



# Temporal and Spatial Analysis of Forward and Backward Microbial Contamination in a Mars Analog Mission

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As human exploration missions to Mars are on the horizon, microbial cross-contamination remains a key issue to address. These issues can be approached today using advances in molecular metagenomics methods, which include rapid and sensitive sequencing platforms for characterizing microbial populations. Combined with analog missions, these methods provide powerful tools for assessing the challenges associated with planetary exploration. Here, we designed a protocol to monitor forward and backward contamination events and progression in an 11-days Mars analog mission in the Ramon crater in Israel. Forward contamination soil samples were collected daily from three sites—two sites in close proximity to the habitat and one isolated site. Backward contamination was determined in samples from nitrile gloves of six analog astronauts before and after extravehicular activities. Temperature, relative humidity and soil composition data were also collected for all sites. Environmental DNA samples were extracted in the main habitat and 16S (bacterial) and 18S (eukaryotic, fungal) rRNA gene amplicons were sequenced and analyzed to study microbial population diversity and composition. Shannon Diversity index analysis and Principal Coordinates analysis (PCoA) of rRNA genes indicated that differences in the diversity and population composition were significant in sites closer to the habitat when compared to a reference site. These samples also demonstrated the introduction of human-associated taxa to the environment. Backward contamination consisted of bacterial taxa found on gloves upon return from EVA and also detected in soil, altogether 44 genera, indicating backward contamination events. To our knowledge, this is the first protocol to utilize advanced molecular technologies to investigate forward and backward contamination in a Mars analog mission.

**Keywords:** planetary protection, contamination, mars, analog research, ramon crater, 16S, 18S

**Abbreviations:** AA, analog astronaut; EVA, extravehicular activity; RA, relative abundance

## INTRODUCTION

Even before the Viking landers successfully landed on Mars in 1976, NASA's principles of planetary-protection policies were conceived and embedded in mission design. The Committee on Space Research (COSPAR, est. 1958) provides recommendations which are then taken by space agencies to create specific mission requirements. COSPAR policies have undergone many iterations, until reaching their current and present configuration (Fisk et al., 2020; NASA, 2021). Prevention of forward and backward contamination is the main goal of planetary-protection policies, to eliminate and reduce the potential of cross-transferring life forms from one planet to another. Such contamination is possible either by carrying terrestrial microorganisms via a spacecraft (forward contamination) or by bringing extraterrestrial microorganisms from other planets or celestial bodies back to Earth (backward contamination), via various mechanisms such as sample return missions (Cockell 2005; Pratt and Smith, 2020). Protection against contamination has significant ethical, scientific and safety related aspects (Rummel, 2001; Schwartz, 2019). Forward and backward contamination is becoming a pronounced risk due to an increasing number of planned spaceflight missions, as highlighted by the National Research Council 2002 Safe on Mars report (National Research Council, 2002), and the 2015 COSPAR workshop (Race et al., 2015).

A recent example of such risk is the case of the Israeli SpaceIL Beresheet lunar lander. Beresheet's hard landing on the Moon in April 2020, while carrying a small payload of tardigrades, raised a vigorous global debate on both ethical and legal implications of a possible forward contamination event (Caplin, 2019; Shahar and Greenbaum, 2020). Although the question whether life arose on Mars is still under investigation (Cabrol, 2018), the potential of Mars-sourced biological cross-contamination via robotic, and in the future, human missions to Mars, stresses the need for a better understanding of the forward and backward contamination potential of such missions (Goh and Kazeminejad, 2004). Several in-depth interdisciplinary analyses of contamination potential were previously done in Mars analog missions, such as the AustroMars simulation, performed in the Mars Desert Research Station (MDRS), located in the Moab desert in Utah, United States (Grömer et al., 2007). An additional approach to study forward contamination was performed under simulated Martian conditions such as desiccation, freezing, Martian-like atmosphere, pressure and UV radiation climate, either in a Mars Simulation Chamber (MSC) (Schuerger et al., 2003) or by using wind tunnels (Van Heereveld et al., 2017) to mimic eolian Martian transport conditions. Most of the current and past studies focus on a single organism, such as *Bacillus subtilis* (Schuerger et al., 2003; Nicholson et al., 2012), or *Staphylococcus xylosum* (Van Heereveld et al., 2017), *Desulfotalea psychrophila* and other sulfate-reducing bacteria (Silver, 2018).

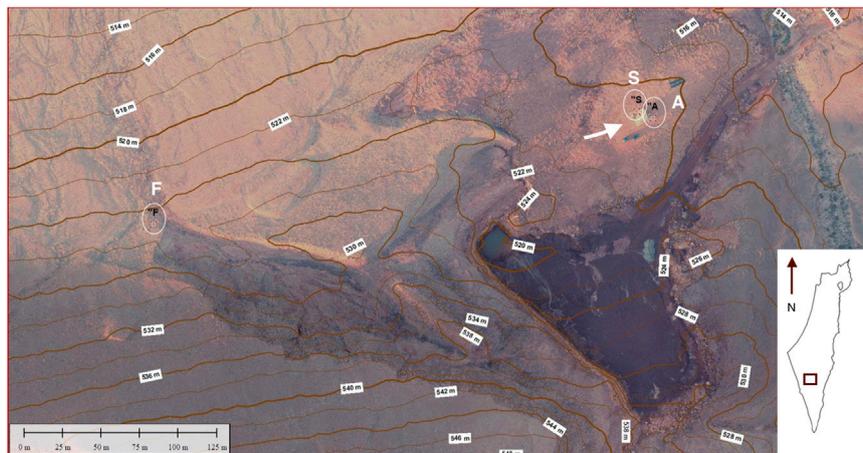
Recent advances in high-throughput molecular biology methods, such as amplicon sequencing, have significantly expanded humanity knowledge on the evolutionary relations and taxonomic structure of life forms and life niches around the world (Liu et al., 2007; Huse et al., 2008), and have enabled deeper understanding of such communities. Characterization of bacterial communities by DNA extraction has already been successfully performed in extreme and pristine environments, such as acidic hypersaline lakes in Australia (Mormile, 2009), the Antarctic Dry Valleys (Cary et al., 2010), the hyper arid Atacama Desert (Piubeli et al., 2015) and the high altitude Andean lakes (Ordoñez et al., 2009). This technology can be efficiently adapted to characterize low-biomass environments and to study delicate shifts in microbial and fungal population dynamics.

Extreme analog environments on earth possess geological or environmental characteristics that are similar to those that exist on an extra-terrestrial planetary body. Therefore, they can serve as a tool to study specific aspects of a space mission to such an extra-terrestrial body (Martins et al., 2017).

In this study, we aimed to investigate the backward and forward contamination potential in a Mars analog. We chose the Ramon crater for an analog mission due to its Mars-like geomorphology and geology, isolation and hyper arid climate conditions (Rubinstein et al., 2019). The Ramon crater is the largest of the three erosional valleys formed along anticlinal axes in the Negev desert in Israel. The crater is 40 km long and 12 km wide, with an area of 241 km<sup>2</sup> (Notesco et al., 2016). The climate of the area is arid to hyper arid and the mean multiannual rainfall is 56 mm in the central part of the crater (Schmidt and Karnieli, 2001). The Ramon crater is well-studied and rich in its geological history, with some unique features such as the largest anticline in the region, exposure of layers from the Triassic period, volcanoes, dikes, sills and basalt flows created from extensive Lower Cretaceous volcanic activity (Schmidt and Karnieli, 2001). It contains marine fossils from the Triassic, Lower Cretaceous, and Upper Cretaceous periods and has additional unique features which have promoted its inclusion in the Negev Craterland Geopark initiative (Finzi et al., 2019).

Our working assumption was as follows: We hypothesized that during the analog mission, soil population composition will shift between sites and between sampling points, and that differences in its diversity and composition will be significant in the sites which are closer to the habitat, compared to the isolated site. We assumed that it will take several days until a forward or backward contamination event will occur and the microbial composition will shift, in all sites. If forward contamination occurs, population diversity and composition will be affected by the artificial introduction of new taxa, potentially human-associated species. New species will be introduced to the habitat, if they remain on the astronaut gloves upon returning to the mock pressure chamber for spacesuit doffing.

We utilized, for the first time to our knowledge, advanced molecular microbiology tools to better understand cross contamination potential. We offer a field demonstration of this methodology, in the longest analog mission performed in Israel to date.



**FIGURE 1** | An orthophoto and elevation model of the habitat and the sampling sites. Sites are highlighted with white circles. Site A is denoted “A,” site F is denoted “F” and site S is denoted “S.” The white arrow points toward the habitat. Small inset shows the general location of the experiment in Israel, Ramon Crater.

**TABLE 1** | Site descriptions for soil samples. SD—sampling day, SS—sampling site.

Site name	GPS coordinates	Color	Texture	Soil type	Moisture (on SD)	Vegetation/lichen
A	34.8587113107, 30.5960945591	Red and light brown	Granular	sandy loam (0% clay, 31% silt and 69% sand)	Dry	None
S	34.8586167386, 30.5961316244	Red and brown with yellow hues	Granular	Loamy sand (0% clay, 22% silt, 78% sand)	Slightly damp	None
F	30.5954698, 34.8558041	Bright yellow and white	Powder	Loam (20% clay, 40% silt, 40% sand)	Slightly damp	Low hanging shrubs located 5 m away from SS

## MATERIALS AND METHODS

### Site Selection and Soil Sampling

D-MARS03 analog mission took place between the 3rd and 14th of March 2019 (Rubinstein et al., 2019) and was situated in a 500 m radius area within Ramon crater, at elevation of 520 m above sea level. During the 11-days mission, six isolated analog astronauts (“Ramonauts,” “AAs”) performed daily EVAs, fully suited with a simulated space suit (~13 kg) and a personal life support system. During EVAs, the AAs collected soil samples for forward analysis and soil characterization. For the initial soil analyses, samples (three samples per site ~0.5 kg each) were transferred to sterile zip lock bags from three different sites: “A”—~0.5 m from the habitat’s airlock entrance, “F”—an undisturbed, isolated site, approximately 280 m west of site A, and site “S”—located at the back of the habitat, ~0.3 m from the sanitary disposal tank, 10.7 m north of site A (see **Figure 1**). The GPS coordinates for each site are detailed in **Table 1**. Plastic jars (non-sterile, 450 ml volume) were used for soil type analysis. Jars were filled with soil (~250 g), and DDW was added to each jar (approx. 350 ml). Jars were shaken vigorously and left to stabilize overnight, at room temperature. Soil texture and type were diagnosed using the available paradigm and texture calculator from the United States Agriculture Department (USDA), Natural

Resources Conservation Service Soils (NRCS) calculator (Natural Resource Conservation Service, 1999). For forward contamination analysis, three soil samples were collected at each site from the top soil layer down to a 3 cm depth, with approximately 10 cm distance between individual samples. The daily sampling time was at 08:00–09:00 AM.

### Nitrile Gloves Sampling

For backward contamination analysis, XXL nitrile gloves (SemperGuard® nitrile powder-free disposable gloves, Sigma-Aldrich) were placed over the AAs’ suit gloves, in the airlock before exiting to an EVA. In brief, a 4 × 4 cm<sup>2</sup> section of the palm of the hand, and then five fingers, were gently swabbed with a sterile cotton swab, which was then stored in RT, inside a sealed tube. A second sampling took place immediately upon the AA’s return to the habitat from EVA, in the simulated airlock area. Then, both samples were extracted as detailed in *Sample Processing and DNA Sequencing*. Control samples were obtained by either using a new pair of disposable nitrile gloves, or by extracting DNA from a sterile swab.

### Sample Processing and DNA Sequencing

In total, 71 soil samples were collected, of which 40 were further used for DNA extraction. All sample manipulations and DNA

**TABLE 2** | Primers used in this study.

Primer	Sequence
CS1-341F	ACACTGACGACATGGTTCTACANNNNCCTACGGGAGGCAG CAG
CS2-806R	TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTA AT
CS1_Euk1391F	ACACTGACGACATGGTTCTACA GTACACACGCCCGTC
CS2_EukBr	TACGGTAGCAGAGACTTGGTCT TGATCCTTCTGCAGGTTTC ACCTAC

extractions were carried out in a laminar flow hood (BSC-8, MRC Ltd.). Genomic DNA was extracted from swabs or soil samples using DNeasy PowerSoil Pro Kit (QIAGEN), as recommended by the manufacturer. Controls for DNA extraction reagents and procedure were either sterile swabs or sterile water to verify the integrity of the extraction in the given conditions. Long-term storage of the samples prior to DNA extraction was avoided, and DNA was immediately isolated from the soil on the same sampling day. No positive control was done for DNA extraction. Extracted DNA samples were stored at  $-20^{\circ}\text{C}$  until further processing. Directly after the mission, PCR amplifications of 16S and 18S rRNA genes were carried out with universal primers containing 5'-end common sequences. Primers used in this study are detailed in **Table 2**. A total of 28–31 PCR cycles ( $95^{\circ}\text{C}$  for 15"s,  $53^{\circ}\text{C}$  for 15"s, and  $72^{\circ}\text{C}$  for 15"s) were conducted using the PCR master mix KAPA2G Fast (Kapa Biosystems), and successful amplification was verified by agarose gel electrophoresis. A control reaction without a template but containing all other reagents was included in each PCR mix. DNA extraction from a human fecal sample was used as a positive control for PCR reactions.

## Data Analysis

Paired-end deep sequencing of the PCR products was performed on an Illumina MiSeq platform at the University of Illinois at Chicago Sequencing Core (UICSQC). Sequencing depth ranged from 5,797 to 145,126 sequences per sample for 16S, and 870 to 95,418 sequences per sample for 18S; Raw data processing was done by the University of Illinois at Chicago Core for Research Informatics (UICCRI). Forward and reverse reads were merged using PEAR (Zhang et al., 2014). Quality trimming based on quality threshold and length parameters was done with  $p = 0.01$  and min length = 100. Adaptor and ambiguous nucleotide trimming was done prior to further applications. Chimeric sequences were identified using the USEARCH (Edgar 2010) algorithm as compared with a reference database (silva\_132\_16S.97 and silva\_132\_18S.97) (Oliver et al., 2017). Sequences were de-replicated and clustered at a threshold of 0.97; sequences appearing fewer than 10 times were excluded. Taxonomic assignments were identified using the USEARCH algorithm against the silva\_132\_16S.97 and silva\_132\_18S.97 reference databases. A table of relative abundance data and associated taxonomic annotation was created after read counts were normalized as a fraction of total sequence counts in each sample. Raw sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive

(<http://trace.ncbi.nlm.nih.gov/Traces/sra/>), and can be accessed under BioProject accession number PRJNA662496 (release date 31-10-2020).

## Statistical Analyses

R vegan (Oksanen et al., 2013) package was used to calculate Shannon diversity index and ANOSIM probability tests. PCoAs were constructed using the base R function "cmdscale" and plotted with ggplot2 package (Wickham 2016). Data rearrangement was done using the Reshape package (Wickham, 2007). Kruskal-Wallis test was conducted using base R functions. The contribution of different variables to the microbiome composition was assayed by PERMANOVA, using the function adonis (vegan) with default parameters and setting temperature, relative humidity and sampling site as independent variables.

## Temperature and Relative Humidity Monitoring

In order to monitor the environmental temperature and relative humidity, three HOBO data loggers (HOBO<sup>®</sup> MX2300 Series Data Logger, HOBO<sup>®</sup> MX2301A Data Logger and HOBOware 3.7.18 by Onset Computer Corporation, Bourne, MA, United States) were placed, one in each site, 3 cm depth, throughout the mission. The loggers were shielded from direct sunlight by a white plastic cover and retrieved at the last sampling day. The data loggers monitored the temperature and relative humidity at all sites continuously.

## RESULTS

### Soil Analysis and Environmental Data Collection

Characterization and analysis of soil composition were performed as described above and the results are detailed in **Table 1**. An orthophoto and elevation model (DEM) of the mission area and sampling sites can be viewed in **Figure 1**. Soil composition was found to be sandy loam in site A, loamy sand in site S and loam in site F. Sites A and S had no vegetation or lichen present, while site F contained low hanging shrubs located 5 m away from the sampling point. Soil type results correspond to previously documented layers of Mishor and Ardon formations, Lower Jurassic, which include mainly sandstone, limestone, siltstone, clay and gypsum (Sneh et al., 1998; Notesco et al., 2015). Environmental data were collected using HOBO data loggers, a well-established tool for environmental temperature and moisture monitoring (Da Cunha, 2015) (results are summarized in **Table 3**). Data collected throughout the mission indicated that the sites differ from each other in terms of average temperature, day and night temperature fluctuations, and relative humidity. While the maximal temperature was equal across all sites, both minimal and mean temperatures were higher in site A (**Table 3**). In terms of relative humidity, site A was drier than sites F and S (52.89 vs. 66.95% and 67.43%, respectively). This observation is expected as site A, located at the entrance of

**TABLE 3** | Summary of environmental temperatures and relative humidity in the different sites throughout the mission.

Site name	Avg. Temp. (°C)	Max. Temp. (°C)	Min. Temp. (°C)	Avg. RH (%)
A	15.009	31.944	8.717	52.892
S	12.269	31.915	7.247	66.959
F	14.52	31.306	7.242	67.437

the habitat, was exposed to direct sunlight most of the day, unlike site S, which was located at the backend side of the habitat, and shaded half of the day. Since it was previously shown that microbial activity and biomass are affected by temperature and moisture in arid environments (Vishnevetsky and Steinberger, 1997), we first examined the correlation of temperature and humidity between all sites. While environmental conditions differed slightly between sampling sites, the changes of both temperature and RH across time were positively intercorrelated between all three sites (Pearson's correlation coefficient 0.8 to 0.87 when correlating RH across sites; 0.61 to 0.87 when correlating temperature). Permutational multivariate analysis of variance (PERMANOVA) indicated that the only 12% of the microbial variance can be explained by RH ( $p = 0.002$ ), and only 9% by temperature ( $p = 0.001$ ), while 37% of the variance is explained by the site ( $p = 0.001$ ).

## Population Diversity Comparison Among Sites Throughout the Mission

To detect forward contamination events, temporal changes in microbial communities were monitored using 16S and 18S rRNA genes analysis.

16S rRNA sequences were clustered at identity threshold of 97%, yielding a total of 1,485 distinct genera in soil samples (Supplementary Table S1). 419 genera were found in all sites (but not in all samples). 197 genera were unique to site F, while 387 and 78 were present in sites A and S only, respectively. A distance matrix, based on pairwise distances between a pair of samples from each site, was calculated and visualized using Principal Coordinates Analysis (PCoA). Comparison of the PCR control and kit control to the soil samples from all sites showed strong separation (ANOSIM R-value = 0.5 for S site, R-value = 0.85 for A site and R-value = 1 for F site, Supplementary Figures S1–S3) between the controls and the samples.

A strong separation (analysis of similarity [ANOSIM] R-value = 0.83,  $p$ -value = 0.001) of soil microbial populations composition between sites was evident (Figure 2A). Notably, the population composition within sites near the habitat exhibited temporal fluctuations; this is reflected in the highly dispersed A and S clusters in Figure 2A. The mean pairwise distances for samples in A and S sites was significantly lower than for the F site (means of 0.52 and 0.72 vs. 0.25 respectively, Kruskal-Wallis  $p = 1.19e^{-10}$ ). Shannon diversity index was calculated based on genera (Figure 2B) and significantly differs among sites F, S, and A ( $p = 0.004$ , Kruskal-Wallis test).

While the Shannon diversity index for bacterial populations (16S) of site F is relatively high and stable across all time points (min = 3.79, max = 4.11, and mean = 3.94), the Shannon diversity index for sites S and especially A fluctuated considerably (min = 2.61, max = 4.772, and mean = 4.05 for site A; min = 2.44, max = 3.61, and mean = 3.15 for site S), with particularly low diversity observed for some samples taken on days 7 and 9. Similar trends were observed in the 18S rRNA diversity analysis; Clustering of sequences at an identity level cutoff of 97% detected a total of 664 unique genera in the soil samples (Supplementary Table S2). Shannon diversity index was calculated based on genera (Figure 2C) with samples from the third days presenting relative lower diversity in site S, and samples from the third and ninth day for site A. PCoA (Figure 2D) indicated a separation (ANOSIM R-value = 0.6,  $p$ -value = 0.001), similarly to the results acquired with 16S sequences.

## Population Dynamics Throughout the Mission Suggest Forward Contamination

To better examine the population composition of each site and each sample, dominant taxa across sites were compared. Bar plots showing detailed taxonomic composition across each site, as a factor of time, are presented in Figures 3A–C. Rare taxa that had a relative abundance (RA) of less than 1% were eliminated from the bar plots to simplify the visualization.

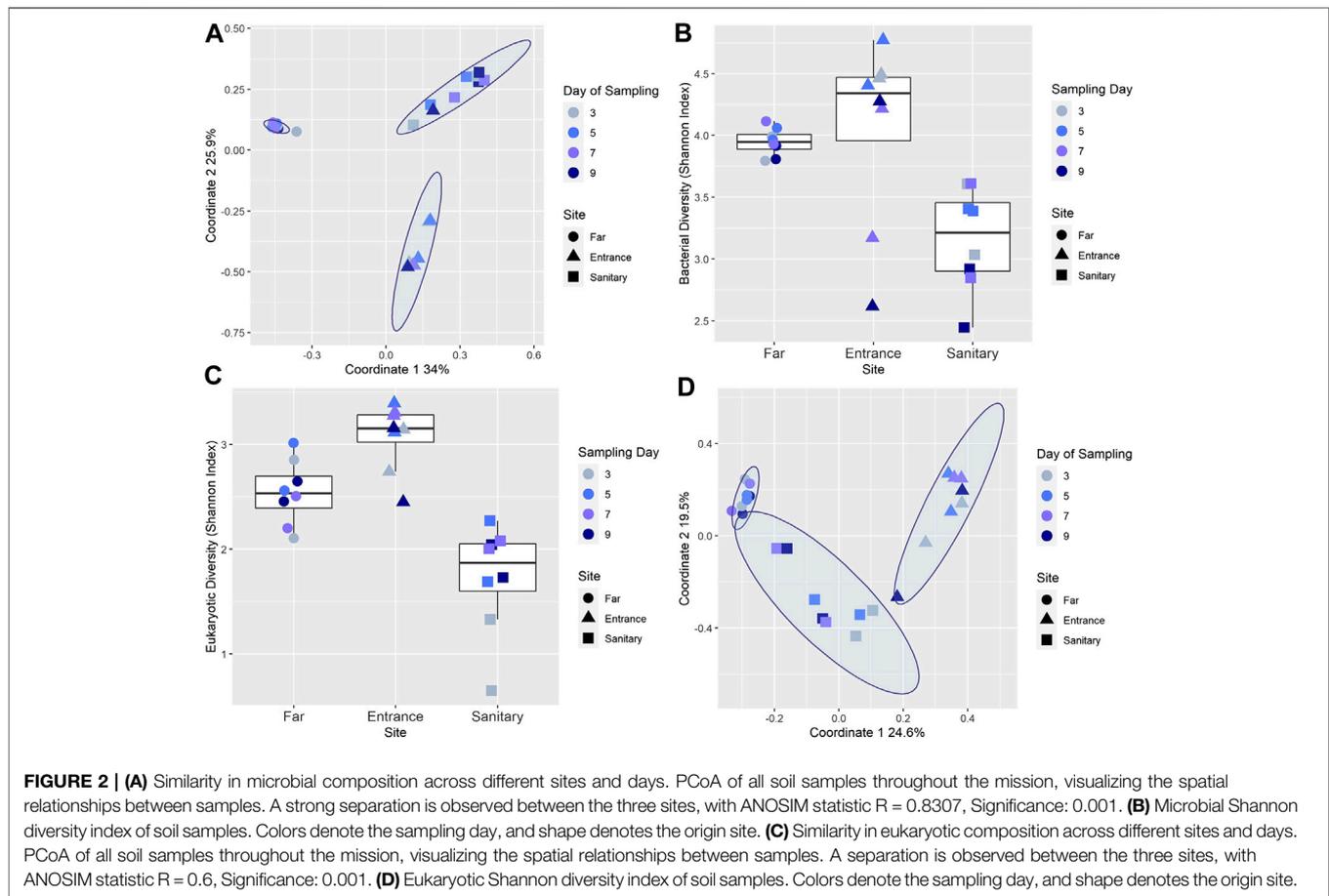
Most genera were present at RA of less than 5% (Supplementary Figure S4).

A certain degree of variation between paired samples (taken from the same location on the same day) is apparent when examining the bar graphs (Figures 3A–C). To further investigate and quantify this variation, we extracted from the Bray-Curtis distance matrix all pairwise distances for paired samples and compared those to all other pairwise distances (random). Overall, smaller pairwise distances are observed for paired samples, with samples taken from the far site having the lowest within-pair variation ( $p$  Kruskal-Wallis = 0.0002;  $p$  Permutations test = 0.15).

In site S (sanitary), which was the least diverse, Moraxellaceae was the dominant bacterial family with the genus *Psychrobacter* most prevalent (mean RA:  $17.8 \pm 9\%$ ). *Actinobacter* was the second most dominant genus with average RA of  $16.4 \pm 6.2\%$  (Figure 3A).

In site A (habitat entrance), Phormidiaceae was the most prevalent family with average RA of 12% ( $\pm 9\%$ ) (Figure 3B). Site F showed the most consistent bacterial and archaeal population composition (Figure 3C). The most dominant families were Nitrososphaeraceae (average RA  $8.7 \pm 1.2\%$ ), Blastocatellaceae ( $6.6 \pm 3\%$ ) and an unidentified family from the class *Armatimonadia* ( $7.7 \pm 2\%$ ). In addition, site F was the only site to show a time affected microbial population shift ( $R = 0.4$ ,  $p = 0.007$ , Supplementary Figure S5–S7).

To detect forward contamination, we first compiled a list of taxa found to be human-associated in previous studies, utilizing the dbBact database (<http://dbbact.org/enrichment>; Supplementary Table S3). These taxa can serve as markers for such contamination. The sum RA of all human associated



genera in each site is presented in **Figure 3D**. Site S presented the highest RA of human associated genera, with samples collected on the seventh day being the most contaminated. As an example, the genus *Citrobacter* had an average RA of  $0.09 \pm 0.015\%$  on the third and fifth days, and on the seventh day its RA in sample 07S1 increased to 38%. Site A was less contaminated, but still had higher RA of human associated genera as compared to site F, which presented very low levels of contamination (**Figure 3D**).

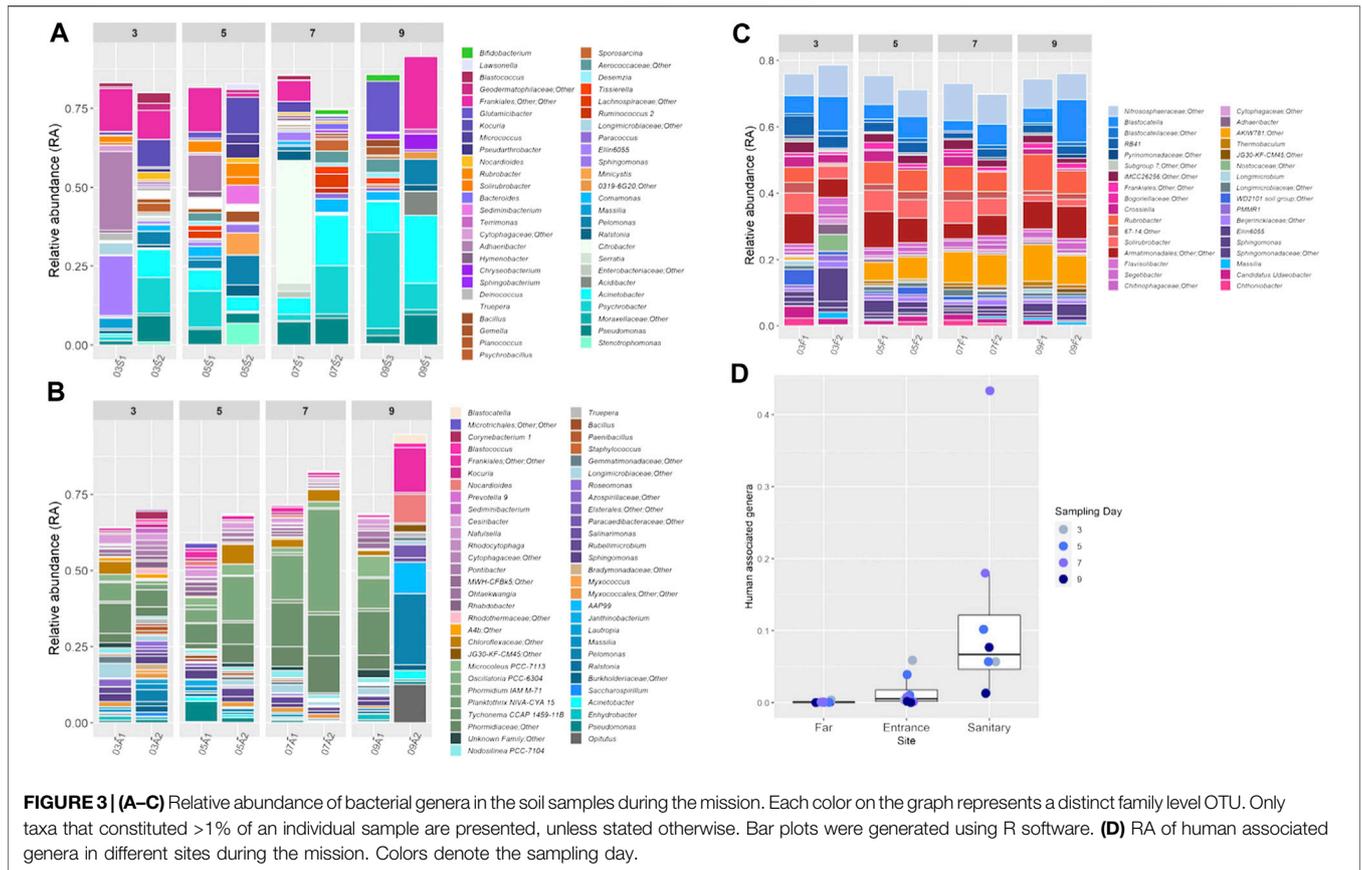
## Introduction of Soil Microorganisms Into the Habitat

The study of backward contamination was designed to detect microorganisms carried on the AA's gloves, upon return from EVAs. Baseline for the nitrile gloves was conducted so each "after EVA" sample was compared and paired to its "before EVA" sample (new glove). In addition, three samples of sterile swabs, DDW, and never worn nitrile gloves were included in the analysis. PCoA indicated that the "before" samples clustered together, along with the controls, suggesting a similar population composition. In contrast, the "after" samples were more dispersed and did not cluster with the controls (**Figure 4A**). The dominant and shared family in both "after" and "before" samples was Moraxellaceae, specifically the genus *Acinetobacter*, with average RA  $9.6 \pm 16\%$  in the "before" samples, and average

RA of  $8 \pm 14.9\%$  in the "after" samples. Analysis of the controls with "after" samples showed a strong separation (ANOSIM  $R$ -value = 0.7,  $p$ -value = 0.003, **Supplementary Figure S8**), while clustering the control samples with the "before" samples, showed a much lower degree of separation (ANOSIM  $R$ -values = 0.26,  $p$ -value = 0.035, **Supplementary Figure S9**).

To detect the most probable candidates for backward contamination, genera detected from "after-EVA" gloves were cross reference with those detected in soil samples, yielding 44 genera that were shared between both sample types (very rare taxa, not reaching a threshold of 0.005 in any sample, were excluded before intersection). We then examined the distribution patterns of these shared genera across three sites. Ten shared genera appeared mainly in the Far site, and belong to commonly arid soil taxa, such as *Chloroflexia* (green non-sulfur bacteria, **Figure 4C**) and *Solirubrobacterales* (**Figure 4D**). These may thus be viewed as classic examples of backward contamination.

A member of the Moraxellaceae family, genus *Psychrobacter* (**Figure 4E**) presented a possible case of a cross backward contamination event (taken from inside the habitat, to the outside, and then back again into the habitat). This genus was found with low RA (0.2%) in the "before" sample taken on the third day, and upon return to the habitat from an EVA, it was found in much higher average RA ( $10 \pm 9\%$  of genera in the



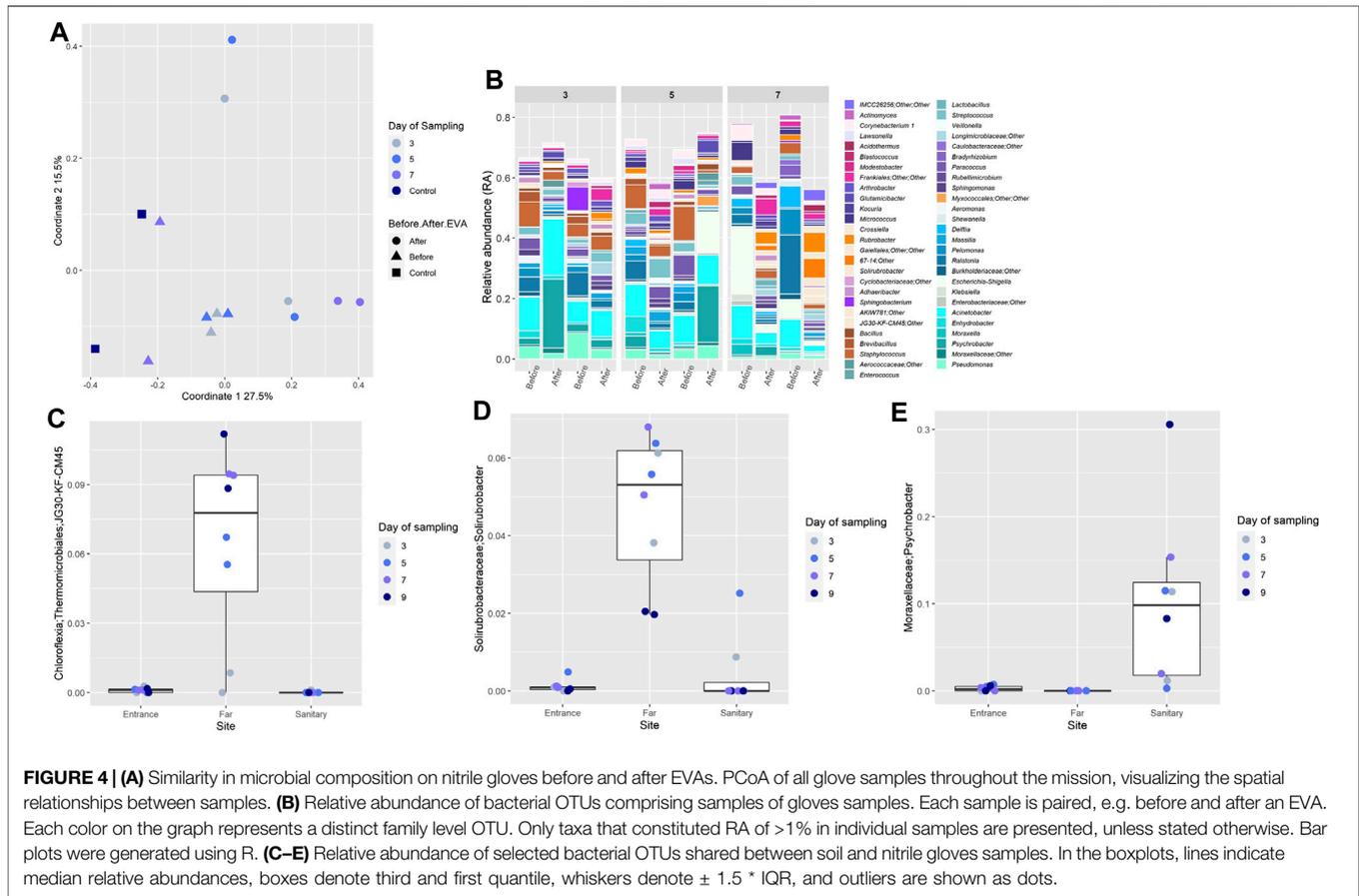
sample). A similar pattern was observed in a sample from the fifth day (3.7% before, 18% after) and the seventh day (0.006% before, 3% after). However, *Psychrobacter* was present in sites F and A with extremely low average RA ( $0.003 \pm 0.006\%$  and  $0.28 \pm 0.29\%$ , respectively), and showed high RA in site S ( $17.8 \pm 9\%$ ), suggesting its source is not in the local environment. Although several *Psychrobacter* species are environmental psychrotrophs (Bowman et al., 1996), some are also known to be human associated (Deschaght et al., 2012; Bonwitt et al., 2018).

## DISCUSSION

Analog missions are powerful platforms to gain broader and deeper understanding of the challenges accompanying human spaceflight and space missions. Forward and backward contamination potential in space exploration missions possess various aspects and hazards that, in order to be handled properly, should be further investigated. As discussed in the 2015 COSPAR workshop, there is a need for research on sampling methodology in low biomass environments, with special attention to determining spatial and temporal detection limits (Race et al., 2015). NASA’s updated policies on biological planetary protection for human missions to Mars (NASA, 2021) focus on the control of forward contamination of Mars, and possible backward contamination in the Earth-Moon system, associated

with human presence in space vehicles. NASA, alongside other key players in this field, continuously advocate for the development of internationally accepted protocols to prevent backward and forward contamination.

In this study, deep amplicon sequencing of the bacterial 16S rRNA and the eukaryotic 18S rRNA genes were used to assess forward and backward contamination potential during a Mars analog mission. Our forward contamination study included two sites with high and almost daily human interruptions: A location very close to the sanitary disposal tanks (S), checked daily by the crew, and a location near the airlock exit (A), which the AAs stepped on, upon exit and return to the habitat. As a reference point, a third isolated site was selected 280 m west of the habitat, and AAs were not allowed to enter its perimeter, except for the purpose of sampling. We hypothesized that differences in the diversity and population composition will be significant in the sites closer to the habitat when compared to the “Far” site, and that presence of human associated microorganisms will be observed. Indeed, sites S and A presented major differences, as compared to site F. Site F, the least visited site, was the only site to present a time dependent population composition change ( $R = 0.4$ , **Supplementary Figure S6**) and maintained a high and consistent diversity. The changes in microbial composition were not correlated to the changes in relative humidity and temperature, according to our statistical analysis. We believe this to be a natural manifestation of the temporal dynamics in



the soil microbial communities' composition, as has been previously shown for bacterial groups in Namib Desert soil (Gunnigle et al., 2017). Site A was located outside the airlock, where the AAs frequently visited, soil and microorganisms carried on the soles of the boots could have been transported to the site from other sites, and from inside the habitat itself. The relatively high diversity score in site A supports our hypothesis and can be explained by the site's location. As for site S, which presented a lower average diversity score, it is important to note that DMARS03 was not the first analog mission to take place in the exact same locale. Thus, pollution may have affected the natural population and its diversity in the soil close to the habitat. The population composition in site S, indicated high RA of several families likely to be human associated, such as *Enterobacteriaceae*, *Moraxellaceae*, and *Pseudomonadaceae*. The fact that some of the samples taken on the same day and from the same site exhibited differences in their population composition highlights the spatial effect on soil sampling, as previously reported (Raynaud and Nunan, 2014).

Cross contamination was also possible between sites, and its level remains unclear. AAs sometimes carried equipment that might have transported microorganisms from one site to the next. Another possible vector for cross contamination transport is the AAs' boot soles, which were not sterilized between sites. This might explain the presence (even at low RA level) of human-associated genera in site F.

We were interested to see if these changes in microbial population will occur within days of establishing a human presence in an isolated environment, based on D-MARS03 mission settings.

Our results indicate that forward contamination occurs within days in the area most adjacent to the habitat and does not manifest itself within days in an isolated location even 280 m away from the habitat. It will take a longer mission to see if changes occur in target areas 200 m or more, from the habitat or activity area of a human crew. Current mission included analog astronaut walking on foot with mock spacesuits on, it did not include any kind of vehicles or mobile robotic components that could've potentially caused forward contamination as well.

Presently, these results show that backward contamination occurred, within a few days, with one dominant genus—*Acinetobacter* (family *Moraxellaceae*). Also, several genera from the "F" site—such as *Chloroflexia* (green non-sulfur bacteria, and *Solirubrobacterales*) were detected.

The case of *Psychrobacter* is intriguing. This genus was present in high RA in site S, in contrast to sites A and F, in which its presence was rare to negligible. This genus was also found on the gloves of almost all AA's upon return from EVAs. This genus might be human associated, since it was specifically enriched in the soil around the sanitary disposal tanks, and exhibited some changes in RA during the mission. Therefore, it is possible that the presence of *Psychrobacter* in site S, is due to forward

contamination, and its return to the habitat is an evidence of backward contamination, hence cross contamination. In order to better handle and reduce cross contamination, an in-depth analysis of possible vectors, that are prone to facilitate the transfer of microorganisms, must be performed.

Investigation of backward contamination is complex; in the current study, we cannot predict which species will colonize the indoors of the habitat. Microbial succession, that should also be considered when examining forward contamination events, is affected by environmental factors, such as pH, salinity, and organic carbon and by stochastic processes (Dini-Andreote et al., 2015). This should be further tested with additional monitoring experiments which will follow the establishment of bacterial species inside the habitat, during a longer period, perhaps in a similar bacterial monitoring method to the one done in Kibō on the ISS (Ichijo et al., 2016). This proof of concept demonstration of cross contamination monitoring can be modified to be used on Mars, with several considerations. While the use of deep sequencing of marker genes is a powerful and sensitive technique to detect and identify bacteria in low biomass settings, it nevertheless possesses inherent technical limitations, which may distort taxonomic information (Salter et al., 2014; Silverman et al., 2019). In particular, the impact of a contaminated extraction kit on low-biomass environmental investigation, was shown to be a possible hazard. Our controls (sterile DDW, sterile cotton swab, sterile nitrile glove) indicated low presence of background DNA, which for most analyses was negligible. However, when examining sensitive questions of contamination, a better and more credible control is needed. Additionally, our suggested method cannot quantify the exact level of contamination. This can be easily solved with addition of a quantitative or semi-quantitative method, such as RT-PCR (Kim et al., 2014). Also, the presented approach is tailored to nucleic acids based genetic material and ribosomal rRNA, yet life that originated elsewhere could be unrecognizable to our technology and methods, rendering it undetectable. Generally, nucleic acids-based detection might not necessarily be the most suitable technique to detect foreign life forms, and different biomarkers should be examined (Davila and McKay, 2014).

Future studies will look for the presence of all the reported genera on the habitat surfaces, and mobile robotic elements or vehicles if those will be used. Also, the impact on habitat systems health and on astronaut health should be investigated in depth regarding these genera.

To conclude, the ability to control and reduce forward and backward contamination is crucial for the future of humankind in space. As our knowledge of the extent and range of possible contamination grows, future space related missions can be utilized to establish the proper protocols to reduce cross contamination events and protect both Earth and extra-terrestrial environments.

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## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject [Accession: PRJNA662496].

## AUTHOR CONTRIBUTIONS

YY conceived and designed the experiment, collected the data, performed the analysis and wrote most of the paper. LR performed data analysis. RA conceived and designed the experiment, wrote small part of the paper and performed other contributions. CG collected the data. GA contributed to data analysis. GY collected the data and performed data analysis and other contributions. OA supported the design and execution of the experiment. All authors reviewed the article and approved submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fspas.2021.589147/full#supplementary-material>.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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