Effective establishment of donor gut microbiota in gnotobiotic mice

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ABSTRACT

Background: Determining whether associations between gut microbiota characteristics and host physiology represent causal relationships is a fundamental challenge for microbiome research. One way these associations can be investigated is to instil donor faecal material into gnotobiotic mice and to assess the extent to which donor phenotype is recapitulated. However, the manner in which this process is performed varies considerably between studies, and assessment of microbiota re-establishment in recipient animals is not always carried out. We report a detailed investigation of microbiome assembly in germ-free mice and compare the effects of single and multiple rounds of faecal gavage, using both native and antibiotic-disrupted donor material.

Results: Levels of bacteria within the faeces of recipient animals increased rapidly following the instillation of donor material. However, considerable instability in microbiota composition continued during the first two weeks post-gavage, with substantial changes in taxon relative abundance occurring in parallel to declining faecal pH. Relative compositional stability was not achieved until day 28 and persistent differences between recipient and donor microbiota remained. These included an increased relative abundance of Bacteroidetes, and a reduced relative abundance of Firmicutes. Of taxa detected in donor material, 52% were represented in stable recipient microbiota following transplantation with native faecal material (single gavage), increasing to 66% following three rounds of gavage. These taxa accounted for 95% and 91% of total donor bacterial abundance, respectively. Performing multiple rounds of gavage significantly increased microbiota similarity between donor and recipient, and significantly reduced within-group dispersion (P<0.05). Instillation of antibiotic-associated microbiota resulted in substantially lower temporal and inter-animal variance, with multiple rounds of gavage providing no substantial benefit.
Conclusions: Microbiome assembly in recipient animals is not immediate and several weeks are required for microbiota stability to be achieved. Multiple rounds of faecal gavage result in greater similarity to donor microbiota and reduced inter-animal variance. The process of donor microbiota re-establishment, and therefore the interval required prior to investigations using recipient animals, is influenced by donor microbiota characteristics.

Key words: germ-free, faecal microbiota transfer, gut microbiota colonisation.
Introduction

Much of our understanding of relationships between the gut microbiome and human physiology comes from observational studies. Having identified associations between gut microbiome traits and host measures, the next challenge is to determine whether these relationships are causal, secondary to observed physiological phenomena, or result from parallel but independent processes.

One of the few experimental strategies available to assess causality in host-microbiome interactions is the instillation of intestinal microbiota from human donors or animal models into germ-free mice, and assessment of whether donor phenotype is recapitulated. This approach has been used, for example, to demonstrate that gut microbiota contributes to obesity in the context of a high fat diet [31], to underdevelopment in the context of a low nutrient density/bioavailability diet [4], and to variation in treatment efficacy in recipients of cancer immunotherapy [18, 34]. The employment of this strategy is becoming increasingly popular due to growing access to gnotobiotic facilities, and evidence that antibiotic depletion of recipient intestinal microbiota in conventional mice prior to faecal transfer represents a relatively poor alternative [13, 37].

Despite the utility of gut microbiota transplantation as a means to understand microbiome-host relationships, there is little consistency in the manner in which the technique is performed. In particular, the number of rounds of gavage employed varies between studies, as does the period allowed to elapse between the final gavage and the initiation of the experiment or assessment. The extent to which donor microbiota are replicated within recipient animals is commonly neglected, and where assessment is performed, it often focuses donor taxon presence/absence, rather than microbiota structure or composition.

Many of the bacterial clades that are closely associated with the regulation of host physiology are obligate anaerobes [33, 46], and are particularly susceptible to loss of viability during the
processing of material for transplant [26, 27]. Not only can failure to establish such taxa in the
gut lumen of gnotobiotic recipient animals lead to divergence in microbiota composition from
donor animals, but it can allow the proliferation of opportunistic facultative anaerobes [22, 42].
Such changes can have profound implications for the metabolic and immuno-regulatory
properties of the gut microbiome [1, 16].
A number of previous studies have aimed to describe the process of intestinal microbiota
assembly in germ-free mice [12, 17]. In particular, Gilliland and colleagues described
microbiota assembly at two mucosal sites, the caecum and the jejunum, during the first 21 days
following the instillation of caecal microbiota, reporting evidence of both ecological succession
and site-specific effects [17]. Aidy and colleagues described microbiome compositional
dynamics and metabolic function in germ-free mice that received a faecal suspension over a 16
day period [12], again with evidence of ecological succession. However, important knowledge
gaps remain, including what the effects of multiple rounds of microbiota instillation are, and
what considerations are necessary when attempting to establish substantially modified donor
microbiota.
We investigated the dynamics of donor microbiome assembly in the gut of recipient germ-free
mice, including a comparison of the effects of single and multiple rounds of gavage.
Assessment of microbiota transplantation was performed using both native and antibiotic-
disrupted gut microbiota, with the latter used to represent changes that are commonly described
in association with pathophysiology [25, 39].
RESULTS

Establishment of normal gut bacterial abundance

In mice that received native donor microbiota (approximately inoculum of $10^5$ bacterial cells), total bacterial load peaked between four days post initial gavage (D4) and seven days post initial gavage (D7), before declining to D14 (Figure 1A). From D14, faecal bacterial load was broadly stable at approximately $5 \times 10^6$ bacterial cells/mg. No significant differences were observed between mice that received one or three rounds of gavage.

Compared to native microbiota, antibiotic-disrupted donor had reduced species richness (Figure S2A-S2B), reduced relative abundance of Firmicutes, and increased relative abundance of Verrucomicrobia and Bacteroidetes (Figure S2C). The pattern of an increase in bacterial load following the instillation of antibiotic-disrupted microbiota was similar to that seen with native microbiota (Figure 1B). The number of rounds of faecal gavage performed was not associated with a significant difference in total bacterial load (Figure 2B).

Donor taxa representation within recipient faecal microbiota

Initial assessment of recipient faecal microbiota was based on the percentage of donor taxa detected. Similar trends were observed for recipients of one and three rounds of gavage, with detected taxa rising steeply from D4 (median, IQR: 1G = 43.1%, 32.8 - 44.0; and 3G = 44.8%, 40.5 - 44.8) but remaining variable throughout the 70-day study (Figure 2A). On average, the percentage of donor taxa detected was higher in recipients of three rounds of gavage than recipients of a single gavage at all time-points bar one (D56). This difference was significant at the end of the study period (median, IQR: 1G = 51.7%, 46.6-60.4; 3G = 65.5%, 62.9-69.0; P <0.05). In recipients of antibiotic-disrupted microbiota, donor taxa detected increased significantly as a result of multiple gavages (Figure 3A). The difference between recipients of
single and multiple rounds of gavage remained significant throughout the 70-day study (median, IQR: 1G= 33.3%, 33.3-37.5; 3G= 37.5%, 37.5-39.6; P < 0.05, except at D42).

The percentage of donor taxa detected in recipient animals fluctuated over the 70-day period of assessment. As mice were housed in a controlled environment in which the only route of bacterial acquisition was faecal gavage, intermittent detection of individual taxa was likely to be due to sampling bias. Assessment of the relative abundance of taxa in relation to their presence or intermittent absence at each timepoint supported this hypothesis (Figure S3), with the latter taxa relative abundance being significantly closer to the threshold of detection (Median, IQR: Present= 0.005, 0.001 - 0.013; Intermittent= 0, 0 - 0.001; Mann-Whitney test, P< 0.05). As a result, we also assessed the percentage of donor taxa detected as a cumulative measure. Again, median percentage of donor taxa detected was lowest at D4 (metrics as above; median, IQR: G1 = 43.1%, 32.8-44.0; G3 = 44.8%, 40.5-44.8) and reached a maximum at D70 (G1 = 81.0%, 79.3 - 84.5; G3 = 91.4%, 88.8 - 94.0) (Figure 2B). The mean percentage of donor taxa detected was significantly higher in recipients of multiple compared to single gavage at all sample points between D21 and D70 (Mann-Whitney test, P< 0.05). Similarly, the median percentage of donor taxa in recipients of single or multiple rounds of gavage with antibiotic-disrupted microbiota was lowest from D4 to D14 (Figure 3B). Multiple gavage was associated with a detection of a higher median percentage of donor taxa compared to single gavage at D21, a difference that persisted throughout the 70 day study (1G= 37.5%, 35.4 - 37.5; 3G= 45.8%, 37.5 - 45.8; P<0.05).

Recipient microbiota was further assessed based on the proportion of donor bacterial abundance represented. Between D4 and D70, bacterial taxa detected in recipient animals represented 89.9 to 95.5% of donor relative abundance (single gavage) or 90.1 to 95.5% (multiple gavage) (Figure 2A). In recipients of antibiotic-disrupted microbiota, detected
bacterial taxa represented 99.8 to 100% (single gavage) and 95.2 to 100% (multiple gavage) between D4 and D70, respectively (Figure 3A).

**Re-establishment of donor microbiota composition**

Recipient-donor microbiota similarity was assessed based on weighted UniFrac distance. Similarity increased in both groups to D35, after which no significant differences were observed between consecutive time points (PERMANOVA P>0.05), with the exception of D70 in the multiple gavage group. From D14, recipients of a single gavage had lower similarity to donor (higher mean weighted UniFrac distance) compared to recipients of multiple gavages (mean ± SD: 0.19 ± 0.05 vs 0.17 ± 0.04, respectively), with the exception of D42 and D70 (Figure 4A). Differences between single and multiple gavage groups achieved significance at D28, D56 and D70 (P<0.05).

Microbiota composition between consecutive timepoints in animals that received antibiotic-disrupted microbiota achieved stability by D35 in recipients of multiple gavage, and D56 in recipients of single gavage (PERMANOVA P>0.05). Recipient-donor microbiota similarity was lower (D14-D70, 0.34 ± 0.02 for single and multiple gavage groups; Figure 4B), although variations in similarity scores across time were substantially less when compared to recipients of native microbiota. No significant differences were observed between recipients of single or multiple rounds of gavage.

Microbiota composition was also assessed based on distance to group centroid. In recipients of native microbiota, distance to centroid increased from D4, peaking at D7, and declining thereafter (Figure 5A). Distance to centroid was higher at all but one time-point (D49) in mice that received one round of faecal gavage compared to those that received three, achieving statistical significance at D14, D28, and D63 (Mann-Whitney test, P<0.05). Distance to group
centroid in recipients of antibiotic-disrupted microbiota did not differ significantly between single and multiple gavage groups (Figure 5B).

**Temporal dynamics in taxon relative abundance**

Temporal changes in taxon relative abundance differed substantially between phylogenetic clades. The relative abundance of Bacteroidales increased substantially from donor values following instillation. Both Bacteroidales and Lactobacillales remained high throughout the study period (Figure 6A), while the relative abundance of Bifidobacteriaceae, Verrucomicrobiales, and Erysipelotrichaceae increased sharply following instillation, peaked at D4, and then declined to near donor levels (Figure 6A-B). Bacillales, Desulfovibrionales, Saccharimonadales, and Anaeroplasmatales were not detectable at D4, but increased gradually over time (Figure 6B), while Peptostreptococcaceae relative abundance declined to D21 and was undetectable thereafter (Figure 6C). Less variation were observed for the relative abundance of other taxa, including Ruminococcaceae. Bacterial family temporal dynamics were broadly consistent between single and multiple gavage groups.

The variation in bacterial family relative abundance was significantly lower in recipients of antibiotic-disrupted microbiota (Figure 7A-B). Dominant families (Lachnospiraceae, Bacteroidales, and Verrucomicrobiales) were broadly stable, although a sustained increase in Bacteroidales was observed between D28 and D35. In contrast to recipients of native donor microbiota, the relative abundance of Ruminococcaceae fell substantially following instillation until D7, before partially recovering. Again, no significant differences were observed between single and multiple gavage groups.

At the phylum level, substantial differences between donor and recipient microbiota were evident at D7, persisting to D70 (Figure S4). In particular, the relative abundance of the most prevalent phylum, Firmicutes, was reduced by an average of 30.3% (3G, D70), while the
relative abundance of the second most prevalent phylum, Bacteroidetes, increased by an average of 29.1% (3G, D70). Phylum-level differences between donor and recipient microbiota were explained largely by the relative abundance of *Lactobacillus* and uncultured members of Muribaculaceae, respectively.

Temporal dynamics in taxon absolute abundance and faecal pH

The absolute abundance of three genera, *Blautia* (Lachnospiraceae), *Bifidobacterium* (Bifidobacteriales) and *Akkermansia* (Verrucomicrobiales), which were dominant members of the bacterial families whose relative abundance changed substantially in recipients of native or antibiotic-disrupted microbiota, were determined by qPCR (Figure S5). Absolute abundance dynamics for these genera were broadly consistent with their relative abundances. Levels of *Bifidobacterium* and *Akkermansia* declined from D4 and were almost depleted by D14 in recipients of the native microbiota, whereas consistent levels of *Blautia* were observed across the timepoints in recipients of the antibiotic-associated microbiota.

Temporal variation in faecal pH was observed in recipients of the native microbiota. Faecal pH decreased following microbiota instillation, from pH 7.1 and pH 7.3 to pH 6.5 and pH 6.4 at D4, in the single and multiple gavage groups respectively (Figure S6). These levels increased by D7 and remained consistent for the multiple gavage group until D28, whereas larger variation across subsequent timepoints were observed for the single gavage group.
Our goal was to characterise microbiota assembly in gnotobiotic mice following the instillation of donor faecal material. In doing so, we aimed to address knowledge gaps relating to how microbiota transplantation is performed in the investigation of causal relationships in host-microbiome associations. Our focus, in particular, was the effect of multiple versus single rounds of gavage, and differences in the dynamics of bacterial colonisation when native or substantially disrupted donor microbiota are used.

In keeping with a previous investigation of microbiota establishment in gnotobiotic mice [17], we observed the total bacterial levels in recipient animals to resemble donor animals rapidly. The rate of microbiota expansion was not affected by the number of rounds of gavage performed. Increases in bacterial diversity during the early stages of gut colonisation are constrained by ecological succession rather than by rate of biomass increase, as described in vaginally-born human infants [43]. Succession involves a process whereby early gut colonisers facilitate the growth of other taxa through modification of growth substrates, production of bioactive metabolites, and alteration of the physicochemical characteristics of the gut environment.

There are many well-described examples of inter-species interactions influencing gut microbiota assembly. For example, members of the Actinobacteria phylum, including bifidobacteria, are able to hydrolyse host glycans to release products including glucose, galactose, lactate and acetate, metabolites that are then utilised by members of the Bacteroidetes and Firmicutes phyla [11, 14]. Antagonistic interactions are also common and important in the suppression of pathogen growth. For example, bacterial fermentation of complex carbohydrates to produce short-chain fatty acids alters intestinal pH and inhibits replication of Enterobacteriaceae, including *Escherichia coli* and *Salmonella* [7], while bacterial conversion of primary bile acids to secondary bile acids inhibits the endospore
germination and vegetative growth of *Clostridioides difficile* [36]. These processes are particularly important in the re-assembly of gut microbiota following antibiotic disruption [6]. The changes in microbiota characteristics that we observed following instillation into germ-free mice were consistent with ecological succession. In mice transplanted with native microbiota, dissimilarity to donor microbiota and within-group dispersion were highest during the initial two weeks period, with changes in keystone bacterial clades consistent with well-described mechanisms of gut bacterial succession. For example, levels of Bifidobacteriaceae were initially high, but became substantially depleted by D14, consistent with the role played by members of this family as primary gut colonisers [21, 41]. In contrast, the relative abundance of Desulfovibrionales increased substantially over the first two weeks post-instillation. Many members of Desulfovibrionales are sulphate-reducers, utilising hydrogen gas produced in the biosynthesis of SCFAs. Levels of *Desulfovibrio*, for example, have been reported to increase with levels of butyrate [12]. The dynamic changes in microbiota structure during early colonisation with native microbiota were also reflected by changes in faecal characteristics, including in pH. The rapid decrease in pH at D4 post-colonisation followed by an increase thereafter might be explained by the high levels of *Bifidobacterium* observed, a taxon capable of the central hexose fermentation pathway (bifid shunt) to produce lactate, which reduces faecal pH [2, 15].

While we did not encounter overgrowth of individual opportunistic taxa, such as *Escherichia coli*, in recipient animals, as reported in previously [17], persistent differences between donor and recipient microbiota composition were evident. Most notable was a reduction in the relative abundance of Firmicutes, the predominant phylum, and an increase in the relative abundance of Bacteroidetes. In addition, several bacterial genera were detected in donor microbiota but not in transplant recipients, including *Anaerofustis, Bacteroides* and *Erysipelothrix*. Why such differences should occur between mice of similar genetic backgrounds is not clear, however,
there are a number of potential contributory factors. For example, the donor transplant material were derived from the caecum, while recipient microbiota composition was assessed based on faecal pellets. Differences in gut physiology in gnotobiotic mice compared with conventional mice have also been described, including in immune mechanisms that are involved in the regulation of intestinal microbiology [19, 23, 28, 40, 45], which could influence colonisation. Approaches used in the conventionalisation of germ-free mice as part of published studies vary considerably. Some investigators have employed single rounds of gavage [4, 31], while others have used multiple (typically three) rounds [13, 18]. Disappointingly, many other studies do not describe donor material preparation or the number of instillations that were performed. In our study, multiple rounds of gavage were associated with modest but consistent increases in the similarity of recipient and donor microbiota, both where native or antibiotic-disrupted microbiota were instilled. These differences are attributable to several bacterial taxa that were detected in recipients of multiple rounds of gavage but not in recipients of single gavage, including *Jeotgalicoccus*, *Lachnospiraceae UC5-12E3* and Mollicutes RF39. Multiple gavage was also associated with reduced inter-animal variance, an important factor when using such animals to investigate host-microbiota interactions.

In addition to investigating microbiota assembly in mice transplanted with native microbiota, we also assessed colonisation dynamics following the instillation of microbiota from antibiotic-exposed animals. In doing so, our aim was to determine whether the dynamics of conventionalisation with substantially-disrupted microbiota, as associated with a number of pathophysiological contexts [4, 31], differs to those with intact gut microbiota. The donor microbiota that resulted from antibiotic disruption exhibited substantially reduced taxa richness, and an increased prevalence of facultative anaerobes. Compared to native microbiota, instillation of antibiotic-disrupted microbiota was associated with greatly reduced temporal variation. The early colonisation period (up to D7) was characterised by the rapid establishment
of predominant taxa \((Lachnospiraceae, Bacteroidales,\text{ and } Verrucomicrobiales)\). These rapidly achieved levels comparable to those in donor material and showed little variation over time. Divergence from donor composition was driven instead by a failure to recover less prevalent taxa (including \(Staphylococcus\) and \(Clostridiales\) \(vadin BB60\)), which were undetectable by D4. In addition, \(Alistipes\) and multiple members of the Lachnospiraceae and Ruminococcaceae families, failed to colonise recipient animals (either single or multiple gavage), while the \(Intestinimonas\) and \(Anaeroplasma\) genera were detected only in multiple gavage recipients. The absence of several members of both the Lachnospiraceae and Ruminococcaceae families can be attributed to competitive interaction between taxa in an altered bacterial community \([35]\). Taxa in these families that successfully colonise recipient mice can also provide compensatory function, including their role in producing important metabolites such as short chain fatty acids by degrading complex polysaccharides \([3]\).

In our study, caecal microbiota were harvested and processed under strict anaerobic conditions. Alternative approaches that utilise stool, or that involve exposure to aerobic conditions, are likely to be associated with lower levels of donor-recipient similarity. Furthermore, our transplantation was between members of the same species, subject to similar environmental and dietary exposures. A growing number of studies involve the instillation of human stool, or stool-derived microbiota, into gnotobiotic mice. Substantial differences in genetics, physiology, anatomy, and diet, would be expected to further reduce associated levels of microbiota recapitulation.

Our study had a number of limitations. For example, we did not attempt to assess all possible gavage schedules or donor microbiota variants. Microbiota assembly dynamics are likely to change with donor microbiome characteristics, and are therefore vary with mouse genetic background \([20]\) and between populations housed at different research facilities \([8, 30]\). What host measures are being assessed, and what the hypothesised mechanisms of host-microbiome
interaction are, will also be important considerations in the design of experiments involving transplanted microbiota.

CONCLUSIONS

In summary, a substantial period (more than 4 weeks) may be required following faecal instillation into germ-free mice to achieve microbiota stabilisation. Establishment of donor microbiota occurs more rapidly in less diverse bacterial communities that result from antibiotic exposure. While multiple rounds of faecal instillation result in greater similarity to donor microbiota, the process of microbiota assembly differs considerably based on the complexity and composition of donor bacterial communities. A failure to understand the extent to which donor microbiota has been established in recipient mice, and the degree of community stability achieved, could contribute to spurious findings.
METHODS

Preparation of donor faecal material for transplantation

Faecal material was obtained from seven week old C57BL/6J donor mice, bred at the SAHMRI Bioresources animal facility, Adelaide, Australia. To induce antibiotic disruption of faecal microbiota, mice received water containing erythromycin ethylsuccinate (20 mg/kg) for 90 days, as opposed to plain water. Mice were killed by carbon dioxide inhalation. Caeca were harvested immediately and transferred to 15% anaerobic glycerol-phosphate buffered saline (PBS). All subsequent processing was performed under anaerobic conditions (10% CO₂, 10% H₂, 80% N₂).

Caecal contents were removed from encasing tissue, weighed, resuspended in 4x (w/v) anaerobic PBS, and homogenised by vortexing. The resulting suspension was passed through a Falcon 100 μM nylon cell filter (Thermofisher Scientific, Waltham, USA) to obtain the non-fibrous content. The supernatant was mixed with an equal volume of 30% anaerobic glycerol-PBS, and stored in a Hungate tube at -80°C until required. Prior to oral gavage, caecal supernatant was diluted with 2x volume of anaerobic PBS (pH 7.2) and sealed in a glass vial within the anaerobic chamber.

Germ-free mice were obtained from the Translational Research Institute (University of Queensland) and housed within germ-free isolators (Park Bioservices LLC, USA) at the SAHMRI Preclinical Imaging and Research Laboratories (PIRL) germ-free facility (Gilles Plains, Adelaide, Australia). All mice were maintained on autoclaved Teklad Global 18% Protein Rodent Diet (Envigo, Huntingdon, UK) throughout the study.

After a 10 day period of acclimatisation, caecal suspensions were instilled into germ-free mice under sterile conditions. Germ-free mice were inoculated with 150 μL of the appropriate donor caecal suspension (containing 10⁵-10⁶ bacterial cells) via an oral gavage. Faecal sampling was performed at baseline and throughout the experiment (Figure S1).
Faecal pellet collection, DNA extraction, and bioinformatic processing

Mice were placed in sterile individual cages for faecal pellet collection. Fresh faecal pellets were transferred aseptically to 1.5 mL Eppendorf tubes and stored at -80°C prior to analysis. Faecal pellets were resuspended in 300 μL of PBS by vortexing, and pelleted by centrifugation at 13 000 × g for 10 min at 4°C. Supernatant was transferred to a sterile 2 mL screwcap tube and stored at -80°C. Pellets underwent DNA extraction by a combination of mechanical and chemical cell lysis methods using the DNeasy PowerSoil kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions and eluted in 100 μL of sterile DNase- and RNase-free water.

Amplicon libraries of the V4 hypervariable region for 16S rRNA gene amplicon sequencing were prepared from DNA extracts using modified universal bacterial primer pairs 515F and 806R [9]. Amplicon libraries were indexed, cleaned, and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol on a 2 x 300 bp Miseq reagent kit v3 at the David R Gunn Genomics Facility, South Australian Health and Medical Research Institute.

Paired-end 16S rRNA gene sequence reads were analysed using QIIME v2.0 [5]. Briefly, de-noising was performed on de-multiplexed sequences using Dada2, with sequence reads truncated at a specific length based on a quality filtering score of 30 to remove low quality sequence region. Taxonomic classification of sequence variants were performed based on a pre-trained classifier composing of the V4 hypervariable region sequences of the SILVA 132 16S rRNA reference database clustered at 97% similarity [29]. All samples were subsampled to 4,859 reads. Sufficient coverage at this depth is confirmed by the rarefaction curve, which reached an asymptote. Taxon relative abundance were used in downstream analyses. Sequence
data is available from the Sequence Read Archive (SRA) repository under the accession number PRJNA592263.

**Quantitative PCR**

Previously described quantitative PCR (qPCR) assays were used to determine the abundance of *Akkermansia muciniphila* [44], *Bifidobacterium* spp [32] and *Blautia* spp. [38], as well as the 16S rRNA gene for total bacterial load [24]. SYBR-based qPCR assays were performed using 0.2 nM of each primer, 1X PowerUP SYBR Green mastermix (Thermofisher Scientific, Waltham, USA), 1 μL of DNA, and sterile DNase- and RNase-free water to make up to a total reaction volume of 35 μL. Each reaction was divided to three, 10 μL, replicate reactions. Total bacterial load was calculated using a standard curve generated from a known concentration of *Escherichia coli* DNA.

**Measurement of faecal pH**

The pH levels of faecal samples were measured using the method described by Xie *et al.*, 2016, with modifications. Faecal samples from each mice were collected and pooled according to cage to a total of approximately 50 – 100mg. Faeces were resuspended in 9x volume of deionized water and the suspension vortexed for 1 min. The pH value of faecal suspension was them measured on a FE20 FiveEasy™ pH meter (Mettler-Toledo AG, Schwerzenbach, Switzerland).

**Statistical analysis**

Bacterial taxa that were not detected in donor material, or present as a single read in only one sample, were removed from recipient microbiota data. Representation of donor taxa in recipient mice was determined based on their presence in more than one mouse per group. Beta diversity
analysis were performed based on weighted UniFrac distances computed between samples using QIIME. Compositional differences between groups and distance to the group centroid were determined based on the PERMANOVA and PERMDISP test, respectively, using PRIMER [10]. Comparison between single and multiple gavage groups was performed by Mann-Whitney test using Graphpad PRISM (v8).
Ethics approval and consent to participate: All animal studies were performed in accordance to comply with the relevant guidelines (Australian Code for the care and use of animals for scientific purposes (8th Edition 2013)) and approved by the South Australian Health And Medical Research Institute Animal Ethics Committee under the study reference SAM378.

Consent for publication: Not applicable.

Availability of data and material: Sequencing data generated are available in Sequence Read Archive (SRA) repository, PRJNA592263.

Competing interests: The authors declare that they have no competing interests.

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Authors’ contributions: JMC and GBR designed the study. JMC performed laboratory work and data analysis. JMC and GBR interpreted the findings and prepared the manuscript.

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REFERENCES

[1] Arthur, J.C., Perez-Chanona, E., Muhlbauer, M., Tomkovich, S., Uronis, J.M., Fan, T.J., Campbell, B.J., Abujamel, T., Dogan, B., Rogers, A.B., et al. (2012). Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 338, 120-123.

[2] Baxter, N.T., Schmidt, A.W., Venkataraman, A., Kim, K.S., Waldron, C., and Schmidt, T.M. (2019). Dynamics of Human Gut Microbiota and Short-Chain Fatty Acids in Response to Dietary Interventions with Three Fermentable Fibers. mBio 10.

[3] Biddle, A., Stewart, L., Blanchard, J., and Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. Diversity 5, 627-640.

[4] Blanton, L.V., Barratt, M.J., Charbonneau, M.R., Ahmed, T., and Gordon, J.I. (2016). Childhood undernutrition, the gut microbiota, and microbiota-directed therapeutics. Science 352, 1533.

[5] Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37, 852-857.

[6] Buffie, C.G., Bucci, V., Stein, R.R., McKenney, P.T., Ling, L., Gobourne, A., No, D., Liu, H., Kinnebrew, M., Viale, A., et al. (2015). Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. Nature 517, 205-208.

[7] Cherrington, C.A., Hinton, M., Pearson, G.R., and Chopra, I. (1991). Short-chain organic acids at pH 5.0 kill Escherichia coli and Salmonella spp. without causing membrane perturbation. J Appl Bacteriol 70, 161-165.

[8] Choo, J.M., Kanno, T., Zain, N.M., Leong, L.E., Abell, G.C., Keeble, J.E., Bruce, K.D., Mason, A.J., and Rogers, G.B. (2017). Divergent Relationships between Fecal Microbiota and Metabolome following Distinct Antibiotic-Induced Disruptions. mSphere 2.

[9] Choo, J.M., Leong, L.E., and Rogers, G.B. (2015). Sample storage conditions significantly influence faecal microbiome profiles. Sci Rep 5, 16350.

[10] Clarke, K.R., and Gorley, R.N. (2006). PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth.

[11] De Vuyst, L., and Leroy, F. (2011). Cross-feeding between bifidobacteria and butyrate-producing colon bacteria explains bifidobacterial competitiveness, butyrate production, and gas production. Int J Food Microbiol 149, 73-80.
[12] El Aidy, S., Derrien, M., Merrifield, C.A., Levenez, F., Dore, J., Boekschoten, M.V., Dekker, J., Holmes, E., Zoetendal, E.G., van Baarlen, P., et al. (2013). Gut bacteria-host metabolic interplay during conventionalisation of the mouse germfree colon. The ISME journal 7, 743-755.

[13] Ericsson, A.C., Personett, A.R., Turner, G., Dorfmeyer, R.A., and Franklin, C.L. (2017). Variable Colonization after Reciprocal Fecal Microbiota Transfer between Mice with Low and High Richness Microbiota. Front Microbiol 8, 196.

[14] Falony, G., Verschaeren, A., De Bruycker, F., De Preter, V., Verbeke, K., Leroy, F., and De Vuyst, L. (2009). In vitro kinetics of prebiotic inulin-type fructan fermentation by butyrate-producing colon bacteria: implementation of online gas chromatography for quantitative analysis of carbon dioxide and hydrogen gas production. Applied and environmental microbiology 75, 5884-5892.

[15] Fushinobu, S. (2010). Unique sugar metabolic pathways of bifidobacteria. Biosci Biotechnol Biochem 74, 2374-2384.

[16] Galipeau, H.J., McCarville, J.L., Huebener, S., Litwin, O., Meisel, M., Jabri, B., Sanz, Y., Murray, J.A., Jordana, M., Alaedini, A., et al. (2015). Intestinal microbiota modulates gluten-induced immunopathology in humanized mice. Am J Pathol 185, 2969-2982.

[17] Gilliland, M.G., 3rd, Erb-Downward, J.R., Bassis, C.M., Shen, M.C., Toews, G.B., Young, V.B., and Huffnagle, G.B. (2012). Ecological succession of bacterial communities during conventionalization of germ-free mice. Applied and environmental microbiology 78, 2359-2366.

[18] Gopalakrishnan, V., Spencer, C.N., Nezi, L., Reuben, A., Andrews, M.C., Karpinets, T.V., Prieto, P.A., Vicente, D., Hoffman, K., Wei, S.C., et al. (2018). Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science 359, 97-103.

[19] Husebye, E., Hellstrom, P.M., Sundler, F., Chen, J., and Midtvedt, T. (2001). Influence of microbial species on small intestinal myoelectric activity and transit in germ-free rats. Am J Physiol Gastrointest Liver Physiol 280, G368-380.

[20] Korach-Rechtman, H., Freilich, S., Gerassy-Vainberg, S., Buhnik-Rosenblau, K., Danin-Poleg, Y., Bar, H., and Kashi, Y. (2019). Murine Genetic Background Has a Stronger Impact on the Composition of the Gut Microbiota than Maternal Inoculation or Exposure to Unlike Exogenous Microbiota. Applied and environmental microbiology 85.

[21] Le Roy, T., Debedat, J., Marquet, F., Da-Cunha, C., Ichou, F., Guerre-Millo, M., Kapel, N., Aron-Wisniewsky, J., and Clement, K. (2018). Comparative Evaluation of Microbiota Engraftment Following Fecal Microbiota Transfer in Mice Models: Age, Kinetic and Microbial Status Matter. Front Microbiol 9, 3289.
[22] Lupp, C., Robertson, M.L., Wickham, M.E., Sekirov, I., Champion, O.L., Gaynor, E.C., and Finlay, B.B. (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe 2, 204.

[23] Manca, C., Boubertakh, B., Leblanc, N., Deschenes, T., Lacroix, S., Martin, C., Houde, A., Veilleux, A., Flamand, N., Muccioli, G.G., et al. (2020). Germ-free mice exhibit profound gut microbiota-dependent alterations of intestinal endocannabinoidome signaling. J Lipid Res 61, 70-85.

[24] Nadkarni, M.A., Martin, F.E., Jacques, N.A., and Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148, 257-266.

[25] Nobel, Y.R., Cox, L.M., Kirigin, F.F., Bokulich, N.A., Yamanishi, S., Teitler, I., Chung, J., Sohn, J., Barber, C.M., Goldfarb, D.S., et al. (2015). Metabolic and metagenomic outcomes from early-life pulsed antibiotic treatment. Nat Commun 6, 7486.

[26] Papanicolas, L.E., Choo, J.M., Wang, Y., Leong, L.E.X., Costello, S.P., Gordon, D.L., Wesselingh, S.L., and Rogers, G.B. (2019). Bacterial viability in faecal transplants: Which bacteria survive? EBioMedicine 41, 509-516.

[27] Papanicolas, L.E., Wang, Y., Choo, J.M., Gordon, D.L., Wesselingh, S.L., and Rogers, G.B. (2019). Optimisation of a propidium monoazide based method to determine the viability of microbes in faecal slurries for transplantation. Journal of microbiological methods 156, 40-45.

[28] Pollard, M., and Sharon, N. (1970). Responses of the Peyer's Patches in Germ-Free Mice to Antigenic Stimulation. Infect Immn 2, 96-100.

[29] Quast, C., Pruesse, E., Yilmaz, P., Gerkin, J., Schweer, T., Yarza, P., Peplies, J., and Glockner, F.O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research 41, D590-D596.

[30] Rausch, P., Basic, M., Batra, A., Bischoff, S.C., Blaut, M., Clavel, T., Glasner, J., Gopalakrishnan, S., Grassl, G.A., Gunther, C., et al. (2016). Analysis of factors contributing to variation in the C57BL/6J fecal microbiota across German animal facilities. Int J Med Microbiol 306, 343-355.

[31] Ridaura, V.K., Faith, J.J., Rey, F.E., Cheng, J., Duncan, A.E., Kau, A.L., Griffin, N.W., Lombard, V., Henrissat, B., Bain, J.R., et al. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science 341, 1241214.
[32] Rinttila, T., Kassinen, A., Malinen, E., Krogius, L., and Palva, A. (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97, 1166-1177.

[33] Riva, A., Borgo, F., Lassandro, C., Verduci, E., Morace, G., Borghi, E., and Berry, D. (2017). Pediatric obesity is associated with an altered gut microbiota and discordant shifts in Firmicutes populations. Environ Microbiol 19, 95-105.

[34] Routy, B., Le Chatelier, E., Derosa, L., Duong, C.P.M., Alou, M.T., Daillere, R., Fluckiger, A., Messaoudene, M., Rauber, C., Roberti, M.P., et al. (2018). Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science 359, 91-97.

[35] Shashkova, T., Popenko, A., Tyakht, A., Peskov, K., Kosinsky, Y., Bogolubsky, L., Raigorodskii, A., Ischenko, D., Alexeev, D., and Govorun, V. (2016). Agent Based Modeling of Human Gut Microbiome Interactions and Perturbations. PloS one 11, e0148386.

[36] Sorg, J.A., and Sonenshein, A.L. (2008). Bile salts and glycine as cogerminants for Clostridium difficile spores. J Bacteriol 190, 2505-2512.

[37] Staley, C., Kaiser, T., Beura, L.K., Hamilton, M.J., Weingarden, A.R., Bobr, A., Kang, J., Masopust, D., Sadowsky, M.J., and Khoruts, A. (2017). Stable engraftment of human microbiota into mice with a single oral gavage following antibiotic conditioning. Microbiome 5, 87.

[38] Suchodolski, J.S., Markel, M.E., Garcia-Mazcorro, J.F., Unterer, S., Heilmann, R.M., Dowd, S.E., Kachroo, P., Ivanov, I., Minamoto, Y., Dillman, E.M., et al. (2012). The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. PloS one 7, e51907.

[39] Sun, L., Zhang, X., Zhang, Y., Zheng, K., Xiang, Q., Chen, N., Chen, Z., Zhang, N., Zhu, J., and He, Q. (2019). Antibiotic-Induced Disruption of Gut Microbiota Alters Local Metabolomes and Immune Responses. Front Cell Infect Microbiol 9, 99.

[40] Thompson, G.R., and Trexler, P.C. (1971). Gastrointestinal structure and function in germ-free or gnotobiotic animals. Gut 12, 230-235.

[41] Turroni, F., Peano, C., Pass, D.A., Foroni, E., Severgnini, M., Claesson, M.J., Kerr, C., Hourihane, J., Murray, D., Fuligni, F., et al. (2012). Diversity of bifidobacteria within the infant gut microbiota. PloS one 7, e36957.

[42] Ubeda, C., Taur, Y., Jenq, R.R., Equinda, M.J., Son, T., Samstein, M., Viale, A., Socci, N.D., van den Brink, M.R., Kamboj, M., et al. (2010). Vancomycin-resistant
Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. J Clin Invest 120, 4332-4341.

Wampach, L., Heintz-Buschart, A., Hogan, A., Muller, E.E.L., Narayanasamy, S., Laczny, C.C., Hugerth, L.W., Bindl, L., Bottu, J., Andersson, A.F., et al. (2017). Colonization and Succession within the Human Gut Microbiome by Archaea, Bacteria, and Microeukaryotes during the First Year of Life. Front Microbiol 8, 738.

Wang, L., Christophersen, C.T., Sorich, M.J., Gerber, J.P., Angley, M.T., and Conlon, M.A. (2011). Low relative abundances of the mucolytic bacterium Akkermansia muciniphila and Bifidobacterium spp. in feces of children with autism. Applied and environmental microbiology 77, 6718-6721.

Wichmann, A., Allahyar, A., Greiner, T.U., Plovier, H., Lunden, G.O., Larsson, T., Drucker, D.J., Delzenne, N.M., Cani, P.D., and Backhed, F. (2013). Microbial modulation of energy availability in the colon regulates intestinal transit. Cell Host Microbe 14, 582-590.

Zeng, Q., Li, D., He, Y., Li, Y., Yang, Z., Zhao, X., Liu, Y., Wang, Y., Sun, J., Feng, X., et al. (2019). Discrepant gut microbiota markers for the classification of obesity-related metabolic abnormalities. Sci Rep 9, 13424.
Figure 1. Bacterial load in faecal samples following establishment of gut microbiota in recipient germ-free mice. Determination of faecal total bacterial load in recipient mice that received (A) intact native microbiota or (B) microbiota derived from antibiotic-exposed mice as a single (1G) or three rounds (3G) of gavage. Bacterial load was determined using quantitative PCR of the 16S rRNA gene. The top and bottom grey lines denote the maximum and minimum bacterial load of donor faecal samples. Solid lines and dotted lines denote median values and interquartile ranges, respectively. Statistical comparison between groups were performed using the Mann-Whitney test at a level of P< 0.05 for significance.
Figure 2. Representation and relative abundance of donor bacterial taxa in mice that received intact native microbiota. (A) Percentage of donor taxa represented in recipient germ-free mice that received one (1G) or three (3G) rounds or faecal gavage with intact microbiota. Representation of the donor taxa (solid line) and their total relative abundances (dashed line) in the recipient mice at each timepoints were determined. (B) The cumulative detection of donor bacterial taxa percentage) in recipient mice. Solid and dashed lines denote median values, dotted lines denote interquartile ranges. Statistical comparison between groups were performed using the Mann-Whitney test at a level of P< 0.05 for significance.
Figure 3. Representation and relative abundance of donor bacterial taxa and in mice receiving material from antibiotic-exposed mice. 

(A) Percentage of donor taxa represented in recipient germ-free mice that received one (1G) or three (3G) rounds of faecal gavage with antibiotic-exposed microbiota. Solid lines denote representation of the donor taxa and dashed lines denote their total relative abundance. 

(B) Cumulative representation of donor bacterial taxa (percentage) in recipient mice. Solid and dashed lines denote median values, dotted lines denote interquartile ranges. Statistical comparison between groups were performed using the Mann-Whitney test at a level of P< 0.05 for significance.
**Figure 4. Weighted UniFrac distances of the donor microbiota and recipient microbiota.**

Weighted UniFrac distance between the microbiota of recipient mice that received (A) intact native microbiota or (B) microbiota derived from antibiotic-exposed mice, and their respective donor microbiota composition throughout the 70 day study period. Recipient mice received either one round (1G) or three rounds (3G) of donor microbiota. Statistical comparison between the groups at each timepoint was performed using the Mann-Whitney test (P< 0.05).
Figure 5. Distance to group centroid of the recipient microbiota. Compositional dispersion of microbiota among recipient mice that received (A) intact native microbiota or (B) microbiota derived from antibiotic-exposed mice were determined based on distances to their respective group centroid throughout the 70 day study period. Recipient mice received either one round (1G) or three rounds (3G) of donor microbiota. Statistical comparison between the groups at each timepoint was performed using the Mann-Whitney test (P< 0.05).
Figure 6. Relative abundance of donor taxa in recipient mice receiving the native microbiota. Donor taxa observed in recipient that received one or three gavages of intact native microbiota were plotted at the order level based on (A) high relative abundance taxa (>0.03 relative abundance), (B) low relative abundance taxa (<0.03 relative abundance). (C)
Bacterial taxa within the Clostridiales order were plotted at the family level. Recipient mice received either one round (1G) or three rounds (3G) of donor microbiota. Solid and dotted lines denote the mean ± SEM values.
Figure 7. Relative abundance of donor taxa in recipient mice receiving the antibiotic-exposed microbiota. Donor taxa observed in recipient that received one or three gavages of the antibiotic-exposed microbiota were plotted at the order level, except for bacterial taxa in the Clostridiales order, which were plotted at the family level. Bacterial taxa were plotted according to (A) high relative abundance (>0.03 relative abundance) and (B) low relative abundance taxa at the order level (<0.03 relative abundance). Recipient mice received either one round (1G) or three rounds (3G) of donor microbiota. Solid and dotted lines denote the mean ± SEM values.
Supplementary Figure 1. Study design for the establishment of gut microbiota in germ-free mice. Germ-free mice (n=7 per group) received either one (1G) or three (3G) rounds of anaerobically-prepared caecal suspension containing approximately $10^5$ bacterial cells. Faecal pellets were collected from individual mice throughout the experiment.
Supplementary Figure 2. Alpha diversities and phylum-level relative abundances of donor microbiota. Alpha diversity measures of (A) observed OTUs and (B) Pielou’s evenness of the native and antibiotic-exposed microbiota of the donor were determined using QIIME2. (C) Relative abundances of phyla (relative abundance >1%) of the native and antibiotic-exposed donor microbiota. The median values are plotted and the error bars represent the interquartile ranges. Statistical comparisons were performed using the Mann-Whitney test at a level of P< 0.05 (denoted as asterisk).
Supplementary Figure 3. Relative abundance of donor bacterial taxa in recipient mice following faecal gavage. Relative abundance of donor taxa observed in recipient mice that received three rounds of faecal gavage with intact native microbiota. Bacterial taxa detected at all nine sampled timepoints following faecal gavage were categorised as consistent (C), while the remaining taxa that were not observed at all timepoints were categorised as intermittent (I). The middle and error bars indicate the median and interquartile ranges, respectively. Statistical comparison between the relative abundance levels of consistent and intermittent taxa were performed using the Mann-Whitney test at a level of P< 0.05 for significance.
Supplementary Figure 4. Relative abundance of donor taxa at the phylum level in recipient mice. Relative abundances of bacterial phyla (present in donor at a relative abundance of >0.01) in recipient mice that received one (1G) or three gavages (3G) of the (A) native or (B) antibiotic-exposed microbiota. Solid and dotted lines denote the mean ± SEM values.
Supplementary Figure 5. Absolute abundance levels of selected taxa in recipient mice.

The absolute levels of (A, B) *Akkermansia muciniphila* (C, D) *Bifidobacterium* spp. and (E, F) *Blautia* spp. were determined by quantitative PCR (qPCR) in recipient mice that received one (1G) or three gavages (3G) of the native or antibiotic-exposed microbiota, respectively. Absolute abundances of each bacterial taxa were normalised against the total bacterial load in the sample based on the delta CT values of the target gene and 16S rRNA gene. Solid and dotted lines denote the median value and interquartile ranges, respectively.
Supplementary Figure 6. Faecal pH levels in recipient mice of the native microbiota.

Faecal samples of recipient mice of the native microbiota at each timepoint were pooled according to their cages and the pH levels analysed. Recipient mice received either one round (1G) or three rounds (3G) of donor microbiota. Solid and dotted lines denote the mean value and standard deviation of pH levels, respectively.