PEER REVIEWED

Model Systems for Studying the Human Gut Microbiome

A variety of *in vivo*, *in silico*, *in vitro*, and *ex vivo* model systems are available to researchers for studying the human intestinal microbiome, its functionalities and complex interactions with the host. Different models serve different purposes, all of them having their relevance as well as limitations and interdependencies. This article provides an overview of the most popular model system concepts, how they are being employed in gut microbiome research, and how they are complementary to each other.

In Silico Modelling

The extreme complexity of our inner microbial ecosystem, the gut microbiome, challenges researchers' abilities to map and understand what specific role organisms in the community have and how they interact with the other community members. Computational, or *in silico,* methods can aid in identifying the metabolic functions and the cross-talk happening between community members and potentially even with the host. Based on the wealth of sequencing and functional profiling data available from organisms identified in the human microbiome, researchers use computational simulations to identify roles and molecular pathways of the microorganisms in the community. Informed by gene expression experiments, it is even possible to use *in silico* methods to model how the community responds to changes in the environment.

Different mathematical techniques are employed for modelling different aspects of the microbiome, spanning from models able to predict ecological population dynamics and spatial structure to models based on genome-scale metabolic networks. Genome-scale metabolic network models are based on collections of metabolic functions derived from the genome of each organism in the network. That enables very detailed analysis of complex cellular processes in the context of a complex community, which can be difficult to get from other model systems. Going forward, it is the hope that computational methods can be utilised to identify *in silico* biomarkers for predicting disease-associated changes in the human microbiome. Furthermore, the computer models may be applied in the field of personalised medicine by the construction of personalised metabolic models.

Despite the potential for discovery and hypothesis generation for subsequent testing in the wet lab models, *in silico* experiments are still relatively underutilised by the scientific community. *In silico* methods modelling complex communities with a large amount of data associated with each organism in the community require considerable computational power, which can be a limiting resource. More importantly, designing and executing computer simulations requires interdisciplinary skills from microbiology, biology and computer technology, which can be a heavy lift to accomplish. Additionally, not all microbial genes have been sequenced; of those with sequences available, less than half have been annotated to a specific function, making *in silico* methods highly dependent on currently available databases and existing knowledge of the microbiome. Hence, it is continuously necessary to inform the computational approaches with experimental data obtained from other model systems, e.g. animal models.[1,2]

In Vitro and *Ex Vivo* Models

Literally meaning "in the glass", in vitro experimentation refers to the study of cell lines, microorganisms or molecules outside of the living context they stem from, e.g. in petri dishes, test tubes, microplates, flasks or the like. *Ex vivo* studies are defined by the removal of tissues or cells from a living organism to enable the greatest similarity to the conditions in the live host, yet happening "out of the living" and thus considered an *in vitro* method.

In vitro bioreactor models of the human gut microbiome are used to mimic microbial processes and physical conditions in the gastrointestinal tract. Such model systems typically consist of different compartments connected in series, with each compartment harbouring human bacteria specific to an intestinal section, i.e. the stomach, or small or large intestine. Enzymatic processes relevant to each intestinal section happen in each compartment. In some systems a mucus layer can be added so the microbiota can adhere to the "gut surface," which is useful for understanding which bacteria are important for shaping the intestinal barrier and mucus layer. Some systems integrate dynamic conditions such as peristaltic movements and absorption of water and nutrients. These "gut-in-a-bottle" models are very good for investigating how food, dietary compounds and drugs metabolise, but lack the immunological and other physiological cross-talk with the host. Because of the large physical setup, it is a notable constraint that in vitro bioreactor systems do not allow for several biological study replicates to be run in parallel under comparable conditions.

The microfluidics-based system is another type of model often referred to as "gut-on-a-chip". In tiny chambers, human and microbial cells can be co-cultured and separated by membranes to mimic the host-microbiome interface. Various technologies exist and they are constantly being refined, adding more details and conditions to the system to make the model as representative as possible of the real-life situation. For instance, investigations are underway to add immune cell populations to a "gut-on-a-chip" to open up avenues of analysing adaptive and innate immune responses to the microbiome.[3] The key limitation of microfluidics-based model systems is that only one organ is simulated and effects on other tissues and organs cannot be measured. While there are development initiatives underway to expand the systems by integrating more organs in the model, the technology is still in its infancy and cannot substitute for animal models. Functions throughout

the entire body are influenced by the host-microbe interaction happening in the gut, including brain development and behaviour. Such systemic effects are still impossible to fully study in microfluidics devices, but these models can serve as very valuable tools for narrowing down compounds before going into animal studies.

Ex vivo models involve removal of living and functional tissue or organs for cultivation in an artificial environment outside the host organism. Three-dimensional cell culture models, so-called organoids, are particularly interesting in human gastrointestinal and microbiome research and have seen rapid progress in recent years. Organoids mimic morphological and functional features of the donor original tissue. The three-dimensional aspect is usually obtained by creating scaffolds of various natural or synthetic materials, e.g. collagen or polymers. The organoids can be derived from human embryonic and induced pluripotent stem cells or in some cases even from tissue biopsies. The cells grow, differentiate and organise in an architectural structure relatively comparable to the *in vivo* situation. The added complexity of organoids compared to more simplistic in vitro methods provides the opportunity to more accurately study concepts like epithelial barrier dynamics, differentiation and proliferation of cells and immune cell crosstalk. Bacteria, such as probiotics, or microbial communities can be added to the system and enables understanding of the molecular processes happening in the host-microbiome interface. The top application area of gastrointestinal organoids is within infectious disease, but the models are also viable for studying inflammation, cancer and the involvement of the microbiome in intestinal development. Additionally, organoids may represent a tractable starting point for the creation of "synthetic" or bioengineered organs for future transplantation into humans, an area known as regenerative medicine. Efforts for refining and expanding on the utilities of organoid models are constantly ongoing, for example by the integration of neurons to model pathways in the enteric nervous system. Nevertheless, outside of the relative high expenses related to the maintenance of organoid models, the key limitation is the lack of a systemic nervous system, vascular, lymphatic and full immune system. The micro-anatomy, i.e. how the cells organise and form structures, can also differ from that of the *in vivo* situation.

All *in vitro/ex vivo* models have in common that they cannot link the microbiome composition to the host phenotype and processes in organs other than the gut. Additionally, these models are biased towards the microorganisms that are able to survive and grow under in vitro conditions, which is currently estimated to be around 20-80% of the human microbiome under standard cultivation conditions.[1,4,5]

In Vivo Models

In vivo models, i.e. animal models, are the only model systems capturing the essence and complexity of a whole organism and the only model able to link the microbiome to phenotype. Behavioural studies are inherently only possible to do in live animals, but also systemic and local physiological processes, such as immune activation orchestrated by many different cell and tissue types, can only fully be studied in whole, living organisms. After all, a functioning body is more than merely the sum of its parts.

Animal model organisms range from invertebrates such as worms and insects, to fish, birds and mammals. For microbiome research, the most popular models include mice and rats, zebrafish and fruit flies. Common to these models is the relative ease with which they can be created and maintained as germ-free, i.e. sterile. Germ-free animals harbour no micro- or macroorganisms, meaning they are completely free from bacteria, viruses and parasites. Pigs are also highly relevant as translational microbiome models for humans and can also be generated as germ-free, but with much less ease than rodents, zebrafish and fruit flies.

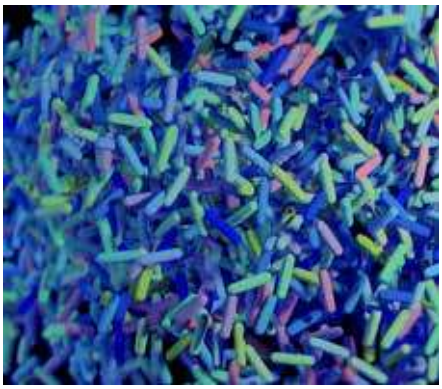
Access to germ-free animals is extremely valuable to researchers engaged in understanding basic mechanistic aspects of the host-microbiome interface, as well as for drug screening and testing. Mice represent the most widely used animal model in biomedical research across disciplines, as well as the most characterised. As such, germ-free mice hold a unique position when it comes to studying the microbiome in a well-known and practical model organism.

Germ-free mice can be colonised with individual, defined bacterial strains (a reductionist approach) or with microbial communities (a holistic approach), e.g. derived from human faecal samples. Both the reductionist and holistic approaches are used for mechanistic and proof-of-concept studies, whereas the holistic approach also has another important application: the creation of laboratory mice with controlled microbiota of interest.

Laboratory rodents display pronounced microbiome variability between different commercial vendors and animal facilities, a fact that has been linked repeatedly to poor reproducibility of studies if performed without consideration to this variability. This is because the microbiota composition of rodents has a significant influence on the phenotype of a wide range of disease models, leading to trouble when trying to replicate experiments across laboratories and across mice from different sources. In some cases, one microbiome may be advantageous for the phenotype of one type of disease model (for example, within infectious disease), but the opposite in another model (for example, within autoimmune and metabolic disease).

Hence, there seems to be an identified need for animal models harbouring microbiomes of relevance to different research applications.[6,7] One reliable way to achieve this would be to colonise germ-free mice with the microbiome of interest and use these mice as the starting point for a breeding colony. If the mice are housed under controlled and protected conditions, the microbiome remains stable over time.[8–10] Such an approach would provide researchers with a steady source to repeatedly obtain mouse cohorts from – for drug candidate testing, for example – without the risk of running into issues with reproducibility due to microbiome variability.

As for any model system, the biggest limitation of using rodents for microbiome research is how well they mimic humans. For instance, the host-microbe interaction is heavily based on activation of the host immune system and there are certainly known differences between the murine and human immune systems. Additionally, or maybe because of this, colonising germ-free mice with human microbiota



does not induce full immune system development in the mouse – a critical limitation to be aware of. To better understand the underlying mechanism behind this phenomenon, *in vitro* cell cultures of human gut epithelial cells or intestinal organoids could be employed.[11]

Zebrafish serve as a very interesting alternative to rodents for gut microbiome research, with a steady increase in their use over the past decade. They are easy to maintain as germ-free, especially in their early life before requiring to be supplied with feed. Zebrafish are unique in that they are transparent until adulthood. Hence, colonisation with microbes can be visualised directly or organogenesis can be monitored. The gastrointestinal tract of zebrafish has many anatomical and physiological traits that make them relevant as models for humans, but they are nevertheless more different than rodents are to us. For instance, zebrafish lack organised lymphoid structures which, in contrast, are highly conserved between mice/rats and humans.

Fruit flies constitute an even simpler model, and one with surprisingly many possible applications. The inherent gut microbiome of fruit flies is very simple, with only 2–20 different bacteria found naturally in the fly gut. The flies can be maintained as germ-free and colonised with specific microbes. By dietary interventions, it is possible to study how different microbes metabolise food and potentially shape innate immunity. Behavioural observations can be done to see how different microbes affect the fly's feeding behaviour. The short generation time, low cost, easy maintenance and possibility to do various interventions make fruit flies tractable, yet relatively underutilised models. A significant limitation is their lack of adaptive immunity and the fact that only aerobic bacteria can colonise the gut. This is in stark contrast to the mouse and human gut microbiome, which are both dominated by anaerobic species.[1,11,12]

In silico, in vitro, ex vivo and *in vivo* models all have their interdependencies and pros and cons when it comes to studying the human gut microbiome. Optimal advancement of microbiome research to enable discovery of microbiome-based therapeutics requires scientists to collaborate across disciplines. Every time an experiment is designed, the choice of model system should thoroughly be scrutinised with consideration to what would be most informative and translationally relevant.

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Alexander Maue

Alexander Maue is Director of Microbiome Products & Services at Taconic Biosciences, a fully-licensed, global leader in genetically engineered rodent models and services with an advanced portfolio of microbiome products and services. Prior to working at Taconic, he was the Head of the Campylobacter Immunology Laboratory at the Naval Medical Research Center in Silver Spring, MD. Trained as an immunologist, his research focus was on enteric diseases and the development of vaccines and therapies to prevent illness.

Email: alexander.maue@taconic.com

Randi Lundberg

Randi Lundberg is Field Applications Scientist at Taconic Biosciences, a fully-licensed, global leader in genetically engineered rodent models and services with an advanced portfolio of microbiome products and services. A veterinarian and PhD in *in vivo* pharmacology from University of Copenhagen, Denmark, Randi specialises in the study of the microbiome in animal models. Prior to joining Taconic, Randi served as laboratory animal veterinarian in the pharmaceutical industry.

Email: