Microbiome

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On-orbit microbial succession patterns of the China Space Station during the construction period

Ying Zhang[1*†], Yuan Peng[1†], Xi Qu[2], Lantao Zhang[2], Tao Wei[1], Hong Wang[1], Zimu Guo[1], Weijie Liu[3*] and Xiang Wang[2*]

Abstract

Background The China Space Station (CSS) modules feature many areas that are difficult to clean and thus susceptible to microbial outbreaks. A new sampling method utilizing an equivalent material sheet was applied to characterize the diversity of microbes that accumulated in inaccessible areas in orbit on the CSS. Equivalent material sheet is a membrane made of the same material as the wall of the module.

Results Fifty samples were collected from interior surfaces (work, sleeping, and sanitary areas) of the Tianhe core module and the Wentian and Mengtian experimental modules, covering three flights by the Shenzhou (SZ)-12 to SZ-14 astronaut crews from 2021 to 2022. The numbers of culturable bacteria and fungi that accumulated during the on-orbit periods of each flight ranged from 0 to 2.83×10^9 colony-forming units/100 cm^2. The number of bacteria detected by quantitative PCR (qPCR) ranged from 1.24×10^5 to 2.59×10^9 rRNA gene copies/100 cm^2, with an average viability of 65.08%. A total of 103 bacterial strains and 27 fungal strains were cultured and isolated. The dominant culturable microorganisms were mainly from the genera *Bacillus*, *Staphylococcus*, *Aspergillus*, *Cladosporium*, and *Penicillium*. High-throughput sequencing results showed that the predominant bacteria were *Pseudomonas*, *Stenotrophomonas*, *Methylobacterium-Methylorubrum*, *Sphingomonas*, *Bacillus*, *Staphylococcus*, and *Nocardiopsis*. The microbial diversity in each module varied significantly with sampling time and sampling area. In the early stage of CSS construction with the SZ-12 crew, the microbial species evenness in the modules was high; later, with the SZ-13 crew, *Pseudomonas* began to appear as the dominant microorganism. More than half (58.80%) of the bacteria on module surfaces originated from the human skin and oral environments. *Lactobacillus* was present in all areas of the three modules at all sampling times. The biomarker bacteria *Stenotrophomonas* sp., isolated from the work area in the Tianhe core module, are typically derived from plants. SourceTracker analysis indicated that most of the microbes in the orbiting CSS came from human bodies, and that microbial diversity was significantly altered with each crew change.

[†]Ying Zhang and Yuan Peng contributed equally to this work.

*Correspondence:
Ying Zhang
zhangying3409@bit.edu.cn
Weijie Liu
leonliu2013@126.com
Xiang Wang
wangxiang0223@hotmail.com

Conclusion Future efforts at microbial prevention and control on orbit should emphasize the human and plant origins of microbes. Information on the microbial diversity in the condensate zone could be useful to guide the development of new strategies to prevent and control microbes during space flight.

Keywords China Space Station, Microbiome, 16S rRNA, Microbial diversity, Built environment

Introduction

The China Space Station (CSS) is a state-level manned space laboratory built by the People's Republic of China. The core module of the CSS was completed in 2021, and in 2022, the Wentian and Mengtian experimental modules were successively docked with the core module. As of 2024, the construction status of the CSS is a "T" configuration of the three modules. The CSS operates at an orbital altitude of 350–450 km and an inclination of 42–43°, and its designed lifespan is 10 years. The internal environment of the CSS is a typical "extreme special environment" [1–3], characterized by low radiation dose, microgravity, long-term sealing, a 24–25 ℃ temperature, and 30–70% humidity. This special environment supports the long-term residence of humans while providing favorable conditions for microbial reproduction.

There are three main sources of microorganisms in space stations: modules, cargo, and humans. Module-source microorganisms are attached to the interior spaces of the modules and enter orbit during module launch. During the early stages of CSS construction, each module was strictly sterilized before launch; however, it was difficult to avoid small amounts of microbial residue [4]. Cargo-source microorganisms, which enter the modules with the cargo or packaging materials, are similarly few in number but difficult to avoid. Human-source microorganisms are brought into the modules by astronauts. For reference, a normal, healthy adult harbors trillions of microorganisms [5].

Early reports on the microbial ecology of the on-orbit Mir space station (MIR) and International Space Station (ISS) indicated the presence of large numbers of microorganisms in long-term on-orbit space stations. Furthermore, these microorganisms were shown to adversely affect the health of astronauts and the safe operation of on-orbit instruments and equipment. The space environment can negatively affect astronaut immune function [6] and may support increases in the pathogenicity and drug resistance of certain microorganisms [7–11]. Pathogenic microorganisms may also contaminate water or food [12]. Therefore, microorganisms in the space environment pose a greater threat to astronaut health than those in the terrestrial environment [13]. Astronauts aboard MIR have experienced microbial infections including conjunctivitis, acute respiratory events, and dental infections [14]. Additionally, microorganisms can corrode spacecraft materials, including metals and a variety of polymeric materials [15–18], posing significant potential hazards to space flight safety.

The MIR was in orbit from 1986 to 2001. During this period, several different forms of microbial sampling were conducted. A 2004 paper reported on the continuous sampling of modules during the 15 years of MIR operation [19]. An article published in 2001 showed that Kawamura et al. conducted a microbiological study on the air, water, and inner walls of the MIR in 1997 [20]. Sampling methods included air vacuum sampling, collection of condensate, and swabbing of the inner walls. In 2004, Ott et al. described their collection of three free condensate samples on the MIR in 1998 [21]. In 2005, Song et al. [22] reported a culture method that was used to study microorganisms in the MIR water system. Because these studies were conducted early on, the detection of microorganisms relied on cultivation methods. However, research has shown that cultivation methods only detect about 1% of microorganisms in typical natural environments [23], with the vast majority of microorganisms being unculturable and thus going undetected. Furthermore, traditional 16S rRNA gene amplification and Sanger sequencing methods are unable to detect all microorganisms from environments with low microbial populations, capturing only a fraction of the more abundant microorganisms [24]. Therefore, the data on microorganisms on the MIR are limited.

The ISS has been in operation from 1998 to the present. Early ISS microbial detection methods were similarly culture based. For example, an article published in 2005 showed that Novikova et al. conducted a 6-year microbiological study on the air and surfaces of ISS starting in 1998 [25]. They used two sampling tools — the SAS air sampler (PBI International, Italy) and surface pipette kits with swabs — for culture and analysis of the numbers and types of bacteria and fungi.

With the rapid development of high-throughput sequencing technology in the twenty-first century [26], microbial analysis methods have gradually been applied in research on the ISS. In 2012, Venkateswaran et al. conducted the first culture-independent 16S rRNA gene detection of microorganisms in the ISS [27], while Lang et al. [28], Sieraf et al. [3], and Madrid et al. [29] reported the results of spatial microbiome research in 2017, 2019, and 2022, respectively. While the findings of these studies

suggest that on-orbit microbial diversity changes over time, the microbial ecology data of the ISS were collected after 14 years of operation; thus, the pattern of microbial change in the early days of the station is unknown. Furthermore, some studies have shown that microbial community structure changes with astronaut activities and time [19, 30], while others found that microbial community structure does not change significantly over time [3, 31]. This paradox of microbial patterns in the space environment requires further elucidation.

This study describes microbial research conducted on the interior surfaces of CSS modules from 2021/06/17 to 2022/12/05. During this period, the Shenzhou (SZ)−12 to SZ-14 crew members successively carried out scientific research missions in orbit. We present here the results of culture and quantitative real-time PCR (qPCR) and microbiomics, revealing for the first time the comprehensive changes in microbial communities within the CSS at the construction stage. Applying state-of-the-art microbial molecular ecology tools, we were able to analyze microbial community succession on the CSS during the construction period, enabling speculation on microbial sources as well as potential hazards. Finally, we offer a theoretical foundation for microbial safety control on the CSS and other manned spacecraft in the future.

Results

Counting of cultivable microorganisms

The number of colony-forming units (CFU) per 100 cm^2 was calculated from colony counts on spread plates. The SZ-12, SZ-13, and SZ-14 flight samples contained 0–8.53×10^6 CFU/100 cm^2, 6.67×10^2 to 2.75×10^5 CFU/100 cm^2, and 0–2.83×10^9 CFU/100 cm^2 of total cultivable bacteria, respectively, and 0–1.62×10^5 CFU/100 cm^2, 0–1.53×10^4 CFU/100 cm^2, and 0–1.01×10^8 CFU/100 cm^2 of total cultivable fungi, respectively. Spread plate counts were used to determine the numbers of microorganisms in the work, sleeping, and sanitary areas obtained using Luria–Bertani (LB) and potato dextrose agar (PDA) during three flight missions (Fig. 1a). We also calculated the average number of cultivable microorganisms in each flight mission in the three regions (Fig. 1b). Contact plate experiments were conducted on SZ-13 and SZ-14 flight samples. The results showed that the numbers of cultivable bacteria in SZ-13 and SZ-14 samples were 0–20 CFU/100 cm^2 and 0–2.35×10^2 CFU/100 cm^2, respectively, and the numbers of cultivable fungi were 0–2.52×10^2 CFU/100 cm^2 and 0–28 CFU/100 cm^2, respectively. All culture count results are shown in Supplementary Table 1.

Identification of cultivable microorganisms

The 130 microorganisms isolated from all samples comprised 103 bacteria and 27 fungi, with 25 bacterial and 18 fungal genera. The phylogenetic trees of cultivable bacteria and fungi are shown in Figs. 2 and 3, respectively. The dominant bacterial genera were *Bacillus* and *Staphylococcus*, accounting for 45.63% and 14.56% of the total bacterial species, respectively. The dominant fungal genera were *Aspergillus*, *Cladosporium*, and *Penicillium*, accounting for 18.52%, 14.81%, and 7.41% of the total fungal species. The accession numbers for the 16S rRNA and internal transcribed spacer (ITS) sequences are shown in Supplementary Table 2. Three genera of bacteria — *Bacillus*, *Pantoea*, and *Pseudomonas* — were

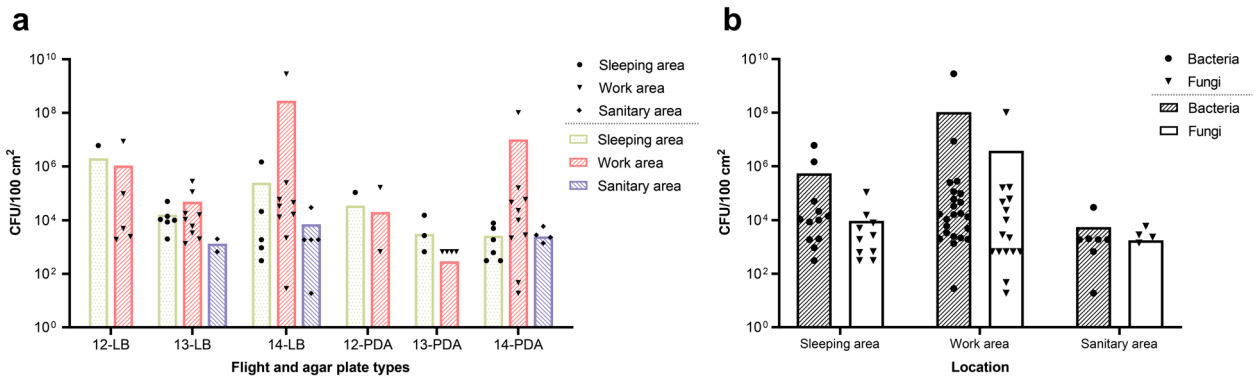

Fig. 1 Numbersof microorganisms obtained using spread plate cultivation. The scatter in the graph represents the number of microorganisms belonging to each of the samples in this grouping. Some samples with a microbial count of 0 cannot be shown (refer to supplementary Table 1) and were counted in the calculation of the mean value. **a** Numbers of microorganisms in the work, sleeping, and sanitary areas of the three flight missions obtained using LB and PDA plates. The green columns represent the mean microbial counts in the sleeping area group, the red columns represent the mean microbial counts in the work area group, and the purple columns represent the mean microbial counts in the sanitary area group. **b** Average level of cultivable microorganisms in the three regions during each flight mission. The columns drawn with a slash represent the average number of bacteria in the group, and the white columns represent the average number of fungi in the group

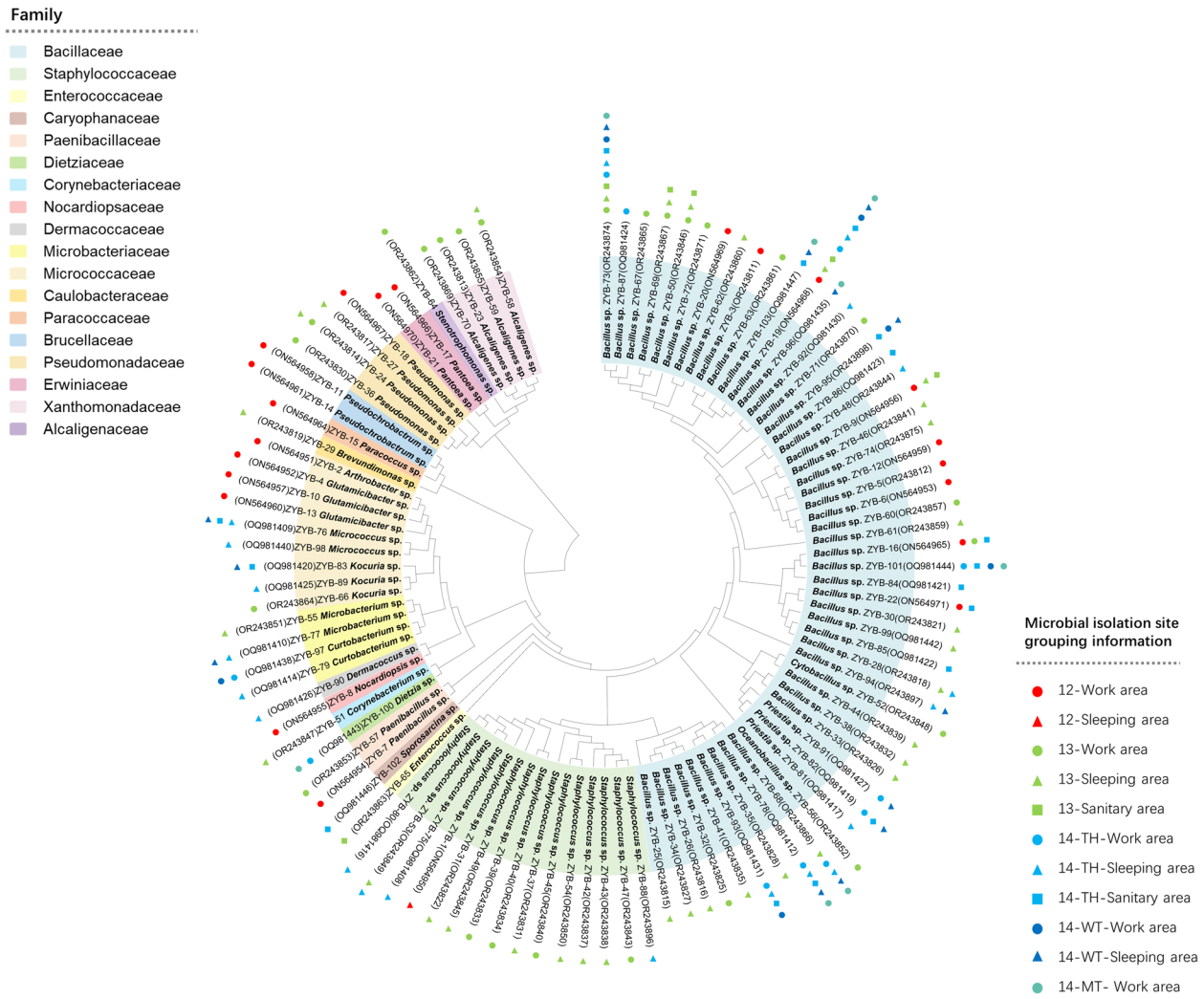

Fig. 2 Phylogenetic tree of culturable bacteria. Individual microorganisms are color-coded by taxonomic family, with 18 families in total. The end of each strain name is labeled with the region of the space station module from which the strain was isolated. There were 11 subgroups: SZ-12 Work area, 12 Sleeping area, 13 Work area, 13 Sleeping area, 13 Sanitary area, 14-TH Work area, 14-TH Sleeping area, 14-TH Sanitary area, 14-WT Work area, 14-WT Sleeping area, and 14-MT Work area. Phylogenetic tree constructed using MEGA software (Version 11.0.13), with the bootstrap replication number set to 1000

obtained from condensate samples using culture-based methods.

Total and viable bacterial counts

Culturable microorganism counting is a simple, easy method that all space stations use to rapidly assess microbial contamination levels on orbit. However, because culturable microorganisms represent a very small percentage of all microorganisms, we also used qPCR to obtain complete information on the total numbers of microorganisms in the samples. For all collected samples, the ITS region was amplified by PCR to detect fungi. But the result showed that no fungi can be detected by PCR.

Figure 4 shows the total and viable bacterial counts in each sample. The average numbers of microorganisms (given in terms of the copy number of rRNA genes) in SZ-12, SZ-13, and SZ-14 samples were 1.11×10^6 copies/100 cm^2, 2.15×10^7 copies/100 cm^2, and 3.26×10^8 copies/100 cm^2, respectively. The average number of bacteria in the three flights showed an upward trend. The average number of microorganisms contaminating the sleeping area was low (9.23×10^6 copies/100 cm^2) compared with that in the sanitary area (6.45×10^8 copies/100 cm^2). Fungi were not detected by qPCR.

The mean numbers of viable bacteria in the SZ-13 and SZ-14 flight samples were 4.99×10^6 copies/100 cm^2 and

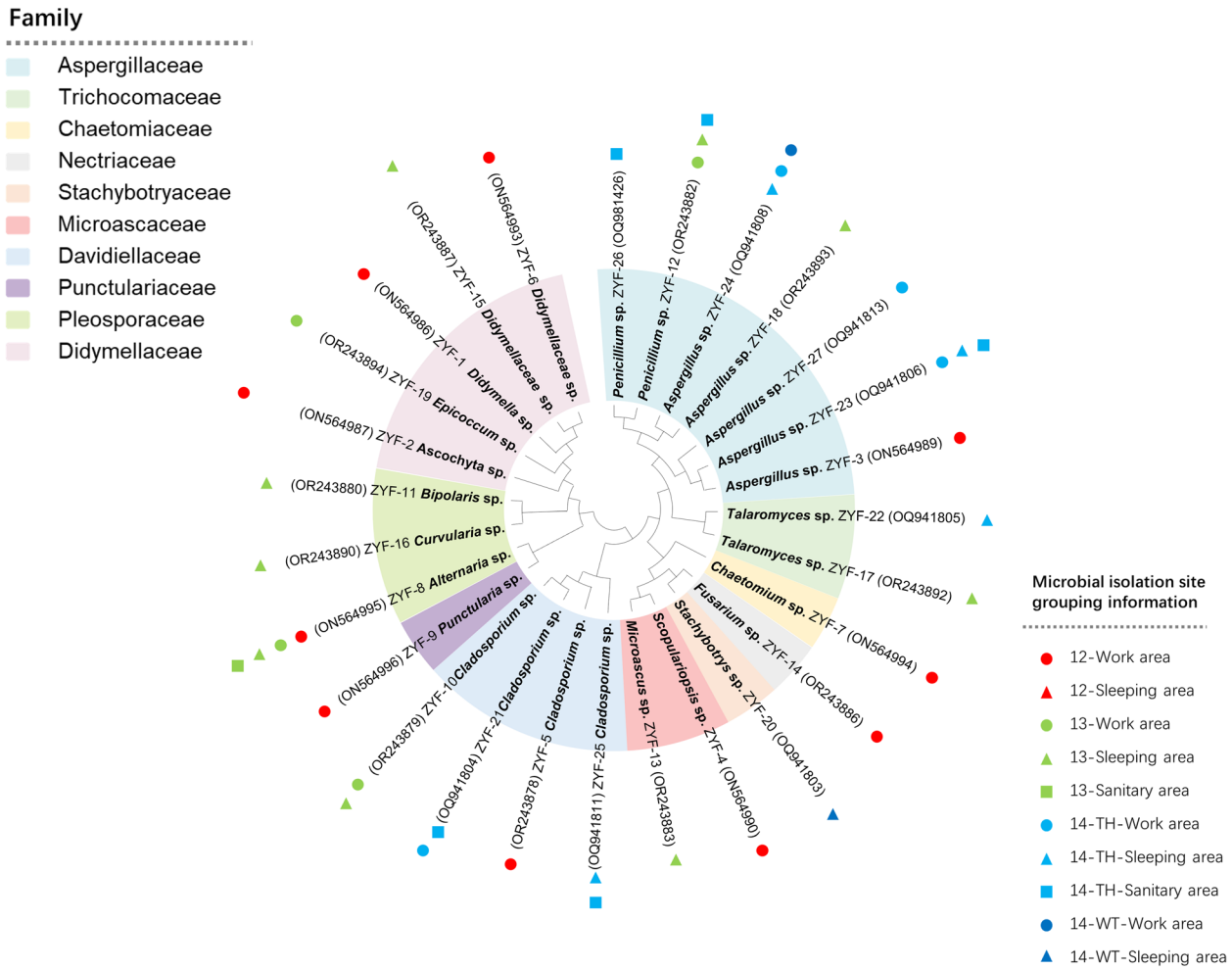

Fig. 3 Phylogenetic tree of culturable fungi. Individual microorganisms are color-coded by taxonomic family level, with 10 families in total. The end of each strain name is labeled with the region of the space station module from which the strain was isolated. There were 11 subgroups: SZ-12 Work area, 12 Sleeping area, 13 Work area, 13 Sleeping area, 13 Sanitary area, 14-TH Work area, 14-TH Sleeping area, 14-TH Sanitary area, 14-WT Work area, 14-WT Sleeping area, and 14-MT Work area. Phylogenetic tree constructed using MEGA software (Version 11.0.13), with the bootstrap replication number set to 1000

1.76×10^8 copies/100 cm^2, respectively. The calculated percentages of viable bacteria in the SZ-13 and SZ-14 flight samples were 60.14% and 69.07%, respectively, revealing that the percentage of surviving bacteria was higher in the SZ-14 flight than in the SZ-13 flight. The average percentage of viable bacteria in all samples was 65.08%, while the mean numbers of viable bacteria in the sleeping, work, and sanitary areas were 7.82×10^6 copies/100 cm^2, 5.94×10^7 copies/100 cm^2, and 3.64×10^8 copies/100 cm^2, respectively. The mean percentages of viable bacteria in these three areas were 68.94%, 59.91%, and 72.49%, respectively. Bacterial survival was found to be lowest in the work area and highest in the sanitary area.

Bacterial sequencing using the Illumina MiSeq system

Bacterial community

The V3–V4 region of the 16S rRNA gene was amplified from the total DNA of all non-propidium monoazide (PMA)-treated bacteria. Twenty-two samples were successfully amplified and sequenced (Table 1). A total of 1,000,027 clean tags with sequence lengths mainly distributed between 400 and 440 bp were obtained, generating 2525 amplicon sequence variants (ASVs). In addition, the sample from the condensate area of SZ-12 flight was successfully amplified to yield 125 ASVs. The ASV and average ASV data within the group corresponding to all samples are shown in Supplementary Table 3. A box plot of the UniFrac distance (Supplementary Fig. 1), a

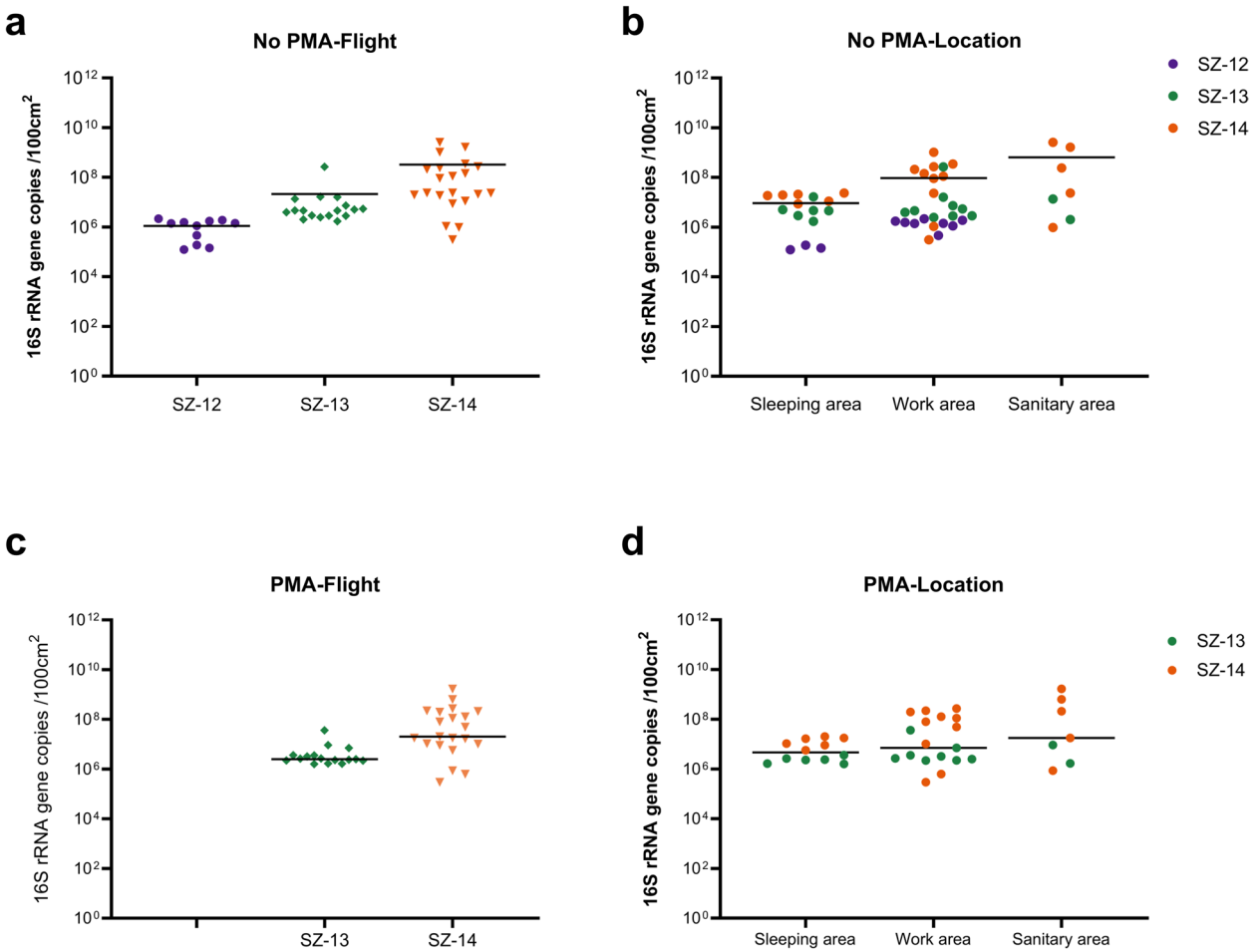

Fig. 4 PCR-based detection ofbacteria in all samples. **a** Total bacterial numbers of the three flight missions (SZ-12, SZ-13, and SZ-14). **b** Total bacterial counts at three sampling sites (Work area, Sleeping area, and Sanitary area). **c** Numbers of total viable bacteria on two flight missions (SZ-13 and SZ-14). **d** Numbers of viable bacteria at three sampling sites (Work area, Sleeping area, and Sanitary area) on SZ-13 and SZ-14

rarefaction curve of 23 samples (Supplementary Fig. 2), a Shannon–Wiener curve of 23 samples (Supplementary Fig. 3), specifications accumulation curves (Supplementary Fig. 4), and rank ambiguity curves (Supplementary Fig. 5) are also provided. The 22 samples were manually divided into seven groups on the basis of the astronauts' activity patterns in different module regions: SZ-12 work area (W_12), SZ-13 sleeping area (S_13), SZ-13 work area (W_13), SZ-14 sanitary area (C_14), SZ-14-TH work area (W_TH_14), SZ-14-WT work area (W_WT_14), and SZ-14-MT work area (W_MT_14).

The bacterial genera and families detected in all of the surface samples are shown in Fig. 5a, b, and c. At the genus level, *Pseudomonas* had the highest proportion, followed by *Stenotrophomonas*, *Methylobacterium-Methylorubrum*, *Sphingomonas*, *Bacillus*, *Staphylococcus*, and *Nocardiopsis*. The most abundant bacteria in the various samples were as follows: SZ-12 work area, *Nocardiopsis* (14.39%), *Staphylococcus* (10.15%), *Lactobacillus* (9.10%), *Paenibacillus* (8.94%), *Muribaculaceae* (8.51%), and *Pantoea* (7.46%); SZ-13 sleeping area, *Pseudomonas* (79.20%), *Ralstonia* (4.27%), *Staphylococcus* (2.35%), *Cutibacterium* (1.33%), *Sphingomonas* (0.90%), and *Streptococcus* (0.76%); SZ-13 work area, *Pseudomonas* (69.06%), *Acinetobacter* (7.89%), *Lysinibacillus* (7.18%), *Ralstonia* (2.11%), *Staphylococcus* (1.70%), and *Lactobacillus* (0.79%); SZ-14 sanitary area, *Sphingomonas* (45.36%), *Bacillus* (39.71%), *Pseudomonas* (1.51%), *Stenotrophomonas* (0.62%), *Blautia* (0.56%), and *Rhodococcus* (0.56%); SZ-14 Tianhe module work area, *Pseudomonas* (40.89%), *Stenotrophomonas* (35.04%), *Curtobacterium* (5.43%), *Brevundimonas* (5.36%), *Bacillus* (1.89%), and *Bacteroides* (1.74%); SZ-14 Wentian module work area, *Methylobacterium-Methylorubrum* (51.77%), *Pseudomonas* (29.41%), *Stenotrophomonas* (8.12%), *Staphylococcus* (2.03%); and SZ-14 Mengtian module work area, *Pseudomonas* (54.13%), *Stenotrophomonas* (17.90%), *Chloroplast* (4.84%), *Acinetobacter* (2.66%), *Bacillus*

Table 1 Sample information

Samples	Flight	Module	Area	Miseq group	Experiment
12–1	SZ-12	Tianhe	Sleeping area	-	Spread plate, qPCR, Illumina MiSeq
12–2	SZ-12	Tianhe	Sleeping area	-	Spread plate, qPCR, Illumina MiSeq
12–3	SZ-12	Tianhe	Sleeping area	-	Spread plate, qPCR, Illumina MiSeq
12–4	SZ-12	Tianhe	Work area	-	Spread plate, qPCR, Illumina MiSeq
12–5	SZ-12	Tianhe	Work area	W_12	Spread plate, qPCR, Illumina MiSeq
12–6	SZ-12	Tianhe	Work area	-	Spread plate, qPCR, Illumina MiSeq
12–7	SZ-12	Tianhe	Work area	-	Spread plate, qPCR, Illumina MiSeq
12–8	SZ-12	Tianhe	Work area	-	Spread plate, qPCR, Illumina MiSeq
12–9	SZ-12	Tianhe	Work area	-	Spread plate, qPCR, Illumina MiSeq
12–10	SZ-12	Tianhe	Work area	W_12	Spread plate, qPCR, Illumina MiSeq
12–11	SZ-12	Tianhe	Work area	-	Spread plate, qPCR, Illumina MiSeq
12–12	SZ-12	Tianhe	Condensate area	-	Spread plate, qPCR, Illumina MiSeq
13–1	SZ-13	Tianhe	Work area	W_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–2	SZ-13	Tianhe	Sleeping area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–3	SZ-13	Tianhe	Sleeping area	S_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–4	SZ-13	Tianhe	Sleeping area	S_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–6	SZ-13	Tianhe	Sleeping area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–7	SZ-13	Tianhe	Sleeping area	S_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–8	SZ-13	Tianhe	Sleeping area	S_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–9	SZ-13	Tianhe	Sanitary area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–10	SZ-13	Tianhe	Work area	W_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–11	SZ-13	Tianhe	Work area	W_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–13	SZ-13	Tianhe	Work area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–14	SZ-13	Tianhe	Sanitary area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–16	SZ-13	Tianhe	Work area	W_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–18	SZ-13	Tianhe	Work area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–19	SZ-13	Tianhe	Work area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–20	SZ-13	Tianhe	Work area	W_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
13–21	SZ-13	Tianhe	Work area	W_13	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–1	SZ-14	Tianhe	Sleeping area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–2	SZ-14	Wentian	Work area	W_WT_14	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–3	SZ-14	Tianhe	Sleeping area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–4	SZ-14	Tianhe	Sleeping area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–5	SZ-14	Wentian	Sleeping area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–6	SZ-14	Tianhe	Sleeping area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–7	SZ-14	Wentian	Sleeping area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–8	SZ-14	Tianhe	Work area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–9	SZ-14	Tianhe	Work area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–10	SZ-14	Tianhe	Sanitary area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–11	SZ-14	Tianhe	Sanitary area	C_14	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–12	SZ-14	Tianhe	Sanitary area	C_14	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–13	SZ-14	Tianhe	Sanitary area	C_14	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–14	SZ-14	Tianhe	Work area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–15	SZ-14	Mengtian	Work area	W_MT_14	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–16	SZ-14	Mengtian	Work area	W_MT_14	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–17	SZ-14	Tianhe	Sanitary area	-	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–18	SZ-14	Tianhe	Work area	W_TH_14	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–19	SZ-14	Wentian	Work area	W_WT_14	Contact plate, Spread plate, qPCR, Illumina MiSeq
14–20	SZ-14	Wentian	Work area	W_WT_14	Contact plate, Spread plate, qPCR, Illumina MiSeq

Table 1 (continued)

Samples	Flight	Module	Area	Miseq group	Experiment
14–21	SZ-14	Tianhe	Work area	W_TH_14	Contact plate, Spread plate, qPCR, Illumina MiSeq

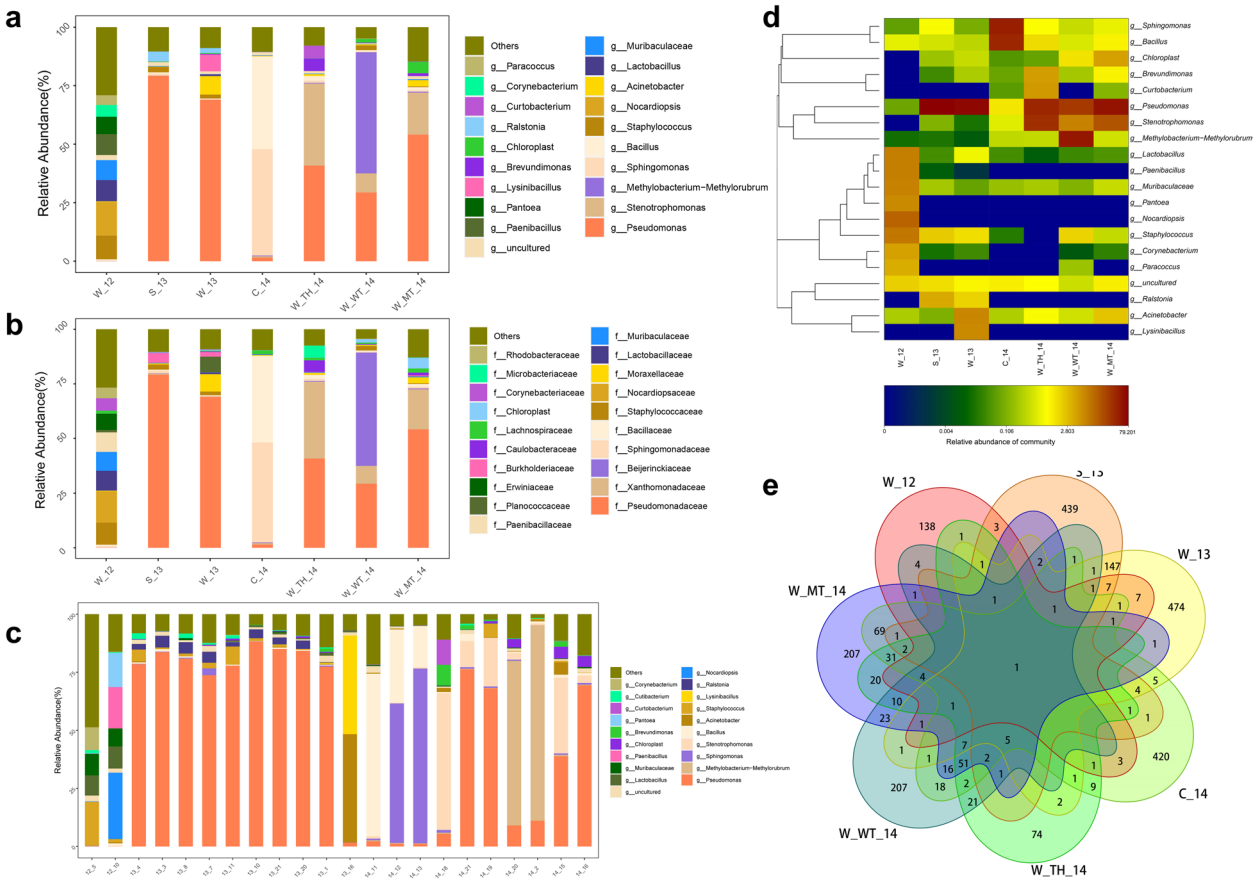

Fig. 5 Bacterial community composition analysis results. **a**, **b** Histograms of each group of samples of genus level (**a**) and family level (**b**) analysis. **c** Histograms of each sample of genus level. **d** Heat map analysis of the top 20 most abundant ASVs. **e** Venn diagram showing the number of unique and shared ASVs for each group

(1.24%), and *Brevundimonas* (1.23%). Figure 5c illustrates the diversity characteristics of each location.

The heatmap in Fig. 5d shows the top 20 most abundant microbial genera across all samples. *Pseudomonas* had high abundance and was detectable in samples from all three flight missions. It had the highest proportions of any microbe in SZ-13 and SZ-14 flight samples, averaging 74.13% and 31.49%, respectively. ASVs specific to each group of samples accounted for 79.80% of the total ASVs (Fig. 5e). Only one ASV, belonging to the genus *Lactobacillus*, was common to all seven groups, suggesting that this bacterial genus was present over time and across different regions of the modules. For the analysis of the single condensate sample collected from SZ-12, we constructed a genus-level evolutionary tree (Fig. 6a) and a Krona species display diagram (Fig. 6b). A total of 41 bacterial genera were detected, with the vast majority belonging to the three phyla: Actinobacteriota, Firmicutes, and Proteobacteria. The dominant genera were *Pantoea*, *Nocardiopsis*, and *Paenibacillus*.

Beta diversity

Figure 6c shows the results of Principal Component Analysis (PCA) of CSS samples from the three flights. Clustering was observed in all three batches of samples, with the SZ-12 sample clusters circled in red ($P<0.05$), the SZ-13 sample clusters (which were the most concentrated) in yellow ($P<0.05$), and the SZ-14 sample clusters

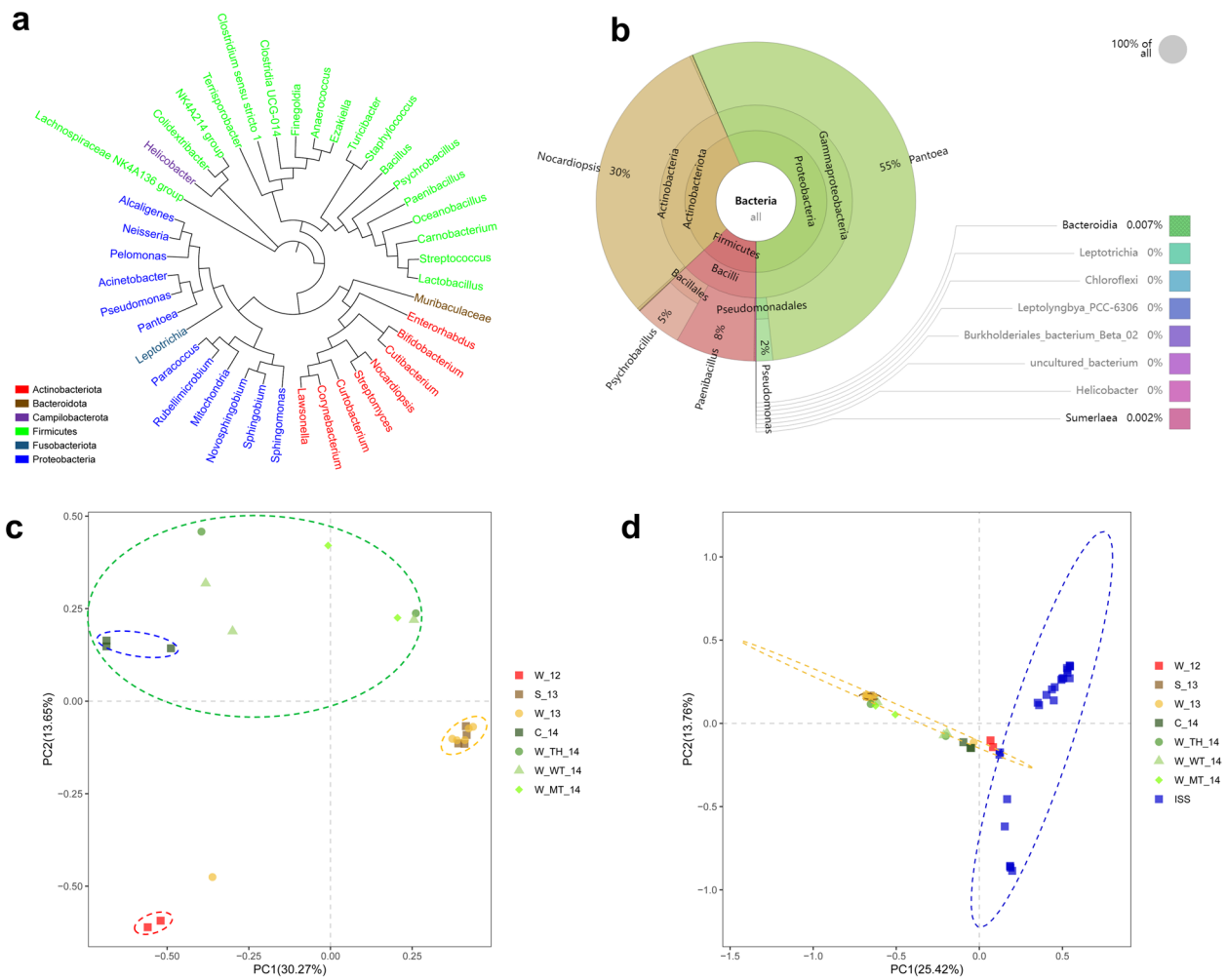

Fig. 6 β diversity analyseis and bacterial community composition analysis of condensate water. **a** Genus-level phylogenetic tree of microorganisms within the condensate samples. **b** Schematic representation of microbial clonal species in the condensate samples. The circles represent the different taxonomic levels from the inside out. The size of each sector represents the relative abundance of the different species. **c** PCA of the seven sample groups (999 permutations; significance threshold, $P < 0.05$). The red, yellow, and green circles represent the SZ-12, SZ-13, and SZ-14 sample clusters, respectively. The blue circle represents the SZ-14 sample cluster collected from the sanitary area. **d** Comparison of PCA of the microbial compositions of CSS and ISS samples (999 permutations; significance threshold, $P < 0.05$). The yellow circle represents SZ-12 to SZ-14 sample clusters collected from the CSS, and the blue circle represents sample clusters collected from the ISS

in green ($P < 0.05$) (Fig. 6c). This indicated that the three flight batches showed significant differences in community structure. The differences among SZ-12 samples were relatively small. Similarly, the clustering relationship of SZ-13 samples was close to that of SZ-12, and the microbial compositions of samples collected from the work and sleeping areas were very closely clustered. The clustering forms of each sample in SZ-14 were relatively loose compared with those in SZ-12 and SZ-13. It is worth noting that the samples in the SZ-14 group showed obvious regional specificity, which was reflected by an individual cluster of the three sanitary area samples (blue circle in Fig. 6c; $P < 0.05$). PCA comparing the CSS and ISS revealed clusters of CSS samples (yellow circle; $P < 0.05$) and ISS samples (blue circle; $P < 0.05$) (Fig. 6d). Data from an analysis of similarities (ANOSIM) test between groups using the Bray–Curtis distances were supportive of the aforementioned results, illustrating that microbial diversity was very different between the two on-orbit space station modules ($P < 0.05$) (Supplementary Table 4).

Alpha diversity

We used the observed species, phylogenetic diversity (PD)_whole_tree, Shannon, and Simpson indexes to describe the alpha diversity in each group of samples

from 3 flights. From the perspective of location, the observed species index of the SZ-12 work area was very low (Fig. 7a). In contrast, while the Simpson index of the SZ-12 workspace was very high (Fig. 7d), indicating a low number of species, the distribution was even, with no dominant population. The remaining six groups of samples exhibited dominant microbial populations.

SourceTracker analysis

Analysis using the SourceTracker algorithm revealed that most of the bacteria in SZ-14 (44.74%) were derived from SZ-13, with a smaller amount (12.67%) from SZ-12, and the remainder (42.59%) coming later, as shown in Fig. 8a. SZ-14 microorganisms from SZ-13 mainly originated from the oral cavity (47.21%) and skin (12.69%), with the remaining 40.10% from unknown sources. Those from SZ-12 included mostly oral (47.05%) and skin (12.65%) microorganisms, with the remaining 40.30% from unknown sources. Microorganisms not originating from SZ-12 or SZ-13 were of three main environmental types — oral (0.27%), skin (57.11%), and soil (0.09%) — with 42.54% from unknown sources. No microorganisms originating from the gut were found. Overall, up to 58.80% of module microorganisms were from the human mouth and skin.

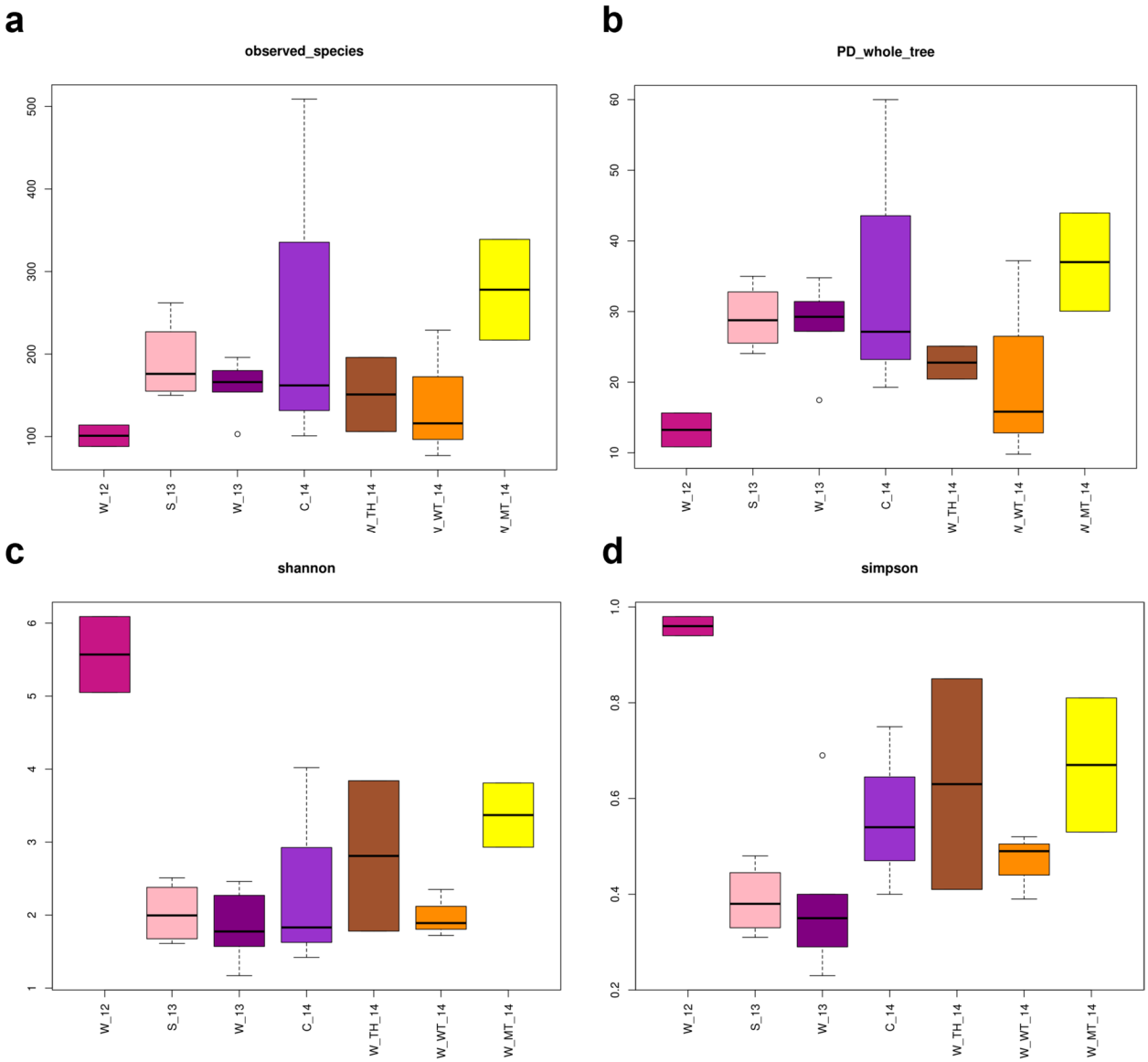

Fig. 7 Bacterial alpha diversity. **a** Observed_species; **b** PD whole tree; **c** Shannon index; **d** Simpson index

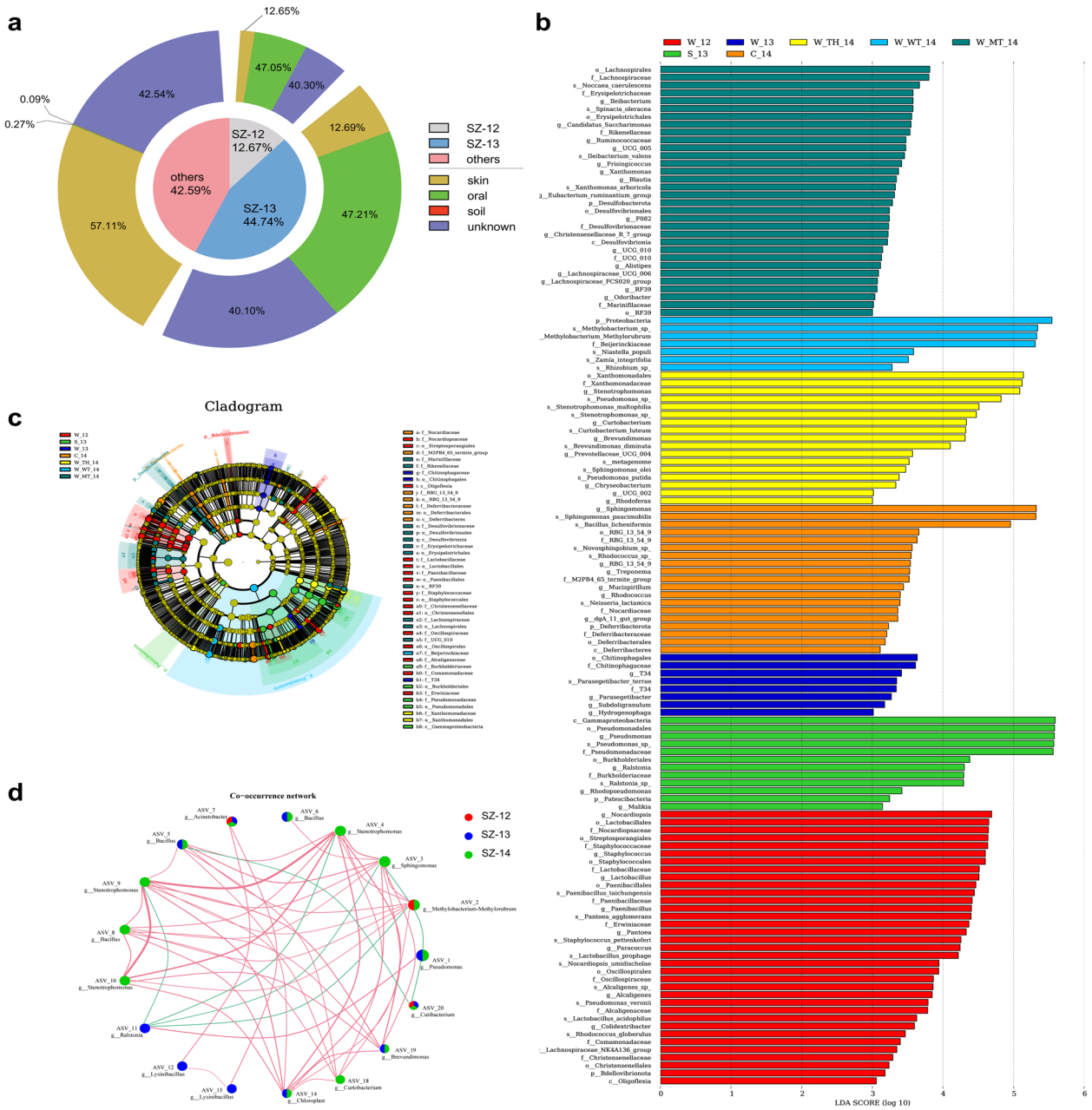

Fig. 8 Bacterial sources, interrelationships, and biomarker bacteria in the different module areas. **a** Results of SourceTracker analysis. The inner circle shows the proportion of microorganisms in SZ-14 that came from SZ-12, SZ-13, and other sources, while the outer circle shows the proportions of microorganisms originating from skin, oral, soil, and unknown environments in each area. **b** LefSe analysis, showing the differences in bacteria among the seven sample groups, using a non-parametric Kruskal–Wallis test threshold of 0.05, a Wilcoxon rank sum test threshold of 0.05, and an LDA score threshold set to 3. **c** Cladogram illustrating the number and phylogenetic status of individual biomarker bacteria. **d** Co-occurrence network diagram. The red line in the graph represents a positive correlation and the green line represents a negative correlation

Linear discriminant analysis (LDA) effect size (LEfSe)

The results of LEfSe performed on the seven groups of samples are shown in Fig. 8b and c. This figure illustrates the genus-level biomarker bacteria identified in the following groups: W_TH_14, *Stenotrophomonas*; W_WT_14, *Methylobacterium* and *Methylobacterium-Methylorubrum*; S_13, *Pseudomonas*; C_14, *Sphingomonas* and *Sphingomonas paucimobilis*. All of these bacteria had LDA scores > 5, indicating that they were all significant biomarkers of the respective source areas.

No biomarkers with LDA scores > 5 were identified in the W_12, W_13, and W_MT_14 groups.

Co-occurrence of microbial taxa

A co-occurrence network graph based on the relationships among the top 20 most abundant ASVs was drawn, with the red line representing a positive correlation and the green line representing a negative correlation (Fig. 8d). This figure illustrates that ASV_1 had the highest abundance and belonged to the *Pseudomonas* genus. It was only positively correlated with ASV_11, ASV_4, ASV_9, and ASV_10, all of which belonged to the genus *Stenotrophomonas* and exhibited additional positive correlations with other ASVs. The network *P* and *r* values can be found in Supplementary Tables 5 and 6, respectively.

Discussion

Our cultivation results showed that the number of culturable microorganisms was very unevenly distributed in the CSS. Furthermore, the number of microorganisms obtained by the spread plate method was higher than that obtained by the contact plate method, as evidenced by the respective orders of magnitude of the results: specifically, ranges of 0–252 CFU/100 cm^2 for contact plates and 0–2.83×10^9 CFU/100 cm^2 for spread plates. The average numbers of bacteria and fungi obtained from contact plate culture were 16.52 CFU/100 cm^2 and 10.67 CFU/100 cm^2, respectively, compared with 5.82×10^7 CFU/100 cm^2 and 2.08×10^6 CFU/100 cm^2, respectively, from spread plate culture. Because of its ease of operation, the contact plate method has been a commonly used tool for the detection of on-orbit microbial counts at the MIR and ISS. However, the microbial counts obtained from the contact analysis are much smaller than the true values, possibly because of the gentle external force used to press on the contact plates, resulting in the collection of a small number of microorganisms from each sample. In addition, because the contact area is small and the colonies may not be uniformly distributed on the culture medium, the colonies are prone to grow into a lawn, resulting in observational errors and failing to reflect the true level of contamination. For the spread plate method in this study, we used a cotton swab to rub the surface of each equivalent material sheet sample, enabling the collection of more microorganisms; after 20 min of full elution and shaking, the collected microorganisms were sufficiently released from the swab. The results obtained from spread plate culture of microbial samples collected from the ISS showed an average of 7×10^5 bacterial CFU/m^2 and a maximum of 7×10^5 CFU/m^2 of fungi [31]. It was reported that the numbers of culturable bacteria and fungi inside the MIR exhibit fluctuations depending on the mission, with bacterial and fungal counts ranging from approximately 10–10^7 CFU/m^3 and 10^2–10^7 CFU/m^3, respectively [19].

It is worth mentioning that the sampling method we used for the analysis of microorganism contamination in the CSS is very different from that used in the MIR and the ISS. The sampling method used in previous studies of microbial contamination in the ISS and MIR was to stick or wipe the module surface directly with contact plates, cotton swabs, or wet wipes, thus focusing on detecting real-time microbial levels within the module. This study represents the first utilization of equivalent material sheets as samples for the collection and study of microorganisms in orbit. Each equivalent material sheet was adhered to the respective module surface for the duration of each flight, and was not subjected to any cleaning operation. Thus, the equivalent material sheets reflected the accumulated levels of microbial contamination of inaccessible areas throughout the flight for each crew.

The dominant bacterial genera obtained by culture-based methods in this study were *Bacillus* and *Staphylococcus*, and the dominant fungal genera were *Aspergillus*, *Cladosporium*, and *Penicillium*. The most abundant culturable bacteria in the ISS were reportedly *Bacillus* and *Staphylococcus*, and the dominant fungi were *Aspergillus niger*, *Penicillium*, and *Malassezia* spp. [2, 32]. In the MIR module, the dominant genera of culturable bacteria were *Staphylococcus*, *Corynebacterium*, *Bacillus*, and *Micrococcus* [19], and the dominant fungus was *Penicillium chrysogenum* [33]. By comparison, the dominant culturable bacteria and fungi in the CSS were very similar to those in the ISS and MIR: namely, *Bacillus*, *Staphylococcus*, and *Penicillium* were culturable in all three space stations, and *Aspergillus* was the dominant fungus in both the CSS and ISS.

Cultivable microorganisms are considered to account for about 1% of the total number of microorganisms in nature. The dominant bacteria in the orbiting CSS identified through high-throughput sequencing methods comprised seven genera: *Pseudomonas*, *Stenotrophomonas*, *Methylobacterium-Methylorubrum*, *Sphingomonas*, *Bacillus*, *Staphylococcus*, and *Nocardiopsis*. Of these, only *Pseudomonas*, *Stenotrophomonas*, *Bacillus, Staphylococcus*, and *Nocardiopsis* were culturable. This suggested that a large number of non-culturable microorganisms also exist in the orbiting space station.

Two research projects, the Microbial Tracking (MT)−1 [3] and MT-2 [31] studies, were conducted in 2015–2016 and 2017–2018, respectively. During this period, seven in-orbit samples were taken from eight fixed points inside the ISS. The results reported for 2019 and 2022 showed no significant differences in the alpha diversity of microorganisms of all samples collected during the seven

flights, indicating that microbial alpha diversity data do not vary with space and time. This conclusion is not entirely consistent with our experimental results. In our study, the uniformity of microbial species in the SZ-12 samples was significantly higher than that in the subsequent SZ-13 and SZ-14 samples. This indicated that, in the early stage of the construction of the CSS, the number of microorganisms in the module was low and the degree of uniformity was high, with dominant species gradually appearing over time in SZ-13 and SZ-14.

Regarding the beta diversity of the seven samples collected in the ISS module, no apparent differences at the microbiome level were observed between locations. However, there were significant differences in the microbiome between flight groups [31]. This finding is partially consistent with our experimental results, in which the microbial community structure showed a similarly significant difference at the three sampling time points. Interestingly, our SZ-14 sample analysis demonstrated that there was also significant variability between samples taken from different zones at the same time point, as evidenced by the significant difference in microbial diversity between the sanitary zone samples and the work and sleeping zone samples on the same flight. Therefore, we speculate that the microbial composition characteristics of the different zones in the CSS may be related to the work–life behavior patterns of the astronauts in each zone. Finally, a comparison of the PCAs of the CSS and ISS showed that there were also significant differences in the microbial communities on the different space stations.

Although some scholars studying microbial contamination on the ISS have argued that microorganisms in the space environment are not necessarily more threatening than those on the ground [34, 35], the indirect effects of microorganisms on human health and their ability to corrode materials in the ISS environment are well documented and should not be ignored. The 16S rRNA gene sequencing results in this study were not 100% certain at the species level, but did support our hypothesis that the following strains have a high probability of being present in the CSS: *Pseudomonas cedrina*, *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus hominis*, *Aspergillus versicolor*, *Aspergillus flavus*, *Cladosporium halotolerans*, and *Cladosporium anthropophilum*.

P. cedrina is easy to grow and forms biofilms on non-biological surfaces [36]. *B. cereus* can proliferate in pre-packaged refrigerated foods to cause foodborne diseases via the production of enterotoxins. Microbial spores can tolerate extreme environments, and the spores of *B. cereus* are more hydrophobic than those of any other *Bacillus* sp., making them easier to adhere to surfaces [37]. *S. aureus* is a conditionally pathogenic bacterium that normally colonizes the skin surface, oropharynx, and upper respiratory tract of the human body and can cause a wide range of infections. It is resistant to antibiotics and has a strong ability to form biofilms [38]. *A. versicolor* produces spore proteins that cause allergies in humans [39], and can survive at high temperatures and produce aflatoxins [40]. *Cladosporium* is a major pathogen of plant and animal diseases that is usually isolated from soil, food, textiles, and other organic matter [41]. It is worthwhile to conduct future research to develop effective methods for eliminating these microorganisms.

The Venn diagram showed that one of the ASVs present in all groups was a bacterium of the genus *Lactobacillus*, which is found in many parts of the human body, playing important roles in the microbiota of the human digestive system and the female reproductive system [42]. *Lactobacillus* form biofilms in the vaginal and intestinal microbiota [43] that enable it to maintain population dominance [44], which is crucial for human health [45]. Additionally, *Lactobacillus* can be sourced from fermented foods, dairy products, fermented vegetables, and meat products [46]. Thus, it can be inferred that the prevalence of *Lactobacillus* in the CSS is related to the astronauts. So far, there have been no reports of *Lactobacillus* bacteria posing a threat to space stations or astronauts in orbit. Based on the above considerations, we may consider not completely removing it from the CSS.

The co-occurrence network diagram showed that *Stenotrophomonas* bacteria were co-cultivated with 24.07% of the microorganisms in the CSS environment, indicating that inhibiting the growth of *Stenotrophomonas* may also inhibit the growth of the others. *Stenotrophomonas* bacteria are ubiquitous in the environment, mainly found in the soil and with plants, and some species have the potential to infect humans or contaminate water bodies [47].

The biomarker bacteria of group W_TH_14 was *Stenotrophomonas* sp. The genus *Stenotropiomonas* is also mainly present in plants and soil but does not have plant pathogenicity. *Stenotropiomonas maltophilia* is considered the most important species, benefiting plant growth and health, but may cause human infection and exhibits extensive drug resistance [47]. Plants that have been cultured in the CSS module include *Arabidopsis*, tomatoes, rice, and cabbage. Microorganisms closely related to plants have become biomarkers of the work area of the Tianhe core module, indicating the need to pay high attention to the prevention and control of microorganisms carried by plant sources in the in-orbit experimental plans. The biomarkers of group W_WT_14 were *Methylobacterium* sp. and *Methylobacterium-Methylorubrum*. A notable feature of *Methylobacterium*, which is commonly found in the atmosphere, soil, and phylloplane,

is the ability to utilize a single carbon substrate as the sole source of carbon and energy [48]. The emergence of biomarker bacteria in the Wentian module work area is sufficient to demonstrate the ability of in-orbit microorganisms to tolerate nutrient-poor environments. The biomarker of group S_13 was *Pseudomonas* sp. *Pseudomonas* is found in a variety of environments, exhibiting diverse metabolic and environmental adaptability [49]. It was the dominant population in the sleeping area of the Tianhe core module of SZ-13, and was also widely present throughout the entire CSS. The biomarker of group C_14 was *Sphingomonas* sp. *Sphingomonas* often coexists with plants and is an opportunistic pathogen that can easily corrode drinking water pipelines made of copper [50]. *Sphingomonas* appeared in the sanitary area inside the Tianhe core module during SZ-14, and its pathogenicity and corrosion abilities are worthy of attention in future research.

The condensate area, which has high humidity and is thus prone to microbial outbreaks, has been a priority area for microbial prevention and control in the space stations of various countries. In this study, we collected samples from the on-orbit condensate area of the CSS for the first time and provided a comprehensive analysis of the microbial diversity. Microorganisms of the genus *Pantoea,* as the most abundant microorganism in the condensate samples, are usually derived from soil and water bodies [51]. Microorganisms of the genus *Nocardiopsis,* as the second most abundant microorganism in the condensate samples, are characterized by tolerance to salt, alkaline, and desiccation conditions [52]. Because *Pantoea* has been cultured, a method to inhibit the propagation of this microorganism could be a valuable contribution to the microbial prevention and control of condensation water.

During the orbiting period of the SZ-14 flight crew, two experimental modules were successively docked with the core module. These two experimental modules carried a large amount of experimental cargo into orbit, theoretically creating a very high likelihood of introducing microorganisms from the new cargo into the SZ-14 samples. Nevertheless, our SourceTracker analysis showed that up to 57.41% of the bacteria in SZ-14 originated from SZ-13 and SZ-12. In addition, the microorganisms originating from human oral and skin environments accounted for up to 58.80% of the microorganisms in the SZ-14 samples. This is similar to results found in the "Home Microbiome Project" which found that the home microbiome was largely sourced from humans [53]. The proportion of microorganisms originating from soil was much lower, and microorganisms from the gut were completely absent. This finding suggested that the microorganisms in the cabin were closely related to human skin contact and the exchange of saliva droplets during the astronauts' conversations. Any untraceable microorganisms may be attributable to differences in microorganisms carried by individual astronauts or by bottlenecks in the analytical methods.

The special structures of space station modules feature many inaccessible areas under the module panels, behind the instruments, in the crevices of various circuit structures, and other spaces, where the accumulation of pathogenic and corrosive microorganisms could ultimately lead to the deterioration of the module environment. Contamination by human-source microorganisms is inevitable, but plant-source microorganisms should be targeted for key prevention and control. Meanwhile, future research should be dedicated to developing new microbial detection devices with ultra-high sensitivity and ultra-rapid results in orbit, as well as surface coating materials for the spacecraft cabin structure that offer long-term active sterilization and self-cleaning capabilities. Such work will be of great practical significance for the prevention and control of microbial contamination of the CSS.

Methods

Sample collection and sampling sites

The equivalent material sheet used for sampling comprised a membrane made of the same material as the wall of the module at the sampling site, with a size of 5 cm × 5 cm (25 cm^2). Specifically, the material composition of the two types of equivalent material sheets is as follows: composite hard material mainly made of polytetrafluoroethylene, and Metas soft fabric woven with chitosan. For sampling, the crew astronauts pasted the equivalent material sheet on the surface of a designated module area on the day of entry, then removed each sheet to a sterile sealed bag 1 to 2 days before the astronauts were due to return to the Earth's surface. The equivalent material sheets descended to the ground with the manned spacecraft and were brought back to the laboratory within 24 h after descent. The equivalent material sheets were kept at room temperature during transportation, without any sterilization. Upon arrival at the ground laboratory, sample processing and microbiological analyses were carried out within 12 h.

The microbial samples in this study were collected from the indicated sampling sites in the Tianhe core module (work, sleeping, and sanitary areas), Wentian experimental module (work and sleeping areas), and Mengtian experimental module (work area) of the CSS. The sample collection sites and other information are shown in Fig. 9 and Table 1, and the sample collection times are provided in Fig. 10. All three batches of samples were collected from three separate SZ-12, SZ-13,

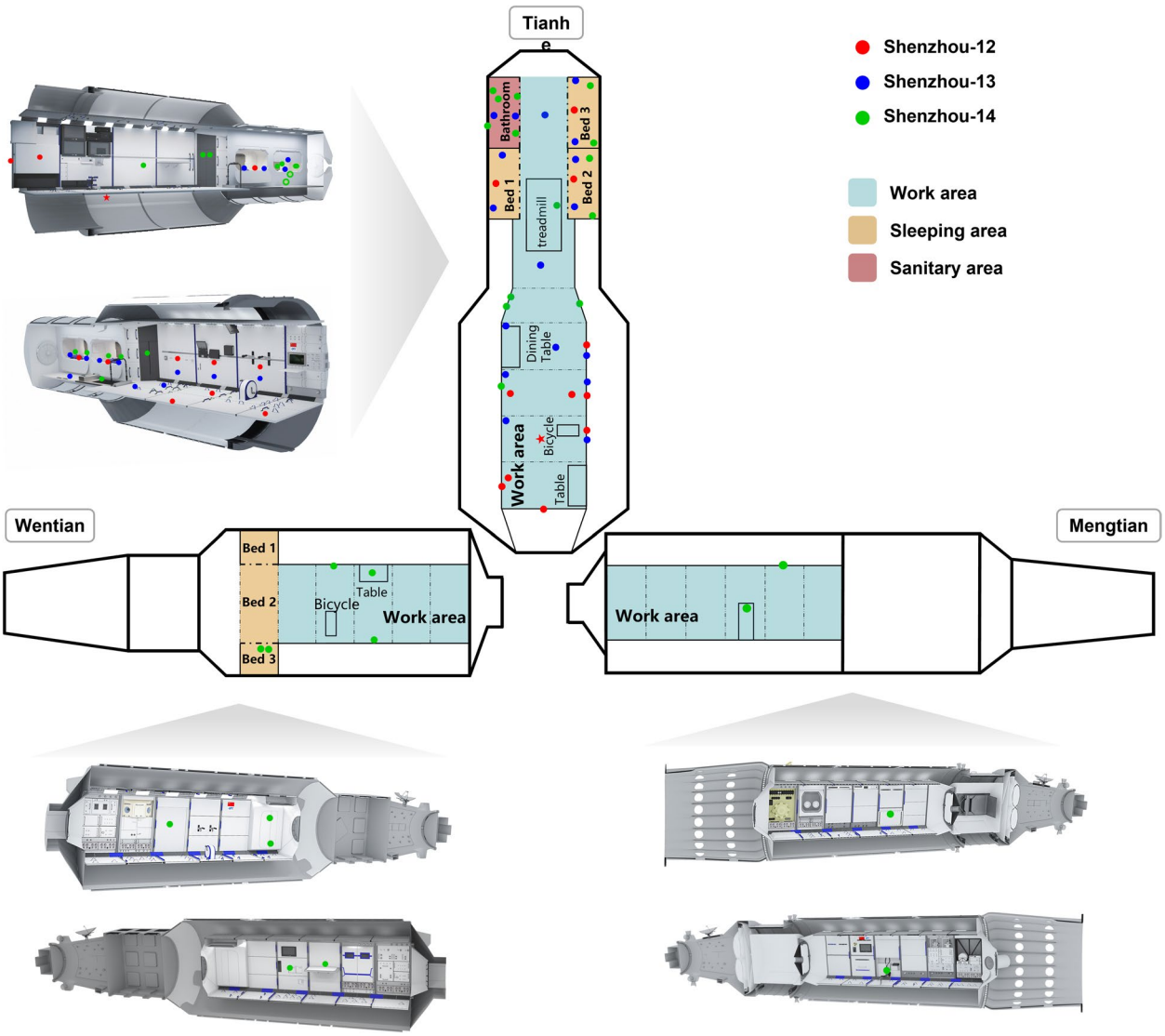

Fig. 9 Schematic illustration of the distribution of sampling sites. The sampling sites for the SZ-12, SZ-13, and SZ-14 flights are labeled as follows: red dots for SZ-12 (with red pentagrams indicating the sampling sites in the condensate region), blue dots for SZ-13, and green dots for SZ-14

and SZ-14, and were placed in orbit for three months, six months, and six months, respectively. For Sampling 1, samples were collected from 12 sites in the Tianhe core module (8 from the work area, 3 from the sleeping area, and 1 from the condensate area). The SZ-12 flight crew entered the on-orbit CSS on 2021/06/17 and pasted equivalent material sheets onto the surface of the module walls. After 90 days (2021/09/15), the sheets were removed and placed in sealed bags, which were brought back to the ground by the SZ-12 human-crewed spacecraft. For Sampling 2, samples were collected from 17 sites in the Tianhe core module (9 from the work area, 6 from the sleeping area, and 2 from the sanitary area). The flight crew entered the on-orbit CSS on 2021/10/16 and pasted equivalent material sheets onto the surface of the module walls. After 181 days (2022/04/15), the sheets were removed and placed in sealed bags, which were brought back to the ground by the SZ-13 human-crewed spacecraft. For Sampling 3, samples were collected from 14 sites in the Tianhe core module (5 from the work area, 4 from the sleeping area, and 5 from the sanitary area), five sites in the Wentian experimental module (3 from the work area and 2 from the sleeping area), and two sites in the Mengtian experimental module (all from the work area). The SZ-14 flight crew entered the on-orbit CSS on 2022/06/05, pasted equivalent material sheets onto the surface of the Tianhe core module walls, and removed them after 181 days (2022/12/03). Similarly, equivalent

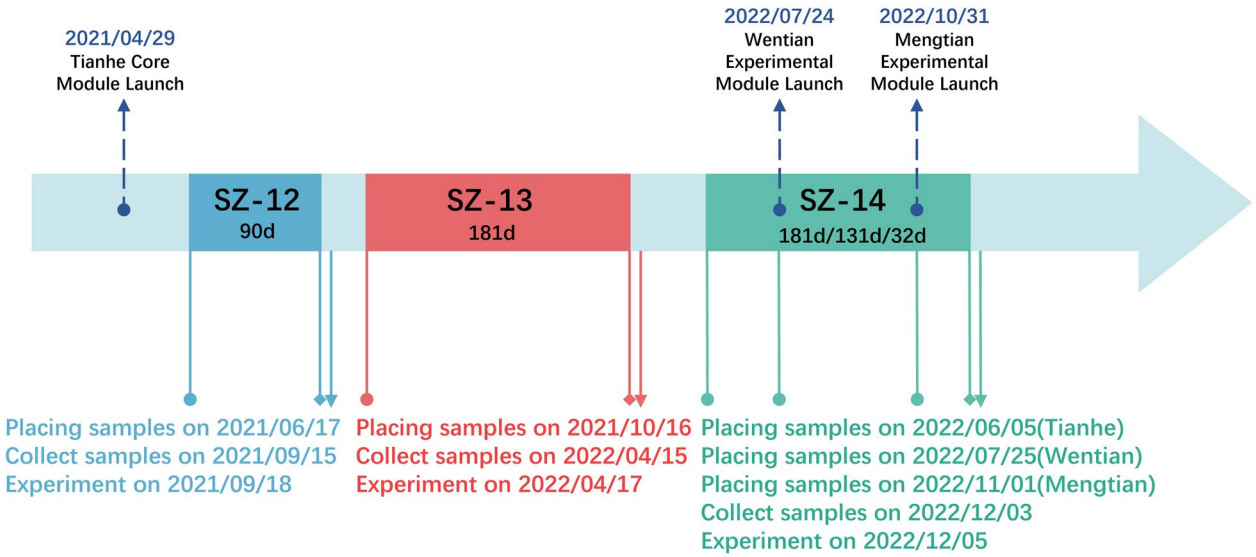

Fig. 10 Schematic timeline of sample collectionduring the SZ-12, SZ-13, and SZ-14 flights. Timeline of the placement of equivalent material sheets, recovery of sample sheets, and arrival at the ground laboratory for subsequent experiments

material sheets were pasted on the wall surfaces of the two areas on the respective days of the docking of the Wentian and Mengtian experimental modules, and were left for 131 days and 32 days, respectively, before being removed. All of the above samples were brought back to the ground by the SZ-14 manned spacecraft.

Sample processing

The sample processing procedure is illustrated in Fig. 11. Sample processing was carried out at an ultra-clean bench in the ground laboratory. First, the equivalent material sheets were pressed onto a contact plate with agar medium, placed into a tube containing sterile phosphate-buffered saline (PBS) solution, vortexed for 20 min, and spun dry by using a mesh liner tube at low-speed centrifugation (3500 rpm/min for 10 min), and finally removed after centrifugation. This yielded a total of 50 tubes of microbial eluates. A portion of these eluates was immediately used as samples for microbial culture. The remaining samples were placed in a refrigerator at 4 °C for subsequent nucleic acid analysis conducted within 24 h.

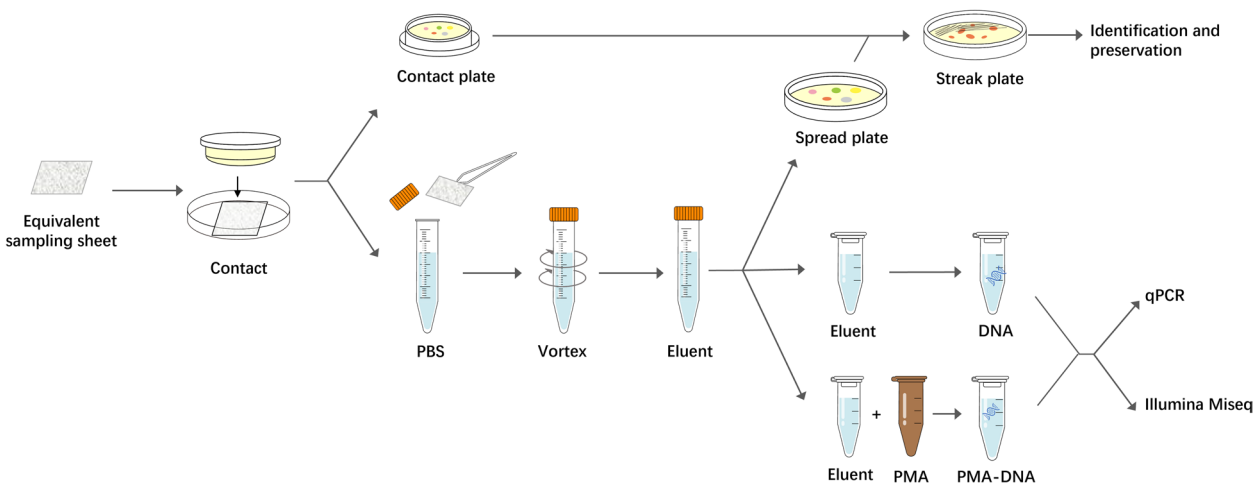

Fig. 11 Sample processing flowchart

Cultivation and counting

For contact plate culture of samples collected and brought back to Earth by the SZ-13 and SZ-14 crews, equivalent material sheets were attached to contact plates containing both LB agar (Cat. No. 22700025, Invitrogen, Carlsbad, CA. USA) and PDA (Cat. No: CM139, Oxford, UK). For spread plate culture, all sample eluents were cultured on LB agar and PDA plates. LB agar plates were cultured at 37 ℃, and colonies were counted at 48 h. PDA plates were cultured at 28 ℃, and colonies were counted at 120 h. The number of microbial CFUs per 100 cm^2 was calculated from the plate culture count results. The data were graphically represented using GraphPad Prism (Version 9.5.1).

Gene sequencing and identification

After colony counting, single colonies were re-streaked on fresh plates to ensure the purity of the culture, and incubated at the appropriate temperature for an appropriate amount of time [31]. DNA was extracted from pure cultures of bacteria and fungi. Bacterial DNA extraction was performed using the TIANamp Bacteria DNA Kit (Cat. No. DP302-02, TIANGEN, Tianjin, China), following the manufacturer's recommended instructions except that 50 μL of molecular grade water (pH 7.0) was used instead of TE buffer in the final wash step for nucleic acids. The extracted bacterial DNA (~ 100 ng/μL; optical density $[OD]_{260}/OD_{280}$ ratio = 1.80–1.96) samples served as a template for subsequent PCR amplification of the bacterial 16S rRNA gene using the 27F and 1495R primers [54]. For fungi, approximately 5 mg (wet weight) of purified fungal cells were initially frozen in liquid nitrogen, followed by homogenization in a ball mill. Fungal genomic DNA was extracted using the DNAsecure Plant Kit (Cat. No. DP320-02, TIANGEN, Tianjin, China), following the manufacturer's instructions. In the final step, 50 μL of molecular grade water (pH 7.0) was used to wash the nucleic acids. The purified DNA sample was utilized for subsequent molecular operations, employing the ITS1 and ITS4 primers [55] to amplify the ITS region between the small and large rRNA subunit genes. After successful amplification of the bacterial and fungal rRNA regions, Sanger sequencing was performed using the ABI3730XL instrument. MEGA software (Version 11.0.13) was utilized for multiple data alignments by CLUSTALW [56], followed by the neighbor-joining method to construct a phylogenetic tree, with the number of bootstrap replications set to 1000. The accession numbers of the bacterial 16S rRNA and fungal ITS sequences are shown in Figs. 2 and 3, respectively.

PMA treatment and DNA extraction

The remaining microbial eluate was divided equally into two portions, transferred to two sterile centrifuge tubes, and centrifuged at 12,000 rpm for 5 min. The supernatant was then removed to obtain a bacterial precipitate. One tube was left untreated, and the other was treated with PMA as follows: 200 μL of 50 μM PMA was added to the centrifuge tube, pipetted up and down to mix well, incubated in the dark for 5 min at room temperature, exposed to light for 15 min, and centrifuged at 12,000 rpm for 5 min. The supernatant was discarded, and the precipitate was washed thrice with sterile PBS buffer and thrice with sterile water to obtain the PMA-treated bacterial precipitate [57]. Only the SZ-12 samples here were not subjected to PMA treatment due to low biomass.

Subsequent DNA extractions using both of the eluate samples were performed by using the E.Z.N.A. Soil DNA Kit (Cat. No. D5625-01, Omega Biotek, Doraville, GA, USA). We slightly modified the manufacturer's protocol as follows: (1) the first step was changed to utilize a 1.5-mL sterile centrifuge tube instead of a Disruptor Tube; (2) the steps involving the addition of 200 μL cHTR Reagent and subsequent centrifugation were omitted; and (3) in the final step, 50 μL of molecular grade water (pH 7.0) was used instead of elution buffer to wash the nucleic acids. The remaining steps were performed in accordance with the standard instructions provided. Extracted DNA (~ 50 ng/μL, OD_{260}/OD_{280} ratio = 1.80–1.96) was used as template for subsequent qPCR. The DNA extracted from the samples that were not treated with PMA was subjected to MiSeq analysis.

qPCR quantification

The copy number of rRNA genes in all samples was quantified using qPCR on a 7300 ABI real-time PCR system (Applied Biosystems, Foster City, CA, USA). The copy number of rRNA genes in PMA-treated samples represents the number of viable bacteria. Each 25-μL reaction mixture included 1 μL of template DNA, 12.5 μL of 2 × SuperReal PreMix Plus (Cat. No. FP205-02, TIANGEN, Tianjin, China), and primers targeting the V3 region (341F [5′-CCT ACG GGA GGC AGC AG-3′] and 534R [5′-ATT ACC GCG GCT G-3′]) [58], each at a final concentration of 0.2 μM. The melting temperature, specificity, and amplification efficiency of the PCR products were assessed. The optimal qPCR conditions were determined to be an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 35 s. Experiments were conducted using the V3 region of the 16S rRNA gene of a standard substance with known dilution, and standard curves were plotted. Three parallel

experiments were conducted for each data point. All qPCR reactions included a set of template-free negative controls, and the accuracy of qPCR was ensured through linear regression analysis ($r^2 > 0.99$). The results were visualized using GraphPad Prism (Version 9.5.1). The number of 16S rRNA gene copies per 100 cm^2 was calculated according to Formula (1):

$$T = t \times A/a \times B/b \times 100/c \quad (1)$$

T: number of 16S rRNA gene copies per 100 $cm.^2$

t: number of 16S rRNA gene copies calculated from the standard curve.

a: amount of DNA used as template for each reaction.

A: total amount of DNA extracted.

b: amount of eluent used for DNA extraction.

B: total amount of eluent.

c: the square measure of the equivalent material sheet (cm^2).

Illumina MiSeq analysis

The V3–V4 region of the 16S rRNA gene in bacterial samples was amplified and sequenced using pair-end (PE) double-ended sequence data. The Illumina MiSeq raw data have been uploaded to the NCBI website, with the accession numbers provided in Supplementary Table 3. The obtained FASTQ data were split into different samples based on the barcode sequence. Pearl software was used to control the quality of the FASTQ data, removing ambiguous bases and primer mismatch sequences. The sequences were trimmed, and bases with quality values lower than Q20 were removed. The paired-end sequences were merged based on the overlap of the reads, setting the minimum overlap to 10 bp and the P value to 0.0001 to obtain the FASTA sequence. VSEARCH software was employed to identify and remove chimeric FASTA sequences using the UCHIME [59] method based on known databases. For unknown databases, the UCHIME de novo method was used to eliminate chimeras while also removing short sequences that did not meet the requirements. The above steps utilized Pearl (v0.9.6) [60], VSEARCH (v2.13.3) [61], and qc_fasta_v2 (v2.0). The FASTQ data were transformed into raw tags by removing barcode and primer sequences, followed by quality control and concatenation. The raw tags were further transformed into clean tags by removing chimeras and short sequences. Using QIIME (v1.8.0) [62] and VSEARCH (v2.13.3) [61], all clean tags were partitioned into ASVs using the UPARSE clustering [60] and UNOISE3 denoising [63] methods. Singletons were removed from ASV data. Subsequently, the ASVs were subjected to bioinformatic statistical analysis [64]. Based on the ASV clustering results, rarefaction analysis was performed using Mothur (v1.48.0) [65]. Rarefaction curves were generated using R (v3.6.0). The reshape2 and ggplot2 packages of R (v3.6.0) were utilized for species composition analysis and creation of bar charts. Distance matrix calculations and clustering analysis were performed using the Bray–Curtis distance metric and the complete linkage clustering method. Heat maps were generated using the vegan, vegadist, and hclust packages in R (v3.6.0). The number of common and unique ASVs across multiple samples was counted, and analyses were conducted using the R (v3.6.0) Venn package to create Venn diagrams. PCA and plotting were executed by analyzing the composition of different sample ASVs (97% similarity) and calculating the Euclidean distance, using the ade4, ggplot2, and ggrepl packages in R (v3.6.0). Raw data collected from ISS samples [3] were downloaded from the NCBI website and analyzed together with CSS samples using PCA. ANOSIM of the Bray–Curtis distances was performed using the vegan package. Alpha diversity analysis was performed on the dataset using QIIME (v1.8.0) [62]. For individual condensate samples, the QIIME (v1.8.0) MAFFT platform was used for sequence alignment, FastTree [66] was used for tree construction, and KronaTools (v2.6) [67] was used to visualize the classification hierarchy and abundance information of the species.

Using SourceTracker (Version: 0.9.1 [Beta]), we filtered out ASVs that satisfied the criteria of P value > 0.05 and correlation value $|R| < 0.6$. We analyzed the proportion of microorganisms in the SZ-14 samples that originated from previous batches of samples, and the proportion of microorganisms originating from four hypothetical source environments, namely soil [68], skin [69], oral [70], and gut [62], which are considered to be important sources of indoor microorganisms [71]. Traceability analysis results were visualized using GraphPad Prism (Version 9.5.1). LEfSe analysis was conducted using Python (3.8.10), with an LDA score threshold set to > 3. The top 20 ASVs by absolute abundance from all samples were selected for correlation analysis. ASVs that met the criteria of P value > 0.05 and $|R| < 0.6$ were selected, and a co-occurrence network graph was then drawn using the igraph and psych packages in R (v3.6.0).

Conclusions

This study is the first to research microbial ecology on the on-track CSS. On the three flight missions that were analyzed, the number of microorganisms in the cabin ranged from 0 to 2.83×10^9 CFU/100 cm^2 and 1.24×10^5 to 2.59×10^9 rRNA gene copies/100 cm^2, with an average viability of 65.08%. The dominant cultivable bacteria included *Bacillus* and *Staphylococcus*, while the main fungal genera included *Aspergillus*, *Cladosporium,* and *Penicillium*. The MiSeq results showed that the dominant bacterial genera in the cabin were *Pseudomonas*,

Stenotrophomonas, *Methylobacterium-Methylorubrum*, *Sphingomonas*, *Bacillus*, *Staphylococcus*, and *Nocardiopsis*. Suggesting that there was a significant difference in microbial diversity between the CSS and ISS. The microbial composition and diversity varied significantly over time and space. More than half (58.80%) of the microorganisms in the cabin originated from the human skin and oral environments, and *Lactobacillus* was identified as the only genus of microorganisms that remained constant in various module regions of the CSS during the construction period. Conditional pathogenic microorganisms of plant and human were found in the CSS. We recommend that future experiments designed to prevent and control microbial contamination in the cabin focus on plant-derived microorganisms.

Abbreviations

CSS	China Space Station
ISS	International Space Station
MIR	MIR space station
SZ	Shenzhou
qPCR	Quantitative real-time polymerase chain reaction
PBS	Phosphate-buffered saline
LB	Luria-Bertani agar medium
PDA	Potato dextrose agar medium
CFU	Colony-forming unit
PMA	Propidium monoazide
ASV	Amplicon sequence variant
PCR	Polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-02025-2.

Supplementary Material 1.

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Authors' contributions
Y.Z. designed the entire study, guided experimental methods, obtained funding, and revised the manuscript. Y.P.completed the experimental operation, analyzed the experimental data, and wrote the manuscript. X.Q.and L.T. Z. assisted in collecting samples. T.W., H.W. and Z.M.G. helped complete the experiment. W.J. L. Participated in the design of the experiment. X.W. participated in the design of the experiments and gave support on the coordination of all resources of the CSS. All authors reviewed the manuscript.

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Data availability
The Illumina Miseq raw data have been uploaded to NCBI website, the access numbers are shown in Supplementary Table 1. The 16S rRNA and ITS gene sequences of the isolated strains have been uploaded to NCBI GenBank, with login numbers listed in Supplementary Table 2.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors have agreed to publish the manuscript.

Competing interests
The authors declare no competing interests.

Author details
[1]School of Life Science, Beijing Institute of Technology, No. 5 Zhongguancun South Street, Beijing 100081, China. [2]Beijing Institute of Spacecraft System Engineering, China Academy of Space Technology, Beijing 100094, China. [3]School of Life Science, Jiangsu Normal University, No.101, Shanghai road, Tongshan district, Xuzhou, Jiangsu Province 221116, China.

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