Microbiota–muscle/immune interactions in rhesus macaque under simulated microgravity revealed by integrated multi-omics analysis

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Abstract

Background Long-term exposure to microgravity during spaceflight has adverse effects on human health including muscle atrophy, impaired immune function, and alterations in gut microbiome profile. Gut microorganisms influence a wide range of host biological processes, but their interactions with skeletal muscle and the immune system under microgravity have yet to be elucidated.

Methods Rhesus macaques (Macaca mulatta) were subjected to –6° head-down tilted bed rest (HDBR) for 6 weeks. Faecal samples, skeletal muscle tissue, and peripheral blood mononuclear cells (PBMCs) were collected for metagenomic, metabolomic, and transcriptomic analyses, respectively, and further integrated for a multi-omics analysis.

Results Head-down tilted bed rest significantly altered taxon abundance (P < 0.05) in 1 class, 5 orders, 11 families, 55 genera, and 122 species of microbes. We also identified the significantly changed metabolites (P < 0.05, fold change >1.2, variable importance in projection >1) in atrophied muscles, including some crucial metabolites (such as L-alanine and l-carnitine) and hub metabolites (such as pyridoxamine and epinephrine) involved in energy metabolism. Transcriptomic analysis of PBMCs revealed genes related to leucocyte activation, differentiation, and interleukin-2 production that were differentially expressed as a result of HDBR exposure (fold change >2 and P < 0.05). By integrating multi-omics analysis, we identified three bacterial genera (Klebsiella, Kluyvera, and Bifidobacterium) that were closely associated with immune dysfunction and five (including Oligella, Sporosarcina, Citrobacter, Weissella, and Myroides) that were associated with abnormal metabolism of amino acids in atrophied muscles induced by HDBR. The reduced abundance of butyrate-producing colon bacteria Eubacterium, Roseburia, and their cross-feeding bacteria Bifidobacteria may contribute to the impaired immune function and muscle atrophy caused by HDBR.

Conclusions This is the first report of the HDBR-associated changes in gut microbiota composition, metabolomics of skeletal muscle, and transcripts of PBMCs in a non-human primate. The underlying microbiota–muscle and microbiota–immune interactions during simulated microgravity imply that modulation of gut microbiota may represent a novel strategy for enhancing the health and safety of crew members during long-term space expeditions.

Keywords Head-down tilted bed rest; Metagenomics; Microbiota; Metabolomics; Transcriptomic; Integrated multi-omics analysis

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Introduction

The harsh environment of space poses a significant physiological challenge to humans and has hindered the progress of deep-space exploration. Astronauts experience a variety of pathophysiological changes during spaceflight, including space motion sickness, spatial disorientation, osteoporosis, muscle atrophy, and impaired cardiac, cognitive, and immune functions. Operation of the International Space Station (ISS) has extended knowledge of the effects of microgravity on human health. For example, many astronauts on the ISS reported ocular issues such as optic disc oedema, globe flattening, hyperopic shifts, and cotton-wool spots. The National Aeronautics and Space Administration (NASA) Twins Study reported increased subfoveal choroidal thickness and peripapillary total retinal thickness. Recently, a case of venous thrombo-embolism during spaceflight was confirmed by radiologists on Earth. Overall, physiological and functional alterations caused by spaceflight are gradually being recognized and characterized.

In the past two decades, various high-throughput omics technologies, such as methyl-CpG-binding domain protein sequencing, RNA sequencing, and mass spectrometry (MS) analysis and metabolomics, have been used to investigate the impact of microgravity on human health. However, each omics technology emphasizes the role of molecules of their corresponding omics layer but miss the complementary effects and interactions between omics layers. Thus, an integrative approach to multi-omics data analysis is needed to fully elucidate microgravity-induced physiological changes.

The gastrointestinal tract harbours complex communities of microbes that play important roles in maintaining human health; these roles include energy extraction, vitamin biosynthesis, protection against pathogens, and development of the innate and adaptive immune systems. Changes in the gut microbiome in response to microgravity have been reported in both humans and rodents, but how these changes relate to other microgravity-induced pathophysiological alterations has yet to be elucidated. The current study aimed to address this issue through an integrative multi-omics analysis—covering metagenomics of faecal samples, metabolomics of skeletal muscle, and transcripts of peripheral blood mononuclear cells (PBMCs)—of rhesus macaques subjected to −6° head-down tilted bed rest (HDBR). The multi-omics data set generated in this study serves as a resource for future investigations on the effects of microgravity on human health and may facilitate the development of potential countermeasures for future spaceflights.

Materials and methods

Animal experiments

A total of five healthy male rhesus macaques aged 4–6 years and weighing 4–8 kg were provided by the Beijing Institute of Xie’erxin Biology Resource (Beijing, China). The animals were acclimatized for 3 months at the Laboratory Animal Center of China Astronaut Research and Training Center prior to being used in experiments. The five fully acclimatized rhesus macaques were then subjected to 42 days of −6° HDBR as previously described. Following HDBR treatment, the animals were individually housed in stainless-steel mesh cages and allowed to recover for 32 days. The animals had unrestricted access to food and water, and their general health was closely monitored for the duration of the study. All procedures were performed in accordance with standard ethical guidelines and were approved by the Institutional Animal Care and Use Committee of China Astronaut Research and Training Center (ACC-IACUC-2014-001).

Sample collection

Under light ketamine sedation, sterile heparinized peripheral blood samples were obtained from the femoral vein of rhesus macaques before (Pre-3), during (H + 25 and H + 40), and after (R + 12, R + 24, and R + 32) HDBR; samples were taken at the same time (10 a.m.) on each day. PBMCs were collected by Ficoll–Hypaque density gradient centrifugation. Faecal samples were also collected from the rhesus macaques before (Pre-2), during (H + 16, H + 30, and H + 42), and after (R + 13, R + 17, and R + 28) HDBR (Supporting Information, Figure S1). To prevent external contamination, only samples from the interior of the faeces, and not exposed to the outside environment, were collected into screw-capped sampling tubes and stored at −80°C before processing for metagenome analysis.

Muscle biopsy

Muscle tissue samples were obtained by biopsy as previously described. Briefly, after general anaesthesia with isoflurane, the pre-HDBR muscle tissue sample (Pre-3) was obtained from the left soleus of each rhesus macaque using an open biopsy technique. At the end of HDBR (H + 42), a biopsy was performed on the right soleus. In addition, 32 days after HDBR, when the pre-HDBR biopsy site in the left soleus was fully healed, the muscle was biopsied at a different site (R + 32) (Figure S1). All muscle samples were immediately frozen in liquid nitrogen for later use.

Transcriptome analysis

Total mRNA of PBMCs was amplified using oligo (dT) primers and sequenced by Complete Genomics (San Jose, CA, USA). At least 20 million reads were generated for each sample. To decrease noise of the sequencing data, SOAPnuke (Version...
1.6.5) was used to filter low-quality reads, which included (i) reads containing sequencing adapter; (ii) reads with a low-quality base ratio (base quality ≤15) more than 50%; and (iii) reads with an unknown base (‘N’ base) ratio more than 10%.\textsuperscript{13} HISAT2 (Version: v2.0.4) was used to map sequence reads to the reference genome [rhesus macaque, assembly Mmul_8.0.1, National Center for Biotechnology Information (NCBI), GenBank accession GCA\_000772875.3], and RNA-Seq by expectation maximization (WGCNA) in the R package.\textsuperscript{15} By selecting the appropriate weighting coefficient to weight the correlation coefficient between metabolites, the WGCNA algorithm could make the metabolite network obey the scale-free network distribution and divide the metabolites with similar expression into the same module. Modules exhibiting high correlation with traits were selected, and the hub metabolite in each module was identified.

**Metabolome analysis**

Metabolite detection was conducted using ultrahigh-performance liquid chromatography (UPLC–MS). UPLC–MS was performed according to previously reported protocols.\textsuperscript{16} Briefly, 25 mg skeletal muscle was homogenized with 800 μL precooled extraction reagent and lysed for 5 min using TissueLyser (JXFSTPRP, China), followed by sonication for 10 min and incubation at −20°C for 1 h. Samples were then centrifuged for 15 min at 25000 g and 4°C, and the supernatant was transferred for vacuum freeze-drying. The resulting metabolites were resuspended in 600 μL of 10% methanol, sonicated for 10 min at 4°C, and then centrifuged for 15 min at 25 000 rpm. The resulting supernatants were transferred to autosampler vials for LC–MS analysis. Quality control samples were included to evaluate data quality, and low-quality samples were removed. The biological reproducibility of the samples was checked according to the features of PCA clustering. Outliers were removed in the subsequent analysis to ensure the reliability of the results. Feature alignment, picking, and identification were performed using Progenesis QI software (Nonlinear Dynamics, Newcastle, UK). MetaX software was used for data cleaning and statistical analysis. Significantly altered features \( P < 0.05, \) fold change \(<1/1.2 \text{ or } >1.2, \) and variable importance in projection (VIP) >1] were identified by combining univariate and multivariate analyses and were annotated using Progenesis QI, the Human Metabolome Database (HMDB) v.3.6, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (www.genome.jp/kegg/).\textsuperscript{17}

Clusters of co-abundant metabolites in muscle tissue were identified by weighted gene correlation network analysis (WGCNA) in the R package.\textsuperscript{18} A soft threshold of \( \beta = 9 \) for muscle features was selected by scale-free topology analysis for signed, weighted features co-abundance correlation network construction. A dynamic hybrid tree-cutting algorithm with deepSplit = 4 and a minimum cluster size of 30 was used for cluster identification. If the biweight mid-correlation between the eigenvectors of two clusters was >0.8, the clusters were considered similar and were merged. Muscle feature clusters were labelled as M1–M6.

Weighted gene correlation network analysis in the R package was used to identify significant differences in metabolic module and hub metabolites.\textsuperscript{18} Spearman correlations between all samples were calculated based on FPKM and were found to be very high (>0.95), and biological replicates clustered together. DESeq2 package in R (Version 3.5.1) was used to identify differentially expressed genes (DEGs), and Gene Ontology (GO) analysis for functional annotation was performed with enrichGO function in R.\textsuperscript{15}

**Metagenome analysis**

Raw reads were obtained by sequencing on an Illumina HiSeq 2000 platform (Expression Analysis, San Diego, CA, USA) at Beijing Genomic Institute. SOAPnake Version 1.6.5 was used to remove adapter contamination, low-quality reads with a quality value <20 and low-quality rate >0.2, and host genomic DNA sequences (rhesus macaque, assembly Mmul_8.0.1, NCBI). The remaining high-quality reads were assembled using metaSPAdes v.3.10.1.\textsuperscript{19} Open reading frames (ORFs) in the contigs of each sample were determined using GeneMark v.2.7.\textsuperscript{20} The nonredundant gene set of all ORFs was clustered using CD-HIT v.4.5.7 based on the nucleotide sequence, with thresholds of 95% identity and 90% coverage. Taxonomic annotation of gene sets was performed using CARMA3 based on BLASTP alignment with bacterial and archaeal genes from the NCBI-NR database.\textsuperscript{21} The gene set was also annotated against the KEGG v.59 database using BLAST v.2.2.23.

The relative abundance of each gene was calculated based on SOAP2 alignment, and species abundance and functional profiles were summarized from their respective genes.\textsuperscript{22} Differential alpha diversity, species, and KEGG orthologues (KOs) at different time points were calculated with the Kruskal–Wallis test using R software (https://www.r-project.org/). Spearman’s correlation coefficient was used to evaluate the relationship between different genera and KOs or metabolic features according to their abundance. VennDiagram in R (Version 3.5.1) was used to generate and visualize the KO functions and genera shared by humans and macaques through comparative analysis with the human gut gene catalogue (ftp://ftp.ncbi.nih.gov/pub/SciRAID/Microbiome/humanGut_9.9M/GeneCatalog/IGC.fa.gz).

Metagenome-assembled genomes (MAGs) were constructed with short reads from single sample, and metaSPAdes v.3.10.1 was used to assemble the
metagenomes by high-quality reads. For each metagenome assembly, the predicted bins from three software tools (CONCOCT v1.0.0, MaxBin2 v2.2.7, and MetaBAT2 v2.12.1) were combined using the metaWRAP v1.1.5. Refinement of MAGs was performed with the bin refinement module. The abundances of the bins across the samples by metaWRAP quant bins module. We kept unique bins at a threshold of ≤10% contamination and completeness of >50% after de-
Figure 2. Representative gut bacteria altered by HDBR and association of differentially abundant bacterial genera with differentially represented KEGG orthologues (KOs). (A) Box plots showing the relative abundance of *Acinetobacter*, *Lactococcus*, and *Bifidobacterium* throughout the experimental period. The x and y axes show different time points and relative abundance (log_{10}) of each genus, respectively. For each inter-quartile range (IQR), the first and third quartiles are shown as boxes and the line inside the box is the median. Data points (circles) outside of the whiskers are the lowest or highest values within 1.5 times the IQR. *P* < 0.05 and **P* < 0.01 compared with P-2 by Wilcoxon test; *P* < 0.05 and ##P* < 0.01 compared with H + 16 by Wilcoxon test. (B) Spearman correlation coefficient heatmap (*P* < 0.05) of 27 differentially abundant genera (x axis) and 442 differentially represented KOs (y axis). Red and blue represent significantly positive and negative correlations, respectively, and blank areas indicate that there was no significant correlation.
replication with dRep v2.5.4.27 MAGs were classified using the Genome Taxonomy Database Toolkit (GTDB-Tk).

Availability of data and materials

All data analysed in this study are included in this published article and its Supporting Information. The metagenomics data of faecal samples and the transcriptomic data of PBMCs generated and/or analysed during the current study are available in the China Nucleotide Sequence Archive (CNSA) database under Accession Number CNP0000147 (https://db.cngb.org/search/?q=CNP0000147). The metabolomics data of skeletal muscle and MAGs sequences of metagenomics are available in CNSA under Accession Number CNP0001527 (https://db.cngb.org/search/project/CNP0001527).

Results

Head-down tilted bed rest-associated changes in the gut microbiome

The effect of HDBR on the gut microbiome was investigated through metagenome sequencing of 35 rhesus macaque gut samples collected at seven time points throughout the whole experiment (Figure S1). A total of 1434.31 million raw reads were generated, and after removing low-quality and host reads, 1379.97 million high-quality reads were obtained (average of 39.43 million reads per sample) (Table S1). A reference library of the rhesus macaque gut metagenome was constructed using all the samples (Table S2); 63.10%, 21.00%, and 2.43% of the genes were annotated at phylum, genus, and species levels, respectively, and 45.51% were annotated to 6631 KOs. Firmicutes and Bacteroidetes were the predominant phyla in the gut of rhesus macaques exposed to HDBR, while Prevotella and Clostridium were the predominant genera (Figure 1A and 1B). Genes related to metabolism constituted the largest proportion of total genes in the KEGG pathway analysis (Figure 1C), and 95.58% of KO functions and 49.48% of genera were shared by rhesus macaques and human gut gene catalogues (Figure S2).

To identify microbial taxa that may be influenced by spaceflight, we compared pre-HDBR (control) and HDBR as well as HDBR and post-HDBR (recovery) gut microbiome profiles with the Kruskal–Wallis test. A number of bacterial taxa were significantly different between control and HDBR samples (1 class, 5 orders, 11 families, 55 genera, and 122 species), and some of these taxa showed the reverse trend during the recovery period (1 class, 1/5 orders, 3/11 families, 24/55 genera, and 72/122 species) (Table S3). For example, the abundance of Acinetobacter and Lactococcus, two genera associated with the regulation of inflammation and protection against infections, was decreased by HDBR but returned to the baseline (pre-HDBR) level after 17 days of recovery (Figure 2A). However, the abundance of some genera was not restored even after 28 days of recovery; for example, the abundance of Bifidobacterium, a beneficial gut bacterium, decreased continuously throughout the experiment (Figure 2A).

There were 537 KOs with significantly altered abundance after HDBR exposure, and these were assigned to 139 genera, of which 27 were significantly altered throughout the experiment (P < 0.05). Correlation analysis showed that 44.76% of pairwise correlations between KOs and specific genera were positive, while 3.38% were negative (R² > 0.3, P < 0.05) (Figure 2B). For example, higher abundance of the genera Myroides and Acinetobacter was associated with increased representation of K00121, K00151, K00276, K00451, and K01555, which are involved in tyrosine metabolism. In addition, we reconstructed MAGs by using metaWRAP in both sample-by-sample binning and co-binning models. A total of 1885 bins were generated, and 608 unique bins were obtained after de-replication. The bin annotated as Myroides also showed similar differential abundance during HDBR (Figure S3).

Metabolic profile associated with head-down tilted bed rest in skeletal muscle

To investigate the metabolic signature associated with HDBR in greater detail, we carried out a UPLC–MS-based metabolomic analysis to quantify metabolites in skeletal muscle tissue of rhesus macaques before, during, and after HDBR (Figure S1 and Table S4). A total of 2525 features were generated and/or analysed during the current study are available in CNSA under Accession Number CNP0000147 (https://db.cngb.org/search/?q=CNP0000147). The metabolomics data of faecal samples and the transcriptomic data of PBMCs are available in CNSA under Accession Number CNP0001527 (https://db.cngb.org/search/project/CNP0001527).

Figure 3 WGCNA of metabolites in atrophied muscle caused by HDBR. (A) Dendrogram of 552 differentially abundant features (DAFs) of muscle clustering in six modules (M1–M6, coloured turquoise, yellow, red, green, blue, and brown, respectively; the grey module includes DAFs that did not meet the clustering criteria). (B) Significantly altered metabolites involved in the tyrosine metabolism pathway. Five metabolites in M1 that decreased in abundance during HDBR and increased during recovery are coloured turquoise; three metabolites in M5 showing the opposite change are coloured blue; and three metabolites in M6 showing a similar change to M1 are coloured brown. The five remaining metabolites coloured grey did not show differential abundance. (C) Abundance of metabolites shown in Figure 2B. *P < 0.05 vs. control group. (D) Heatmap of DAFs in M1 down-regulated during HDBR and up-regulated during recovery in muscle. (E) Correlation of all muscle DAFs in M1 showing good similarity. M1–M6 are coloured turquoise, yellow, red, green, blue, and brown, respectively; the grey module includes DAFs that did not meet the clustering criteria. (F) Abundance of l-alanine and l-carnitine in HDBR-induced muscle atrophy. *P < 0.05 vs. control group. (G) Epinephrine and pyridoxamine were identified as the hub metabolite for M1 and M6, respectively. Correlations of M1 and M6 DAFs were calculated by WGCNA; a larger node size indicates a greater number of neighbours for the node.
were identified through the metabolic analysis, of which 356, 287, and 100 were differentially abundant features (DAFs) in the HDBR vs. control, HDBR vs. recovery, and recovery vs. control comparisons, respectively ($P < 0.05$, fold change $>1.2$, VIP $>1$) (Figure S4A). Further analysis focused on the 154 DAFs that were significantly altered in HDBR samples relative to the control and recovery samples ($P < 0.05$) (Figure S4B). KEGG pathway analysis revealed that the Top 5 pathways most affected by HDBR were tyrosine metabolism; biosynthesis of amino acids; protein digestion and absorption; alanine, aspartate, and glutamate metabolism; and tryptophan (Table S5). These pathways were consistent with the accelerated protein degradation occurring in HDBR-induced muscle atrophy.

The interaction of these DAFs was evaluated by WGCNA. A total of 552 DAFs in muscle were classified into six modules (Figure 3A). DAFs involved in tyrosine metabolism (mcc00350) were mainly distributed in three modules (M1, M5, and M6) (Figure 3B and 3C). DAFs in M1 showed co-abundance in muscle, with the levels of all these metabolites decreased during HDBR but reversed in the recovery group (Figure 3D and 3E). Consistent with the known metabolic and functional changes in skeletal muscle under microgravity, the DAFs in M1 contained some critical amino acids involved in gluconeogenesis [e.g. L-alanine (KEGG compound C00041, HMDB00161)] and fatty acid transport [e.g. L-carnitine (C00318, HMDB00062)] (Figure 3F). Epinephrine (C00788, HMDB00068) was identified as the hub metabolite of M1 (Figure 3G). Pyridoxamine (C00534, HMDB01431), a form of vitamin B6 that participates in free radical scavenging, was identified as the hub metabolite of M6 (Figure 3G).

Transcriptional signature associated with head-down tilted bed rest in peripheral blood mononuclear cells

PBMCs were collected from the five rhesus macaques throughout the experiment to evaluate the effect of HDBR on gene expression in immune cells (Figure S1). A total of 779.09 million reads were obtained at six time points (Table S6). By comparing every two time points (fold change $>2$ and $P < 0.05$) and removing duplicate genes, 65 DEGs (Figure S5A) were detected. These DEGs were significantly enriched in 44 biological processes ($P < 0.05$), of which 41 were related to immune regulation, including leucocyte activation (GO:0002694), T-cell differentiation (GO:0045580), and interleukin (IL)-2 production (GO:0032663) (Figure 5B and Table S7). Furthermore, 35 of the 65 DEGs were down-regulated by HDBR, which was consistent with previous findings that immune function is impaired by simulated or actual microgravity.

Integrated analysis of omics data

To clarify the functional implications of microgravity-associated changes in the gut microbiome, a multi-omics analysis was performed with combined metagenomic, transcriptomic, and metabolomic data from faecal samples, PBMCs, and muscle tissue, respectively (Tables S8–S10). As shown in Figure 4, 16 of the 27 genera that were differentially represented in faecal samples during HDBR were closely associated with 27 of the 65 DEGs in PBMCs, with Klebsiella, Kluyvera, and Bifidobacterium showing the highest correlations. More specifically, the abundance of Bifidobacterium was positively correlated with the expression of ENSMMUG00000015297 (cluster of differentiation 69), ENSMMUG00000045565 (DNA damage-inducible transcript 4), ENSMMUG00000010956 (suppressor of cytokine signalling 1), ENSMMUG000000008869 (TNF-α-induced protein 3), and ENSMMUG0000011607 (nuclear receptor subfamily 4 group A member 2), and negatively correlated with ENSMMUG00000005378 (N-acetyltransferase 9) and ENSMMUG000000005325 (solute carrier family 5 member 10). The abundance of Delftia, Stenotrophomonas, Acinetobacter, and Comamonas was positively correlated with ENSMMUG0000038412 (docosahexaenoic acid omega-hydroxylase CYP4F3, LOC718349) and negatively correlated with ENSMMUG0000010256 (early growth response 2) and ENSMMUG0000008817 (zinc finger protein 441).

Integration of metagenomic data from faecal samples and metabolomic data from atrophied muscle showed that 25 of the 27 differentially represented genera were significantly correlated with 174 of the 372 differentially expressed metabolites in muscle (Figure 5), with Oligella, Sporosarcina, Citrobacter, Weissella, and Myroides showing the highest correlations. Specifically, the abundance of Oligella was positively correlated with 45 metabolites and negatively correlated with 12 metabolites, while the abundance of Myroides was negatively correlated with leucodopachrome (C05604, HMDB04067) and dopaquinone (C00822, HMDB01229), which are involved in tyrosine metabolism (mcc00350). Some bacterial genera were closely correlated with crucial metabolites identified in HDBR-induced muscle atrophy. For example, the abundance of Oligella was positively correlated with L-alanine, whereas that of Citrobacter

Figure 4 Spearman’s rank correlations between differentially abundant intestinal bacteria and differentially expressed genes (DEGs) in PBMCs during HDBR. The x and y axes show the 16 genera and 27 DEGs that were significantly correlated ($R^2 > 0$ and $P < 0.05$). Red and blue colours represent positive and negative correlations, respectively, and grey indicates that there was no significant correlation. The relative abundance of each genus is annotated on its right.
was negatively correlated with this metabolite; *Lactococcus* showed positive correlation with l-carnitine; and *Providencia* was positively correlated with p-cresol (C01468, HMDB01858), a metabolite of tyrosine (Figure 5).

Microbiota-derived short-chain fatty acids (SCFAs) are thought to mediate interactions between gut bacteria and other tissues.29 For example, butyrate is a major SCFA produced by the gut microbiota and prevents excessive inflammation by stimulating the function of M2 macrophages and regulatory T cells and inhibiting neutrophil infiltration.30 Moreover, administration of butyrate increased muscle mass and cross-sectional area in aged mice.31 In the current study, the abundance of butyrate-producing colon bacteria such as *Eubacterium* and *Roseburia* spp. and their cross-feeding bacteria *Bifidobacterium* was reduced during HDBR (Figure 2A). The abundance values of KOs about the butyrate production pathways also showed decreased trend during HDBR (Figure S6 and Table S11). These results suggest that a lower level of butyrate may contribute, at least in part, to the impaired immune function and muscle atrophy caused by HDBR (Figure S7). 3-Hydroxyphenylacetate can be transformed into 3,4-dihydroxybenzenacetic acid (3,4DPHAA; C01161, HMDB01336)—a metabolite involved in tyrosine metabolism in skeletal muscle—by the enzyme 4-hydroxyphenylacetate 3-monoxygenase (EC: 1.14.14.9), which is produced by *Providencia rettgeri*.32 The level of 3,4DPHAA was increased during HDBR and returned to the baseline level during the recovery phase, reflecting the changes in the abundance of *P. rettgeri* in the gut and suggesting that this bacterium influences tyrosine metabolism in HDBR-induced muscle atrophy via 3,4DPHAA (Figure S7). The enzyme tryptophan 2,3-dioxygenase (EC: 1.13.11.11) produces N1-formylkynurenine (C02700, HMDB60485), which is transformed into formylanthranilic acid (C05653, HMDB04089) by kynureninase (EC: 3.7.1.3); formylanthranilic acid is involved in tryptophan metabolism. Both tryptophan 2,3-dioxygenase and kynureninase are produced by gut microbes such as *Myroides* and *Comamonas*,33 which both showed significant changes in abundance during HDBR (Figure S7). Collectively, these findings indicate that changes in the microbiota community composition following exposure to HDBR influence amino acid metabolism in skeletal muscle as well as immune function in rhesus macaques.

**Discussion**

Long-term spaceflight is recognized to have a broad spectrum of deleterious effects on mammalian health. For example, intestinal microbiota homeostasis has been reported to be disturbed in both humans and rodents exposed to microgravity.3,30 However, the human study involved only a single set of twins,3 while the findings of the rodent study were not fully applicable to humans because of interspecies physiological differences.30 The limited availability of biological samples from astronauts means more animal studies—especially in non-human primates—are needed to clarify the impact of microgravity on mammalian physiological functions. HDBR, which simulates weightlessness by inducing head-ward fluid shifts, is considered the best integrated ground-based model for spaceflight.34 This study comprised a longitudinal analysis of changes in the gut microbiome in response to HDBR. Consistent with findings from NASA’s Twins Study,3 microgravity induced a decrease in the abundance of beneficial gut bacteria such as *Bifidobacterium* and an increase in that of opportunistic pathogens such as *Escherichia coli* in the current study. The lower abundance of *Bifidobacterium* during HDBR was also observed in faecal samples and continued to decrease during the 28 day recovery period. However, HDBR does not fully simulate the environmental conditions encountered during spaceflight and has relatively modest effects on the gut microbiome. HDBR caused no changes at the phylum level, and only one bacterial class showed differential abundance in faecal samples of rhesus macaques. In contrast, a 1 year spaceflight altered the abundance of three phyla and three classes of bacteria in the faecal microbiome of astronauts.3 Surprisingly, HDBR in rhesus macaques did produce more differentially abundant families (11 vs. 8), genera (55 vs. 13), and species (122 vs. 36) of bacteria in faecal samples than microgravity did in humans.3 Given that microbiota community composition is strongly influenced by diverse factors such as diet, lifestyle, and medication use, thus the specific diet and nutritional supplementation for astronauts may potentially alleviate the negative effects of microgravity on gut microbiome composition.

In addition to nutrient absorption, intestinal microorganisms maintain human health through bidirectional interactions with host biological processes including the immune system.35 Studies in pathogen-free or gnotobiotic animals have demonstrated that the intestinal immune system influences the compartmentalization of commensal microbiota and microbial community composition; reciprocally, gut bacteria affect the development of organized lymphoid structures and intestinal and systemic immune cell function.35 In the current study, HDBR reduced the abundance of butyrate-producing colon bacteria including *Eubacterium*, *Roseburia*, and *Bifidobacterium*. The SCFA butyrate is believed to mediate communication between commensal bacteria and the immune system.30 Butyrate suppresses the

**Figure 5** Spearman’s rank correlation between differentially abundant intestinal bacteria and metabolites in muscle. The x and y axes represent 25 genera and 174 metabolites with significant correlations ($R^2 > 0$ and $P < 0.05$). Red and blue represent positive and negative correlations, respectively, and white indicates that there was no significant correlation. The relative abundance of each genus is annotated on its right.

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production of pro-inflammatory cytokines released by M1 macrophages and neutrophils while activating Tregs, stimulating the production of the anti-inflammatory cytokine IL-10, and increasing the expression of interferon (IFN)-γ and granzyme B in cytotoxic T lymphocytes and Tc17 cells.30,36 Simulated or actual microgravity can weaken immune function and increase susceptibility to infection as a result of reduced lymphocyte proliferation and IFN-γ production1; hence, the reduced abundance of butyrate-producing microbiota may contribute to the immune dysfunction caused by HDBR. Besides changes in *Bifidobacterium*, the current study revealed that the genera *Klebsiella* and *Kluyvera* were closely associated with immune dysfunction. Species of the genus *Klebsiella* cause pneumonia, urinary tract infections (UTIs), bloodstream infections, and sepsis, while *Kluyvera* is an opportunistic pathogen that infects hosts under various conditions.37 It remains to be determined how changes in the abundance of these two genera influence host susceptibility to infection.

The relationship between intestinal microbiota community composition and skeletal muscle has recently been investigated,38,39 and reduced muscle mass was found to be closely correlated with a specific gut microbiome signature.38,39 For example, muscle atrophy in ghrelin-null mice was accompanied by selective depletion of butyrate-producing bacteria such as *Clostridium* XIVa and *Roseburia*,39 and there is evidence of an association between frailty and reduced abundance of butyrate-producing bacteria.40 Administration of probiotics such as *Lactobacillus reuteri* and *Faecalibacterium prausnitzii* was shown to alleviate the loss of muscle mass.51–53 In addition, butyrate treatment increased muscle mass and cross-sectional area in aged mice.31 Based on these previously reported findings, a reasonable conclusion is that muscle atrophy caused by HDBR can be partly attributed to the reduced abundance of butyrate-producing bacteria. Five other bacterial genera (*Oligella*, *Sporosarcina*, *Citrobacter*, *Weissella*, and *Myroides*), which have been mainly implicated in UTIs, were closely related to the abnormal metabolism of amino acids in HDBR-induced muscle atrophy, although the mechanistic basis for this association is unclear. It is possible that intestinal mucosal barrier disruption and enhanced intestinal permeability under microgravity result in the release of lipopolysaccharide and pro-inflammatory cytokines by these pathogens, which may promote muscle atrophy.35 In support of this theory, bacterial-derived indoxyl sulfate was reported to enhance the expression of myostatin and atrogin-1 in atrophic skeletal muscle.44

In this study, we further tried to reconstruct MAGs from the metagenome data using metaWRAP in both sample-by-sample binning and co-binning models. A total of 1885 bins were generated, and 608 unique bins were obtained after de-replication. Some species, such as butyrate-producing bacteria *Eubacterium*, were reconstructed and showed differential abundance during HDBR. *Myroides* was also generated in all of the samples and showed similar trend during HDBR as previously mentioned. However, genomes of some species were not reconstructed might due to the relatively low relative abundances of these species. For instance, according to the mapping results of the clean reads to the assembled contigs, the relative abundances of the genes annotated to *Roseburia* and *Bifidobacteria* were lower than 0.1%. There should be more species of which their genomes remained to be constructed, and higher sequencing depth is needed to get more high-quality MAGs in the future study.

In summary, this study utilized a multi-omics approach to describe microbiota–immune/microbiota–muscle interactions in rhesus macaques under simulated microgravity. Despite the phylogenetic proximity, results from the study may not be completely recapitulated in humans. Nevertheless, this research has extended our understanding of the effects of gut microbiota on host physiology under microgravity and provided a novel strategy to enhance adaptation of astronauts in space, for instance, using dietary supplementation to increase the abundance of butyrate-producing microorganisms in the intestine during space missions.

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**Conflict of interest**

The authors declare no competing financial interests.

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**Ethics statement**

The authors certify that they comply with the ethical guidelines for authorship and publishing of the *JCSM Rapid Communications*.45
Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Schematic of the 42-day HDBR protocol. Sample collection time points are shown.

Figure S2. Venn diagram of gut bacteria KEGG ortholog (KO) functions and genera shared by rhesus macaques and humans. Each circle represents the number of genes or annotated functions for humans (left circle) and rhesus macaques (right circle).

Figure S3. Relative abundance values of Myroides in faeces from rhesus macaque subjected to HDBR. By using MetaWRAP software, the genus Myroides was reconstructed in all of samples and showed differential abundance during HDBR.

Figure S4. Changes in metabolic profile of skeletal muscle induced by HDBR. (A) Venn diagrams of differentially abundant features (DAFs) identified by univariate and multivariate analyses in muscle. (B) Heatmap of 154 DAFs induced by HDBR in atrophied muscle.

Figure S5. Differentially expressed genes (DEGs) induced by HDBR and enriched biological processes. (A) Histograms of DEGs between two time points. Up- and down-regulated DEGs are colored in orange and blue, respectively. (B) Gene Ontology (GO) analyses of 65 DEGs showing significant enrichment of several biological processes.

Figure S6. Relative abundance values of the KEGG orthologies (KOs) involved in the butyrate production pathway during HDBR.

Figure S7. Model of interactions between gut microbiota, skeletal muscle, and the immune system in rhesus macaques subjected to HDBR. HDBR increases the abundance of P. rettgeri, which produces 4-hydroxyphenylacetate 3-monoxygenase (HpAB), an enzyme that transforms 3-hydroxyphenylacetate into 3,4DPHA. Excess 3,4DPHA is absorbed by muscle and is involved in tyrosine metabolism in the process of muscle atrophy. In addition, an increased abundance of Myroides and Commononas leads to increased production of tryptophan 2,3-dioxygenase (TDO2) and kynureninase (KYNU), which are critical for tryptophan metabolism. The HDBR-induced decrease in the abundance of butyrate-producing colon bacteria such as Eubacterium, Roseburia, and Bifidobacterium results in a reduced amount of butyrate being released into the circulation, which contributes to impaired immune function and muscle atrophy.

Table S1. Metagenome sequencing data before and after quality control and after removing host data.

Table S2. Reference gene catalog of the Macaca mulatta gut metagenome.

Table S3. Abundance of 55 genera of gut bacteria with significantly altered abundance during HDBR.

Table S4. Number of features before and after quality control in skeletal muscle.

Table S5. Top enriched pathways associated with the 154 DAFs.

Table S6. PBMC transcriptome sequencing data before and after quality control.

Table S7. GO enrichment analysis of 65 DEGs in PBMCs.

Table S8. Meta data of Metabolomic from CNGB database.

Table S9. Meta data of Metagenome from CNGB database.

Table S10. Meta data of Transcriptome from CNGB database.

Table S11. P-value and the log fold change value in Figure S6.

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