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REVIEW

Experimental design considerations in microbiota/inflammation studies

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There is now convincing evidence that many inflammatory diseases are precipitated, or at least exacerbated, by unfavourable interactions of the host with the resident microbiota. The role of gut microbiota in the genesis and progression of diseases such as inflammatory bowel disease, obesity, metabolic syndrome and diabetes have been studied both in human and in animal, mainly rodent, models of disease. The intrinsic variation in microbiota composition, both within one host over time and within a group of similarly treated hosts, presents particular challenges in experimental design. This review highlights factors that need to be taken into consideration when designing animal trials to investigate the gastrointestinal tract microbiota in the context of inflammation studies. These include the origin and history of the animals, the husbandry of the animals before and during experiments, details of sampling, sample processing, sequence data acquisition and bioinformatic analysis. Because of the intrinsic variability in microbiota composition, it is likely that the number of animals required to allow meaningful statistical comparisons across groups will be higher than researchers have generally used for purely immune-based analyses.
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INTRODUCTION

The gut microbiota is made up of complex interacting populations of microbes, usually dominated not only by bacteria but also archea, eukaryotes and viruses. There is a rapidly growing body of evidence that demonstrates that the microbiota has diverse and wide-ranging roles in many biological functions of the host. The integration of host and microbiota is so important and fundamental that it has led to the concept of the metaorganism.[1] Technological advances in DNA sequencing have driven the development and broad application of culture-independent methods of microbiota analysis that have revealed much greater diversity and complexity within the microbiota than had been apparent from older culture-dependent studies of microbiota.

The development of the host immune system is among the many important functions that have now been attributed to the microbiotas found within and on the body of humans and other animals. In turn, the host immune system influences the composition of the microbiota. Inflammation is an important protective response produced when the host detects potentially pathogenic or foreign biochemical signatures. An appropriate inflammatory response helps to protect the host from invading pathogens. However, if the host immune system has not been properly trained, an inappropriate inflammatory response against self-molecules or elements within the commensal microbiota can ensue, which can result in profound health effects. There is thus a balancing act between the maintenance of commensal bacteria and the elimination of pathogenic bacteria, which is reflected in the balance of the host response between tolerance and immunity. Inappropriate inflammatory responses have a role in a range of disorders including inflammatory bowel disease (Crohn's disease, ulcerative colitis), irritable bowel syndrome, infectious enterocolitis, obesity, metabolic syndrome, asthma and type 1 diabetes (T1D). Rodent models of many of these diseases have been developed and have been extensively used to investigate the role of the immune system in the development and manifestation of the diseases. More recently, these same rodent models have been adapted to study the role that the gut microbiota/host interaction may have in these inflammatory diseases. Because of the complex interplay of microbiota with other factors such as food and water quality, the sensitivity of microbiota to other host factors such as hormone levels and neural activity and the transferability of microbiota, great care must be taken in designing and interpreting the results of studies investigating the role of microbiota in inflammatory disease models.

In a well-designed study, it is relatively easy to demonstrate correlations between various biological parameters (e.g. a disease outcome or a change in an immune cell population) and changes in microbiota composition. The challenge is to determine the causal relationships in such interactions and devise realistic ways to avoid or resolve the negative impacts of inappropriate interactions of microbiota with the host immune system.

PITFALLS TO AVOID WHEN USING HOSTS WITH DIFFERENT HISTORIES

In studies of the mouse immune system, it has been a common practice to do comparative studies on mouse lines with various gene knockouts. In the past, it has been assumed that genetics, age, diet,

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environment and experimental treatment were the main drivers of differences between animals. However, recently, it has become clear that the gastrointestinal microbiota represents a significant source of variation between animals, with a very strong influence on metabolite concentrations in the body.[2] Changes in metabolites may have consequential effects on the response to drugs and toxins.[2] When integrating microbiota studies into experiments probing the immune system, it is necessary to carefully consider the origins of the gene-knockout lines. Often these might be sourced from different facilities. It is now well established that even the same line of mice from different facilities can have very different microbiotas,[3–5] and, indeed, the microbiota can be significantly different in a single mouse line within the one facility.[6] This is also true of other animals.[7] These different microbiotas can result in different patterns of immune cell interaction.[5] Such heterogeneity in microbiota compositions can lead to difficulties with the reproducibility of experiments, but this is often not obvious as there are very few examples of studies in which replicate experiments have been reported. Therefore, special precautions need to be taken to ensure the microbiota changes attributed to immune system interactions are truly due to this rather than a reflection of different origins and hence different starting microbiotas of the gene-knockout lines. There are a number of ways in which these legacy effects can be minimised, including cross-fostering, extended cohousing (coprophagy) and heterozygote matings to generate the test mice. An example of the importance of these considerations can be seen in the work of Ubeda *et al.*,[8] in which they found that, contrary to previously published work, the differences in microbiota in TLR-deficient mice were largely a reflection of their husbandry history rather than genotype.[8]

VARIABILITY, STABILITY AND REPRODUCIBILITY

Intestinal microbiota is highly variable between and within individuals[9–11] and responds to environmental changes and diet almost immediately, within 1–3 days.[12,13] Faith *et al.*[9] sampled faecal microbiota from 37 adults multiple times over 5 years; although there was considerable variability in microbiota composition between people, the microbiota within individuals remained relatively stable, with ~60% of strains persisting in the individual over the time of sampling and 70% over 1 year. This stable core of strains was present in individuals alongside elements of the microbiota that were more prone to fast and plastic response to stimuli. In agreement with Turnbaugh *et al.*,[11] Faith *et al.*[9] reported that bacterial strains varied between the families but were maintained within the family. Opposite to the familial microbiota similarity, unrelated individuals shared only ~30% of strains in their gut. This may suggest that the loss of microbiota due to extreme events such as antibiotic treatments can be replenished from the family reservoir of microbiota via the process of cohousing and contribute to microbiota stability. Furthermore, there is evidence for substantial microbiota variation in each individual, both temporal and spatial.[11,12] It may seem confusing and contradictory to refer to microbiota as stable over time but at the same time highly variable; however, the core microbiota represents a stable component in the community and is often defined as microbiota highly shared between individuals, as opposed to a highly responsive variable component of the community that is capable of mounting a very fast response to any external stimuli and thus allows the community to adapt to new conditions.[14] Despite the well-accepted designation of core microbiota, others[11] have suggested that defining the core microbiota as a set of stable and abundant taxa shared between individuals may be wrong, as they failed to detect one single abundant phylotype shared between 154 humans. They suggested that core microbiota exists at the gene level or at the level of metabolic functions, preserved in majority by different bacteria, rather than in the specific bacteria present.

In situations where plasticity and dynamics of the gut are disturbed, diseases and dysbiosis follow, thus variability and dynamics are often the key responses to treatment, but at the same time they represent a problem from the experimental design perspective. Reproducibility, or the lack thereof, can be a significant problem in microbiota studies. Very different starting microbiota will respond to treatments in very different ways; planting the same type of seed in a tropical forest and a desert is unlikely to result in similar outcomes! Because of the very significant founder effect in the development of gut microbiota, small stochastic variations in the initial microbiota composition can have profound and long-lasting effects on overall microbiota composition. The variability reported in humans is also paralleled in multiple mouse studies[3] and in trials from other animals.[7] Surprisingly, the practice of repeating animal trials is not at all customary in mouse model studies. In other animal models identical repeated trials gave very different results[15] because of the large impact of the variable microbiota backgrounds. In addition to the lack of multiple trials, there is a tendency in mouse studies to use a very small number of animals per treatment, often less than five. Considering inter- and intraindividual differences observed and the intrinsically high variation in microbiota, the statistical power of analysis in trials with low levels of replication would be very low and unreliable and hence confirmatory studies starting with animals from different parents with very different microbial cohort is very unlikely to confirm the result. In experimental designs that include microbiota analysis, it is essential to take all potential levels of influence and variance on the microbiota into account when interpreting results and designing confirmatory studies.

METHODOLOGICAL CONSIDERATIONS FOR MICROBIOTA SAMPLING AND ANALYSIS METHODS

In addition to the design principles that should be applied to the animal trial components of experiments, there are also a series of variables in the physical processing of samples and the bioinformatic approaches to the interrogation of microbiota profiling data that need careful consideration. In most studies, faeces have been used as a convenient sample for microbiota analysis, but it should be acknowledged that faeces do not necessarily represent the most accurate estimation of relevant microbiota. Often the key functional responses that are under study occur in one or more sections of the gastrointestinal tract. The small intestine carries a lower bacterial load and less complex bacterial community than the large intestine; therefore, if small intestinal gut function and hence small intestine microbiota is a significant aspect of a study, then faecal sampling may not be appropriate. Similarly, on a finer scale, the microbiota intimately associated with the gut mucous layer may be more relevant than microbiota present in the gut lumen. Differences in precise position of sample can be important—there are gradients within the gut for pH, nutrient levels, bile acid, oxygen, interacting immune cells and mucous composition,[16,17] which can all affect the local microbiota.

Time of sampling can also be important—there are rapid changes in composition soon after birth, which gradually stabilise over time, generally over several weeks in mice,[18] but several years in humans. Sampling at early times can result in even greater microbiota variability as the normal inter- and intraindividual variability is compounded by differences in temporal development of microbiota and an amplification of stochastic differences. Sample handling and storage conditions can influence the exact outputs from microbiota

sequencing, whether 16S rRNA gene based or whole metagenome. For example freeze-drying, as against immediate processing or freezing, did subtly change the apparent composition but did not adversely affect analysis of infant faecal samples.[19] There are many different DNA extraction methods and kits available and each is likely to produce subtly different results. Lysis method can be a particularly important issue with methods of different efficacy potentially producing substantially different results.[20] Although there are differences in the DNA yields of different bacterial types with different methods, each particular method is generally stable, thus the most important advice is to ensure that the same methods are used for all samples within any one study. There are real dangers in using materials that have been collected and/or processed in different laboratories or in the one laboratory using different methods. For microbiota phylogenetic comparisons using amplified 16S rRNA genes, there are different sets of primers available that amplify different regions of the 16S rRNA gene. Results are only comparable if the same primers for the same region and the same amplification protocols are used—sequencing of different variable regions will give different results.[21]

Following generation of raw sequence data for microbiota characterisation, the data are then processed and analysed to identify the characteristics of the microbiota, either a phylogenetic characterisation of the microbes present or a characterisation of the metabolic potential of the microbiota. Whichever path is chosen, there are a range a bioinformatic tools and analysis pipelines that can be used. The application of different software tools for tasks such as removal of sequences artefacts, chimera checking, sequence clustering, operational taxonomic unit (OTU) picking and taxonomic classification can all introduce variables that need to be controlled or at least standardised. Often the final goal of microbiota characterisation is to compare across groups to detect shifts in composition—again there are many different tools that can be used and each is likely to give at least subtly different results and sometimes substantially different results.[22] As with the sampling and DNA preparation methods, the most important thing to do with the downstream bioinformatic analysis is to standardise on one approach for the data collection and analysis within a study.

A number of papers have claimed to have identified the temporal stability of specific strains and sharing of specific strains across groups of animals or humans based on 16S rRNA gene sequencing results. It should be recognised that, in general, 16S analysis cannot differentiate bacteria down to the strain level; it only discriminates clusters of similar sequences that are grouped together into OTUs. Each OTU may be comprised of many distinctive strains of bacteria, thus it is wrong to infer that a shared OTU means the sharing of a specific microbial strain. It must also be appreciated that many bacterial species are highly diverse, for example, *Escherichia coli* strains can have $>40\%$ difference in gene content,[23] but would not be identified as different by 16S sequencing. Therefore, when interpreting and reporting results, care needs to be taken in the language used and claims made in regard to the information generated from 16S rRNA gene studies of microbiota composition.

ANIMAL HUSBANDRY: COHOUSING, MATERNAL EFFECTS, CROSS-FOSTERING, FEEDING, HANDLING AND GROUP ASSIGNMENT

Care must be taken with a number of aspects of animal husbandry such as caging, feeding, handling and assignment of littermates to groups. Parent of origin, sex, cohort, family (nested with parent of origin) and litter (nested with cohort) all have effects on the microbiota.[3,24] Cage sharing (cohousing) of mice that belong to the same treatment reduces natural microbiota variation via coprophagy and direct animal to animal contact, which will in turn result in strong clustering of mice owing to cohousing rather than the treatment.[25] This phenomenon has such a strong effect on intestinal microbiota of cohoused animals that it can be used in animal trials instead of faecal transplant. Cohousing is now widely accepted as a method of normalising microbiota communities before trial treatments being applied. Cohousing provides continual seeding rather than the single inoculation usually used with faecal transplant. Macia *et al.*[26] showed that high dietary fibre protects from dextran sulphate sodium-induced colitis via microbiota short-chain fatty acid production and their interaction with GPR43 and GPR109A.[26] Their wild-type mice fed a high fibre diet were protected from colitis and showed improved clinical symptoms; however, this protection could not occur in $Gpr43^{-/-}$ mice. However, cohousing of WT with $Gpr43^{-/-}$ mice significantly improved colitis symptoms in $Gpr43^{-/-}$ mice. After cohousing $Gpr43^{-/-}$ mice, the microbiota was significantly altered with a very strong increase in the number of observed species present in the $Gpr43^{-/-}$ mice. Cohousing of obese and lean mice was able to prevent increases in adiposity and weight in obese mice and transformed the metabolic profile of obese mice to resemble that of lean mice, an effect driven by the transfer of specific bacteria from the lean to the obese microbiota.[27] Unfortunately, the effect of rodent cohousing is often ignored in microbiota experiments, with many manuscripts failing to provide any detail of animal husbandry, focussing their methodology on treatments, feed and the number of animals per treatment instead of mouse housing. This has resulted in published reports of very strong clustering of mice microbiota, based on treatment, that are often too perfect to be credible. Some earlier published studies need to be considered in light of our current understanding of the effects that cohousing can have on the outcomes and interpretation of microbiota studies. Fortunately, most researchers are taking measures to eliminate the effects of cohousing by exchanging the litter between the cages or using single animal housing combined with the randomising of littermates across treatment groups or ensuring multiple balanced number of cages and sampling for each treatment. Whichever way is used to control the effects of cohousing and coprophagy, the details of animal husbandry should be reported and acknowledged as potential influences on microbiota data and treatment outcomes.

These cohousing effects are also obvious in human studies. Song *et al.*[28] inspected the microbiota of 60 families and showed that there were very strong effects of cohabitation in humans, with the strongest effects of cohabitation noted by similarities in skin microbiota.[28] This cohabitation effect even extended to their dogs! Other studies, in humans[29,30] and mice,[31] demonstrated that genetically related family members had much higher number of shared species in the gut microbiota than unrelated controls, regardless whether they cohabitate or not.

Although cohousing is a relatively simple method for normalising intestinal microbiota communities in mice before an experiment, there are practical limitations in how it can be applied, in particular it is usually necessary to wean mice before cohousing can be used. At this stage, the natural microbiota can be resistant to change, either by cohousing or direct inoculation with faecal material or defined cultures. An alternative approach is to influence the very first colonisation of the mouse gut and introduce permanent changes by cross-fostering of pups. Daft *et al.*[32] swapped pups between non-obese diabetic (NOD) and non-obese diabetic-resistant mouse mothers and concluded that nursing mother and not the birth mother determines the pup microbiota. They also noted that NOD mice fostered by

non-obese diabetic-resistant mothers had reduced (although not statistically significant) incidence of T1D. The change introduced persisted out to 32 weeks of age, when the experiment was terminated. Org *et al.*[33] investigated microbiota variability in 110 inbred strains of mice to find that genetic background accounts for the abundance of some of the most common bacteria within the microbiota. They performed cross-fostering between two extreme strains discordant for fat gain under a high-fat, high-sucrose diet and found that male and female pups responded differently to cross-fostering, with initial weight gain in the lean SWR strain, occurring in both male and female, followed by females regaining the SWR phenotype and microbiota by 8 weeks of age. In contrast, male cross-fostered SWR pups gained significantly more weight than SWR controls and showed significantly increased levels of plasma triglycerides, similar to the fat-prone nursing mother strain. Clearly, the interplay between gut microbiota and host genetics is important and cross-fostering cannot override all aspects of the parental influence.

Maternal effects can have a strong influence on the phenotypic expression of microbiota–host interactions. Thorburn *et al.*[34] demonstrated that high fibre or high acetate diets protected mice from the development of asthma. A maternal diet high in fibre or acetate also prevented the onset of asthma in the offspring. They elegantly demonstrated that the pup protection was independent of microbiota and mediated *in utero*, by cross-fostering protected offspring from high fibre or acetate-fed mothers to nursing control mothers. The pups were still protected despite being fostered by controls. Control offspring cross-fostered to high fibre/acetate mothers remained susceptible to asthma, despite adopting microbiota from the foster asthma-protected mother.

Cross-fostering has been a useful option for experimental purposes, but there are some practical issues to consider when contemplating such experiments. Cross-fostering is most successful for pups born within 1 day (and a maximum of 2 days) for each mother and this requires a large breeding stock and coordinated breeding. Additionally, most cross-fostering experiments are performed after Caesarean section birth, which requires high levels of skill and poses higher risk for the mother and the pups. There is a lack of cross-fostering data on vaginal birth owing to pup rejection issues that may follow. In addition, success of cross-fostering may differ depending on mouse strains and pup sex;[32,33] thus, unless the maternal effect is of central importance, as in Thorburn *et al.*,[34] cohousing remains a much easier alternative.

MANIPULATION OF THE GUT MICROBIOTA

Considering the inter- and intrapersonal and temporal variability in gut microbiota and the statistical power needed to distinguish whether variations in microbiota composition associated with different aspects of health and disease are significant or are simply the product of bias and natural variation is often hard to confirm. To advance from correlative studies that indicate that there may be significant involvement of microbiota in inflammatory processes and to test the hypothesised bases of this involvement, it is likely necessary to manipulate the gut microbiota. There are many ways that this can be achieved and the method of choice depends very much on what the researcher is aiming to achieve.

Germ-free, gnotobiotic and xenografted model systems

Germ-free (GF) mouse experiments have had an important role in microbiota research. The ability to observe a condition suspected of being influenced by microbiota, without the presence of any micro-organisms, can provide clear answers regarding the role of the microbiota communities. One of the most cited early GF microbiota experiments was performed by Bäckhed *et al.*,[35] who reported that GF mice were protected against obesity induced by a high-fat, sugar-rich Western-style diet. Similarly, Wen *et al.*[36] used specific pathogen-free mice to first show that mice without MyD88 protein do not develop T1D. However, if grown in the absence of microbiota, in GF conditions, they did progress to T1D. These and many other GF model experiments have provided a tool in connecting the microbiota with a number of human conditions and had a role in the growth of this research area.

GF mice, completely devoid of all microbiota, have also been a useful tool to investigate the effects of controlled repopulation of microbiota, ranging from single strains such as specific probiotics (monoassociated) to collections of strains (e.g. Schaedler flora) or to complete faecal transplants. The transfer of known microbes into GF animals produces gnotobiotic animals carrying defined microbiotas. Exogenous bacteria can be administered via a number of different routes including in feed or water, rectal, intranasal and, most commonly, by direct oral gavage. Similar to GF mice, gnotobiotic mice never properly develop a fully functional immune system. It has become increasingly clear that the immune system requires modulation by, and interaction with, a full and species appropriate intestinal microbiota.[37,38]

Xenografting of human microbiota into GF animals has facilitated the laboratory investigation of human microbiota in a tractable model system. Transferring human donor intestinal microbiota into GF mice can transfer some disease phenotypes and as such can be used to generate useful animal models of human diseases. In recent years, humanised mouse models have become widely used, including in the study of intestinal disease,[39] antibiotic treatment and faecal transplant,[40] gluten-induced immunopathology[41] and obesity,[42] as well as investigating interactions, gene expression or metabolomics in the gut.[43–45] Humanising mouse microbiota changes the metabolic profile of the recipient mice with the majority of the donor metabolomics profile reproduced in the corresponding humanised mice.[45] Humanised mice, however, cannot fully reproduce human microbiota composition, partly due to the different gut anatomy, surface molecules, immune cells and genetics, but additionally because there are host specific strains of bacteria. Such host specificities have been shown to have profound effects. Tannock *et al.*[46] showed that during ileal content transplant into GF mice or rats, the filamentous segmented bacteria were able to adhere to the epithelium of mouse recipients only if the donor was mouse and in rats only if the donors were rats, but colonisation with material coming from the different host was not possible. Strain host specificity and between-strain difference is a well-known issue for cross-species probiotic usage as strains beneficial to their original host may not have any effects on other host species.[47] This may be one of the reasons that the immune system in humanised mice does not properly mature,[48] as human microbiota may not contain mouse-specific immune-modulating phylotypes or the human-derived strains may not have the same effect on mice.

Specific pathogen-free animals have been used in microbiota studies, but these are generally less useful than gnotobiotic animals because they do have a complex native microbiota. They are really of use only if the goal is related to the specific pathogens, which are absent from the animals.

Antibiotic administration

The recent advances in our understanding of the importance of a healthy gut microbiota have highlighted why the unnecessary use of

antibiotics can compromise general gut health and led to dysbiosis. The major perturbations in gut microbiota that result from antibiotic use have been used to study the microbiota function and to develop models for some human diseases. For example, *Clostridium difficile* infection and the inflammatory sequelae are commonly precipitated by the use of antibiotics,[49] and rodent models of the disease have been generated in a similar manner.[50] Similarly, antibiotics have been used to precipitate disease in a number of disease models including those for colitis,[51] obesity[52] and T1D[53] and T2D.[52] Because of the extensive and largely indiscriminate remodelling of the microbiota that occurs following antibiotic use, this approach is not broadly applicable for general studies of the effects of microbiota on inflammation. Antibiotic use is largely restricted to those models specifically designed to investigate the role that antibiotic use in humans may have on disease development.

Faecal microbiota transplantation

Faecal microbiota transplantation (FMT) is a treatment where faecal bacteria from a donor are transplanted into a recipient. This is closely related to the xenografting models mentioned above, except that we have used xenograft in the context of GF animals, whereas FMT is into a recipient that already carries its own, often dysfunctional, microbiota. FMT is a clinically proven treatment able to restore microbiota and consequently intestinal health with high efficacy while bypassing the need to even identify the cause of dysbiosis.[54] It has been of particular clinical relevance for the treatment of potentially deadly *C. difficile* infection and inflammatory bowel disease.[55] FMT has been used experimentally in inflammatory disease models to demonstrate that microbiota transplant can restore health or transmit susceptibility to disease. For example, it has been shown that NOD mice susceptible to T1D harbour a dysfunctional microbiota rich in pathobionts that, upon FMT, initiates insulitis in diabetes-resistant mice.[56] Female NOD mice are more susceptible to T1D and have higher incidence of up to 80% as opposed to males that remain relatively protected. FMT from male NOD mice conferred protection on female recipients.[57] Perhaps, the most cited FMT experiments are those investigating the ability of intestinal microbiota to influence weight control. Microbiota transfer from obese mouse donors into GF mice resulted in an obese phenotype.[58] Transfer of intestinal microbiota from human adult female twins, discordant for obesity, into GF mice also demonstrated the transmissibility of the obese phenotype.[27]

Dietary manipulation

Diet is one of the variables with the strongest influence on intestinal microbiota. This is not surprising given that the food eaten is the main substrate, along with host mucin, for microbial growth in the gut and, just as for *in vitro* growth in a Petri dish, bacteria will only grow in a medium that meets their minimum growth requirements. Changes in diet influence the gut microbiota almost immediately.[13,59] Turnbaugh *et al.*[13] showed, in mice populated with human microbiota, that a switch from a low-fat, plant polysaccharide-rich, balanced diet to a high-fat, high-sugar, 'Western' diet caused major microbiota shifts within a day and changes stabilised after a week. Wu *et al.*[60] investigated short-term versus long-term dietary changes in humans and demonstrated that the intestinal microbiota changed rapidly, but fairly modestly, following dietary change, but suggested that radical changes would only result from extended periods of altered diet. Some diets have a strong therapeutic potential such as diets rich in short-chain fatty acids that can delay colitis,[26] asthma[34] or obesity.[61] This indicates that diet design has a profound influence on intestinal microbiota, metabolism and metabolites circulating in the blood. Thus, selection of appropriate diets can be critical to the outcomes of microbiota studies.

In experimental settings in animal models, all the above methods of microbiota manipulation can be considered and used; however, when it comes to applying any of the findings in humans, the only viable methods of microbiota manipulation are likely to be via the use of dietary manipulation, prebiotics and probiotics, plus, in extreme cases, faecal microbiota transfer. Therefore, once the involvement of microbiota has been clearly delineated, perhaps by using some of the more extreme microbiota manipulation methods, it is important for researchers to consider the more difficult task of modulating disease and immune outcomes via the methods than can be more readily adopted for practical use in real target populations, whether humans or other animals.

MICROBIOTA STUDIES MAY REQUIRE LARGER GROUPS THAN OFTEN USED IN TRADITIONAL IMMUNE STUDIES: STATISTICAL CONSIDERATIONS

In traditional studies of immune mechanisms and inflammatory diseases in mouse models, the group sizes rarely exceed 12 animals and are sometimes as low as 3. Because of the inherent variability in microbiotas between animals, as well as temporal variation and strong responsiveness to many environmental stimuli, it is necessary to use larger treatment groups in experiments to achieve sufficient statistical power to draw valid conclusions. For example, using the R pwr package, which implements the methods of Cohen,[62] to estimate sample size with a default significance level of 0.05 and existing microbiota sets to estimate effect size, the required number of samples (mice) in microbiota experiments is often >50. Low number of samples in combination with high variability may account for a number of conflicting reports in nearly all the main spheres of microbiota research. The issue of underpowered studies in biological research has recently been highlighted[63] and a number of authors have discussed the issue of determining adequate sample sizes to allow robust assessments of experimental effects.[64,65]

Lack of replicate trails is another major issue in the experimental design. With sequencing prices falling, the excuse for analysis of low number of samples and lack of replicate trails is disappearing, and combined with revolution in the diversity and ease of use of analysis tools, it is hoped that much more abundant, reliable and reproducible data will be generated in the future.

IS THE MOUSE AN INFORMATIVE MODEL FOR THE EFFECTS OF HUMAN MICROBIOTA?

Just as there are significant differences between the development and action of the mouse and human immune systems, there are also important differences in microbiota development and function and the gut environment in which it develops. Differences in anatomy, including the relative size and function of different parts of the GIT, lack of appendix in mice and the much more important role of the caecum are all likely to modify the host–microbiota interaction as is the different distribution of some key cell types (e.g. paneth cells and goblet cells). Dietary differences are also likely to result in significantly different responses from the microbiota, whether native or implanted. Human diets are usually much more diverse than the standard mouse chow that is routinely used in experimental settings. Although the basic nutritional content of mouse chow diets should be fairly standardised, the reality is that, unless special precautions are taken, each batch can vary in composition as it is often formulated with different components depending on the relative cost of raw materials (e.g. grains). Availability and feeding habits are also very different with

constant *ad libitum* feeding often used for mice, but with pulsed food consumption in humans. It is important to be aware of these differences and the impact they may have on the interpretation of experimental results. Nguyen *et al.*[66] have comprehensively reviewed these issues. Despite these differences, the mouse is a powerful tool that has given deep insights into the causal linkages between inflammatory diseases and microbiota.

CONCLUSIONS

The realisation that many inflammatory diseases involve interactions between the host and microbiota has led to an explosion in the number of studies that include microbiota manipulation and analysis in experimental designs. The above discussion makes it clear that this sort of research requires careful planning and carriage to ensure that meaningful results are generated. In most inflammatory diseases associated with disturbances in microbiota, the interaction is likely to be complex and depend on the overall structure, complexity and biochemical potential of the microbiota. Such interactions can often be identified by finding correlations between microbiota composition and disease or immune outcomes, but then careful studies, usually involving deliberate manipulation of the microbiota, are required to identify causal links. Attention to detail in choosing experimental model systems with appropriate animals, under appropriate husbandry conditions, using appropriate sampling approaches and analysis methods will reward researchers with informative and reproducible outcomes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

1 Bosch TCG, McFall-Ngai MJ. Metaorganisms as the new frontier. *Zool Jena Ger* 2011; **114**: 185–190.
2 Holmes E, Nicholson J. Variation in gut microbiota strongly influences individual rodent phenotypes. *Toxicol Sci* 2005; **87**: 1–2.
3 Hufeldt MR, Nielsen DS, Vogensen FK, Midtvedt T, Hansen AK. Variation in the gut microbiota of laboratory mice is related to both genetic and environmental factors. *Comp Med* 2010; **60**: 336–342.
4 Ericsson AC, Davis JW, Spollen W, Bivens N, Givan S, Hagan CE *et al.* Effects of vendor and genetic background on the composition of the fecal microbiota of inbred mice. *PLoS ONE* 2015; **10**: e0116704.
5 Ivanov II, de Llanos Frutos R, Manel N, Yoshinaga K, Rifkin DB, Sartor RB *et al.* Specific microbiota direct the differentiation of Th17 cells in the mucosa of the small intestine. *Cell Host Microbe* 2008; **4**: 337–349.
6 Hoy YE, Bik EM, Lawley TD, Holmes SP, Monak DM, Theriot JA *et al.* Variation in taxonomic composition of the fecal microbiota in an inbred mouse strain across individuals and time. *PLoS ONE* 2015; **10**: e0142825.
7 Stanley D, Geier MS, Hughes RJ, Denman SE, Moore RJ. Highly variable microbiota development in the chicken gastrointestinal tract. *PLoS ONE* 2013; **8**: e84290.
8 Ubeda C, Lipuma L, Gobourne A, Viale A, Leiner I, Equinda M *et al.* Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. *J Exp Med* 2012; **209**: 1445–1456.
9 Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL *et al.* The long-term stability of the human gut microbiota. *Science* 2013; **341**: 1237439.
10 Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature* 2012; **489**: 220–230.
11 Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE *et al.* A core gut microbiome in obese and lean twins. *Nature* 2009; **457**: 480–484.
12 Candela M, Biagi E, Maccaferri S, Turroni S, Brigidi P. Intestinal microbiota is a plastic factor responding to environmental changes. *Trends Microbiol* 2012; **20**: 385–391.
13 Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* 2009; **1**: 6ra14.
14 Shade A, Handelsman J. Beyond the Venn diagram: the hunt for a core microbiome. *Environ Microbiol* 2012; **14**: 4–12.
15 Stanley D, Hughes RJ, Geier MS, Moore RJ. Bacteria within the gastrointestinal tract microbiota correlated with improved growth and feed conversion: challenges presented for the identification of performance enhancing probiotic bacteria. *Front Microbiol* 2016; **7**: 187.
16 Pang W, Vogensen FK, Nielsen DS, Hansen AK. Faecal and caecal microbiota profiles of mice do not cluster in the same way. *Lab Anim* 2012; **46**: 231–236.
17 Donaldson GP, Lee SM, Mazmanian SK. Gut biogeography of the bacterial microbiota. *Nat Rev Microbiol* 2016; **14**: 20–32.
18 Schloss PD, Schubert AM, Zackular JP, Iverson KD, Young VB, Petrosino JF. Stabilization of the murine gut microbiome following weaning. *Gut Microbes* 2012; **3**: 383–393.
19 Lewis ZT, Davis JCC, Smilowitz JT, German JB, Lebrilla CB, Mills DA. The impact of freeze-drying infant fecal samples on measures of their bacterial community profiles and milk-derived oligosaccharide content. *PeerJ* 2016; **4**: e1612.
20 Brooks JP, Edwards DJ, Harwich MD Jr, Rivera MC, Fettweis JM, Serrano MG *et al.* The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiol* 2015; **15**: 66.
21 Ibarbalz FM, Pérez MV, Figuerola ELM, Erijman L. The bias associated with amplicon sequencing does not affect the quantitative assessment of bacterial community dynamics. *PLoS ONE* 2014; **9**: e99722.
22 Westcott SL, Schloss PD. *De novo* clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. *PeerJ* 2015; **3**: e1487.
23 Jackson SA, Patel IR, Barnaba T, LeClerc JE, Cebula TA. Investigating the global genomic diversity of *Escherichia coli* using a multi-genome DNA microarray platform with novel gene prediction strategies. *BMC Genomics* 2011; **12**: 349.
24 Benson AK, Kelly SA, Legge R, Ma F, Low SJ, Kim J *et al.* Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci USA* 2010; **107**: 18933–18938.
25 McCafferty J, Muhlbauer M, Gharaibeh RZ, Arthur JC, Perez-Chanona E, Sha W *et al.* Stochastic changes over time and not founder effects drive cage effects in microbial community assembly in a mouse model. *ISME J* 2013; **7**: 2116–2125.
26 Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S *et al.* Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat Commun* 2015; **6**: 6734.
27 Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL *et al.* Cultured gut microbiota from twins discordant for obesity modulate adiposity and metabolic phenotypes in mice. *Science* 2013; **341**: 6150.
28 Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D *et al.* Cohabiting family members share microbiota with one another and with their dogs. *eLife* 2013; **2**: e00458.
29 Khachatryan ZA, Ktsoyan ZA, Manukyan GP, Kelly D, Ghazaryan KA, Aminov RI. Predominant role of host genetics in controlling the composition of gut microbiota. *PLoS ONE* 2008; **3**: e3064.
30 Zoetendal EG, Akkermans ADL, Vliet WMA, Visser JAGM, de Vos WM. The host genotype affects the bacterial community in the human gastrointestinal tract. *Microb Ecol Health Dis* 2001; **13**: 129–134.
31 Ussar S, Griffin NW, Bezy O, Fujisaka S, Vienberg S, Softic S *et al.* Interactions between gut microbiota, host genetics and diet modulate the predisposition to obesity and metabolic syndrome. *Cell Metab* 2015; **22**: 516–530.
32 Daft JG, Ptacek T, Kumar R, Morrow C, Lorenz RG. Cross-fostering immediately after birth induces a permanent microbiota shift that is shaped by the nursing mother. *Microbiome* 2015; **3**: 17.
33 Org E, Parks BW, Joo JWJ, Emert B, Schwartzman W, Kang EY *et al.* Genetic and environmental control of host-gut microbiota interactions. *Genome Res* 2015; **25**: 1558–1569.
34 Thorburn AN, McKenzie CI, Shen S, Stanley D, Macia L, Mason LJ *et al.* Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. *Nat. Commun* 2015; **6**: 7320.
35 Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc Natl Acad Sci USA* 2007; **104**: 979–984.
36 Wen L, Ley RE, Volchkov PV, Stranges PB, Avanesyan L, Stonebraker AC *et al.* Innate immunity and intestinal microbiota in the development of type 1 diabetes. *Nature* 2008; **455**: 1109–1113.
37 Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 2012; **489**: 231–241.
38 Round JL, Mazmanian SK. The gut microbiome shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009; **9**: 313–323.
39 Collins J, Auchtung JM, Schaefer L, Eaton KA, Britton RA. Humanized microbiota mice as a model of recurrent *Clostridium difficile* disease. *Microbiome* 2015; **3**: 35.
40 Hintze KJ, Cox JE, Rompato G, Benninghoff AB, Ward RE, Broadbent J *et al.* Broad scope method for creating humanized animal models for animal health and disease research through antibiotic treatment and human fecal transfer. *Gut Microbes* 2014; **5**: 183–191.
41 Galipeau HJ, Cox JE, Rompato G, Benninghoff AD, Ward RE, Broadbent J *et al.* Intestinal microbiota modulates gluten-induced immunopathology in humanized mice. *Am J Pathol* 2015; **185**: 2969–2982.
42 Respondek F, Gerard P, Bossis M, Boschat L, Bruneau A, Rabot S *et al.* Short-chain fructo-oligosaccharides modulate intestinal microbiota and metabolic parameters of humanized gnotobiotic diet induced obesity mice. *PLoS ONE* 2013; **8**: e71026.
43 Kashyap PC, Marcobal A, Ursell LK, Larauche M, Duboc H, Earle KA *et al.* Complex interactions among diet, gastrointestinal transit, and gut microbiota in humanized mice. *Gastroenterology* 2013; **144**: 967–977.
44 Lu L, Yu Y, Guo Y, Wang Y, Chang EB, Claud EC. Transcriptional modulation of intestinal innate defense/inflammation genes by preterm infant microbiota in a humanized gnotobiotic mouse model. *PLoS ONE* 2015; **10**: e0124504.

45 Marcobal A, Kashyap PC, Nelson TA, Aronov PA, Donia MS, Spormann A *et al.* A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. *ISME J* 2013; **7**: 1933–1943.
46 Tannock GW, Miller JR, Savage DC. Host specificity of filamentous, segmented microorganisms adherent to the small bowel epithelium in mice and rats. *Appl Environ Microbiol* 1984; **47**: 441–442.
47 Oh PL, Benson AK, Peterson DA, Patil PB, Moriyama EN, Roos S *et al.* Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. *ISME J* 2010; **4**: 377–387.
48 Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB *et al.* Gut immune maturation depends on colonization with a host-specific microbiota. *Cell* 2012; **149**: 1578–1593.
49 Owens RC, Donskey CJ, Gaynes RP, Loo VG, Muto CA. Antimicrobial-associated risk factors for *Clostridium difficile* infection. *Clin Infect Dis* 2008; **46**: S19–S31.
50 Lawley TD, Young VB. Murine models to study *Clostridium difficile* infection and transmission. *Anaerobe* 2013; **24**: 94–97.
51 Munyaka PM, Eissa N, Bernstein CN, Khafipour E, Ghia J-E. Antepartum antibiotic treatment increases offspring susceptibility to experimental colitis: a role of the gut microbiota. *PLoS ONE* 2015; **10**: e0142536.
52 Rajpal DK, Klein J-L, Mayhew D, Boucheron J, Spivak AT, Kumar V *et al.* Selective spectrum antibiotic modulation of the gut microbiome in obesity and diabetes rodent models. *PLoS ONE* 2015; **10**: e0145499.
53 Candon S, Perez-Arroyo A, Marquet C, Valette F, Foray A-P, Pelletier B *et al.* Antibiotics in early life alter the gut microbiome and increase disease incidence in a spontaneous mouse model of autoimmune insulin-dependent diabetes. *PLoS ONE* 2015; **10**: e0125448.
54 Sha S, Liang J, Chen M, Xu B, Liang C, Wei N *et al.* Systematic review: faecal microbiota transplantation therapy for digestive and nondigestive disorders in adults and children. *Aliment Pharmacol Ther* 2014; **39**: 1003–1032.
55 Borody TJ, Paramsothy S, Agrawal G. Fecal microbiota transplantation: Indications, methods, evidence, and future directions. *Curr Gastroenterol Rep* 2013; **15**: 337.
56 Brown K, Godovannyi A, Ma C, Zhang Y, Ahmadi-Vand Z, Dai C *et al.* Prolonged antibiotic treatment induces a diabetogenic intestinal microbiome that accelerates diabetes in NOD mice. *ISME J* 2016; **10**: 321–332.
57 Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U *et al.* Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* 2013; **339**: 1084–1088.
58 Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006; **444**: 1027–1131.
59 Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X *et al.* Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J* 2011; **5**: 220–230.
60 Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA *et al.* Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011; **334**: 105–108.
61 Lin HV, Frassetto A, Kowalik EJ Jr, Nawrocki AR, Lu MM, Kosinski JR *et al.* Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. *PLoS ONE* 2012; **7**: e35240.
62 Cohen J. *Statistical Power Analysis for the Behavioral Sciences*, 2nd edn. Lawrence Erlbaum Associates: Hillsdale, NJ, USA, 1988.
63 Button KS, Ioannidis JPA, Mokrysz C, Nosek BA, Flint J, Robinson ESJ *et al.* Power failure: why small sample size undermines the reliability of neuroscience. *Nat Rev Neurosci* 2013; **14**: 365–376.
64 Fosgate GT. Practical sample size calculations for surveillance and diagnostic investigations. *J Vet Diagn Invest* 2009; **21**: 3–14.
65 Charan J, Kantharia ND. How to calculate sample size in animal studies. *J Pharmacol Pharmacother* 2013; **4**: 303–306.
66 Nguyen TLA, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? *Dis Model Mech* 2015; **8**: 1–16.