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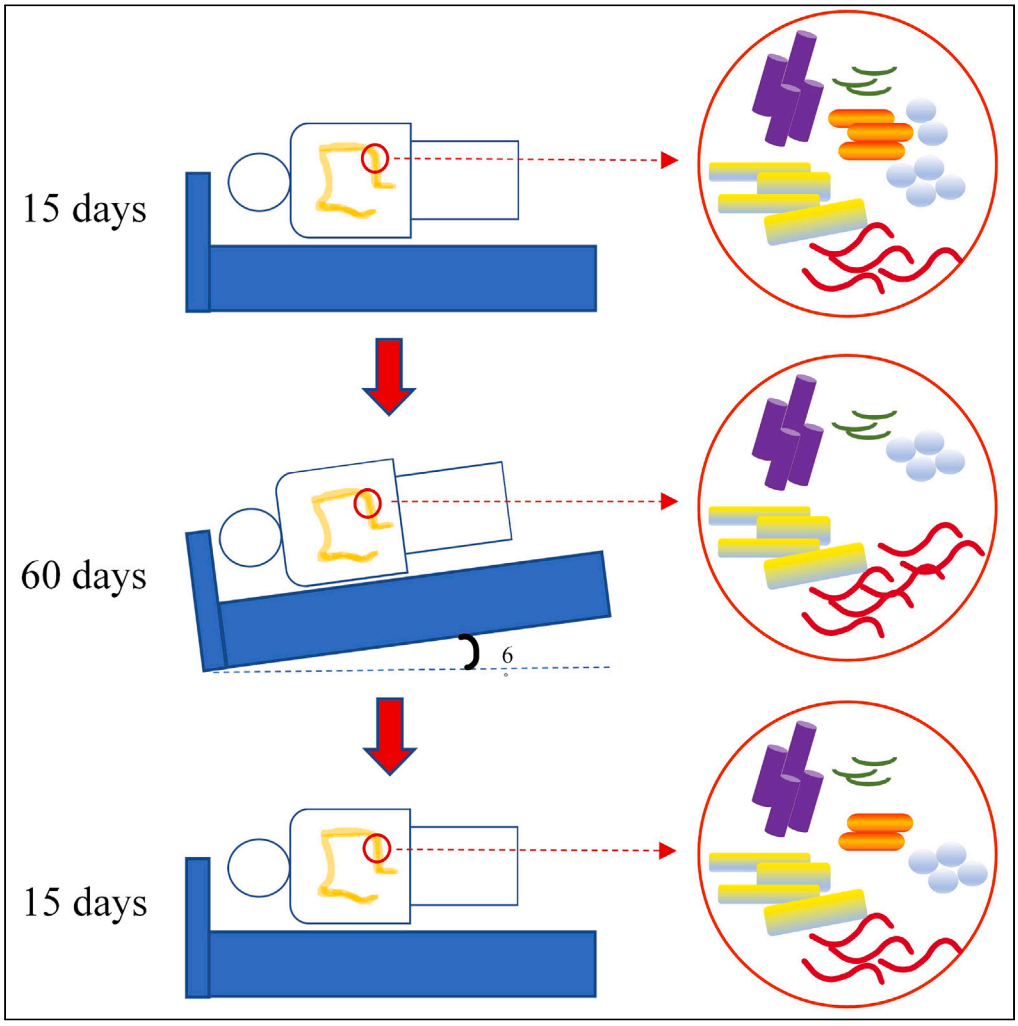


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## Highlights

The composition and function of gut microbiota were affected by HDBR

Resistance and virulence genes of gut microbiota were affected by HDBR

HDBR was a simulation of how spaceflight affects the human gut microbiota

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# Effects of 60 days of 6° head-down bed rest on the composition and function of the human gut microbiota

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## SUMMARY

**Spaceflight is rigorous and dangerous environment which can negatively affect astronauts' health and the entire mission. The 60 days of 6° head-down bed rest (HDBR) experiment provided us with an opportunity to trace the change of gut microbiota under simulated microgravity. The gut microbiota of volunteers was analyzed and characterized by 16S rRNA gene sequencing and metagenomic sequencing. Our results showed that the composition and function of the volunteers' gut microbiota were markedly was affected by 60 days of 6° HDBR. We further confirmed the species and diversity fluctuations. Resistance and virulence genes in the gut microbiota were also affected by 60 days of 6° HDBR, but the species attributions remained stable. The human gut microbiota affected by 60 days of 6° HDBR which was partially consistent with the effect of spaceflight, this implied that HDBR was a simulation of how spaceflight affects the human gut microbiota.**

## INTRODUCTION

The human gut microbiota is a large and diverse microbial community and the interaction with humans is important in human physiology.[1–5] The gut microbiota are sensitive to endogenous and environmental factors,[6] specific human experiences and environments can enormously affect the gut microbiota.[7–11] Spaceflight was rigorous and dangerous environment which can negatively affect astronauts' health and the entire mission. The NASA Twins study has reported the impact of long-term spaceflight on the human microbiome.[12] Our previous study showed that the composition and function of the human gut microbiota were enormously affected by short-term spaceflight.[13]

Spaceflight is limited and rare, in order to disclose the effect of spaceflight on human physiology, HDBR was used as a microgravity simulation model which can well simulate the specific environment of confinement, stress, and the altered physical condition of microgravity.[14] HDBR is characterized by immobilization, inactivity, and confinement, which induce upward fluid shift, unloading the body's upright weight, absence of work against gravity, reduced energy requirements and reduction in overall sensory stimulation. There are some studies have illustrated that the effect of HDBR on immune, cardiovascular, muscular, and skeletal systems;[15–17] however, no study focuses on the effect of HDBR on gut microbiota. To confirm the influence of simulated long-term spaceflight on the human gut microbiota, Turroni et al. characterized the human gut microbiota in the 520-day' MARS500 Project.[18] However, the MARS500 Project just simulated the restricted environment of confinement and stress, and the altered physical condition of microgravity was lacked. In order to explore the simulated short-term microgravity on human physiology, the Earth Star International Bed Rest Experiment Project was carried out by China. The study provided the opportunity to confirm whether the changes of gut microbiota affected by 60 days of 6° HDBR were consistent with short-term spaceflight, which is critical for the research of physiology and psychology affected by gut microbiota under spaceflight environment.

## RESULTS

### The composition of the human gut microbiota was markedly affected by 60 days of 6° head-down bed rest

The detail of volunteers, study design, and fecal collection in the Earth Star International Bed Rest Experiment Project were shown in Figure 1A. We first characterized the gut microbiota compositions by 16S

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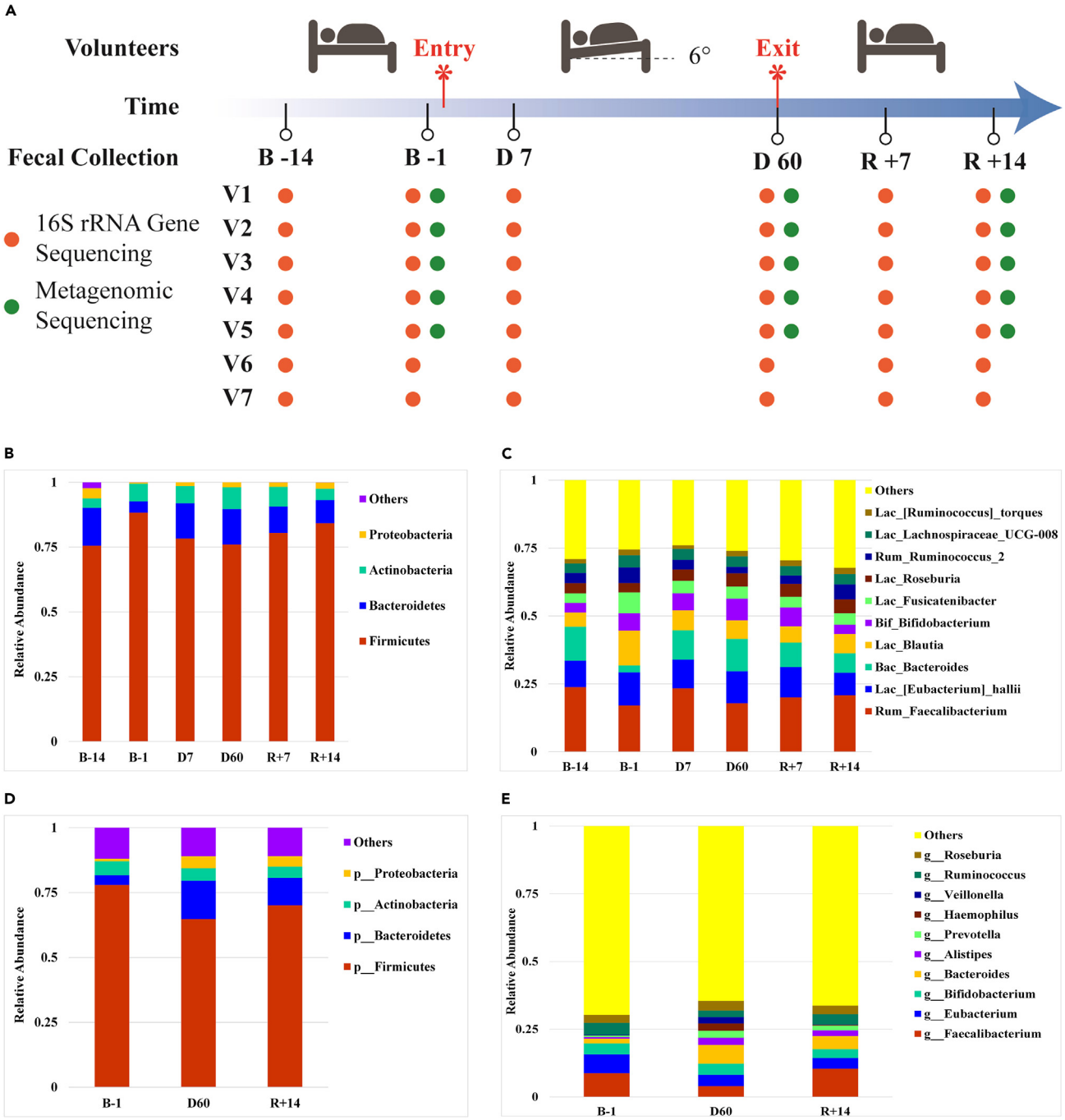


**Figure 1. Relative abundances in the gut microbiota at the phylum and genus levels by 16S rRNA gene sequencing and metagenomic sequencing**

(A) Overview of the volunteers, study design and fecal collection in the study.

(B) Top four phylum abundances in gut microbiota at six time points by 16S rRNA gene sequencing.

(C) Top ten genus abundances in gut microbiota at six time points by16S rRNA gene sequencing.

(D) Top four phylum abundances in gut microbiota at three-time points by metagenomic sequencing.

(E) Top ten genus abundances in gut microbiota at three-time points by metagenomic sequencing.

rRNA gene sequencing and metagenomic sequencing at the phylum and genus levels, the gut microbiotas at both the phylum and genus levels fluctuated obviously after 60 days of 6° HDBR and had varying degrees of recovery after two weeks horizontal bed rest (Figures 1B–1E). We found fluctuations in the top four

microbial abundances of the gut microbiotas at the phylum level (Figures 1B and 1D), we also showed the top four phylum abundances in each volunteer's gut microbiota at six or three-time points by 16S rRNA gene sequencing and metagenomic sequencing (Figures S1A and S2A). We then characterized the changing trend of the top four microbial abundance before and after 60 days of 6° HDBR by 16S rRNA gene sequencing and metagenomic sequencing (Figures S1B and S2B). Among them, the *Firmicutes* abundance was decreased in D60 compared to B-1 and R+14. While the *Bacteroidetes* and *Proteobacteria* abundance was increased in D60 compared to B-1 and R+14. We found fluctuations in the top ten microbial abundances of the gut microbiotas at the genus level (Figures 1C and 1E), we also showed the top five genus abundances in each volunteer's gut microbiota at six or three-time points by 16S rRNA gene sequencing and metagenomic sequencing (Figures S1C and S2C). We then characterized the change trend of the top five microbial abundances before and after 60 days of 6° HDBR by 16S rRNA gene sequencing and metagenomic sequencing (Figures S1D and S2D). Among them, the *Bacteroides* abundance was increased in D60 compared to B-1 and R+14. While The *Blautia* abundance was decreased in D60 compared to B-1 and R+14. In addition, 60 days s HDBR also greatly affected the gut microbiota abundances at the other levels (class, order, family, and species; Figures S3 and S4).

## 60 days of 6° head-down bed rest influenced species diversity and differences in the gut microbiota

The Shannon index based on the genus profile was used to estimate the alpha diversity of each sample. Shannon Index is defined as:

$$\text{Shannon Index} = -\sum_{i=1}^{s}(p_i \log 2\, p_i)$$

where s is the number of OTUs and $p_i$ is the proportion of the community represented by OUT.

The α-diversity at the genus level was gradually increased after volunteers participated in the Project (Figure 2A). The unweightedβ-diversity was decreased significantly in D7 and D60 compared to B-1 and after two weeks horizontal bed rest the β-diversity had a good degree of recovery (Figure 2B). To explore the differences among species that were affected by 60 days of 6° HDBR in the gut microbiota, T test, and LEfSe analysis was used to obtain the species biomarkers. We compared the gut microbiota compositions of B-1 with D60 and displayed the 95% confidence intervals and p values of the species at both the phylum and genus levels Figures 2C and 2D). At the phylum level, the abundance of *Firmicutes* was decreased significantly and the abundances of *Bacteroidetes* and *Proteobacteria* were increased significantly after 60 days of 6° HDBR. At the genus level, the abundance of *Blautia* and *unidentified*_Erysipelotrichaceae was decreased significantly and the abundances of *Bacteroides*, *Lachnoclostridium*, *Klebsiella*, *Parabacteroides*, and *Pantoea* were increased significantly after 60 days of 6° HDBR. We compared the gut microbiota compositions of B-1 with D60 and displayed the LDA score of the species, we showed the distribution diagram of species differences at all levels and the cladogram based on different species (Figures 2E and 2F). After 60 days of 6° HDBR, the abundances of 11 species were increased, while the abundances of 2 species were decreased at all levels.

## 60 days of 6° head-down bed rest significantly affected the gut microbiota function

To investigate the influence of 60 days of 6° HDBR on the gut microbiota function, we blasted Metagenomic sequencing Unigenes to the KEGG, eggNOG, and CAZy databases, and the relative abundances of KEGG (Figure 3A), eggNOG (Figure 3B) and CAZy (Figure 3C) at level 1 are shown in Figure 3. 60 days of 6° HDBR markedly affected the gut microbiota function, especially the relative abundance of envelope biogenesis genes in eggNOG level 1and the relative abundance of glycoside hydrolases and glycosyl transferases genes in CAZy level 1, however, the relative abundance of environmental information processing genes in KEGG level 1 had no significant change (Figure S5).

## Antibiotic resistance genes and virulence genes in the gut microbiota affected by 60 days of 6° head-down bed rest and the species attributions were stable

Our previous studies on gut microbiota in space have revealed that spaceflight has an important impact on bacterial resistance genes and virulence genes. Thus, we analyzed the resistance genes and virulence genes in the gut microbiotas. The total resistance genes in the gut microbiota were no significant differences between B-1 and D60 (Figure 4A). Some AROs had altered markedly after 60 days of 6° HDBR; for

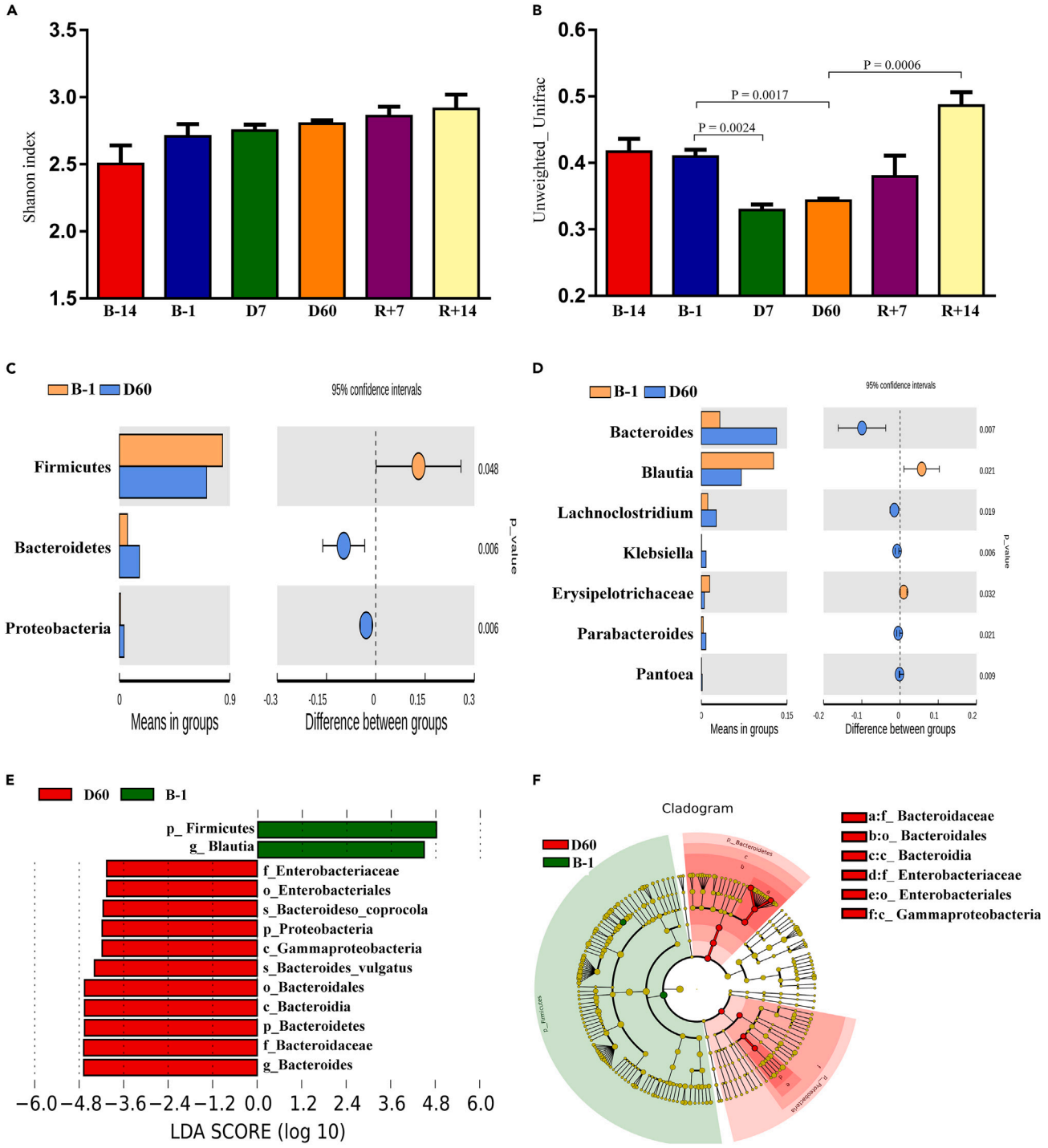


**Figure 2. Comparison of diversity at the genus level and the species in the gut microbiota**

(A) Comparison of α-diversity across six time points.

(B) Comparison of β-diversity across six time points.

(C) The different species at the phylum level compared B-1 to D60.

(D) The different species at the genus level compared B-1 to D60.

(E) Distribution diagram of the LDA score and results of the LEfSe analysis based on the LDA score to screen the species biomarkers. LDA scores of the above 4 species between B-1 and D60 differed significantly.

(F) Cladogram based on different species. Red and green nodes represent the microorganisms that played important roles in each group.

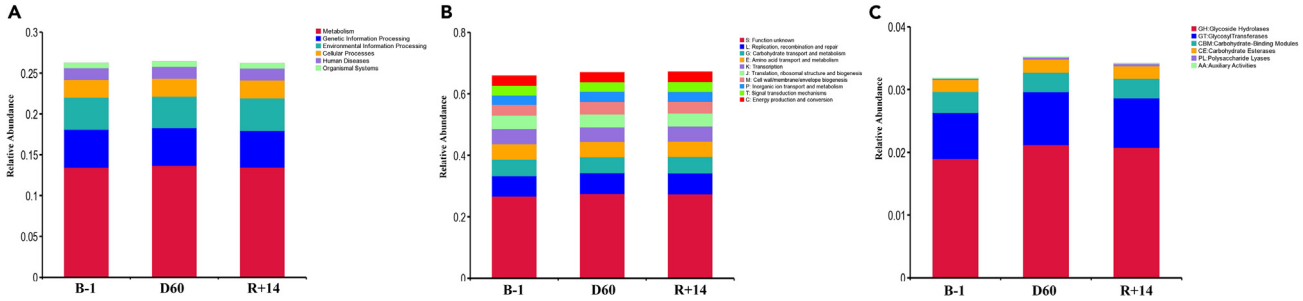


**Figure 3. Relative abundances of KEGG**
(A), eggnog (B) and CAZy (C) level 1 in gut microbiota.

example, the abundance of tetQ, which is related to tetracycline resistance, was increased in D60 compared with that in B-1 and R+14 (Figures S6A and S6B). Thus, the species abundance and resistance genes in the gut microbiotas were both influenced by spaceflight; therefore, we analyzed the relationship between species attribution at the phylum level with the AROs distribution (Figures 4B–4D). We found that no matter how the species attribution fluctuated the ARO distribution for each species remained stable and the AROs were mainly come from *Firmicutes* and *Proteobacteria*. The total number of virulence genes in the gut microbiota was increased in D60 compared with that in B-1 (Figure 4E). Some VFs altered markedly after 60 days of 6° HDBR; for example, the abundance of VF0044, which is related to LPS phase variation and leads to host immune defenses and allow the colonization of different host microenvironments, was increased in D60 compared with that in B-1 and R+14 (Figures S6C and S6D). The species abundance and virulence genes in the gut microbiotas were both influenced by spaceflight, therefore, we analyzed the relationship between species attributions at the phylum level with the virulence genes distribution (Figures 4F–4H). We found that no matter how the species attribution fluctuated, the virulence genes distribution for each species remained stable and the virulence genes were mainly come from *Firmicutes* and *Bacteroidetes*.

## DISCUSSION

Spaceflight can negatively affect astronauts health and the entire mission,[19,20] The NASA twins study and our previous study showed that the human gut microbiota were enormously affected by spaceflight,[12,13] However, the spaceflight is limited and rare. The Earth Star International Bed Rest Experiment Project provided an invaluable opportunity to explore the simulated short-term spaceflight on the gut microbiota. This study was the first to elucidate the impact of simulated short-term spaceflight on the human gut microbiota, as previous studies only simulated the specific environment of confinement and stress.[18,21,22] Our study demonstrated that the composition and function of the human gut microbiota were markedly affected by 60 days of 6° HDBR.

Turroni's and Hao's studies found that the composition and function of gut microbiota fluctuated markedly after simulated spaceflight.[18,22] However, our study showed that some results were different from their even though some results were opposed to their, this difference mainly resulted from these studies only simulated the specific environment of confinement and stress. Our results demonstrated that the Shannon index of the gut microbiota was gradually increased after volunteers participated in the project, the increased Shannon index after volunteers participated in the project suggests a possible healthy microflora because of the adaptation. These results were different from our previous study of true spaceflight, we speculate that the reasons for this difference mainly resulted from astronauts had a good adaptation before spaceflight and true spaceflight was more rigorous and dangerous environment. By analyzing the species biomarkers affected by simulated spaceflight, we observed that after HDBR, at the phylum level, the *Bacteroidetes* and *Proteobacteria* abundance increased, while the *Firmicutes* abundances decreased (Figure 2C). So the ratio of *Firmicutes* abundance to *Bacteroidetes* abundance (F/B ratio) was decreased, which was consistent with our previous true spaceflight study,[13] while, was opposed to NASA twins study.[12] At the genus level, The increased abundance of *Bacteroides* after HDBR was consistent with our previous study which showed that the abundance of *Bacteroides* was increased after spaceflight.[13] As we know, *Bacteroides* in the human gut are efficient degraders of dietary fiber and can efficiently produce propionate

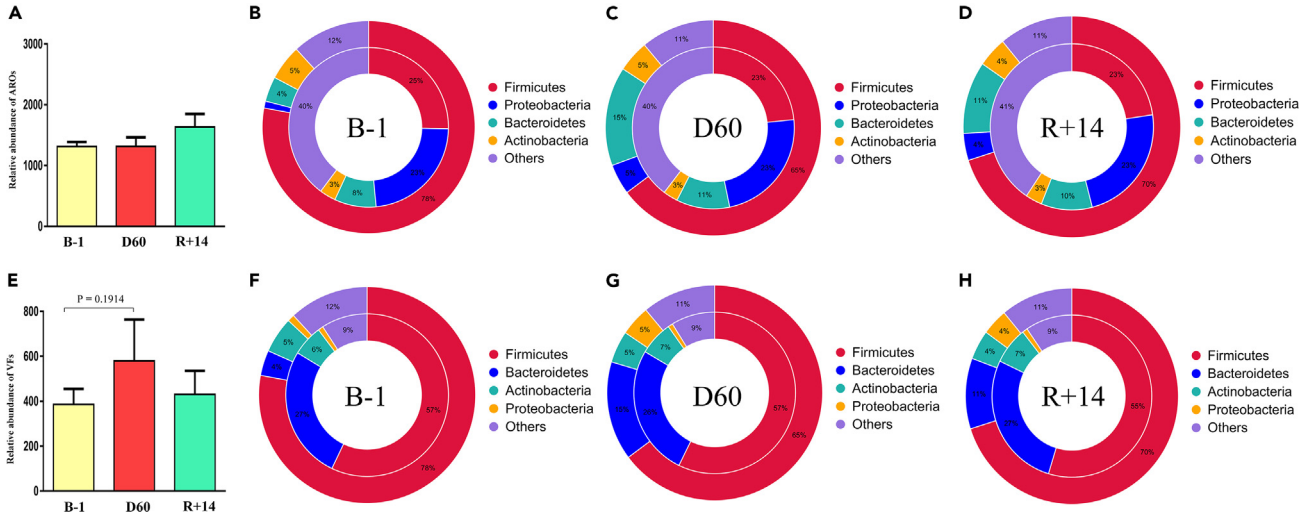


**Figure 4. Antibiotic resistance ontologies (AROs) and virulence factors (VFs)affected by the 60 days of 6° HDBR, and the relationship between species attributions at the phylum level and the antibiotic resistance distribution or the virulence factors distribution in the gut microbiota. The inner circle is the ARO or VF distribution in each species, and the outer circle is the distribution of the gut microbiota in a group**

(A) Total number of AROs affected by the 60 days of 6° HDBR at three-time points. The relationship between AROs distribution and species attribution in B-1(B), D60(C) and R+14(D) group.

(E) Total number of VFs affected by the 60 days of 6° HDBR at three-time points. The relationship between VFs distribution and species attribution in B-1(F), D60(G) and R+14(H) group.

and phenolic acids; this genus is an opportunistic pathogen that often breeds rapidly under several stress conditions.[23–26] The conditions of HDBR induced immune system injury, and as an opportunistic pathogen, the *Bacteroides* abundance increased prospectively, which is consistent with Turroni's study.[18] The abundance of *Blautia* was decreased significantly after HDBR, *Blautia* is performing 7α-dehydroxylation from primary to secondary bile acids which provide an energy advantage in healthy human flora.[27–29] The decreased abundance of *Blautia* after HDBR indicating the presence of an "unnormal flora" in human gut. Our results revealed that 60 days of 6° HDBR markedly affected the composition and function of the human gut microbiota which was partially consistent with the effect of spaceflight on human gut microbiota, implying that the HDBR well simulated the effect of spaceflight on human gut microbiota.

## Limitations of the study

Because of the limited number of subjects and the unique environment, we could not distinguish the influences of immobilization, inactivity, isolation, stress, and other factors on the human gut microbiota during the project. more studies are warranted to analyze the mutualistic relationship between humans and the gut microbiota under unique environmental conditions. Because of the limit of fecal samples, we didn't have enough fecal samples to detect the change of metabolites, more research is needed to understand the effect of HDBR on gut microbiota' metabolites.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106615.

## ACKNOWLEDGMENTS

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## AUTHOR CONTRIBUTIONS

YXL, ZZL, and GL analyzed the volunteers' data and wrote the article. HYL, PC, and YZL collected the fecal samples from the volunteers. RKD, GCJ, LL, and XHC played a key role in microbiome analyses. YPH, JX, SKL, HWZ, and YXL interpreted the data and designed the structure of the article. All authors read and approved the final article.

## DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest with the contents of this article.

## INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Deposited data | | |
| Raw and analyzed data | This paper | https://db.cngb.org/cnsa/ |

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Zizhong Liu (liuzizhong911@163.com).

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

- The 16S rRNA gene sequencing and metagenomic sequencing data of this study have been deposited in the CNSA. CNGBdb: CNP0002834, The DOI is listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

Seven healthy Asian men were enrolled in the Earth Star International Bed Rest Experiment Project and were classified as the control group (mean ± SEM, age, 30 ± 1 years; weight, 62 ± 1 kg; height, 169 ± 1 cm). These volunteers had no history major disease and used no antibiotics before the experiment. The experimental procedures and risks were explained to the volunteers, and the written consent was signed by each participant. The experimental procedures were approved by the ethical committee of the China Astronaut Research and Training Center (Reference no.HYK-Z040FB1A). The Earth Star International Bed Rest Experiment Project was carried out by the China Astronaut Research and Training Center (Beijing, China). All volunteers spent 90 days in the Bed Rest Study which including three periods, 15 days horizontal bed rest before the HDBR (B-15 to B-1), 60 days during the HDBR (D1 to D60) and 15 days horizontal bed rest recovery after the HDBR (R+1 to R+15). All volunteers were permitted to have a daily 10-min stand for defecation. Nicotine, alcohol, tea and caffeine-containing drinks were prohibited during the experiment. The subjects take 2400 –2900 kcal calories a day followed the same diet and the water intake was not controlled in the experiment. During the experiment, all volunteers were required get up at 6:30 am and go to bed and lights out at 10:30 pm, the room temperature was 23°C–25°C. The gut microbiotas of the seven subjects from the Earth Star International Bed Rest Experiment Project were tracked over six time points. Fecal samples were collected and characterized by 16S rRNA gene sequencing and metagenomic sequencing.

### METHOD DETAILS

#### Sampling

Fecal samples were collected in the morning from seven volunteers (V1, V2, V3, V4, V5, V6 and V7) and each person at six time points, including two weeks before HDBR (B-14), one day before HDBR (B-1), one week during HDBR (D7), the last day during HDBR (D60), recovered one week after HDBR (R+7) and recovered two weeks after HDBR (R+14). All fecal samples were collected in sterile vials and immediately transferred to the −80°C freezer.

#### Fecal DNA extraction

Volunteers' stool samples (180–220mg) were weighed in 2 ml microcentrifuge tubes and placed on ice. Total DNA from fecal microbiotas was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN,

Germany) per the manufacturer's instructions (see the QIAamp Fast DNA Stool Mini Kit Handbook, www.qiagen.com/handbooks). Degradation degree and potential contamination of the DNA were analyzed using 1% agarose gels. DNA purity was determined using the NanoPhotometer® Spectrophotometer (IMPLEN, CA, USA), and the DNA concentration was measured using the Qubit® dsDNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA).

### Fecal 16S rRNA gene sequencing and data analyses

Fecal DNA was diluted to 1ng/μL using sterile water according to the concentration. 16S rRNA genes of distinct regions(16SV3-V4) were amplified used specific primer with the barcode. The PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) was used to generate Sequencing libraries following manufacturer's recommendations and index codes were added. Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system were used to assess the library quality. The libraries were sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated. FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) was used to merge paired-end reads, and the splicing sequences were called raw tags. According to the QIIME 2 (V2020.11, https://qiime2.org/) quality controlled process, the raw tags were performed to obtain the clean tags. All the sample details of the quality of their assemblies are present in Table S1. The UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) was used to compared the clean tags with the reference database(Silva database, https://www.arb-silva.de/) and to obtain the effective tags. Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) was used to perform sequences, and the sequences with ≥97% similarity were assigned to the same OTUs. Taxonomic information was annotate by the Silva Database (http://www.arb-silva.de/), and the MUSCLE software (Version 3.8.31, http://www.drive5.com/muscle/) was used to study phylogenetic relationship. OTUs abundance information was normalized by the sample with the least sequences. All alpha diversity indices were calculated with QIIME 2 (Version 2020.11) and displayed with R software(Version 2.15.3). QIIME 2 software (Version 2020.11) was used to calculate the beta diversity on both weighted and unweighted Unifrac.

### Fecal metagenomic sequencing and data analyses

Because of the limit of fecal samples, just five volunteers' stool samples were analyzed by metagenomic sequencing. One microgram of qualified DNA was used to construct the library. DNA samples were fragmented to 350 bp by sonication, then the DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. Libraries were analyzed for size distribution using the Agilent2100 Bioanalyzer (Agilent, USA) and quantified via real-time PCR. The libraries were sequenced on an Illumina HiSeq platform. Raw data obtained from the Illumina HiSeq sequencing platform were preprocessed by Readfq (V8,https://github.com/cjfields/readfq), and the acquired clean data were used for subsequent analysis. The clean data were analyzed using SOAPdenovo software (V2.04, http://soap.genomics.org.cn/soapdenovo.html), and the Scaftigs were obtained. The Scaftigs (≥ 500 bp) were used to predict the open reading frame (ORF) using MetaGeneMark (V2.10; http://topaz.gatech.edu/GeneMark/) and CD-HIT software (V4.5.8; http://www.bioinformatics). The website, org/cd-hit, was used to obtain the initial gene catalog from the predicted ORF. Clean data from each sample were mapped to the initial gene catalog using SoapAligner software(soap2.21; http://soap.genomics.org.cn/soapaligner.html). The obtained gene catalog (Unigenes) was eventually used for subsequent analyses. The obtained Unigenes were used to blast the sequences for the bacteria, fungi, archaea and viruses, which were extracted from the NR database (V20161115; https://www.ncbi.nlm.nih.gov/) of NCBI using DIAMOND software (V0.7.9; https://github.com/bbuchfink/diamond/). We used the lowest common ancestor (LCA) algorithm to obtain the number of genes and abundance information for each sample in each taxonomic hierarchy (kingdom, phylum, class, order, family, genus, and species). DIAMOND software (V0.7.9) was used to blast Unigenes to functional databases, including the KEGG (V201609,http://www.kegg.jp/kegg/), eggNOG (V4.5; http://eggnogdb.embl.de/#/app/home) and CAZy databases (V20150704; http://www.cazy.org/), for the blast results, and the best blast hit was used for subsequent analysis. The Unigenes were blasted to the CARD and VFDB databases using DIAMOND software to analyze the resistance and virulence genes (V0.7.9).

## QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as mean±standard deviation(SD). One-way ANOVA was used to assess between-group differences, Graphpad Prism version 7.0c was used to follow by post-hoc pairwise repetitive comparisons with Turkey test.