus* is the most effective (Wu *et al.*, 2018; Graves *et al.*, 2019). 2. The immune monitoring mechanism on normal oral microbiota and symbiotic bacteria mediate the catabolism of healthy periodontal tissue in the regulation of alveolar bone homeostasis (Irie *et al.*, 2014). 3. Changes in microbial community composition

or bacterial pathogenicity are related to other dental diseases (such as gingivitis, periodontitis and dental plaque) (Marchesan *et al.*, 2018; Graves *et al.*, 2020). GF animals make it possible to study various pathogenesis events in vivo and provide a scientific basis for the prevention of oral diseases.

## Discussion

After more than a century, extraordinary achievements have been made in the development of GF animals. GF and gnotobiotic animal models provide relatively ideal experimental tools for the occurrence and development of microbe-related diseases (Figure 3). Fig.1 provides an overview. In 2007, the number of international GF animal-related SCI papers began to exceed 100 (103) and showed a rapid growth trend. This is the beginning of the New Year of 2022 and statistics show that the number of SCI papers published in 2021 has slightly decreased. I think this is partly due to incomplete data statistics during this period and also indicates that the application of germ-free animals has reached a relative peak. In the past two years, the corona virus disease 2019 (COVID-19) spread all over the world, many countries and industries shut down and it is understandable that the number of papers published by SCI has indeed declined. However, the future development of GF animals has potential and is worth looking forward to. GF animal models, such as GF rats and mice, have been widely used in the study of human and animal diseases in the immune system (Björkholm *et al.*, 2009; Vaishnavi *et al.*, 2011; Geva-Zatorsky *et al.*, 2017), digestive system (Costello *et al.*, 2009; Grover and Kashyap, 2014; Al-Asmakh and Zadjali, 2015), nervous system (Foster *et al.*, 2016; Luczynski *et al.*, 2016) and reproductive system (Chen *et al.*, 2020). Undoubtedly, GF animals have contributed greatly to life science research and gnotobiotic biotechnology is particularly important in identifying the main subgroups of functional microorganisms, the microbial genes and products that mediate these functions and the host pathways that transduce these microbial signals (Rogala AR *et al.*, 2020) but there is still plenty of room for improvement.



**Figure 3** GF animal technology is an important way to study the relationship between microbiota and disease

Perspectives on the application of GF animals in the research of microbe-related diseases indicate that the physiological structure (skin, coat, or oropharynx and others) of GF animals (such as mice), co-eating behavior and social lifestyles are different from those of humans. Meanwhile, the objective factors, such as individual life pressure, emotional fluctuation, and diet status, undoubtedly affect the human microbial community. These differences lead to the difficulty in the full simulation of the real situation in humans by human-derived microbiota animal models. The experimental results obtained cannot be directly used in the clinic. Furthermore, although the information obtained by comparing GF animals with SPF animals or bacterial colonization can provide clues to the occurrence and development of diseases, such as gut-brain axis and tumor and liver diseases, the underlying mechanism is still unknown. Therefore, translating the results of research using aseptic animals to specific methods of disease prevention and control is difficult.

Many studies have shown that the regulation of intestinal microorganisms on host metabolism and diseases occurs in the gut; moreover, such microorganisms can use their metabolites (such as short-chain fatty acids) to act on remote target tissues and even form two-way regulatory pathways with multiple remote tissues and organs (such as gut-liver-brain, gut-lung, and enteropancreatic axes) (Kamvissi et al., 2015; Dumas et al., 2018; Tremblay et al., 2021; Yao et al., 2021). The latest research by Dr. Lanjuan Li and Prof. Siew C has shown that the occurrence and development of COVID-19 and secondary bacterial infections are significantly associated with intestinal microbiota, and the gut-pulmonary axis is the structural basis of interaction between the novel coronavirus and intestinal microbiota (Tang et al., 2020; Zuo et al., 2020). Moreover, the improvement of intestinal microbiota may be a new way to prevent and treat COVID-19. However, to further discover how novel coronavirus and the intestinal microbiota can achieve long-distance communication, we need to prepare humanized animal models for research on the pathogenesis of novel coronavirus. Besides COVID-19, the mechanism research, drug preclinical testing and vaccine evaluation of many infectious diseases are inseparable from GF animal models, especially humanized animal models based on GF animals. Thus, with the continuous development and improvement of single or multiple humanized mouse models, such as the humanization of pathogenic factors (bacteria, viruses, and others), the humanization of intestinal microorganisms and the immune system, as well as GF animal technology, can be further fully applied to various studies involving the intestines. The effectiveness of preclinical research results of many diseases will increase, thereby providing more reliable information for clinical research and ultimately helping humans overcome many difficult diseases.

In conclusion, the germ-free animal is a powerful experimental tool for the study of the relationship between flora and host health. We have reviewed the development history of germ-free animals and provide an overview of their application in medical research, such as intestinal flora, immunology and oncology. We

provide references for research on microbe-related diseases.

### Acknowledgements

This work was supported by National Key Research and Development Program of China (2021YFA1301100, 2021YFA1301101), Zhejiang Provincial Natural Science Foundation of China under Grant No. LXR22H160001 to Yifeng Gu, National Natural Science Foundation of China (81874142 and 82073041 to Yifeng Gu; 81790630, 81790631, and 81330011 to Lanjuan Li), Research Project of Jinan Microecological Biomedicine Shandong Laboratory under Grant No. JNL-2022028C to Yifeng Gu.

**Compliance with ethics guidelines:** This article does not contain any studies with human or animal subjects.

**Declaration of competing interest:** Rui Guo, Yifeng Gu and Lanjuan Li declare that they have no conflict of interest.

### References

- Al-Asmakh M, Zadjali F 2015. Use of Germ-Free Animal Models in Microbiota-Related Research. *J Microbiol Biotechnol.* 25: 1583-1588.
- Albhaisi SAM, Bajaj JS, Sanyal AJ. Role of gut microbiota in liver disease. (2020). *Am J Physiol Gastrointest Liver Physiol* 318: G84-98.
- Ansaldo E, Slayden LC, Ching KL, Koch MA, Wolf NK, Plichta DR, Brown EM, Graham DB, Xavier RJ, Moon JJ, Barton GM 2019. Akkermansia muciniphila induces intestinal adaptive immune responses during homeostasis. *Science.* 364: 1179-1184.
- Arvidsson C, Hallén A, Bäckhed F 2012. Generating and analyzing germ-free mice. *Curr Protoc Mouse Biol.* 2: 307 - 316.
- Bajaj JS, Heuman DM, Hylemon PB, Sanyal AJ, White MB, Monteith P, Noble NA, Unser AB, Daita K, Fisher AR, Sikaroodi M, Gillevet PM 2014. Altered profile of human gut microbiome is associated with cirrhosis and its complications. *J Hepatol.* 60: 940-947.
- Baker JA, Ferguson MS, Tenbroeck C 1942. Growth of Platyfish (*Platylocilus maculatus*) Free from Bacteria and Other Microorganisms. *Exp Biol Med.* 51: 116-119.
- Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy KD, Verdu EF, Collins SM 2011. The intestinal microbiota affect central levels of brain-derived neurotrophic factor and behavior in mice. *Gastroenterology.* 141: 599-609, 609.e1-3.
- Bhattarai Y, Kashyap PC 2016. Germ-Free Mice Model for Studying Host-Microbial Interactions. *Methods Mol Biol* 1438: 123-135.
- Björkholm B, Bok CM, Lundin A, Rafter J, Hibberd ML, Pettersson S 2009. Intestinal microbiota regulate xenobiotic metabolism in the liver. *PLoS One.* 4: e6958.
- Björkholm BM, Guruge JL, Oh JD, Syder AJ, Salama N, Guillemin K, Falkow S, Nilsson C, Falk PG,

- Engstrand L, Gordon JI 2002. Colonization of Germ-free Transgenic Mice with Genotyped *Helicobacter pylori* Strains from a Case-Control Study of Gastric Cancer Reveals a Correlation between Host Responses and HsdS Components of Type I Restriction-Modification Systems. *J Biol Chem.* 277: 34191-34197.
- Canesso MCC, Lacerda NL, Ferreira CM, Gonçalves JL, Almeida D, Gamba C, Cassali G, Pedrosa SH, Moreira C, Martins FS, Nicoli JR, Teixeira MM, Godard ALB, Vieira AT 2014. Comparing the effects of acute alcohol consumption in germ-free and conventional mice: the role of the gut microbiota. *Bmc Microbiol.* 14: 240.
- Chen HJ, Antonson AM, Rajasekera TA, Patterson JM, Bailey MT, Gur TL 2020. Prenatal stress causes intrauterine inflammation and serotonergic dysfunction, and long-term behavioral deficits through microbe- and CCL2-dependent mechanisms. *Transl Psychiatry.* 10: 191.
- Chen HJ, Antonson AM, Rajasekera TA, Patterson JM, Bailey MT, Gur TL 2015. Microbiota Protects Mice Against Acute Alcohol-Induced Liver Injury. *Alcohol Clin Exp Res.* 39: 2313-2323.
- Chen ZR, Jin SF, Ma WB, Jiang RL 2021. Intestinal microecology: A crucial strategy for targeted therapy of liver diseases. *Hepatobiliary Pancreat Dis Int.* 20: 499-500.
- Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R 2009. Bacterial community variation in human body habitats across space and time. *Science.* 326: 1694-1697.
- Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, Codagnone MG, Cusotto S, Fulling C, Golubeva AV, Guzzetta KE, Jaggar M, Long-Smith CM, Lyte JM, Martin JA, Molinero-Perez A, Moloney G, Morelli E, Morillas E, O'Connor R, Cruz-Pereira JS, Peterson VL, Rea K, Ritz NL, Sherwin E, Spichak S, Teichman EM, van de Wouw M, Ventura-Silva AP, Wallace-Fitzsimons SE, Hyland N, Clarke G, Dinan TG 2019. The Microbiota-Gut-Brain Axis. *Physiol Rev.* 99: 1877-2013.
- Dumas A, Bernard L, Poquet Y, Lugo-Villarino G, Neyrolles O 2018. The role of the lung microbiota and the gut-lung axis in respiratory infectious diseases. *Cell Microbiol.* 20: e12966.
- Fessler J, Matson V, Gajewski TF 2019. Exploring the emerging role of the microbiome in cancer immunotherapy. *J Immunother Cancer.* 7: 108.
- Foster JA, Lyte M, Meyer E, Cryan JF 2016. Gut Microbiota and Brain Function: An Evolving Field in Neuroscience. *Int J Neuropsychopharmacol.* 19: pyv114.
- Frank DN, Pace NR 2008. Gastrointestinal microbiology enters the metagenomics era. *Curr Opin Gastroenterol.* 24: 4-10.
- Geuking MB, Cahenzli J, Lawson MA, Ng DC, Slack E, Hapfelmeier S, McCoy KD, Macpherson AJ, 2011. Intestinal Bacterial Colonization Induces Mutualistic Regulatory T Cell Responses. *Immunity.* 34: 794-806.
- Geva-Zatorsky N, Sefik E, Kua L, Pasman L, Tan TG, Ortiz-Lopez A, Yanortsang TB, Yang L, Jupp R, Mathis D, Benoist C, Kasper DL 2017. Mining the Human Gut Microbiota for Immunomodulatory Organisms. *Cell.* 168: 928-943.e11.
- Gong S, Lan T, Zeng L, Luo H, Yang X, Li N, Chen X, Liu Z, Li R, Win S, Liu S, Zhou H, Schnabl B, Jiang Y, Kaplowitz N, Chen P 2018. Gut microbiota mediates diurnal variation of acetaminophen induced acute liver injury in mice. *J Hepatol.* 69: 51-59.
- Gonzalez FJ, Jiang C, Patterson AD 2016. An Intestinal Microbiota-Farnesoid X Receptor Axis Modulates Metabolic Disease. *Gastroenterology.* 151: 845-859.
- Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, Prieto PA, Vicente D, Hoffman K, Wei SC, Cogdill AP, Zhao L, Hudgens CW, Hutchinson DS, Manzo T, Petaccia de Macedo M, Cotechini T, Kumar T, Chen WS, Reddy SM, Szczepaniak Sloane R, Galloway-Pena J, Jiang H, Chen PL, Shpall EJ, Rezvani K, Alousi AM, Chemaly RF, Shelburne S, Vence LM, Okhuysen PC, Jensen VB, Swennes AG, McAllister F, Marcelo Riquelme Sanchez E, Zhang Y, Le Chatelier E, Zitvogel L, Pons N, Austin-Breneman JL, Haydu LE, Burton EM, Gardner JM, Sirmans E, Hu J, Lazar AJ, Tsujikawa T, Diab A, Tawbi H, Glitza IC, Hwu WJ, Patel SP, Woodman SE, Amaria RN, Davies MA, Gershenwald JE, Hwu P, Lee JE, Zhang J, Coussens LM, Cooper ZA, Futreal PA, Daniel CR, Ajami NJ, Petrosino JF, Tetzlaff MT, Sharma P, Allison JP, Jenq RR, Wargo JA 2018. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science.* 359: 97-103.
- Gordon HA, Pesti L 1971. The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriol Rev.* 35:390-429.
- Graves DT, Corrêa JD, Silva TA 2019. The Oral Microbiota Is Modified by Systemic Diseases *J Dent Res.* 98: 148-156.
- Graves DT, Ding Z, Yang Y 2020. The impact of diabetes on periodontal diseases. *Periodontol.* 2000. 82: 214-224.
- Grover M, Kashyap PC 2014. Germ-free mice as a model to study effect of gut microbiota on host physiology. *Neurogastroenterol Motil.* 26: 745-748.
- Gustafsson BE 1959. Lightweight stainless steel systems for rearing germfree animals. *Ann. NY Acad. Sci.* 78: 17-28.
- Hapfelmeier S, Lawson MA, Slack E, Kirundi JK, Stoel M, Heikenwalder M, Cahenzli J, Velykoredko Y, Balmer ML, Endt K, Geuking MB, Curtiss R 3rd, McCoy KD, Macpherson AJ 2010. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science.* 328: 1705-1709.
- Hartmann P, Chen WC, Schnabl B 2012. The intestinal microbiome and the leaky gut as therapeutic targets in alcoholic liver disease. *Front Physiol.* 3: 402.
- Hartmann P, Chu H, Duan Y, Schnabl B 2019. Gut microbiota in liver disease: too much is harmful, nothing at all is not helpful either. *Am. J. Physiol. Gastrointest. Liver. Physiol.* 316: G563-573.
- Hooper LV, Littman DR, Macpherson AJ 2012. Interactions between the microbiota and the immune system. *Science.* 336: 1268-1273.

- Hu FL, Chen HT, Guo FF, Yang M, Jiang X, Yu JH, Zhang FM, Xu GQ 2021. Biliary microbiota and mucin 4 impact the calcification of cholesterol gallstones. *Hepatobiliary Pancreat Dis Int.* 20: 61-66.
- Irie K, Novince CM, Darveau RP 2014. Impact of the oral commensal microbiota on alveolar bone homeostasis. *J Dent Res.* 93: 801-806.
- Jin C, Lagoudas GK, Zhao C, Bullman S, Bhutkar A, Hu B, Ameh S, Sandel D, Liang XS, Mazzilli S, Whary MT, Meyerson M, Germain R, Blainey PC, Fox JG, Jacks T 2019. Commensal Microbiota Promote Lung Cancer Development via  $\gamma\delta$  T Cells. *Cell.* 176: 998-1013.
- Kamvissi V, Salerno A, Bornstein SR, Mingrone G, Rubino F 2015. Incretins or anti-incretins? A new model for the "entero-pancreatic axis". *Horm Metab Res.* 47: 84-87.
- Kirk RG 2012. "Life in a germ-free world": isolating life from the laboratory animal to the bubble boy. *Bull Hist Med.* 86: 237-275.
- Knudsen LE, Smith A, Törnqvist E, Forsby A, Tähti H 2019. Nordic symposium on "toxicology and pharmacology without animal experiments-Will it be possible in the next 10 years?". *Basic Clin Pharmacol Toxicol.* 124: 560-567.
- Konturek PC, Harsch IA, Konturek K, Schink M, Konturek T, Neurath MF, Zopf Y 2018. Gut-Liver Axis: How Do Gut Bacteria Influence the Liver?. *Med Sci (Basel).* 6: 79.
- Lahiri S, Kim H, Garcia-Perez I, Reza MM, Martin KA, Kundu P, Cox LM, Selkrig J, Posma JM, Zhang H, Padmanabhan P, Moret C, Gulyás B, Blaser MJ, Auwerx J, Holmes E, Nicholson J, Wahli W, Pettersson S 2019. The gut microbiota influences skeletal muscle mass and function in mice. *Sci Transl Med.* 11: eaaan5662.
- Li H, Limenitakis JP, Greiff V, Yilmaz B, Schären O, Urbaniak C, Zünd M, Lawson MAE, Young ID, Rupp S, Heikenwälder M, McCoy KD, Hapfelmeier S, Ganal-Vonarburg SC, Macpherson AJ 2020. Mucosal or systemic microbiota exposures shape the B cell repertoire. *Nature.* 584: 1-5.
- Liu R, Kang JD, Sartor RB, Sikaroodi M, Fagan A, Gavis EA, Zhou H, Hylemon PB, Herzog JW, Li X, Lippman RH, Gonzalez-Maeso J, Wade JB, Ghosh S, Gurley E, Gillevet PM, Bajaj JS 2020. Neuroinflammation in Murine Cirrhosis Is Dependent on the Gut Microbiome and Is Attenuated by Fecal Transplant. *Hepatology.* 71: 611-626.
- Llopis M, Cassard AM, Wrzosek L, Boschat L, Bruneau A, Ferrere G, Puchois V, Martin JC, Lepage P, Le Roy T, Lefèvre L, Langelier B, Cailleux F, González-Castro AM, Rabot S, Gaudin F, Agostini H, Prévot S, Berrebi D, Ciocan D, Jousse C, Naveau S, Gérard P, Perlemuter G 2016. Intestinal microbiota contributes to individual susceptibility to alcoholic liver disease. *Gut.* 65: 830-839.
- Lu Y, Fan C, Li P, Lu Y, Chang X, Qi K 2016. Short chain fatty acids prevent high-fat-diet-induced obesity in mice by regulating G protein-coupled receptors and gut microbiota. *Sci Rep.* 6: 37589.
- Luczynski P, McVey Neufeld KA, Oriach CS, Clarke G, Dinan TG, Cryan JF 2016. Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. *Int J Neuropsychopharmacol.* 19: pyw020.
- Macpherson AJ, Hunziker L, McCoy K, Lamm R 2001. IgA responses in the intestinal mucosa against pathogenic and non-pathogenic microorganisms. *Microbes Infect.* 3: 1021-1035.
- Macpherson AJ, Yilmaz B, Limenitakis JP, Ganal-Vonarburg SC 2018. IgA Function in Relation to the Intestinal Microbiota. *Annu Rev Immunol.* 36: 359-381.
- Manolios N, Geczy CL, Schrieber L 1988. High endothelial venule morphology and function are inducible in germ-free mice: a possible role for interferon-gamma. *Cellular Immunol.* 117: 136-151.
- Marchesan J, Girnary MS, Jing L, Miao MZ, Zhang S, Sun L, Morelli T, Schoenfisch MH, Inohara N, Offenbacher S, Jiao Y 2018. An experimental murine model to study periodontitis. *Nat Protoc.* 13: 2247-2267.
- Maschirow L, Suttorp N, Opitz B 2019. Microbiota-Dependent Regulation of Antimicrobial Immunity in the Lung. *Am J Respir Cell Mol Biol.* 61: 284-289.
- Mező C, Dokalis N, Mossad O, Staszewski O, Neuber J, Yilmaz B, Schnepf D, de Agüero MG, Ganal-Vonarburg SC, Macpherson AJ, Meyer-Luehmann M, Staeheli P, Blank T, Prinz M, Erny D 2020. Different effects of constitutive and induced microbiota modulation on microglia in a mouse model of Alzheimer's disease. *Acta Neuropathol Commun.* 8: 119.
- Miyakawa M 1959. The Miyakawa remote-control germfree rearing unit. *Ann N Y Acad Sci.* 78: 37-46.
- Murdoch CC, Rawls JF 2019. Commensal Microbiota Regulate Vertebrate Innate Immunity-Insights From the Zebrafish. *Front Immunol.* 10: 2100.
- Nobili V, Mosca A, Alterio T, Cardile S, Putignani L 2019. Fighting fatty liver diseases with nutritional interventions, probiotics, symbiotics, and fecal microbiota transplantation (FMT). *Adv Exp Med Biol.* 1125: 85-100.
- Nuttall GHF, Thierfelder H 1897. Thierisches Leben ohne Bakterien im Verdauungskanal. *Biol Chem.* 23, 62-73.
- O'Hara AM, Shanahan F 2006. The gut microbiota as a forgotten organ. *Embo Rep.* 7: 688-693.
- Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, Glickman JN, Siebert R, Baron RM, Kasper DL, Blumberg RS 2012. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science.* 336: 489-493.
- Paik J, Pershutkina O, Meeker S, Yi JJ, Dowling S, Hsu C 2015. Potential for using a hermetically-sealed, positive-pressure isocage system for studies involving germ-free mice outside a flexible-film isolator. *Gut Microbes.* 6: 255-265.
- Pasteur L 1885. Observations relative a la note de M. Duclaux *Compt Rend Acad.* 100.
- Planer JD, Peng Y, Kau AL, Blanton LV, Ndao IM, Tarr PI, Warner BB, Gordon JI 2016. Development of the gut microbiota and mucosal IgA responses in twins and gnotobiotic mice. *Nature.* 534: 263-266.
- PLEASANTS JR 1959. Rearing germfree cesarean-born rats, mice, and rabbits through weaning. *Ann N Y Acad Sci.* 78:116-126.

- Qv L, Yang Z, Yao M, Mao S, Li Y, Zhang J, Li L 2020. Methods for Establishment and Maintenance of Germ-Free Rat Models. *Front Microbiol.* 11: 1148.
- Ramírez-Labrada AG, Isla D, Artal A, Arias M, Rezusta A, Pardo J, Gálvez EM 2020. The Influence of Lung Microbiota on Lung Carcinogenesis, Immunity, and Immunotherapy. *Trends Cancer.* 6: 86-97.
- Reikvam DH, Erofeev A, Sandvik A, Grcic V, Jahnsen FL, Gaustad P, McCoy KD, Macpherson AJ, Meza-Zepeda LA, Johansen FE 2011. Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression. *PLoS One.* 6: e17996.
- Reyniers JA 1932. The use of germ-free guinea pigs in bacteriology. *Proc. Ind. Acad. Sci.* 42:35-40.
- Reyniers JA, Trexler PC, Ervin RF 1946. Rearing germ-free albino rats. *Lobund reports.* 1: 1-84.
- Rogala AR, Oka A, Sartor RB 2020. Strategies to Dissect Host-Microbial Immune Interactions That Determine Mucosal Homeostasis vs. Intestinal Inflammation in Gnotobiotic Mice. *Front Immunol.* 11: 214.
- Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillère R, Fluckiger A, Messaoudene M, Rauber C, Roberti MP, Fidelle M, Flament C, Poirier-Colame V, Opolon P, Klein C, Iribarren K, Mondragón L, Jacquilot N, Qu B, Ferrere G, Clémenson C, Mezquita L, Masip JR, Naltet C, Brosseau S, Kaderbhai C, Richard C, Rizvi H, Levenez F, Galleron N, Quinquis B, Pons N, Ryffel B, Minard-Colin V, Gonin P, Soria JC, Deutsch E, Lorient Y, Ghiringhelli F, Zalcman G, Goldwasser F, Escudier B, Hellmann MD, Eggermont A, Raoult D, Albiges L, Kroemer G, Zitvogel L 2018. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science.* 359: 91-97.
- Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, Challis C, Schretter CE, Rocha S, Gradinaru V, Chesselet MF, Keshavarzian A, Shannon KM, Krajmalnik-Brown R, Wittung-Stafshede P, Knight R, Mazmanian SK 2016. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell.* 167: 1469-1480. e12.
- Schaeck M, De Swaef E, Van den Broeck W, Van Nevel S, Boon N, De Geyter N, Morent R, Demeestere K, Duchateau L, Coulombet C, Haesebrouck F, Decostere A 2016. Germ-free sea bass *Dicentrarchus labrax* larval model: a valuable tool in the study of host-microbe interactions. *Dis Aquat Organ.* 117: 177-185.
- Schindler D, Gutierrez MG, Beineke A, Rauter Y, Rohde M, Foster S, Goldmann O, Medina E 2012. Dendritic cells are central coordinators of the host immune response to *Staphylococcus aureus* bloodstream infection. *Am J Pathol.* 181: 1327-1337.
- Schnabl B, Brenner DA 2014. Interactions Between the Intestinal Microbiome and Liver Diseases. *Gastroenterology.* 146: 1513-1524.
- Schwarzer M, Tlaskalova-Hogenova H, Leulier F, Schabussova I 2021. Editorial: Employing Experimental Gnotobiotic Models to Decipher the Host-Microbiota Cross-Talk in Health and Disease. *Front Immunol.* 12: 729052.
- Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM, Zink EM, Casey CP, Taylor BC, Lane CJ, Bramer LM, Isern NG, Hoyt DW, Noecker C, Sweredoski MJ, Moradian A, Borenstein E, Jansson JK, Knight R, Metz TO, Lois C, Geschwind DH, Krajmalnik-Brown R, Mazmanian SK 2019. Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. *Cell.* 177: 1600-1618.
- Shoemark DK, Alen SJ 2015. The microbiome and disease: Reviewing the links between the oral microbiome, aging, and Alzheimer's disease. *J Alzheimers Dis.* 43: 725-738.
- Singh V, Yeoh BS, Chassaing B, Xiao X, Saha P, Aguilera Olvera R, Lapek JD Jr, Zhang L, Wang WB, Hao S, Flythe MD, Gonzalez DJ, Cani PD, Conejo-Garcia JR, Xiong N, Kennett MJ, Joe B, Patterson AD, Gewirtz AT, Vijay-Kumar M 2018. Dysregulated Microbial Fermentation of Soluble Fiber Induces Cholestatic Liver Cancer. *Cell.* 175: 679-694.
- Slack E, Hapfelmeier S, Stecher B, Velykoredko Y, Stoel M, Lawson MA, Geuking MB, Beutler B, Tedder TF, Hardt WD, Bercik P, Verdu EF, McCoy KD, Macpherson AJ 2009. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science.* 325: 617-620.
- Sobhani I, Bergsten E, Couffin S, Amiot A, Nebbad B, Barau C, de'Angelis N, Rabot S, Canoui-Poitrine F, Mestivier D, Pédrón T, Khazaie K, Sansonetti PJ 2019. Colorectal cancer-associated microbiota contributes to oncogenic epigenetic signatures. *Proc Natl Acad Sci U S A.* 116: 24285 - 24295.
- Tang L, Gu S, Gong Y, Li B, Lu H, Li Q, Zhang R, Gao X, Wu Z, Zhang J, Zhang Y, Li L 2020. Clinical Significance of the Correlation between Changes in the Major Intestinal Bacteria Species and COVID-19 Severity. *Engineering (Beijing).* 6: 1178-1184.
- Temraz S, Nassar F, Nasr R, Charafeddine M, Mukherji D, Shamseddine A 2019. Gut Microbiome: A Promising Biomarker for Immunotherapy in Colorectal Cancer. *Int J Mol Sci.* 20, 4155.
- Torres J, Hu J, Seki A, Eisele C, Nair N, Huang R, Tarassishin L, Jharap B, Cote-Daigneault J, Mao Q, Mogno I, Britton GJ, Uzzan M, Chen CL, Kornbluth A, George J, Legnani P, Maser E, Loudon H, Stone J, Dubinsky M, Faith JJ, Clemente JC, Mehandru S, Colombel JF, Peter I 2020. Infants born to mothers with IBD present with altered gut microbiome that transfers abnormalities of the adaptive immune system to germ-free mice. *Gut.* 69: 42-51.
- Tremblay A, Lingrand L, Maillard M, Feuz B, Tompkins TA 2021. The effects of psychobiotics on the microbiota-gut-brain axis in early-life stress and neuropsychiatric disorders. *Prog Neuropsychopharmacol Biol Psychiatry.* 105: 110142.
- Trexler PC 1959. The use of plastics in the design of isolator Systems in germfree vertebrates: present status. *Ann N Y Acad Sci.* 78: 1299-1302.
- Trexler PC, Reynolds LI 1957. Flexible film apparatus for the rearing and use of germfree animals. *Appl Microbiol.* 5: 406.

- Ulmer WT, Weller W, Reif E 1964. Animal Experiments relating to the Prophylaxis of Silicosis with Aluminium Chloride. *Int Arch Gewerbepathol Gewerbehyg.* 20: 482-495.
- Uzbay T 2019. Germ-free animal experiments in the gut microbiota studies. *Curr Opin Pharmacol.* 49, 6-10.
- Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, Ley R, Wakeland EK, Hooper LV 2011. The Antibacterial Lectin RegIII $\gamma$  Promotes the Spatial Segregation of Microbiota and Host in the Intestine. *Science.* 334: 255-258.
- Vétizou M, Pitt JM, Daillère R, Lepage P, Waldschmitt N, Flament C, Rusakiewicz S, Routy B, Roberti MP, Duong CP, Poirier-Colame V, Roux A, Becharaf S, Formenti S, Golden E, Cording S, Eberl G, Schlitzer A, Ginhoux F, Mani S, Yamazaki T, Jacquilot N, Enot DP, Bérard M, Nigou J, Opolon P, Eggermont A, Woerther PL, Chachaty E, Chaput N, Robert C, Mateus C, Kroemer G, Raoult D, Boneca IG, Carbonnel F, Chamaillard M, Zitvogel L 2015. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science.* 350: 1079-1084.
- WANG J 2006. Establishment of germfree rats and their techniques. Zhejiang university.
- Wang JW, Kuo CH, Kuo FC, Wang YK, Hsu WH, Yu FJ, Hu HM, Hsu PI, Wang JY, Wu DC 2019. Fecal microbiota transplantation: Review and update. *J Formos Med Assoc.* 118 Suppl 1:S23-S31.
- Weisburger JH, Reddy BS, Narisawa T, Wynder EL 1975. Germ-free status and colon tumor induction by N-methyl-N'-nitro-N-nitrosoguanidine. *Proc Soc Exp Biol Med.* 14: 1119-1121.
- Wong SH, Zhao L, Zhang X, Nakatsu G, Han J, Xu W, Xiao X, Kwong TNY, Tsoi H, Wu WKK, Zeng B, Chan FKL, Sung JY, Wei H, Yu J 2017. Gavage of Fecal Samples From Patients With Colorectal Cancer Promotes Intestinal Carcinogenesis in Germ-Free and Conventional Mice. *Gastroenterology.* 153: 1621-1633.
- Wostmann BS 1996. Germfree and Gnotobiotic Animal Models: Background and Applications Informa Healthcare.
- Wu H, Zeng B, Li B, Ren B, Zhao J, Li M, Peng X, Feng M, Li J, Wei H, Cheng L, Zhou X 2018. Research on oral microbiota of monozygotic twins with discordant caries experience - in vitro and in vivo study. *Sci Rep.* 8: 7267.
- Wu YY, Westwater C, Xiao E, Dias Corrêa J, Xiao WM, Graves DT 2018. Establishment of oral bacterial communities in germ-free mice and the influence of recipient age. *Mol Oral Microbiol.* 33, 38-46.
- Xia J, Jiang S, Lv L, Wu W, Wang Q, Xu Q, Ye J, Fang D, Li Y, Wu J, Bian X, Yang L, Jiang H, Wang K, Yan R, Li L 2021. Modulation of the immune response and metabolism in germ-free rats colonized by the probiotic *Lactobacillus salivarius* LI01. *Appl Microbiol Biotechnol.* 105: 1629-1645.
- Xiao E, Mattos M, Vieira GHA, Chen S, Corrêa JD, Wu Y, Albiero ML, Bittinger K, Graves DT 2017. Diabetes Enhances IL-17 Expression and Alters the Oral Microbiome to Increase Its Pathogenicity. *Cell Host Microbe.* 22: 120-128.e4.
- Xiao W, Dong G, Pacios S, Alnammary M, Barger LA, Wang Y, Wu Y, Graves DT 2015. FOXO1 deletion reduces dendritic cell function and enhances susceptibility to periodontitis. *Am J Pathol.* 185: 1085-1093.
- Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, Nagler CR, Ismagilov RF, Mazmanian SK, Hsiao EY 2015. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell.* 161: 264-276.
- Yao M, Qv L, Lu Y, Wang, Berglund, B, Li L 2021. An Update on the Efficacy and Functionality of Probiotics for the Treatment of Non-Alcoholic Fatty Liver Disease. *Engineering.* 7, 8.
- Zhang X, Coker OO, Chu ES, Fu K, Lau HCH, Wang YX, Chan AWH, Wei H, Yang X, Sung JY, Yu J 2021. Dietary cholesterol drives fatty liver-associated liver cancer by modulating gut microbiota and metabolites. *Gut.* 70: 761-774.
- Zheng P, Zeng B, Liu M, Chen J, Pan J, Han Y, Liu Y, Cheng K, Zhou C, Wang H, Zhou X, Gui S, Perry SW, Wong ML, Licinio J, Wei H, Xie P 2019. The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. *Sci Adv.* 5: eaau8317.
- Zheng P, Zeng B, Zhou C, Liu M, Fang Z, Xu X, Zeng L, Chen J, Fan S, Du X, Zhang X, Yang D, Yang Y, Meng H, Li W, Melgiri ND, Licinio J, Wei H, Xie P 2016. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. *Mol Psychiatry.* 21: 786-796.
- Zuo T, Zhang F, Lui GCY, Yeoh YK, Li AYL, Zhan H, Wan Y, Chung ACK, Cheung CP, Chen N, Lai CKC, Chen Z, Tso EYK, Fung KSC, Chan V, Ling L, Joynt G, Hui DSC, Chan FKL, Chan PKS, Ng SC 2020. Alterations in Gut Microbiota of Patients With COVID-19 During Time of Hospitalization. *Gastroenterology.* 159: 944-955.e8.