Examination into the HI-SEAS IV built environment reveals differences in the microbial diversity and composition of plastic and wood surfaces

Diane Li, Kyle Ching*, Wesley J. Hunt*

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

* These authors contributed equally to this work

SUMMARY The conditions within a confined built environment designed for long-term habitation during space travel can influence the microbiomes of the abiotic surfaces, emphasizing the necessity of regular microbial screens. The recent Hawaii Space Exploration Analog and Simulation (HI-SEAS) IV study examined the microbiome of a confined environment built to mimic a habitat on Mars. Temporal variations in microbial diversity were identified within the HI-SEAS built environment, but the factors associated with the observed microbial dynamics had yet to be explored. Here, we identified these factors by investigating the potential effect of resupply events and surface material on microbial diversity and composition. We found that resupply events had no significant effect on the alpha or beta diversity of the microbiome within the HI-SEAS built environment, but that plastic and wood surfaces exhibited significant differences in alpha and beta diversity. Together, our study provides insights into the considerations for monitoring microbial communities within a confined habitat designed for space exploration.

INTRODUCTION

Space exploration is the next frontier for humanity. In the confined setting during space travel, the microbiomes of humans and the surrounding environment inevitably interact for prolonged periods of time (1). Stringent cleaning procedures are implemented in such confined spaces for the purpose of maintenance (2). However, this may select for opportunistic pathogens and resistant microorganisms (2), creating a need for microbial screening of the confined built environment to ensure the safety of the crew (3). Indeed, the International Space Station (ISS) is routinely monitored for microbial burden (4).

A model for studying the microbiome in space. The Hawaii Space Exploration Analog and Simulation (HI-SEAS) dome is a confined habitat built to simulate Mars and Moon exploration missions (5). Mahnert et al. recently analyzed the microbial dynamics of both skin and abiotic surfaces during the HI-SEAS IV mission (5). To generate the dataset (European Nucleotide Archive (ENA) accession code ERP118380), microbiome samples were regularly collected from the habitat dome (5). The study provided important insights into the shifts in the microbiomes associated with a space analog habitat (5). The authors found that the microbial diversity of the habitat surfaces fluctuated over the duration of the mission (5). However, the authors did not examine the potential factors that could explain the trends in microbial dynamics associated with the abiotic surfaces. In the present study, we explored two factors that could influence the microbiomes of the HI-SEAS built environment: the resupply events and the material of abiotic surfaces. Identification of the factors affecting the microbiome within a confined environment in the context of space travel could highlight some critical considerations for minimizing the risks associated with space exploration (2-4).

Resupply events. During the fourth HI-SEAS mission, nine resupply events occurred (5).

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Address correspondence to: https://jemi.microbiology.ubc.ca/

Interestingly, the alpha diversity of the built environment was found to fluctuate over time (5). Thus, we were interested in examining whether these fluctuations coincide with the resupply events. Because the HI-SEAS dome was a highly confined environment with the crew physically isolated from the rest of the world (5), these resupply events were likely the only cross-over between the external environment and the HI-SEAS habitat. It would seem possible that the introduction of supplies could have disrupted the microbiome within the HI-SEAS built environment. However, previous research analyzing the effect of resupply events onboard the International Space Station (ISS) suggested that commercial resupply vehicles (CRV) do not significantly alter the microbiome of the station (6). CRV's are prepared in cleanrooms where the sterility of the room is closely controlled (6). Additionally, the ISS environment is routinely sanitized and monitored for microbial contamination (6). Considering that the HI-SEAS IV mission was designed to simulate life in space, it is likely that a similar practice of resupply cargo decontamination may have been adopted. Weekly cleanings conducted during the HI-SEAS IV mission might function similarly to those implemented at the ISS, which could contribute to the control of microbial levels (6). Based on the findings related to the ISS, which suggest that cleaning practices mitigate contamination by resupply cargo (6), we hypothesized that there is no relationship between resupply events and the microbial diversity and composition of the HI-SEAS built environment. Our hypothesis contrasted with our initial interest in examining the potential role of resupply events in altering the microbiome of abiotic surfaces. Therefore, our investigation into the changes in microbial diversity and composition in relation to resupply events aimed to clarify this contradiction.

Material of abiotic surfaces. Another factor that could affect the microbial communities harbored by the abiotic surfaces is the surface material. The abiotic surfaces sampled during the HI-SEAS IV mission were comprised of either wood or plastic (5). Mahnert et al. identified that the alpha diversity was significantly different between samples obtained from these materials (5). However, the authors did not directly assess the beta diversity of the built environment with respect to surface material (5). We were therefore interested in examining the potential influence of surface material on beta diversity as well as alpha diversity. A study examining the decontamination of cutting boards produced from different materials indicated that more microbes could be recovered from plastic than wood boards (7). Additionally, wooden surfaces do not support the growth of certain microorganisms due to the antimicrobial characteristics of wood (8). Furthermore, a microbiome study on the confined analog habitat used for National Aeronautics and Space Administration (NASA) astronaut training suggested that different surface materials harbor distinct microbial communities (9). Taking into consideration the properties of wood and plastic, as well as previous findings related to the microbiome within a confined analog habitat (9), we hypothesized that there is a relationship between surface material and microbial diversity and composition.

METHODS AND MATERIALS

Data collection and availability. The current amplicon sequence data was generated by Mahnert et al. over the duration of the HI-SEAS IV mission (5). On a biweekly basis, swabs were used to sample four abiotic surface locations within the HI-SEAS built environment: toilet bowl, kitchen floor, main room desk, and bedroom desk (5). On a given sampling day, only one surface sample was collected from each abiotic surface. Field controls were prepared by sampling the air within the built environment using swabs (5). Together, a total of 111 swabs were acquired. The DNA was extracted from the samples, the V4 region of the 16S rRNA gene was amplified using the 515F/806R primer pair, the amplicons were sequenced on Illumina, and the resulting sequences were demultiplexed on QIIME2 (5, 10). We retrieved the amplicon sequence data from the European Nucleotide Archive (ENA) (accession no. ERP118380).

Associated metadata was found on Qiita (https://qiita.ucsd.edu/) (study ID 12858). The metadata includes additional information pertaining to the samples such as sampling day,

resupply timing, and surface material of the location from which the sample was obtained. Resupply timing refers to the timing at which both skin and abiotic surface samples were obtained relative to the nine resupply events that occurred over the duration of the HI-SEAS IV mission (5). With regards to surface material, the abiotic surface locations were categorized as either plastic or wood. Of the four abiotic surfaces, the toilet bowl, bedroom desk and main room desk were comprised of plastic while the kitchen floor was comprised of wood (5).

Quality control. Using QIIME2 version 2020.8 (10), the demultiplexed sequences were truncated to 220 bases to remove low quality bases and denoised using DADA2 to correct for sequencing errors as well as to define a set of amplicon sequencing variants (ASV's) (11). The sequences of the ASV's were listed in a representative sequences file and a summary of the abundance of sequences assigned to each ASV was outlined in a features table. These steps are described in Supplemental Script 1.

Generation of a phylogenetic tree and taxonomic classification. In order to compute the Unweighted UniFrac diversity metric, which considers phylogenetic distance (12), a rooted phylogenetic tree was generated with the representative sequences using FastTree 2 (13). The representative sequences were then assigned taxonomy using a Naive Bayes Classifier pre-trained with the Silva 138 99% OTU's reference (14-18). This classifier was trained to recognize the region of the 16S rRNA gene amplified with the 515F/806R PCR primer pair used to generate the current amplicon sequence data (5, 14-15). These steps are described in Supplemental Script 1.

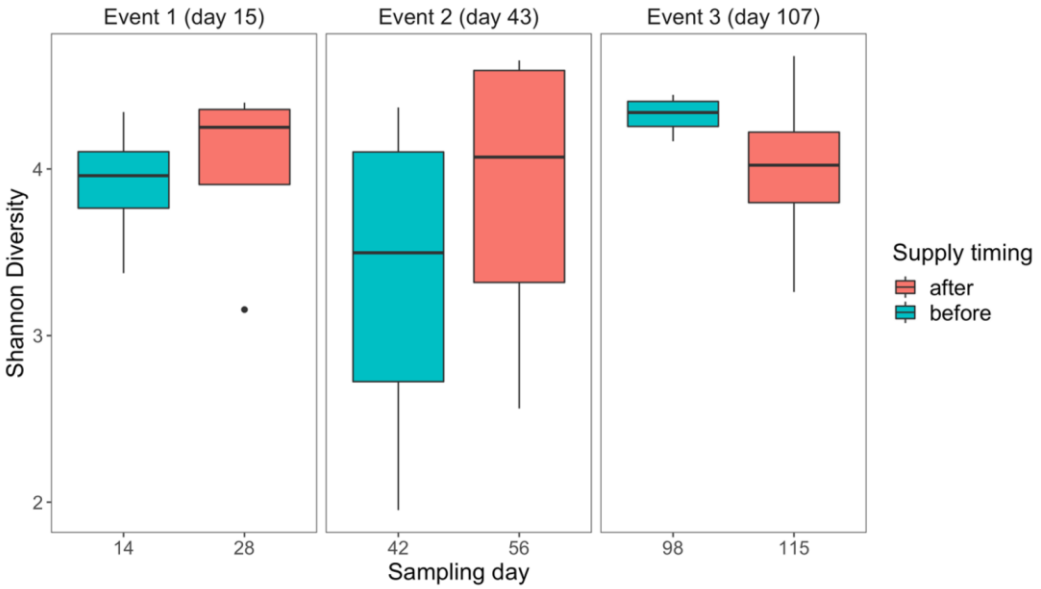

FIG. 1 Alpha diversity of abiotic surfaces is not significantly different before and after resupply events. Boxplots comparing the Shannon diversity of surface samples obtained on the closest sampling days before or after three resupply events. Colours indicate whether the sample was acquired before or after the resupply event. q-values = 0.83 for each resupply event (pairwise Kruskal-Wallis tests, $\alpha = 0.05$). n = 4 for each sampling day.

Filtering of the dataset. The features table was further filtered on QIIME2 (10) to remove low abundance ASV's that accounted for less than 0.005% of total sequences. Filtered features tables were then generated by selecting metadata categories of interest. To assess the effect of resupply events on the microbial diversity and composition within the built environment, the features table was further filtered by abiotic surface samples and on the closest sampling days (days 14, 28, 42, 56, 98, 115) surrounding three resupply events (days 15, 43, 107) (resupply event filtered features table). To assess the effect of surface material on the microbial diversity and composition within the built environment, the features table was further filtered by abiotic surface samples and by surface material (plastic or wood) (surface material filtered features table). Lastly, the taxonomic classification for each ASV was used to remove any mitochondrial sequences from the filtered features tables. All filtering steps are detailed in Supplemental Script 1.

Alpha rarefaction. Alpha rarefaction curves were generated on QIIME2 (10) using the filtered features tables with mitochondrial sequences removed to determine a rarefaction depth for each filtered dataset. To maximize the number of ASV's and samples retained for statistical analysis, the rarefaction depth for the resupply event-filtered features table was chosen to be 20,706 reads/sample, and the rarefaction depth for the surface material-filtered features table was chosen to be 30,000 reads/sample. These steps are described in Supplemental Script 1.

Analysis of alpha and beta diversity. Alpha and beta diversity metrics were computed on QIIME2 (10) with the resupply-filtered features table and the surface material-filtered features table with mitochondrial sequences removed. We assessed Shannon's diversity as the alpha diversity metric and Unweighted UniFrac as the beta diversity metric. The rarefaction depths determined for each filtered dataset was specified for the computation of diversity metrics.

Statistical tests were performed to assess the differences in alpha and beta diversity between surface samples obtained before and after each resupply event and between plastic and wood surfaces. Pairwise Kruskal-Wallis tests (19) and pairwise PERMANOVA tests (20) were performed for the statistical analysis of alpha and beta diversity metrics, respectively. Steps pertaining to alpha and beta diversity analysis on QIIME2 are described in Supplemental Script 1. Diversity metrics were also computed and visualized on R using the R packages tidyverse, vegan, ape, phyloseq, ggplot2, and ggthemes (21-28). Steps pertaining to alpha diversity metric computation and visualization on R are outlined in Supplemental Script 2 while steps pertaining to beta diversity metric computation and visualization on R are described in Supplemental Script 3.

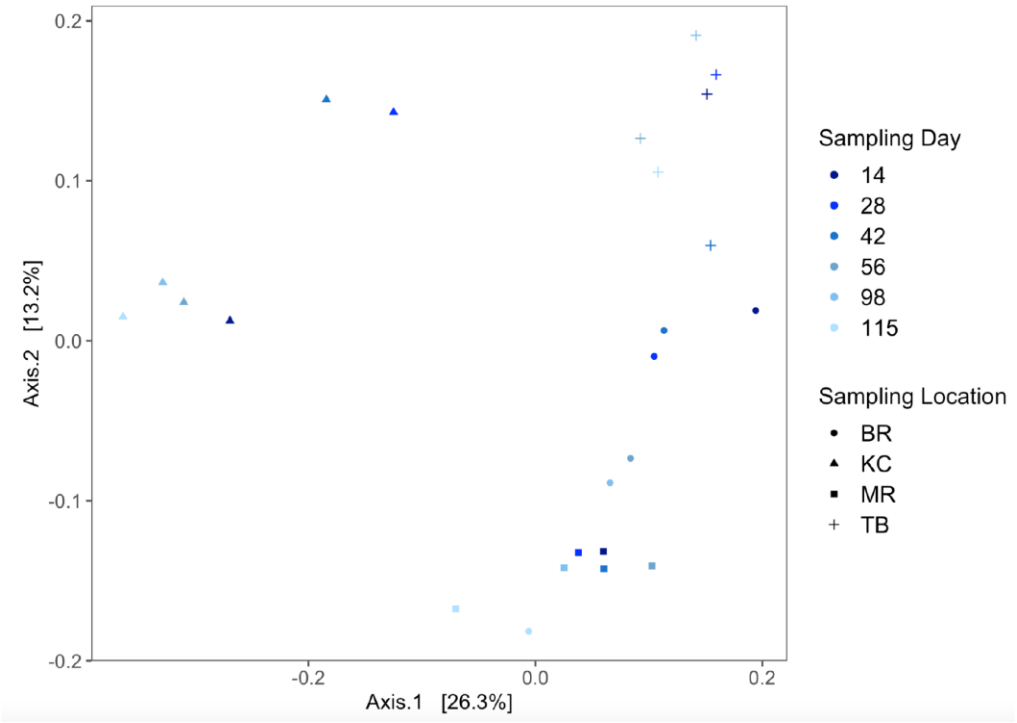

FIG. 2 Beta diversity of abiotic surfaces is not significantly different before and after resupply events. Principle coordinate analysis (PCoA) plot based on Unweighted UniFrac distance. Colors indicate the closest sampling days occurring before or after three resupply events. Shapes indicate the sampling location (BR = bedroom desk, KC = kitchen floor, MR = main room desk, TB = toilet bowl). q-values = 0.93 (pairwise PERMANOVA tests, $\alpha = 0.05$). n=4 for each sampling day.

RESULTS

Resupply events had no significant effect on the alpha or beta diversity of the microbiome within the HI-SEAS built environment. We were first interested in exploring the potential influence of the resupply events on the structure of microbial communities within the HI-SEAS built environment. In particular, we examined the effect of resupply events on the alpha diversity within the HI-SEAS built environment by comparing the Shannon diversity of abiotic surfaces before and after three resupply events. The Shannon diversity of the abiotic surfaces did not change significantly following each

event (q-values > 0.05, pairwise Kruskal-Wallis tests) (Figure 1), suggesting that the microbial diversity of abiotic surfaces was not affected by the individual resupply events.

In addition to alpha diversity, we also evaluated the beta diversity of the abiotic surfaces based on Unweighted UniFrac distance. Principle Coordinate Analysis (PCoA) plots revealed distinct clustering by the location of each surface within the built environment across all sampling days (Figure 2). This indicated that the beta diversity at each location remained similar over time. Consistent with the observed clustering by location, no significant differences in beta diversity were observed for abiotic surfaces before and after each resupply event (q-values > 0.05, pairwise PERMANOVA tests). This suggested that the microbial composition of abiotic surfaces was not significantly affected by the resupply events.

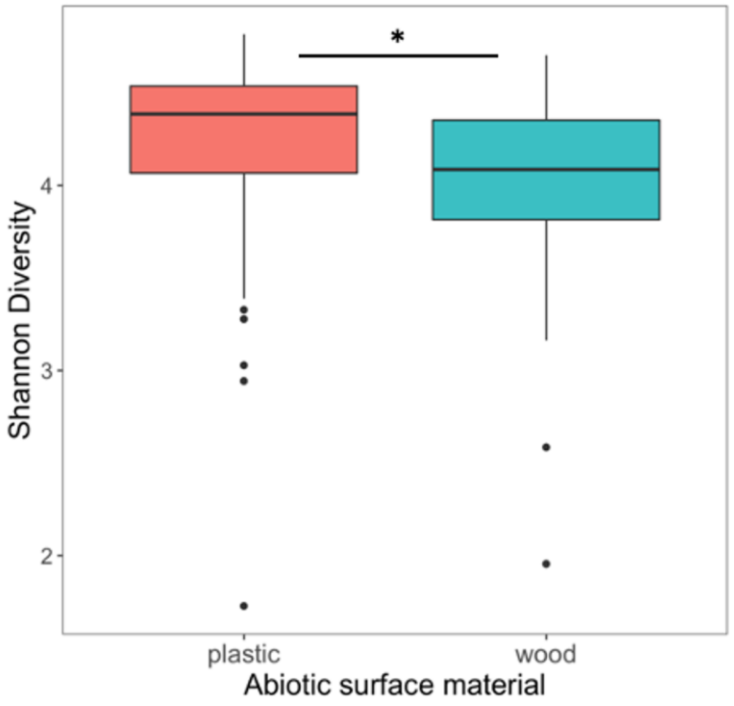

FIG. 3 Alpha diversity is significantly higher on plastic than wood surfaces. Boxplots comparing the Shannon diversity of plastic and wood surfaces. p-value = 0.0070 (* indicates statistically significant difference, pairwise Kruskal-Wallis test, $\alpha = 0.05$). n = 72 for plastic surfaces. n = 26 for wood surfaces.

Plastic and wood surfaces within the HI-SEAS built environment exhibited significant differences in alpha and beta diversity. We further assessed the microbial diversity and composition of the abiotic surfaces within the HI-SEAS built environment by investigating the effect of different surface materials on alpha and beta diversity. The surfaces within the HI-SEAS built environment were comprised of either wood or plastic. Comparison of Shannon diversity indicated that the alpha diversity of the microbial community on plastic surfaces was significantly higher than that of wood surfaces ($p < 0.05$, pairwise Kruskal-Wallis test) (Figure 3).

Assessment of beta diversity through PCoA plots based on Unweighted UniFrac distance showed a distinct clustering of samples by surface material (Figure 4), suggesting that the microbial composition differed between plastic and wood surfaces. Comparison of Unweighted UniFrac distance revealed that the difference in microbial composition between plastic and wood surfaces was indeed significant (q-value < 0.05, pairwise PERMANOVA tests).

DISCUSSION

In our study, we aimed to elucidate the factors influencing the microbial diversity and composition of the skin and built environment during the HI-SEAS IV mission, namely the effects of resupply events and abiotic surface material. Through analysis of the HI-SEAS IV microbiome amplicon dataset generated by Mahnert et al. (5), we found that the alpha and

beta diversity of the built environment was significantly affected by surface material, but not resupply events.

Changes in diversity with resupply events. We first investigated how resupply timing affected the microbial diversity and composition of the built environment. Of the nine resupply events that occurred during the HI-SEAS IV mission, we focused our analyses on three. Our diversity analyses suggested that the resupply events did not affect the microbial diversity or composition of the abiotic surfaces within the confined environment. Interestingly, the composition of the microbiome at each abiotic surface location remained similar over time. This is consistent with previous analyses on the present dataset, such that distinct microbial taxa were found to be associated with each surface location (5). These results corroborate with our hypothesis that there is no relationship between resupply events and the microbial diversity or composition of the built environment.

Several factors could explain the lack of significant changes in alpha and beta diversity following the HI-SEAS IV resupply events. In terms of resupply vehicles, research has indicated that sanitation practices for ISS resupply cargo can curb microbial transfer (6). Generally, microbial levels in space vehicles are controlled before flight by the application of heat, radiation or chemical agents (29). Considering that the HI-SEAS IV dome simulates space habitation (5), it is likely that similar protocols for cargo decontamination were established to limit the introduction of microbes into the confined environment, thereby minimizing any changes to existing microbial communities.

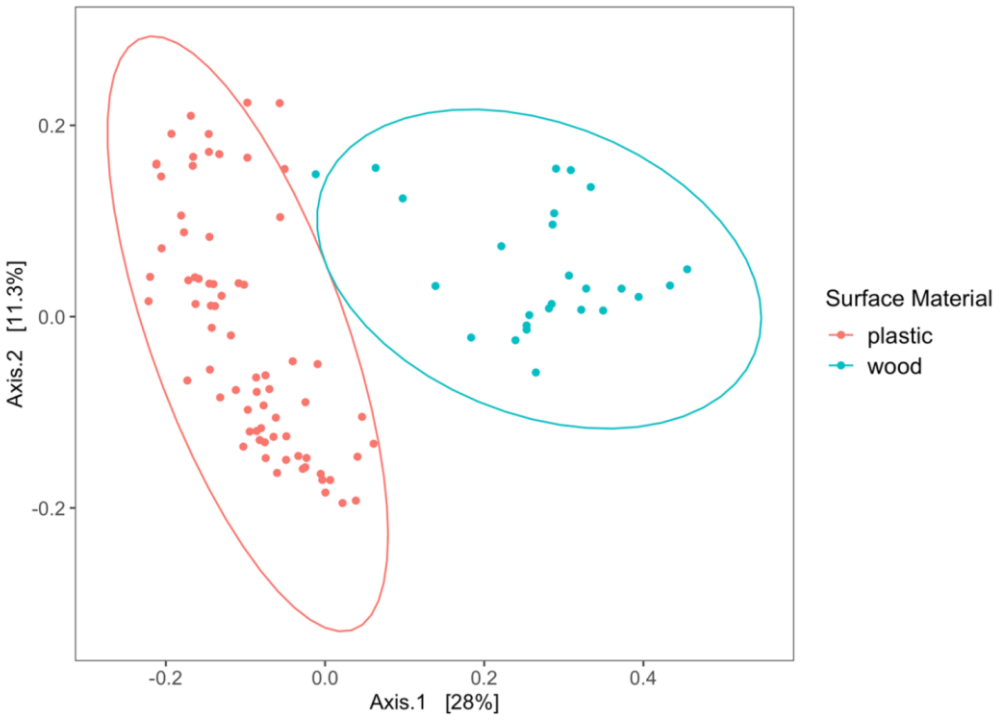

FIG. 4 Beta diversity is significantly different between plastic and wood surfaces. Principle coordinate analysis (PCoA) plot based on Unweighted UniFrac distance. Colours indicate the surface material (plastic or wood). q-value = 0.001 (pairwise PERMANOVA test, $\alpha = 0.05$). n = 72 for plastic surfaces. n = 26 for wood surfaces.

Apart from cargo resupply sanitization measures, missions onboard the ISS are known to tabulate bacterial and fungal levels (3). Over the duration of spaceflight missions, disinfectant wipes are utilized on contaminated surfaces to bring bacterial levels within acceptable limits (30). Although information regarding microbial monitoring was not provided for the HI-SEAS IV mission, the potential for contamination was still a consideration as surfaces were cleaned on a weekly basis (5). A previous study has indicated that sublethal levels of disinfectant can select for resistant bacterial species (31). Mahnert et al. examined the patterns of microbial resistance throughout the HI-SEAS IV mission and identified temporal fluctuations in the antimicrobial resistance marker encoding a class 1 integrase within the built environment (5). Integrases are crucial enzyme components of integrons, which are genetic elements that obtain and express exogenous

genes including antibiotic-resistance cassettes (32). In particular, a class 1 integrase gene was found to be significantly associated with a gene encoding resistance to sulphonamide antibiotics (33). Based on this finding, it is possible that resistant strains were enriched on abiotic surfaces within the HI-SEAS IV dome following each scheduled cleaning event. Surface sanitation may have controlled the population of microbes present, offering an additional explanation for our findings that microbial diversity and composition on abiotic surfaces did not change significantly following the introduction of supplies. However, this would require further investigation.

Changes in diversity with surface material. We also aimed to identify whether surface material affected the microbial diversity and composition of the HI-SEAS built environment. Our findings indicated that both alpha and beta diversity were significantly affected by surface material. In particular, alpha diversity was significantly higher on plastic than wood surfaces. This is consistent with our hypothesis that there is a relationship between surface material and microbial diversity and composition. Our alpha diversity results are also consistent with previous analyses using the current HI-SEAS IV dataset, where alpha diversity was found to be significantly different between wood and plastic surface samples (5).

The significant shifts in microbial diversity and composition between wood and plastic samples can be attributed to the properties of the materials. Wood surfaces can generate antimicrobial compounds of different chemical classes including tannins, phenols, and terpenoids (8, 34). Pinosylvin, which is an antimicrobial derived from wooden material, was found to impede the growth of certain Gram-positive bacteria including *Bacillus cereus* and *Staphylococcus aureus* as well as specific Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas fluorescens* (35). Mahnert et al. investigated the built environment of the HI-SEAS IV mission and observed that distinct microbial communities inhabited each surface type (5). The presence of the genera *Brevundimonas* and *Achromobacter* were characteristic of the kitchen floor, which was composed of painted plywood, the only wood surface that was sampled (5). Considering that members of both genera are known to exhibit resistance against multiple antibiotics (36-37), it is possible that the antimicrobial effect of wood surfaces selected for certain bacterial strains. Altogether, the antimicrobial compounds derived from wood presents an explanation for our observations that microbial diversity was lower on wood than plastic surfaces.

Materials composed of plastic can affect microbial growth in different ways than wooden materials. A study on acrylonitrile butadiene styrene (ABS) plastics has demonstrated that specific microbes including *Enterococcus faecium* can remain on plastic surfaces for more than a year (38). Furthermore, plastics with increased surface indentations can retain more cells, promoting increased microbial colonization (39). As well, certain plastics can leach carbon compounds that encourage bacterial growth (40). Research comparing the decontamination of plastic and wooden cutting boards has indicated that more bacteria are recovered from plastic than wooden boards (7). Properties of plastics including surface texture and the ability to leach bacterial nutrients offer reasoning for the differences in microbial diversity and composition that we observed between plastic and wood surface samples.

Limitations In relation to the evaluation of the effect of resupply events, there existed a period of time between when the resupply events occurred and when the abiotic surface samples were acquired. Because the surfaces were not sampled immediately prior to and following the resupply events, the influence of resupply events on the changes in microbial diversity and composition within the built environment may have been masked by any changes to the abiotic surface microbiomes within that time frame. Furthermore, the regular sanitation practices introduced a confounding variable as it is known that disinfection can select for particular microbial groups (31). Thus, the observed patterns of alpha and beta diversity may be impacted by factors apart from the resupply events.

In terms of surface material, the plastic surfaces sampled in this dataset varied in their specific composition. For example, toilet bowls were comprised of high-density plastic while desks were made of plastic laminated fiberboard (5). Different types of plastics have

been shown to be inhabited by different bacterial groups (41), and so the variation in plastic types assessed complicates the analysis of microbial diversity and composition. Additionally, the wood surface we assessed in our present study was also described to be painted (5). Several nanomaterials exhibit antimicrobial properties and are integrated into paint formulations as a preventative measure against the biodeterioration of surfaces (42). As such, it is possible that the microbiome on the wood surface within the HI-SEAS IV dome was impacted by the additives within the paint in addition to the intrinsic properties of wood. Lack of data pertaining to the specific composition of the paint layer limits our ability to account for the effect of this confounding variable on microbial community structure.

Conclusions In this study, we aimed to elucidate the factors that influenced the microbial diversity and composition of the abiotic surfaces within the confined living environment designed for the HI-SEAS IV mission. We focused our analyses on the following potential factors: resupply events and abiotic surface material. Our findings suggested that resupply events were not associated with significant changes to the alpha and beta diversity of the built environment. However, microbial diversity and composition was significantly influenced by surface material, with plastic surfaces possessing a significantly higher alpha diversity than wood surfaces. These findings provide grounds for future studies into the critical factors influencing the microbial dynamics within confined built environments, in the context of space travel.

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Future Directions Our investigation into the effect of resupply events on microbial diversity and composition focused on just three out of nine resupply events. While our current findings suggested that the occasional introduction of supplies do not significantly alter the alpha and beta diversity of the built environment, it would be worthwhile to determine whether this trend can also be observed for other events. Considering previous findings on the ISS related to the microbiome of the resupply vehicles and their influence on the microbiome of the ISS (6), microbial studies on future HI-SEAS missions could aim to acquire microbiome profiles of the systems used for the replenishment of supplies. Comparison of the microbiomes within the confined HI-SEAS dome to that of the resupply systems could allow for a more intimate assessment of the potential perturbations to the HI-SEAS microbiome.

Our study also identified significant differences in alpha and beta diversity between plastic and wood surfaces. However, our scope of study did not extend into an examination of the microbial taxa associated with each surface material. It would therefore be interesting to conduct a differential abundance analysis of taxa based on surface type. In addition, it would be useful to acquire more detailed information regarding the properties of the wooden and plastic surfaces in future HI-SEAS missions, such as the specific composition of each plastic as well as the paint constitution applied to the wooden surface. It has been shown that different plastic surfaces harbor different microbial taxa and that the presence of nanomaterials in paints can dictate bacterial survival (41-42). Therefore, consideration for the variability within each surface material could offer a more descriptive explanation for any taxa identified.

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CONTRIBUTIONS

Diane Li: Contributed to the analysis of alpha and beta diversity. Generated all figures. Composed the methods and results sections. Contributed to the discussion, conclusions, limitations, and future directions.
Kyle Ching: Contributed to the analysis of alpha and beta diversity. Composed the discussion, conclusion, limitations, and future directions.
Wesley J. Hunt: Contributed to the analysis of alpha and beta diversity. Composed the abstract and introduction. Contributed to other sections outlined in the study.
All authors were equally involved in editing the final manuscript.

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