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Standardization in host–microbiota interaction studies: challenges, gnotobiology as a tool, and perspective

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Considering the increasing list of diseases linked to the commensal microbiota, experimental studies of host–microbe interactions are of growing interest. Axenic and differently colonized animal models are inalienable tools to study these interactions. Factors, such as host genetics, diet, antibiotics and litter affect microbiota composition and can be confounding factors in many experimental settings. The use of gnotobiotic mice harboring defined microbiotas of different complexity plus additional housing standardization have thus become a gold standard to study the influence of the microbiome on the host. We highlight here the recent advances, challenges and outstanding goals in gnotobiology with the ambition to contribute to the generation of reliable, reproducible and transferrable results, which form the basis for advances in biomedical research.

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Introduction

Mammalian body surfaces get colonized by trillions of microorganisms at birth, leading to a complex and diverse ecological community of commensal, symbiotic and pathogenic microorganisms, referred to as microbiota. The microbiota encompasses bacteria, viruses, archaea, protozoa and fungi. The number of bacteria harbored by humans is estimated to equal the amount of human cells [1] and a fragile equilibrium is established between the microbiota and its host. The microbiota represents a source of essential nutrients and profoundly shapes host immunity. Studying host–microbial mutualism faces numerous hurdles due to the vast diversity of the microbiota. In addition, 60–80% of its bacteria are not cultivable [2]. The recent development of 'omics technologies and downstream analyses provides deep insight into the operating mode of both the host and the microbiota. This includes a better understanding of the involvement of the microbiota in the pathogenesis of various diseases, such as intestinal bowel disease, autoimmunity, and allergies [3–7]. Nevertheless, the molecular mechanisms of host–microbial interactions remain largely elusive. In order to gain insight into so far unraveled aspects of the host–microbial relationship, we take advantage of animal models that reduce multidimensionality and offer reproducibility. In this review, we provide an overview on available animal models and standardization processes for microbiota research, including their advantages and limitations.

Genetics have a substantial impact on host physiology [8], hence genetic diversity impairs standardization. At the beginning of the 20th century, William Castle achieved pioneer work in the field of mouse genetics. He addressed the inheritance of coat colour in mice and created the first inbred strain used in research, the DBA mouse [9]. Later, with the development of transgenic mouse technologies, precise engineering of the host genetics became possible, ensuring standardization of the genetic background [10,11]. While the use of defined genetic backgrounds is a gold standard, controlling for microbial diversity is in the fledgling stages. The need for microbial standardization arose in the last two decades, as contradictory results regarding the role of the microbiota in the development of host immunity and diseases appeared. Both in humans and in rodents [12], microbiota composition depends on multiple parameters, such as genetics [13], diet [14,15], drinking water [16], maternal and cage effects [17,18], presence of pathobionts [19], housing conditions (temperature, moisture, pressure) [20,21], and circadian rhythm [22]. It became apparent that the composition of the microbiota is linked to host biology [7,23–28] and efforts are made to standardize protocols for microbiota-related studies [29••].

Standardization minimizes the occurrence of confounding factors. The complexity of host–microbiota interactions remains however to be overcome. Two complementary approaches are commonly applied for host–microbiota interaction studies. The 'bottom up' strategy takes advantage of gnotobiology by using defined and

simple microbiota consortia, whereas the 'top down' approach involves more complex models.

In the following, we describe how to standardize the microbiota of inbred animals following the 'bottom up' or the 'top down' approach, including an overview of existing gnotobiotic animal models, their advantages and limitations. In a second part, we focus on procedures to monitor the hygiene status of such models.

'Top down' approach: specific-pathogen free rodents

The human gut harbors around 500 different bacterial species. Firmicutes and Bacteroidetes are the predominant phyla, besides Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobacteria. Although the murine gut contains less than 100 different species, the human phyla are represented [30]. Thus, mice harboring a natural complex microbiota represent well the human situation.

Specific pathogen-free (SPF) hygienic status of rodent husbandries was widely implemented approximately 15 years ago. Conventional animal facilities with undefined presence of pathogens were substituted by SPF facilities being now the most common hygiene condition in animal studies. This change in paradigm was promoted by the use of susceptible immunocompromised animal models, where the impact of pathogens has become more significant [31]. Health monitoring for breeding colonies has been introduced to confirm that animals are devoid of certain pathogens [32].

By generating inbred SPF animals, biologists achieved an experimental tool that represents well the natural situation. It is however to mention that the microbiota composition and immune phenotype of SPF animals differ from wild mice [33•,34•,35•,36•]. Wild mice exhibited a generally more activated/memory immune phenotype, although some aspects such as the release of pro-inflammatory cytokines following *in vitro* stimulation were diminished in wild mice [35•,36•]. Microbiota composition in wild mice was also considerably different from that of laboratory mice [34•]. Clearly, it is difficult to conclude if the phenotypes are the results of the broader genetic diversity of a non-inbred population, a more diverse microbiota, and thus a better immune homeostasis, or simply reflect a history of an uncontrollable series of infections. To exclude the latter two, Rosshardt *et al.* have used the approach of reconstituting germ-free (GF) laboratory mice with wild microbiota, which protected from viral infection and colorectal cancer.

Despite enormous advances in 'omics technologies, characterization of the microbiota is still a field to be fully explored. The undefined nature and almost endless inter-individual diversity of the SPF microbiota remains a limitation in host–microbiome investigations, although best resembling the human microbiota.

Multiple factors (e.g. environmental and genetic) contribute to microbiota variability both within and among different vivaria. A nowadays broadly used strategy consists of re-deriving strains by two-cells stage embryo transfer into axenic recipients. Subsequent colonization with a facility-specific cocktail of bacteria avoids the introduction of pathogens or pathobionts and homogenizes colonization within the facility. Animals are then housed in individually ventilated cages to reduce microbial fluctuations and to steady environmental factors, such as noise, moisture and temperature. In addition, diet and drinking water are sterilized according to standard operating procedures [37]. Nevertheless, cage, maternal, and genetic impacts on microbial composition remain the quicksand of contradictory results. Extensive strategies have been implemented [38•,39•], such as littermate controls and cohousing for several generations (see also Figure 1). Inconsistency between different facilities and vendors remains, despite the efforts to standardize all SPF procedures, as the scientific community still lacks a common SPF standard cocktail.

The variability and undefined nature of the SPF microbiota represent an enormous disadvantage for predictive bioinformatic models, as the number of variables tends to be unpredictable.

'Bottom up' approach: gnotobiotic mouse models

Gnotobiology represents an attractive strategy in our quest for standardization of animal models. In gnotobiotic animals, the microbiota composition is by definition known (from 'gnotos' (greek) = known). GF mice are the basis for the generation of the different gnotobiotic models. They are held in flexible film isolators kept under positive pressure. Chow, bedding, drinking water and any other material are heat-sterilized or irradiated before import to the isolator, enabling a sterile milieu inside of the isolator. Additional standardization of the environment is possible through control of pressure within the isolators, noise, light cycles, and moisture in the room. This infrastructure originally developed to preserve the sterility of GF mice (described by Smith *et al.* [40] and Macpherson *et al.* [41]) has been successfully adapted for gnotobiotic animal holding. Hence animals harboring a defined microbiota (gnotobiotic animals) can be kept in isolators at the defined hygiene status over generations. Others and we have developed gnotobiotic models of different degrees of microbial complexity (see also Figure 2).

Monocolonization

Monocolonizations represent a simple gnotobiotic model to study host–microbiota interactions. The effect of a single defined bacterium on host physiology can be