Multimicrobial Kombucha Culture Tolerates Mars-like Conditions Simulated on Low Earth Orbit

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Abstract

A kombucha multimicrobial culture (KMC) was exposed to simulated Mars-like conditions in low Earth orbit (LEO). The study was part of the BIOlogy and Mars EXperiment (BIOMEX), which was accommodated in the European Space Agency's EXPOSE-R2 facility, outside the International Space Station. The aim of the study was to investigate the capability of a KMC microecosystem to survive simulated Mars-like conditions in LEO. During the 18-month exposure period, desiccated KMC samples, represented by living cellulose-based films, were subjected to simulated anoxic Mars-like conditions and ultraviolet (UV) radiation, as prevalent at the surface of present-day Mars. Postexposure analysis demonstrated that growth of both the bacterial and yeast members of the KMC community was observed after 60 days of incubation; whereas growth was detected after 2 days in the initial KMC. The KMC that was exposed to extraterrestrial UV radiation showed degradation of DNA, alteration in the composition and structure of the cellular membranes, and an inhibition of cellulose synthesis. In the "space dark control" (exposed to LEO conditions without the UV radiation), the diversity of the microorganisms that survived in the biofilm was reduced compared with the ground-based controls. This was accompanied by structural dissimilarities in the extracellular membrane vesicles. After a series of subculturing, the revived communities restored partially their structure and associated activities.

1. Introduction

Until recently, scientists assumed that microorganisms lived a selfish lifestyle, acting solely on behalf of their own survival (Manning and Kuehn, 2013). Based on this assumption, astrobiological studies on low Earth orbit (LEO) have mainly focused on microbial monocultures. Using this approach,

numerous studies have demonstrated the capability of microbial cultures to survive in the space environment (for reviews see Taylor, 1974; Horneck *et al.*, 2010). For example, in 1992, the EURECA facility exposed a suite of organic material and microorganisms to the conditions of space for 11 months (Dose *et al.*, 1995). This was followed by the development of the short-term exposure facilities Biopan (*e.g.*, de la Torre *et al.*, 2010) and Biokont (Ponizovskaya *et al.*, 2017). These facilities were used to investigate the effect of spaceflight conditions on the biological properties of microbes and small multicellular organisms for periods <2 months. More recently, with the installation of spaceflight hardware on the outside of the International Space Station (ISS), for example, BIORISK (Baranov *et al.*, 2009), EXPOSE-E (Rabbow *et al.*, 2012), and EXPOSE-R (Rabbow *et al.*, 2015), new evidence has been obtained regarding long-term survival of prokaryotic and eukaryotic organisms in LEO.

Astrobiology experiments in LEO can also be used to investigate the effect of simulated Mars surface conditions (*e.g.*, using an optical filter that cuts out short wavelengths [<200 nm] and exposing the samples in a Mars-like atmosphere that is dominated by carbon dioxide). Investigating the survival of microorganisms, and their associated biomarkers, under Mars-simulated surface conditions is a focal point of astrobiological research. This is due to the impact this has on finding evidence of life (Vago *et al.*, 2017) and planetary protection issues raised from unmanned and future human exploration missions to potentially habitable environments on Mars (Rettberg *et al.*, 2016). Ground-based simulation experiments have shown that in Mars analog soils spore forming microorganisms, for example, *Bacillus* and *Enterococcus*, can survive and pose a risk to surface sites where liquid brines are present (*e.g.*, Nicholson *et al.*, 2012; Schuerger *et al.*, 2017). To date, the number of microorganisms that have been screened to determine their survivability under Mars-simulated conditions is limited, and studies have predominantly focused on monocultures (for review see Olsson-Francis and Cockell, 2010).

In this article, the survival of a microbial community organized within a natural microecosystem is investigated under Mars-simulated conditions in LEO. This was a part of the BIOlogy and Mars EXperiment (BIOMEX), which was exposed to LEO on the EXPOSE-R2 facility outside the ISS. In natural microecosystems, it is widely accepted that microbial survival strategies are directed by the cooperation of different species against harsh environmental stressors, competitors, or predators (Kreft, 2004; Özkaya *et al.*, 2017). Such a social activity organizes microorganisms into stratified communities, which form biofilms that improve survival (Prakash *et al.*, 2003; Shuryak *et al.*, 2017). The first exposure experiments in space, using microbial communities, led to the conclusion that microbial cells were better protected in space if they were living as natural assemblages embedded in rocks; for example, the endolithic phototrophic community exposed to LEO for short (Olsson-Francis *et al.*, 2010) and long durations (Cockell *et al.*, 2011). Furthermore, a symbiotic partnership of prokaryotes and eukaryotes, as known for lichens, is beneficial for survival in LEO (*e.g.*, de la Torre *et al.*, 2010; Onofri *et al.*, 2012). These results are consistent with the concept of "massapanspermia" (Kawaguchi *et al.*, 2013), which suggests that microbial assemblage is a putative advantage toward microbial survival during interplanetary journeys.

To investigate the effect of simulated Mars-like conditions in LEO on community survival, a kombucha multimicrobial culture (KMC), which is a natural assemblage of probiotic bacteria and yeasts, was used as a self-regulative microecosystem (Kozyrovska *et al.*, 2012; Kukharenko *et al.*, 2012). Previous work has shown that sessile kombucha community members are protected by a cellulose-based film against harsh environmental factors (Williams and Cannon, 1989; Kato *et al.*, 2007). Preliminary ground-based experiments demonstrated that a KMC tolerated a low dosage of ultraviolet (UV)-C radiation, high vacuum, temperature cycling (between −10°C and +45°C), a Mars-like atmosphere and pressure (Podolich *et al.*, 2017a). In this article, we examined the effect of simulated Mars-like conditions, in LEO, on desiccated and partly mineralized (through living)

cellulose-based KMC biofilms, which contained both multicomponent prokaryotic and eukaryotic assemblages.

2. Materials and Methods

2.1. Microorganisms

KMC *Medusomyces gisevii* Lindau IMBG1 was obtained from the Institute of Molecular Biology and Genetics (Kyiv, Ukraine). It was maintained in black tea medium (*Camellia sinensis*) with white sugar (3% w/v). Previous phylogenetic analysis has demonstrated that the community was highly diverse (Reva *et al.*, 2015). Culturing (using methods described in Section 2.2) identified the yeast species: *Pichia* sp.; *Bretanomyces/Dekkera anomala*; *Zygosaccharomyces bailii*, and the bacterial species: *Komagataeibacter saccharivorans*; *Komagataeibacter intermedius*, and *Gluconobacter oxydans.*

2.2. Cultural media and cultivation conditions

The KMC was grown in filter-sterilized (0.2 µm Millipore filter) black tea (Lipton, 1.2%, w/v) with white sugar (3%, w/v) (BTS) medium, or with BTS and autoclaved apple juice (3%, final concentration). The pH of the medium was adjusted to 2.9 with acetic acid.

For isolation and cultivation of acetobacteria, nutrient media such as minimal medium A, Lysogeny broth (LB) (Miller, 1972), and HS (Hestrin and Schramm, 1954) were used. *Lactobacillus* was selected by using the medium MRS (de Man *et al.*, 1960). Yeast were isolated on Glucose Yeast Peptone agar (HiMedia Laboratories, India). After initial enrichments, the antibiotics cycloheximide (100 µg/mL; Sigma-Aldrich), which was selected for prokaryotes, and ceftriaxone (50 µg/mL; Roche Biochemicals), which was selected for yeast, were added to the relevant media to avoid contamination. The cultures were incubated at 28°C without shaking. One gram of wet KMC pellicle was homogenized in a sterile mortar with 0.2 mL of 0.9% NaCl. The homogenate was serially diluted into the medium and incubated at 28°C without shaking.

2.3. Biomineral sample preparation and exposure

Anorthosite (obtained from the Penizevitchi deposit, Ukraine) was crushed to produce a powder (fragment size between 0.1 and 1.0 mm), which was sterilized by autoclaving at 120°C for 40 min. The sterilized anort hosite powder was added to the BTS medium (20.0%), and the KMC was grown in the supplemented BTS medium at 28°C.

The cultures were grown until pellicles were formed, which were ~0.7 mm in diameter. The pellicles were mixed with an anorthosite egg white mixture to ensure that the culture was integrated with the mineral samples and desiccated, as described (Podolich *et al.*, 2017a). The samples were shipped from Kyiv to the German Aerospace Center (DLR) in Cologne.

Under sterile conditions, the KMC samples were integrated into their appropriate positions in the flight tray 2, which was used to study the effect of simulated Mars conditions. In orbit, tray 2 was kept in an inner artificial Mars atmosphere, consisting of a gas mixture composed of 95.55% CO_2, 2.70% N_2, 1.60% Ar, 0.15% O_2, and ~370 ppm H_2O (Praxair Deutschland GmbH) at a pressure of 980 Pa. The upper layer was exposed to extraterrestrial solar UV by using cutoff optical filters, with wavelengths >200 nm (UV-C) as prevalent on the martian surface. Therefore, the top-level KMC (tKMC) samples were exposed to UV-C radiation, whereas the middle (mKMC) and bottom (bKMC) levels were kept in darkness (protected from UV radiation) for reference (Supplementary Fig. S1; Supplementary Data are available online at https://www.liebertpub.com/suppl/doi/10.1089/ast.2017.1746). In parallel, a ground-based control

experiment (Mission Ground Reference) was carried out in the Planetary and Space Simulation Facilities at DLR in Cologne, Germany. Exposure conditions and the layout of the Mission Ground Reference experiment have been described in detail previously (Rabbow *et al.*, 2017).

2.4. Survival postexposure microorganisms

Survival of the KMCs after exposure was investigated by using growth experiments. Samples were transferred into sterile glass vials containing 5 mL of BTS medium under aseptic conditions. The cultures were incubated at 28°C until biofilms were visible.

The biofilms were rehydrated with 0.2 mL of 0.9% NaCl and disintegrated by shaker, which released the embedded KMC. The samples were then serially diluted (using 0.9% NaCl) and spread onto selective media with appropriate antibiotics (as described in Section 2.2). The ground-based samples were analyzed in parallel.

The survivability and resuscitation of the KMCs (including the laboratory and transportation controls) from a viable-but-non-cultivable (VBNC) state were also performed. For this, the KMC was serially diluted in BTS medium and incubated at 28°C for 2 weeks. The survivability of the culturable microorganisms was determined by using selective agar (as described in Section 2.2). This procedure was repeated three times until biofilms were formed.

Each of the isolates were identified by morphological features (Brenner, 1997), and confirmed with sequencing of the 16S rRNA (bacteria) and 26S rDNA (yeasts) genes, as previously described (Reva *et al.*, 2015). The sequence data have been submitted to the Genbank NBCI database under accession numbers: MG980168, MG650185, MG650186, MG650187, MG650188, MG993350, and MG993351.

2.5. DNA integrity postexposure

The DNA stability was examined by using a PCR approach, which was based on the inability of damaged DNA to amplify. This method is used as an alternative to the DNA comet assay when hydrolysis of microbial cells is problematic, for example, in fungi (Selbmann *et al.*, 2011). In this study, a mixed microbial community was assessed, which comprised yeasts and bacteria. The assay used was based on the principle that there is a higher probability of DNA damage in larger DNA regions. Hence, when these regions are analyzed by PCR, it is more likely to result in a decrease in amplicon size and/or yield.

For this analysis, total DNA was extracted from the KMC samples at the stage of biofilm formation. The DNA was extracted with the innuSPEED Bacteria/Fungi DNA isolation kit (Analytik Jena AG, Germany), and nucleic acids were quantified and qualified with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The bacterial 16S rRNA gene was amplified with primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 531R (5'-ACGCTTGCACCCTCCGTATT-3') and 8F and 1494R (5'-ACCTTGTTACGACTT-3'). The PCR mix (final volume 20 µL) contained 10 pmol of each primer, 20 ng of template DNA, and the BioMix buffer (Neogen, Ukraine). Amplification was carried out with a Thermal Cycler T-CY (CreaCon Technologies, The Netherlands) equipped with a heated lid. The PCR conditions were as follows: denaturing at 95°C for 5 min, denaturing at 94°C for 45 s, annealing at 56°C for 30 s, and elongation at 72°C for 60 s. The last three steps were repeated 30 times, and the final elongation was performed at 72°C for 10 min. Band intensity was measured and compared with the ImageJ software (https://imagej.net/).

2.6. DNA metabarcoding of the revived multiculture and bioinformatic analysis

Total DNA from the planktonic cells were isolated by using the innuSPEED Bacteria/Fungi DNA isolation kit (Analytik Jena AG, Germany) at the stage of biofilm formation (3 months after inoculation) and at stationary phase (6 months after inoculation). The DNA was sequenced with Illumina MiSeq technology (Macrogen, Seoul, Korea). The 337F (5'-GACTCCTACGGGAGGCWGCAG-3') and 805R (5'-GACTACCAGGGTATCTAATC-3') primers were used for partial sequencing of the 16S rRNA gene. The ITS was sequenced with the primers 4R (5'-TCCTCCGCTTATTGATATGC-3') and 3F-(5'-GCATCGATGAAGAACGCAGC-3').

Quality control was performed by a locally installed FastQC program. Poor quality reads with Phred quality score <20 (this corresponds to a *p*-value ≥0.05) were filtered out. Read binning was performed by using the online RDP Release 11 Classifier pipeline (Wang *et al.*, 2007). Filtered metagenomic datasets were deposited in the MG-RAST database server (www.mcs.anl.gov/project/mg-rast-metagenomics-rast-server) under IDs: mgm4776118.3, mgm4776119.3, mgm4776120.3, mgm4776121.3, mgm4776122.3, and mgm4776123.3.

Simpson's diversity indices (SDIs) of sequencing data were calculated as follows:

$$SDI = 1 - \frac{\Sigma n(n-1)}{n(n-1)},$$

where n is the number of reads binned to a specific taxonomic unit; N is the total number of binned reads.

In this experiment, SDI measured the probability that two randomly selected reads will be binned to two different species.

The Shannon diversity coefficient (H_a) was calculated as

$$H_a = -\sum_{i=1}^{s} p_i ln(p_i),$$

where S is the number of taxa identified in a sample and p_i is the frequency of the i's taxon in the sample. The Shannon coefficient is basically the entropy of the sample.

Jaccard distances (JD) between sequence data were calculated by the following equation:

$$JD = 1 - \frac{n}{N},$$

where n is the number of taxonomic units shared by two samples and N is the total number of taxonomic units identified in both samples.

2.7. Extracellular membrane vesicle isolation and visualization

KMC samples (3–7 day old) were centrifuged at 17,000 × g for 20 min at 4°C. The supernatant was collected and further ultracentrifuged at 100,000 g for 1 h, at 4°C (Beckman Instruments, Inc., L8M, rotor 55.2 Ti). The pellet was resuspended in sterile phosphate-buffered saline (PBS), pH 7.4. For vesicle preparation, the cell suspension was filtered through a 0.20 μm filter (Minisart, Sartorius, Germany).

For scanning electron microscopy (SEM), 2 µL of sample (2 mg/mL in protein) was added onto formvar-coated copper grids, and contrasted with 2.0% aqueous uranyl acetate for 30 s and dried in darkness for 20 min (reagents from Serva, Germany). The samples were analyzed by SEM (Mira 3 LMU: Tescan s.r.o., Czech Republic) using the SEM detector in bright field mode at various magnifications with an accelerating voltage of 10 kV.

The size of the extracellular membrane vesicles (EMVs) was measured using dynamic light scattering (DLS) (Zetasizer Nano ZS) and Zetasizer software (Malvern Instrumental Ltd.).

2.8. The electrophoresis mobility shift assay

Electrophoresis mobility shift assay (EMSA) is a rapid and sensitive method to detect nucleic acid complexation (Fried and Crothers, 1981; Garner and Revzin, 1981). Extracellular vesicles (5 mg/mL) were suspended in PBS (pH 7.4) and filtered (0.20 µm). Various concentrations (12.5–1.25 mg/mL) of EMSA were added to 300 ng of pTZ19R* DNA (Fermentas, Lithuania) or its *Eco*RI-linearized form. The final reaction mixture (20 µL) was incubated for 1 h at 37°C in diethyl pyrocarbonate (DEPC)-treated water. After incubation, the samples were run on an electrophoresis gel (1.2%) in tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.0). The stained gel was visualized by using a UV light and photographed on a UV transilluminator. The interaction of the EMVs with the DNA was indicated by a slower mobility of their complexes in the gel. All experiments were carried out in triplicate.

2.9. Attenuated total reflection Fourier-transform infrared spectroscopy

An attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) was used to obtain information regarding the macromolecular composition of the EMVs. The ATR method allowed the thickness of the investigated material to be increased due to the multiple reflectance of a beam from a prism surface. A 0.2–0.3 µL of EMV sample (2 mg/mL of protein) in PBS (pH 7.4) was mounted on a KRS-5 prism and covered by a second prism. The IR absorption analyses were carried out with a Bruker-113v FTIR. The measurements were performed at room temperature in the range of 500–4000/cm with a spectral resolution of 1.0/cm. The accuracy of line position was ±1/cm.

2.10. Statistical tests

Results are expressed as mean ± SEM of independent experiments. The difference between groups was analyzed by two-tailed Student's t test, and values of $p \leq 0.05$ were considered significant.

3. Results

3.1. Survival of the KMC community

Previous work has shown that a KMC normally forms a biofilm within 2 days postinoculation; however, it can take up to 2 months to form biofilm in stress-exposed KMC samples (Podolich *et al.*, 2017a, 2017b). In this study, during the recovery process, the KMC samples that had returned from LEO and also the laboratory and transportation control samples, as well as ground-based experiment ones, had completely blackened and become nontransparent after 7 days of culturing. This was despite the fact that the color of the samples was initially light brown (Fig. 1A). The change in color signaled the initiation of the metabolic activity; that is, bioleaching of elements in the anorthosite. At this stage, all of the KMCs were dominated by *Bacillus subtilis*.
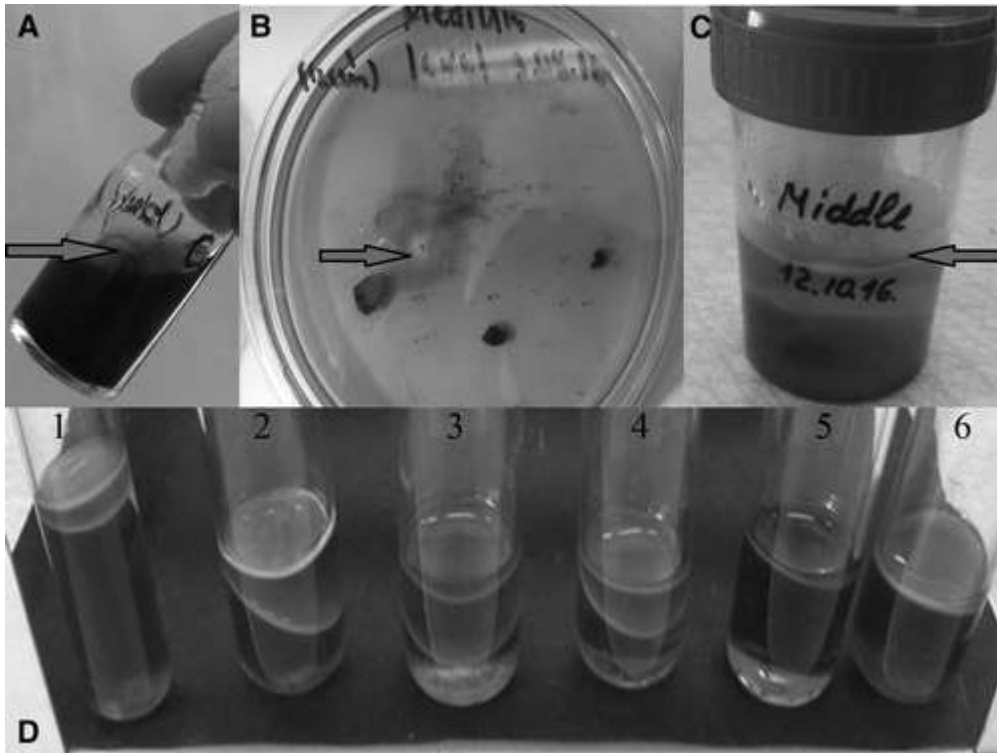
FIG. 1. Postexposure analysis of the KMC. **(A)** Biomineral samples of KMC (7 mm) were inoculated into a nutrient medium. **(B)** Once the medium turned black (due to metabolic activity), serial dilutions were carried out, and the samples were spread onto agar plates. The arrow indicates where a gel formation is occurring. **(C)** Serial dilutions, with the addition of sterile organics and lowering of the pH, resulted in a balance of a bacterial–yeast community, which finally produced a typical cellulose-based pellicle (indicated by the arrow). **(D)** Postflight analysis of cellulose synthesis by *Komagataeibacter intermedius* reisolated from the exposed KMC samples. Bacterial strains were grown in sterile HS (Hestrin and Schramm, 1954). Cellulose-based pellicle appeared after 3 days in all strains of the *Komagataeibacter* genus (from the control and exposed samples), except for *K. intermedius*, which was isolated postexposure. KMC = kombucha multimicrobial culture.

In the laboratory and transportation controls, after 13 days, a gel-like formation was observed on the vial walls, which was level with the surface of the medium and around the mineralized cellulose membrane retrieved from the samples (Fig. 1B). For the samples returned from LEO the addition of apple juice to the BTS medium increased microbial growth and resulted in significant revival of the mKMC populations. Colony-forming units (CFUs) on selective media demonstrated that the cellulose-synthesizing acetic acid bacteria reached 1.8×10^2 CFU/mL; whereas no acetic acid bacteria were recovered in the tKMC and bKMC samples.

After 22 days, a thin transparent gel-like film was observed floating on the surface of the mKMC culture medium; however, the film formed differed in thickness and transparency from the pellicle produced from the initial KMC. The partnering yeast growth was stimulated by successive dilutions and the addition of apple juice to the medium. Finally, for the tKMC- and bKMC-exposed samples, a decrease in pH to 2.9 was enough to balance the community and for a typical pellicle to form after 60–62 days (Fig. 1D). The KMC samples from the ground-based simulation experiment formed a cellulose-based pellicle a few days later.

3.2. Phylogenetic analysis of returned cultures

Total DNA was isolated from the KMC samples after recovery of the pellicle on the surface of the liquid culture. All of the KMCs recovered from the biomineral samples, including the laboratory

control and space-exposed samples, showed reduced species diversity compared with the initial KMC. This was confirmed by calculating the Simpson (SDI) and Shannon (H_a) diversity coefficients (Fig. 2).
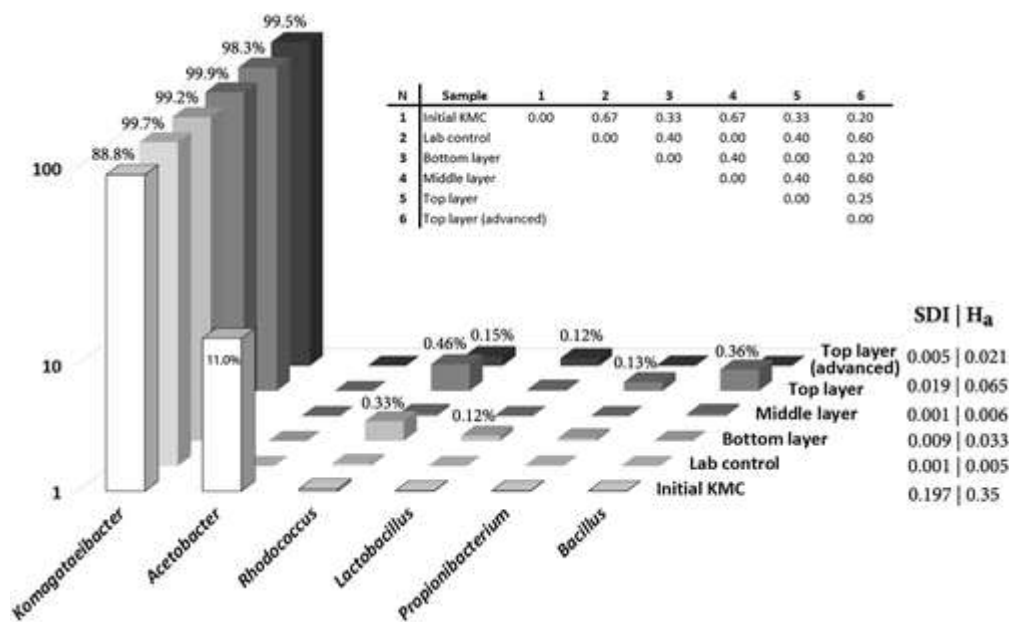

FIG. 2. Percentage of species identified in the KMC samples exposed to Mars-like conditions, in LEO, including UV radiation (the top layer), no UV radiation (the middle layer), and vacuum (the bottom layer). As controls, the initial and laboratory controls are included. Percentages were derived from 16S rDNA amplicon-based metagenomics. For each sample, the Simpson and Shannon diversity indices were calculated. The table demonstrates the calculated Jaccard distance coefficients between the samples. LEO = low-Earth orbit; UV = ultraviolet.

DNA read binning by the RDP pipeline and local BLASTN allowed identification of microorganisms to the genus level and a tentative species identification. The genus *Komagataeibacter* was the dominant bacterial taxon in all of the KMCs. For example, the genus represented between 88.8% (in the initial KMC) and 99.9% (in the mKMC) of the sequences. Read binning by BLASTN showed the highest similarity to 16S rDNA sequences of the species *Komagataeibacter europaeus*, close relative of *Komagataeibacter xylinus* and *K. intermedius* (Yamada *et al.*, 2012), and also to *K. saccharivorans* that is common in KMCs (Reva *et al.*, 2015). In the original KMC sample, the second most abundant taxon (11% of the binned reads) belonged to the genus *Acetobacter* but was not identified in any of the returned biomineral samples from the BIOMEX experiment, and only a few reads were binned to this genus in the laboratory control.

Interestingly, the samples from the tKMC, mKMC, and bKMC were diverse due to a higher frequency of reads binned to *Rhodococcus*, *Propionibacterium*, *Lactobacillus*, and *Bacillus* species. For example, the frequency of DNA reads associated with the genus *Rhodococcus* increased from 0.03% in the control samples to 0.33–0.46% in the tKMC, mKMC, and bKMC samples. These reads showed sequence similarity to several uncultured *Rhodococcus*. Other groups, which were identified as *Lactobacillus acidophilus* and uncultured *Propionibacterium* sp., had an increased number of binned reads in the exposed samples (from 0.004% in the control to 0.01% and 0.2%, respectively). The tKMC samples were contaminated by *Bacillus* spp. (0.36%). *Bacillus* species were not observed in the initial or the laboratory control KMCs (their presence in the exposed samples was confirmed by culturing). The majority of the *Bacillus* belonged to the species *B. subtilis* group, but at least 10% belonged to *B. pumilus*.

The yeast reads represented in the revived planktonic culture belonged to *Saccharomycetales* (*Zygosaccharomyces*) and *Pulchromyces.*

3.3. Viable postexposure microorganisms

The KMC core members of three yeast genera *Pichia*, *Brettanomyces/Dekkera*, and *Zygosaccharomyces*, as well as bacterial genus *Komagataeibacter* and *Gluconobacter,* were identified in the original culture (Reva *et al.*, 2015). In all of the exposed samples, *D. anomala*, as well as *G. oxydans* and *K. saccharivorans,* were not recovered. The only microorganisms that survived were the acetic acid bacterial species *K. intermedius*, and the yeast species *Z. bailii* and *Pichia manshurica*. Although opportunistic community members *B. subtilis* and *Bacillus pumilis* were also detected, they were not cultured in the controls. The pH of the medium was adjusted to avoid the overgrowth of *Bacillus* spp. The attempts to isolate *L. acidophilus* on MRS medium from the tKMC, mKMC, and bKMC samples after exposure were unsuccessful. Although characteristic catalase-positive colonies appeared on selective agar, they did not grow after transfer onto a new plate.

3.4. DNA integrity assay

After exposure the KMC DNA could accumulate lesions (*e.g.*, bulky adducts and single-strand breaks), which inhibit or block the action of DNA polymerases (Ponti *et al.*, 1991). Some of these lesions are repaired through nucleotide excision, if a repair system is not damaged. The integrity of DNA templates isolated from exposed and control samples was assessed by amplifying two 16S rDNA regions of different lengths (as the majority in KMCs was represented by bacteria after formation of a pellicle). PCR resulted in amplicons of the correct size of DNA being obtained. The electrophoretic agarose gel analysis showed that the 16S rDNA 523 bp fragment was well represented in all samples (Fig. 3A). However, bacterial community members from the tKMC accumulated more DNA lesions in the 1486 bp band than the mKMC and bKMC community members (Fig. 3B).
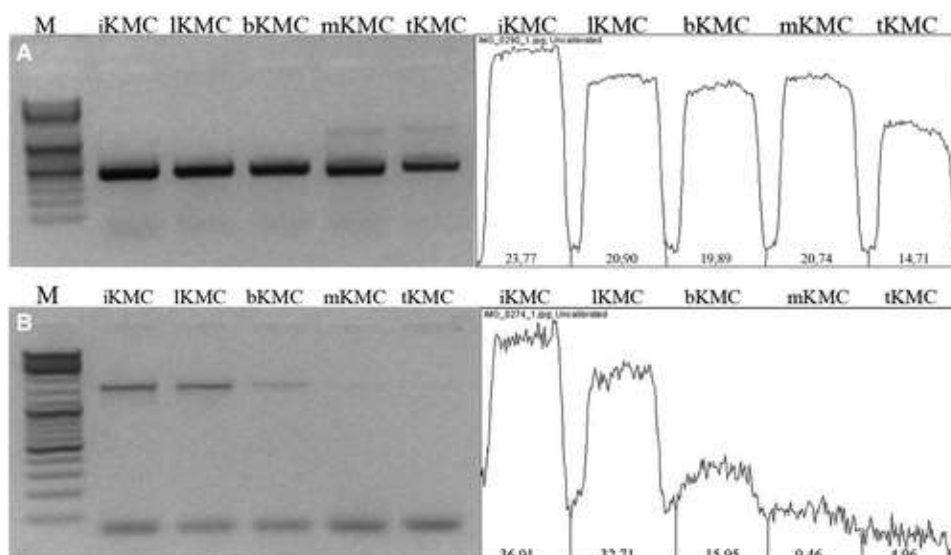

FIG. 3.   Assessment of DNA damage postexposure by amplification of the long DNA regions. Electropherograms in agarose (1.2%) (on the left) and densitograms (on the right) of 523 bp **(A)** and 1486 bp PCR products **(B)**. Lanes: M = GeneRuler DNA Ladder Mix (Thermo Scientific); iKMC = the initial kombucha culture (as a control); lKMC = a laboratory kept reference; bKMC = a bottom-level sample; mKMC = a middle-level sample; tKMC = a top-level sample.

3.5. The KMC metavesicleome morphology and functionality

3.5.1. Size distribution of the EMV populations in the KMC samples

A few species of bacteria and yeasts in an active KMC produced EMVs that range in size from 20 to 200 nm. Six to seven EMV populations consisted of 50% of metavesicleome, with a diameter of 141 and 164 nm (Supplementary Fig. S2A). After revival, the laboratory control KMC produced EMV populations that resembled the initial KMC (Supplementary Fig. S2B). The EMVs of the tKMC, mKMC, and bKMC samples were isolated and tested after the appearance of cellulose pellicle (2 months after inoculation). EMVs from the tKMC were characterized by both a small-size fraction (13–50 nm) and three larger fractions (122, 141, and 164 nm) (Supplementary Fig. S2C). The bKMC and mKMC specimens did not possess small-size fraction vesicles but contained two larger populations with diameters of 164 and 190 nm, which demonstrated a shift in the average sizes (Supplementary Fig. S2D).

Vesicle preparations were controlled by SEM. The results represented in Figure 4A–E match, in general, the results obtained from DLS for each KMC variant. However, fused vesicles in the tKMC are observed in Figure 4A–C; while the laboratory control produced EMVs with diameters of ≥500 nm (Fig. 4E). The majority of vesicle populations tested appeared to be single membrane; however, outer–inner bacterial vesicles were detected (Fig. 4F). Some deformations in vesicles and their aggregations were observed in the tKMC-exposed samples (Fig. 4C, F).
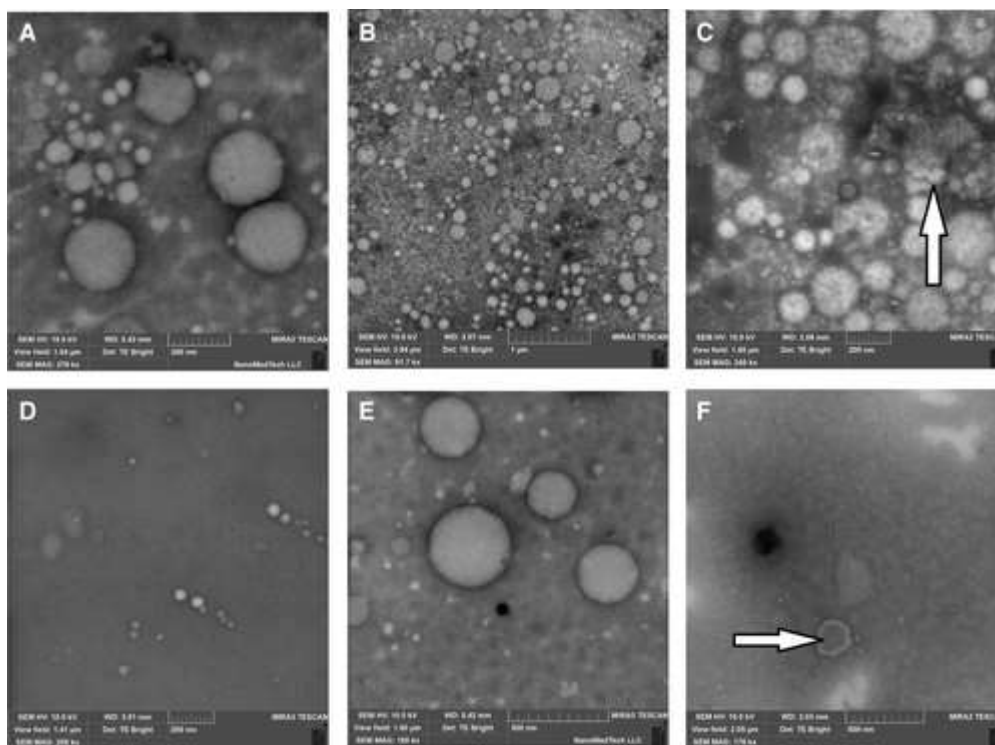

FIG. 4. SEM of the EMVs. **(A)** Population of the initial KMC-derived EMVs. **(B, C)** populations of EMVs from the KMC exposed to UV (the aggregations of EMVs indicated by an arrow). **(D)** The populations of EMVs derived from the KMC exposed on the bottom ("dark") layer. **(E)** EMVs from the laboratory control KMC samples. **(F)** A deformation of outer–inner membrane vesicle from bKMC (indicated by an arrow). Size bars denoted in figures. EMV = extracellular membrane vesicle.

3.5.2. The DNA-binding capacity of EMVs postexposure

In this study, the interaction of the KMC EMVs with different forms of the plasmid pTZ19R* DNA was examined. The electrophoretic patterns showed that at concentrations of EMVs between 12.5 and 1.25 mg/mL, the formation of EMVs–DNA complexes in a KMC was probable. This was demonstrated by a lower mobility in the agarose gel compared with the control DNA (Fig. 5A, B, 7–12). The mobility change was dependent on the EMV dose and the interactions with supercoiled, opened, and linearized plasmid DNA forms. The most pronounced shifts in the mobility of the DNA–EMVs complexes were seen after interaction of the EMVs with DNA linearized with *Eco*RI (Fig. 5B, 7–12).
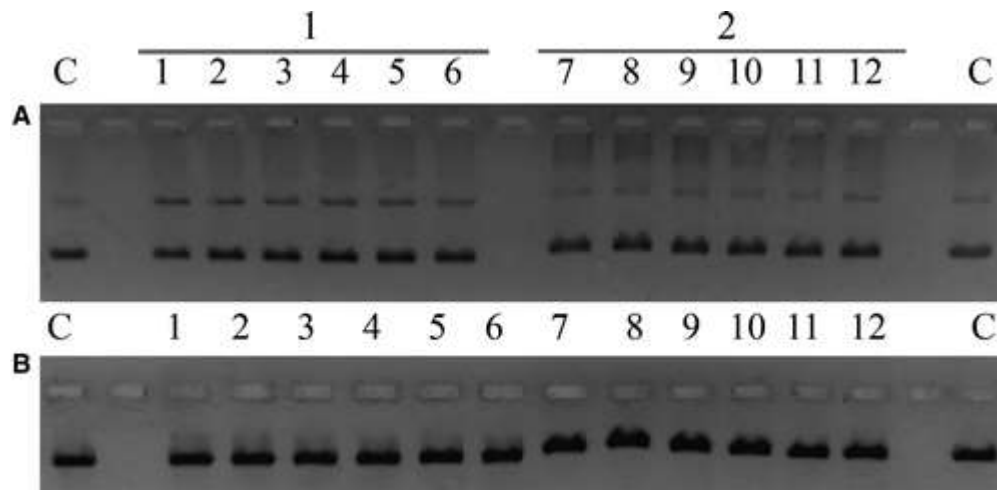

FIG. 5. Effect of interaction of **(A)** plasmid pTZ19R* DNA and **(B)** the *Eco*RI-linearized form with EMVs from KMC exposed to Mars-like conditions, in LEO (1) and from the initial KMC (2). Lane C is the reference plasmid pTZ19R* (supercoiled and open circular forms).

The combination of different forms of the pTZ19R* DNA with EMVs isolated from the tKMC (exposed to Mars-like stressors) did not exhibit the mobility shift (Fig. 5A, B, 1–6). The role of the EMV lipids and proteins in the interaction with the DNA will be assessed in our future research.

3.5.3. Effect of exposure on the cellular membranes

Bacterial EMVs originate from the outer/inner membrane by budding, and therefore have a very similar composition to the latter, which confers information on the structure of the bacteria (Guerrero-Mandujano *et al.*, 2017). Information regarding the effect of exposure on the microbial cellular membranes of the returned KMC was obtained by using the ATR-FTIR absorption spectra of the EMVs.

In this study, the ATR-FTIR absorption spectra of the EMVs from the tKMC, which were exposed to Mars-like conditions, were used to identify any effects associated with proteins/lipoproteins (1550–1660/cm), lipids (2880–2950/cm), and carbohydrates (925–1111/cm) (Fig. 6). Significant changes in the spectra were observed in the range of 2820–2950/cm (methylene groups in amino acids or fatty acids), 1570–1740/cm (amide I, amide II, and C = O group), and 900–1150/cm (carbohydrates, alkenes, and amino acids) for the returned samples compared with the initial KMC. The increase in band intensity at 1731/cm (lipid group C = O) was detected for the EMVs extracted from the tKMC, mKMC, and bKMC samples. The additional absorption bands with maxima at ~982 (carbohydrates) and 920/cm (alkenes) were registered for the EMVs from the bKMC samples, compared with EMVs derived from the initial KMC (Fig. 6A, spectrum 3). These features were less pronounced in the tKMC samples (Fig. 6A, spectrum 2). Symmetric phosphate stretching modes (probably, originated from

phospholipids or the phosphodiester groups in nucleic acids) in the EMVs from the initial KMC had maximum absorption at 1076/cm and were weakly detected in the EMVs spectra of the tKMC, mKMC, and bKMC samples. Bending (scissoring) vibration of the lipid acyl CH_2- groups (1455/cm), which was specific for the KMC EMVs, was recorded in the tKMC, mKMC, and bKMC samples with higher intensity than in the EMVs from the initial KMC. In general, shifts were observed for most of the absorption bands in the samples returned from LEO compared with the initial KMC. This indicated the presence of internal stresses in the samples exposed to Mars-like factors regardless of exposure to solar radiation. After a series of resubbing of the KMCs, the ATR-FTIR spectra of the EMVs from the exposed samples increasingly resembled the spectra of the control samples (Fig. 6B, spectrum 4).
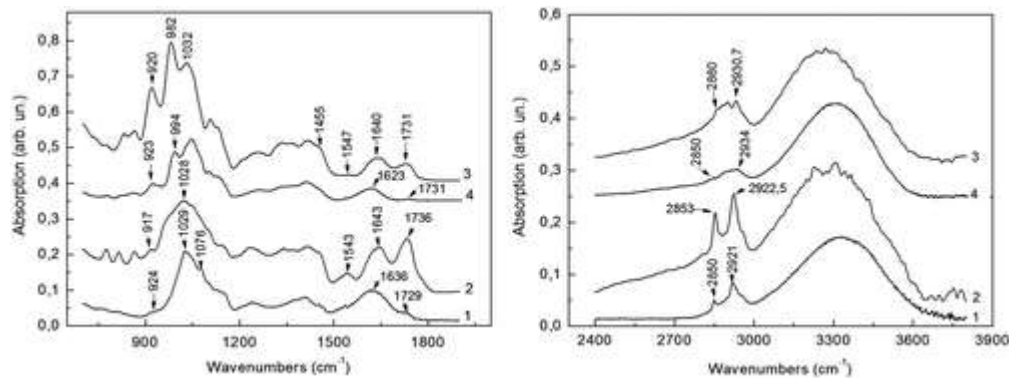

FIG. 6. Fragments of the ATR-FTIR absorption spectra (400–4000/cm) for EMVs originated from KMCs. (1) EMVs from the initial KMC; (2) EMVs from revived KMC after the exposure at the top level of the carrier; (3) EMVs from revived KMC after the exposure at the bottom level of the carrier; (4) EMVs from revived KMC after the exposure at the top level of the carrier and a further series of cultivations.

4. Discussion

In this study, the tolerance of a community of prokaryotes and eukaryotes to Mars-like environmental stressors in LEO was tested. Living desiccated kombucha pellicle samples were exposed outside of the ISS in a three-level carrier, which enabled discrimination between effects uniquely created by Mars-like solar irradiation and other Mars-like environmental factors. In postflight analyses, the focus was on studying the microbial survival and activity.

4.1. Microbial community survivability

The KMC was considered reactivated and functional if the yeast and bacterial community members formed together in a microecosystem and were able to produce a cellulose-based biofilm. In any KMC, the community structure may vary, but the metabolic partnering of core microorganisms, sugar-fermenting yeasts and cellulose-synthesizing acetic acid bacteria of the *Komagataeibacter* genus, is an absolute requirement.

The exposure experiment demonstrated that the Gram-negative acetic acid bacteria and certain species of yeast, which were part of a mineralized cellulose biofilm, were able to survive desiccation and Mars-like simulation conditions. It is known that acetic acid bacteria evolve under stringent unstable conditions in the environment and, as a result, have acquired adaptive mechanisms, including altering their membrane lipid composition (Trcek *et al.*, 2007). For instance, the synthesis of sphingolipids, which are ubiquitous components of the eukaryote cell membranes but are rarely found in bacterial cells, is related to the regulation of stress response elements under high temperature and low pH in acetic acid bacteria (Ogawa *et al.*, 2010). Yeast community members *Z.*

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bailii and *P. manshurica* also successfully survived within the kombucha biofilm after exposure to Mars-like conditions; however, another community member, *D. anomala*, did not survive. The survival of *Z. bailii* was shown in an earlier space experiment (Grigoryev *et al.*, 1972). In our case, survival may have been due to the KMC being desiccated in a mineralized cellulose matrix, which is known to protect cells from harmful (germicidal) UV doses (Williams and Cannon, 1989). It was also shown that in a dried state, microorganisms were significantly more UV tolerant than in a hydrated state (Bauermeister *et al.*, 2011).

Compared with the initial KMC, phylogenetic analysis showed a significant reduction in species diversity after exposure to LEO. The community was also different in the desiccated samples, which were kept in the laboratory during the flight experiment, implying that desiccation by itself affected the microbial composition of the KMC. Common for all the KMC samples exposed to conditions in LEO (tKMC, mKMC, and bKMC) was the absence of the *Acetobacter* representative, which was frequent in the initial KMC and reduced in the laboratory control. Surprisingly, the exposure of the KMC samples to space irradiation (tKMC) resulted in additional taxa, which were rare or absent in the initial KMC. A significant increase in the number of *Bacillus*, *Rhodococcus*, *Propionibacterium*, and *Lactobacillus* species was also observed. We hypothesize that these bacterial species were able to survive the Mars-like conditions in LEO, and hence were more readily revived due to their tolerance to the harsh conditions.

Rehydration of the KMC samples after returning from LEO, as well as the transportation and laboratory controls, showed a delay in the revival capacity. This was especially notable for the tKMC samples, which were exposed to UV radiation. It is known that harsh conditions such as desiccation and UV radiation induce the VBNC state in bacteria as a survival strategy (Vriezen *et al.*, 2012; Zhang *et al.*, 2015). The resuscitation of latent cellulose-synthesizing bacteria and yeast species in all of the samples returned from LEO, and in the transportation controls, occurred after subculturing with dilutions and correction for pH, which appeared to be promoted by a metabolic partnering. Cooperative metabolic interactions of bacterial and yeast populations led to a better survival/revival of the KMCs: the yeasts were involved in the fermentation of sugar to produce ethanol and carbon dioxide, and the acetic acid bacteria then fermented the ethanol and produced acetic acid. The reactivation of all samples returned from LEO happened 2 months later than in the laboratory control samples, and the survival capacity was the same as the ground-based simulation experiments performed earlier (Podolich *et al.*, 2017b).

Summarizing, we may assume that the survival of the KMCs after exposure to the harsh Mars-like conditions in LEO occurred due to a complex of resistance factors, including (1) evolutionary acquired adaptive/protective mechanisms, such as a cellulose-based film formation and a specific cell membrane lipidome; (2) metabolic partnering; (3) desiccation of the KMC film during sample preparation, leading to the priming of community members for better survival in the stressful environment. In ground-simulated astrobiological experiments, biofilm formation proved to be advantageous for surviving simulated Mars-like conditions for both monoculture (Frösler *et al.*, 2017) and microbial assemblages (Baqué *et al.*, 2013; Podolich *et al.*, 2017a).

In members of the microbial community that survived exposure in LEO, the genomic DNA was damaged. However, the DNA integrity was dependent on the sample location: DNA from the bacteria isolated from KMC exposed to UV irradiation (tKMC) was damaged to the highest extent. This is even taking into account an attenuated UV-C spectral region (200–280 nm), that is, in which the 100–200 nm range was filtered out. UV-C radiation is detrimental to living cells because it is directly absorbed by DNA, resulting in the formation of promutagenic cyclobutane pyrimidine dimers and single-strand breaks in the sugar-phosphate backbone of DNA (Pfeifer, 1997). This diminished

DNA integrity in the tKMC samples may explain the lower resuscitation capacity of the community members of these samples.

4.2. Morphology and activity of revived kombucha community members

Exposure to Mars-like conditions in LEO affected the EMVs produced by members of the KMC. Exposure resulted in EMVs exhibiting changes in the membrane lipidome, a decreased intensity of P–O–C vibrations, and an appearance of new bands that may reflect the destructive transformations of phospholipids and glycolipids.

There were also distinct differences in the ATR-FTIR absorption EMVs spectra between the three sets of exposed samples (tKMC, mKMC, and bKMC). In the tKMC samples, the UV irradiation increased absorption intensities of the $C=O$, CH_3, and methylene groups, showing pronounced alteration in the lipid. The UV-C impact may also result in the alteration of fatty acid membrane composition (Chatti *et al.*, 2015). Ionizing radiation is known to cause chemical transformations (breaking of bonds, knocking-out of components from bonds, changing the number and arrangement of bonds, etc.) (Mozumder, 1999). The knock-out components can remain in vicinity to the location of the knock-out event and form new bonds with "neighbors," or can diffuse over the sample and interact with other components to form new bonds. The energy received by the tKMC from the UV-irradiation could enhance its diffusion process, and additional absorption might occur as a result of an increase in the concentration of molecules (a change of the bonds) responsible for this absorption. Ionizing radiation induces lipid peroxidation, leading to an increase in membrane permeability, disruption of ion gradients, and other transmembrane processes (Reisz *et al.*, 2014). According to the current model, the plasma membrane is patchy, with distinct segregations of ordered liquid (rich in sterol, sphingolipids, and hopanoids) and disordered domains (rich in unsaturated lipids) (Huang and London, 2016). The perturbations in membrane structure caused by environmental stressors can lead to considerable changes in microbial fitness. In our case, we observed changes in cellulose-synthesizing activity from bacterial members of the tKMC. The substantially lowered cellulose yield observed could be explained not only by downregulated activity of the cellulose synthase but also by perturbations in the cellular membrane and an abnormal extrusion process of cellulose through the membrane outside of a cell.

The EMVs ATR-FTIR spectra derived from the non-UV-exposed samples showed the appearance of vibrations specific for alkenes and carbohydrates, which may testify some disorder in structured microdomains in the cell membrane. In the "dark" samples, cosmic ionizing radiation and other factors, apparently, led to a switch off of the bonds near a location of the knock-out component with the formation of alkenes. Ionizing radiation also damaged or inactivated other biopolymers, including proteins. These alterations in membranes could result in induced changes in the membrane fluidity, passive and active transport through the membrane, and in interactions of EMVs with biopolymers, nanobiostructures, cells, etc. The loss of the vesicle membrane capacity to bind plasmid DNA may be explained by alterations in the EMVs membrane composition of the postexposed "dark" KMC samples, by losing either the negative charge or DNA-binding proteins. Notably, the EMVs' changed membranes were restored partially after a few generations, and this may mean that the revived microorganisms lost their epigenetic memory for exposure to Mars-like conditions in LEO. It is known that bacteria use postreplicative DNA methylation for the epigenetic control of DNA–protein interactions (Casadesús and Low, 2006; Willbanks *et al.*, 2016). DNA methylation patterns can split clonal bacterial populations into epigenetic lineages that keep memory about a stressful environment, which may be translated in changed microbial fitness.

Together these results strongly indicate that the impact of Mars-related conditions, simulated in LEO, has led to changes in the microbial membrane composition. The rearrangements in the cell

membrane normally lead to defects in cell–cell signaling processes and transduction pathways, in which EMVs are involved, for example, in biofilm formation and protein secretion (López and Kolter, 2010). In the context of microbial communities, the EMVs contribute to interactions between cells of community members (Manning and Kuehn, 2013). Their role is also to transport safely different forms and types of complex biological information (*e.g.*, transcription regulators, enzymes, ATP, genetic materials, etc.) from microbial cells to the external environment or to the cells of other organisms (Guerrero-Mandujano *et al.*, 2017; Jan, 2017). The present data showed changes in the microbial EMVs of exposed KMC samples induced by Mars-like conditions, in LEO, compared with initial KMC.

KMC is well known as the producer of a popular soft drink (kombucha tea), commercially available and claimed to be beneficial for human health (Jayabalan *et al.*, 2014; Vīna *et al.*, 2014; Villarreal-Soto *et al.*, 2018). We have been able to brew kombucha tea from cultures that have been flown in space and exposed to Mars-like simulation conditions. The demonstrated robustness of KMC under simulated Mars conditions and its capacity to produce probiotic and prebiotic products demonstrated the importance of continuing to study the safety and functionality of kombucha cultures returned from space journey.

It is well known that space-related or simulated stressful conditions promote changes in microbial characteristics (Nickerson *et al.*, 2004; Wilson *et al.*, 2007; Abshire *et al.*, 2016) and host–microbial interactions (Kordyum *et al.*, 1983; Casaburi *et al.*, 2017). It is expected that a consumption of extra microbial cells with probiotics or synbiotics (a combination of probiotic and prebiotic) formulations would balance and replenish exhausted astronaut's gut microbiota; however, its impact should be well examined. In spite of a keen interest for the use of probiotics in the diet of astronauts (Voorhier and Lorenzi, 2016; Castro-Wallace *et al.*, 2017), the question of the in-flight safety of probiotic microbes remains unanswered. Moreover, there is little progress in the design, stability, and survival of probiotic strains under space and Mars-related stressful conditions. Further profound systemic studies will be required to determine the safety and stability of probiotic microorganisms. Microbes may not be the only players in probiotic products that affect the recipient's biology, and a safety of their EMVs should be evaluated according to the changed conditions.

5. Conclusion

A biofilm form of a KMC community was used as a terrestrial microecosystem model to study survival in Mars-like conditions simulated in LEO. Importantly, in the postflight period, the KMC restored the minimal community structure needed for the synthesis of cellulose and a restoration of the microecosystem by means of revival of the latent microorganisms. The KMC samples exposed to Mars-like environmental conditions were distinguished by (1) a longer period of resuscitation of their community members, (2) DNA degradation, and (3) defects in cellular membranes. The survival of the microbial community structure and associated activities were dependent on exposure to UV radiation. The "dark" samples, which had been fully protected against solar light, displayed a higher rate of survival than the top-located KMCs. The robustness of the self-regulated KMC microecosystem can be explained by the protective properties provided by the desiccated, mineralized cellulose-based biofilm, and by metabolic partnering. The results from this study demonstrate that a multimicrobial KMC can survive exposure to Mars-like environmental conditions in LEO. The work provides support to the Panspermia theory, contributes to the issue of planetary protection, and could help judge the possibilities for Mars habitability, which are in line with the initial goals defined for the BIOMEX project.

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Author Disclosure Statement

No competing financial interests exist.

Abbreviations Used

BIOMEX	BIOlogy and Mars EXperiment
CFU	colony-forming units
DLS	dynamic light scattering
EMSA	electrophoresis mobility shift assay
EMV	extracellular membrane vesicle
ISS	International Space Station
JD	Jaccard distances
KMC	kombucha multimicrobial culture
LEO	low Earth orbit
PBS	phosphate-buffered saline
SDI	Simpson's diversity indices
SEM	scanning electron microscopy
UV	ultraviolet
VBNC	viable-but-non-cultivable

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