

**Impact of a model used to simulate socio-environmental stressors encountered during
spaceflight on murine intestinal microbiota**

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Background: During deep-space travels, crewmembers face various physical and psychosocial stressors that could alter gut microbiota composition. Since it is well known that intestinal dysbiosis is involved in the onset or exacerbation of several disorders, the aim of this study was to evaluate changes in intestinal microbiota in a ground-based murine model mimicking psychosocial stressors encountered during a long-term space mission.

Results: We demonstrate that 3 weeks of exposure to Chronic Unpredictable Mild Stress (CUMS) induce significant change in intracaecal β -diversity characterized by an important increase of the *Firmicutes/Bacteroidetes* ratio. These stress-induced alterations are associated with a decrease of *Porphyromonadaceae*, particularly of the genus *Barnesiella* that is a major member of gut microbiota in mice, but also in human, where it is described as having protective properties.

Conclusions: These results raise the question of the impact of stress-induced decrease of beneficial taxa, support recent data obtained with in-flight experimentations or gravity change models, and emphasize the critical need for further studies exploring the impact of spaceflight on intestinal microbiota in order to propose strategies to countermeasure spaceflight-associated dysbiosis and its consequences on health.

Keywords: gut microbiota, Chronic Unpredictable Mild Stress, spaceflight, *Barnesiella*

Background

Gut microbiota (GM) is a complex microbial ecosystem whose balance and homeostasis are essential to the well-being of the host. Its composition is affected by numerous intrinsic and extrinsic factors such as antibiotics or diet [1,2]. Recent works have also shown that host stress, particularly chronic stress, has profound effects on the composition and organization of GM [3–9]. Stress is a physiological adaptive response to aversive stimuli (endogenous or exogenous, psychological or physical, real or perceived) that represent a common life experience of one's daily life [2,10–12]. Chronic or excessive stress could be involved in the onset or exacerbation of chronic disorders such as anxiety and depression or intestinal bowel diseases (IBD) [13]. Indeed, more and more studies suggest a link between these pathologies and intestinal dysbiosis [2,12–15]. The sympathetic nervous system as well as the hypothalamic-pituitary-adrenal (HPA) axis represent the main biological stress axes and are strongly involved in the bidirectional communication between the gut and the central nervous system, also called brain gut axis [7,8,11,12,16]. This could explain how host stress impacts intestinal bacteria. Stress-induced modulation of GM could also be explained by the alteration of local immunity, intestinal motility, mucin secretion or visceral perception [5,17]. Furthermore, stressor exposure is able to directly affect GM composition by the action on bacteria of stress mediators released in the lumen, such as glucocorticoids and catecholamines that are known to modulate bacterial growth, virulence and gene expression [6,11,18–20].

During spaceflight, astronauts face chronic or intermittent stressors of psychosocial (confinement, isolation, sleep deprivation, persistent circadian misalignment) or physical (gravitational changes, radiations) origin [21]. These stressors, associated to dysregulation of the

immune system [22,23], put astronauts at high risk of developing intestinal dysbiosis and a recent study on International Space Station crew members described an alteration of the composition of astronauts' microbiome during space travel [24]. Such dysbiosis could have an impact not only on immune system efficiency, but also on energy intake, nutrients assimilation and intermediary metabolisms such as those of antibiotics [25]. As imbalance in GM could be correlated with a shift from a healthy state to a diseased state, it is important to evaluate the status of GM in response to stressors encountered during long-duration space missions [26]. Recently, we showed that hypergravity disrupts murine intestinal microbiota in a G-level-dependent manner [3]. In this study, we now evaluated the impact of a model used to mimic psychosocial stressors encountered during a long-term space mission [27]. This model involves the exposure of mice to chronic unpredictable psychosocial and environmental stressors of various nature and mild intensity separated by resting periods (CUMS model, [Fig. 1A](#)).

Results

21 days of CUMS exposure do not induce a major stress response

Male mice were divided in two groups: 10 mice submitted to 21 days of CUMS and 10 controls placed in another room of the animal facility. Animals presenting injuries, such as bites that could induce inflammation, were discarded resulting in ten CUMS mice and seven controls at the end of the experiment. To evaluate stress, mice were weighted at the end of the experimental procedure and the amount of corticosterone in peripheral blood was quantified by ELISA. [Figures 1B and 1C](#) show that these two parameters were similar in both groups of mice. We also determined thymus weight since it is well known that stress induces its involution. This organ

weight was normalized to body weight (**Fig. 1D**). Again, no statistically significant difference could be noted between the two groups of mice.

Intestinal microbiome β -diversity is significantly modified by CUMS

The quantification of the number of 16S rRNA encoding gene copies per mg of intracaecal content revealed that bacterial load was not significantly affected by CUMS exposure (CUMS: $1.12 \cdot 10^8 \pm 1.34 \cdot 10^7$ vs. controls: $1.39 \cdot 10^8 \pm 1.67 \cdot 10^7$, $p=0.19$) (**Fig. 2A**). Our pyrosequencing experiments generated an average of 9,024 reads per sample (ranging from 4,276 to 24,502) with a mean length of 527 bp (ranging from 517 to 533 bp). Individual rarefaction curves (**Suppl. Figure S1**) showed that the mean numbers of observed operational taxonomic units (OTUs), that is 140 taxa (ranging from 61 to 210 OTUs), reached in all samples a plateau of approximately 5,000 sequence reads. The read coverage was therefore sufficient to capture most of the bacterial diversity of each intracaecal microbiome.

The within-sample diversity (α -diversity) indicated no significant difference between CUMS and control mice (**Fig. 2B**). This suggests that CUMS mice had no change in microbial richness and evenness. However, in terms of β -diversity, Principal Component Analysis (PCA) showed distinct clustering between samples from control and CUMS mice indicating a significant change in microbiome composition (**Fig. 2C**, PERMANOVA $p=0.029$).

CUMS modified caecal microbiome composition.

A more in-depth taxonomic analysis of bacterial types revealed several changes in the microbiome composition, and variations appeared at different phylogenetic levels. Nine divisions

were identified by pyrosequencing. In all samples, the majority of caecal bacteria (ranging from 92 to 98% of total 16S) belonged either to the *Firmicutes* (ranging from 49.3 to 94.4%) or to the *Bacteroidetes* phylum (ranging from 2.5 to 46.8%), with a small proportion (2-8% of the identified sequences) of bacteria from seven others phyla: *Actinobacteria*, *Candidatus Melainobacteria*, *Candidatus Saccharibacteria* (TM7), *Cyanobacteria*, *Proteobacteria*, *Tenericutes* and *Verrucomicrobia* (Suppl. Database S1). Moreover, 16 classes, 26 orders, 53 families, and 123 genera were identified.

CUMS led to an increase of the *Firmicutes* phyla ($p=0.0041$) and a decrease of the *Bacteroidetes* taxa ($p=0.0062$) compared to control mice (Figure 3A). These alterations induced a significant rise of the *Firmicutes/Bacteroidetes* ratio from 2.28 ± 0.38 in controls to 11.75 ± 3.43 in CUMS mice (Figure 3B, $p=0.00072$). The gain of *Firmicutes* in CUMS mice was not clearly associated to the expansion of distinct genera, except the *Clostridiales* members *Anaerotruncus*, *Coprococcus* and *Sporobacter* (Figure 3C), but seemed rather to be due to a general moderate rising of several taxa within the phylum. Concerning the diminution of *Bacteroidetes*, it is clearly linked to a significant decrease of *Porphyromonadaceae* ($p=0.022$) and *Flavobacteriaceae* ($p=0.073$) with the corresponding impacted genera being *Barnesiella*, *Prevotella*, *Coprobacter*, *Porphyromonas*, *Pricia*, *Parabacteroides*, *Dysgonomonas* and the vanishing of *Nonlabens* and *Maribacter* (Figure 3C, Suppl. Database S1). We also noticed the lowering of another *Bacteroidetes* (*Candidatus Armantifilum* and *Odoribacter*) and of members of the genus *Akkermansia*.

At the species level, of the 389 taxa assigned, 275 species were found in control mice and 337 species in the CUMS mice, corresponding to 223 species recovered in both groups (Fig. 3D). Among them, only 27 were shared by all animals (*core microbiome*).

Discussion

It is increasingly evident that chronic psychosocial stresses influence intestinal homeostasis. Such alterations in microbiome composition can lead to local or central dysregulations that could be involved in the onset or exacerbation of chronic disorders such as IBD or psychiatric disorders [2,12,28,29]. During spaceflight, astronauts are subjected to various physical and psychosocial stressors which could lead to dysbiosis, in a context associated with limited medical procedures and facilities. Whether space travel affects intestinal equilibrium has not been thoroughly investigated because of constraints imposed by in-flight experimentation [30]. To overcome these limitations, ground-based experiments have been conducted to explore intestinal diversity in mice, mainly based on gravity modulation [3,31]. However, gravity changes are not the only stressors encountered during space missions. Consequently, in this study, we used an easy-to-implement model (CUMS), involving the chronic exposure of mice to multiple unpredictable mild environmental and psychosocial stressors, to simulate socioenvironmental stresses encountered during a spaceflight and explore their effects on GM composition. Indeed, we previously showed that this model replicates some spaceflight-induced immunological changes observed in astronauts [27]. CUMS is also recognized as a reliable and effective rodent model of depression [9,12,16,29,32–34].

Our results revealed that after 3 weeks of CUMS exposure, a duration chosen to simulate a six-month flight at the human scale [35], there was no significant change in murine caecal bacterial load. Additionally, no statistically significant modification of the α -diversity was observed in CUMS mice by comparison to controls, indicating that the within-community diversity was not altered

by this model of chronic stress. Although these results are in agreement with other studies using variants of the rodent CUMS model [9,29], they are discrepant when compared to other works describing a decrease of α -diversity [14,16,33,34]. Such differences could be explained by variation in the CUMS protocols (species, strains, age, gender and feeding conditions of rodents, type of stressors, duration of exposure to individual stress), the origin of the samples (fecal or intraluminal), or protocol parameters (DNA extraction method, PCR parameters) [3].

However, significant change in intracaecal global β -diversity was observed after CUMS treatment. Indeed, an important increase of the *Firmicutes/Bacteroidetes* ratio was observed in CUMS mice, consistently with other reports using variants of the rodent CUMS model [9,16,29,33]. Within the *Bacteroidetes* phylum, we observed a decrease of *Porphyromonadaceae* that has already been noted with other chronic stress such as restraint stress [36] and multifactorial model of early-life adversity [37]. Within this family, the greatest impact of CUMS was observed on the relative abundance of *Barnesiella* sp., a genus composed of *Barnesiella intestinihominis* and *Barnesiella viscericola*, belonging to the core microbiome of mice and human gut. These species are described as having beneficial effects, such as protecting effects against colitis [38], enhancing the efficacy of antitumor treatments [39] and conferring resistance to intestinal colonization by pathogenic microorganisms [40]. These data raise the question of the impact of the decrease of this major member of GM in CUMS mice.

On the other hand, the increase of *Firmicutes* in CUMS mice cannot be statistically correlated with the increase of specific OTUs. This lack of correlation could be due to high interindividual variability in GM illustrated by the small number of species shared by all animals, stressed or not, suggesting the existence of only a reduced core microbiome. Such variability could also explain

the lack of statistical significance at low taxa level and the fact that the impact of CUMS was manifest only at the phylum level. It is noteworthy that CUMS is associated with the appearance of several new taxa (114, [Fig. 3D](#)), mainly belonging to *Firmicutes*, among them various OTUs of *Lactobacillus* with a great interindividual variability. Some protective taxa appeared (*Lactobacillus johnsonii*) while other decreased (*Lactobacillus murinus*), potentially offsetting each other. Interestingly, we observed opposite results when using a 3G-hypergravity model with a lowering of *L. johnsonii* and a rise of *L. murinus* [3]. Moreover, 3G-hypergravity was associated with increased bacterial load and α -diversity, as well as with a significant impact on the relative abundance of 50 intestinal species, whereas 2G-hypergravity seemed to modulate only moderately the GM composition. As described for the 2G-hypergravity model, the moderate alteration of GM observed with the CUMS model could be explained by a lower activation of the HPA axis without elevation of corticosterone level in mice sera. This hypothesis is supported by higher serum corticosterone concentrations noticed in mice exposed to 3G during 21 days [41], as well as during the first two weeks of exposition to the chronic mild stress model (CMS) which is more intense than CUMS because of water and food deprivation periods [42]. So, as previously reported for the TCR β repertoire [43,44], socio-environmental stressors seem to have less impact on intestinal microbiota than gravity changes.

Conclusions

The results of the present study demonstrate that 3-weeks of exposure to chronic unpredictable psychosocial and environmental stressors alter mice GM, although at a lower extent than physical stressors encountered during deep-space exploration such as gravity changes. Such alteration of

GM must receive attention and should be monitored in crewmembers, especially since it has been recently shown that a fecal transfer of GM from CUMS mice to healthy mice induces despair-like behaviors associated with alterations in serotonin pathway [34]. Furthermore, these data provide additional arguments to the countermeasure protocol proposed by experts against spaceflight-associated perturbations to the immune system [23]. Their recommendations include physical and psychological exercises for stress management, pre- or probiotics supplementation and dietary approaches, that could also permit to limit dysbiosis and its consequences on health. Finally, note that the results of this study go beyond astronaut health protection because the CUMS model can also be used to study the impact of everyday life stresses and it is well established that stress can contribute to the development or aggravation of several pathologies [2,45].

Methods

Animals. C57BL/6j male mice (8-week-old, mean body mass of 20 g) were purchased from Charles River (Les Oncins, France). On arrival, animals were housed for 5 days in groups of five in standard cages in the animal facility of the INSERM UMR 894 laboratory (Paris). They were housed in a quiet room under constant conditions (22°C, 50% relative humidity, 12-h light/dark cycles with dark periods from 8 pm to 8 am) with free access to standard food and water. Then, mice were randomly divided in two groups housed in two separate rooms: one control group and one group subjected to CUMS for 21 days. Experimental procedures were carried out in conformity with the National Legislation and the Council Directive of the European Communities on the Protection of Animals Used for Experimental and Other Scientific Purposes (2010/63/UE). The CUMS protocol

was approved by the French Ministry of Research (authorization 00966.02).

Exposure to chronic unpredictable mild psychosocial and environmental stressors (CUMS model). Isolated animals (one mouse per cage) were subjected during 21 days to different unpredictable mild psychosocial and environmental stressors, according to Pardon *et al.* (2000) [46]. The CUMS procedure presented in [Figure 1A](#) was scheduled over a 1-week period and repeated throughout the 3 weeks of experimentation. Stress periods were always separated by stress-free intervals of at least 2 h to avoid any habituation process. The control group was left undisturbed in another room of the animal facility, five mice per standard cage (37.5 cm x 21.5 cm x 18 cm). Animals presenting injuries (such as bites that could induce inflammation) were discarded resulting in 7 Control mice and 10 CUMS mice.

Sample collection. At the end of the experiment, CUMS and control mice were anesthetized using isoflurane, weighed and then put to death by cervical dislocation. All samples were immediately processed to avoid degradation and/or contamination. The intestine was dissected in by excising the entire caecum. Samples were opened longitudinally and their contents were removed by two successive washes in DEPC (1%)-treated PBS. Intra-luminal contents were immediately frozen in liquid nitrogen and stored at -80 °C until DNA isolation.

Corticosterone quantification. Corticosterone was quantified in serum samples without any extraction procedure using the Corticosterone Enzyme Immunoassay kit (ArborAssays, Ann Arbor, MI, USA). Samples were analyzed in duplicate. Absorbance at 405 nm was measured and

concentrations, calculated from a standard curve established using calibrators, were expressed as ng/ml.

DNA isolation. Whole genomic DNA was extracted from caecal samples (50 mg) using the Fast DNA SPIN kit for Soil (MP Biomedicals, Santa Ana, CA, USA) [47] after bead beating with the FastPrep-24 Instrument (MP Biomedicals) at 6.0 ms⁻¹ for 40 s, according to manufacturer's instructions. Purified DNA was resuspended in sterile deionized DNase/pyrogen-free water, analyzed by spectrophotometry (NanoDrop 2000C; Labtech, Heathfield, East Sussex), and frozen (-20°C) until analysis.

Fecal microbiota sequencing. Barcoded primers Bact-515F (5'- GTGCCAGCMGCNGCGC-3') and Bact-1061R (5'-CRRACAGAGCTGACGAC-3') described by Klindworth *et al.* (2013) [48] were used for the initial amplification of the V4-V6 region of the 16S rRNA gene as previously described [3]. PCR reactions contained 2.5 U of *Taq* DNA Polymerase (Invitrogen, Cergy Pontoise, France), 5 µl of 5X buffer, 75 nmol MgCl₂, 1 µl of 10 mM dNTPs, 1 µl of each primer (50 µM) and 50 ng of DNA. Three PCR reactions were run for each sample as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 45 s, 60°C for 45 s, 72°C for 45 s and a final extension at 72°C for 5 min. PCR reactions from the same sample were pooled, purified using the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France) and quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) using the dsDNA HS Assay Kit (Life Technologies). To ensure equal representation of each sample in the sequencing run, each barcoded sample was standardized by calculating equimolar amounts (100 ng/sample) using the SequalPrep Normalization Plate Kit (Invitrogen)

prior to pooling. Pooled samples of the 16S rRNA gene multiplexed amplicons were sequenced on a Roche 454 Genome Sequencer FLX Titanium instrument using the GS FLX Titanium XLR70 sequencing reagents and protocols (Beckman Coulter Genomics, Danvers, USA).

Amplicon sequencing data analysis. Analysis of amplicon sequencing data was carried out using the MEGAN pipeline [49]. After demultiplexing, combined raw sequencing data plus metadata were filtered to exclude low-quality reads. Next, data were denoised and clustered using the MIRA 4 software (<http://mira-assembler.sourceforge.net>). Sequences with $\geq 98\%$ similarity were binned and assigned to the same OTU to approximate species-level phylotypes. Representative sequences of each OTU, derived from clusters or singletons, were assigned at different taxonomic level by using the Ribosomal Database Project II Classifier [50]. To avoid a potential bias linked to variation of sequence coverage between samples, the data were normalized to 100000 sequences per samples. Rarefaction curves were constructed to evaluate sequencing depth. Relative abundances of each OTU were compared according to the different experimental conditions. Bacterial richness and diversity across samples were estimated by calculating the following indexes as previously described [3]: Shannon index, Evenness index, OTU's number, Simpson's index of diversity, and Simpson's reciprocal index. PCA was conducted to appreciate overall distance between microbial communities, using relative abundance and taxa-to-taxa distance estimates. Obtained 16S rRNA gene sequences have been deposited into NCBI's Sequence Read Archive database (<https://www.ncbi.nlm.nih.gov/sra>) under accession number SRP153311.

Analysis of intracaecal bacterial load by qPCR. The amount of total bacteria was assessed by

amplifying 0.5 ng of DNA extracted from each fecal sample with pan-bacterial primers targeting the 16S rRNA gene as previously described [3]. Briefly, PCR assays were performed using the MESA FAST qPCR MasterMix for SYBR Assay as recommended by the manufacturer (Eurogentec, Seraing, Belgium). DNA extracted from the *Barnesiella intestinihominis* DSM 21032^T strain using the QIAamp DNA Mini Kit (Qiagen) was used to establish the standard curves. All assays were performed in triplicate. The following thermocycling conditions were applied with the MyiQ™2 real-time PCR system (Bio-Rad Laboratories): initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were obtained immediately after the amplification under the following conditions: 70 cycles of 10 s with an increment of 0.5 °C/cycle starting at 60 °C.

Statistical analysis. Comparison of bacterial loads quantified by qPCR, relative abundances, and phylogenetic diversity indexes were performed using the Mann-Whitney U test with a significance level α of 0.05. *p*-values comprised between 0.05 and 0.10 indicate trend. The *p*-values were adjusted for multiple hypotheses testing using the False Discovery Rate method [51] for all the results within each taxonomy level. The PERMANOVA analysis (99 permutations) was conducted on dissimilarity indices produced by the Bray-Curtis method [52]. The β -diversity PCA was produced using Marti Anderson's procedure for the analysis of multivariate homogeneity of group dispersions [53]. All the analysis were performed using R version 3.5.0 (<https://www.R-project.org/>).

List of abbreviations

CMS: chronic mild stress

CUMS: chronic unpredictable mild stress.

GM: gut microbiota

HPA: hypothalamic-pituitary-adrenal

IBD: intestinal bowel disease

LDA: linear discriminant analysis

OTUs: operational taxonomic units

PCA: principal component analysis

TCR: T cell receptor

Declarations

Ethics approval and consent to participate. Experimental procedures were carried out in conformity with the National Legislation and the Council Directive of the European Communities on the Protection of Animals Used for Experimental and Other Scientific Purposes (2010/63/UE). The CUMS protocol was approved by the French Ministry of Research (authorization 00966.02).

Consent for publication. Not applicable.

Availability of data and materials. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files). 16S rRNA gene sequences are

available through the NCBI's Sequence Read Archive (SRA) database
(<https://www.ncbi.nlm.nih.gov/sra>) under accession number SRP153311.

Competing interests. The authors declare that they have no competing interests.

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Authors' contributions. CA conceived, designed and performed experiments, analyzed data, and wrote the manuscript. LC assisted with and performed experiments, and analyzed data. MW designed and performed statistical analysis. AL assisted with experimental design and analysis, and corrected the manuscript. LL designed animal experimentation and supervised mice treatments. CLF performed experiments and analyzed data. NA assisted with statistical design and corrected the manuscript. CCG analyzed data, and wrote the manuscript. JPF assisted with experimental conceiving, design and analysis, and corrected the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Figure Legends

Fig. 1. Stress status of mice. (A) CUMS protocol, (B) body weights, (C) thymus weights (normalized to body weight), (D) serum corticosterone concentrations in control and CUMS mice.

Fig. 2. Comparison of microbiota diversity between CUMS and control mice. (A) Total bacterial load quantification by qPCR corresponding to the total number of 16S rRNA gene copies per mg of intracaecal content of mice subjected to CUMS (n=10) and control mice (n=7) ($p=0.19$), and (B) α -diversity indexes: Observed OTUs (richness, $p=0.73$), Evenness ($p=0.52$), Shannon index ($p=0.84$), Simpson index of diversity ($p=0.69$), and Simpson's reciprocal index ($p=0.81$). Statistical analyses were done using the Mann-Whitney U test. The upper and lower ranges of the box represent the 75% and 25% quartiles, respectively. Error bars reflect standard error of the mean. (C) PCA of microbiomes from CUMS vs. control mice ($Pr(>F)=0.029$). The variance explained by each of the main two dimensions of the PCA is indicated in parentheses on the axes.

Fig. 3. Differential abundance of bacterial taxa. (A) Mean relative abundance (%) of bacterial phyla and (B) *Firmicutes/Bacteroidetes* ratio in the caecal content of mice subjected to 21 days of CUMS compared to controls. A significant decrease of *Bacteroidetes* ($p=0.006$) and a significant increase of *Firmicutes* ($p=0.004$) were noted in CUMS mice by comparison to control mice. This was associated to a significant increase of the *Firmicutes/Bacteroidetes* ratio ($p=0.00072$) in CUMS mice. Statistical analyses were done using the Mann-Whitney U test. * $p < 0.01$; ** $p < 0.001$. (C) Differentially abundant main genera in control mice (green) and CUMS mice (red) identified using Linear Discriminant Analysis (LDA) Effect Size (LEfSe) analysis. (D) Schematic representation of

core microbiome at the species level. Green circle: number of species shared in all control mice. Red circle: number of species shared in all CUMS mice. Intersections and numbers inscribed within refer to shared species and in parentheses shared with all mice.

Supplementary Fig. S1. Rarefaction curves of bacterial 16S rRNA gene sequences obtained from (A) control and (B) CUMS mice, and used to evaluate if further sequencing would likely detect additional taxa, indicated by a plateau.

Supplementary Database. Statistical analysis of intracaecal microbiomes comparing relative abundance using the nonparametric Mann-Whitney U test at the phylum level (Supplementary Dataset S1_a), at the class level (Supplementary Dataset S1_b), at the order level (Supplementary Dataset S1_c), at the family level (Supplementary Dataset S1_d), at the genus level genera (Supplementary Dataset S1_e) and at the species level (Supplementary Dataset S1_f).