Title: Microbiome dynamics during the HI-SEAS IV mission, and implications for future crewed missions beyond Earth

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Abstract:

Background

Human health is closely interconnected with its microbiome. Stable microbiomes in, on and around the human body will be key for safe and successful long-term space travel. However, longitudinal dynamics of microbiomes inside confined built environments are still poorly understood. Herein we used the Hawaii Space Exploration Analog and Simulation IV (HI-SEAS IV) mission, a one year-long isolation study, to investigate microbial transfer between crew and habitat, in order to understand adverse developments which might occur in an outpost on the Moon or Mars in the future.

Results

Longitudinal profiles of the 16S rRNA gene revealed significant differences in microbial diversity and composition between samples of the built environment and its crew. While microbial profiles from individual crew members were highly dynamic, the microbiome on built environment surfaces remained more stable. Especially within the first 200 days, archaeal signatures of *Methanobrevibacter* were regularly transferred between crew members, but did not impact the microbiome on habitat surfaces. In contrast to a rather stable microbial diversity recovered from surfaces of the habitat, microbial diversity from the crew's skin increased over time. Quantitative observations based on qPCR supported observations of dissimilarity between the built environment and its crew and was also used to track the propagation of antimicrobial resistances in the habitat. Together with functional and phenotypic predictions, quantitative and qualitative data both supported the observation of a delayed longitudinal homogenization between the crew and their habitat, that was mainly caused by the hygiene infrastructure.

Conclusions

The study highlights main routes of microbial transfer, interaction of its crew and origins of microbial dynamics in an isolated set-up. We identified key targets of microbial monitoring, and emphasize the need for defined baselines of microbiome diversity and abundance on surfaces and skin. Targeted manipulation to counteract adverse developments of the microbiome will be a highly important strategy to ensure safety during future space endeavors.

Keywords

confined built environments, isolation, HI-SEAS, skin microbiome, indoor microbiome, 16S rRNA gene amplicons, qPCR, antimicrobial resistances, longitudinal, phenotype predictions

Background

This decade may see the beginning of a sustainable human presence on the Moon. The US government stated their commitment to lead such an endeavor in the Space Policy Directive-1 [1] and current goals include landing a crew at the Moon's south pole by 2024, before establishing a sustained presence there by 2028 [2]. Since 2015, ESA has been strongly advocating its “Moon Village” concept, a large collaborative undertaking that would lead to a permanent presence on the Moon [3]. In addition, the latest Council at ministerial level anticipates a strong involvement of ESA in the US-led Moon program [4]. Other collaborators include JAXA and CSA. While lunar exploration could greatly benefit different areas of science and technology in itself [5,6], the Moon is expected to serve as a testing ground for crewed missions to Mars. Reaching the red planet is also the stated goal of private spacecraft companies, notably SpaceX [7] who aims for a landing as early as in the 2020s.

In such endeavors, microorganisms will inevitably co-travel with the crew: they are thought to outnumber human cells in our bodies [8] and each individual releases millions of them every hour [9]. A microbe-free crewed mission is unrealistic, unethical and undesirable, as our microbiome is essential to our health [10]. Microbial communities may however pose, if inadequately managed, serious threats to future missions.

The most obvious threats to the crew’s health are (opportunistic) pathogens. This risk is exacerbated by crewmembers’ confinement and promiscuity, limited treatment options, increased microbial transmission in microgravity [11], restricted hygienic practices, potentially increased virulence and decreased antibiotic susceptibility of bacteria in space [11–14], lowered immune responses of astronauts attributed to microgravity, radiation and stress [11,12,15–17], and lack of competition to human-borne pathogens from environmental microorganisms [18]. While no life-threatening infections have been reported during spaceflight so far, (opportunistic) pathogens, which are part of the normal human-associated microbial diversity, were detected on the International Space Station (ISS) [13,19]. However, such pathogens might have caused tens of minor medical incidents—among which are urinary tract, upper respiratory tract and subcutaneous skin infections—beyond Earth [11,16].

The threat increases when emergency returns become impossible, notably on a Mars journey, which is expected to last at least 520 days [20]. Even in a relatively mild disease scenario, the

resulting decrease in productivity may be a significant loss given the high value of astronaut working time.

Another threat comes from microorganisms' interference with equipment. First, some microorganisms, referred to as technophiles, can colonize industrial materials and lead to hardware malfunction, degradation of structural materials and corrosion of metal parts [21]. Technophiles were found onboard the Mir station [22,23] and onboard the ISS [13,19,24], leading to damage of various systems [25,26]. Second, microbial contaminants could interfere with biological life-support systems [27] — which could greatly contribute to the feasibility of long-term missions on the Moon or Mars [28–30] — by harming the system-relevant organisms through competition and/or toxicity or by making food products unsuitable for crew consumption.

Finally, microorganisms brought alongside the crew could interfere with the search for life on Mars [31,32]. One strategy to mitigate the risk of contamination could be to increase our knowledge of, and to catalogue, the microbial communities we are carrying [33,34]: this would help to discriminate between endogenous life and our microbiome in case of an ambiguous discovery and to assess the risk of contaminants to adapt to some local (micro)environments. While Mars's surface appears hostile even to microbial life, it cannot be excluded that some extremophiles may reach niches where they could remain active [32].

In any case, microbial communities will be critical components of future space endeavors, with a potentially large influence on mission success [35]. Their importance is reflected in space agencies' efforts to characterize them onboard the ISS [13,19,24,36–40].

However, microbial monitoring on the ISS is constrained by logistical and funding challenges. An alternative is to study microbiome nature and dynamics in similar, closed systems (such as submarines and polar stations) or specific, ground-based analogues of long-term crewed spaceflight [18,41]. While those analogues differ in some important aspects (e.g., gravity and radiation) from spaceflight itself, they place a small, isolated crew in combinations of the following: long-term confinement, high workloads, restricted waste disposal, limited hygiene and/or low air or water quality. They offer the possibility to comprehensively monitor related medical and psychological issues.

In the past, several studies on the indoor and human microbiome were conducted in settings simulating missions to future facilities on the Moon or Mars, for instance: Mars500 (520 days)

[41,42], the Antarctic base Concordia (one year) [43], the inflatable lunar/Mars analogous habitat (ILMAH) (30 days) [44] and the biological life-support testbed “Lunar Palace 1” (LP1; 105 days; [27]. For all these studies, individual parameters have to be critically considered due to variations in methodologies, environmental conditions, activity, geographical location, architectural design, baseline-diversity of microorganisms, contaminants from crew members and cargo such as food and scientific equipment.

Another opportunity arose in 2015-2016: as part of the Hawaii Space Exploration Analog and Simulation IV (HI-SEAS IV) mission, six people spent one year in isolation in a dome (diameter: 11 meters) located at 2.5 km of altitude on the barren slopes of the Mauna Loa volcano, primarily for NASA Behavioral Health and Performance (BHP) research [45]. Over a period of 336 days, swab and wipe samples from habitat surfaces and crew skin were taken in order to assess the microbial community fluctuation, as well as the interactions of surface and skin microbiomes.

Our main hypotheses were that i) the microbiome of the HI-SEAS habitat would follow a longitudinal homogenization between individual crew members, but also the surrounding built environment, ii) overall microbial diversity would be depleted and iii) the microbiome would resemble those of other long time experiments in isolated confined built environments (ICE) on Earth and on the ISS.

Methods

Setting of the HI-SEAS IV mission

The one-year Hawaii Space Exploration Analog and Simulation IV (HI-SEAS IV) mission took place from August 28th, 2015 to August 28th, 2016 [45] in the HI-SEAS habitat, an 11 meter in diameter spherical shaped dome located at 2.5 km of altitude on the barren slopes of the Mauna Loa volcano (Supplementary Fig. S1). Operated by the University of Hawaii, and funded by NASA, this habitat served primarily for NASA Behavioral Health and Performance (BHP) research. During HI-SEAS IV, six crew members (3 males and 3 females) selected for their astronaut-like profile, four from the US and two from Europe (France and Germany), spent a year there in conditions mimicking those of a Mars mission. Time was mostly spent on research work (including outside work, wearing mock spacesuits), test subject duties, physical exercise and household chores. The crew was physically isolated from other human beings for the whole mission duration and communications had a high latency (20-minute delay in both directions). The diet was mostly composed of dehydrated food, canned food, occasional fermented food (yoghurt, bread, and cream cheese) made from rehydrated products and rare vegetables grown on site.

Participants typically showered 1 to 3 times a week, for an average duration of 1.5 to 2 minutes under running water (Heinicke et al., submitted); bodies were then washed with Equate's Sensitive Skin Body Wash, and hair with Garnier Fructis' Pure Clean Clear 2in1. In-between showers, participants occasionally used disinfecting wipes (mainly, Kirkland's Extra Large Disinfecting Wipes). Hands were routinely washed with Dr. Bronner's 18-in-1 Hemp Peppermint Pure-Castile liquid soap, and disinfected with germ's hand sanitizer.

A general cleaning of the habitat was performed every Sunday. Most hard surfaces were then cleaned with Simple Green's cleaner, the kitchen floor with Comet's bleach-based powder, and floors aside from the bathroom and kitchen were only vacuumed. Dishwashing was performed by hand. Clothes were washed with Kirkland's UltraClean laundry detergent, either by hand or in a washing machine.

Sources of voluntarily introduced microorganisms included the following. Fermented products (sourdough bread, tempeh, cream cheese, kombucha, and yoghurt) were prepared using

commercial microbial mixes. Toilets were composting toilets, maintained with Sun-Mar's Microbe Mix and Sun-Mar's compost swift. Cyanobacteria (*Anabaena* sp. PCC 7120 and *Chroococcidiopsis* sp. CCMEE 029) were used for research purposes. Part of the kitchen waste was processed in a bokashi composting system (purchased from Each One Teach One Farms, Hawaii).

Sampling

Microbiome samples were taken every other week. Habitat/furniture surface samples were taken with swabs at 4 different locations. Skin surface (front torso) samples were taken with wipes from each crew member. In-mission sampling occurred from September 4th, 2015 to August 5th, 2016, and an extra series of skin wipe samples was performed after mission completion. The baseline (day 0) was defined as the day of the first sampling event.

The swab samples were taken from (Supplementary Fig. S2):

- the front part of the (composting) toilet bowl (high density plastic), in the upstairs bathroom,

- the kitchen floor (painted, waterproof plywood), in an area (between the fridge and another piece of furniture) where dust tended to accumulate,

- the desk (medium density fiberboard overlaid with plastic laminate) in one of the bedrooms, and

- one desk (medium density fiberboard overlaid with plastic laminate) in the main room.

For each habitat/furniture surface sample, a swab (552C regular swab; ethylene oxide sterilized, Copan, Brescia, Italy) was moistened with autoclaved, deionized water (ELGA's Vision 125 Deionizer). An area of 5 × 5 cm was sampled in three directions (horizontal, vertical and diagonal). The swab was turned between each change in direction. The swab was then broken at the predetermined breaking point and placed back into its original container. Field controls were performed during each sampling session by waving the swab in the air for a few seconds instead of sampling a surface.

Prior to samplings of skin surfaces, sterile 50-ml tubes were filled with one wipe (TX3211, SterileWipe LP, Texwipe) and 10 ml of autoclaved, deionized water. Once the wipes were homogeneously moistened, crewmembers sampled their own skin following oral and written

instructions. Briefly: they put on gloves, cleaned them with ethanol, took the wipe out of the tube, put it flat on their hand, wiped their torso up and down and left and right, folded the wipe over the target surface, wiped again with one of the clean sides, wiped with the other side and put the wipe back into the original tube. 20 ml of water were then added to each tube before storage. Field controls were performed during each sampling session by waving the wipe in the air for a few seconds instead of wiping skin.

Swab and wipe samples were stored at -20°C after sampling, shipped to Europe in dry ice after mission completion and then stored at -80°C until analysis.

DNA extraction

A total of 63 wipes were thawed at 4 °C overnight before transferring them to DNA-free bottles (baked at 250 °C for 24 h) filled with PCR-grade water. The bottles were sonicated for 120 s ± 5 s with a maximal power of 240 W and a frequency of 40 kHz, and vortexed at maximum speed for 1 min. The biomass-containing water suspension was concentrated to 200–500 µl using UV sterilized Amicon filters (Amicon Ultra 15 ml, 50 K, Merck Millipore).

DNA was then extracted from cells suspended and concentrated from wipes, and from all surface swabs plus 7 swab field controls (for a total of 111 swab samples), using Qiagen's DNeasy PowerSoil Kit.

16S rRNA gene amplicons

Microbial profiles were based on amplicons targeting the V4 region of the 16S rRNA gene. The common primer pair F515-R806 [46] with tags for Illumina sequencing were used to cover most bacterial and some archaeal taxa (Supplementary Table S1). 25 µl of the PCR reaction mix contained a final concentration of 200 mM each of forward and reverse primers (0.4 µl of 10 µM stock each), 0.1 µl TaKaRa ExTaq polymerase (5 U/µl, Clontech, Japan), 2 µl ExTaq buffer with $MgCl_2$ (10x), 1.6 µl dNTP mix (2.5 mM), 2µl of template DNA and 18.5 µl PCR grade water. PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s, elongation at 72 °C for 90 s and a final elongation step at 72 °C for 10 min. PCR fragments were evaluated for

product size and quantity by agarose gel (3%) electrophoresis at 70 V for 30 min. Libraries for Illumina MiSeq sequencing were prepared by the Core Facility Molecular Biology at the Center for Medical Research at the Medical University Graz, Austria and covered biological samples, field blanks, extraction blanks as well as no-template controls of PCRs. For the NGS library, DNA concentrations of the generated amplicons were normalized with a SequalPrep™ normalization plate (Invitrogen). After normalization of PCR products, each sample was indexed with a unique barcode sequence using 8 cycles of indexing PCR. Indexed samples were then pooled and purified by gel cuts. Finally, the library was sequenced on an Illumina MiSeq instrument and the MiSeq Reagent Kit v3, 600 cycles (2 × 301 cycles).

Statistics and Bioinformatics

After sequencing, resulting fastq files were processed with Qiime2 versions 2018.6 – 2020.6 [47]. Demultiplexed reads were denoised with DADA2 [48] and an ASV (amplicon sequence variant) feature table was created after truncating forward reads at position 200 and reverse reads at position 150. Potential contaminants were identified and removed with Decontam [49] and its prevalence method with default settings (method="prevalence", neg="is.neg", threshold=0.5). Representative sequences were classified by a Naïve Bayes trained classifier [50] based on Silva 128 [51,52] and a rooted phylogenetic tree for phylogenetic diversity measures was created with Fasttree [53]. Core metrics for alpha and beta diversity (including metrics for richness, evenness, diversity and distances) were calculated including phylogenetic measures like UniFrac [54] at a depth of 3,000 sequences per sample. Diversity analysis covered displays and statistics like rarefaction curves, principal coordinate analysis (PCoA), procrustes analysis, biplots, Mantel tests [55], Kruskal-Wallis, bioenv [56], Spearman rank correlations [57] or PERMANOVA. Meta-analysis of longitudinal microbial diversity inside built environments was conducted in Qiita [58] and used datasets of the following publicly available Qiita studies: 2192, 10423, 11740 and 12858. All selected studies targeted the V4 region of the 16S rRNA gene and were processed with Qiita standard workflows (trimmed to 150 bp, used Deblur for denoising of the ASVs and Greengenes 13_8 for closed reference taxonomy assignments). Longitudinal analysis was based on the q2-longitudinal plugin available in Qiime2 [59] and covered calculations of feature-volatility, linear-mixed-effects modeling, pairwise-differences, pairwise-distances, Wilcoxon signed rank tests and Mann-Withney U tests. Supervised classification and regression of sample metadata was conducted

with the q2-sample-classifier plugin [60] with settings for optimized feature-selection and parameter tuning for RandomForest regression and classifications. Differential abundance and composition of features was determined with balances in gneiss [61], ancom [62] and feature rankings (feature differentials and loadings) available from aldex2 [63], songbird [64] and deicode [65] were partly visualized in qurro [66]. Microbial contributions of different sources and sinks were predicted with SourceTracker2 (https://github.com/biota/sourcetracker2; [67]) at a source and sink depth of 3,000 sequences per sample. Potential phenotypes and functions were predicted with PiCrust2 (https://github.com/gavinmdouglas/q2-picrust2; [68] using the custom tree pipeline and BugBase [69–71]. Further statistics and visualizations were conducted in R [72] using the libraries ggplot2 and streamgraph.

Data availability

All amplicon raw data is available at the European Nucleotide Archive ENA (EMBL-EBI ERP118380). In addition to raw data, processed data is available in Qiita (study id 12858; https://qiita.ucsd.edu/; [58]).

qPCR (16S rRNA genes and resistance genes)

Overall microbial load was determined by qPCR of the 16S rRNA gene. Two setups were used to quantify Bacteria and Archaea separately (primer pair 331f-797r for bacteria [73] and primer pair A806f-A958r for archaea, Supplementary Table S2). For each setup 10 µl of the SYBR Green Supermix (Biorad) contained 5 µl of SsoAdvanced Universal qPCR Kit MM 2x, 0.3 µl each of forward and reverse primers (10 µM), 3.4 µl of PCR-grade water and 1 µl of template DNA. qPCR runs were then carried out on a Bio-Rad CFX96 thermocycler with the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 54 °C for bacteria and 60 °C for archaea for 60 s, elongation at 72 °C for 90 s and a final elongation step at 72 °C for 10 min. Quantifications of 16 qPCR runs relied on serial dilutions of a cloned 16S rRNA gene of *Escherichia coli* and *Nitrososphaera viennensis*, respectively for bacteria and archaea, into StrataClone vector pscA_AmpKan according to manufacturer instructions. For reliable quantifications a minimum reaction efficiency of 0.8 and a correlation coefficient above 0.9 as well as clean melting curves were

required. Counts in negative and no-template controls were subtracted from actual samples and extrapolated per m^2. Finally, qPCR counts were displayed as volatility plots including linear regressions with time.

In addition to quantifications of the microbial load, monitoring of antimicrobial resistances was based on the following four selected resistance genes: *blaOXA* (class A beta-lactamase; [74], *int1a* (class 1 integrase; [75], *qacEΔ1* (biocide resistance gene, quaternary ammonium compound-resistance; [74,76] and *tetM* (tetracycline resistance; [74,77] (Supplementary Table S3). As individual standards were not available, all quantifications were based on relative proportions and serial dilutions (1:10, 1:100, 1:1000) of the genomic DNA of four cultures (*Acinetobacter* sp., *Escherichia coli*, *Enterococcus* and *Pseudomonas aeruginosa*) with reported presence of these resistances. qPCR runs were prepared with the STARlet pipetting robot (Hamilton, Germany) for the Bio-Rad CFX384 instrument. PCR conditions were set to initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s and a final elongation step at 72 °C for 30 s. All 14 qPCR runs were normalized internally (according to qPCR counts of each individual standard), counts from negative controls and no-template controls were subtracted from actual samples and then extrapolated per m^2. Similarly, as done for qPCR counts of the 16S rRNA gene, antimicrobial resistances were also displayed as volatility plots with linear regressions.

Microbial nomenclature

Throughout the manuscript we refer to the nomenclature assigned by the Silva 128 release. A special case is the genus *Propionibacterium*. We are aware that skin-associated representatives of this genus were renamed in recent releases of the Silva database to the genus *Cutibacterium* [78]. However, despite the fact that human skin is a reasonable source for the detected signatures of *Propionibacterium* / *Cutibacterium*, we decided to use the former annotated name by the referenced Silva database release 128.

Results

Overview of the microbiome of the built environment and its occupants

Samples were taken from four representative locations (toilet bowl, kitchen floor, desk in one of the bedrooms, desk in the main room) within the confined built environment and from the skin (front torso) of six isolated crew members. Sampling was performed at 27 time points spanning one year. Besides amplicon sequencing, all samples plus laboratory controls (n=186 in total) were subjected to quantitative PCR (qPCR) on the 16S rRNA gene to assess the overall bacterial load, and on four representative resistance genes (*blaOXA*, *int1a*, *qacEΔ1*, *tetM*) to assess the progression of microbial resistances on skin and surfaces over time.

Along with the samples, 16 types of metadata of environment and crew members were recorded (selected numerical metadata is listed in Supplementary Table S4). The crew was composed of three male and three female members (crewID A-F), with an average age of 30+/-4 and an average body size of 176 cm +/-9 cm. During the isolation and confinement, hygiene practices were restricted. On average, crew members showered preferentially on Saturdays, every 5.4 +/- 1.8 days (60.67 +/- 15.7 times) for about 1 min and 42 seconds +/- 47 seconds. However, individual showering practices differed. For instance, some crew members showered shorter, but more frequently and others showered longer, but only a few times during the isolation period. The diet was mostly composed of dehydrated food, but the crew was allowed to bring beneficial microbes into the habitat, e.g., starters for sourdough bread, tempeh, cream cheese, kombucha and yoghurt. Toilets were composting toilets. Cyanobacteria (*Anabaena* sp. PCC 7120 and *Chroococcidiopsis* sp. CCMEE 029) were used for research purposes. Part of the kitchen waste was processed in a bokashi composting system.

No direct or real-time contact to other humans except to crew members was allowed, and the extravehicular activities foresaw donning of a mock spacesuit that prevented exposure to open air and direct sunlight (described in [79]. Nine resupply events happened during the isolation period (on days 15, 43, 79, 107, 148, 185, 223, 258, 303, 335). A total of 132 samples were processed before and 43 samples after a resupply event. Temperature was stable over time (mean temperature 18 °C +/- 1 °C). CO_2 levels were always in the recommended range for indoor environments (400 - 1,000 ppm) with an average of 662 ppm +/- 62 ppm.

Denoising of demultiplexed amplicon data with DADA2 resulted in 10,016 unique features (ASVs). In an initial step, we analyzed the processed controls (sampling blanks, process controls, no-template controls), which showed significantly lower microbial diversity than actual samples (pairwise Kruskal-Wallis P = 1.8*10E-4; H' ~5.7 vs. 7.0; rarefaction depth of 7,850 sequences). Moreover, microbial composition differed significantly between samples and controls, according to weighted UniFrac metrics (PERMANOVA P = 0.001). To clean the dataset, contaminants were identified from processed controls with decontam [49] and subsequently removed from the dataset. All subsequent analyses were performed with the cleaned dataset, which contained 3,077,780 sequences (median frequency was 17,533 sequences per sample). According to rarefaction curves, sequencing depth was of sufficient quality, as the Shannon diversity metric (H) plateaued at ~2,500 sequences.

Since we were interested in the characteristics of the microbiome profile of the different sample groups, the dataset was divided into different surface types (crew [skin], built environment) and sample locations (e.g., individual crew members and locations within the facility).

The overall microbial diversity and composition of biotic (skin) and abiotic surfaces differs significantly

In the first step, we compared the alpha diversities (based on Shannon index) of all sample types. Samples from the crew's skin showed significantly lower diversity than samples from surfaces of the built environment (pairwise Kruskal-Wallis P = 7.3*10E-16; mean H' ~6.2 vs. 7.5) (Fig. 1A). Significant differences were also detected in the diversity index of five crew members (pairwise Kruskal-Wallis of crew member A and B: q-value 1.6*10E-3; A and D: q-value 1.3*10E-3; A and F: 7.5*10E-3; C and F: 2.7*10E-2; Fig. 1C).

Notably, the microbial diversity on the crew's skin varied more (mean H' ~5.0 in samples from crew member F to ~6.7 for crew member A) than that on abiotic surfaces (mean H' ~7.2 in samples of the kitchen floor to ~7.6 in bedroom samples; Fig. 1B and Fig. 1C).

With respect to alpha diversity, the built environment surfaces showed only significant differences between bedroom and kitchen floor samples (pairwise Kruskal-Wallis q-value 4.5*10E-2; Fig. 1B), while richness was variable (Supplementary Fig. S3). Alpha diversity was

also significantly different according to type of surface material (plywood vs. polymer; pairwise Kruskal-Wallis q-value 4.5*10E-2; Supplementary Fig. S4).

Beyond alpha diversity, the microbiome profile of samples from built environment surfaces was significantly different from that of crew skin samples (weighted UniFrac distances, PERMANOVA q-value = 3*10E-3) and samples clustered separately in PCoA plot analysis (Fig. 1G and Fig. 1H). Further significant differences were found between all four locations of the HI-SEAS habitat (ranging from q-values of 1.8*10E-3 to 3.3*10E-3, PERMANOVA pairwise testing). However, the microbial composition was not significantly different between individual crew members despite highly explained variability along PCoA Axis 1, indicating dynamic changes of microbial composition on skin samples over time (see below).

Supported by extensive metadata analysis, the factors time (P = 0.04, Spearman rank correlation of Shannon diversity with time) and sampling location (P = 9.4*10E-19, Kruskal-Wallis test of all groups, see above for more details) were identified to have a significant impact on microbial diversity and the microbial profile, whereas the microclimate of the habitat revealed no significant influence.

Metadata predictions based on Random Forest classifiers and regressors showed high overall accuracy estimates of 95% for the sampling environment (skin samples vs. built environment samples), and the day of sampling (R=0.77, P = 2.3*E10-8). Thus, our subsequent analyses focused on the impacts of time and sampling location.

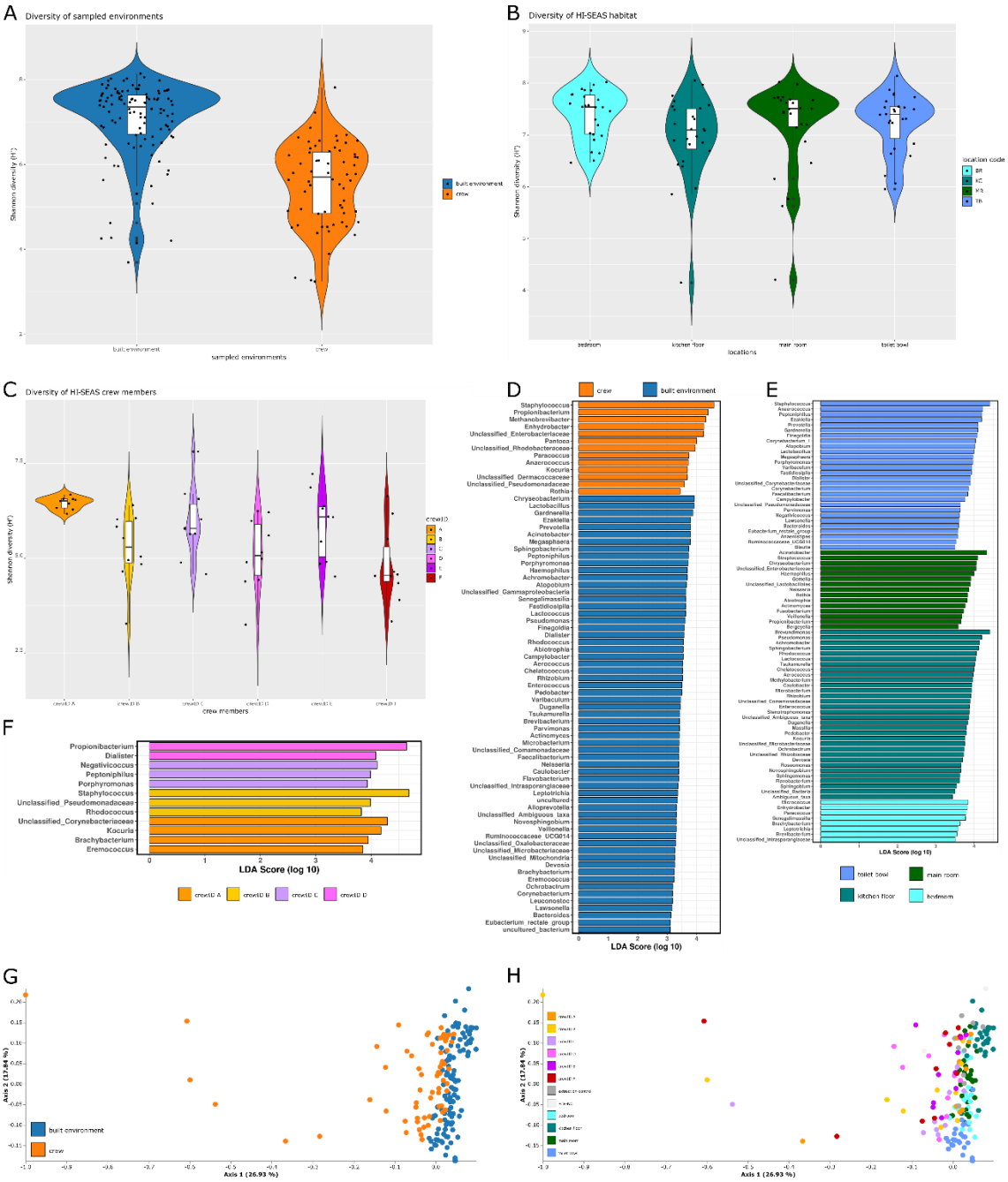


Fig. 1: Microbiome profile of crew and built environment samples, all time points included. A to C: violin plots (kernel probability density of Shannon values, displaying the alpha diversity) including individual data points and a box with median and interquartile range. A: Sampled surface types: skin of crew and locations of the built environment. B: Built environment surfaces: BR (desk in bedroom), KC (kitchen floor), MR (desk in main room), TB (toilet bowl). C: Samples from the individual crew members (A-F). D: Linear discriminant analysis effect size (LEfSe) on samples from crew and built environment. E: LEfSe on samples from built environment surfaces. F: LEfSe on samples from individual crew members. G to H: Beta diversity represented by PCoA plots based on weighted UniFrac metrics and a rarefaction depth of 3,000 ASVs per sample. G: Microbial profile of samples from built environment and crew. H: Microbial profile of the individual crew members and sampling locations of the built environment.

Each surface was characterized by a specific set of microbial signatures, which can be predicted with high accuracy

As selected surfaces of the built environment (desk in a bedroom, kitchen floor, desk in the main room, toilet bowl) and the crew's skin showed a significantly different composition (see below), we were interested in a detailed analysis of the characteristic features.

Overall, the skin samples were characterized by high abundance of *Staphylococcus, Propionibacterium, Enterobacteriaceae, Enhydrobacter*, and *Methanobrevibacter* signatures (LEfSe analysis, Fig. 1D), whereas the built surfaces were characterized by the presence of *Chryseobacterium, Lactobacillus, Gardnerella, Prevotella*, and *Acinetobacter*.

Indicative microbial signatures were identified for the toilet bowl (*Staphylococcus, Anaerococcus*), the main room desk (*Acinetobacter, Streptococcus*), the kitchen floor (*Brevundimonas, Achromobacter*), and the bedroom desk surface (*Enhydrobacter, Micrococcus*; Fig. 1E).

As observed for habitat surfaces, individual crew members revealed typical microbiome profiles with *Propionibacterium* being indicative for crew member D, *Peptoniphilus* for crew member C, *Staphylococcus* for crew member B and *Kocuria* for crew member A (Fig. 1F). Remarkably, accuracy of metadata prediction based on RandomForest classifications was possible for certain individuals (e.g. crew member D with 100%) or distinct surfaces of the built environment (e.g. kitchen floor and the toilet bowl both 100% accuracy) (Supplementary Figure S5).

Microbial diversity on skin increased during the isolation over time

Overall, the longitudinal microbial diversity in samples from skin showed a steady increase over time (mean Shannon H' 4.9 to 6.4), whereas the increase in microbial diversity on built environment surfaces was lower (mean Shannon H' 6.4 to 7.3). The microbial diversity from built environment surfaces was subject to greater fluctuations throughout the time period (Fig. 2A). This observation, however, could be due to a higher number of analyzed built environment samples. Increasing microbial diversity on skin was also confirmed by linear mixed effect models which tested whether Shannon diversity changed over time in response to the sampling

locations (Supplementary Fig. S6). An increase in microbial diversity on skin was observed for most crew members (C, D, E, and F; mean Shannon H' 5.1 to 6.5; highest increase for individual C from 5 to 7.8). However, almost no change was visible for individual A, and a slight decrease was observed for individual B (mean Shannon H' 5.5. to 4.8; Fig. 2B).

The microbial diversity on different locations inside the HI-SEAS habitat changed as well (Fig. 2B). The largest fluctuations were detected for samples of the main room, and a slight increase in microbial diversity was visible for all locations apart from the toilet bowl. In the latter case, microbial diversity decreased by 1 log (mean Shannon H' 7.9 to 6.8), possibly due to more rigorous cleaning procedures.

Other metrics describing the alpha diversity of all samples, such as richness (92 to 213.67) and phylogenetic diversity estimates (7.6 to 14.7), followed a similar pattern, while Pielou's evenness remained constant over the entire isolation period (0.8 to 0.84; Supplementary Fig. S7 and Supplementary Fig. S8).

Temporary dynamics of microbial diversity were investigated by pairwise difference comparisons of samples from individual time points. Significant differences were only evident between day 210 and day 252 for skin samples (Kruskal-Wallis test for multiple groups, $P = 0.02$) and between skin and built environment surface samples (Mann-Whitney U test, FDR $P = 0.03$).

Furthermore, correlating patterns of microbial diversity were analyzed by Spearman rank correlations. Significant positive correlations of microbial diversity were evident between crew member C and D ($P = 0.05$, $rho = 0.65$), C and E ($P = 8.8*10E-4$, $rho = 0.9$), and D and E ($P = 0.02$, $rho = 0.73$). Crew member C also showed significant positive correlations of microbial diversity with the kitchen floor ($P = 0.01$, $rho = 0.82$). In contrast to these positive correlations, crew member D and E showed significant negative correlations of microbial diversity with samples from the bedroom surfaces ($P = 0.04$ and $P = 0.03$, $rho = -0.7$ and $rho = -0.75$, respectively).

Observations of microbial composition followed a similar pattern as described for microbial diversity. Hence, composition of skin samples (weighted UniFrac distances) changed to larger magnitudes than those from built environment surfaces along PCoA Axis1. Largest shifts on crew's skin were visible between day 0 and day 210 (with a maximum at day 84 of -0.3) (Fig.

2D), especially for crew member B. In contrast, almost no changes along PCoA Axis 1 were visible for crew member D and E (Fig. 2E).

Pairwise distance comparisons of microbial composition (weighted UniFrac distances) at individual time points showed significant differences between day 84 and day 126 (Kruskal-Wallis test for multiple groups, $P = 0.03$), and between skin and built environment surface samples (Mann-Whitney U test, FDR $P = 0.04$).

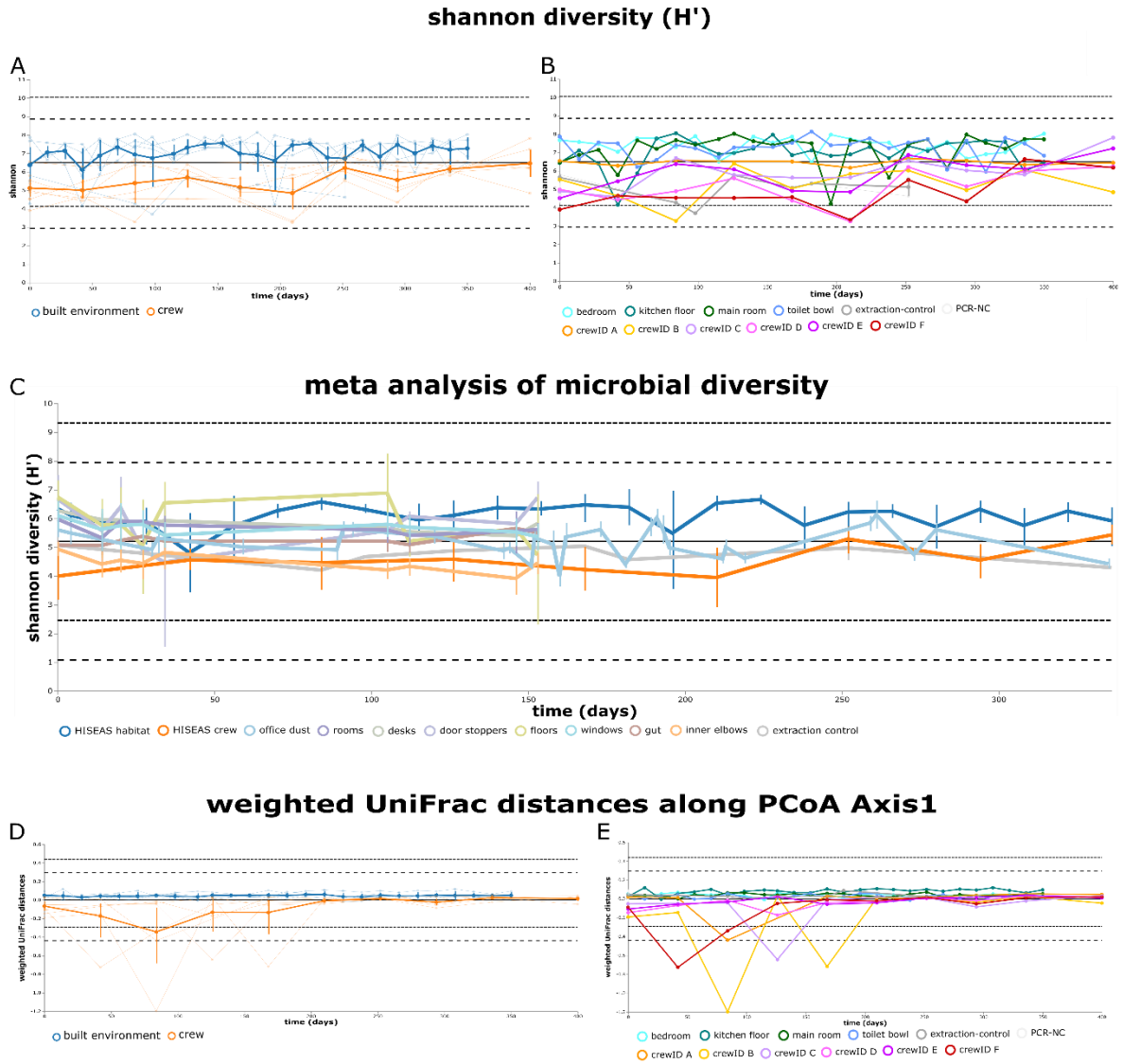


Fig. 2: **Longitudinal development of diversity and composition.** A: Volatility analysis of Shannon diversity resolved to sample type (built environment and human skin). B: Volatility analysis of Shannon diversity resolved to individual crew members and sampled locations within the HI-SEAS habitat. C: Meta volatility analysis of longitudinal microbial diversity of samples taken within the HI-SEAS habitat and inside other built environments. D: Volatility analysis along PCoA Axis 1 showing weighted UniFrac distances for different sample types (built environment and human skin). E: Volatility analysis along PCoA Axis 1 showing weighted UniFrac distances for individual crew members and sampled locations within the HI-SEAS habitat.

Comparison with other built environment studies indicates an atypical increase of skin diversity under isolation

For a suitable evaluation of our observations, we performed a meta-analysis of longitudinal microbial diversity patterns inside different built environments. This analysis (see Material & Methods for more details) covered more than 3,400 samples and 10 different sample types (front torso skin, confined habitat surfaces, office dust, room surfaces, desk surfaces, door stoppers, floors, windows, fecal samples and skin samples from the inner elbow) from four longitudinal studies inside the built environment. Public studies from Qiita were selected based on three criteria: first they had to be longitudinal, second they had to be conducted in a built environment setting and third they had to cover samples from human sources beside built environment surfaces. According to these criteria we included a longitudinal analysis of microbial interactions between humans and the indoor environment [80], a longitudinal assessment of the influence of lifestyle homogenization on the microbiome of United States Air Force Cadets [81], and a study which identified geography and location as the primary drivers of office microbiome composition [82]. Our meta-analysis confirmed that microbial diversity progressions in the HI-SEAS habitat were exceptional. While all other sample types showed a decrease in microbial diversity, only samples from the human gut of US air force cadets (mean Shannon H' 5.1 to 5.5) and skin samples of the HI-SEAS crew (mean Shannon H' 4.9 to 5.4) showed a steady increase over time (Fig. 2C).

Microbial dynamics during isolation was driven by specific taxa

For a higher resolution of microbial composition over time, the isolation period was grouped into four phases (phase 1: days 0 - 84, phase 2: 84 - 210, phase 3: 210 - 294 and phase 4: 294 - 336). To get insights into microbiome evolution after isolation, skin samples from the post-mission control (day 400) were also studied.

According to differential abundance analysis of all samples using balances in gneiss (Fig. 3A), higher proportions were visible for *Staphylococcus*, *Propionibacterium* and *Methanobrevibacter* (phase 1). Between day 84 and day 210 (phase 2), only *Stenotrophomonas* and unclassified Enterobacteriaceae showed higher proportions at the latter time point. Later on, unclassified Dermacoccaceae, *Propionibacterium*, *Kocuria* and unclassified Rhizobiaceae showed increasing proportions, while *Streptococcus* and *Fusobacterium* showed decreasing

proportions (phase 3). During phase 4, *Methylobacterium populi*, *Streptococcus*, *Brevundimonas*, *Pseudomonas*, *Lactococcus*, *Sphingomonas* and *Cloacibacterium* revealed lower proportions than unclassified Enterobacteriaceae and *Staphylococcus*.

After the isolation period, increasing proportions of *Acinetobacter*, *Propionibacterium*, *Rhizobium* and *Methylobacterium populi* were prevalent on the skin of the crew, while signatures of *Pseudomonas*, *Corynebacterium* or unclassified *Intrasporangiaceae* decreased (Supplementary Fig. S9).

Dynamics of representative skin, GIT/UGT and environment-associated microbial taxa

In a next step, we selected 15 microbial genera and families which were indicative of either skin (*Acinetobacter, Staphylococcus [aureus], Brevundimonas, Kocuria, Propionibacterium, Streptococcus, Kytococcus,* Dermacoccacae*),* gastrointestinal/urogenital tract (*Gardnerella, Lactococcus, Methanobrevibacter, Faecalibacterium*, Enterobacteriaceae) or environment and water (*Pseudomonas, Enhydrobacter)*, to assess the dynamics of those microbial signatures. Our feature selections were supported by higher rankings in differential abundance tests based on gneiss, aldex2 and songbird, and feature loadings based on deicode. Grouping these representative features into the categories skin, GIT/UGT (gastrointestinal/urogenital tract), and environmental was based on empirical data from literature [10].

Representing the skin microbial taxa, *Acinetobacter*, despite being recognized as a typical skin microbial taxon, shows higher relative abundances on built environment surfaces (especially in the main room and bedroom). Crew member E showed over proportional prevalence at the beginning and together with crew member A also at the end of the isolation period (Fig. 3B).

Staphylococcus aureus was mainly present on human skin (crew members D, E and F), but has also been detected on surfaces inside the habitat. Interestingly, exchange with bedroom surfaces seems obvious, since higher proportions of *S. aureus* on skin and bedroom surfaces seem to be coupled (Fig. 3B and Supplementary Fig. S10). *Brevundimonas* was clearly associated with the kitchen surfaces and could have been transferred to the toilet bowl (between day 70 and day 84) and main room (between day 238 and day 252). Crew member F might have picked up this taxon from the kitchen, for instance between day 238 and day 252 and again between day 294 and day 336. Relative proportions of *Kocuria* were clearly correlated with time by linear regression models and showed the highest value for importance (0.3). It seems that this signature was introduced by crew members A and E into the habitat and then

546 transferred to almost all investigated built environment surfaces, as well as crew members B
547 and C. In contrast, *Propionibacterium* did not manifest itself on built environment surfaces
548 after being introduced by crew member D and was recovered only from other skin samples
549 over time. On the other hand, signatures of *Streptococcus* could not be linked to a defined
550 human source and established itself on bed and main room surfaces. *Kytococcus* was associated
551 with crew members A and D at the beginning. Later on, only single events of high proportions
552 were visible on the skin of crew member B or sampled bedroom surfaces, suggesting that this
553 taxon could not establish itself in the confined habitat. Dermacoccaceae could have been
554 continuously transferred between skin and built environment surfaces with a peak in the
555 bedroom and on crew member D at the end of the confinement period. After *Kocuria*,
556 Dermacoccaceae showed the highest importance (0.1) in linear regression models.

557 As a representative of the GIT/UGT, *Gardnerella* showed a consistent presence despite varying
558 proportions on the surface of the toilet bowl. Likewise, signatures of *Lactococcus* revealed a
559 single peak on the kitchen floor after day 50, but did not establish itself on the skin of any crew
560 member. In contrast to *Faecalibacterium*, *Methanobrevibacter* were not consistently recovered
561 from the toilet bowl, but were clearly associated with some of the crew members. As biplot
562 analyses identified Euryarchaeota (in particular *Methanobrevibacter* sp.) as the main reason
563 for compositional changes around day 84, this genus was analyzed further. In general,
564 signatures of *Methanobrevibacter* were highly associated with the human crew within the first
565 210 days (Fig. 3B and Supplementary Fig. S11). However, these signatures were not common
566 on built environment surfaces and were only observed on the toilet bowl and the kitchen floor
567 on day 115 and on day 224. This pattern was different from that of other archaeal lineages
568 (Euryarchaeota, Thaumarchaeota and Woesarchaeota), which showed scattered peaks on built
569 environment surfaces but not in skin samples. Enterobacteriaceae showed only single events
570 of prevalence on the toilet bowl and were mainly associated with skin samples of crew
571 members D, E and F between day 100 and day 250.

572 Despite representing an environment-associated taxon, *Enhydrobacter* was present on all crew
573 members to varying proportions and was regularly transferred to the toilet bowl. *Pseudomonas*
574 was obviously introduced by crew member B and D, then transferred to the kitchen floor and
575 further on to crew member E and F.

576

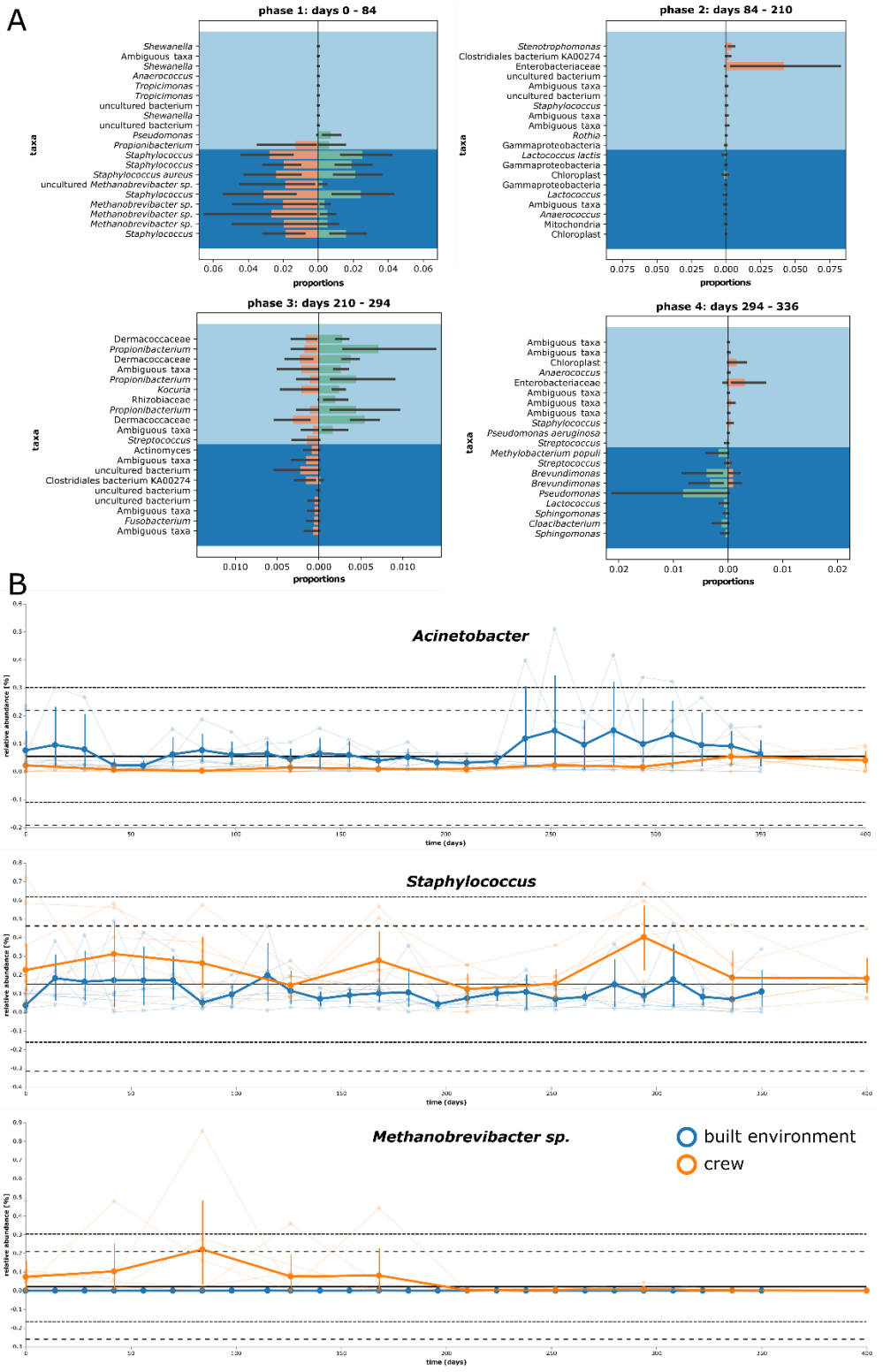
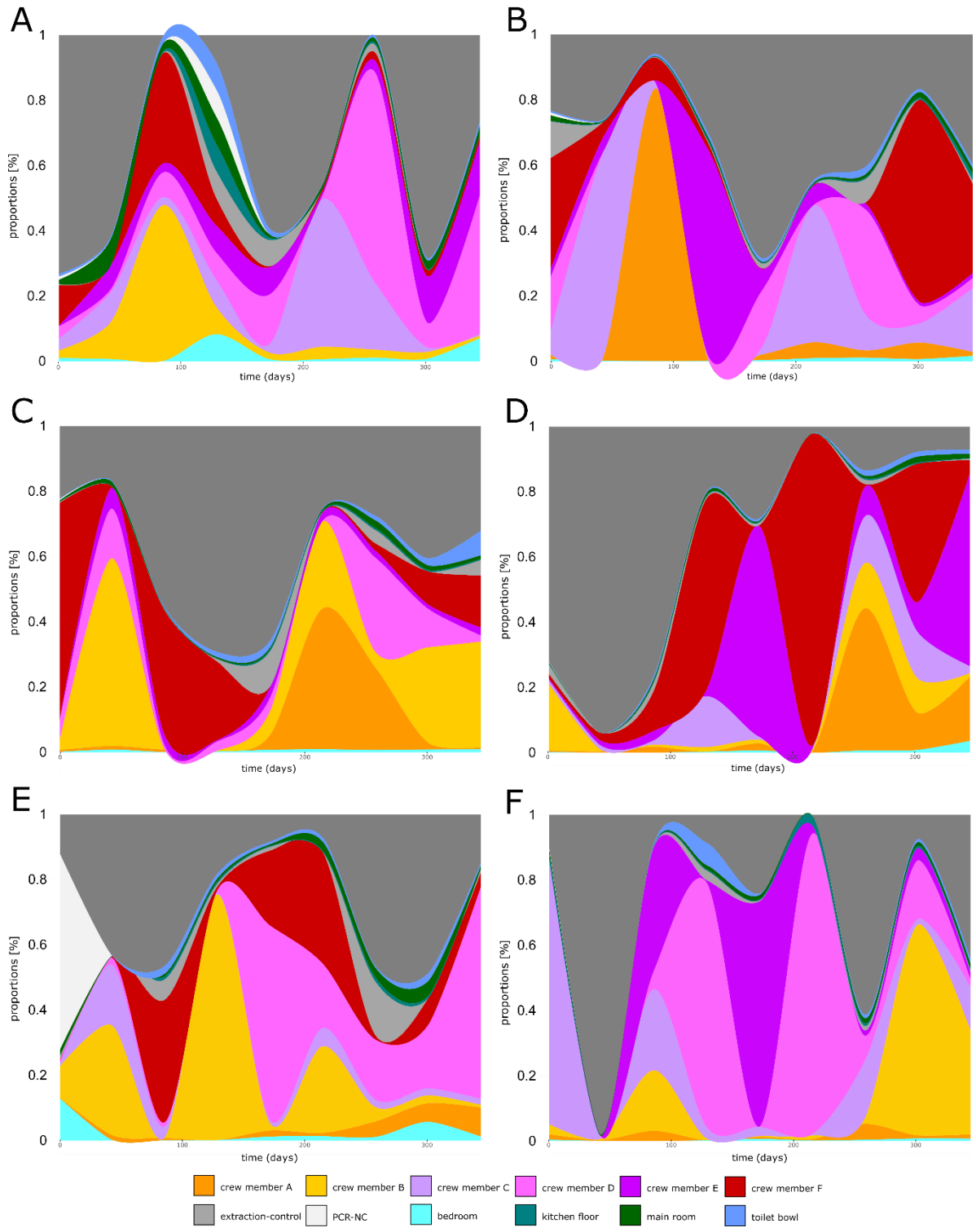


Fig. 3. **Microbial dynamics in distinct phases and for representative features.** A: Proportion plots of differential feature abundances using balances in gneiss on genus level grouped into four distinct phases. Background color of numerators light blue and for denominators dark blue. B: Volatility analysis based on linear regression models with time of *Acinetobacter*, *Staphylococcus* and *Methanobrevibacter* sp.

Shared occupancy influences the microbiome composition and function of the crew skin and abiotic surfaces

Source tracking of microbial signatures with Sourcetracker2 identified human skin as the main source of microbial dispersal. Noteworthy, the intensity of microbiome exchange was heterogeneous among the possible pairs of crewmembers. In more detail, crew member F showed the highest interactive profile (13.4%) of all crew members. This was also supported by redundancy analysis (RDA) revealing pairwise microbial exchange for two pairs of crewmembers as significant parameters on respective skin microbiome profiles ($p = 0.002$ RDA). Sampled locations of the built environment played only a minor role in overall microbial dispersals. Maximal microbial contributions on the crew reached only proportions of 1.2% in case of the main room. Interestingly, crew gender showed different microbial associations for bedroom and the kitchen floor samples. Nevertheless, microbial interaction profiles were highly person-specific as well as dynamic over time. Overall, trends were difficult to delineate (Fig. 4).

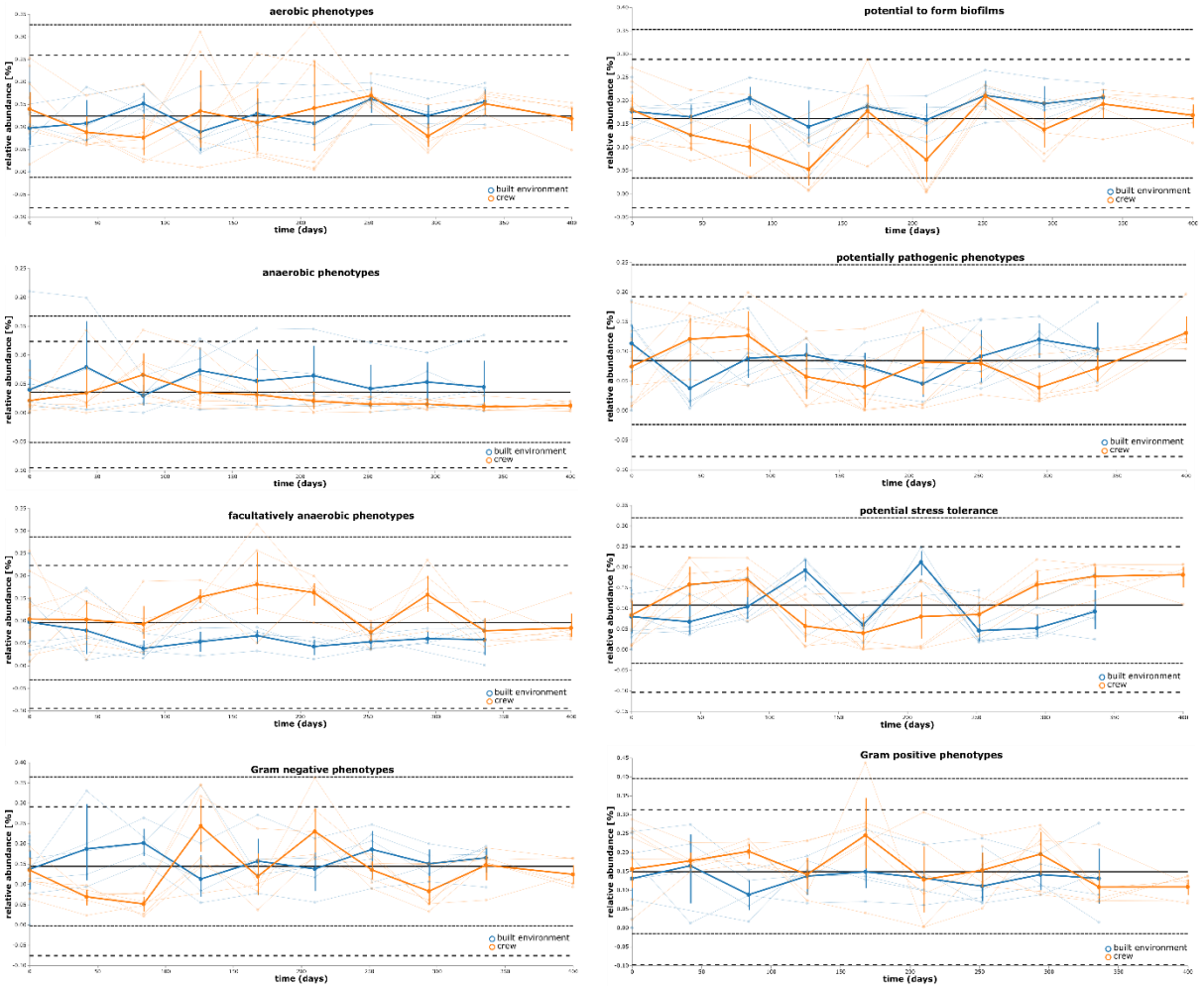


598

599 Fig. 4 **Source tracking of microbial signatures** according to SourceTracker2. Panels A to F show respective
600 crew members A to F as a sink of microbial dispersal.

601

Further on, we were interested in whether microbial profiles and interactions between crew members and surfaces inside the HI-SEAS habitat had an impact on potential phenotypes predicted with Picrust 2 and BugBase. According to these predictions, most phenotypes showed higher proportions (e.g., potential mobile elements, potential pathogens, potential stress tolerance and especially facultative anaerobes) or recurring peaks (Gram-positive and Gram-negative phenotypes and aerobes) on samples from the crew's skin. However, the potential to form biofilms showed a constant maximum in samples retrieved from the kitchen floor (global median 0.18, maximum 0.27, cumulative average decrease/increase -0.25/0.24) and anaerobes were increasing on the toilet bowl (minimum 0.06, maximum 0.21) while decreasing on human skin (0.02 to 0.01). Interestingly, potential pathogens showed an anti-cyclic pattern of samples from the built environment versus samples from the crew (Fig. 5).



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615 Fig. 5. **Linear regression with time of predicted phenotypes from BugBase** shown as volatility plots (sorted
616 according to regression with time).

617

Fluctuation of microbial quantity correlates with the presence of certain antimicrobial resistance genes and microbial phenotypes

Quantitative PCR was used to assess bacterial and archaeal abundance, and its dynamics inside the HI-SEAS habitat and the skin of its isolated crew members. As observed for microbial profiles, bacterial abundance changed to a larger extent for samples from the crew's skin than from built environment surfaces. In general, two main phases of differing bacterial load could be determined. In the beginning (days 0 - 42) and between days 126 and 210, bacterial abundance on human skin was much lower than on selected locations of the built environment (respective mean difference for the two phases: 12.5 and 22.5%). The largest dynamics were observed around day 28 and day 182 (change in relative proportions by 43%; Fig. 6A). On the other hand, archaeal abundance peaked around day 84 (82%) but varied to much lesser extents, especially in the mid-term of the isolation period (Supplementary Fig. S12).

In addition, four markers for antimicrobial resistance (*blaOXA* - class A beta-lactamase, *int1a* - class 1 integrase, *qacEΔ1* - biocide resistance gene, quaternary ammonium compound and *tetM* - tetracycline resistance) were selected to analyze dynamics of microbial resistances in a quantitative way. *TetM* was most predictive for the factor time (importance = 0.2) and was constantly more abundant in skin samples between day 140 and day 294. Interestingly, beta-lactamases showed the opposite pattern, with lowest proportions between day 140 to day 294. *Int1a* gene abundance was highly dynamic over the whole time frame (highest global variance of 0.06) and showed peaks on built environment locations (toilet bowl and kitchen floor) on day 140, but also on human skin (especially crew member C and D) on day 308. Highest and lowest abundances of *qacEΔ1* regularly alternated between samples of the built environment and from human skin. Nevertheless, all four targeted resistance genes showed high dynamics and potential transfer between skin and the built environment (Fig. 6B).

Finally, predicted phenotypes were correlated both with each other and with obtained quantitative information (16S rRNA gene copies and selected resistance genes). While all quantitative data could be significantly positively correlated with each other (especially class A beta-lactamases with biocide resistance of quaternary ammonium compounds - P = 5.0*E10-15, rho = 0.66), and biocide resistance of quaternary ammonium compounds with tetracycline resistance - P = 3.2*10E-8, rho = 0.5), comparisons of the qualitative information showed both positive and negative correlations. Significant positive correlations were evident between aerobes and potential biofilm formers (P = 4.9*E10-12, rho = 0.60), as well as potential

650 pathogens and stress tolerance (P = 2.1*E10-13, rho = 0.63). On the contrary, significant
651 negative correlations were observed between aerobes and facultative anaerobes (P = 2.2*E10-
652 16, rho = -0.67), as well as between potential biofilm formers and Gram-positives (P =
653 1.1*E10-8, rho = -0.51). However, significant correlations between quantitative and qualitative
654 measures were scarce. Only the overall bacterial load (16S rRNA gene copy numbers) showed
655 significant positive correlations with anaerobes (P = 5.6*E10-4, rho = 0.32) and significant
656 negative correlations with aerobes (P = 5.3*E10-3, rho = -0.26).

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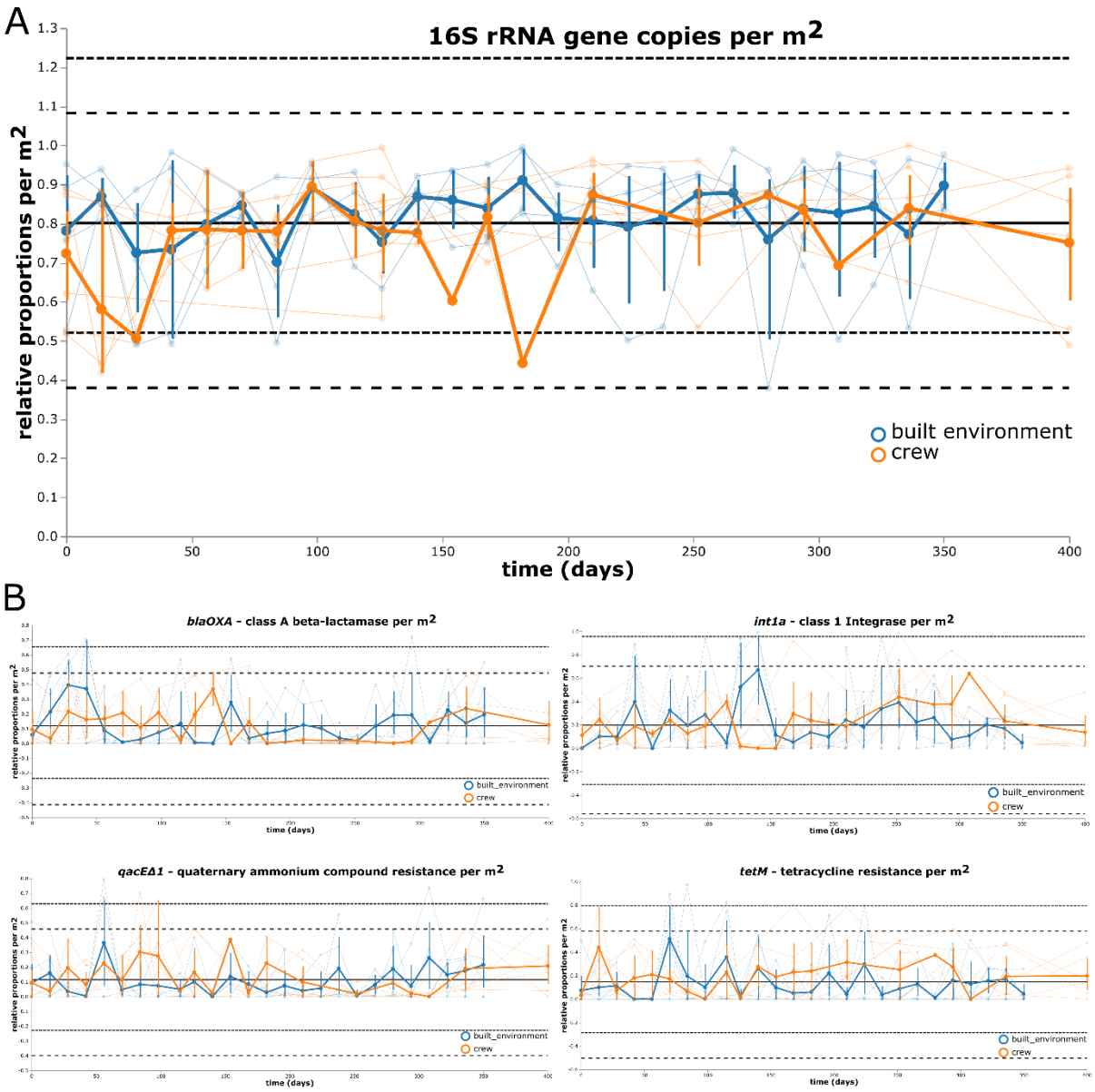

Fig. 6. **Overall bacterial load and abundance of selected antimicrobial resistances.** A: Volatility analysis of bacterial 16S rRNA gene copies per m^2 according to qPCR. B: Linear regression volatility analysis of selected resistance gene copies per m^2 according to qPCR.

Discussion

Human well-being is inseparably linked with its microbiome. Thus, the dynamics of the microbiome in and around a human being is subject of research for the preparation of human long-term spaceflight and settlement in remote locations such as a future Martian outpost [7]. For such studies, Earth-based models are indispensable including a monitoring of the longitudinal microbial dynamics of an isolated crew with its confined environment beside social analysis of team cohesion and performance. Numerous suitable model environments were investigated in the past (besides the one studied herein), but greatly differed in terms of setup and study design. For instance, the Concordia research station in Antarctica comprised separate buildings, accommodated 16-32 occupants during the sampling period and was microbially monitored for 365 days [43]. The US inflated lunar/Mars analog habitat (ILMAH) provided 300 m^3 space for three occupants and was investigated for 30 days [44]. Another example is the Mars500 habitat located in Moscow, Russia, which included four modules with a total volume of 550 m^3 and housed six participants for 520 days [41].

All isolated and confined built environment (ICE) models represented a unique testbed for assessing the microbial interaction of an isolated crew inside a confined built environment. In contrast to other studies in the MoBE (microbiology of built environments) field, its reduced set of potential environmental variables that could drive the microbiome in a longitudinal context allows distinct assumptions about where microbial signatures originated, as well as when, where and why they were transferred through time and space [83].

Unexpectedly, our study revealed a highly dynamic skin microbiome of the HI-SEAS crew despite its isolated and confined setup. In contrast to the study by Sharma and co-workers [81] in which longitudinal homogenization of microbial composition was visible *ab initio*, we observed a retarded longitudinal homogenization between skin and built environment samples only after 210 days. Nevertheless, traces of the skin microbiome were clearly visible on the studied surfaces and as described before occupancy was identified as the major microbial source [9,84–86]. Compared to microbial interaction profiles from human skin, building surfaces seem to play a more passive and subordinated role for spreading and dispersal in this habitat and confirmed again that humans dominate microbial communities on indoor surfaces [87,88]. Accordingly, the kitchen floor surface stood out with its low microbial diversity. This

observation could be associated with the low interaction frequency of this specific location (floor surface) in contrast to the regularly touched desk or toilet surfaces in the habitat.

In contrast to other isolation experiments like the Mars500 study [41] in which a significant decrease in microbial diversity was observed, the microbial diversity on the HI-SEAS surfaces remained rather constant and even increased in samples from crew skin. Confirmed by our meta-analysis based on four selected longitudinal studies from built environment settings, increasing microbial diversity on human skin and built environment samples was not reported in offices or homes [80,82]. However, this observation could be the result of a human occupancy in confined and in more isolated built environments as this pattern was also observed in samples from crew members at the ISS (International Space Station) for samples of the forearm and gut [89] or in fecal samples during the cohabitation of US Air Force cadets [81]. Crew members C, D, E, and F started with a relatively low range of microbial diversity on their skin compared to representative studies of the skin microbiome[90,91]. This rather low microbial diversity might result from extensive personal hygiene cleaning procedures before entering the HI-SEAS habitat with a limited allowance of daily hygiene procedures.

The sources of microorganisms are not completely clear; the steady increase on the crew's skin could result from interactions with the higher microbial diversity present in their habitat (refer to Fig. 2A and 2B), regular resupply events, complications and elaborate cleaning of the composting toilet (for details see below) or dispersal of microbial food supplements used to ferment food. In addition to these potential microbial sources, the altered hygiene regime during isolation has probably contributed to an increase or stabilization of skin microbiome composition. The most stable skin microbiome was observed for a crew member, who applied extremely short showers (average duration of 40 seconds; Supplementary Table S4), compared to the other crew members (overall average showering time of 102 seconds), which revealed a more fluctuating microbial profile. Another impact of hygiene regiments on the skin microbiome was indicated by the higher abundance of quaternary ammonium compound resistance genes on the skin of individual crew members. This observation reflected the application of disinfecting cleaning wipes (which contained such compounds) on the crew's skin in-between the showering events.

The most striking observation was the delayed longitudinal homogenization and very dynamic developments of the skin microbiome in the first half of the isolation period (days 0 – 210). These unstable profiles correlated with complications of the composting toilet, which required

a manual cleaning and emptying rotationally by all the crew members. Dispersal of gut/urogenital-associated microorganisms from the composting toilet might have even been supported by the use of a fan to reduce the odor. An obvious indicator species of these complications was *Methanobrevibacter*. As verified by biplots, specific filtering of the dataset and significant correlations of quantitative data (bacterial and archaeal abundance) with an anaerobic predicted phenotype, this archaeon of the human gut explained most of the dynamics in this timeframe and delayed the expected longitudinal homogenization process of its microbiome. *Methanobrevibacter* represents the most common archaeal genus of the human gut and has an important role at the end of the intestinal food chain where it facilitates the fermentation process of bacteria by removing hydrogen from the system (an overview is given in [92]. Obviously, the increased prediction of anaerobic phenotypes correlated very well with the increased signatures of *Methanobrevibacter* in the same timeframe. Later on, the signatures of *Methanobrevibacter* increased relatively on samples of the toilet bowl while decreasing on human skin as soon as the toilet issue was fixed. Most of the other predicted phenotypes showed higher relative proportions and dynamics on skin samples, which might be due to the reported temporal and personalized variability on human skin samples [90,93]. In addition, the anti-cyclic pattern of potential pathogens might picture regular transfer events between skin and desk surfaces in the habitat, which were described before [81].

Besides the remarkable traces of gut-associated archaea, other selected bacterial indicator species were helpful to interpret microbial dynamics in the HI-SEAS habitat. Hence, most skin-associated bacteria like *Staphylococcus aureus, Brevundimonas, Kocuria, Propionibacterium, Streptococcus, Kytococcus* and Dermacoccacae could be easily traced in the habitat, were most frequently exchanged with the desk surface in the bedroom and were transferred more likely between crew members who also had close physical interaction with each other. Their presence on different built environment locations were often transient and higher proportions were usually detected in samples from the crew. This observation was also confirmed by source tracking analysis, which showed higher microbial transfer between crew members A and D, B and C, or E and F, or low microbial transfer between crew members A and F, or C and E. This microbial transfer pattern corresponds to the reported interaction preference of male with female crew members during the isolation period. These results underline the important impact of the personal microbial cloud [94], the importance of direct physical interaction [80,95,96] as well as cohabitation of crew members [97] and the low microbial input from selected built environment locations [88] to the overall microbiome of the HI-SEAS habitat.

Propionibacterium could act as an example for the low input of the built environment microbiome, since this bacterium was only recovered from skin samples and was never observed on surfaces of the HI-SEAS habitat. This might be due to its strict anaerobic lifestyle, that seems to prefer the oxygen depleted pores of human skin and much less those relatively smooth oxygen rich building surfaces and materials.

However, an opposite profile was observed for signatures of *Acinetobacter*. Although described as a skin-associated microbe [98], *Acinetobacter* established itself much better in various locations of the main room and bedroom. As this bacterium is also frequently detected on surfaces of public and private buildings, as well as hospitals or cleanrooms [99–102] it is tempting to speculate whether *Acinetobacter* could be perceived as an indicator species for confined built environments and to a lesser extent only as a skin-associated bacterium.

Peaks of signatures from *Lactococcus* on the kitchen floor might be due to its usage in fermenting yoghurt, cream cheese (*Lactococcus lactis* subsp. lactis and cremoris), tempeh or kombucha. The idea of using microbial cultures as part of the food supply, or entire artificial ecosystems for life support [28,103], is of particular importance in ICE and might be key to colonize another celestial body in the solar system. Nevertheless, the exact composition of commercially available products must be well-characterized to mitigate potential risks for the crew.

Although a number of metadata about health and medication of the crew were obtained, the information was not precise enough for correlations with the microbiome. Crew members provided medication data, but only two reported treatments could be associated with increases in tetracycline resistance and bacterial abundances, potential pathogens or stress tolerance. Hence, in contrast to the study by Abeles and co-workers [104] who could follow specific treatment courses in the microbiome, our dataset was not comprehensive enough to make such assumptions. On the contrary, recordings of the microclimate produced numerous values, but was rather constant and did not represent a major driving factor of the microbiome (only up to 9%) as investigated by bioenv analysis.

The presented study has limitations and serves only partially as a basis for meaningful recommendations for future attempts to sustain a safe microbial environment in a human outpost on the Moon or Mars. First of all, no baseline (i.e. before the start of the isolation experiment) of the indoor and skin microbiome was defined. In addition, information on all

791 microbial sources were limited and only one body site was selected for monitoring potential
792 microbial transfer with the environment. Additional human samples would have been
793 supportive to draw conclusions about an individual's health status (e.g. fecal samples) or draw
794 detailed conclusions of frequently touched surfaces inside the habitat (e.g. hand samples).

795 Nevertheless, the study benefits from its defined confined setup with limited amount of
796 confounding environmental variables, a defined set of occupants, the deduced prediction and
797 tracking potential of microbial transfer in a remarkable level of detail, the correlation of
798 qualitative and quantitative microbial and resistance data and our pre-informed knowledge of
799 microbial hotspots on desks, in bedrooms, or the toilet bowl on ICE locations from the Mars500
800 project [41].

801

Conclusions

From a microbiological point of view, the risk of infection or transfer of antimicrobial resistant pathogens to crew members was found to be extraordinarily low and is thus in agreement with recent information from the ISS [24]. However, although the risk was deemed to be low, the need for monitoring microbial dynamics inside isolated and confined habitats became obvious, in order to understand the impact of special events like the contamination caused by the composting toilet. Moreover, baselines of the microbiome inside the habitat, its crew and post sampling events would greatly improve evaluations of the impact of confinement on the crew and habitat surfaces. Most comparable studies, either ground-based like Mars500 [41,105] or in space aboard the ISS [24,89,106], showed a similar picture of longitudinal homogenization, composition and diversity of microbiomes.

However, in many cases these studies suffered from a lack of appropriate controls, or baselines, which are a central point of management of metadata or interpretations. For future experiments, it is key to determine a standard operating procedure regarding sampling intervals, methods suitable for microbial monitoring, distinct knowledge on pre- or probiotics used for food production, stabilization of the gut microbiome or even personalized collections for autologous fecal microbial transplants in case of worrisome developments leading to a microbial dysbiosis. Hence, future ICE missions in preparation for crewed mission to the Moon or Mars in the upcoming decades should emphasize on actual tests on a microbial warning system that could be based on automatic sampling technologies as developed by BiomeSense (https://www.biomesenseinc.com/) and predictive models comparing expected and true microbial compositions in the habitat and its crew. Furthermore, manipulations of the microbiome would be essential to stress the microbiome by different levels of desired (reduce potential pathogens and technophilic microorganisms) and unintentional perturbations through defined cleaners and antibiotics, and following restorations of a beneficial community on the surfaces of the habitat, as well as different body sites including the crew's gut microbiome. One key question for future related studies is how diverse a microbiome needs to be and which actual composition it needs to have on surfaces, and on and in the human body, to be self-sustaining over longer timeframes, or how it can be stabilized and restored after critical perturbations. The answers to these questions are a crucial prerequisite for the planning of future human long-term missions in space or on other celestial bodies in our solar system.

List of abbreviations

ANCOM: Analysis of composition of microbiomes

ASVs: Amplicon sequence variants

BHP: Behavioral Health and Performance

CSA: Canadian Space Agency

FDR: False discovery rate

HI-SEAS: Hawaii Space Exploration Analog and Simulation mission

ICE: Isolated and confined built environments

ILMAH: Inflatable lunar/Mars analogous habitat

ISS: International Space Station

JAXA: Japan Aerospace Exploration Agency

LEfSe: Linear discriminant analysis Effect Size

MoBE: Microbiome of the built environment

NASA: National Aeronautics and Space Administration

PCR: Polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

PERMANOVA: Permutational multivariate analysis of variance

QIIME: Quantitative Insights Into Microbial Ecology

RDA: Redundancy analysis

SD: Standard deviation

Declarations

Ethics approval and consent to participate

The study proposal was reviewed and approved by an institutional review board (JSC IRB - Study Protocol Number 1648: Key contributors to the maintenance and regulation of team function and performance on long duration missions).

Consent for publication

All authors gave their consent for publications.

Availability of data and materials

All the raw and processed data used for analyses in this study have been deposited in QIITA under the study ID 12858 (https://qiita.ucsd.edu/; Gonzalez et al., 2018) and EBI-ENA under the accession number ERP118380.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AM, CM-E, CV wrote the manuscript. AM, CM-E analyzed the data. CV, PS, DBi, CM-E did the study design. CV, KK, LW, DBr, TG, CK, MB did the work in the wet-lab. All

authors improved the manuscript by their specific input and also approved the final version of the manuscript.

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Figure and Table legend

Fig. 1: Microbiome profile of crew and built environment samples, all time points included. A to C: violin plots (kernel probability density of Shannon values, displaying the alpha diversity) including individual data points and a box with median and interquartile range. A: Sampled surface types: skin of crew and locations of the built environment. B: Built environment surfaces: BR (desk in bedroom), KC (kitchen floor), MR (desk in main room), TB (toilet bowl). C: Samples from the individual crew members (A-F). D: Linear discriminant analysis effect size (LEfSe) on samples from crew and built environment. E: LEfSe on samples from built environment surfaces. F: LEfSe on samples from individual crew members. G to H: Beta diversity represented by PCoA plots based on weighted UniFrac metrics and a rarefaction depth of 3,000 ASVs per sample. G: Microbial profile of samples from built environment and crew. H: Microbial profile of the individual crew members and sampling locations of the built environment.

Fig. 2: Longitudinal development of diversity and composition. A: Volatility analysis of Shannon diversity resolved to sample type (built environment and human skin). B: Volatility analysis of Shannon diversity resolved to individual crew members and sampled locations within the HI-SEAS habitat. C: Meta volatility analysis of longitudinal microbial diversity of samples taken within the HI-SEAS habitat and inside other built environments. D: Volatility analysis along PCoA Axis 1 showing weighted UniFrac distances for different sample types (built environment and human skin). E: Volatility analysis along PCoA Axis 1 showing weighted UniFrac distances for individual crew members and sampled locations within the HI-SEAS habitat.

Fig. 3. Microbial dynamics in distinct phases and for representative features. A: Proportion plots of differential feature abundances using balances in gneiss on genus level grouped into four distinct phases. Numerators are colored in light blue, denominators in dark blue. B: Volatility analysis based on linear regression models with time of *Acinetobacter*, *Staphylococcus* and *Methanobrevibacter sp.*

Fig. 4 Source tracking of microbial signatures according to SourceTracker2. Panels A to F show respective crew members A to F as a sink of microbial dispersal.

Fig. 5. Linear regression with time of predicted phenotypes from BugBase shown as volatility plots (sorted according to regression with time).

Fig. 6. Overall bacterial load and abundance of selected antimicrobial resistances. A: Volatility analysis of bacterial 16S rRNA gene copies per m^2 according to qPCR. B: Linear regression volatility analysis of selected resistance gene copies per m^2 according to qPCR.