Routine assessment of the gut microbiome to promote preclinical research reproducibility and transparency

The irreproducibility of preclinical, biomedical research is becoming increasingly problematic, as recently highlighted by Omary et al., in the June issue of Gut. As discussed, variations in study design, mouse strain, sex and age are important factors that should be adequately described to promote study reproducibility. In line with recent speculation, authors also emphasised the gut microbiome as a potential confounder underlying inconsistencies in preclinical research data.

Recently, there has been a large influx of studies reporting dysbiotic changes in many preclinical models of human disease, both GI and non-GI. Although informative, much of this research continues to be associative. To dissect causative disease mechanisms, the impact of benign environmental factors relating to study design and rodent husbandry must be acknowledged.

Based on twin studies, it is understood that a core subset of bacteria are hereditary. However, environmental factors are thought to contribute more heavily to the composition of the gut microbiome. For example, in vivo transfer of genetically distinct embryos results in similar microbial profiles regardless of genetic background. Similarly, relocation of infant mice dramatically changes the native microbial community. In adult mice, viable counts of the total bacterial load have shown large differences in the gut microbiome among animals from different facilities and even different breeding rooms within the same facility. This critically highlights the need to routinely characterise the composition of the gut microbiome to promote study reproducibility.

We have previously shown that the genetic knockout of the innate immune receptor, Toll-like receptor 4 (BALB/c-Tlr4−/−billy), alters the composition of the caecal microbiota. Following introduction of a new breeding facility for this genetically modified strain, we assessed the composition of the gut microbiome in this new population. In keeping with previous recommendations, efforts were made to reduce environmental confounders. Female mice (n=12, BALB/c background, 18–20 g, 8–10 weeks) were group housed in ventilated cages, in the same rack/room, with six animals per cage. All animals were allowed to acclimatise for 1 week (at the University of Adelaide) during which they were exposed to the same dark/light conditions (12 hours) and given access to water and food ad libitum. The first population of BALB/c-Tlr4−/−billy mice were obtained from the University of Adelaide Laboratory Animal Service (TLR4KO1). The second population of BALB/c-Tlr4−/−billy mice were sourced from University of Newcastle (TLR4KO2). Both populations were originally sourced from Osaka, Japan. The caecal contents were aseptically collected and sent for genetic sequencing at the Australian Genomics Research Facility.

Consistent with previous in vivo research, our data indicate that breeding facility alters the composition of the gut microbiome. TLR4KO1 has significantly lower levels of Bacteroidetes compared with TLR4KO2 (**p=0.009, figure 1). TLR4KO1 mice
displayed higher levels of gram-positive Actinobacteria (TLR4KO1 2.23±0.63%; TLR4KO2 60.38±0.07%, *p=0.03) and the pathogenic microbe, Proteobacteria (TLR4KO1 3.54±0.90%; TLR4KO2 1.03±0.25%, *p=0.03). Fewer total species were also recorded in TLR4KO1 (total=69) compared with TLR4KO2 (total=104), although no changes were seen in alpha diversity (figure 2). Given efforts to reduce additional confounders, this supports previous research showing altered microbial phenotype in mice from different breeding facilities and even different rooms within the same facility and suggests that the gut microbiome reflects the cumulative effects of various environmental factors. The mechanism(s) underlying these changes are unclear; however, our results highlight the importance of consistent rodent husbandry when designing and conducting preclinical studies, and the need to routinely characterise the composition of the gut microbiome. This is especially important with current calls to address the irreproducibility of preclinical research and to standardise reporting of animal research data presentation.

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**Figure 1** Breeding conditions affect gut microbiome composition in the BALB/c mouse. (A) Relative abundance (%) of bacteria phyla in n=6 TLR4KO1 and n=6 TLR4KO2 mice. (B) Mean percentage of each bacteria phyla in TLR4KO1 and TLR4KO2 populations. Differences were identified in Actinobacteria (TLR4KO1 2.23±0.63%; TLR4KO2 60.38±0.07%, *p=0.03), Bacteroidetes (TLR4KO1 13.34±3.47%; TLR4KO2 27.75±2.75%, *p=0.009) and Proteobacteria (TLR4KO1 3.54±0.90%; TLR4KO2 1.03±0.25%, *p=0.03). Data presented as individual data points with mean±SEM.

**Figure 2** Shannon’s diversity index for TLR4KO1 and TLR4KO2 caecal microbiome species. An unpaired t-test with Welch’s correction showed no significant difference between populations of BALB/c-Tlr4−/− mice. Data presented as individual data points with mean±SEM; n=6 per group, p >0.05.
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