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Research Article

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Metagenomic sequencing reveals high reproducibility of human donor microbiota transplanted into germ-free mice via lower gut route

Yapeng YANG[1,2,3], Xiang TAN[2], Zeyue ZHANG[2], Lifeng LIANG[4], Zhifeng WU[2], Jinhui HE[2], Yuqing WANG[2,3], Miaomiao DONG[2], Jixia ZHENG[2], Hang ZHANG[2,5], Shuaifei FENG[2], Wei CHENG[2], Bota CUI[6], Hong WEI[2,5]✉, Qinjin LI[1]✉

[1]*College of Animal Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China*
[2]*State Key Laboratory of Agricultural Microbiology, College of Animal Sciences and Technology, Huazhong Agricultural University, Wuhan, 430070, China*
[3]*Central Laboratory, Clinical Medicine Scientific and Technical Innovation Park, Shanghai Tenth People's Hospital, Tongji University, Shanghai, 200072, China*
[4]*Precision Medicine Institute, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510080, China*
[5]*Yu-Yue Pathology Scientific Research Center, Chongqing 401329, China*
[6]*Medical Center for Digestive Diseases, The Second Affiliated Hospital of Nanjing Medical University, Nanjing, 210011, China*

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Abstract: Human flora-associated (HFA) mice are often used to simulate the structure of human intestinal microbiota and to study the causal relationship between diseases and gut microbiota. However, several factors affect the colonization efficiency of human microbiota in germ-free (GF) mice, and the differential effects of gavage and lower gut transplantation on colonization are still unclear. In this study, we explored the reproducibility of recipient-to-donor gut microbiota community structure and function under different transplantation routes and the differences in microbial colonization between recipients via gavage (GT_mice group) and the lower gut (LGT_mice group). High-throughput sequencing of the metagenome was performed on the feces of each subject, and the composition of microbiome of each group was analyzed. As expected, the introduction of human fecal microbiota into GF mice via lower gut transplantation had a high transfer efficiency, which was evident from the similar species community structure to the donor (Adonis R^2, LGT_mice group – Donor group = 0.71396; GT_mice group – Donor group = 0.774095) and the higher bacterial colonization rate. The findings provide unique insights into improving the accuracy of constructing humanized microbiota transplantation models, aiding our understanding of the relationships between the human gut microbiota and disease.

Key words: Fecal microbiota transplantation; Germ-free mice; Lower gut; Gavage; Metagenome

1 Introduction

The composition of the human gut microbiota is affected both by the environment and genetics. In turn, biochemical homeostasis is regulated by complex interactions between the gut microbiota and the innate immune system (Benson et al., 2010; Li and Hotamisligil, 2010; Vijay-Kumar et al., 2010; Wos-Oxley et al.,

✉ Qinjin LI, liqinjin0809@163.com
✉ Hong WEI, weihong63528@163.com
Qinjin LI, https://orcid.org/ 0009-0005-0924-5113
Hong WEI, https://orcid.org/0000-0001-6069-1067

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2012). The gut microbiota is critical for regulating host physiology via influencing metabolism and immunity (Cebra, 1999; Backhed et al., 2004). Although numerous studies have offered preliminary support for a link between host pathophysiology and gut microbiota composition (Ley et al., 2006; Turnbaugh et al., 2008; Wang et al., 2012), it has been challenging to prove that specific disease-associated gut microbes are pathogens. Besides, sampling challenges, ethical concerns and the enormous inter-individual variations in microbial composition limit the study of innate and modified *in situ* human gut microbial populations (Marteau et al., 2001; Pang et al., 2007; Becker et al., 2011). To demonstrate the causative effect of human intestinal microbiota on the phenotype, many studies have transplanted gut microbiota into germ-free (GF) animals to attempt to transfer the host phenotype (Le Roy et al., 2013; Chassaing et al., 2015; Nagao-Kitamoto et al., 2016; Le Roy et al., 2018; Li and Wei, 2019). In addition, numerous studies have shown that certain phenotypic or metabolic profiles can be transmitted by transplanting human fecal microbial communities into GF animals (Ley, et al., 2006; Turnbaugh et al., 2006).

Owing to their commercial availability, high reproductive rate, and well-characterized and controllable anatomical and genetic background, mice are the most widely used laboratory animal species. Various studies using microbiota transplantation in GF mouse models have reported variations in the colonization rate of human donor gut microbiota in the gastrointestinal tract of GF mice after transplantation (Turnbaugh et al., 2009; Chung et al., 2012; Wos-Oxley, et al., 2012). Strong selection pressure from the host's genetics, or the applied cage and isolator are likely among the many factors that lead to this huge variance in colonization (Rawls et al., 2006; Campbell et al., 2012; Arrieta et al., 2016). Also, the microbiota of human flora-associated (HFA) mice and donors differ due to genetic and nutritional variations, and the ability to "recapture" the donor microbiota has only been partially successful (Arrieta, et al., 2016). Because of their simplicity of collection and the lack of significant ethical problems, human feces has been the primary transplantation material in the majority of studies on microbiota transplantation (Li et al., 2016; Kumar et al., 2017). According to earlier studies, the fecal community, which is derived from the large intestinal community rather than the small intestinal community, has a bulk of microbial species with a diversity of functions (Looft et al., 2014; Li et al., 2020). Compared to the hindgut, the small intestine has a shorter transit time, lower pH, and higher concentrations of oxygen and antimicrobials, making it a demanding microenvironment for microbial life (Donaldson et al., 2016; Martinez-Guryn et al., 2019). The conditions created by gastric acid also affect the ability of bacteria to survive (Ramai et al., 2019). Given its simplicity, the majority of investigations to date have used gavage to transplant human microbiota into GF mice (Zhou et al., 2019; Fouladi et al., 2020). This raises the possibility that the stomach and small intestine environments may destroy some of the bacteria that pass through after gavage. Therefore, we hypothesized that lower gut transplantation would more effectively replicate the donor microbiota.

The efficiency of donor bacterial colonization in recipient mice is an important indicator of the suitability of the animal model. Despite numerous studies on HFA mice, it is still unknown what range of human bacterial genera and species can be successfully established in mice via different delivery routes, and differences in the similarity between their microbes and human donor gut microbes are also yet to be explored. This study attempts to fill this knowledge gap by providing a detailed map of how human fecal microbial communities change when they are inoculated into the gut of GF mice via different delivery routes, offering guidance for the selection of accurate HFA mice models.

2 Materials and methods

2.1 Experimental animals and treatments

Eight-week-old GF C57BL/6 male mice were acquired from the Germ-Free Animal Platform of Huazhong Agricultural University. The mice were housed in an isolator with sterile conditions (temperature, (25±2) °C; relative humidity, 45%–60%; photoperiod, 12 h/d; light hours, 06:30–18:30), and had free access to sterile food and water. According to the experimental strategy, C57BL/6J GF mice were split into two groups: lower gut transplantation group (LGT_mice, n=9) and gavage transplantation group (GT_mice, n=10). All experiments were performed in accordance with the Huazhong Agricultural University of Health Guide for the Care and Use of Laboratory Animals. Prior to the end of the experiment, mouse fecal samples were collected in isolators,

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passed out through isolators, and transported on ice to refrigerators set at −80 °C. The samples were then kept on dry ice for metagenomic sequencing.

2.2 Human–donor bacteria inoculation

Healthy donor fecal suspensions were obtained from the Chinese fmtBank (Nanjing, China). In short, the methodology for microbiota preparation involves the use of an automated microbiota purification system, followed by centrifugation and three cycles of resuspension within a dedicated and specifically designed laboratory space that adheres to good manufacturing practice standards (Cui et al., 2016; Zhang et al., 2019; Zhang et al., 2020). To ensure homogeneity under anaerobic conditions, a suspension of feces was prepared by mixing 500 mL of saline with every 100 g of feces. This fecal suspension was then processed automatically through scheduled microfiltration within the machine. The fecal microbiota suspension after microfiltration was carried out for centrifugation for 3 min at a speed of 700*g* (TDZ5-WS, XIANGZHI, Changsha, China). The obtained supernatant was aspirated. Subsequently, resuspension was carried out by adding a 9 g/L saline glycerol solution (concentrated at 15% in glycerol) to the microbiota precipitation, resulting in a final bacterial concentration of 1×10^9 CFU/mL.

The bacterial suspension was aseptically transferred into the GF isolator using a validated sterilization protocol. In short, the outer port of the isolator was opened and a sterile-sealed microcentrifuge tube containing the bacterial suspension was introduced into the transfer chamber. The tube surface was disinfected with peracetic acid, followed by sealing of the outer port and a 1-hour sterilization period to eliminate potential contaminants. Subsequently, the inner port was opened under strict aseptic conditions and the microcentrifuge tube was retrieved for downstream procedures (e.g., gavage or enema).

GF C57BL/6 mice were inoculated with a healthy human bacterial solution via gavage and lower gut transplantation (**Fig. 1**). Recipient mice were gavaged with 100 μL bacterial suspension in a GF isolator, and proper esophageal insertion was confirmed via visual/gentle resistance checks to avoid perforation. After the completion of the gavage operation, mice were monitored for distress. To promote the intestinal evacuation of feces before inoculation, mice were fasted for 12 h. The syringe was connected to the enema hose and the bacterial solution was drawn up. The anuses of mice were then lubricated by applying glycerol. After inserting approximately 5 cm of the enema hose slowly into the anal opening in the same direction, 100 μL of the bacterial solution was injected. Administration was performed slowly to avoid reflux or trauma. The mice were then lifted up by the tail for approximately 30 s to 1 min to prevent the bacterial solution from flowing out. The animals were monitored for rectal prolapse, bleeding or behavioral distress to ensure the quality control of experimental procedures and avoid the negative effects of handling. The mice were given enemas every other day a total of three times. The mice in the GT_mice group were administered 100 μL via gavage every other day for a total of three times. Four weeks after colonization, all mice were euthanized with CO_2, followed by cervical dislocation.

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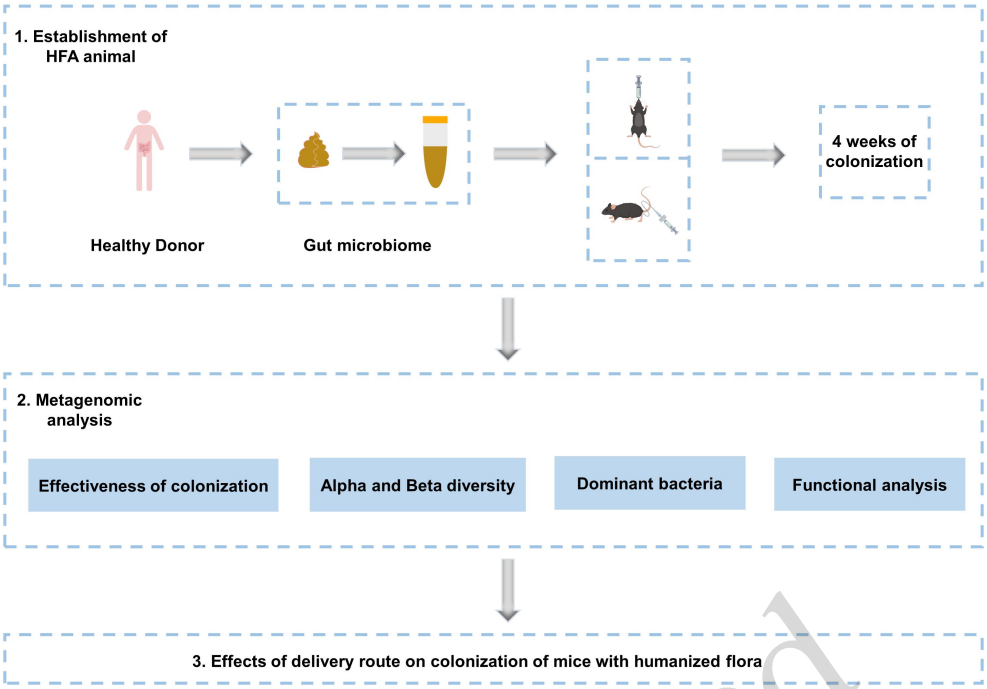

Fig. 1 Study design. The reproducibility of recipient-to-donor microbial community structure and function in fecal microbiota transplantation studies under different delivery routes analyzed by metagenomics. The mice in the image are sourced from Figdraw (https://www.figdraw.com/).

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2.3 Metagenomic sequencing and analysis

Mouse fecal samples were collected in a clean cage inside the isolator and placed on dry ice for DNA extraction (Cetyltrimethylammonium Bromide, CTAB) and sequencing. The input material for the DNA sample preparation was 1 μg of total DNA per sample (OD value range: 1.8–2.0). Sequencing libraries were generated using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's instructions, and index codes were added to match the sequences to each sample. In short, the DNA sample was first lysed into 350 bp-sized fragments through sonication; then, these fragments were end-polished, A-tailed and ligated with the full-length adaptor for Illumina sequencing, followed by further PCR amplification. Finally, the PCR products were purified using the AMPure XP system, and the size distribution of the libraries was analyzed using an Agilent 2100 Bioanalyzer and quantified using real-time PCR. sOn a cBot Cluster Generation System, index-coded samples were clustered in accordance with the manufacturer's recommendations. Library preparations were sequenced on an Illumina HiSeq platform after cluster creation, and paired-end reads were produced. To determine the species abundance data for further investigation, filtering host reads were compared with the Chocophlan (version MPa_V30_Chocophlan_201901) database using the MetaPhlAn3 (version 3.0.7) software. To obtain the UniRef database of gene family annotations and pathway abundance information from the gene family-based MetaCyc database (https://metacyc.org/), the pan-gene database, ChocoPhlAn (version mpa_v30_CHOCOPhlAn_201901) was compared to the Uniref90 (version 201901) database using the HUMAnN (version v3.0.0. alpha.4) software. MetaCyc is a comprehensive reference database of metabolic pathways and enzymes in all domains of life (Caspi et al., 2020). Beta diversity was visualized using principal coordinate analysis (PCoA) or Principal Component Analysis (PCA), and the data were imported into RStudio (4.1.1) and plotted using ggplot2 (3.3.5) for the mapping of PCoA, alpha diversity (Shannon's Diversity Index) and heatmap. In addition, the significance between groups and their degree of variance was examined using permutational multivariate analysis of variance (PERMANOVA, Adonis analysis). To analyze the degree of differences between groups by delivery route and their statistical significance using a permutation test, total variance was decomposed using a distance matrix based on Bray-Curtis distance. Wekemo Bioincloud (https://www.bioincloud.tech) was used to complete the PCA, Venn diagram and linear discriminant analysis effect size (LEfSe, (Segata et al., 2011)). P-values were corrected for multiple testing using the BH method.

2.4 Analysis of colonization rate

The number of donor genera or species was normalized to 100%, and colonization efficiency was evaluated by calculating the number of overlapping genera or species between the donor and the two groups of recipients.

2.5 Statistical analysis

The Kruskal-Wallis test was used to examine differences among three groups in the alpha diversity indices (Shannon's Diversity Index). Data was analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Mann-Whitney U Test was employed for statistical analysis between the two groups. All data was represented as mean ± SEM. A *P*-value of ≤0.05 was considered statistically significant.

3 RESULTS

3.1 The efficiency of microbial colonization is significantly affected by the delivery route

To explore the effect of delivery route on fecal microbiota transplantation (FMT) colonization efficiency, we examined the number of genera and species in the donor and those that were transferred to the recipient. As shown in **Fig. 2a**, at the genus level, we detected 44 genera in the donors, of which 24 and 21 genera were colonized in the LGT_mice group (colonization rate: 54.55%, **Fig. 2b**) and GT_mice group (colonization rate: 47.73%, **Fig. 2b**), respectively. In the donor, we found 98 species at the species level (**Fig. 2d**), of which 41 and 35 species could colonize in the LGT_mice group (colonization rate: 41.84%, **Fig. 2e**) and GT_mice group (colonization rate: 35.71%, **Fig. 2e**), respectively. The colonization rate of genera and species showed that lower gut transplantation was significantly better than the gavage transplantation ($P<0.0001$ for genera and $p < 0.001$ for species) (**Figs. 2c and 2f**). In conclusion, lower gut transplantation was more reproducible for donor genera and species compared to gavage transplantation.

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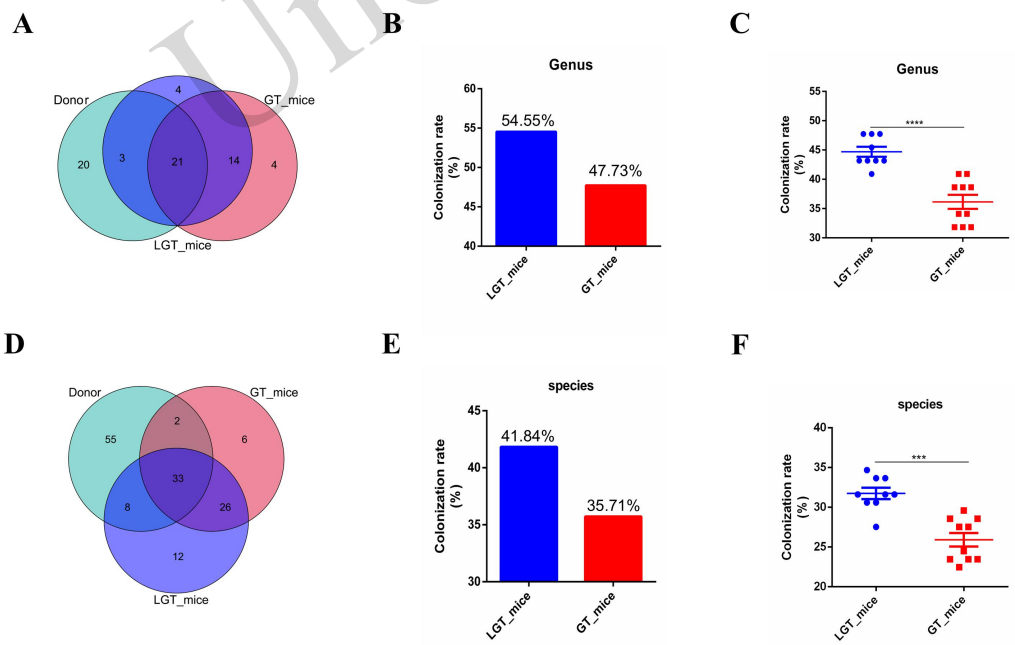

Fig. 2 Analysis of donor bacterial colonization rate in recipient mice. (A) Microorganisms that are unique and shared at the genus level; (B) Analysis of the overall colonization rate of genera in each group of recipient mice; (C) Analysis of differences in bacterial colonization of genus among groups of recipient mice; (D) Microorganisms that are unique and shared at the species level; (E) Analysis of the overall colonization rate of species in each group of recipient mice; (F) Analysis of differences in bacterial colonization of species among groups of recipient mice (Lower gut transplantation group, LGT_mice, *n*=9; Gavage transplantation group, GT_mice, *n*=10). *** $p < 0.001$, **** $p < 0.0001$.

3.2 The alpha diversity of fecal bacteria in the LGT_mice group was higher than that in the GT_mice group

Next, we analyzed the differences in the number of fecal genera and species and the alpha diversity between the GT_mice and LGT_mice groups (**Fig. 3a-d**). The number of genera and species in the LGT_mice group was significantly higher than that in the GT_mice group (genera, p = 0.03; species, p = 0.019), and the number of genera and species in the donor group was also significantly higher than that in the GT_mice group (genera, p = 0.002; species, p = 0.002). Meanwhile, there was no significant difference in the number of genera and species between the donor and LGT_mice groups. The Shannon index was used to evaluate the alpha diversity of fecal genera and species, and the results showed no significant difference between the GT_mice and LGT_mice groups at the genus level (p = 0.627) (Shannon index**, Fig. 3c**). The alpha diversity of fecal bacteria was significantly lower in the GT_mice group compared to the donor group (p = 0.011). At the species level (Shannon index**, Fig. 3d**), compared to the GT_mice group, both the donor and LGT_mice groups had significantly higher alpha diversity levels (Donor-GT _mice group, p = 0.002; LGT_mice group-GT_mice group, p = 0.019), while there was no significant difference in alpha diversity between the donor and LGT_mice groups (p = 0.449). These results suggest that the alpha diversity of fecal bacteria in the LGT_mice group was higher than that in the GT_mice group.

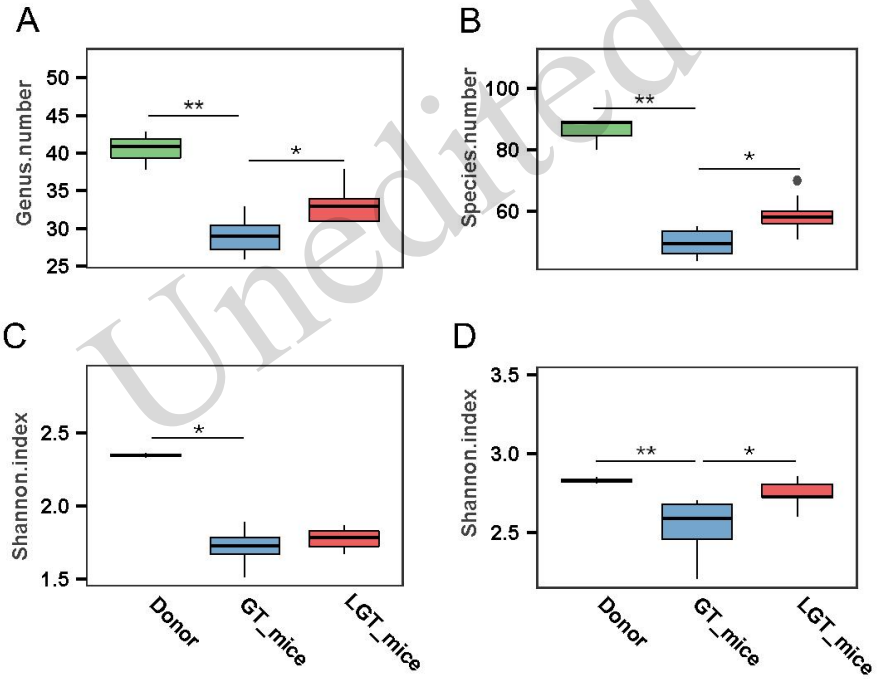

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Fig. 3 Analysis of Alpha diversity in GT_mice and LGT_mice groups. (A) Number of genera; (B) Number of species; (C) Alpha diversity of genera; (D) Alpha diversity of species (LGT_mice, *n*=9; GT_mice, *n*=10). * p < 0.05, ** p < 0.01.

3.3 Lower gut transplantation has better donor microbial community reproducibility

Principal Component Analysis (PCA) descending sorting was used to analyze the beta diversity of the community structure in the donor, LGT_mice, and GT_mice groups. The results showed that, at the species level, the communities of both recipient groups were significantly separated from the donor community (**Fig. 4a)** and the communities of GT_mice group was separated from the LGT_mice community (**Fig. 4b)**. The Bray-Curtis distance between the LGT_mice group and the donor was smaller than the Bray-Curtis distance between the GT_mice group and the donor **(Fig. 4c, species)**. Correlation analysis of the Bray-Curtis distance revealed that the distance between LGT_mice group and the donor group was smaller than the distance between

the GT_mice group and the donor group, as depicted in the heatmap **(Fig. 4d, species)**. The quantification of group differences by the ratio of group variance to total variance variation (R^2) results showed that at the species level (R^2, which is the ratio of grouping variance to total variance, indicates the degree of explanation of differences in samples by different groupings, larger R^2 indicates higher explanation of differences by grouping factors), the difference between the LGT_mice group and the donor group was smaller than the difference between the GT_mice group and the donor group **(Table 1)**. These results indicate that the community structure after lower gut transplantation is more similar to that of the donor than that after the gavage transplantation approach.

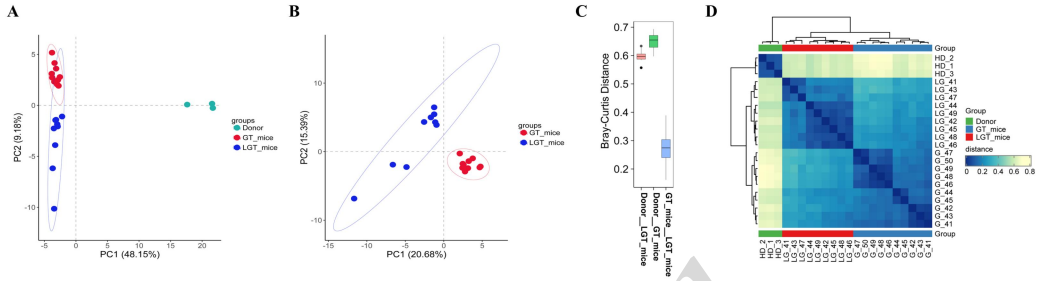

Fig. 4 Microbiota community structure analysis. (A) PCA analysis of Donor group, LGT_mice group and GT_mice group; (B) PCA of LGT_mice group and GT_mice group; (C) The Bray-Curtis distance of species among groups; (D) Heatmap of groups based on Bray-Curtis distance. (LGT_mice, *n*=9; GT_mice, *n*=10. Donor group: HD_1-HD_3; LGT_mice group: LG_41-LG_49; GT_mice group: G_41-G_50).

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Table 1 Comparison of the global bacterial communities between recipient models and the donor using Adonis[a]

Group	Sums Of Sqs	F. Model	R^2	Pr (>*F*)
Donor-LGTmice	1.721156	24.96009	0.71396	0.002997
Donor-GTmice	2.01522	37.69303	0.774095	0.006993
LGTmice-GTmice	0.162124	2.159669	0.11272	0.084915

[a]R^2, Variation(R^2), which is the ratio of grouping variance to total variance, indicates the degree of explanation of differences in samples by different groupings. larger R^2 indicates higher explanation of differences by grouping factors. Pr (>*F*), which represents significant *P*-value, and $P<0.05$ indicates significant differences.

3.4 Analysis of bacterial markers in recipients under different delivery routes

To further analyze the bacterial markers between the two groups of recipients, the LEfSe was used to identify the significantly different phylum, genus and species markers between the donor, GT_mice and LGT_mice groups. As shown in **Fig. 5a**, at the phylum level (LDA > 4), Firmicutes and Actinobacteria were enriched in the donor group **(Fig. S1a)**. Bacteroidetes and Proteobacteria were enriched in the LGT_mice group **(Fig. S1a)**. Verrucomicrobia was enriched in the GT_mice group **(Fig. S1a)**. At the genus level (**Fig 5b, S1b**) (LDA > 2), 29 genera, including *Prevotella*, were significantly enriched in the donor group. Fifteen genera, including *Bacteroides*, were significantly enriched in the LGT_mice group. Fourteen genera, including *Alistipes*, were enriched in the GT_mice group. At the species level (**Fig. 5c, S1c**) (LDA > 3), 33 species, including *Megamonas funiformis*, were significantly enriched in the donor group. Ten species, including *Bacteroides ovatus*, were significantly enriched in the LGT_mice group, and 12 species, including *Bacteroides cellulosilyticus*, were enriched in the GT_mice group. The above results suggest that different delivery routes result in significantly different bacterial markers.

Furthermore, the bacterial colonization characteristics were distinct under different transplantation routes. The Venn plot shows the number of species that differed between the donor and two groups of recipients **(Fig. 3d)**. *Bifidobacterium bifidum* and *Bacteroides plebeius* were detectable in fecal samples from the donor and GT_mice groups, whereas *Prevotella copri*, *Prevotella sp AM42_24*, *Eubacterium eligens*, *Blautia obeum*,

Ruminococcus torques, *Dorea longicatena*, *Megamonas funiformis*, and *Megamonas hypermegale* were detectable in fecal samples from the donor and LGT_mice groups. We also found 44 species present in the recipient group but not in the donors, of which six species were present only in the GT_mice group and 12 species in the LGT_mice group **(Table S1)**, indicating the differential bacterial colonization in recipients under different delivery routes.

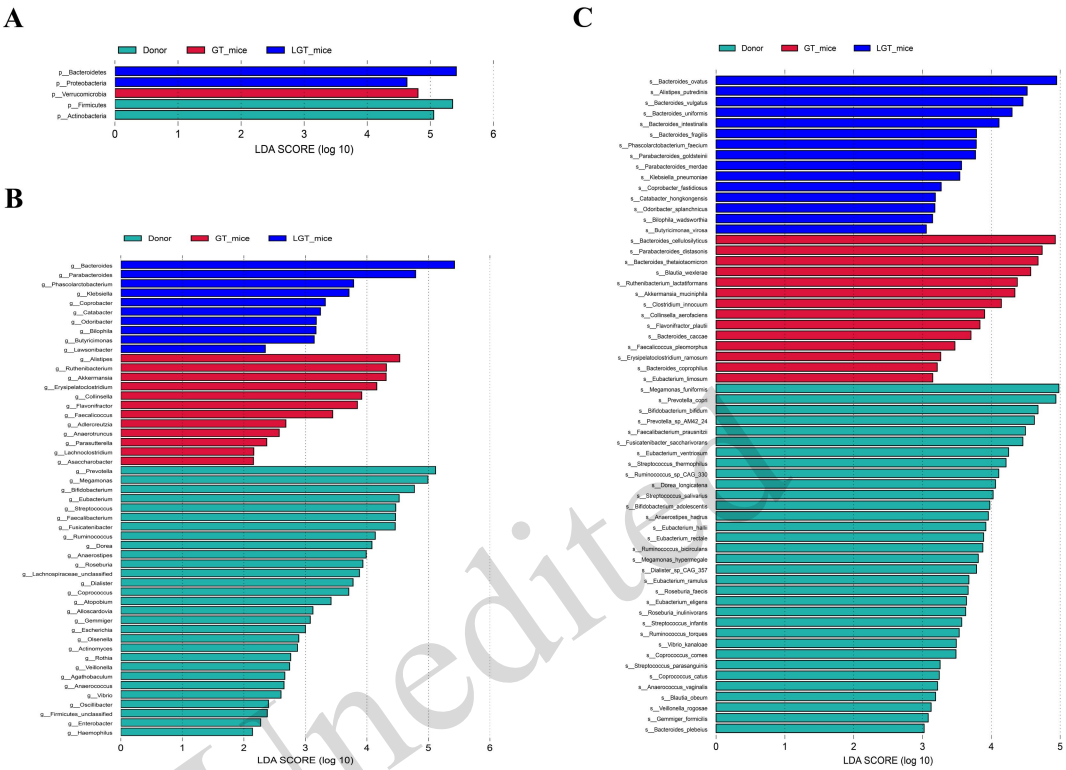

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Fig. 5 Analysis of bacterial markers in recipients under different delivery routes. (A) Difference analysis at the phylum level, LDA > 4; (B) Difference analysis at the genus level, LDA > 2; (C) Difference analysis at the species level, LDA > 3 (LGT_mice, *n*=9; GT_mice, *n*=10).

3.5 Analysis of the microbial metabolic functions of each group

Based on the MetaCyc database, sequencing data was annotated with the identified metabolic pathways. There were 344 shared metabolic pathways between the donor and the two recipient groups, and the number of metabolic pathways specific to each group was seven, eight and seven in the donor, LGT_mice and GT_mice groups, respectively **(Fig. 6a)**. Beta diversity analysis showed that the functional structures of the donor were significantly different from those of the two recipient groups, while there was no significant difference between the functional structures of the LGT_mice and GT_mice groups (p = 0.2587) (**Fig. 6b, Table 2**). Compared to the LGT_mice group, the donor and GT_mice groups had a greater variety of metabolic pathways (**Fig. 6c**). However, the alpha diversity of functions was not significantly different between the LGT_mice and GT_mice groups, and both were higher than that of the donor group (Shannon index, **Fig. 6d**). Adonis analysis showed that the microbial metabolic pathway function of the LGT_mice group was substantially similar to that of the donor group (donor-GT_mice, R^2 = 0.829455; donor-LGT_mice, R^2 = 0.704776). More importantly, L-isoleucine biosynthesis I (from the threonine pathway) and L-valine biosynthesis pathways were the among the top five dominant metabolic pathways in the donor and LGT_mice groups **(Table S2)**. In contrast, the top five dominant metabolic pathways of the GT_mice group were different from those of the donor group **(Table S2)**. These results suggest that the community function of microbiota after lower gut transplantation is more similar to that of the donor organism.

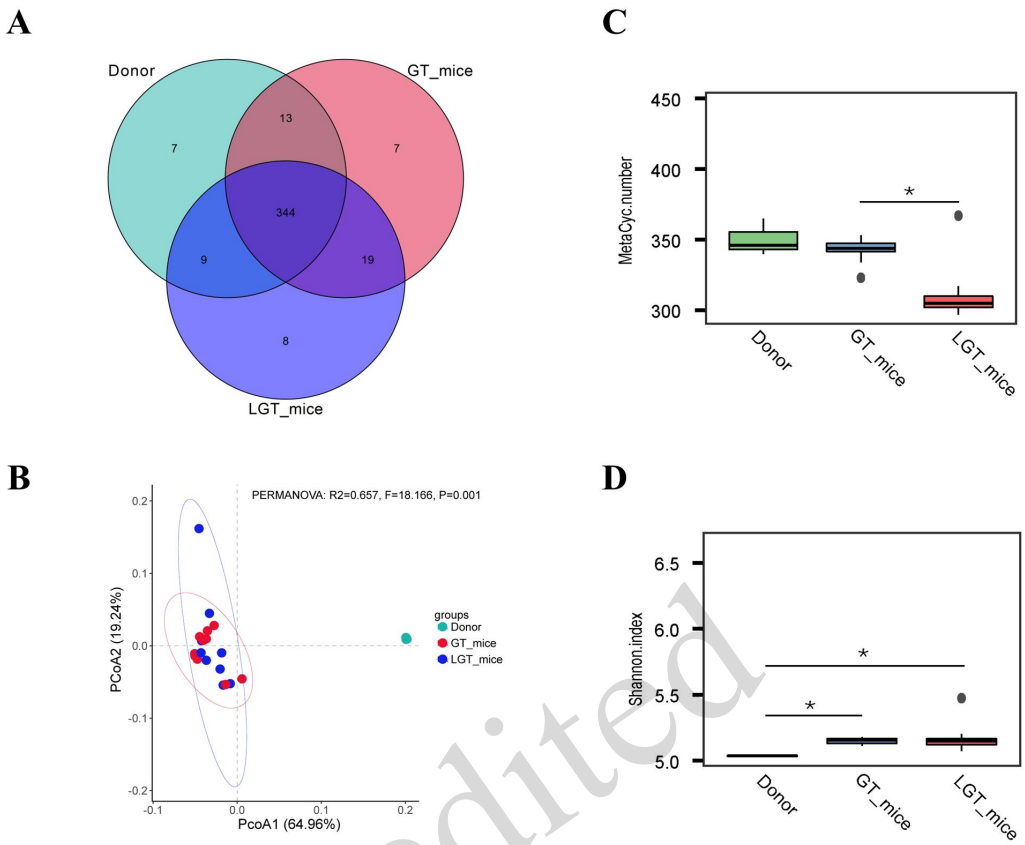

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Fig. 6 Analysis of microbial metabolic functions of each group. (A) Venn diagram showing the common metabolic pathways and unique metabolic pathways among donor and recipients; (B) Comparison of metabolic pathway numbers among donor and recipients; (C) Comparison of metabolic alpha diversity analysis among donor and recipients; (D) PCoA of metabolic pathway among three groups; (E) Dominant metabolic pathway among three groups (LGT_mice, *n*=9; GT_mice, *n*=10). * $p < 0.05$.

Table 2 Comparison of the metabolic pathway between recipients and the donor using Adonis

Group	Sums Of Sqs	F. Model	R^2	Pr(>F)
Donor-LGT_mice	0.12033317	23.87254	0.704776	0.002997
Donor-GT_mice	0.12881504	53.49928	0.829455	0.006993
LGT_mice-GT_mice	0.005665817	1.259338591	0.068969562	0.258741259

3.6 Functional differences in each group

To identify functional biomarkers of the MetaCyc pathways in each group, we performed a functional analysis to find differences among the three groups using LEfSe (LDA > 3). As shown in **Fig. 7**, the dominant metabolic pathways in the donor, GT_mice and LGT_mice groups included 43, such as L-arginine biosynthesis IV archaebacteria, 16, such as L-histidine degradation III, and 32, such as inosine-5-phosphate biosynthesis I, metabolic pathways, respectively. These results suggest that different delivery routes result in significantly differences in the dominant bacterial functions between recipients.

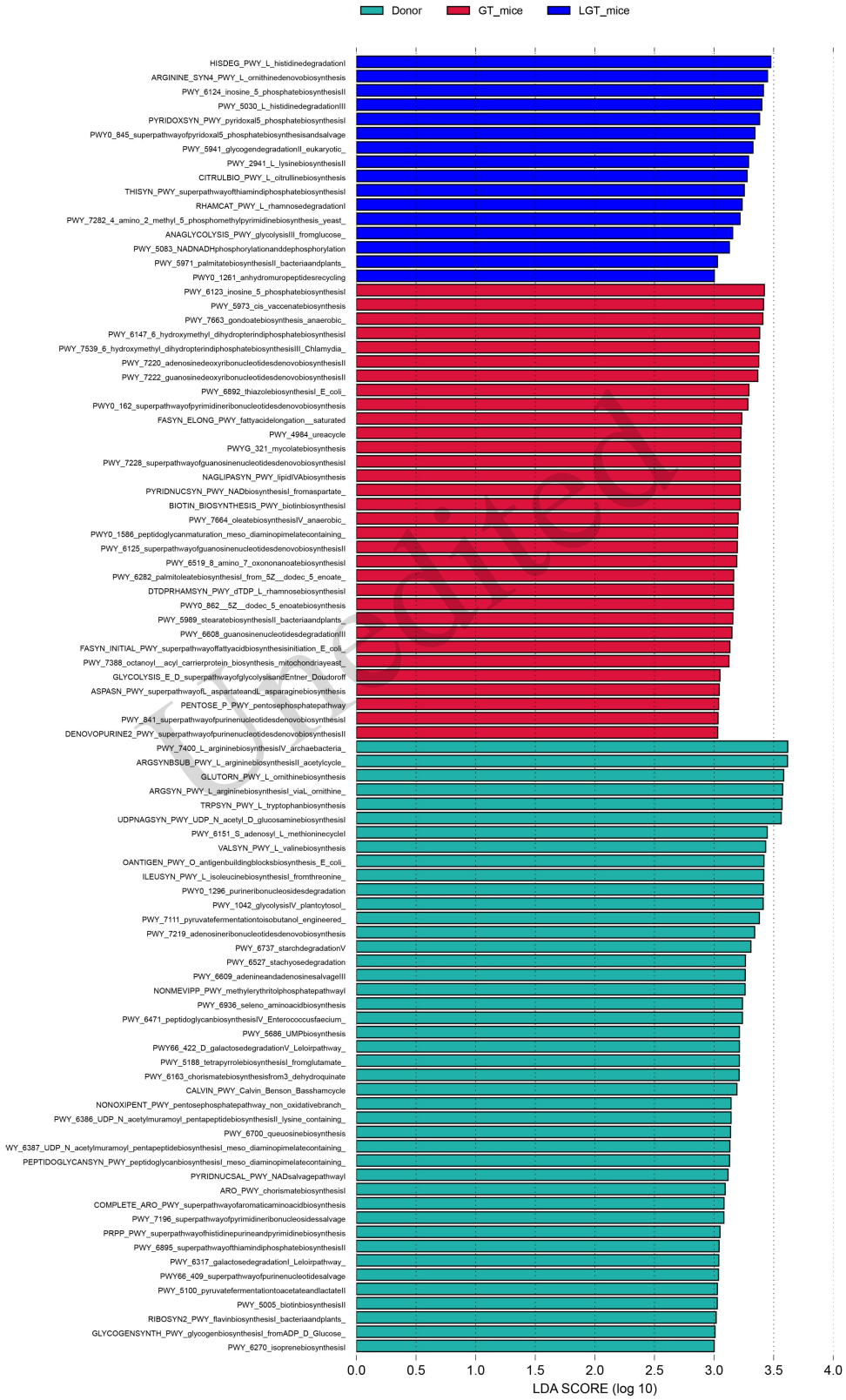

Fig. 7 Functional differences in each group. LEfSe analysis of differential metabolic pathway, LDA > 3.

4 Discussion

Research on microbiota is currently shifting from relevance to causality, and mechanistic investigations show how members of the complex microbial community affect the physiology of the host (Fritz et al., 2013). One of the most desirable models for simulating human intestinal flora composition, which may be utilized to investigate the effects of a disease-associated gut microbiota on the host, involves GF mice that are colonized with human fecal flora. The HFA mouse model used in most studies was constructed by gavage (Zhou, et al., 2019; Fouladi, et al., 2020). However, the small intestine environment and gastric acid make it difficult for microorganisms to survive (Donaldson, et al., 2016; Martinez-Guryn, et al., 2019; Ramai, et al., 2019). In this study, human donor microorganisms were implanted into GF mice via two different transplantation routes. We discovered that the introduction of human fecal microbiota into GF mice by lower gut transplantation had a high transfer efficiency, which could be demonstrated by the similar community structure and dominant metabolic pathways to the donor and the higher bacterial colonization rate and diversity. Our results also showed that the two groups of HFA mice had different bacterial markers and functions as a result of the transplantation routes. Interestingly, consistent with other studies (Turnbaugh, et al., 2009; Wos-Oxley, et al., 2012), members of Bacteroidetes colonized GF mice more successfully than those of Firmicutes, indicating a general selection of the mouse intestine for particular human microbiota members.

The gut microbiota profile of HFA mice may not precisely match that of a human donor owing to genetic and dietary variations (Arrieta, et al., 2016). This is in line with our findings, that is, the metabolic pathways and community structures of the donor and recipient groups differ significantly. However, Silley (2009) pointed out that the major problem lies in the accurate understanding of a model's limitations, and if these are considered accordingly, the model can be helpful even if it does not perfectly represent the human gut microbiota (Silley, 2009). Compared to the large intestine, the mammalian foregut is more acidic, transitions more quickly, and contains stronger gradients of oxygen and antimicrobials (Donaldson, et al., 2016; Li, et al., 2020). Previous research has demonstrated that transplanting microbiota from the large intestine improves colonization of the recipient's large intestine (Li, et al., 2020). The large intestinal microbiota makes up a major proportion of the microbial species in the fecal community, while the small intestinal microbiota is somewhat underrepresented (Looft, et al., 2014; Li, et al., 2020). In the present study, human donor fecal bacteria transplanted via the lower gut had better transfer efficiency relative to that of human donor fecal bacteria passing through gastric acidic and small intestinal environments, as demonstrated by the similarity of community structure with the donor, the high colonization rate of genera and species, species alpha diversity, and the two identical dominant metabolic pathways.

The introduction of human fecal microbiota into GF mice resulted in distinct clustering patterns dependent on the delivery route. Microorganisms may colonize the small and large intestines after gavage dosing; *Alistipes* has been detected in both small intestine (Wang et al., 2020) and large intestine samples (Huang et al., 2020); *Anaerotruncus* has been found in the small intestine of mice (Huang, et al., 2020). In the present study, these two genera appeared to be genus markers in the GT_mice group. Apart from the colon, the small intestine is one of the areas where *Akkermansia*-like sequences (such as those from the phylum Verrucomicrobia and/or *Akkermansia* spp. sequences described in the literature) have been discovered (Geerlings et al., 2018), indicating that members of Verrucomicrobia colonize a variety of sites. In this work, Verrucomicrobia, *Akkermansia* and *Akkermansia muciniphila* were abundant as bacterial markers in the GT_mice group. In addition, some strictly anaerobic bacteria, such as *Bacteroides cellulosilyticus* (Robert et al., 2007), were identified as bacterial markers in the GT_mice group. This shows that the intestinal bacteria in mice in the gavage group had dispersed throughout the gastrointestinal tract. Complex non-digestible polysaccharides are largely fermented by community members in the hindgut, thus facilitating the dominance of fermentative polysaccharide-degrading anaerobes, especially those of *Bacteroidaceae* (Donaldson, et al., 2016). According to a previous study, recipients' large intestines prefer to reconstitute more saccharolytic anaerobes generated

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from exogenous large intestinal communities than their small intestines, such as Bacteroidetes, which are capable of breaking down indigestible carbohydrates (Li, et al., 2020). The feces of human donors consist mainly of large intestine microorganisms (Li, et al., 2020); hence, it is likely that the bacteria following lower gut transplantation primarily colonize in large intestine. In the present study, Bacteroidetes were considerably enriched in the LGT_mice group at the phylum level. At the genus and species level, bacteria such as *Bacteroides, Bacteroides ovatus*, *Bacteroides vulgatus*, and *Alistipes putredinis* were significantly enriched as LGT_mice group bacterial markers. Interestingly, consistent with the study by Fouladi et al. (Fouladi, et al., 2020), we found that six bacterial species, including *Enterococcus hirae*, only colonized the GT_mice group, while 12 bacterial species, including *Bacteroides intestinalis*, only colonized the LGT_mice group. Meanwhile, none of these bacteria were detected in the donor. These bacteria may have existed in the donor at levels below the metagenomic sequencing detection limits, but they may have multiplied after they were introduced into the GF gut (Fouladi, et al., 2020).

Clarifying the functional role of gut microbiota in the onset and maintenance of associated disease is crucial for the development of effective therapeutics (Fouladi, et al., 2020). Therefore, when using HFA mice for research, it is important to determine whether a particular microbe (which may be what we wish to examine) can colonize GF mice via the gavage route or the lower gut route. In the present study, we found that some bacteria were present in the donor and GT_mice groups, but not in the LGT_mice group, such as *Bacteroides plebeius*, which may be strains that grow in the presence of bile (Kitahara et al., 2005). Bile is synthesized by the liver and secreted into the duodenum to mediate physiological functions (Tanimizu et al., 2016), which provides a growth environment for *B. plebeius*, as evidenced by its bile tolerance (Kitahara, et al., 2005). Oral gavage administration may therefore to enable it to colonize the small intestine. Conversely, the introduction of bacterial consortia via lower gut transplantation might fail to deliver the inoculum to the proximal small intestine due to the unidirectional gastrointestinal peristalsis. This mechanical limitation could result in the absence of small intestine-colonizing bacteria in fecal samples from the LGT_mice group, potentially explaining why *B. plebeius* was only detected in GT_mice group. In addition, we discovered that some bacteria were present in the donor and LGT_mice groups, but not in the GT_mice group, such as *Dorea longicatena*, which may be related to the fact that these are obligate anaerobes (Taras et al., 2002). However, more research is needed to make better use of HFA mice because different microbes could only colonize the GT_mice or LGT_mice groups. Notably, several specific common species were identified in the two groups that were transplanted into GF mice from the donor sample. Future research employing human fecal microbiota and GF mice may benefit from using these taxa because they may have clinical implications (Fouladi, et al., 2020). In addition, the number of genera and species colonized via lower gut transplantation was not significantly different from that of the donor, suggesting that lower gut transplantation is preferable when GF mice are used for overall microbial community transfer.

The metabolism of simple carbohydrates and amino acids is primarily carried out by small intestinal bacteria (Zhao et al., 2015; Donaldson, et al., 2016; Martinez-Guryn, et al., 2019). The enzyme involved in guanosine ribonucleotide *de novo* biosynthesis (PWY-7221) can use glutamine or ammonia as substrates (Oliver et al., 2014). In the present study, PWY-7221 was the dominant metabolic pathway in the GT_mice group. Free citrulline is formed mainly by the catabolism of amino acids in the small intestine (Windmueller and Spaeth, 1981). The superpathway of L-citrulline metabolism (PWY-5004) was only found in the GT_mice group, suggesting that microorganisms may have colonized the intestine through gavage to perform this function. Additionally, we discovered bacterial metabolic pathway markers in the GT_mice group, including the superpathway of glycolysis and the Entner-Doudoroff pathway (GLYCOLYSIS-E-D) associated with glycolysis (Entner and Doudoroff, 1952), which may be due to bacteria reaching the large intestine in the gavage group. Complex polysaccharide fermentation is favored by the large-intestinal microbial community (Zhao, et al., 2015; Donaldson, et al., 2016; Martinez-Guryn, et al., 2019). Bacterial metabolic pathway markers in the LGT_mice group included glycogen degradation II (PWY-5941), which is involved in glycogen

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metabolism (Francois and Parrou, 2001). Furthermore, we discovered that metabolic functions were present only in the LGT_mice group, such as the reductive acetyl coenzyme A pathway I (homoacetogenic bacteria) (CODH-PWY), which has been documented in several types of organisms and is best studied in homoacetogenic bacteria (strict anaerobes that can synthesize acetate from H_2 and CO_2) (Kathrin Jansen, 1982; Ljungdahl, 1986; Diekert and Wohlfarth, 1994). Since these pathways are exclusive to anaerobiosis and may not be able to develop in the small intestine environment, they were only present in the LGT_mice group. Further research is needed to clarify these functional distinctions.

There are certain limitations of our study. First, germ-free mice were inoculated with human microorganisms to emulate the human microbiota, and the replication of the human microbiota was constrained due to the differences in gastrointestinal and physiology between humans and mice (Arrieta, et al., 2016; Fouladi, et al., 2020). Second, the fidelity of human microbiota reconstruction in GF mice is limited by interspecies divergence in gastrointestinal physiology (e.g., immune responses, metabolic networks), which, despite these inherent constraints, may alter microbial functionality and further drive functional adaptations of the transplanted microbiota. Future investigations should utilize animal models with a closer anatomical resemblance to the human gastrointestinal tract.

5 Conclusions

The current study demonstrated that different human gut microbial community delivery routes can be transferred to recipient mice with differing degrees of efficiency, offering adding to the evidence that the delivery method may significantly affect the makeup of the human gut microbiota. To this end, our experimental results recommend the use of GF mice to investigate the human intestinal communities via lower gut transplantation. Notably, the differences in colonization under different transplantation routes indicate the selection of microorganisms by the recipient host under different transplantation routes, which facilitates the use of the corresponding transplantation method when examining specific microorganisms. Overall, this work provides new perspectives in how to improve the accuracy of constructing humanized models of gut microbiota transplantation.

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Data availability statement

The datasets generated and analyzed during the current study are available in the NCBI repository, Submission ID: SUB14159019, BioProject: PRJNA1066070.

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Author contributions

Yapeng YANG, and Hong WEI designed the experiment. Qinjin LI, Yapeng YANG, Xiang TAN, Lifeng LIANG, Yuqing WANG, Zeyue ZHANG, Jinhui HE, Hang ZHANG, Zhifeng WU, Miaomiao DONG, Shuaifei FENG, and Wei CHENG performed the animal trials, sample collection, and data analysis. Healthy donor bacterial solution was prepared by Bota CUI. Yapeng YANG drafted the manuscript. Qinjin LI and Hong Wei revised the manuscript. All authors contributed to the article and approved the submitted version.

Compliance with ethics guidelines

Yapeng YANG, Xiang TAN, Zeyue ZHANG, Lifeng LIANG, Zhifeng WU, Jinhui HE, Yuqing WANG, Miaomiao DONG, Jixia ZHENG, Hang ZHANG, Shuaifei FENG, Wei CHENG, Bota CUI, Hong WEI, and Qinjin LI declare that they have no conflict of interest. All experimental methods were performed according to the Huazhong Agricultural University of Health Guide for the Care and Use of Laboratory Animals. The animal experimental ethics number for this study is HZAUMO-2023-0027.

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Supplementary information

Fig. S1; Tables S1 and S2

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