Shotgun metagenomic analysis of kombucha mutualistic community exposed to Mars-like environment outside the International Space Station

Aristóteles Góes-Neto[a], Olga Kukharenko[b], Iryna Orlovska[b], Olga Podolich[b], Madangchanok Imchen[c], Ranjith Kumavath[c], Rodrigo Bentes Kato[a], Daniel Santana de Carvalho[a], Sandeep Tiwari[a], Preetam Gosh[d], Bertram Brenig[e], Vasco Azevedo[a], Oleg Reva[f], Jean-Pierre P. de Vera[g], Natalia Kozyrovska[b], Debmalya Barh[h*]*

[a]*Institute of Biological Sciences, Universidade Federal de Minas Gerais (UFMG), Av. Antonio Carlos, 6627, Pampulha, Belo Horizonte, MG, Brazil. arigoesneto@icb.ufmg.br (A.G-N.)*

[b]*Institute of Molecular Biology and Genetics of NASU, Acad. Zabolotnoho str., 150, 03680 Kyiv, Ukrain hikia48@gmail.com (O.K.), i.vviki@ukr.net (I.O.), podololga@ukr.net (O.P.), kozyrna@ukr.net (N.K.)*

[c]*Department of Genomic Science, School of Biological Sciences, Central University of Kerala, Padanakkad P.O., Kasaragod, Kerala-671320, India. anokimchen@gmail.com (M.I.), rnkumavath@gmail.com (R.K.), rnkumavath@cukerala.ac.in (R.K.)*

[d]*Department of Computer Science, Virginia Commonwealth University, Richmond, VA, 23284, USA. preetam.ghosh@gmail.com (P.G.)*

[e]*Institute of Veterinary Medicine, Burckhardtweg, University of Göttingen, Göttingen, Germany. bbrenig@gwdg.de (B.B.)*

[f]*Centre for Bioinformatics and Computational Biology, Dep. of Biochemistry, Genetics and Microbiology; University of Pretoria, Pretoria, South Africa. oleg.reva@up.ac.za (O.R.)*

[g]*Institute of Planetary Research, German Aerospace Center, Germany. jean-pierre.devera@dlr.de (J-P.P.V.)*

[h]*Centre for Genomics and Applied Gene Technology, Institute of Integrative Omics and Applied Biotechnology (IIOAB), Nonakuri, Purba Medinipur, West Bengal, India, dr.barh@gmail.com*

*Corresponding author: Debmalya Barh *(dr.barh@gmail.com)*
*Co-corresponding author: Aristóteles Góes-Neto (arigoesneto@icb.ufmg.br)

Originality significance statement: Kombucha is a multispecies microbial ecosystem, mainly composed by acetic acid bacteria (AAB) and osmophilic acid-tolerant yeasts, characterized by a liquid phase covered by a cellulose biofilm, which produces a probiotic drink and nanocellulose polymer. Kombucha has been recently proposed to be exploited as a possible living system to be used as functional fermented product to improve astronauts' health under both space exploration and planetary colonization and also as an efficient source of bacterial cellulose. Furthermore, Kombucha can be used as a model system for addressing important questions about the evolution of cooperation and conflict in diverse multispecies systems. Our work was the first in the world to study this multispecies microbial ecosystem in the space in the International Space Station, under real Mars-like conditions. Moreover, we used shotgun metagenomics approach to analyze both structure and function of not only bacterial and fungal communities but also the viral ones, which is also a study that has never been done on this multispecies microbial ecosystem. Altogether, we understand that our study is enough original and significant in order to place it within the top 10% of current research in environmental microbiology.

ABSTRACT

Kombucha is a multispecies microbial ecosystem mainly composed of acetic acid bacteria and osmophilic acid-tolerant yeasts, which is used to produce a probiotic drink. Furthermore, Kombucha Mutualistic Community (KMC) has been recently proposed to be used during long space missions as both a living functional fermented product to improve astronauts' health and an efficient source of bacterial nanocellulose. In this study, we compared KMC structure and functions before and after samples were exposed to the space/Mars-like environment outside the International Space Station in order to investigate the changes related to their re-adaptation to Earth-like conditions by shotgun metagenomics, using both diversity and functional analyses of Community Ecology and Complex Networks approach. Our study revealed that the long-term exposure to space/Mars-like conditions on low Earth orbit may disorganize the KMC to such extent that it will not restore the initial community structure; however, KMC core microorganisms of the community were maintained. Nonetheless, there were no significant differences in the community functions, meaning that the KMC communities are ecologically resilient. Therefore, despite the extremely harsh conditions, key KMC species revived and provided the community with the genetic background needed to survive long periods of time under extraterrestrial conditions.

Keywords: Kombucha multimicrobial culture, space/Mars stressors, metagenome, community structure and function.

INTRODUCTION

In the era of deep space exploration, it is crucial to understand the effects of exposure to stressful space and/or Mars conditions on microbial organisms. Effects of spaceflight have been mostly investigated on microbial monocultures (Horneck *et al*., 2010; de Vera *et al*., 2019; Dey, 2019; Ott *et al*., 2020). Nonetheless, most species thrive in multispecies consortia, and exploration of multispecies communities remains an interesting area of investigation due to the multiple mechanisms of spaceflight tolerance (May *et al*., 2019; Podolich *et al*., 2019; Sobisch *et al*., 2019), such as the formation of protective biofilm and cooperative sustainability of multispecies consortium structure. Hence, complex microbial communities can potentially serve as models for studies of multispecies cooperation, as well as to test the dynamics of biochemical and physiological traits that benefit complex communities of microorganisms under extraterrestrial settings.

Resistance and survival of microbial assemblages exposed to cosmic radiation in microgravity or simulated Martian environments would also provide vital knowledge on the possibility of finding life in other planets (Vago *et al*., 2017). Moreover, microbial consortia (or their monocultures) will be crucial players in biotechnology, allowing the production of key-products needed for space crews (Lopez *et al*., 2019). The application of such microbial communities in extraterrestrial habitats for food and health, as well as for waste recycling, is absolutely necessary as microorganisms are an inalienable component of Bio-Regenerative Life Support System (BRLSS) (Huang *et al*., 2018; Ilgrande *et al*., 2019).

Acetic acid bacteria (AAB) and osmophilic acid-tolerant yeasts are the main microorganisms associated with Kombucha Mutualistic Community (KMC), which is known to produce both a probiotic drink and nanocellulose polymer (May *et al*., 2019). It is traditionally prepared by black or green tea fermentation by adding a pellicle, a cellulose-based biofilm, with the aforementioned entrapped microorganisms (from older, already established cultures) as a starter culture. Kombucha has originated as a ‘medicinal tea’ from Manchuria in about 220 B.C. (Jayabalan *et al*., 2014). Recently, KMC-based soft drinks have become quite popular in the Western Hemisphere as a part of the functional food movement (Kapp and Sumner, 2019; Kim and Adhikari, 2020), and the United States Food and Drug Administration has found no hygiene violations in kombucha tea (Centers for Disease Control and Prevention, 1996). Furthermore, the social biochemistry of kombucha as a functional fermented product could be beneficial for astronauts' health under both space exploration and planetary colonization (Kozyrovska and Foing, 2010; Kozyrovska *et al*., 2012) and could be

implemented in BRLSS. KMC is also an efficient source of bacterial cellulose, a multipurpose biopolymer, which is valuable for healthcare and many industrial technologies (Gallegos *et al*., 2016; Azeredo *et al*., 2019; Sales *et al*., 2019; Choi and Shin, 2020; Perugini *et al*., 2020).

Our previous studies have emphasised to unravel the core and accessory microbial members of KMC (Reva et al., 2015; Zaets et al., 2016). The core bacterial community is dominated by AAB such as *Komagataeibacter* spp. (formerly known as *Gluconacetobacter*, Acetobacteraceae family), *Acetobacter* spp., and *Gluconobacter* spp. (Jayabalan et al., 2014). Nonetheless, the relative dominance of yeast genera in KMC, such as *Zygosaccharomyces*, *Candida*, *Torulaspora*, *Pichia*, *Brettanomyces* (*Dekkera*), *Schizosaccharomyces*, and *Saccharomyces* varies based on the variants of KMC (Mayser et al., 1995; Teoh et al., 2004; Jayabalan et al., 2014).

Until quite recently, reports on KMC were conducted using culturable studies, and the full landscape of the microbial communities was still unknown. High-throughput sequencing technologies combined with a suite of computational pipelines opened a new era of microbial ecology (Quince et al., 2017; Almeida and De Martinis, 2019), and, with the advent of cost effective modern next-generation sequencing platforms, an increasing number of KMC community analysis has been published (Marsh et al., 2014; Reva et al., 2015; Chakravorty et al., 2016; Zaets et al., 2016; De Filippis et al., 2018; Coton et al., 2017; Podolich et al., 2019). Although such studies provided a profound knowledge on the KMC, the application of PCR for the amplification of 16S rRNA gene metabarcoding could introduce biases (Brooks et al 2015).

Shotgun metagenomics allows to overcome the challenges that affect both assembly-based and mapping-based metagenomic profilings (Laudadio et al., 2019), particularly, of high-complexity samples (Walsh et al., 2016; Seol et al., 2019; Sirén et al., 2019), including KMC (Arkan et al., 2020). Hence, we have implemented a shotgun metagenomic approach to gather an unbiased snapshot of the community structure and functionality in KMC that were previously exposed to space and different levels of Mars-like conditions, simulated on low Earth orbit outside the International Space Station (ISS), within the BIOMEX project (deVera et al., 2019; Podolich et al., 2019).

The purpose of this study was to compare postflight KMC samples structure and function with those KMCs that passed through a prolonged period of adaptation to normality. The following questions were addressed in this study: (i) are there significant differences in the taxonomic composition, richness, and relative abundance in the bacterial, yeast and viral subcommunities between postflight KMC samples, the ones that were continuously

subcultured after the space and Mars conditions (KMC_c), and the reference samples (KMC_4 and KMC_5); (ii) are there significant differences in the community functions between these aforementioned sample groups?

RESULTS

KMC structure variation among distinct conditions in space and postflight

This study aimed to elucidate changes in microbial subcommunities (bacterial, yeast and viral) of the postflight KMCs designated KMC_1b, which was exposed at the top unshelled support, KMC_2b exposed at the middle support and KMC_3b exposed at the bottom support of the sample carrier outside the ISS compared with laboratory-stored desiccated reference sample KMC, analogical to spaceflight samples, as well as to the other reference sample, the initial KMC. After landing, postflight KMCs were reactivated and produced revived communities. After a series of subculturing, the revived communities have partially restored their structures and functions. Nevertheless, the experimental KMC samples differed from the reference ones by a higher taxonomic richness of bacterial and yeast inhabitants. Some of these newly found species most likely could be considered as either successful survivors, which were in low numbers in the initial KMC but propagated themselves owing to better resistance capacities; or these surviving species are environmental contaminants taking the opportunity of the decreased self-maintenance capacity of the semi-open space-exposed KMC. Moreover, diversity differences between reference samples (KMC_4 and KMC_5) are related to the fact that laboratory-kept reference samples (KMC_4) were desiccated and, thus, so far stressed, in contrast to the initial KMC variant (KMC_5). It was assumed that the initial KMC structure would be restored with time. The current study uses KMC lineages derived from the experimental cultures and designated as KMC_1c, KMC_2c and KMC_3c, which were continuously cultivated in laboratory conditions for 2.5 years after having been sent to the ISS. Annotation of the shotgun metagenomics reads generated from all the KMC samples using MG-RAST identified several bacterial, yeast and viral species. Bacterial reads dominated the postflight KMCs by making up to 99.88% of the total number of reads. It might be supposed that the applied DNA extraction procedure was more efficient for bacterial rather than yeast cells. Nonetheless, in samples KMC_1c, KMC_2c and KMC_3c, yeast reads comprised values as high as 14.65%, 2.78% and 8.6% respectively. It indicates an increase in the yeast subcommunities in laboratory-cultivated postflight KMCs that was not the case with the initial KMC, laboratory-kept KMC, and the experimental samples revived after the space exposure experiments. Viral reads encompassed 0.01% of the total number of reads in all the samples,

and, specifically, in all the laboratory-cultivated postflight KMCs (1c, 2c, 3c). An increase in the relative abundance of viruses was observed in the postflight KMCs (1b, 2b, 3b) when compared with the laboratory-cultivated postflight KMCs.

Alpha diversity

Space/Mars-like exposure stressors caused compositional alterations in KMC communities (Figure 1 and Table S1). Generally, the diversity of the exposed KMC communities decreased, whereas the dominance of the major *Komagataeibacter* spp. increased. On the other hand, the number of minor species belonging to *Bacillus*, *Pseudomonas* and other taxa has increased. This indicates an induction of decaying of the stable KMC consortium comprised of cellulose producing bacteria, mainly *Komagataeibacter* spp., and yeasts after the space exposure. The bacterial richness and the Shannon diversity of the space-exposed KMC samples have increased. Surprisingly, further 2.5-year cultivation of the experimental samples did not bring them to the initial KMC structure. Contrary, the bacterial richness and diversity kept growing in KMCs (1c, 2c, 3c) , whereas the structure of the control community KMC_4c, the descendant of the initial KMC cultivated for 2.5 years, appeared to be more stable showing stronger resistance to environmental contamination (Figure 1A). Nevertheless, the bacterial richness of KMC_4c was higher compared to the initial KMC. It may be assumed that the long cultivation of KMC in laboratory allows enriching the community with opportunistic bacterial species that were at minority levels in the initial KMC or appeared through KMC contamination. The space/Mars-like stressors reduced profoundly the KMC stability, making it more vulnerable.

The diversity of the yeast component of KMCs has not been affected significantly by the space exposure compared to the laboratory reference sample KMC_4b (Figure 1B and Table S1). The laboratory cultivation of KMCs led to an increase in the dominance of abundant yeast species that was associated with declining species diversity of the yeast component of KMC. Analysis of the viral populations of KMC showed a significant increase of viral diversity in the cultivated experimental KMCs. It may also indicate a higher vulnerability of the space-exposed KMC consortia to phage infections (Figure 1C).

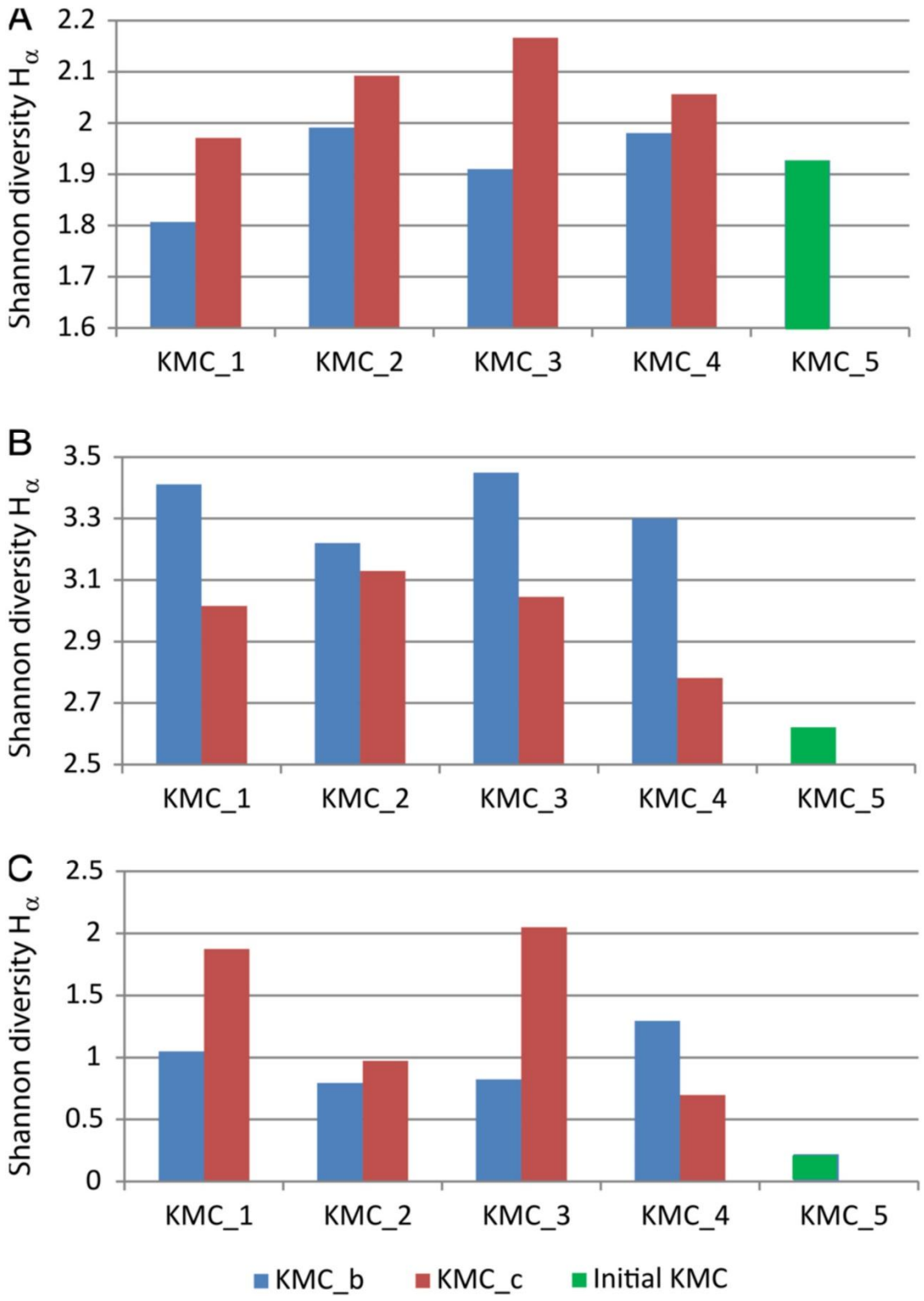

Fig. 1. Alternation of H_α-diversity of bacterial (A), yeast (B) and viral (C) components in samples exposed on the International Space Station during (ISS) (KMCb1, b2, b3 and the laboratory control b4 – blue columns); respective descendant cultures after 2.5-year cultivation in the laboratory (KMC_ c1, c2, c3 and b4 – red columns) and the initial KMC_5 – green column. KMC_1 – location of KMC samples on the unprotected top level outside the ISS; KMC_2 – on the protected middle level; KMC_3 – on the protected bottom level; KMC_4 – laboratory-kept KMC samples during the exposure experiment; KMC_5 – the initial KMC sample.

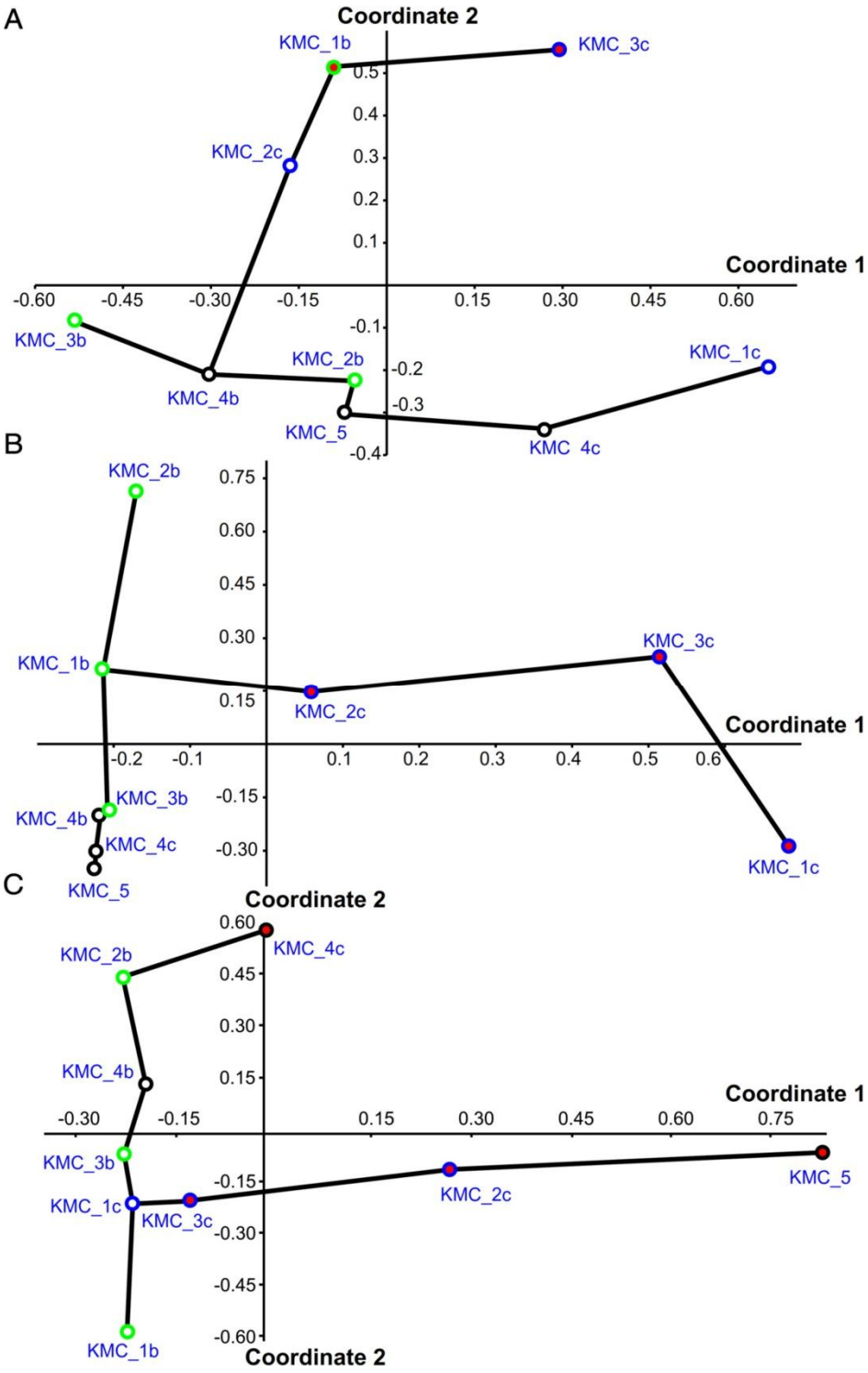

Fig 2. Principal coordinate analysis (PCoA) plotting of Kombucha Microbial Communities (KMC) exposed on the International Space Station during 18 months (1.5 years) in the space/Mars-like conditions (KMC_b) and further 2.5-year cultivation in the laboratory after the revival of KMC samples (KMC_c) based on beta-diversity comparison of bacterial (A), yeast (B) and viral (C) subcommunities of these consortia. KMC samples are represented by round nodes linked by graphs of the minimal spanning tree. Nodes located outside of the estimated 95% confidence areas are depicted by the red colour of filling while those within the 95% confidence are marked by open cycles. Nodes representing repetitions of different experiments are labelled accordingly. Additionally, nodes of KMC samples 1b, 2b and 3b have green outlines; and the KMC samples 1c, 2c and 3c have blue outlines.

Beta diversity

Beta diversity comparison by principal coordinate analysis (PCoA) showed rather random ungrouped distribution of the samples compared with their bacterial subcommunities (Fig. 2A). Usually, the three experimental samples KMCs (1c, 2c and 3c), which were cultivated for 2.5 years after the exposure, were more distant from both the initial KMC (KMC_5) and the other reference sample KMC_4b than their progenitor KMCs (1b, 2b and 3b) sampled immediately after their return from the ISS. It suggests that the exposed KMCs became more vulnerable to random environmental opportunists, particularly the bacterial genus *Pseudomonas*.

The previous analysis on the alpha diversity of yeast KMC species showed a trend towards stabilization of their populations in the experimental KMCs after 2.5 years of laboratory cultivation (Fig. 1B). Nonetheless, the PCoA ordination of the samples revealed that the exposed KMCs did not regain the initial composition. Conversely, each postflight KMCs has developed its own specific yeast community clearly distinguishable from the initial KMC (Fig. 2B). Moreover, PCoA ordination of the viral subcommunities also revealed a haphazard grouping (Fig. 2C).

Community taxonomic composition

Bacterial KMC subcommunity

The most abundant bacterial taxa in all KMC samples were representatives of genera *Komagataeibacter*, *Gluconobacter*, and *Acetobacter*, all of them belonging to the family Acetobacteraceae (Figure 3A-B). Representatives of this family made 81-84% of the total bacterial reads. Postflight KMCs and laboratory-kept references differed by the relative representation of different genera of bacteria. *Komagataeibacter* was enriched in KMC_1b and KMC_3b, while *Gluconobacter* was depleted; however, it was not the case in KMC_2b. *Acetobacter* was depleted in all the space/Mars-like-exposed samples. Meanwhile, opportunistic proteobacteria *Pseudomonas, Acidovorax, Ruegeria, Methylocella, Magnetospirillum* and *Burkholderia* were enriched in all the experimental samples after a 2.5-year cultivation. Nonetheless, they constitute only a minor component of experimental KMC samples, indicating a general increase of either the resuscitated or incoming microbial organisms in KMCs.

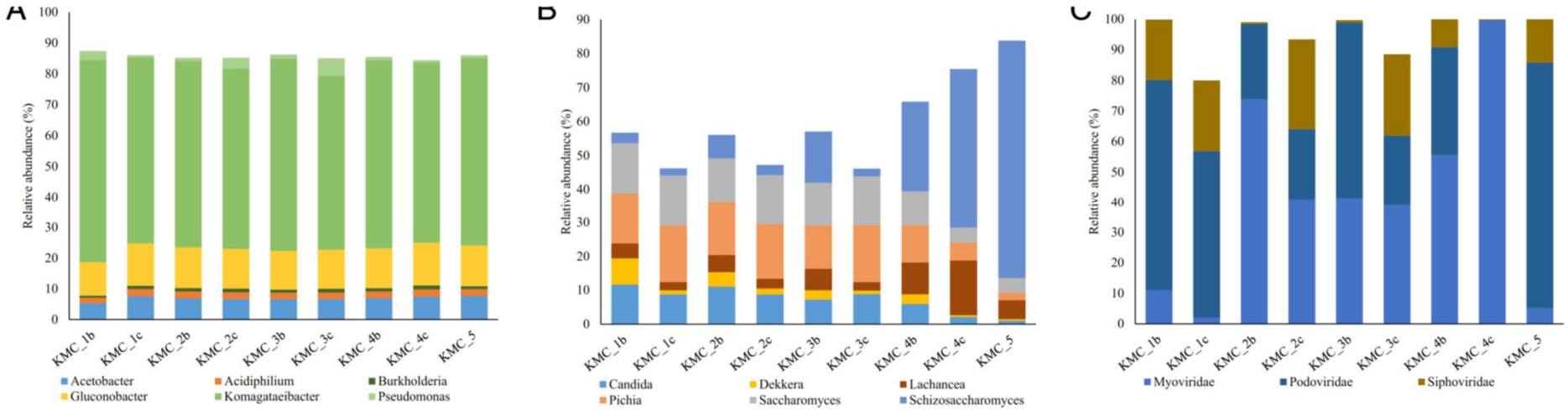

Fig 3. Stacked bar charts presenting the bacterial (A), yeast (B) and viral (C) components of postflight kombucha microbial communities (KMC-b) and corresponding KMCs cultured within 2.5 years (KMC_c). (1: top, UV-unprotected level; 2: middle, UV-protected level; 3: bottom, UV-protected level, 4: laboratory-kept KMC samples during the exposure experiment; 5: the initial KMC sample). The bacterial genera above 1% relative abundance, and dominant yeast and viral families are shown in the figure.

Yeast KMC subcommunity

Yeast subcommunity of KMC also experienced a significant alternation after the space-exposure and laboratory cultivation (Figure 3C-D). Yeasts of the genus *Schizosaccharomyces* were dominant in the initial KMC samples, making up 38% of the total number of yeast reads. During the cultivation in laboratory, the relative abundance of *Schizosaccharomyces* decreased to 25%. Space exposure has reduced the fraction of *Schizosaccharomyces* in KMC populations, depending on the sample location, from 11.1% in KMC exposed on the protected bottom supporter to 1.9% in KMC exposed on the UV-illuminated top carrier and 5.7% from the middle level. Postflight recovery of the experimental KMCs and further cultivation at laboratory conditions did not restore the initial abundance of *Schizosaccharomyces*. Conversely, it declined and stabilized at ~2% in all the KMC_c cultures.

Significant changes also occurred with *Lachancea*. Representatives of this yeast genus constituted 3.5% of the initial KMC yeast subcommunity, but increased in number during laboratory cultivation to 7-12%. After the space exposure, the relative abundance of these yeasts decreased to 2.8% on the top level and ~4% in the shelled supports. The relative abundance kept declining in further 2.5 years of cultivation to ~2.5% in all the KMC_c cultures. Both *Schizosaccharomyces* and *Lachancea* yeast taxa were replaced in the space exposed KMCs with representatives of several other genera. The space exposure was most beneficial for *Brettanomyces/Dekkera*, which increased from barely detectable minority in the initial KMC to 10.4% on the top level of KMC exposure, 6.3% on the middle support and 1.8% on the bottom level. Nonetheless, these yeasts did not withstand further 2.5 years cultivation in laboratory and dropped below 1% in all the KMC_c cultures.

Another yeast genus, *Pichia*, also beneficiated from the space exposure and increased its relative abundance from ~5% in the reference KMC to 9-13% after the exposure. After 2.5 years of laboratory cultivation its relative abundance increased up to 14-15%. In a similar manner, the space exposure has stimulated the growth of *Candida* and *Debaryomyces* from around 1% initially to respectively 6-9% and ~4% after the exposure. These yeasts maintained the same level of abundance after 2.5 years of laboratory cultivation.

Yeasts of the *Saccharomyces* genus were abundant in the initial and cultivated reference KMCs, making up 10% of the yeast subcommunity. Space exposure did not change this level of abundance of *Saccharomyces*, and it increased in KMC_c cultures up to 13-14% of the total yeast subcommunity. *Saccharomyces* replaced the initially most abundant genus *Schizosaccharomyces*, but only to some extent, far below the level of 20-40% maintained by *Schizosaccharomyces* in the initial KMC.

Viral KMC subcommunity

Tailed bacteriophages of the order *Caudovirales* were the most prevalent and relatively abundant in all the KMC samples regardless if they were those that were submitted to space/Mars conditions or the ones that remained under laboratory conditions on Earth. Among the *Caudovirales*, the families *Myoviridae* (long contractile tailed bacteriophages), *Podoviridae* (short-tailed bacteriophages), and also, but in a minor representation, *Siphoviridae* (long non-contractile tailed bacteriophages) were found (Fig. 3C; Fig. S1E and F). Besides bacteriophages, some other DNA viruses usually reported for animals, plants and algae were also detected, but all of them with very low relative abundances when compared with the tailed bacteriophages (Fig. S1E and F).

Representation of functional genes in KMC samples

Functional analysis was performed to compare relative abundances of genes of different functional categories in KMC by aligning the generated DNA reads against reference genomes. Taxonomic alternations in KMC caused by the space exposure and further laboratory cultivation may reflect a proliferation of species possessing certain genes that increase the tolerance to this harsh stress condition, while other species, not bearing some of these genes, proportionally decreased their representativeness in the community.

In order to investigate the potential resistance to UV radiations, we examined the UvrABC system that is responsible for nucleotide excision repair. Interestingly, UvrABC system was the highest in the postflight top level sample, which was exposed to maximum radiation (Figure 4A). Nonetheless, it should be noted that, on average, UvrABC system was enriched in all the samples both in postflight and lab-kept reference but dropped significantly after reactivation.

KMC produces cellulose-based pellicle due to acetic acid bacteria that protects kombucha community from harsh environmental factors. The enzymes required for initial steps of cellulose synthesis, phosphoglucomutase *pgm* (EC 5.4.2.2), UTP-glucose-1-phosphate uridylyltransferase UGP2 (EC 2.7.7.9), and cellulose synthase *bscA* (EC 2.4.1.12) were practically unchanged and kept the relative gene abundances at the level of the initial KMC ecotype. It was noted; however, that the abundance of these genes has decreased to some extend in KMC_c samples (Figure 4B) that might indicate a depressed capacity to produce cellulose by these consortia.

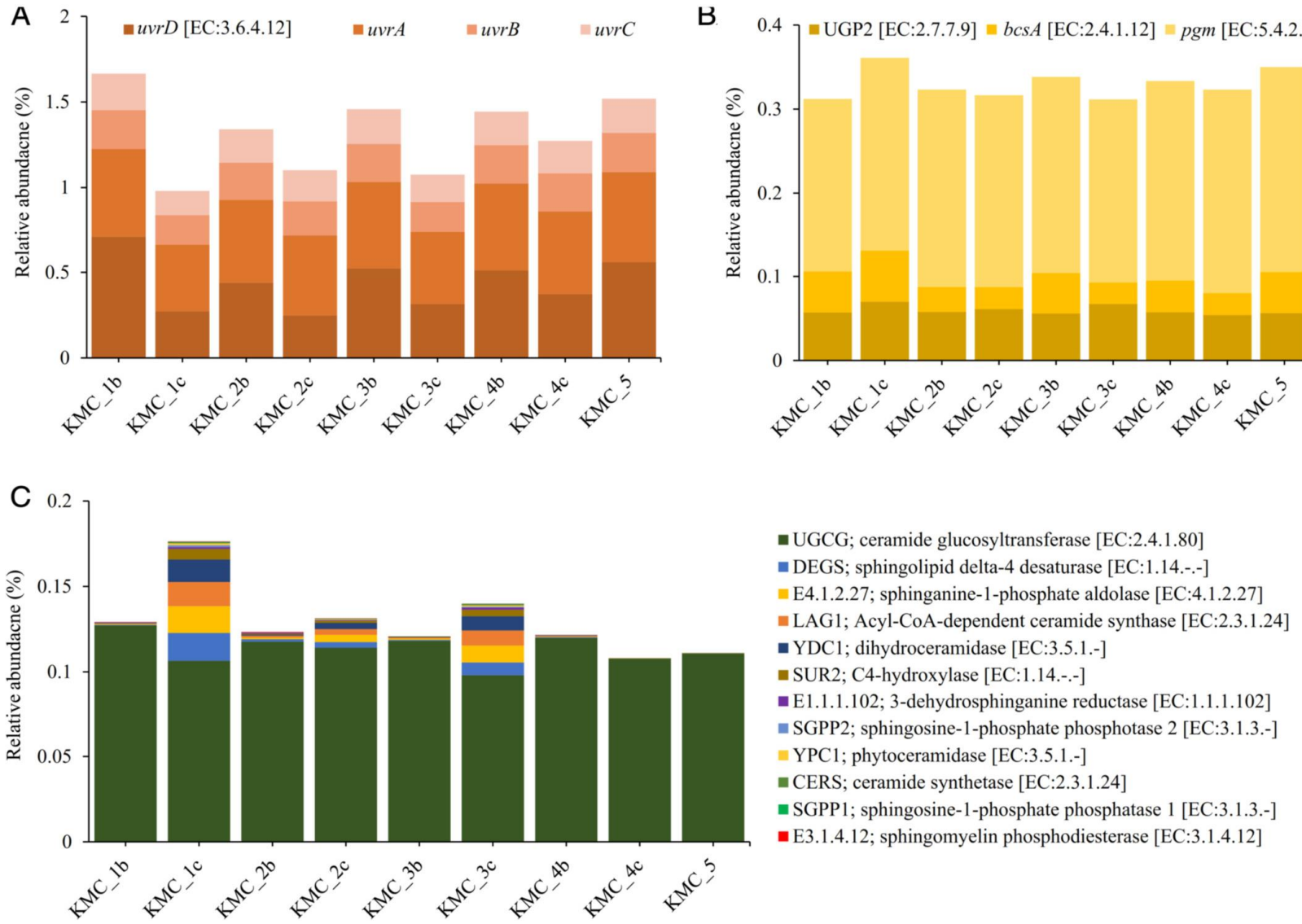

Fig 4. The relative abundance of functional genes involved in (A) UvrABC system, (B) cellulose synthesis and (C) ceramide and sphingolipid biosynthesis.

The presence of sphingolipids in AAB, which are rarely found in other prokaryotic species, could indicate its importance to survive in low pH and high concentration of acetic acid (Goto and Nakano, 2008). The key enzyme catalysing the synthesis of sphinganine, and several other genes involved in ceramide production normally are overexpressed when exposed to low pH or heat stress (Ogawa et al., 2010). Nevertheless, sphinganine can be toxic to yeasts as well as to AAB. Hence, the synthesis of sphingolipids could serve as a way to dispose excess sphinganine (Mao et al., 1999). Similarly, we also observed an enrichment of 3-dehydrosphinganine reductase (EC 1.1.1.102) and several other related genes involved in ceramide and sphingolipid biosynthesis (Figure 4C). The enrichment was most prominent in the KMC_c samples cultured within a prolonged period of time.

Functional clustering of the genes/proteins related to amino acid metabolism, carbohydrate metabolism, translation, energy metabolism, replication and repair, membrane transportation, and nucleotide metabolism has also been analysed by the network method. We plotted the relative read abundance of the metagenomics data as a network to investigate the functions with higher/lower abundance in different KMC samples, as well as identify those gene functions with similar relative read abundance. The network analysis showed that in all the samples both amino acid metabolism and signal transduction related functions were among the most abundant ones. Furthermore, in all the cases, there were very few nodes with high relative read abundance, and a high number of nodes with low relative read abundance. Although the network topology was similar across all the samples, KMC_1b /c was the only sample where a gene function exhibited relative read abundance above 0.9 compared to the remaining samples, while the highest relative read abundance function was at the 0.8-0.89 range. All the networks show a high number of connections among nodes with relative read abundance between 0.10 and 0.49, as well as some connections between communities. This indicates similar relative read abundance within and between these communities (Figure 5).

Sample KMC_1b/c also exhibited a difference among the most abundant functions, showing replication and repair related functions in the 0.7-0.79 range (Figure 5A). The most abundant replication and repair related functions in the remaining samples were retrieved in the 0.5-0.59 range (Figures 5B-E). Another striking difference was that sample KMC_1b/c was the only sample in which a membrane related function was retrieved in the 0.6-0.69 range. In the other samples, the same function was found at lower ranges (< 0.49) (Figure 5). The lack of connections across nodes with relative read abundance greater than 0.50 indicates that the nodes within these abundance levels were not as similar and that specific genes had their relative read abundance modified. This pattern was not seen in the other networks. These

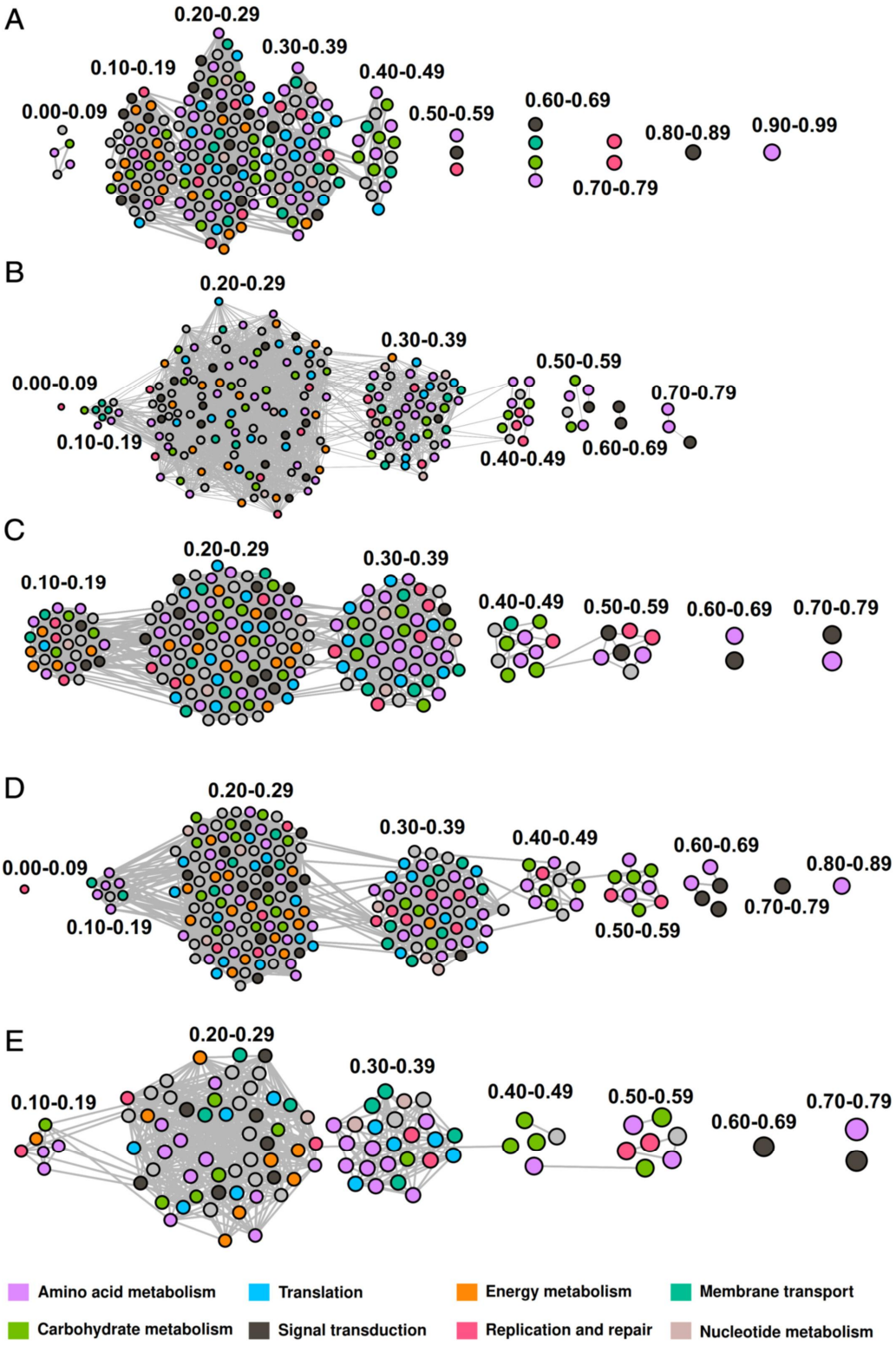

Fig 5. Networks representing function abundance of space/Mars-like exposed KMC samples: (A) KMC_1b/c (top), (B) KMC_2b/c (middle) and (C) KMC_3b/c (bottom). Networks, representing function abundance of ground reference samples: (D) KMC_4 b/c, (E) KMC_5. Node size represents the relative read abundance of genes in a certain function. Colours represent different categories of functions. The range of relative read abundance is shown above each community (values rounded to two decimal points on the community legend with '9' as the repeating decimal).

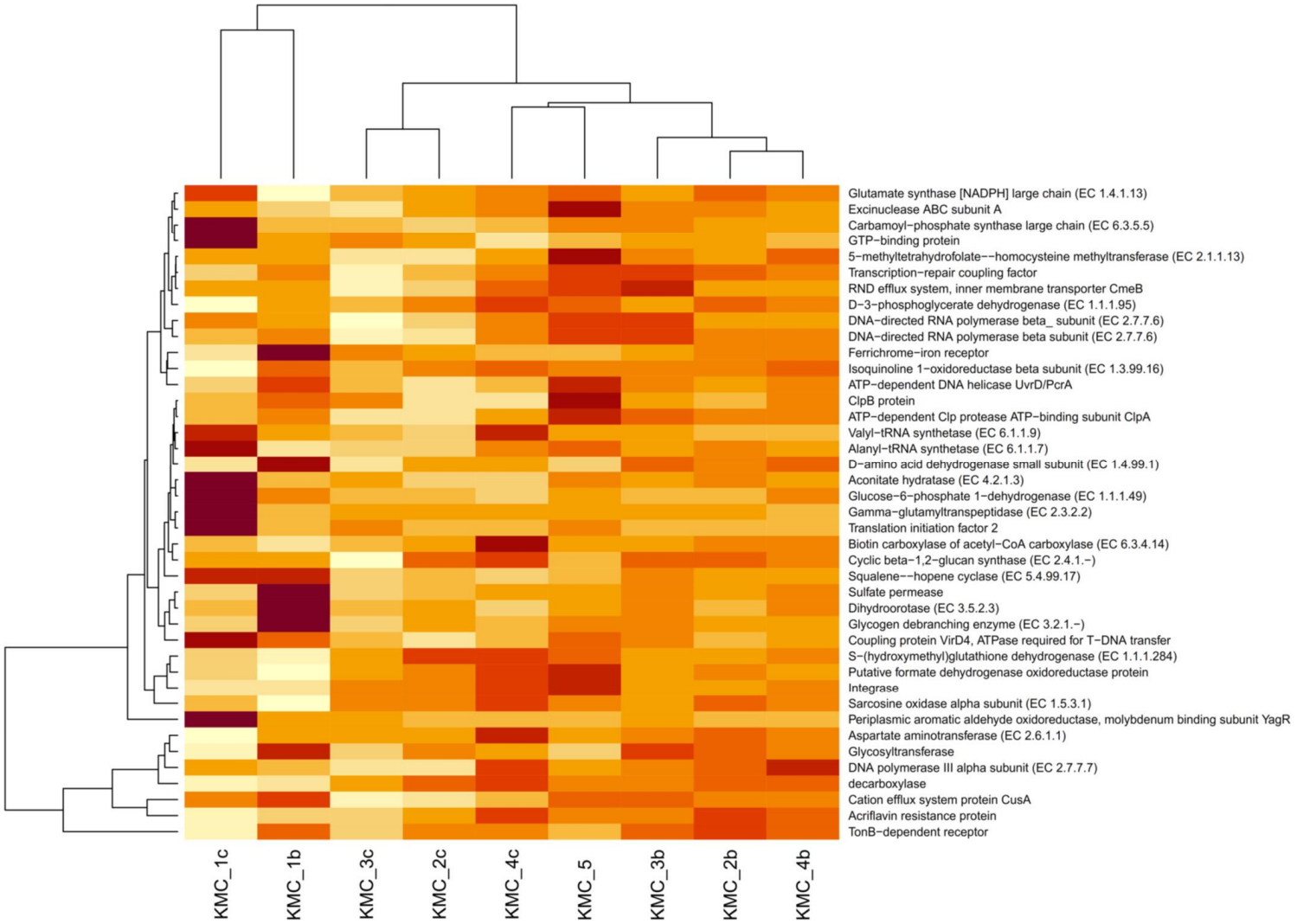

Fig 6. Top 10 abundant functions in each dataset produced 41 functional genes. Hierarchical clustering with heatmap shows that the KMC_1b/c (unprotected top level) clustered separately from the other datasets.

results suggest that the changes in relative read abundance of KMC_1b/c could possibly be linked to the higher stress levels due to being exposed to cosmic radiation.

The sample KMC_2b/c exhibited a topology that seemed to be an intermediate between samples KMC_1b/c and KMC_3b/c, while sample KMC_3b/c, KMC_4b/c, and KMC_5 had very similar topologies, with the same functions (amino acid metabolism and signal transduction) highly frequent (Figure 5). This result suggests that the less exposed to the cosmic radiation a sample was, the more similar it was to the Earth control samples. On the other hand, KMC_1b/c had different highly frequent functions (amino acid metabolism, signal transduction and replication and repair). Although KMC_1b/c exhibited the same functions highly frequent as the other samples, it also had a high frequency of replication and repair functions (>0.7). The other samples displayed frequencies of replication and repair related functions between 0.4 and 0.59.

Top 10 functions from all the samples were extracted and merged to construct a colour matrix and its corresponding biplot cluster dendrogram. The KMC samples formed two main clusters using the protein-coding genes as attributes. The first main cluster grouped together the UV-irradiated postflight KMC-1b and its corresponding serial cultured KMC-1c whereas the other main cluster grouped together all the other KMC samples (KMC_2-KMC_5). When the samples are used as attributes to cluster the protein-coding genes, two main clusters were also retrieved. One formed by only seven proteins, and the other grouping all the other proteins (Figure 6).

Variation patterns of the dominant and cellulose-producing *Komagataeibacter* species in KMCs

The analysis of returned samples showed that the KMC key microorganisms, the bacterial species of the genus *Komagataeibacter*, remained alive within the cellulose pellicle after being exposed to space and Mars-like conditions, and this fact was critical for forming kombucha microbial community *per se* and for cellulose production. Cellulose-based pellicle-forming *Komagataeibacter* species of the post-flight bacterial subcommunities were then predicted with metagenomic approach. The species *K. saccharivorans, K. hansenii, K. rhaeticus, K. oboediens, K. intermedius* and *K. europaeus* were detected in all the KMC samples (Figure 7). In UV-protected exposed KMCs, KMC_2b and KMC_3b, *K. saccharivorans* was the dominant species. In addition, the relative abundance of *K. hansenii* and/or *K. rhaeticus* were substantially altered in the KMC_2c and KMC_3c compared to the

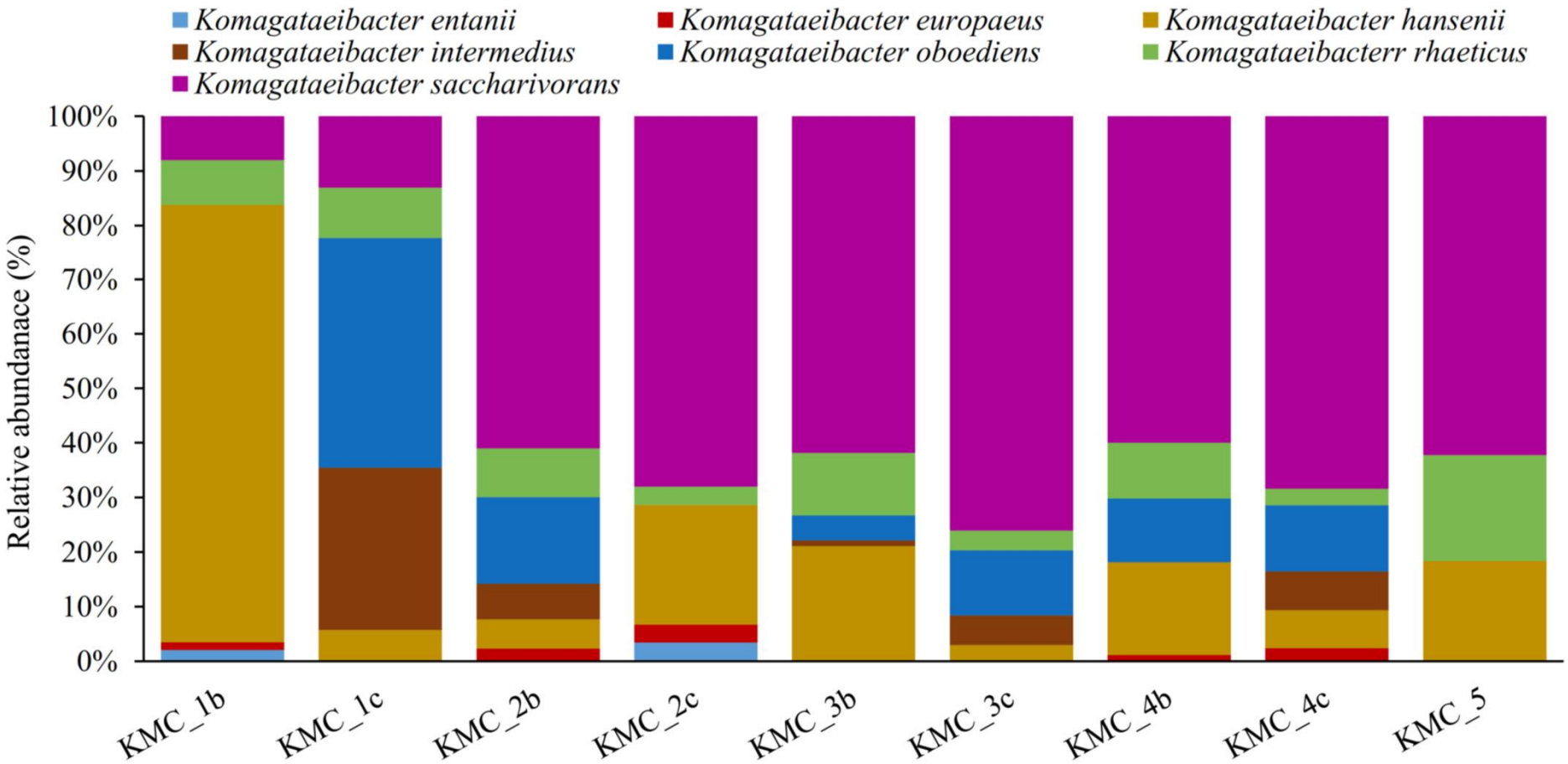

Fig 7. *Komagataeibacter* species in kombucha multimicrobial culture (KMC) samples exposed outside the International Space Station to Mars-like conditions at top, middle and bottom levels of the sample carrier (KMC_1, KMC_2, KMC_3) in the BIOMEX astrobiological project based on the shotgun metagenomics analysis. *Komagataeibacter* species from initial KMC and from KMC samples kept in the laboratory during the space experiment designated as KMC_4 and KMC_5 respectively. KMC samples reactivated after a 1.5 year period of conservation designated as KMC_1b, KMC_2b, KMC_3b and KMC_4b, and after 2.5 years of serial subcultures designated as KMC_1c, KMC_2c, KMC_3c and KMC_4c.

initial KMC. Similarly, *K. oboediens*, *K. intermedius*, and *K. europaeus* were enriched to the level of detectability in all the exposed KMCs, including the KMC_4c.

More striking changes were deduced in *Komagataeibacter* of the UV-irradiated top layer KMC_1b, where there was a reduction of *K. saccharivorans* and a dominance of *K. hansenii*. After prolonged cultivation of the returned KMCs, *K. oboediens*, *K. intermedius* and *K. europaeus* were enriched, which were not found in ground reference KMC_5. Nevertheless, the most impressive transformations in *Komagataeibacter* relative abundances were noticed in the UV-irradiated top layer KMC_1c after the prolonged cultivation: *K. hansenii* reduced its relative abundance, *K. saccharivorans* relative abundance increased, but was not restored, and *K. oboediens* and *K. intermedius* enhanced their competitiveness.

DISCUSSION

Herein, we performed a shotgun metagenomic analysis in order to compare the community structures and functions among (i) post-flight KMC samples (KMC_b) previously exposed to different levels space/Mars conditions, simulated on low Earth orbit outside the International Space Station for 1.5 year, (ii) KMC samples (KMC_c) that passed through a prolonged period of adaptation to normal through 2.5 years, and (iii) the corresponding reference KMC samples (KMC_4 and KMC_5). Our study comprised alpha and beta diversity analyses for the comparison of the taxonomic composition, richness, and relative abundance of bacterial, yeast, and viral components, and community functions using both traditional and complex network approaches. Until date and as far as we know, our study is the second one dealing with shotgun metagenomics of kombucha, after that from Arikan et al. (2020) but the first one on analyzing the virome of this symbiotic multimicrobial system.

Regardless of the layer position in sample carrier on the EXPOSE-R2 facility outside the ISS, all the KMC samples that were continuously subcultered (KMC_c) experienced shifts in the bacterial, yeast, and viral subcommunities when compared to the corresponding postflight samples (KMC_b): (i) an increase of Shannon diversity indexes in the bacterial, and (ii) a decrease of these diversity indexes in the yeast subcommunities. At a first glance, this shift in the diversity might be probably associated to the harsh selective pressures of Space/Mars conditions leading to a possible permanent change in the structure of the communities; however, the same trends in both bacterial and yeast subcommunities occurred in the laboratory-kept reference KMC_4, which was submitted to the same period of serial cultivations and did not underwent the strong abiotic stresses, except desiccation. Furthermore, based on beta diversity analyses, there is no marked ordination between postflight reactivated

samples (KMC_b), and those that underwent serial cultivations after reactivation (KMC_c). Nevertheless, the diversity of viral subcommunities was strikingly distinct. In all the KMC_c samples, the Shannon diversity indexes were higher than the corresponding KMC_b samples whereas there was a sharp decrease in the lab kept reference (KMC_4c). This pattern observed in viral subcommunities may be possibly associated to the increase of the diversity of their bacterial hosts.

Kombucha biofilm is a three-dimensional structure, comprising biomacromolecules such as cellulose, other polysaccharides and proteins, exogenic DNA, extracellular membrane vesicles, inorganic inclusions, as well as microbial cells, bacteria and yeasts, and, also, viruses (Podolich et al., 2017). Previously, temperate bacteriophages of Myoviridae, identified as one of the dominant *Komagateibacter* species of kombucha biofilm after prophages induction using a chemical stressor, increased the production of outer membrane vesicles, which collected and engulfed formed phage particles and, thus, act in a defensive manner, preventing bacteriophage spread in bacterial population (Kharina et al., 2015). In fact, in our study, the most prevalent and relatively abundant bacteriophages of the experimental samples were those from *Myoviridae* and *Podoviridae* families. Altogether, these findings suggest that, after simulated Martian conditions, kombucha multimicrobial consortia are much more prone to bacteriophage induction/infection and proliferation, which may also have contributed, besides the selective pressures of abiotic stresses, for the observed changes in bacterial taxonomic composition.

One of the most dramatic changes in both postflight (KMC_b) and serially cultivated (KMC_c) compared to reference, laboratory KMCs (KMC_4 and KMC_5), was the very sharp decrease in the relative abundance of one of the dominant yeast genus: *Schizosaccharomyces*. Yeast species in kombucha multimicrobial systems (under normal, non-stressed conditions) are usually much more variable than bacterial species, and mainly include yeasts in the genera *Zygosaccharomyces*, *Schizosaccharomyces*, *Brettanomyces*/*Dekkera*, *Saccharomyces*, *Candida*, *Torulaspora* and *Pichia*, besides other less representative genera (Reva et al., 2015; May et al., 2019; Laureys et al., 2020). Space/Mars conditions severely affected *Schizosaccharomyces*. It was unable to restore its original condition in terms of niche repartition in the kombucha community even after the 2.5-year of serial cultivation under normal conditions. *Schizosaccharomyces* is significantly more resistant to UV or ionizing radiation than *Saccharomyces* since, unlike *Saccharomyces*, *Schizosaccharomyces* has an extra pathway (UVER) for excision of UV photoproducts in addition to nucleotide excision repair (NER) (Forsburg, 2005; McCready et al., 2000). As the relative abundance of *Saccharomyces* practically remained similar in control (KMC_4 and KMC_5), postflight (KMC_b), and serial

culturing after postflight (KMC_c) samples, the most plausible explanation was the high sensitivity of *Schizosaccharomyces* to the Mars-like organo-mineral mixture, most probably to the anorthosite rock powder. This hypothesis; however, must be experimentally tested further.

The repertoire of cellulose-synthesizing *Komagataeibacter* species in returned KMCs, both reactivated and serially cultured, was depended on their location outboard the ISS and previously influenced stressors. In UV-protected KMCs, KMC_b, the *K. saccharovorans* population increased abundance and occupied a leading position. Nonetheless, in UV-irradiated KMC_1b, *K. hansenii* was predominant. The prevalence of either of them was strongly affected probably not only by stressors under a period of exposure, and their competitiveness could depend probably on the kombucha community structure occurred after spaceflight. It is interesting to note that, in all experimental KMC samples, including the laboratory kept reference, bacteria of the genus *Komagataeibacter* that were minoritarily represented in the wild type KMC, *e.g.*, *K. oboediens*, *K. intermedius* and *K. europaeus*, raised their populations to a level of detectability. Although they may be not detected in the original consortia used as first inoculum, latent microbial community composites boost populations under favorable conditions, (De Filippis et al., 2018).

Both complex network and multivariate clustering analyses retrieved strong patterns related to the community functions: a marked distinction of KMC_1 samples from all the other samples, and, especially of the UV-irradiated top layer postflight KMC_1b sample. Not only the complex network topology was considerably distinct, but also these samples formed a rather distinct cluster. KMC_1(b/c) samples displayed the highest relative read abundance of replication and repair, and membrane-related functions. Furthermore, these UV- irradiated samples (KMC_1b and KMC_1c) displayed the highest read relative abundance of the protein coding gene of SHC, squalene-hopene cyclase (EC 5.44.99.17). SHCs primarily synthesize two hopanoids, diploptene and diplopterol, which are also known as C30 hopanoids (Belin et al., 2018). These hopanoid lipids occur in several bacterial species and are quite similar to eukaryotic sterols. Hopanoids modulate the fluidity and permeability of membranes and are directly related to stress tolerance (Belin et al., 2018). Several studies in many bacterial species show that hopanoid lipids facilitate bacterial survival under diverse kinds of physical-chemical stress conditions, such as high temperatures, low pH, high osmotic pressures, and during chemical treatments; however, the specific set of conditions for which these hopanoid lipids are really beneficial may vary among the distinct bacterial strains (Flesch et al., 1987; Horbach et al., 1991; Schmerk et al, 2011; Kulkarini et al, 2015; Welander et al., 2009). Therefore, we

hypothesize that hopanoid lipids may have a crucial importance in tolerance of Mars-like stress conditions. The next steps in our research program will address this important finding.

CONCLUSION

Our study revealed that after the long-term exposure to space/Mars-like conditions on low Earth orbit, KMC core microorganisms of the community were maintained. Harsh extraterrestrial stressors, however, may disorganize the KMC to such extent that it will not restore the initial community structure. Furthermore, there were significant differences in the taxonomic composition, richness and relative abundance in the bacterial, yeast and viral subcommunities between postflight KMCs, the ones that were continuously subcultured after the space and Mars-like conditions, and the reference samples. Nonetheless, there were no significant differences in the community functions, which may mean that the KMC communities are ecologically resilient. Therefore, in spite of the extremely harsh conditions, key KMC species revived and provided the community with needed gene sets for the formation of a cellulose-based three-dimensional framework that allows the survival of the multimicrobial community during long periods of time under extraterrestrial conditions.

MATERIALS AND METHODS

Spaceflight exposure treatments

Dried KMC pellicles (7-mm-diameter disks), embedded into organo-mineral mixture, were exposed to simulated Mars-like conditions (atmosphere of 95.55 % CO_2, 2.70 % N_2, 1.60 % Ar, 0.15 % O_2, ~ 370 ppm H_2O and a pressure of 980 Pa) in the three-layer sample carrier mounted on the EXPOSE-R2 facility outside the ISS (Podolich et al., 2019). During 2.5-year exposure at the ISS (18 months outside and 7 months inside the station), samples located on the unprotected top level received total UV (>200 nm) fluencies of about 4.92×10^2 kJ/m^2 and 0.5 Gy of cosmic ionizing radiation (de Vera et al., 2019). The middle and bottom levels were maintained in darkness (protected from UV radiation); however, in Mars-like atmosphere and pressure, for reference. During the exposure period, all the KMC samples were kept dried at temperature fluctuations from +50 to -20 ^{0}C and therefore were also subjected to desiccation stress. Three sets of analogically prepared KMC samples were maintained in laboratory under room temperature in darkness, as references.

Sample preparation

For shotgun metagenomic analyses, we used (i) postflight revived KMCs that were conserved after reactivation, designated as KMC_b (1 – a top-; 2 – a middle-; 3 – a bottom-located KMC) and a laboratory-stored desiccated KMC (designated as KMC_4b) and (ii) a set of the same samples aliquots, continuously cultured monthly during 2.5 years in sweetened black tea (Podolich *et al.*, 2019), designated as KMC_c. The initial KMC ecotype, which had served for sample preparation for the space experiment, was used as a reference (designated as KMC_5). The postflight KMC samples (KMC_1b, KMC_2b and KMC_3b), as well as a laboratory, kept KMC (KMC_4b) were stored in a freezer at −80°C.

Culturing conditions

KMC samples from all three levels of exposure (top, middle, and bottom) as well as laboratory-kept reference, were reactivated with filtered black tea infusion (0.5% brew, 7.0% sugar) at room temperature (average 21°C). After the consortium restoration, aliquots of the culture from each sample (10%) were transferred to a new flask of 50 mL for serial culturing in parallel with initial KMC ecotype.

Metagenomic DNA isolation, shotgun sequencing, and annotation

Total DNA from the planktonic cells of KMC were isolated by using the innuSPEED Bacteria/Fungi DNA isolation kit (Analytik Jena AG, Germany) during the stage of biofilm formation. The nucleic acids were quantified and qualified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). A 450 bp library was prepared from genomic DNA with the NEBNext Fast DNA Fragmentation and Library Preparation Kit (New England Biolabs, Ipswich, NE, USA) following the manufacturer's instructions. Library quality was evaluated with Agilent 2100 Bioanalyzer, and shotgun metagenomic sequencing was performed using an Illumina HiSeq 2500.

Raw fastq files were annotated using the widely used public annotation server Metagenome Rapid Annotation using Subsystem Technology (MG-RAST v4.0.3) (Meyer *et al.*, 2008). The raw data were subjected to quality control by trimming low-quality sequences (Phred score <30) (Cox *et al.*, 2010) and removal of artificially duplicated reads with Duplicate Read Inferred Sequencing Error Estimation (Gomez-Alvarez *et al.*, 2009; Keegan *et al.*, 2012). The annotation was performed against RefSeq (Wilke *et al.*, 2012) and KEGG (Kanehisa, 2002) database. The annotated data were merged and downloaded from the server in tsv (tab-separated values) format for analysis in R (R Core Team, 2020). In order to avoid the

background noise, only the genera above 0.1% in, at least, one sample were considered for further analysis. Data were analysed using R packages, such as Phyloseq v1.3 (McMurdie and Holmes, 2013) and Microbiome v1.8 (Lahti *et al.*, 2017). For the most relatively abundant taxa, such as the pellicle-forming *Komagataeibacter* in KMC, reliable taxonomic resolution could be achieved at the species level with confidence. The raw data are publicly available in MG-RAST server under the project 'KMC_Project', with accession ID mgp91475, and the following accession numbers of the samples: mgm4865605.3, mgm4865606.3, mgm4865607.3, mgm4865608.3, mgm4865609.3, mgm4865610.3, mgm4865611.3, mgm4865612.3, mgm4865613.3, as well as under the following BioProject access numbers (NCBI): PRJNA636820, PRJNA636837, PRJNA636891, PRJNA637016, PRJNA637018.

The OTU matrix was initially normalized using the rarefy_even_depth function in Phyloseq as recommended in the literature (Weiss *et al.*, 2017; Estaki *et al.*, 2020). The rarefied datasets were transformed using *transform_sample_counts* in Phyloseq to obtain the relative abundance of each metagenome.

KMC taxonomic diversity

In order to evaluate and compare predicted microbiomes of different KMC cultures, alpha- and beta-diversity analyses of KMC communities were performed using statistical algorithms implemented in the program PAleontological STatistics (PAST) 4.02 (http://folk.uio.no/ohammer/past/) (Hammer et al., 2001). Alpha-diversity indexes, such as Chao-1 species richness, dominance, evenness and Shannon H diversity, were estimated separately for the bacterial, fungal, and viral KMC subcommunities.

Network analysis

Network plots were generated using the relative abundance data from all the samples: KMC_1b exposed at top position, KMC_2b – middle position, and KMC_3b – bottom position, on the sample carrier, comparing them to the initial ecotype iKMC (KMC_5) and the laboratory-kept (KMC_4b) control samples, which were also cultured during 2.5 years postflight KMCs, and designated (KMC_1c, KMC_2c, KMC_3c) and lab control (KMC_4c). The nodes represent functional genes from a specific sample (*i.e.*, function x in sample KMC_1 is a node, while function x in sample KMC_2 is a different node). These nodes were connected by edges that were placed when the relative read abundance value of two or more nodes were within the same range of ±0.01. For instance, two nodes with relative abundance of 0.232 and 0.241 were considered connected, because 0.232±0.01 and 0.241±0.01 are within the same

interval. After generating the networks, they were plotted using GePhi v.0.9.2 (Bastian, Heymann and Jacomy, 2009) and the nodes were sized based on their relative abundance value: nodes with higher abundance were also bigger.

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