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Relationship between the Gut Microbiome and Energy/Nutrient Intake in a Confined Bioregenerative Life Support System

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ABSTRACT Recent studies have suggested that the gut microbiome is modified in space analogs and that human health can be affected during actual spaceflight. However, the relationship between the gut microbiome and dietary intake in simulator subjects and astronauts remains unclear. Bioregenerative life support systems (BLSSs) are confined and self-sufficient ecosystems that enable exploration of this issue. Here, we correlate changes in gut microbes to the nutrient types present in controlled diets within subjects cohabitating in a BLSS. A metagenome-wide association study (MWAS) was performed on 55 shotgun-sequenced fecal samples longitudinally obtained from healthy Chinese subjects ($n = 4$ in total, $n = 2$ per sex) subjected to a 60-day BLSS stay and a specialized diet. Each food item was categorized based on nutrient type according to the Chinese Food Ingredients List (https://wenku.baidu.com/view/3f2b628488eb172ded630b1c59eef8c75fbf9514.html?from=search). The physical parameters of each subject fluctuated within normal medical ranges. Sex- and individual-specific differences and a trend of individual convergence of the gut microbiome in the BLSS were observed. Depletion of bacterial taxa such as *Faecalibacterium prausnitzii*, *Bifidobacterium longum*, and *Escherichia coli* and functional modules such as short-chain fatty acid (SCFA) production, as well as an increase in an unidentified *Lachnospiraceae* and glutamate/tryptophan synthesis, were observed in the BLSS. Correlation analysis showed that these compositional and functional changes were associated with energy/nutrient intake during the BLSS stay. Our findings suggest that the gut microbiota is a useful indicator for monitoring health and that individual nutritive diets should be considered according to sex and individual differences in simulations or in spaceflight.

IMPORTANCE The gut microbiome shows individual specificity and is affected by sex, environment, and diet; gut microbiome imbalance is related to cancer, cardiovascular diseases, and autoimmune diseases. Astronauts are faced with a challenging environment and limited diet in outer space. Recent studies indicate that the gut microbiome is altered in space simulators and space, but what happens to intestinal microorganisms when astronauts cohabitate in a self-sufficient ecosystem in which they plant and cook food is unclear. Bioregenerative life support systems (BLSSs) are ideal devices to investigate the above issues because they are closed and self-sufficient. Four healthy Chinese subjects cohabitated in a confined BLSS for 60 days, during which their physical parameters and energy/nutrient intake were recorded. We performed a metagenome-wide association study (MWAS) on 55 shotgun-sequenced fecal samples longitudinally obtained from the subjects. Alterations occurred in the gut microbial composition and function, and their relationships with energy/nutrient intake were explored.

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Human health is dramatically affected by outer space because of its hard-vacuum conditions, microgravity, electromagnetic radiation, and the required changes in the diets of astronauts (1). During both short- and long-term spaceflights and missions, the suitable amount of food, water, and oxygen and the proper temperature required to sustain astronauts' health must be ensured (2). Ground-based space simulators are an efficient way to explore the physical and psychological conditions of crew members during real spaceflight. The National Aeronautics and Space Administration (NASA)'s analogs, including Human Exploration Research Analog (HERA), Antarctic stations, Concordia, Aquarius/NASA Extreme Environment Mission Operations (NEEMO), Desert Research and Technology Studies (Desert RATS), NASA Space Radiation Lab (NSRL), parabolic flights, *in situ* resource utilization (ISRU), and Human Exploration Spacecraft Testbed for Integration and Advancement (HESTIA), are the most popular analogs used to simulate human reactions and for technological demonstrations under isolation and confinement in extreme and hostile environments to support the next generation of human exploration missions. However, these analogs are not bioregenerative and self-sufficient, and subjects must take food and other necessities with them, which is not practical for long-term and long-distance travel in space. Unlike these analogs, bioregenerative life support systems (BLSSs) are small, balanced, manmade ecosystems aimed at supporting life on space stations and spaceflights; these ecosystems include human beings, animals, plants, and microorganisms that are integrated with mechanical and physicochemical hardware to satisfy the environmental control functions of the space station and close the food cycle (3). Chinese Lunar Palace 1 (LP1) is a closed, ground-based BLSS designed to simulate the lunar environment, consisting of two plant cultivation cabins and an integrated cabin with four bedrooms, a washroom, a living room, and a waste treatment cabinet (4). LP1 integrates highly efficient plant cultivation, animal protein production, urine nitrogen recycling, and bioconversion of solid waste, representing a further step toward next-generation manned space exploration in which crew members plant and cook food themselves instead of consuming vacuum-packaged products.

The gut microbiome plays an important role in modulating human health and is susceptible to a variety of factors, such as the environment (5), diet (6), and sex (7). Changes in the composition and function of the gut microbiome are closely related to chronic diseases such as cancer, autoimmune diseases, and cardiovascular diseases (8), as well as mental disorders such as anxiety and depression (9). During space simulator experiments or real spaceflights, the crew members must stay in isolation, with limited space and altered diets. Studies have shown that long-term consumption of vacuum-packaged products may promote the growth of anaerobic bacteria, affecting the composition of the gut microbiome (10). In addition, the confined living conditions may also influence the appetites of astronauts (11) and the efficacy of energy harvest from food (12). However, few studies have investigated the influence of energy and dietary nutrient intake on the gut microbiome in either analogs or real spaceflights. BLSSs are highly closed and self-sufficient ecosystems in which there is no exchange of matter and energy, but electricity is provided via outside sources, and the crew members plant and cook food according to a special formula, making it possible to record and investigate the effect of energy/nutrient intake on the gut microbiome.

In this study, four healthy Chinese volunteers ($n = 2$ per sex) were enrolled in the BLSS for 60 days without interruption after physical examination and psychological assessment. The energy and nutrient type of each food item were classified according to the Chinese Food Ingredients List (https://wenku.baidu.com/view/3f2b628488eb172ded630b1c59eef8c75fbf9514.html?from=search), and the physical indicators of the subjects were recorded and calculated. Fourteen fecal samples from each subject

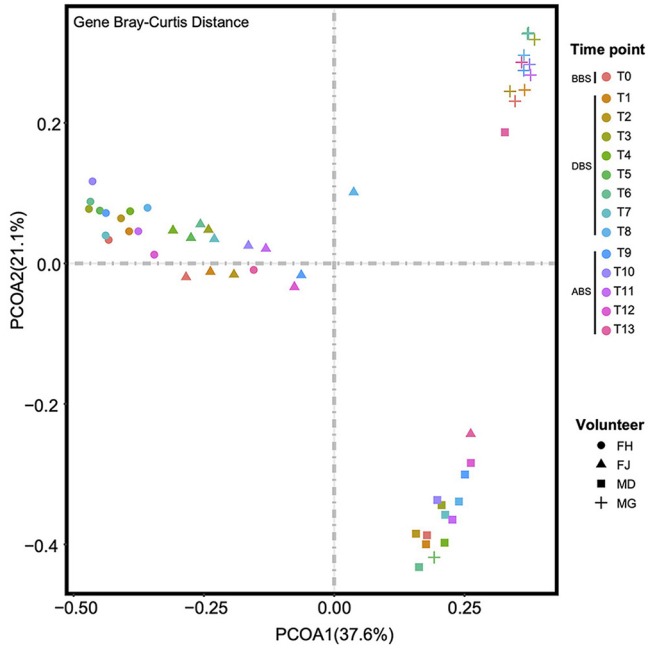

FIG 1 PCoA of the gut microbiome based on gene Bray-Curtis distances for all samples. Different shapes represent samples from different subjects (circle, FH; triangle, FJ; square, MD; cross, MG). Different colors indicate the time points. BBS, DBS, and ABS represent the baseline, during the BLSS stay, and after leaving the BLSS, respectively.

were longitudinally collected over the week before entering the BLSS, during the stay, and after leaving the BLSS. A metagenome-wide association study (MWAS) (13) was performed on 55 shotgun sequencing data sets. Our study illustrated the changes in gut microbial composition and function over time and investigated the relationships between these changes and the intake of different energy/nutrient types. Our results will not only play a crucial role in providing useful information and strategies to improve similar studies but will also provide important tips and guidance for the maintenance of human physiological and mental health in next-generation space explorations.

RESULTS

Overall changes in the gut microbiome over time. Samples from each subject were divided into three stages: baseline (T0, BBS), during the BLSS stay (T1 to T8, DBS), and after leaving the BLSS (T9 to T13, ABS). Changes over time in the gut microbiota were examined by shotgun metagenome sequencing of DNA extracted from longitudinally collected fecal samples from the four subjects. High-quality sequencing reads (see Table S1a in the supplemental material) were aligned to the latest human gut microbial gene set (14) to obtain the gene profile. The taxonomic profiles were calculated by MetaPhlAn2 (15) and are shown in Table S1b to d. For functional changes, putative amino acid sequences translated from the gene catalog (14) were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (16), and 9,793 KEGG orthologs (KOs) were identified. Then, 104 gut metabolic modules (GMMs; evaluating gut metabolic potential and anaerobic fermentation capacity; Table S1e) and 55 gut-brain modules (GBMs; describing the relationship between the gut microbiome and mental health; Table S1f) were annotated according to Vieira-Silva et al. (17) and Valles-Colomer et al. (18).

Sex- and individual-specific differences were observed in our study at the gene and genus levels by principal coordinate analysis (PCoA) (Fig. 1, gene level; Fig. S1c, genus level). Additionally, a trend of gut microbiome compositional convergence was observed during the subjects' stay at the BLSS (Fig. 1; Fig. S2a [species level] and b [genus

level]). In addition, the Shannon index displayed a slight difference among the BBS, DBS, and ABS samples (Fig. S1b to c; $P < 0.05$, tested with the Kruskal-Wallis rank sum test). To further illustrate the differences at the species level, GMMs and GBMs were compared among the BBS, DBS, and ABS samples (Tables S2 to S4; $P < 0.05$, tested with the Kruskal-Wallis rank sum test). Then, the top 20 species and genera were selected based on their mean relative abundances (Fig. 2; Fig. S3) to explore the characteristics of highly abundant intestinal microorganisms. At the genus level, female H (FH) had *Prevotella* as the dominant microbial genus during the experiment. For female J (FJ), *Prevotella* was the most abundant genus in BBS and DBS, which shifted to *Bacteroides* and *Faecalibacterium* at ABS T9 and T10. For male D (MD), the abundance of the dominant genus in BBS, *Bacteroides*, decreased in DBS, while the abundance of *Eubacterium* and *Roseburia* increased, and then, the dominant genus shifted to *Faecalibacterium* at ABS T11 and T12. The predominant genus of male G (MG) shifted from *Faecalibacterium* in BBS to *Bacteroides* as soon as he entered the facility, and this change was sustained. At the species level, *Prevotella copri* was the major species for female H (FH) and female J (FJ) in DBS. Male D (MD) had additional species, including *Prevotella stercorea*, *P. copri*, *Roseburia intestinalis*, and *Faecalibacterium prausnitzii*. Male G (MG) had a high relative abundance of *Alistipes onderdonkii* and *Bacteroides* spp. such as *B. ovatus* and *B. cellulosilyticus*.

Dietary nutrient intake is related to gut microbiome composition. We were interested in the effects of dietary nutrient intake on the gut microbiomes of subjects, since BLSSs are ideal environments to record and calculate the intake of various nutrients and energy sources. The physical parameters, including body mass index (BMI), blood pressure (BP), and heart rate (HR), of the subjects fluctuated within normal reference ranges (Table S5a). An average of 7 hours of total sleep time (TST) and 2 hours of deep sleep time (DST) were observed for all subjects, which is normal for adults, and the females seemed to wake up less during the night in our study (Table S5b), which might be due to an increase in *P. copri* abundance and tryptophan synthesis (as well as a decrease in propionate synthesis I in females in DBS), which were negatively correlated with awakenings at night (AWNs) (Fig. S4). Difference analysis (two-tailed Student's *t* test, $P < 0.05$) revealed that all of the subjects had a higher percentage of carbohydrates and lower fat levels in DBS samples than in samples collected outside the BLSS. Additionally, all subjects consumed *Tenebrio molitor* as a source of protein and edible fungi at the T5, T6, and T8 time points during their stay in the BLSS (Table S5c).

Permutational multivariate analysis of variance (PERMANOVA) was performed to analyze the influence of the intake of food energy sources (Table S5e) and various dietary nutrients (Table S5f) on the gut microbiome. The energy sources and nutrients that had a significant effect ($P < 0.01$, $q < 0.01$) on the gut microbiome are shown in Table S5d. To investigate the relationship between these significant changes in energy/nutrient intake and changes in species ($P < 0.05$; union set of the Kruskal-Wallis rank sum test among BBS, DBS, and ABS in all female and male samples), Spearman's rank correlation was performed. The coefficient of association (rho) is shown by a heatmap (Fig. 3; +, $P < 0.05$; *, $P < 0.01$). Except for vitamin B_5 and vitamin B_9, intake of all the significantly related energy sources/nutrients was negatively correlated with *Bacteroides intestinalis*, *P. copri*, and *Coprobacter fastidiosus* but positively associated with *Anaerostipes hadrus* and *Bacteroides vulgatus*. The abundance of *B. intestinalis*, which is a human colonic microorganism and upregulates multiple endoxylanases during growth on xylan, decreased in DBS samples from females (19). The abundance of *P. copri*, which is involved in dietary fiber-induced improvement in glucose metabolism (20) and is enriched in some elderly rheumatoid arthritis (RA) patients (21), was increased in DBS samples from females. *C. fastidiosus* is a novel member of the family *Porphyromonadaceae* that was isolated from infant feces (22). *A. hadrus* is a dominant species within the human colon that produces butyrate (23) and was depleted in DBS samples from females. *B. vulgatus* can reduce gut microbial lipopolysaccharide produc-

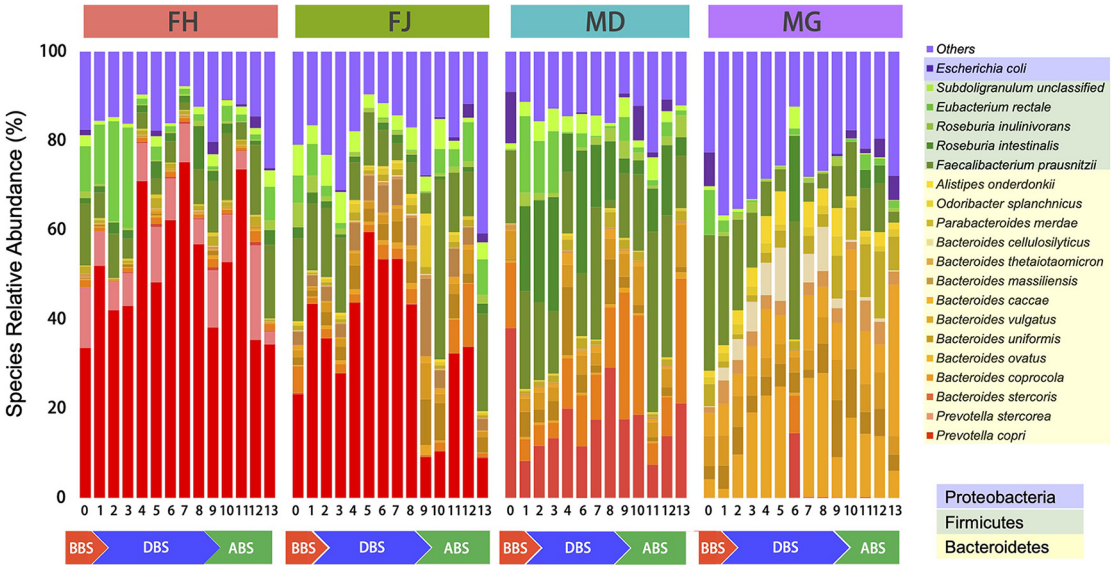

FIG 2 The top 20 species of the gut microbiome based on mean relative abundance. BBS, DBS, and ABS represent the baseline, during the BLSS stay, and after leaving the BLSS, respectively. The colors of the boxes shown below the figure key indicate the phylum that each genus belongs to.

tion and inhibit atherosclerosis (24) and was depleted in DBS samples from males. In addition, an unidentified *Lachnospiraceae* species, a fiber specialist (25), was enriched in DBS samples from all subjects. The abundance of *F. prausnitzii*, a well-known butyrate producer, was positively correlated with available (digestible) carbohydrate and water-from-food levels and decreased in DBS samples from females. The abundance of *Clostridium leptum*, which can metabolize plant polysaccharides (26), was negatively correlated with amino acid content and decreased in DBS samples from all subjects. In addition, some poorly correlated and possibly pathogenic bacteria, such as *Escherichia coli* (some types can cause illnesses in humans, including diarrhea, abdominal pain, fever and vomiting [27]), *Enterobacter cloacae* (might be opportunistic and infectious [28]), and *Veillonella atypica* (might cause infection [29]), were also depleted in DBS samples.

Functions inferred from metagenomic information. To further illustrate the associations between energy/nutrient intake and the gut microbiome, significantly changed ($P < 0.05$; union set of the Kruskal-Wallis rank sum test among BBS, DBS, and ABS samples at the all, female, and male levels) gut microbiome functional modules were characterized and associated with energy/nutrient intake (Fig. 4; +, $P < 0.05$; *, $P < 0.01$).

Functional GMMs, including rhamnose degradation, pyruvate dehydrogenase complex, glycine degradation, and histidine degradation, which were depleted in females, and 4-aminobutyrate degradation, which was depleted in DBS samples from males, were positively related to almost all energy/nutrient intake categories except for vitamin B_5 and vitamin B_9. Meanwhile, the methionine degradation II pathway, arabinose degradation, fructan degradation, and triacylglycerol degradation were enriched in all individuals, the threonine degradation I pathway and the butyrate production II pathway were depleted in all individuals, and the glutamate degradation III and galacturonate degradation I pathways were depleted in DBS samples from males and showed strong negative associations with various phenotypes.

GBMs, including the glutamate degradation II pathway, the glutamate synthesis II pathway, and tryptophan synthesis, showed strong negative associations with most of the phenotypes, while the acetate synthesis II pathway and the propionate synthesis I pathway showed the opposite trend. For glutamate, which is a transmitter in the healthy brain and has excitatory effects on nerve cells (30), synthesis increased and

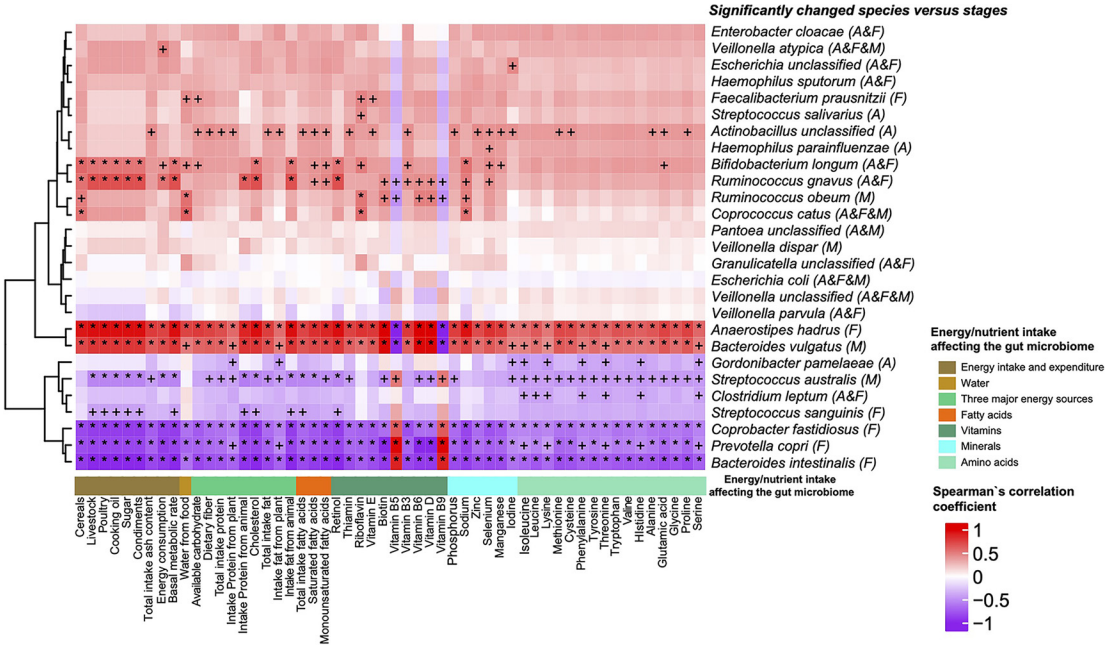

FIG 3 Heatmap of the Spearman's rank correlation between changed species (*y* axis, by the Kruskal-Wallis rank sum test among stages and Wilcoxon rank sum test between stages, $P < 0.05$; the letters A, F, and M in brackets after the species name indicate whether the species was significantly changed in all subjects, female subjects, and male subjects, respectively) and energy/nutrient intake, which might have an influence on the gut microbiome (*x* axis, assessed by PERMANOVA based on the gut microbial gene profile, $q < 0.01$). Red indicates positive associations, while purple indicates negative associations. +, $P < 0.05$; *, $P < 0.01$. The *x* axis shows energy intake and expenditure (brown), water (light brown), three major energy sources (light green), fatty acids (orange red), vitamins (dark cyan), minerals (cyan), and amino acids (turquoise) from left to right.

degradation decreased in DBS samples from males. Tryptophan, an essential compound that can be synthesized by bacteria in humans, acts as a precursor to the neurotransmitter serotonin, the hormone melatonin, and vitamin B_3 (31). Tryptophan synthesis was significantly elevated in DBS samples and decreased in ABS samples from all subjects. Acetate, propionate, and butyrate are important SCFAs synthesized by colonic bacteria in humans, and their synthesis decreased in DBS samples.

DISCUSSION

In short- and long-term space travel or space simulators, crew members are exposed to harsh space environments and experience changes in dietary models that can potentially affect the composition and function of their gut microbiomes, which may have a negative effect on their health. Previous studies performed on simulated or real outer-space conditions have suggested that space travel may cause both compositional (2, 32, 33) and functional (34–37) changes in the gut microbiome. However, it is not clear what happens to the human gut microbiome when humans are in a simulated outer-space environment with specialized diets and closed sources of vital necessities such as water and oxygen. This study uses an MWAS to characterize the unique features of microecological diversity, composition, and functional changes in the gut microbiota and their associations with dietary nutrient and energy intake on four healthy Chinese adults cohabitating in a confined BLSS for 60 days.

In our study, the monitored physical parameters, such as BMI, weight, sleep, BP, and HR, were shifted within normal medical ranges, which indicated that the subjects were all in a healthy physiological state. Additionally, crew members showed neither behavioral disturbance nor psychological distress during their stay in the BLSS (38). For the gut microbiome, we did not find significant changes that adversely affected human health, but a trend of gut microbial convergence was observed for each subject during their stay in the BLSS, which was in accordance with a previous long-term (6- to 12-month) space exploration study performed by Voorhies et al. (33). This might be due

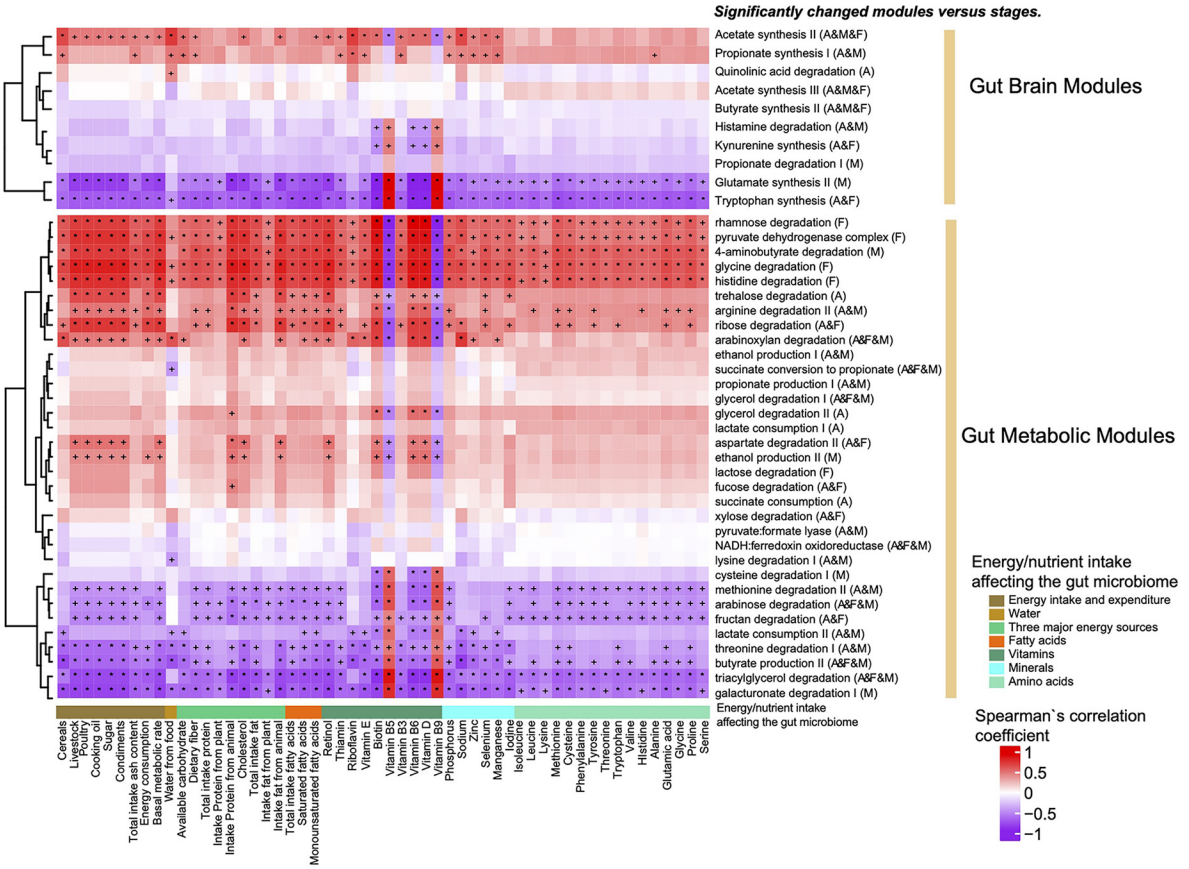

FIG 4 Heatmap of the Spearman's rank correlation between changed functional modules (*y* axis, GBMs, and GMMs using the Kruskal-Wallis rank sum test among stages and Wilcoxon rank sum test between stages, $P < 0.05$; the letters A, F, and M in brackets after the species name indicate whether the species was significantly changed in all subjects, female subjects, and male subjects, respectively) and energy/nutrient intake, which might affect the gut microbiome (*x* axis, assessed with PERMANOVA based on the gut microbial gene profile; $q < 0.01$). Red indicates positive associations, and purple indicates negative associations. +, $P < 0.05$; *, $P < 0.01$. The *x* axis shows energy intake and expenditure (brown), water (light brown), three major energy sources (light green), fatty acids (orange red), vitamins (dark cyan), minerals (cyan), and amino acids (turquoise) from left to right.

to either the synchronization of diets during the BLSS stay or an increase/decrease in the abundance of some bacterial taxa, such as *P. copri*, *Eubacterium rectale*, *Bacteroides stercoris*, and *B. ovatus*, as well as the interactions between diets and gut microbiomes. However, we did not observe a similar gut microbial composition across subjects, which might be a result of the short-term (60-day) cohabitation of the subjects. In addition, we found individual- and sex-specific trends in the gut microbiome in our study, which were consistent with the results obtained in a normal environment (7, 39). Not coincidentally, interindividual variability was also found by Jin et al. (40) among six Japanese men who explored Antarctica for 3 months, during which period a reduction in *Bifidobacterium* species abundance was observed, just as in our study. The difference was that we did not observe an increase in the abundance of *Bacteroides thetaiotaomicron*, which is thought to proliferate during emotional stress and might be a microbial cause of nonapparent emotional changes in subjects between being inside and outside the BLSS (38). According to our findings, cohabitation for 60 days in a confined BLSS does not compromise the compositional and functional layout of the individual- and sex-specific microbiota, suggesting the resilience of the individuality of the gut microbial ecosystem, which was consistent with the previous long-term Mars500 experiment (2).

The gut microbiome is known to be influenced by the environment and diet (41). We found certain significantly changed genera, species, and functional modules between samples collected inside and outside the BLSS, especially between DBS and ABS

samples (Table S2), which suggested that the gut microbiome can be shifted by the BLSS and the specialized diet in the BLSS. This finding was consistent with the previous NASA Twins study (32), in which the gut microbial community structures of the twin subjected to long-term flight showed a significant difference between in-flight samples and the pre- and postflight combined samples, but those of the twin on the ground did not. We tried to investigate the relationship between significant changes in the gut microbiome across the BLSS and energy/nutrient intake in the BLSS based on a previous study (42). During their stay in the BLSS, all the subjects had a specialized dietary pattern, with a significantly increased intake of carbohydrates and decreased intake of fat, which has been reported to be beneficial for human health (43). Consistent with expectations, the relative abundance of bacteria that feed on fiber and carbohydrates, such as *Lachnospiraceae* (25) and *P. copri* (20), was increased in DBS samples. Interestingly, we also observed a decrease in the abundance of possible pathogenic microbes, such as *E. cloacae* (28) and *V. atypica* (29), in DBS samples, which might be due to the high-carbohydrate/low-fat diet. However, the decreased abundance of some SCFA-producing bacteria, such as *B. longum* (44) and *F. prausnitzii* (45), in DBS samples might explain the decrease in the synthesis of SCFAs, such as propionate and butyrate.

Infection/inflammation and cardiovascular diseases are the main threats faced by astronauts. Increased abundance of RA-related bacteria, such as *C. leptum*, and decreased abundance of *Enterobacter* spp. and *B. vulgatus* in DBS samples might also be risk factors for infection/inflammation and acute arterial events (24, 46). Additionally, the decreased capacity for SCFA synthesis during the BLSS stay may also indicate a decreased capacity for anti-inflammatory activity. Sleep disorders represent another problem faced by astronauts in space, not only affecting cognitive abilities and overall health but also disrupting the body's circadian rhythm, leading to fatigue and mood changes, as well as metabolic disorders, heart disease, and gastrointestinal problems and, in turn, to accidents on the job. The increased relative abundance of *Haemophilus parainfluenzae* and *Haemophilus sputorum* (Fig. S4a), which were negatively correlated with TST but positively correlated with AWNs, in DBS samples from all the subjects might have affected the sleep quality of the subjects. In addition, alterations in some GBMs, such as the synthesis of propionate, butyrate, and glutamate, in our study also indicated that the gut microbiome may play a role in mental disorders such as anxiety (47) and depression (48) in those confined in the BLSS, although there were no obvious emotional changes in the subjects (38). In addition, specific links between significantly changed gut microbes/functional modules and energy/nutrient intake passed from BBS to DBS to ABS samples, as described in Results, also show that the gut microbiota should be monitored in those confined in BLSSs.

Notably, the general and sex-specific trends described in this study do not necessarily reflect what happens in other subjects because of the individual-specific resilience of the gut microbiome. We will consider adding more space-related risk factors, such as weightlessness and electromagnetic radiation, to the BLSS to simulate a more realistic space environment. In addition, the crops and fruits in this experiment need proper temperature and humidity conditions, and we will introduce some drought-tolerant and hypoxia- and low-temperature-resistant plants such as millet and highland barley into the BLSS in the future. Moreover, the protein supply in the experiment mainly came from *T. molitor* cultivated in the BLSS and vacuum-compressed chicken and pork brought in from outside; we will try to cultivate some other animals that can provide nutrition to humans to achieve complete closure of the BLSS. In addition, we believe that it would be beneficial to add more samples to the BBS group in the future.

In summary, our results revealed the compositional and functional changes in the gut microbiota and their relationships with dietary nutrient and energy intake in a BLSS. Sex- and individual-specific differences in the gut microbiome indicated that sex and individuality should be considered when designing similar simulated experiments or real space missions. Moreover, changes in some specific bacteria, including *B. longum*, *F. prausnitzii*, *E. coli*, and *E. cloacae*, and functional modules, such as SCFA production,

also indicated that it might be helpful to balance the gut microbiome and maintain subject health by providing prebiotics/probiotics and individual dietary guidance depending on individual gut microbiome characteristics.

MATERIALS AND METHODS

Statement of institutional review board approval. This study was approved by the Bioethics Committee of Beihang University and BGI-Shenzhen. All subjects voluntarily participated in this project and signed an informed consent form.

Volunteer enrollment, sample collection, and phenotype determination. Four healthy volunteers (female H [FH], female J [FJ], male D [MD], and male G [MG]) with ages from 23 to 27 years and BMIs from 18.5 to 22.9 were enrolled after taking a general physical examination and profile of mood states (POMS) psychological assessment. Fecal samples were collected weekly during the BLSS stay. One and five samples were obtained before and after the BLSS stay, respectively. In total, 14 samples were obtained for each subject, immediately stored at −80°C, and transferred to the laboratory on dry ice when the experiment was complete.

Various phenotypic metadata (Table S5a and b) were recorded during the BLSS stay, including body indexes (body weight [BW], fat-free body weight [FFBW], BMI, systolic BP [SBP], diastolic BP [DBP], HR), sleep indicators (TST, DST, AWN), total carbohydrate, fat, and protein intake ratios during the whole experiment (Table S5c), total energy intake and consumption, and basal metabolic rate (BMR). Consumption of multitudinous dietary nutrients, including water, vitamins, minerals, amino acids, fatty acids, and fat/protein from animals/plants was recorded via a food frequency questionnaire (FFQ) (Table S5e to h) and calculated according to the Chinese Food Ingredients List (version 2015).

DNA preparation and metagenomic sequencing. DNA extraction was performed using the phenol/trichloromethane method after thawing samples on ice. Extracts were treated with DNase-free RNase, and DNA quality was measured using agarose gel electrophoresis and a Qubit 3.0 fluorometer (Thermo Fisher, Waltham, MA, USA). A paired-end metagenomic sequencing strategy was performed on a BGI-SEQ500 platform (49) (insert size, 350 bp; read length, 100 bp).

Quality control and host genome filtering. The raw reads that had 50% low-quality bases (quality ≤20) or more than five ambiguous bases were excluded. The remaining reads were mapped to the human genome (hg19) using SOAP v2.22 (-m, 100; -x, 600; -v, 7; -p, 6; -l, 30; -r, 1; -M, 4; -c, 0.95), and the matching reads were removed (49). An average of 6.5 gigabytes of data was generated for each sample (Table S2).

Taxonomic profiling of metagenomic samples. The high-quality nonhuman reads were defined as clean reads and aligned against the latest human gut microbial 11.4-M (14) gene catalog through SOAP v2.22 (-m, 100; -x, 600; -v, 7; -p, 6; -l, 30; -r, 1; -M, 4; -c, 0.9) to generate the gene profile. MetaPhlAn2 (15) (–input_type fastq –ignore_viruses –nproc 6) was used to generate phylum, genus, and species profiles from the clean reads.

Acquisition of gut metabolic/brain module profiles. Putative amino acid sequences were translated from the gene catalogs and aligned against the proteins or domains in the KEGG databases (release 79.0, with animal and plant genes removed) using BLASTP v2.26 (default parameters except -m, 8; -e, 1e-5; -F, -a, 6; and -b, 50). Each protein was assigned to a KEGG ortholog group on the basis of the highest scoring annotated hit(s) containing at least one segment pair scoring over 60 bits. The relative abundance profile of KOs was determined by summing the relative abundance of genes from each KO using the mapped reads per sample (40). The abundance of each GMM (-a, 2; -d, GMM.v1.07.txt; -s, average) and GBM (default parameters) was calculated as shown previously (17, 18).

PERMANOVA of the effects of various phenotypic metadata on the gut microbiome. PERMANOVA (50) was performed on the gene abundance profile of the samples to assess the effect of the intake of each energy and nutrient type listed in Table S5e to h, and the results are shown in Table S5d. We used Bray-Curtis distance and 9,999 permutations in R (v3.10, vegan package, R Project for Statistical Computing) (51). The *P* values were adjusted with the Benjamini-Hochberg correction.

Richness and diversity analysis. Alpha diversity (within samples) at the gene and genus levels was quantified with the Shannon index using relative gene abundance profiles. Beta diversity (between samples) at the gene and genus levels was calculated based on Bray-Curtis distance (R v3.2.5, vegan package 2.4-4).

Correlation analysis. Spearman's rank correlation was used to evaluate the relationships between the changed phenotypes (evaluated with PERMANOVA) and the changed gut microbiota (at the species level) as well as similarities between samples. Coefficient of association (rho) results are shown by heatmaps (Fig. 3 and 4), and the significance is indicated by the *P* value (+, $P < 0.05$; *, $P < 0.01$).

Statistical analysis. The changes in the gut microbiome composition and functional modules for subjects among different stages (BBS, DBS, and ABS) were evaluated using the Kruskal-Wallis rank sum test. The ratios of carbohydrate, fat, and protein intake inside and outside the BLSS were calculated using Student's *t* test.

Data availability. The high-quality nonhuman reads that support the findings of this study have been deposited in the CNSA (https://db.cngb.org/cnsa/) of CNGBdb under the accession number CNP0000408.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.3 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.04 MB.
SUPPLEMENTAL FILE 5, XLSX file, 0.03 MB.
SUPPLEMENTAL FILE 6, XLSX file, 0.1 MB.

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