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# Considering the microbiota to achieve reduction in the numbers of animals used in scientific studies

**Axel Kornerup Hansen**

*Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark*

**Abstract**

Elimination of pathogens by laboratory rodent commercial vendors has substantially improved standardized conditions as well as laboratory animal welfare. However, pathogens are also important for basic activation and functioning of the immune system with consequential influences on the symbiotic bacteria composition in the individual microbiota. One of the reasons for failures of translating results from preclinical research to the clinical phase in some studies could be due to unintentional selection processes. Some recommendations are provided to increase researchers' awareness on this point, together with a practical checklist to optimize information from microbiota knowledge.

**Key words**

- 3R Principle
- laboratory rodents
- symbiotic bacteria composition
- intra-individual variations

## SPF ANIMALS

In 1957 Russell and Burch introduced the concept of the 3R's: Replacement, Reduction and Refinement [1]. A few years later Henry Foster, the founder of the US breeding company Charles River, initiated the production of rodents free of well-defined specific pathogens (SPF) by the use of caesarian section and barrier protection [2]. Since then long lists of pathogenic and opportunistic microorganisms have been eliminated from rodent breeding colonies, and laboratory rodents purchased from commercial breeders today are extremely clean compared to fifty years ago [3].

Maybe these rodents are too clean. There is no doubt that this elimination of pathogens has improved laboratory animal welfare substantially, because before that studies were commonly terminated due to fatal diseases among the animals, such as Tyzzer's disease, ectromelia and murine colonic hyperplasia, and it has had importance for reduction in the individual studies due to reduced inter-individual variation and model interference. However, pathogens are also important for the basic activation and the function of the immune system [4]. Thus, it is argued that one of the reasons for failures of translating results from preclinical research to the clinical phase in some studies is due to lacking pathogen stimulation [4]. True is it that the immune system of adult laboratory mice often looks more like the immune system of a newborn than that of an adult human being [4], which may also be due to the lack of the symbiotic bacteria found in the microbiota of wild mice.

## THE IMPACT OF THE MICROBIOTA

When a scientist buys a mouse from a commercial vendor, he or she will get one genome with a little more than 20 000 functional genes. However, with the mouse the scientist will also get $10^{14}$ microbes, i.e. bacteria, archea, protozoans and phages with more than 1 million functional genes. Dependent on which vendor and which room at this vendor the mouse comes from, these microbiotas will differ, and often this difference will be larger than e.g. what a change in diet can induce [5]. It is clearly documented that the microbiota composition is of importance for the development of human diseases, and so, it is also important for the modelling of diseases in animals. For example, in humans there is a strong correlation between microbiota composition and clinical parameters of type 2 diabetes and obesity [6], and this is fully comparable to mouse models, in which 30-40 % of the inter-individual variation in key clinical parameters of type 2 diabetes can be ascribed to gut microbiota variation [7]. This is even higher in models of atopic dermatitis [8], while it is somewhat lower in models of neuro-psychiatric diseases [9]. As inter-individual variation is one of the factors determining group sizes in animal studies, there is no doubt that inter-individual microbiota variation therefore leads to higher group sizes or lack of power in studies.

## ANIMALS WITH A UNIFORM MICROBIOTA

One way to solve the problem could therefore be to produce animals inoculated with a uniform microbiota

*Address for correspondence*: Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, Section of Experimental Animal Models, University of Copenhagen, Ridebanevej 9, DK-1870 Frederiksberg C, Denmark. E-mail: akh@sund.ku.dk.

to achieve animals with little inter-individual variation or to do selective breeding to produce animals with a uniform microbiota. However, this is not a simple task. Selective breeding selecting the most similar animals for further mating does not seem to reduce the inter-individual microbiota variation [10], and inoculation with selected microbiotas does not lead to a clustering of 100% between donor and recipient, and therefore inoculated animals still exhibit inter-individual microbiota variation. In principle, animals with a standardized and uniform microbiota already do exist in the form of gnotobiotic animals. However, these animals if not being fully germ-free have a very little diversity in their microbiota, and this will probably also be the case for those animals produced on the basis of tailor-made microbiotas.

## SPECIFIC KEY BACTERIA

There are key bacteria, which are essential for the expression of a specific phenotype in the animals, e.g. a human disease, or which are crucial for the response to certain interventions tested on the animals [3]. Littman's group experienced that they were unable to induce the adoptive transfer model of inflammatory bowel disease in mice from one vendor compared to their normally used vendor due to the absence of *Segmented filamentous bacteria* (SFB's) in the new mice [11]. A popular issue in the food industry is to develop oligosaccharides to increase the abundance of the anti-inflammatory bacterium *Bifidobacterium*. However, often mice from commercial breeders have no *Bifidobacterium* spp. at all, and therefore testing oligo-saccharides on such animals may give false negative results [3]. The incidence of type 1 diabetes in non-obese diabetic mice is to a high degree determined by the presence of *Akkermansia muciniphila* [3]. Therefore, to avoid wasting animals and to increase the chance of producing translational and reproducible results rats and mice used for microbiota sensitive studies should be checked for the presence or absence of key species [3].

## HUMANIZED MICROBIOTA MICE

Mice and humans on a phylum level may appear to have a very similar microbiota, but on genus and species level they are quite different [12]. Inoculation with a human microbiota may obviously be seen as a way to make the mouse a more translational model for humans. However, inoculation with human microbiotas in mice fail to stimulate the immune system, so a microbiota humanized mouse may look as naïve in its immune system as a germ-free mouse [13].

## INCORPORATING MICROBIOTA DIVERSITY IN DATA EVALUATION

It is, therefore, not just around the corner to answer to the microbiota challenge in animal research by the production of animals with a fixed microbiota. The opposite approach may seem much more attractive, i.e. to strive for animals with a high diversity in microbiota and to monitor and describe it. Costs for microbiota characterizations have decreased rapidly over the last decade. Much of the routine health monitoring of today can be done by more cost-efficient PCR methods compared to the so far applied bacterial cultivation, and the agent screening list can be reconsidered because many of the pathogens are seldom found in laboratory animals nowadays [3]. Therefore, if routine health monitoring is reorganized, it should not be difficult to find resources for current screening of the microbiota of animals, both on a colony level and down to the individuals of each study. It should be possible in some studies to convert maybe 40 % of the uncontrolled variation into controlled variation. In addition, it will also reveal important information on the impact of various microorganisms on the development of diseases. So, it will add to reduction both by allowing a smaller group size, because inter-individual variation is controlled, and because more information per animal is created.

## ENVIRONMENTAL IMPACT ON THE MICROBIOTA

However, controlling the microbiota of the animals is not enough if one wants to use microbiota considerations to strive for reduction. A range of environmental factors can currently change the microbiota, and although likely to create a lot of information, daily microbiota screens are not realistic. In addition, even though this could be done by a daily sampling of feces, key differences between the animals are often better revealed by a characterization of the cecal microbiota, i.e. it can only be done at termination [14]. Therefore, attempts should be done to keep the microbiota stable during the experiment. Stress is known to change the microbiota [15], and both during breeding and during

**Table 1**
Microbiota considerations to be made prior to an animal study

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|---|
| Consider if there are any essential microbes, which need to be present or absent in the animal model, and if so obtain information from the breeding colony on the status of these microbes. |
| Consider if a specific diet composition is essential for the animal model. |
| Freeze a batch of the selected diet for the interior study and rethaw it weekly during the study. |
| Check that experimental design allows testing for the cage factors impact on the primary read-out. |
| Check that there are no known stressors in the animal room for the entire study period.<br>Check if staff has been appropriately trained for the procedures<br>Check if the animals need to be trained for the procedures |
| Consider if a microbiota characterization before, during and after the study will be beneficial for the data evaluation and if so plan sampling, and how to incorporate the characterization in the data evaluation. |

the experiment environmental stressors such as poor housing, noise and untrained staff should be avoided, which is also relevant from a refinement point of view. Even commercial laboratory animal diets contains a range of micronutrients with impact on the microbiota, which vary substantially between batches, and therefore it should be ensured that the same batch is fed throughout the study. This may be achieved by freezing the entire amount of diet for the entire study, and at every feeding, thaw the amount of diet needed.

## CONCLUDING REMARKS

Increased awareness of the impact of the microbiota is likely to result in reduction in the spirits of Russell and Burch, i.e. we can use fewer animals and/or obtain more information in each individual study. However, from an overall point of view it may not reduce the total number of animals used. Increased knowledge on the microbiota has also revealed new potentials in research, and the use of animal studies within the food industry, which has not used that many animals before, has increased in the search for functional foods, which can increase the abundances of microbes beneficial for humans.

To obtain the full potential of microbiota knowledge in animal studies the simple checklist in *Table 1* may be applied.

### *Conflict of interest statement*

The author declares no conflict of interest.

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