Implicated Connections Between The Gut Microbiome and Bone Homeostasis within Spaceflight

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**GRAPHICAL ABSTRACT**

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**Summary**

Mankind’s exploration into the cosmos coincides with increasing space travel durations and demands upon the human body. Understanding how the microbiome may impact human physiological homeostasis is one of the essential tasks in managing deep-space travel associated health risks. As part of the National Aeronautics and Space Administration (NASA) led Rodent Research (RR) 5 mission, which involved the first live-animal return (LAR) rodent flight in the US, evaluation of the possible connection between gut microbiome and bone homeostasis was enabled using a non-ovariectomy rodent model with exacerbated osteoporosis through microgravity exposure. The gut microbiome from the following four rodent groups were analyzed: 1) ISS, constant microgravity exposure (9 weeks total) at the International Space Station before termination; 2) ISS\_G, the terrestrial control for ISS; 3) LAR, initial microgravity exposure (4.5 weeks total), then returned alive to earth for recovery (4.5 weeks) before termination; 4) LAR\_G, the terrestrial control for LAR. We demonstrate the ISS group maintained elevated alpha and beta diversity associated with microgravity relative to the control ISS\_G. In comparison with ISS, the LAR group’s gut microbial diversity re-established to pre-flight levels after returning to Earth. Metagenomic and LC-MS metabolomic analyses showed that the ISS group had elevated metabolite production detected in the blood plasma sera relative to ISS\_G. The gene clusters responsible for production of these metabolites were functionally assigned to the highly enriched *Lactobacillus murinus* and *Dorea sp.* within the ISS group. These metabolites, such as lactic acid, leucine/isoleucine, and glutathione, have been reported to directly influence bone formation in terrestrial studies through known mechanisms. We also found differential elevated serum levels of Osteocalcin, a sensitive biomarker for osteoblastic activity, and reduction in Tartrate-resistant acid phosphatase (TRACP 5b), a reliable osteoclastic biomarker, associated with bone formation and resorption, respectively, in ISS versus ISS\_G. These data suggest a modulatory effect of specific bacterial secondary metabolites on host bone homeostasis,which provides not only a potential molecular connection between gut microbiome and bone homeostasis, but also new avenues for designing molecular therapeutics to mitigate osteoporosis.

**Introduction**

Low-Earth orbit space travel induces great stress to the human body, including galactic cosmic radiation, sleep deprivation, psychological stress, and microgravity ([Voorhies and Lorenzi, 2016](#_ENREF_107); [Afshinnekoo et al., 2020](#_ENREF_1)). The physical elements which greatly alter the homeostasis of human physiology normally experienced on Earth and microgravity are correlated with human ([Garrett-Bakelman et al., 2019](#_ENREF_37); [Voorhies et al., 2019](#_ENREF_108); [Liu et al., 2020](#_ENREF_60); [Urbaniak et al., 2020](#_ENREF_105)) and rodent microbiome ([Ritchie et al., 2015](#_ENREF_86); [Jiang et al., 2019](#_ENREF_49)) alterations; although, the health consequences of microbiome shifts within this environmental context remain vastly underexplored. Furthermore, the difficulty of consistently obtaining microbiome samples at desired time-points and reliance of culture-dependent assessment ([Taylor et al., 1977](#_ENREF_98)), hindered rapid exploration. Prior to the Rodent Research (RR) mission series (RR-1and 5), which was initiated by Center for Advancing Science in Space (CASIS) and National Aeronautics and Space Administration (NASA) Ames Research Center in 2014 and is conducted onboard the International Space Station U.S. National Laboratory, animal research in space has been limited to less than 3 weeks of spaceflight onboard space shuttles with limited sample size (N=6-8), particularly for studying bone homeostasis ([Vico and Hargens, 2018](#_ENREF_106); [Coulombe et al., 2020](#_ENREF_21)). As such, these studies were incomparable to human low-earth orbit durations and due to the fact NASA’s development of Rodent Habitat in 2014 ([Ronca et al., 2019](#_ENREF_87); [Choi et al., 2020](#_ENREF_20)), superseding the Animal Enclosure Module (AEM) ([Moyer et al., 2016](#_ENREF_77)), enabled long-duration rodent research onboard the International Space Station with a large sample size and a variety of experimental procedures ([Ronca et al., 2019](#_ENREF_87); [Choi et al., 2020](#_ENREF_20)). Benefitting from this technology, the RR-5 mission signifies the United States of America’s first live animal-return (LAR) of rodents from the International Space Station **(CITE RR5 primary Ting,Kwak,Soo paper)**. The primary objective of the RR-5 mission was to investigate if BP-NELL-PEG systemic therapy combats long-duration spaceflight-induced osteoporosis ([Shi, 2019](#_ENREF_93)). As a microbiome focused ancillary study, the gut and oral microbiome ecology and their intersection with the bone homeostasis axis was examined. RR-5 provided a unique opportunity to test the use of a non-ovariectomy BALB/c rodent as a potential *in vivo* rodent model system for evaluating the effects of microgravity on the host’s microbiome and bone homeostasis because of notable as well as reproducible exacerbated bone-loss in mammals ([Vico and Hargens, 2018](#_ENREF_106); [Coulombe et al., 2020](#_ENREF_21)).

Understanding the dynamic interactions among the commensal microbiome will be important for developing therapies in order to maintain healthy homeostasis for future space travel, including those of increased duration ([Voorhies and Lorenzi, 2016](#_ENREF_107); [Afshinnekoo et al., 2020](#_ENREF_1)). Innovations in genomic science and technology have enabled vast improvements in understanding the effect of the microbiome on both health and disease-associated states, such as the landmark NIH human microbiome project ([Peterson et al., 2009](#_ENREF_81)). Bacterial dysbiosis of the commensal microbiota, which are essential mutualists within the human gut ([Bäckhed et al., 2005](#_ENREF_8); [Ley et al., 2006a](#_ENREF_57); [Turnbaugh et al., 2007](#_ENREF_102); [Qin et al., 2010](#_ENREF_83)), is correlated with multiple diseases ranging from various gastrointestinal (GI) disease states ([Manichanh et al., 2006](#_ENREF_68); [Frank et al., 2007](#_ENREF_33); [Sartor, 2008](#_ENREF_89); [Willing et al., 2009](#_ENREF_109); [Saulnier et al., 2011](#_ENREF_90)), altered bone homeostasis ([Sjögren et al., 2012](#_ENREF_94); [Hernandez et al., 2016](#_ENREF_42); [Li et al., 2016](#_ENREF_59); [Schwarzer et al., 2016](#_ENREF_92); [Yan et al., 2016](#_ENREF_110); [Guss et al., 2017](#_ENREF_39)), various types of cancer ([Schwabe and Jobin, 2013](#_ENREF_91)), obesity ([Ley et al., 2006b](#_ENREF_58); [Turnbaugh et al., 2006](#_ENREF_103)), diabetes ([Larsen et al., 2010](#_ENREF_54); [Giongo et al., 2011](#_ENREF_38)), neurodevelopmental disorders ([Hsiao et al., 2013](#_ENREF_44)), and even depression ([Luna and Foster, 2015](#_ENREF_64)). In addition to the most recent NASA Twin study ([Garrett-Bakelman et al., 2019](#_ENREF_37)) other long term human astronaut studies ([Voorhies et al., 2019](#_ENREF_108)), have repeatedly shown intriguing increases in gut bacterial diversity in-flight relative to terrestrial conditions. This observation was reproducibly recapitulated in rodents from the NASA’s RR-1 mission ([Jiang et al., 2019](#_ENREF_49)). Lastly, while future studies with greater sampling sizes and temporal durations, such as the RR-7 mission and the NASA Astronaut Microbiome project already underway ([Voorhies and Lorenzi, 2016](#_ENREF_107)), there remains no reliable or well established rodent research model to test hypotheses generated from observational studies in humans and bone homeostasis. This study aimed to bridge this current knowledge gap.

**Results**

**Longitudinal assessment of the LAR microbiome on Earth reveals reduction in microbiome diversity relative to microgravity (Experiment 2).**

The RR-5 mission **(CITE RR5 primary Ting,Kwak,Soo paper)** housed 40 rodents onboard the International Space Station for 4.5 weeks, after which 20 rodents were live returned (Live-Animal Return group, or LAR) to Earth and delivered to the University of California, Los Angeles (UCLA) to examine the effect of recovery for additional 4 weeks. The other 20 rodents remained in the International Space Station for a full 9 week duration (Full-Term Flight group, or ISS). 40 matching ground control groups were housed in the Kennedy Space Center (KSC) in the International Space Station Environmental Simulator (ISSES), LAR\_G and ISS\_G, with LAR\_G group being delivered to UCLA at the same time as LAR group (see graphical abstract) The rodents that did not receive drug therapy, non-treatment (phosphate buffered saline injected) control groups were analyzed pre-flight, Live Animal Return to Earth (4.5 weeks), and at the terminal time-point (post-launch, week 9) of the study.Corresponding oral swabs and fecal pellets provided the microbiome samples and were analyzed using 16S rRNA amplicon sequencing for the LAR and LAR\_G groups (Figure 1). To assess biodiversity of these communities, taxonomically assigned reads were converted to Hill Numbers ([Hill, 1973](#_ENREF_43)) or ‘Effective Number of Species/Genera’, which has recently been reported to offer a more biologically intuitive microbiome compositional metric that is a mathematically unified set of diversity measures as well as obeys *the replication principle or doubling property*, as previously described ([Jost, 2007](#_ENREF_50); [Chao et al., 2010](#_ENREF_17); [Chao et al., 2014](#_ENREF_18); [Hsieh et al., 2016](#_ENREF_45)). This approach enabled a robust analysis of 16S rRNA phylogenetic data throughout the study by factoring in relative abundance and phylogenetic diversity accordingly. In previous human studies, elevated microbial diversity was observed in flight, but returned to pre-flight levels post-Earth return after varying durations in recovery ([Garrett-Bakelman et al., 2019](#_ENREF_37); [Voorhies et al., 2019](#_ENREF_108)). Live Animal Return to Earth (4.5 weeks) analysis demonstrated the LAR flight group re-established to pre-flight levels after returning to Earth, comparable in alpha (Figure 1A, 1E, 1I) and beta diversity (Figure 1B, 1F, 1J) as well as with respect to the Firmicutes/Bacteroidetes ratio (Figure 1C,1G,1K), a well-accepted metric for gut dysbiosis in GI disease states ([Peterson et al., 2008](#_ENREF_80)), in the gut (Figure 1) and oral (Figure S1) microbiome composition relative to LAR\_G. This observation held true until termination (week 9), indicating a stabilization of the gut microbiome, when assessing fresh fecal samples (Figure 1, Tables S1-S3), fecal samples obtained at necropsy (Figure S2A, Table S4), as well as samples obtained from oral swabs (Figure S1A-J, Tables S5-7). Additionally, LAR to Earth analysis revealed enrichment of gut microbiome genera *Lactobacillus, Ruminiclostridum 9, Shuttleworthia,* and *Acetatifactor* with loss of *Escherichia-Shigella*, *Hungatella*,and *Acetatifactor*, that were not differently abundant by termination (Figure 1).

**Microgravity influences microbial diversity of the ISS flight group (Experiment 1).** The most recent assessment of the rodent gut microbiome exposed to microgravity was of modest sample size (N=6) and involved only a short duration exposure of 37 days ([Jiang et al., 2019](#_ENREF_49)). To gain insights into the effects of longer-term exposure to microgravity, the gut microbiome of the ISS group in the present study, which remained in the International Space Station until termination (week 9), was compared to its terrestrial corresponding ground control, ISS\_G. Fecal samples were collected at necropsy at termination after euthanasia. Evaluation of the microbiome diversity via 16S rRNA amplicon sequencing of the ISS group and its corresponding ground control, ISS\_G, revealed statistically significant increases in overall alpha and beta diversity compared to the ISS\_G control group as previously observed ([Jiang et al., 2019](#_ENREF_49)), albeit with no change in genera richness, that were correlated with microgravity exposure (Figure 1). Hill number analysis determined a mean of 72 genera, genera richness, that were detected in the ISS group relative to 73 genera in the ISS\_G (Figure 2A). However, the observed Hill numbers calculated using the Shannon Diversity index, 7.4, and Simpson Diversity index, 3.7, alpha diversity indices were modestly increased in the ISS group relative to ISS\_G with 4.63, and 2.29 respectively. Community structure analysis revealed increases in beta diversity between ISS and ISS\_G (Figure 2B). Furthermore, the ISS group showed a significant increase in the relative abundance of the phylum *Firmicutes* relative to *Bacteroidetes* (Figure 2C) compared to ISS\_G. A comprehensive analysis of differential genera abundance was carried out using the *metacoder* package as part of the R suite([Team, 2013](#_ENREF_99); [Foster et al., 2017](#_ENREF_32)). This analysis revealed several enriched genera within the ISS group relative to ISS\_G, including: *Ruminiclostridium 9*, *Romboustia*, *Clostridium Sensu Stricto 1*, and *Shuttleworthia* as well as loss of *Hungatella* and *Lachnospiraceae UCG-001* (Figure 2D, Table S8). These data suggest a dramatic shift in gut microbiome composition and is consistent with the previous reports in both human astronauts ([Garrett-Bakelman et al., 2019](#_ENREF_37); [Voorhies et al., 2019](#_ENREF_108)) and rodents ([Jiang et al., 2019](#_ENREF_49)).

**Significant microbiome compositional structure alterations detected between the ISS and LAR group at termination (Experiment 1 and 2 comparison).** To determine the microbiome diversity of the ISS group relative to the live animal return (LAR) cohort, fecal samples collected at necropsy after termination were additionally processed, sequenced, and analyzed as stated in the methods. We observed statistically significant increases in alpha (Figure 3A) and beta (Figure 3B) diversity measures of the ISS grouped compared to LAR. The observed Hill numbers from genera richness analysis demonstrated a mean of 69 genera that were detected in the ISS group relative to 61 genera in the LAR. The Shannon Diversity index, 7.4, and Simpson Diversity index, 3.7, indices were significantly increased in the ISS relative to the LAR group with a Shannon index, 2.9, and Simpson index, 2.2. Community structure analysis observations revealed increases in beta diversity, between ISS and LAR. Furthermore, there was a statistically significant increase in the relative abundance of the phyla Firmicutes relative to Bacteroidetes (Figure 3C) in the ISS relative to the LAR group. Additionally, differential genus abundance comparisons of genera using *metacoder* ([Foster et al., 2017](#_ENREF_32)) revealed several enriched genera in the ISS group relative to LAR (Figure 3D, Table S8) Intriguingly, ISS vs LAR comparisons at termination showed enrichment of *Lactobacillus* akin to LAR and LAR\_G at post-flight return.

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Figure. 1 Longitudinal Analysis of the Fecal Microbiome in the LAR Flight Group versus LAR\_G Ground Control.

(A, E, I) Longitudinal differences in alpha diversity using richness, Shannon, and Simpson diversity orders based upon sample-based rarefaction and extrapolation as previously described ([Hsieh et al., 2016](#_ENREF_45)) for Pre-Flight, Live Animal Return to Earth, and the Termination of the study (See methods). The Wilcoxon Rank-Sum statistical test was employed to compare LAR vs LAR\_G groups for alpha diversity analysis. Non-statistical significance is not indicated.

(B, F, J) Beta diversity analysis between LAR and LAR\_G groups showing a statistically significant difference between the LAR and LAR\_G. Nonparametric multivariate analysis of variance (PERMANOVA) was used to indicate with p value for statistical comparison of variance using distance matrices between LAR and LAR\_G groupings.

(C, G, K) Analysis of Firmicutes to Bacteroidetes ratios between the LAR and LAR\_G groups. The Wilcoxon Rank-Sum statistical test was employed to compare LAR vs LAR\_G where significance is indicated with p value.

(D, H, L) Analysis of genera enriched or lost in the LAR compared with LAR control group. Taxa enriched or lost in the ISS group at a threshold of p<0.05 compared to taxa present in the LAR group are represented in the *metacoder* heat tree by a color intensity Log2 median ratio scale.

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Figure 2 Alpha Diversity, Beta Diversity, and Comparative Compositional Shift Analysis in the of the Fecal Microbiome in the ISS Flight group versus ISS\_G Ground Control.

(A) Bacterial genera richness, Shannon, and Simpson diversity orders based upon sample-based rarefaction and extrapolation as described ([Hsieh et al., 2016](#_ENREF_45)). The Wilcoxon Rank-Sum statistical test was employed to compare ISS vs ISS\_G groups for alpha diversity analysis and statistical significance is designated with asterisks indicated at threshold of \*P ≤ 0.05, \*\*P ≤ 0.01.

(B)Beta diversity analysis between ISS and ISS\_G groups showing a statistically significant difference between the ISS and ISS\_G groups. Nonparametric multivariate analysis of variance (PERMANOVA) was used to calculate a statistical comparison (p= 0.016) between the ISS and ISS\_G groupings using distance matrices.

(C) *Firmicutes* to *Bacteroidetes* ratios between the ISS and ISS\_G groups. The Wilcoxon Rank-Sum statistical test was employed to compare ISS vs ISS\_G groups where significance is indicated with asterisks indicated at threshold of \*P ≤ 0.05.

(D) Analysis of genera enriched or lost in the ISS compared with ISS\_G control group. Taxa enriched or lost in the ISS group at a threshold of p<0.05 compared to taxa present in the ISS\_G group are represented in the *metacoder* heat tree by a color intensity Log2 median ratio scale.

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Figure 3 Alpha Diversity, Beta Diversity, and Comparative Compositional Shift Analysis in the of the Fecal Microbiome in the ISS Flight Group versus LAR Flight Return Group.

(A) Statistically significant differences found in bacterial genera richness, Shannon, and Simpson diversity orders based upon sample-based extrapolation as described ([Hsieh et al., 2016](#_ENREF_45)). The Wilcoxon Rank-Sum statistical test was employed to compare ISS vs LAR groups for alpha diversity analysis and statistical significance is designated with asterisks indicated at threshold of \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001.

(B) Represents Beta diversity analysis between ISS and LAR groups showing a statistically significant difference between the ISS and LAR groups. Nonparametric multivariate analysis of variance (PERMANOVA) was used for statistical comparison (p= 0.001) using distance matrices between ISS and LAR groupings.

(C) Represents analysis of Firmicutes to Bacteroidetes ratios between the ISS and LAR groups. The Wilcoxon Rank-Sum statistical test was employed to compare ISS vs LAR groups where significance is indicated with asterisks indicated at threshold of \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001, \*\*\*\*P ≤ 0.0001).

(D) Represents analysis of genera enriched or lost in the ISS compared with LAR control group. Taxa enriched or lost in the ISS group at a threshold of p<0.05 compared to taxa present in the LAR group are represented in the *metacoder* heat tree by a color intensity Log2 median ratio scale.

**Metagenomic analysis of the flight (ISS) and ground control (ISS\_G) groups reveals gene cluster enrichment of lactic acid, glutathione, and isoleucine/leucine production (Experiment 1).**

In addition to applying amplicon sequencing strategies to investigate taxonomic differences between the ISS and ISS\_G, whole genome shotgun (WGS) metagenomic sequencing was also performed in order to help elucidate the functional capacity and differential gene abundance between these groups. Using the Metagenomic Intra-Species Diversity Analysis System (MIDAS) we carried out a robust species and gene level metagenomic analysis of fecal microbiome obtained from ISS\_G/ISS rodents. MIDAS utilizes a universal set of single copy marker genes to determine relative abundance of species level assignments. Here we identified several differentially abundant species between ISS\_G and ISS (Figure 4A, Table S10) as well as determined that beta diversity between samples was significantly different between groups (Figure 4B). A total of 65 species clusters was identified within this WGS analysis, with 51 species being shared between the ISS\_G and ISS group and 7 unique species within each group respectively. Some of the most differential abundant species between ISS\_G and ISS included *Lactobacillus murinus* and *Dorea sp.* (Figure 4C, D). Species clusters assigned by MIDAS were compiled to generate pangenomes and used to map the remaining unassigned reads. A total of 396,080 genes were identified with 65,547 of those differentially abundant when compared by EdgeR differential analysis using weighted trimmed mean of the log abundance ratios (TMM). Due to the large number of differentially abundant genes, a strict cutoff was applied to isolate the most significantly different genes between ISS\_G and ISS rodent and to identify associated metabolic functions. We used gene assigned enzyme commission numbers (EC’s) to compare gene differences between the ISS\_G and ISS groups (Figure 4A, D Table S11). Multiple metabolic pathways were greatly enriched within the ISS group when compared to the ISS\_G, even though the ISS group had 4,762 fewer genes per sample on average (Figure 4E). Lactate (L), Malate (M), Glutathione (G), Leucine/Isoleucine (L/I), and Butanoate metabolism (BM) was significantly enriched within the ISS group (Figure 4F). Thus, we pulled all of the EC’s associated with these pathways from all 396,080 genes and aimed to identify differentially abundant L, M, G, L/I, and BM associated EC’s as well as the taxonomic contribution to those EC’s within each group (Figure 4F, Figure S11). Of the 5 identified differentially abundant EC’s associated with BM, 60% were only found in one species – *Lactobacillus murinus*. We mapped the differentially abundant EC’s for the BM pathway using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Metacyc. This result indicated that *L. murinus* provides a unique function within the ISS gut microbiome community in which it converts Pyruvate to either meso-(R,S) 2,3-butanediol or para (R,R) 2,3-butanediol under aerobic and/or anaerobic conditions (Figure 4F). The product, 2,3-butanediol, and its role in gut health and gut microbiome research is not well understood or discussed in the literature. This is a novel observation and warrants further investigation.

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Figure 4 *Lactobacillus murinus and Dorea sp.* Contribute Differentially Abundant Genes Encoding Butanoate Metabolic Enzymes Between ISS and ISS\_G rodent as well as Provides Unique Function Within the Community.

(A) Observed diversity of Genes. A total of 1,571,490 genes were detected with an average of 81455.2 gene hits in the ISS\_G and 76693.8 gene hits in the ISS groups. No statistical significance between ISS and ISS\_G was observed (Wilcox Rank Sum Test Adjusted by FDR, p = 0.80). Shannon diversity of Genes. A total of 1,571,490 genes were detected with an average Shannon Index value of 8.790 in the ISS\_G and 8.78 in the ISS groups. No statistical significance between ISS and ISS\_G was observed (Wilcox Rank Sum Test Adjusted by FDR, p = 0.97)

(B) Principal component analysis (PCA) was determined using Midas species TMM normalized counts by group, ISS\_G and ISS. Marginal boxplots show the differences in PC distances for PC1 and PC2 by group (Wilcoxon Test, adjusted by FDR, See Methods). All boxplots show median and lower/upper quartiles; whiskers show inner fences (see Methods). All heatmaps show individual scales by EC or taxonomy. Stars show false discovery rate (FDR) corrected statistical significance (FDR: \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001, \*\*\*\*P ≤ 0.0001).

(C) Relative Abundance of Top 9 Changing Species between ISS and ISS\_G. Enriched Species Lactobacillus murinus and Dorea spp. are highlighted.

(D) Relative Abundance of Select Species. Parabacteroides goldsteinii made up nearly 84% of the total relative abundance for both ISS and ISS\_G and was excluded from the top 10 most changing bacterial species as no significant difference between ISS and ISS\_G was observed (Wilcox Rank Sum Test Adjusted by FDR, p = 0.97). Dorea Sp. and Lactobacillus murinus species were significantly enriched in the ISS cohort (p =0.0000087 and p = 0.0000075 respectively, Wilcox Rank Sum Test Adjusted by FDR: \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001, \*\*\*\*P ≤ 0.0001).

(E) Volcano plot of all genes between ISS and ISS\_G. Log Fold Change of genes were then plotted against the adjusted p value for significance. Significant changing genes are highlighted in red, non-significant changing genes are highlighted in blue (significance was determined as p < 0.05 by VommLimma Differential Expression analysis adjusted by FDR). Select ECs associated with short chain fatty acid synthesis and bone homeostasis in mammals were unbiasedly identifies and highlighted if enriched in the ISS group.

(F) Taxonomic contributions to associated genes for SCFA and Bone homeostasis in ISS. Major SCFA and Metabolic Pathways are highlighted: Lactate, Glutathione, Leucine/Isoleucine, Butanoate and Malate. Log Fold Change of different EC gene hits are shown for all members that contribute genes to this pathway from the community in relationship to ISS\_G levels. Additionally, individual ECs contributed by enriched Dorea sp. and Lactobacillus murinus are highlighted with statistical inference. All p values were calculated using the non-parametric Wilcox Rank Sum Test Adjusted by FDR: \*P ≤ 0.05, \*\*P ≤ 0.01, \*\*\*P ≤ 0.001, \*\*\*\*P ≤ 0.0001).

**Altered bone biomarkers and plasma metabolome of the ISS Flight and ISS\_G ground control groups (Experiment 1)**

Terrestrial studies have demonstrated that altered microbiome compositions are strongly implicated with regulating changes observed in bone mineral density and bone matrix ([Sjögren et al., 2012](#_ENREF_94); [Hernandez et al., 2016](#_ENREF_42); [Guss et al., 2017](#_ENREF_39)), including the use of probiotics ([Li et al., 2016](#_ENREF_59); [Schwarzer et al., 2016](#_ENREF_92)), and increases short-chain fatty acids, particularly acetic acid, propionic acid, and butyric acid ([Yan et al., 2016](#_ENREF_110); [Lucas et al., 2018](#_ENREF_63)). Additionally, elevated circulation of many osteoblastic formation biomarkers such as propeptides of type 1 collagen (P1NP), hydroxyapatite binding matrix proteins such as osteocalcin (OCN), and decreases in osteoclastic resorption markers CTX-1, carboxy terminal crosslinked telopeptides of type 1 collagen, as well as histological TRAP, tartrate-resistant acid phosphatase, staining of osteoclasts were found in this association ([Yan et al., 2016](#_ENREF_110); [Lucas et al., 2018](#_ENREF_63)). While no histological changes when assessed for OCN and TRAP were observed **(CITE RR5 primary Ting,Kwak,Soo paper)** elevated serum changes for OCN and TRAP5b were detected when comparing ISS to ISS\_G (Figure 5A), albeit P1NP abundance were not significantly different statistically. When evaluating whether these bone biomarkers, OCN and P1NP, were correlated with the abundance of acetic acid, propionic acid, and butyric acid, between the ISS and ISS\_G groups, no differential abundances were detected (Figure 5B). Intriguingly, statistically significant increases in abundance of lactic and malic acid were associated with microgravity exposure (Figure 5B, Table S12) as demonstrated in the most recent NASA Twin Study ([Garrett-Bakelman et al., 2019](#_ENREF_37)). While cellular metabolism of osteoblasts and mammalian cells certainly produces lactate broadly, gene cluster enrichments encoding lactate-dehydrogenase solely map to *L. murinus* and *Dorea sp*. (Figure 4F) and may contribute overall elevated levels detected in the serum.The untargeted metabolomic analysis (Figure 6A,6B) demonstrated many statistically significantly differentially abundant metabolites in the ISS group relative to ISS\_G (Figure 6, Table S12), such as the antioxidant glutathione, which has been shown to influence redox homeostasis that is essential for osteoblastic function ([Lee et al., 2017a](#_ENREF_55)) and recently, survival of implanted osteoblast precursors in a murine-bone regeneration model ([Stegen et al., 2016](#_ENREF_96)). However, not only was glutathione highly abundant in the ISS group serum relative to ISS\_G (Figure 6C), but gene cluster enrichment for glutamate-cysteine ligase, a crucial enzyme for glutathione synthesis, was functionally enriched in the ISS group and associated with the significant increase in *L. murinus* (Figure 4F), suggesting an additive contribution to serum levels of glutathione along with hepatically derived glutathione. To evaluate microbial metabolite mediators correlated with the reflected increase of OCN or decrease in TRAP, two interesting branch-chain amino acids (BCAAs), leucine and isoleucine, were found to be enriched in the ISS group sera (Figure 6C). Similarly, gene cluster enrichments for acetolactate synthase and Leucyl-tRNA synthetases, responsible for microbial synthesis of BCAAs ([Umbarger and He, 1978](#_ENREF_104); [LaRossa et al., 1987](#_ENREF_53)), were directly associated with *L. murinus* and *Dorea sp.* (Figure 4F)*.* The previous RR-1 predicted relative decreases in microbial associated putrescine degradation pathways, however, the differential abundance between the ISS and ISS\_G was not statistically significant (Figure 6A, 6B).

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Figure 5 Bone Biomarker and Short-Chain Fatty Acid Profiling of Blood-Plasma Serum of ISS vs ISS\_G

(A) Represents ELISA analysis of bone biomarkers P1NP, OCN, and TRAP in ISS vs ISS\_G groups. Asterisks indicates a threshold of \*P ≤ 0.05, \*\*P ≤ 0.01. Non-significance is indicated by ns.

(B) Absolute quantification of short-chain fatty acids detected via Liquid-Chromatograph Mass Spectrometry. Asterisks indicates a threshold of \*P ≤ 0.05, \*\*P ≤ 0.01. Non-significance is indicated by ns.

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Figure 6Untargeted metabolome profiling of plasma serum via Liquid-Chromatograph Mass Spectrometry in ISS vs ISS\_G

(A) Metabolite abundance and relative fold change (Log2 mean ratios) in ISS vs. ISS\_G

(B) Statistically significantly enriched metabolites in the ISS vs. ISS\_G group. Abundance and individual statistical indications listed in Table S12

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Figure S1 Longitudinal Analysis of the Oral Microbiome in the LAR Flight Group versus LAR\_G Ground Control.

(A, D, G)) Represents the sequence sample based upon sample size rarefaction and extrapolation for timepoints Pre-Flight, Post-Flight Return, and the Termination of the study and differences found in bacterial richness, Shannon, and Simpson diversity orders based upon sample-based rarefaction as described ([Hsieh et al., 2016](#_ENREF_45)). The Wilcoxon Rank-Sum statistical test was employed to compare LAR vs LAR\_G groups for alpha diversity analysis and statistical significance is designated with p value.

(B, E, G) represents Beta diversity analysis between LAR and LAR\_G groups showing a non- statistically significant difference between the LAR and LAR\_G groups represents analysis of species enriched or lost in the LAR compared with LAR\_G control group.

(C, F, I) Taxa enriched or lost in the LAR group at a threshold of p<0.05 compared to taxa present in the LAR\_G group are represented in the *metacoder* heat tree by a color intensity Log2 median ratio scale.

**Map

Description automatically generated**

Figure S2 Fecal Necropsy Termination Analysis of Enriched or Lost Genera Evaluating Caging Effects.

(A-C) (A) Analysis of genera enriched or lost in the LAR vs compared with LAR\_G group at necropsy, (B)Analysis of genera enriched or lost in the LAR vs compared with baseline group, and (C)Analysis of genera enriched or lost in the LAR\_G vs compared with baseline group. Taxa enriched or lost in the LAR group at a threshold of p<0.05 compared to taxa present in the LAR\_G group are represented in the *metacoder* heat tree by a color intensity Log2 median ratio scale.

(D) Analysis of genera enriched or lost in the ISS vs compared with baseline group.

(E)Analysis of genera enriched or lost in the ISS\_G vs compared with baseline group.

**Discussion**

To our knowledge, this is the first live animal return study in the United States ever conducted by NASA/CASIS which sought to evaluate the influence of microgravity on the microbiome in a rodent model system. Furthermore, this study used the largest rodent cohort (N=10) and longest temporal duration of microgravity exposure to date (9 Weeks). Importantly, there are limitations to the interpreted outcomes worth noting, such as the influence of cosmic radiation, which is known to negatively disrupt the GI epithelial integrity and presumably the microbiota-mucosal interface ([Kumar et al., 2018](#_ENREF_52)), that have been shown to alter the fecal microbiome in terrestrial studies mimicking space-flight induced radiation ([Casero et al., 2017](#_ENREF_15)). In fact, previous shuttle era (Space Shuttle Atlantis STS-135) missions studied how dietary regimens with varying iron (pro-oxidant/free radical inducer) along with microgravity and cosmic radiation affected the microbiome and colonic function ([Ritchie et al., 2015](#_ENREF_86)). Therefore, it is an important variable to consider when interpreting low-earth orbit microbiome data, though *Jiang et al*. 2019 eloquently demonstrated that the gut microbiome diversity increases observed were markedly different from that of the insignificant correlative spaceflight-induced effects and cosmic radiation. It has been well established that behavioral factors such as coprophagy ([Bo et al., 2020](#_ENREF_11)) and iatrogenic effects such as transportation ([Ma et al., 2012](#_ENREF_65); [Bidot et al., 2018](#_ENREF_10); [Montonye et al., 2018](#_ENREF_74)), vendor source ([Ivanov et al., 2008](#_ENREF_48); [Hufeldt et al., 2010](#_ENREF_46); [Denning et al., 2011](#_ENREF_26); [Ericsson et al., 2015](#_ENREF_29); [Hart et al., 2018](#_ENREF_41)), as well as environmental, stress, caging, bedding, ventilation, and husbandry effects can affect the rodent microbiome composition ([Bendtsen et al., 2012](#_ENREF_9); [Franklin and Ericsson, 2017](#_ENREF_34); [Bidot et al., 2018](#_ENREF_10); [Ericsson et al., 2018](#_ENREF_30)). To account for these variables for the ISS and ISS\_G groups, the RR-5 mission utilized the NASA Rodent Habit ([Ronca et al., 2019](#_ENREF_87); [Choi et al., 2020](#_ENREF_20)), the next generation of the Animal Enclosure Module (AEM), a sophisticated rodent housing unit (with a built in air-particle filter more selective than HEPA-0.1 micron), to conduct rodent spaceflight research and has been successfully used in over 26 rodent spaceflights ([Moyer et al., 2016](#_ENREF_77)). A recent study performed robust analyses assessing animal health and wellness, including animal growth and body masses, organ masses, rodent food bar consumption ([Sun et al., 2010](#_ENREF_97)), water consumption, and blood contents and found AEM housed rodents to be in normal biological ranges when compared to traditional vivarium housed rodent ([Moyer et al., 2016](#_ENREF_77)).

Considering all these factors and the consistent genetic background and well-controlled design of the current study, the gut microbiome of the ISS group at week 5 is plausibly similar as the LAR group (Figure 1, Figure 2). Thus, the argument could be made that microgravity-exposed rodents are sensitive to microbial compositional disturbances, such as resulting in the rapid loss or enrichment of certain existing species compared to humans ([Garrett-Bakelman et al., 2019](#_ENREF_37); [Voorhies et al., 2019](#_ENREF_108)) and leading to a quick reverse in microbial diversity to a level comparable to the LAR\_G group (Figure 1). Furthermore, *Voorhies et al*. 2019 did find increases in richness as measured by the Shannon Diversity index associated with microgravity which was reduced upon returning to Earth within 60 days, which was observed more quickly by 30 days in the present study at termination (Figure 1A, E, I). However, despite reversal of diversity and observed persistent compositional differences (Figure S2), it is difficult to directly conclude the health consequences other than the observation of a persistent microbiome shift longitudinally. Despite, the inability to sample the LAR group inflight, the LAR did not show any changes in the diversity within oral microbiome throughout (Figure S1A, D, G), corroborating the human astronaut tongue microbiome analyses ([Voorhies et al., 2019](#_ENREF_108)); thus, a shift in the oral community towards dysbiosis was not observed and may suggest the oral microbiome is more stable and less influenced by microgravity exposure unlike the gut microbiome that shows clear shifts in community composition and function (Figure S1B, E, G, Figure 4).

Under these notions, findings from the present study, along with previous studies ([Garrett-Bakelman et al., 2019](#_ENREF_37); [Jiang et al., 2019](#_ENREF_49); [Voorhies et al., 2019](#_ENREF_108)), of increased microbial diversity in the ISS group is partially surprising considering that microgravity, along with other stress-based flight factors such as radiation, could promote a dysbiosis with the microbiome. Terrestrially speaking, the well-accepted concept in ecological gut microbiome dynamics, which is the positive correlation between biodiversity and ecosystem stability, associates decreases in gut microbial diversity as one of the hallmarks of GI diseased states, such as Inflammatory Bowel disease or Crohn’s disease ([Frank et al., 2007](#_ENREF_33); [Willing et al., 2009](#_ENREF_109); [Mosca et al., 2016](#_ENREF_76)). However, the opposite association is found in oral disease states when evaluating the microbiome ([Zheng et al., 2015](#_ENREF_113); [Lee et al., 2017b](#_ENREF_56); [Gao et al., 2018](#_ENREF_36); [Tsai et al., 2018](#_ENREF_101)) and has empirically been shown to influence GI disease states, such as colitis ([Kitamoto et al., 2020](#_ENREF_51)). Thus, despite susceptibility to these space-flight stressors in rodents and humans alike, it is unclear how these data can be reconciled solely based upon relative abundance or consequential functional outcomes; thus, these paradigms may not hold true in the absence of terrestrial gravitational forces. Nonetheless, long-term exposure to microgravity and low-earth orbit or beyond the Van Allen radiation belt could impose significant adverse effects, such as systematic and local microenvironmental changes, including known associated dysregulated immune states ([Crucian et al., 2008](#_ENREF_25); [Crucian et al., 2013](#_ENREF_22); [Chang et al., 2015](#_ENREF_16); [Crucian et al., 2015](#_ENREF_23); [Crucian et al., 2018](#_ENREF_24)), that are likely to impact host-microbiomes, and induce adaptive and pathophysiological changes in digestive structures and physiology ([Rabot et al., 2000](#_ENREF_85); [Arun, 2004](#_ENREF_7)). Naturally, assessing causal outcomes of dysbiosis, such as inflammation in the colonic epithelial layer, in the ISS group relative to ISS\_G was of high priority, but impossible due to the primary objective of RR-5, which required freezing the carcasses prior to flight return. Thus, the tissue was compromised from the freeze-thaw event and could not be reliably assessed (data not shown). However, the previous STS-135 mission, which only had 13 days of microgravity exposure, did not find significant changes in colonic intestinal injury or inflammatory infiltration ([Ritchie et al., 2015](#_ENREF_86)).

Increasing evidence suggests that an increased *Firmicutes*/*Bacteroidetes* ratio has been widely considered a signature of gut dysbiosis and correlated with disease states, such as inflammatory bowel disease ([Peterson et al., 2008](#_ENREF_80)) and obesity ([Ley et al., 2006b](#_ENREF_58)). In this study, an increase in relative abundance of Firmicutes, as well as decrease in *Bacteroidetes* (F/B ratio >1), was associated with microgravity exposure (Figure 1C) and is consistent with the pattern of microbiome flux observed in the RR-1 microgravity related rodent study ([Jiang et al., 2019](#_ENREF_49)) and two pivotal human astronaut studies ([Garrett-Bakelman et al., 2019](#_ENREF_37); [Voorhies et al., 2019](#_ENREF_108)). Furthermore, when comparing the ISS vs LAR groups to review longitudinal effects of recovery on Earth, we found the F/B ratio in the ISS group is increased relative to the LAR group (Figure 3C) as seen in the NASA Twin study ([Garrett-Bakelman et al., 2019](#_ENREF_37)). To provide further insight into this observed increase in the F/B ratio, deep metagenomic sequencing revealed for the first time that *Lactobacillus murinus*, a commensal rodent gut bacterium and of the Firmicutes phylum, is enriched in the ISS rodents in comparison to the ISS\_G cohort. Interestingly, Dorea sp. was also found to be enriched within the ISS cohort. Notably, Dorea sp. was only observed in 10% of the ISS\_G cohort, and at very low relative abundance, 0.00124%. Yet was found within 100% of the samples from the ISS group and increased relative abundance of 0.48% on average (Figure 4C, D). Though it is worth noting that due to the Rodent Hardware system automatically removing urine and fecal waste via constant air-flow in microgravity, the ISS and LAR group mice were not coprophagic during flight, which may explain some diversity differences and is a known factor for influencing the gut microbiome in rodents ([Bo et al., 2020](#_ENREF_11)). Conversely, this enabled a rare and true host selection event absent of exogenous microbial colonization as evidenced by a relatively similar microbiome composition, albeit with minor alterations in microbial genetic composition between the ISS and ISS\_G groups (Figure 4A,D). This selection under microgravity could have enabled an opportunistic selection of *L. murinus and/*or *Dorea spp*, which may promote intraspecies growth and indirect benefit to the rodent host. These differentially abundant species may also provide a unique metabolic function for host bone homeostasis through the lactate, branch-chain amino acids, and glutathione metabolic pathway within ISS rodents (Figure 4F, Table S11). Previously, probiotic therapy in rodents through introduction of single *Lactobacilli* species, such as *Lactobacillus helveticus* ([Narva et al., 2004](#_ENREF_78)), *Lactobacillus reuteri* 6475 ([McCabe et al., 2013](#_ENREF_69)), *Lactobacillus rhamnosus* ([Li et al., 2016](#_ENREF_59)) or a part of cocktails such as VSL#3 ([Li et al., 2016](#_ENREF_59)), have been shown to promote increases in bone mineral density.

It was recently shown that the ISS group relative to ISS\_G, the non-treatment controls in the RR-5 study, displayed flight associated longitudinal decreases in bone mineral density in the tibia and lumbar vertebrae, as well as decreases in femur trabecular thickness, chondrogenesis, and increases in bone marrow adipogenesis **(CITE RR5 primary Ting,Kwak,Soo paper)**. Bone marrow adipogenesis, which is inversely correlated with bone formation ([Meunier et al., 1971](#_ENREF_73); [Burkhardt et al., 1987](#_ENREF_12); [Dragojevič et al., 2011](#_ENREF_27)), was highly increased in the ISS group versus ISS\_G**(CITE RR5 primary Ting,Kwak,Soo paper)**..Intriguingly, terrestrial studies demonstrated increases in obesity were correlated with increases in *Lactobacilli* species ([Turnbaugh et al., 2006](#_ENREF_103); [Armougom et al., 2009](#_ENREF_5)). These functional outcomes associated with the observed changes in the gut microbiome is in congruence with emerging research linking the gut microbiome to bone homeostasis ([Hernandez et al., 2016](#_ENREF_42); [Pacifici, 2018](#_ENREF_79)) through immune system effectors ([Sjögren et al., 2012](#_ENREF_94); [Yan et al., 2016](#_ENREF_110); [Guss et al., 2017](#_ENREF_39)), vitamin and nutrient deficiencies ([Guss et al., 2019](#_ENREF_40)), endocrine regulation ([Charles et al., 2015](#_ENREF_19)), and energy metabolism through SCFAs ([Yan et al., 2016](#_ENREF_110); [Lucas et al., 2018](#_ENREF_63)). Interestingly, in terrestrial rodent models, it has been shown that administration of SCFAs (acetic/propionic/butyric acid), as well as microbial metabolites, increased serum levels of insulin growth factor-1, IGF-1, a hormone that directly affects and leads to skeletal growth ([Yan et al., 2016](#_ENREF_110)) and decreased CTX-1, and NF-Kβ factors responsible for osteoclastic activity ([Yan et al., 2016](#_ENREF_110); [Lucas et al., 2018](#_ENREF_63)). Moreover, SCFAs, primarily produced by gut microbiota, are known immunomodulators ([Arpaia et al., 2013](#_ENREF_6); [Furusawa et al., 2013](#_ENREF_35); [Smith et al., 2013](#_ENREF_95)) and regulate systemic bone mass as well as prevent bone loss ([Lucas et al., 2018](#_ENREF_63)). Our results showed no differential abundances of acetic/propionic/butyric acid in the ISS vs ISS\_G, however, elevated levels of lactic and malic acid were found as previously reported ([Garrett-Bakelman et al., 2019](#_ENREF_37)). The authors suggested this could be due to a switch from aerobic to anaerobic metabolism, partially influenced mandated increased exercise in flight. Additionally, SCFAs are known to be produced by the gut microbiome ([Macfarlane and Macfarlane, 2003](#_ENREF_67); [Morrison and Preston, 2016](#_ENREF_75)), including lactic acid/lactate ([Russell et al., 2011](#_ENREF_88)). Notably, lactate-dehydrogenase, which converts pyruvate into lactate and has been shown to be a product of energy metabolism in normal osteoclastic function ([Indo et al., 2013](#_ENREF_47); [Ahn et al., 2016](#_ENREF_2); [Lee et al., 2017a](#_ENREF_55)) is highly enriched in the ISS cohort and most functionally associated with *L. murinus* and *Dorea spp*. (Figure 4F), thus, potentially driving this observation. The untargeted metabolomic analysis demonstrated statistically significantly enriched metabolites in the ISS group relative to ISS\_G (Figure 6C, Table S12). Of note, the antioxidant glutathione, which is primarily hepatically derived ([Lu, 1999](#_ENREF_62)) and synthesized by some gut microbiota ([Loewen, 1979](#_ENREF_61)), has been shown to influence redox homeostasis that is essential for osteoblastic function ([Lee et al., 2017a](#_ENREF_55)) and recently, survival of implanted osteoblast precursors in a murine-bone regeneration model ([Stegen et al., 2016](#_ENREF_96)). Furthermore, the previous RR-1 study indicated positively correlated inferred microbial gene-encoding glutathione-glutaredoxin redox enzymes with hepatic expression of redox associated genes, albeit non-significant once accounting for experimental variables that suggests independent regulation between the microbiome and the liver ([Jiang et al., 2019](#_ENREF_49)). Another important nutritional component to bone maintenance is adequate bioavailability of amino acids. A recent protein intake study was demonstrated to be critical for osteoblast differentiation ([MacDonell et al., 2016](#_ENREF_66)). Leucine/isoleucine are well known to be actively imported into osteoblasts along with chondrogenesis resulting from the activation of the osteoblastic transcription factor ATF4, the cognate ligand of RSK2, and mutations in this pathway (Coffin–Lowry syndrome) are associated with skeletal abnormalities ([Yang et al., 2004](#_ENREF_111); [Elefteriou et al., 2006](#_ENREF_28); [Lee et al., 2017a](#_ENREF_55)). *L. murinus* and *Dorea spp*. Displayed gene cluster enrichments for acetolactate synthase and Leucyl-tRNA synthetases and are believed to contribute to the increase in leucine/isoleucine detected in the ISS sera relative to ISS\_G in the current study (Figure 6C).

The novel insights into *Lactobacillus murinus* and *Dorea sp.* metabolism provide new avenues for osteoporosis therapeutics, perhaps through a live-engineered biotherapeutic chassis. There is a great need for the expansion of the current arsenal of treatment modalities for prevention and treatment of osteoporosis ([Pacifici, 2018](#_ENREF_79)). Most importantly, however, these data encourage higher resolution and targeted analyses of microgravity exposure aimed to elucidate the benefits and consequences of these microbial compositional shifts at the bone homeostasis axis upon safely returning to earth, longer durations in low-Earth orbit, traversing past the Van Allen radiation belt, and beyond.

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**AUTHOR CONTRIBUTIONS**

**STAR\*Methods**

**Experimental Design and Timeline**

All animals were handled in accordance with the institutional guidelines of the Institutional Animal Care and Use Committee (IACUC) of the National Aeronautics and Space Administration (NASA)and the University of California, Los Angeles (UCLA). Animals were housed in a light- and temperature-controlled environment and given moisturized food or food and water *ad libitum*. The animals used in this study are from the Rodent Research (RR) 5 mission with its primary objective to test an osteoporosis therapy, BP-NELL-PEG ([Shi, 2019](#_ENREF_93)) **(CITE RR5 primary Ting,Kwak,Soo paper)**.. In this study, only animals that received control PBS therapy were used, and thus only control animals and procedures are described in the methods below.

32-week-old BALB/c female mice were obtained from Taconic Biosciences (New York, U.S.A.) and acclimated in the Kennedy Space Center in Florida, U.S.A. for 2 weeks in vivarium cages and provided with water and standard chow. Baseline animals (n=20) were examined and euthanized immediately before rocket launch. Experimental animals were randomly assigned to the following groups (n=10/group): Live-Animal Return group (LAR), Full-Term Flight group (ISS), Ground control group for LAR (LAR\_G), and Ground control group for ISS (ISS\_G). Flight groups (LAR and ISS) were flown to space via SpaceX Dragon (CRS-11) and housed onboard the International Space Station. Matching ground control groups (LAR\_G and ISS\_G) were housed at the NASA Kennedy Space Center, where CO2, temperature, and humidity were matched to that of International Space Station in the International Space Station Environmental Simulator (ISSES). At week 5 post-flight, LAR group was returned live to Earth (UCLA) from the International Space Station, and LAR\_G group mice were shipped to UCLA from KSC/ISSES. LAR and LAR\_G groups were both kept at UCLA to examine the effects of recovery for 4 additional weeks. ISS and ISS\_G groups remained in the Space Station and the Kennedy Space Center, respectively, for the 4 weeks. All animals were euthanized at termination (9 weeks post-launch).

**Animal Husbandry**:

All Flight (ISS and LAR) and Ground groups (ISS\_G and LAR\_G) were housed in the NASA Rodent Habitat ([Ronca et al., 2019](#_ENREF_87); [Choi et al., 2020](#_ENREF_20)) and provided with NASA Rodent Food Bar ([Sun et al., 2010](#_ENREF_97)) until delivery to UCLA, where they were placed in standard vivarium cages and provided with water and standard chow. All rodents continued with the same diet (NASA Rodent Food Bar ([Sun et al., 2010](#_ENREF_97))) throughout the entire study (Table S13). Throughout the duration of the experiment (9 weeks total), animals received phosphate-buffered saline (PBS) injection peritoneally every 2 weeks. LAR and LAR\_G groups were sampled pre-flight and immediately upon live-return (4.5 weeks post-Live Animal Return to Earth), and at terminal time-point (9 weeks post-flight). Individual fresh fecal samples and oral swabs were acquired pre-flight from each mouse, post live animal return to Earth (24 hours post arrival), and at termination (week 9) for time-point microbiome analysis. ISS and ISS\_G groups were euthanized in the Space Station and the Kennedy Space Center, respectively, and delivered to UCLA as frozen carcasses (at -80 °C). Thus, samples were acquired after carcasses were thawed for tissue harvest for all collaborating teams at termination during necropsy utilized for analysis (Figure 2).

**Fecal Sample Acquisition and Harvesting**

Nominally, two freshly harvested fecal samples or more per mouse (in individual, autoclaved cages) were collected and placed in 2.0 mL sterile, nuclease free (RNAase/DNAase), non-pyrogenic, polypropylene Corning Cyrogenic vials. Samples were promptly “flash frozen” with liquid nitrogen for 15 minutes – one hour (within 15 minutes or less of collection) and stored in a -80°C freezer. One fecal pellet (equivalent in mass) was used per mouse for DNA extraction and sequencing of the 16S ribosomal RNA gene was then performed as previously described([Tong et al., 2014](#_ENREF_100)). Briefly, bacterial DNA was isolated using the QIAGEN Power Fecal DNA isolation kit (QIAGEN, Cat. No. 12955-4) with bead beating. All procedures were approved under IACUC Protocol Number: NAS-16-001-Y1.

**Oral Sample Acquisition and Harvesting**

The molars, cheeks and tongue (lingual and buccal) regions of the LAR and LAR\_G rodents (3 swabs per mouse within each cage) were used as a consistent anatomical position for oral microbiome sampling. The rodents were anesthetized via ketamine and sampled. Specifically, Plasdent Maxaplicator Super fine (2 mm) swabs were used for collection (MPN# 600-R-2). DNA samples were processed using the Lucigen - Master Pure DNA isolation kit (Ca. No. MC85200). The experimental PBS controls and baseline, avoiding influence of the NELL-1 treatment. All procedures were approved under IACUC Protocol Number: NAS-16-001-Y1.

**Sequence Quality Control and Noise Reduction of Fecal Microbiome Samples**

The V4 region of the 16S rRNA gene was amplified and barcoded using 515f/806r primers then 2x150bp (paired-end) sequencing was performed on an Illumina HiSeq 2500 platform ([Caporaso et al., 2012](#_ENREF_14)). The reads of amplicons from the V4 region of 16S rRNA were processed using the DADA2 package ([Callahan et al., 2016](#_ENREF_13)) following a standard workflow of quality trimming, de-replicating, DADA2 denoising, read-pair merging and chimera removal steps with the following parameter settings: For quality trimming, truncLen=c(151, 144), maxEE=c(Inf, Inf), minQ=c(0, 0); for error rate learning and DADA2 denoising, selfConsist = TRUE, pool=TRUE; for chimera removal, method = "pooled". A total of 454 distinct amplicon sequence variants (ASVs) were identified by DADA2 among all samples. The distinct sequences were sorted according to their total counts in all samples in descending order and assigned a numeric sequence ID, e.g. seq1, seq2, …, seq454, representing from the most abundant sequence to the least abundant sequence in terms of total read count in all samples.

**Taxonomy Assignment of Fecal Microbiome Samples**

Fecal ASV sequences were searched against a reference sequence set containing 16S rRNA sequences from all named prokaryotes downloaded from the SILVA high quality ribosomal RNA database(v132)([Quast et al., 2012](#_ENREF_84)) using “blastn”. The best hit covering >= 95% of the query length was identified for each sequence. If the best hit shares >= 98% identity with query sequence, the query sequence is assigned the taxonomy of the hit to the species level. If the sequence identity between the query and the hit is greater than 97% but less than 98%, the query sequence is assigned taxonomy of the hit to the genus level. If a sequence does not have any hit with >= 97% identify, the taxonomy was not assigned. The genus level read count data, generated by the "tax\_glom" function of Phyloseq (see methods) were used in this analysis.

**Sequencing Noise Reduction and Sequence Quantification of Oral Samples**

The sequencing of the V1-V3 region ([Allen et al., 2016](#_ENREF_4)) of 16S rRNA was completed using a custom protocol ([F. Escapa et al., 2020](#_ENREF_31)) and on the Illumina Mi-Seq platform. The 100x401 uneven paired-end reads of amplicons from the V1-V3 region of 16S rRNA were processed using the DADA2 package ([Callahan et al., 2016](#_ENREF_13)) for quality trimming, de-replicating, and DADA2 denoising with default settings and following parameters: truncLen=c(100,200), maxN=0, maxEE=c(2,2), truncQ=2. Due to the sequencing gap between the two reads, the read pairs were concatenated by inserting 10 Ns in between two reads with the “justConcatenate=TRUE” option in the DADA2 “mergePairs” function. Chimera were then removed with the “removeBimeraDenovo” function using the "consensus" method. A total of 1,708 ASVs were identified and were subject to taxonomy assignment.

**Taxonomy Assignment of Oral Samples**

Oral ASV sequences were searched against a collection of species level full length 16S rRNA reference sequences consisting of the HOMD RefSeq V15.1, HOMD RefSeq Extended V1.11, GreenGene Gold and NCBI 16S rRNA Reference collections, based on a specie-level taxonomy assignment algorithm([Al-Hebshi et al., 2015](#_ENREF_3)) (Detailed algorithm and reference sequences available online at <http://www.homd.org/ftp/NGS_Pipeline/Species_Level_BLASTN/>).

**Bacterial Diversity and Statistical Analyses**

Samples that were obtained for oral and gut microbial community analysis assessed for the microbiome composition resulting from microgravity exposure. We profiled the microbial abundance and diversity of the flight groups ISS and LAR, as well as ground controls, ISS\_G and LAR\_G, avoiding influence of the NELL-1 treatment. All diversity and statistical analyses were done under the R statistical environment (version 3.6.1) ([Team, 2013](#_ENREF_99)). ASV count data, along with taxonomy assignment and sample meta information (Supplemental Tables 2-4), were imported into R using the *PhyloSeq* package ([McMurdie and Holmes, 2013](#_ENREF_71)). Count data were sub-sampled into various comparison groups (for example, to compare ISS and ISS\_G ground control only relevant samples were included). After sub-sampling, ASVs with fewer than 10 reads in at least 2 samples, or fewer than 100 reads across all samples, were excluded, using the “filter\_taxa” function. Count data were then rarefied to the same sampling depth based on the minimal sample sum among the comparison group using the “rarefy\_even\_depth” with the parameters “sample.size=minimal\_sample\_sum” and “replace=F” ( where minimal\_sample\_sum = 40,400 and 18,000 for ISS group, LAR Fecal and Oral group, respectively, as well as their controls).

Alpha diversity analysis was conducted by using the mean ASV counts (or species or genus counts) for each group as input to calculate Hill Numbers (or effective number of ASVs, species, or genera) using the R package*, iNEXT*([Hsieh et al., 2016](#_ENREF_45)). The “iNEXT” function was used with the parameters “q=c(0,1,2),datatype='abundance', nboot=100”. In this function, the Hill Number numerical equivalents were generated for interpolation of sample based rarefaction (solid line segment) and extrapolation curves with 95% confidence intervals (shaded region) and the “ggiNEXT” function was used to generate plots to illustrate a) q=0 (genera or species richness), q=1 (Shannon index), q=2 (Simpson index), and sample completeness curves ([Hsieh et al., 2016](#_ENREF_45)). To assess the statistical significance of the difference in alpha diversity between two groups of samples, read count data of individual samples were subject to iNEXT to calculate the Hill Numbers. Hill Numbers at a fixed read count depth of 40,000, 18,000 for oral samples, were then extracted from the iNEXT results, so that all samples would have a Hill Number at the same sampling depth for fecal samples. Hill numbers between two groups of samples were then statistically assessed for their difference using the R functions to perform unpaired two-sample statistical tests either by 1) “t.text” function for Student’s T-test, or 2) “wilcox.test” function for Wilcoxon ranksum test.

Beta diversity was assessed by non-metric multidimensional scaling (NMDS) - rarefied count data of the comparison group were subject to the “ordinate” R function specifying “NMDS” as the ordination method and “bray” specifying Bray-Curtis as the distance calculating function. To assess whether the variation in distances can be explained by the test groups, the “adonis” R function (analysis of variance using distance matrices, a form of nonparametric multivariate analysis of variance) were used to partition sums of squares and calculate the R2 and *p* values (R2 is the portion of the variants that can be explained, and *p* value indicating the possibility of the result by chance). To identify and illustrate differentially abundant microbes at various taxonomic ranks (i.e., from ASV to domain) the “compare\_group” function in the R package *metacoder(*[*Foster et al., 2017*](#_ENREF_32)*)* was used to determine the differences in median abundances between two groups of samples. The *p* values were measured using the Wilcoxon Rank-Sum test, followed with adjustment for multiple comparisons using the “fdr” method. Abundance ratios of taxa with non-significant *p* value (>0.05) were set to zero so that they will not show in the final differential abundance taxonomy tree, which was compiled with the “heat\_tree” function in the *metacoder*. Firmicutes/Bacteroidetes ratios were calculated by agglomerating the read count data to the phylum level using the “tax\_glom” function provided by the R *PhyloSeq* and *ggplot2* packages ([McMurdie and Holmes, 2013](#_ENREF_71)), and the log2 count ratios between the two phyla were calculated for each sample. Wilcoxon Rank-Sum test was then performed to evaluate the difference between the test groups.

**Metagenome Analysis of ISS vs ISS\_G Groups**

Whole genome shotgun (WGS) sequencing was performed at the University of Washington’s Northwest Genomics Center (NWGC) on 10 biological replicates for both ISS and ISS\_G rodents respectively (N=20). Sequencing libraries were generated using KAPA HTP Library Preparation Kits (07961901001, Roche) and sequenced on an Illumina NovaSeq 6000 System using a S Prime flow cell configured for 300 cycles which resulted in a total of 1,342,813,654 reads with an average of 70,674,403 reads per sample. Raw paired-end reads were then filtered, trimmed for quality, and screened against a mouse (C57BL) reference database using kneadData([McIver et al., 2018](#_ENREF_70)). Filtered reads were then analyzed using the Metagenomic Intra-Species Diversity Analysis System (MIDAS) (database v.1.2, Species Coverage Cutoff 0.01, Merge Sample Depth Cutoff 1.0) (kkerns85/midas\_nextflow.git). Species, gene, and single nucleotide polymorphism (SNP) analysis were performed (database v.1.2, Species Coverage Cutoff 0.01, Merge Sample Depth Cutoff 1.0). Species were identified by clustered sub-species (>95% ANI) and assigned by a reference genome for that cluster. A total of 67 species clusters were identified. Genes were identified and mapped to pangenomes of these sub-species clusters and then annotated using an in-house annotation pipeline utilizing the Pathosystems Resource Integration Center (PATRIC) Database (v. 3.6.5). Differential species abundance was determined using mean relative abundance between ISS and ISS\_G after imposing a cutoff of a row-summed relative abundance ≥ 0.0001 and prevalence ≥ 20% of the total samples (4/20): resulting in a total of 50 species clusters that were then plotted using R (v. 15.6.0; Clustvis([Metsalu and Vilo, 2015](#_ENREF_72))). Differential gene abundance was analyzed using counts per million (CPM) normalized gene counts using the online web server Degust([Powell, 2015](#_ENREF_82)) which calculated differential abundance by Voom/Limma (Min Gene Read Count 1.0, Min Gen CPM 1.0). Out of 396,080 total genes identified by MIDAS, 64,574 genes were determined to be differentially abundant. Use of a cutoff allowed for more stringent analysis of differentially abundant genes between ISS and ISS\_G rodents (FDR P-value > 0.001, abs Log Fold Change = 3, > 8X Coverage), which resulted in 651 highly differentially abundant genes. Using the midas\_merge function we were able to determine the coverage of genes by taxonomic group. In order to better investigate this, we utilized RNASeq2G (unpaired, group 0, min read count 10, normalized using trimmed mean of M values (TMM), and log normalized using local polynomial regression, Loess([Zhang et al., 2017](#_ENREF_112))) within R. TMM normalized gene counts were then used to determine differences in Enzyme Commission numbers (EC’s) for various KEGG annotations. Heatmaps were generated using Clustvis([Metsalu and Vilo, 2015](#_ENREF_72)) in R. TMM normalized gene counts were then converted to using a row z score (x – (mean(x)) / std(x)). Statistical analysis of these differences in EC’s was determined using non-parametric Kruskal-Wallis anova (p values adjusted by FDR). For statistically significant groups, the non-parametric Wilcoxon Ranked Sum test was applied (p values adjusted by FDR). EC pathways were mapped using Kyoto encyclopedia of genes and genomes (KEGG) and Metacyc. An Rmarkdown will be made available if this is integrated into the publication and made publicly available and linked to the manuscript. Supplemental tables will be provided for raw reads, species and gene counts, normalization methods, and statistics.

**LC-MS Analysis of Rodent Serum Plasma**

**ELISA Analysis of Rodent Serum Plasma Bone Biomarkers**

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