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REVIEW ARTICLE

The role of the gut microbiota on animal model reproducibility

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Abstract

The gut microbiota is composed of approximately 10^{10}-10^{14} cells, including fungi, bacteria, archaea, protozoa, viruses, and bacteriophages; their genes and their various metabolites were found throughout the gastrointestinal tract. It has co-evolved with each species to assist with day to day bodily functions, such as digestion, metabolism of xenobiotics, development of mucosal immunity and immunomodulation, and protection against invading pathogens. Because of the significant beneficial impact that gut microbiota may have, there is interest in learning more about it and translating these findings into clinical therapies. Results from recent studies characterizing the gut microbiota of various species have demonstrated the range of influences that may affect gut microbiota diversity, including animal strain, obesity, types of enrichment used, bedding and housing methods, treatment with antimicrobials, vendor source, specific animal housing, diet, and intercurrent disease. Relatively little is known about the functional consequences of alterations of the gut microbiota and exactly how changes in richness and diversity of the microbiota translate into changes in health and susceptibility to disease. Furthermore, questions have been raised as to whether germ-free or even ultraclean, barrier-raised mice are relevant models of human disease, given their significantly reduced gut microbiota diversity and complexity compared with conventionally housed mice. In addition, evidence suggests that the specific anatomical location selected for assessing the gut microbiota has a highly significant effect on study outcomes, in that bacterial phyla change significantly along the gastrointestinal tract. This paper will explore animal model reproducibility in light of this information about the gut microbiota.

KEYWORDS
animal models, gastrointestinal microbiota, mice, reproducibility

1 | INTRODUCTION: THE GUT MICROBIOTA IN HEALTH AND DISEASE

The gut microbiota refers to the community of microorganisms inhabiting a defined environment along the gastrointestinal tract, and includes bacteria, fungi, protozoa, archaea, and yeasts. As greater than 98% of isolated genetic sequences present in the gut come from bacteria, the term and the focus of most research in this area largely refer to the bacteria present within the gastrointestinal tract.[1,2(p117)] Of all regions of the body, the gut contains the most

abundant and complex microbiota with over 500 bacterial species identified to date, many of which remain unclassified, and the significance of the whole still remains poorly understood, despite more than a decade of intensive research.[3(p279)] In an adult human, the gut microbiota is thought to consist of 10^{10}-10^{14} bacteria, weighing up to 2 kg and exceeding the weight of the liver, and for that reason has been termed the "neglected organ" as it has only recently been investigated for its role in health and disease.[4,5]

The enteric microbiota is thought to play a significant role in nutrient digestion and uptake, synthesis of volatile fatty acids, amino acids, and vitamins, and maintenance of intestinal mucosal integrity and gut peristalsis, as well as aiding in development of the enteric immune system, including organisation of Peyer's patches and isolated lymphoid follicles and epithelial secretion of antimicrobial peptides.[6-8] In addition, the microbiota helps to protect the body from pathogenic organisms by competing for defined metabolites, which can significantly affect expression of pathogen virulence genes and bacterial growth rates, as well as altering signaling pathways controlling host gene expression and immune cell response via release of various proteins and short chain fatty acids, which act as signaling molecules.[3,9,10,11(p3)] The intestinal microbiota also communicates with other organ systems including the brain, lungs, skin and liver, influencing their function in newly discovered ways and highlighting the possible contributions of gastrointestinal dysbiosis to other bodily conditions.[11(p5)] The specific make-up of the gut microbiota is influenced by numerous factors, including host genotype, age, diet, localized inflammation, antimicrobial use, and direct invasion of pathogenic organisms.[2] In animals and humans, alterations in the gut microbiota have been linked to several important diseases and conditions, including obesity, Crohn's disease, diabetes mellitus, ulcerative colitis, and some forms of neoplasia, making it an important area of current research.[12-16]

Microbiota research takes advantage of bacterial expression of a 16s rRNA gene that is unique to prokaryotes, which can be used as a marker gene to describe bacterial populations in samples.[17] The 16S rRNA gene is composed of both highly conserved and highly variable regions that allow for specific species identification. In particular, the V4 region is one of the variable regions that allows for precise bacterial species identification and is frequently characterized in microbiota studies.[18] With advances in culture-independent analytical techniques, bacterial DNA can be extracted directly from tissue or fecal samples and can be rapidly analyzed to describe taxonomic diversity, richness, and distribution, as well as investigating aspects of functional metagenomics, the examination of biological functions of a bacterial community.[19] Once the relative abundance and distribution of different bacteria is known in a population of healthy individuals, then changes in the microbiota can be studied in response to diet, treatment, infectious disease, or other manipulations of the microbiota.

While not a new approach for treating refractory gastrointestinal conditions, microbiome research has provided a better mechanistic understanding of the potential benefits of transfaunation of fecal material.[20] There is significant ongoing interest in evaluating the therapeutic outcome associated with manipulating the gut microbiota in disease states. A number of successful case reports have been described for fecal bacteriotherapy as treatment for *Clostridium difficile* infections as well as other chronic inflammatory gastrointestinal conditions and these trials now require FDA approval and must be run under Good Clinical Practice conditions.[21,22]

2 | MODELING CHANGES IN THE GUT MICROBIOTA IN ANIMAL SPECIES

Because of the burgeoning interest in the gut microbiota as it relates to human and animal health, particularly in the area of antimicrobial use in the agricultural animal sector, there has been significant interest in finding suitable animal models in which to study the effects of microbiota changes. In humans, the dominant gut bacterial phyla are Firmicutes and Bacteroidetes, and various animal species have been examined for suitability.[23(p3)] Humanized germ-free rats inoculated with fecal microbiota from human donors have been found to have more similar Firmicutes: Bacteroidetes phylogenetic ratios than mice.[24] This is thought to be because rats have more similar baseline gut microbiota to humans than mice, which allows for more stable expression and establishment of the inoculated bacteria.[25] However, there are not as many genetic variants of rats available, limiting their utility in studying specific disease conditions.[26] Similarly, the dominant gut bacterial phyla in guinea pigs, another rodent species, are also Firmicutes and Bacteroidetes.[27(p2)] Despite this, the richness of bacteria in human samples is lower than for guinea pigs (commonly found when comparing the microbiota of an omnivore to an herbivore) and the relative abundance of shared bacterial genera is markedly different between each species. In addition, the metabolic functions of the microbiota differ substantially between guinea pigs and humans, consistent with highly different diets, digestive processes, and mechanisms for nutrient extraction.[27(p6)]

There has also been significant interest in larger mammalian models of gut microbiota. Both minipigs and conventional swine have been studied as a model for the human gastrointestinal microbiome, and in both species of pigs, Firmicutes and Bacteroidetes phyla predominate, similar to humans.[28,29] Generally, the microbiota of pigs share similar diversity patterns and similar dominant phyla as in humans, but there are also a number of distinctive genera that are exclusive to pigs.[30] Dogs also have similar dominant phyla in their gut microbiota to humans, but differ substantively in specific genera and relative abundance within different phyla.[31-35] Finally, nonhuman primate species, such as macaques, have distinctly different gut microbiota compared to both the mouse and human, including complete lack of some major genera.[36,37] They are thought to be less relevant models for studying the human gastrointestinal microbiome.[38]

Because of the various differences described above, there has been no overwhelmingly obvious alternative to the use of mice in modeling the human gut microbiota. At first glance, the mouse and human microbiota look quite similar—in both the gut microbiota are dominated by the same two phyla, Firmicutes and Bacteroidetes.

However, when drilling down further to look at the specific bacteria, despite an 89% similarity in overall bacterial genera between clean laboratory mice and humans, a number of human-specific genera are completely absent in mice, including ones linked to gut health in humans, making direct microbiota comparisons from laboratory mice questionable for modeling human gut health and disease.[23,39] Mice can be used to evaluate general mechanisms influencing microbiota composition and there is significant interest in studying the mouse gastrointestinal microbiota as a readily manipulable model for mimicking human disease conditions. In particular, there has been an explosion of interest in using germ-free mice to study transfers of the gut microbiota and its variations, particularly since genetic tools exist for mice to layer this with overall genetic alterations, knockouts, conditional expression of genes, etc.

The study of germ-free (ie, axenic) mice has helped to define the role that the enteric microbiota plays in shaping the enteric immune system. In general, germ-free mice are significantly more susceptible to pathogenic infections and the development of illnesses from these infections.[40(p3)] Studies have demonstrated that, when compared to their normal counterparts, germ-free mice have a number of significant physiologic and functional differences in their gastrointestinal tract.[41] For example, they tend to have fewer and less cellular Peyer's patches, smaller mesenteric lymph nodes that are both less cellular and contain fewer plasma cells, fewer $CD8^+$ intestinal epithelial T cells, reduced expression of MHC class II molecules within the intestinal epithelial cells, and reduced production of secretory IgA by B cells.[40(p2)] Thus, it is important to keep these differences in mind when working with these models and determining translational significance of any results.

3 | REPRODUCIBILITY AND THE GUT MICROBIOTA

Concern about experimental reproducibility has reached near epic proportions in North America amongst the funding agencies and various science councils, and should be of concern for all those involved with conducting, collaborating, or facilitating research.[42,43] This concern extends to studies surrounding the gut microbiota. A major consideration when conducting microbiota studies is that by its very nature, these are population studies and require large sample sizes to ensure relevance of findings. Furthermore, efforts to describe population characteristics may be less useful then are evaluations of population dynamics and studies of microbial functional genomics. In humans, there are significant differences in the specific composition of the gut microbiota between individuals in a population and within the same individuals over time, largely driven by resource availability.[44] Yet, the overall microbiota within a healthy adult remains relatively consistent with approximately 60% stability of major phylotypes over a 3-year period, such that samples obtained from the same individual over time will vary but are generally more similar to each other than to samples from other individuals.[45(p5)] Healthy adults do share common bacterial phyla; however, the richness and diversity of phylotypes vary markedly across the population. Perhaps more interesting, the functional microbial genomics (that is, central metabolic processes and other functions that are carried out by the gut microbiota) across different individuals are remarkably similar, despite significant differences in discrete gut microbiota composition, leading to conceptualization that humans share a functional core microbiome rather than an overall core microbiota.[45(p3)] This is an important consideration for appropriate modeling of gut dynamics and when considering reproducibility of these types of studies.

To ensure appropriate diversity in findings and relevance of results, animal, and specifically, mouse, microbiota studies should contain sufficiently large numbers of samples from different animals, and to avoid pseudosampling, samples should be obtained from animals across many different cages or housing environments. This approach takes into consideration the subtle and expected variations in microbiota that are inherent in genetically identical offspring from different dams, fed and housed in identical environments.[46(p2),47(p323)]

The evolutionary differences between humans and mouse models must also be considered. While it is true that mice can be manipulated genetically and microbiologically, and humanized to some extent, at core, they remain a mouse. The mammalian host and its associated gut microbiota have co-evolved across millions of years.[47(p319)] Despite our ability to maintain axenic mice inoculated with human microbiota by housing them in tightly controlled environments, there are still significant differences in long term colonization of these mice compared with the microbiota of the original human donors. For example, compared with engrafting mouse microbiota-associated bacteria into mice, engraftment of fecal microbial communities from human feces into germ-free mice results in only a partial resemblance to the donor microbiota, favoring those bacterial phylotypes adapted to the donor species.[48(p576)] The microbial dysbiosis known to occur in certain pathological states (eg, obesity) will, in most cases, experience an ecological shift after engraftment to mice, one that may not be representative of the community associated with the original donor's pathology.[48(p576)] For example, human microbiota-associated mice harboring healthy vs disease-associated microbiotas may have compositional dissimilarities, but these may reflect new ecological patterns of the engrafted microbial communities rather than those representative of the pathological state. This demonstrates that interpretation and reproducibility of these types of studies can be quite challenging and there is a need to be cautious to prevent over interpretation of results. As a further example, when using ultra-clean specific pathogen-free mice to evaluate the effects of vaccination against certain health conditions involving gut immunity, a very different response is obtained from mice compared with adult outbred humans. When these same mice are reconstituted with a more diverse array of gut bacterial microbiota from conventional or pet store mice, the overall gut immunologic response more closely mimics the "conventional" adult human immunologic response. In addition to raising questions about reproducibility, these types of studies have raised questions as to value of using "clean"

laboratory mice compared with those from a conventional source, such as a pet store.[47(p324),48(p577)]

4 | INFLUENCES ON THE GUT MICROBIOTA OF MICE

If mice or other animals are going to be used as models to study the human gut microbiota, it is critical that we understand the differences between these species and the sources of variation that may ultimately impact reproducibility and translatability of results. The major sources of variation include animal vendor, strain or stock, age, diet and its treatment, time of day for microbial sampling, environment (eg, single vs pair vs group-housed, type of bedding and other in-cage resources provided), recent transportation, health status of animals, recent treatment with antimicrobials for surgery or other conditions, and water source and treatment.[49-51] Studies have demonstrated different inbred strains of mice have different gut microbiota and it changes over time, and even inbred mice from the same sources will have different absolute characteristics in their core microbiota.[46(p3),51(p8)] Furthermore, just as the collective human gut microbiota modulates overall gene expression in human cells, the gut microbiota in mice modulates gene expression in intestinal cells and thus helps to determine overall mouse phenotype, making animals more or less susceptible to certain conditions, such as development of colon cancer following exposure to an initiating agent. This helps to explain some of the variability seen within models but is not necessarily reassuring when trying to work with these models.[49(p207)]

Not surprisingly, antimicrobial treatment of mice can lead to profound alternations in the gut microbiota, especially when they are administered orally.[52] In general, at least two to 3 weeks are needed for the microbiota to return to near normal composition, following short term administration; however, this is dependent on the specific bacteria evaluated, the broad-spectrum nature of the antimicrobials administered, and the duration of treatment.[49(p211),53] The specific changes and return to original microbiota cannot be assumed and should be specifically evaluated if this is an important component of the study.[49(p211)]

Age of the mouse under study is a critical consideration in gut microbiota experiments. As in humans, few, if any, bacteria are present in the fetal mouse gut at birth and the gastrointestinal immune system is immature.[54,55] Upon birth, the neonate is inoculated with microorganisms from the dam and the environment, and it rapidly develops an immune system that enables the pup to fight infections.[49(p210),56,57(p7)] Genetic background will determine gut microbial profiles as well as disease susceptibility states and will further alter the gut microbiota. After weaning, the dramatic change in diet from one that is primarily milk-based to one that is primarily plant-based, induces a novel surge in microbiota development and maturation of the immune response. At this time point, the microbiota is fully established but still susceptible to changes in its composition by manipulation (eg diet) or natural influences.[57(p7)] When the mouse reaches adulthood around 8 weeks, the gut microbiota displays a relatively stable homeostatic state; however, even in adult mice when it appears that the gut microbiota is more stable, when examined closely and when the same genetically identical mice are followed across time, there is remarkable variation in the gut microbiota over time as is seen in humans, in which each individual microbiota may be as unique as a fingerprint.[2(p119),57(p8)] These taxonomic differences seen with age in mice are complemented by bacterial-encoded functional differences in the intestines, such as changes in gut mucosal immunity, digestion efficiency, and xenobiotic metabolism.[2(p122)]

One final consideration influencing studies of the gut microbiota of mice is the spatial organization of various bacterial communities along the gastrointestinal tract. This is a factor that complicates analysis of results in that there is a gradual increase in bacterial species richness, abundance and diversity from the small intestine through to the large intestine of adult humans and mice.[50(p152),58-60] Thus sample type collected for extraction and analysis is critical. When considering the principles of the three R's, and in particular, refinement, there is considerable interest in using fecal samples from research mice because sample collection is noninvasive. However, it is recognized that the bacterial content of the feces is more representative of that found in the distal large intestine.[61] If an investigator wishes to study a condition primarily affecting the upper gastrointestinal tract, then they will still need to euthanize animals to collect content or tissue samples from those specific sites, since feces will not be representative of small intestinal microbiota. Animal ethics committees will need to understand that not all gut microbiota studies using animal models will result in refinement of sample collection or reduction in animal use.

5 | CAN MICE BE USED TO MODEL THE HUMAN GUT MICROBIOTA?

When considering all of these variables, a researcher might question whether mice can be used at all to model the human gut microbiota. As for any research in which one animal is used as a proxy for another to study physiology or disease, it comes down to understanding similarities and differences between the two species and taking a conservative approach when drawing general conclusions about the translatability of findings. The use of murine models, including genetically altered and gene targeted mice, for gut microbiology studies permits investigators to ask questions and introduce interventions that cannot be practically studied in humans, a highly diverse outbred population. Mice have similar dietary preferences to humans (ie, they are omnivorous) and generally have comparable gastrointestinal anatomy and physiology. Other confounding variables can be controlled in murine studies making it easier to interpret results and a range of genetic and microbial interventions can be used.[23(p3)] Investigators need to be aware of specific physiologic and anatomic differences between mice and humans, including higher metabolic rates in mice and recognize that the complex diet,

environments and experiences of humans can never be adequately modelled in mice. The very fact that inbred mice are more homogenous genetically than humans limits the types of questions that can be asked of murine populations.[23(p8)] Furthermore, it is important to remember that hosts and their gastrointestinal microbiota have co-evolved over time, developing a complex and symbiotic relationship. It is not realistic to think that simple translocation of bacteria from one species to another will result in a closely replicative model. Even between strains of mice, it may not be possible to predict shifts in the gut microbiota with treatment or transplantation, thus all results will be highly strain- or stock- dependent.

Other experimental considerations that will impact the results and determine reproducibility relate to handling and storage of samples, analytical approaches including amplified regions for bacterial identification, sequencing methods, and final data cleaning and comparisons.[62,63]

There is significant and fierce competition for funding and resources between research laboratories but the community has experienced the dangers of overstating the results of comparative biology studies, especially when inappropriate experimental design is used. It is critical for reproducibility to consider study design carefully for experimental microbiota research and to clearly define the specific questions that are being asked.

In terms of overall study design considerations, general principles of experimental design apply. When genetic mutants are used, there should be selective breeding of siblings, standardization of extrinsic variables, and an effort to maximize the number of cages from which samples are drawn.[2(p125)] Recommendations have been developed to control for variations in microbiota.[2(p125)] To summarize, when studying the effect of a particular treatment outside of antimicrobials or probiotics, investigators should use mice with a defined microbiota (ie, isobiotic mice) or homogenize the gut content of mice to be used by co-housing them for 3-4 weeks. The treatment groups should be mixed within each cage, where possible. To study the impact various antimicrobials or probiotics on the animal model or gut microbiota, the microbiota should be homogenized and then cage mates should be redistributed across cages just prior to study and the number of cages again maximized. For studying the impact of host genetics on common microbial background, then heterozygous littermates should be used as controls and the microbiota homogenized between mice by co-housing them for 3-4 weeks. Finally, to study the effect of host genetics on microbiota composition, heterozygous littermates should be used as controls and wild-type and genetically altered mice should be co-housed over several cages.[2(p125)] When transplanting microbiota from human donors into mice, it is important to keep in mind the significant inter-individual variation in the microbiota and not to assume that a single human sample is representative of even a fraction of the human population. It is preferable to use pooled samples from at least several donors, some healthy and some diseases, for meaningful comparative studies.

For any scientific study to be broadly useful, it must be replicable and reproducible. Particularly in an area fraught with so many subtleties of research it is critical that researchers plan their studies carefully and report their methodology as completely as possible, in accordance with the PREPARE and ARRIVE guidelines.[64,65] It is also critical that datasets be published in open repositories to permit others to compare and review methods of analysis.[66,67]

6 | FUTURE CONSIDERATIONS FOR MICROBIOTA RESEARCH TO ENSURE RELEVANCE

Study of the gut microbiota is of high relevance to developing a deeper understanding of many aspects of health and disease in humans and animals but this type of research requires a thoughtful approach to ensure relevance of the data generated. As more is understood about the interplay between bacterial inhabitants of the gut microbiota, it will be important to examine the relevance and influence of other gut microbiota components, such as bacteriophages, viruses, and fungi. Furthermore, to truly understand the functional significance of qualitative and quantitative taxonomic changes in the gastrointestinal microbiota, sequencing results will need to be complemented with metabolomics and other related approaches.

7 | CONCLUSION

Study of the gastrointestinal microbiota is an exciting area of research with potential clinical relevance for many medical conditions of humans and animals. As detailed, it is important to thoughtfully consider aspects of study design, model selection, and the specific research questions to ensure research reproducibility and translational relevance.

CONFLICT OF INTEREST

None.

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