ASTROBIOLOGY
Volume 17, Number 5, 2017

DOI: 10.1089/ast.2016.1480

Kombucha Multimicrobial Community under Simulated Spaceflight and Martian Conditions

O. Podolich,[1] I. Zaets,[1] O. Kukharenko,[1] I. Orlovska,[1] O. Reva,[2] L. Khirunenko,[3] M. Sosnin,[3] A. Haidak,[1] S. Shpylova,[1] E. Rabbow,[4] M. Skoryk,[5] M. Kremenskoy,[5] R. Demets,[6] N. Kozyrovska,[1] and J.-P. de Vera[7]

Abstract

Kombucha microbial community (KMC) produces a cellulose-based biopolymer of industrial importance and a probiotic beverage. KMC-derived cellulose-based pellicle film is known as a highly adaptive microbial macrocolony—a stratified community of prokaryotes and eukaryotes. In the framework of the multipurpose international astrobiological project ''BIOlogy and Mars Experiment (BIOMEX),'' which aims to study the vitality of prokaryotic and eukaryotic organisms and the stability of selected biomarkers in low Earth orbit and in a Mars-like environment, a cellulose polymer structural integrity will be assessed as a biomarker and biotechnological nanomaterial. In a preflight assessment program for BIOMEX, the mineralized bacterial cellulose did not exhibit significant changes in the structure under all types of tests. KMC members that inhabit the cellulose-based pellicle exhibited a high survival rate; however, the survival capacity depended on a variety of stressors such as the vacuum of space, a Mars-like atmosphere, UVC radiation, and temperature fluctuations. The critical limiting factor for microbial survival was high-dose UV irradiation. In the tests that simulated a 1-year mission of exposure outside the International Space Station, the core populations of bacteria and yeasts survived and provided protection against UV; however, the microbial density of the populations overall was reduced, which was revealed by implementation of culture-dependent and culture-independent methods. Reduction of microbial richness was also associated with a lower accumulation of chemical elements in the cellulose-based pellicle film, produced by microbiota that survived in the post-test experiments, as compared to untreated cultures that populated the film. Key Words: BIOlogy and Mars Experiment (BIOMEX)—Kombucha multimicrobial community—Biosignature—Biofilm—Bacterial cellulose. Astrobiology 17, 459–469.

1. Introduction

Within the last decade, the European Space Agency has initiated a series of space biology exposure experiments that were designed to exploit multi-user exposure facilities such as EXPOSE, which was installed on the International Space Station (ISS), and BIOPAN, on the retrievable satellite Foton. The general motivation has been to perform multipurpose assessments of terrestrial (micro)organisms in the open space environment. These platforms facilitate analysis of the impact of spaceflight factors (vacuum, cycling temperature drops, cosmic radiation) on terrestrial organisms. EXPOSE allows users to carry out different astrobiological experiments for extended periods of exposure to space conditions and solar radiation outboard of the ISS (Rabbow *et al.,* 2012, 2015). Experiments conducted on BIOPAN and EXPOSE-E facilities have shown remarkable survival characteristics of several of a varying number of organisms, for example, lichens and prokaryotic and eukaryotic phototrophs (Cockell *et al.,* 2011; Brandt *et al.,* 2015). A more recently deployed platform, EXPOSE-R2, was installed outside the ISS to provide an environment for four sets of experiments from August 2014 to February 2016.

Microorganisms, spores, protocell nanoglobules as probable ''seeds'' of life in the Universe, or their remnants can be recognized by their either unchanged or diagenetically changed

[1]Institute of Molecular Biology & Genetics of NASU, Kyiv, Ukraine.
[2]Pretoria University, Bioinformatics Center, Pretoria, South Africa.
[3]Institute of Physics of NASU, Kyiv, Ukraine.
[4]Radiation Biology Department, Institute of Aerospace Medicine, German Aerospace Center (DLR), Cologne, Germany.
[5]NanoMedTech LLC, Kyiv, Ukraine.
[6]ESA/ESTEC, Noordwijk, the Netherlands.
[7]Institute of Planetary Research, German Aerospace Center (DLR), Berlin, Germany.

but still detectable markers (Cohn, 1876; Arrhenius, 1903; de Gregorio *et al.,* 2013; Chen and Walde, 2010; Floss *et al.,* 2014; Saha *et al.,* 2014; Gill and Forterre, 2016). A number of molecular and biochemical markers associated with the viability of different forms of living organisms have been selected for study. In the framework of the multipurpose international project "BIOlogy and Mars Experiment (BIOMEX)," a variety of organo-mineral samples were integrated into the EXPOSE-R2 platform to study the vitality of prokaryotic and eukaryotic organisms and the stability of organic biomolecules as putative biomarkers (de Vera *et al.,* 2012). The definition of biomarkers may vary in the context of a number of disciplines, but here the definition includes molecules or compounds of biological origin that are indicative of life-associated activities (Lovelock, 1965; Aerts *et al.,* 2014; Georgiou and Deamer, 2014).

Microbial cellulose has been identified as a significant extracellular matrix component of biofilms, which plays a key role in colonization of extreme environments by prokaryotes (Ross *et al.,* 1991; Romling and Galperin, 2015). In harsh environments, cellulose-forming bacteria can endure dry, cold, osmotic, or heat stresses by way of protective biofilms. A biofilm is a structured microbial community enclosed in a self-produced polymer matrix that adheres the community to surfaces such that it can occupy particular eco-niches (Costerton *et al.,* 1999). The capability to synthesize cellulose has been documented in a wide variety of bacteria, including cyanobacteria, which occupy practically all terrestrial eco-niches in nature (see rev. by Romling and Galperin, 2015) and artificial confined environments (Hu *et al.,* 2015). Cellulose is believed to have appeared as early as 3.0–3.5 billion years ago (Nobles *et al.,* 2001) and is considered one of the most abundant polymers in nature (Ross *et al.,* 1991). In our previous studies, microbial cellulose produced by a kombucha microbial community (KMC) was substantiated as a possible biosignature of bacterial activity (Kukharenko *et al.,* 2012; Zaets *et al.,* 2014). During the preparatory stage of the spaceflight experiment BIOMEX, a series of ground-based tests (Experiment Verification Tests and Science Verification Tests, EVTs and SVTs, respectively) was performed, which indicated an optimal scenario for the development of organo-mineral specimens and associated methods of postflight analyses for the study of pellicle microbiota and cellulose structural integrity. Desiccated and partly mineralized, though living, cellulose-based pellicle films, which comprise multicomponent prokaryotic and eukaryotic microbial assemblages residing in a latent form, were used in preflight tests.

2. Materials and Methods

2.1. Microorganisms

The kombucha microbial culture (KMC) *Medusomyces gisevii* Lindau IMBG1 was obtained from the collection of microorganisms of the Institute of Molecular Biology and Genetics (Kyiv, Ukraine). This culture was maintained in a filter-sterilized black tea (*Camellia sinensis*) (Lipton, 1.2%, w/v) with white sugar (3%, w/v) (BTS) at 28°C.

2.2. Cultural media and cultivation conditions

Nutrient media A, LB (Miller, 1972), and HS (Hestrin and Schramm, 1954) were used for bacterial growth; for yeast culturing, glucose yeast peptone medium (HiMedia Laboratories, India) was used. Antibiotics cyclohexymide (100 μg/mL, Sigma-Aldrich) against yeasts and cephtriaxon (50 μg/mL, Roche Biochemicals) against bacteria were applied to avoid culture contamination. Identification of isolated bacteria and yeasts was based on morphological and cultural characteristics and then proven by PCR and sequencing of amplified genetic markers (see below).

2.3. Geological samples

Anorthosite rock samples were obtained from the Penizevitchi deposit (Ukraine) (Mytrokhyn *et al.,* 2003). Rocks were fragmented in particles of 0.1–1.0 mm, sterilized by autoclaving at 120°C for 40 min, and then added to KMC culture as an additive (20.0%).

2.4. Biomineral sample preparation

2.4.1. Experiment Verification Test 1 (EVT-1). For EVT-1, aliquots of sterile powder of anorthosite were mixed in a sterile mortar with a sterile dry egg white powder in proportion 1:1. All steps in this section were performed under aseptic conditions. The mixture was homogenized by stirring in the mortar. KMC samples grown in BTS for 7 days under 28°C were pelleted (5000*g,* 2 min, +4°C). The pellets were stirred with the mixture of rock and egg white powder (1:10) followed by adding minced pellicle. With a sterile spatula, the mixture was filled into the holes of the sterile (autoclavation) device for tablet fabrication that was constructed for this study (Supplementary Fig. S1.A, available online at www.liebertonline.com/ast). KMC samples in the form of tablets were collected in sterile Petri dishes and kept at 28°C for 1–2 days for desiccation (Supplementary Fig. S1.B). For the repeated experiment on EVT-1, KMC was grown in BTS supplemented with the anorthosite powder (20% v/v) in stationary conditions, and the 7-day-old KMC pellicle fragments were mixed with the anorthosite and egg white mixture.

2.4.2. Experiment Verification Test 2 (EVT-2). KMC was grown in BTS supplemented with the anorthosite powder (20% v/v) in stationary conditions, and the 21-day-old KMC pellicle fragments were mixed with the anorthosite–egg white mixture to integrate them with the mineral samples.

2.4.3. Science Verification Tests. KMC was grown in BTS supplemented with the anorthosite powder (20% v/v). The 21-day-old KMC biofilm fragments ($d = 7$ mm) were built inside the organo-mineral mixture as described above. Samples were delivered to Cologne by fast post and accommodated in 16-well aluminum sample carriers with flat lower surfaces provided by the German Aerospace Center (DLR).

2.5. Isolation of microorganisms from biomineral samples

The tablets were placed into 5 mL of sterile sugared tea infusion (BTS) and kept overnight; the next day the tablets were minced, and samples were inoculated into BTS and incubated for 30 days under stationary conditions as was the case

for KMC culturing mentioned above. Aliquots of the culture were plated on the selective agar media with appropriate antibiotics for bacterial or fungal growth (see Section 2.2).

2.6. Isolation of cultivable forms of KMC members entrapped in the cellulose-based biofilm

For isolation of microorganisms, 1 g samples of wet KMC pellicle were homogenized in a sterile mortar with 0.2 mL of 0.9% NaCl. The homogenate was serially diluted in the same solution and spread on selective media as mentioned in Section 2.5.

2.7. Species identification

The cultivable kombucha community members were identified by morphological features and confirmed by sequencing and analysis of the PCR products of marker genes (16S rRNA for bacteria and 26S rRNA for yeasts), as has been described previously (Reva *et al.,* 2015).

2.8. Randomly Amplified Polymorphic DNA (RAPD) fingerprinting

Total DNA samples from treated and untreated organo-mineral specimens were isolated with the innuSPEED Bacteria/Fungi DNA isolation kit (Analytik Jena AG). The nucleic acids were quantified and qualified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Two RAPD primers, OPO10 (5′-TCAGAGCGCC-3′) for proteobacteria (Lee *et al.,* 2012) and OPX-03 (5′-TGG CGCAGTG-3′) for yeasts (Echeverrigaray *et al.,* 2000), were chosen for amplifying kombucha microbial DNA in the experiment, as it provided reproducible and discriminatory banding patterns. The PCR mixture consisted of a 100 ng microbial genomic DNA, 2.0 μL 10×PCR buffer with 20 m*M* $MgCl_2$, 2.0 μL 10 m*M* dNTPs, 1 U Taq polymerase, and 20 p*M* RAPD primer. Sterile ultrapure water was added to a final volume of 20 μL. The reaction was run for 40 cycles in the following condition: denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 1 min. An initial denaturation for 4 min at 95°C and a final 7 min extension at 72°C were applied. Products of the amplifications were resolved on 2.5% agarose gel, stained with ethidium bromide, and visualized under UV light. The gels were photographed and analyzed with the PyElph 1.4 program. RAPD patterns were clustered by using the unweighted pair-group method with arithmetic average (UPGMA).

2.9. Biofilm microscopic analysis

2.9.1. Confocal scanning laser microscopy (CSLM). Samples were fixed in formaldehyde vapor for 1 h and stained with calcofluor (excitation 405 nm, filter BP 420–480), ethidium bromide (Sigma, USA; excitation 514 nm, filter BP 530–600 nm), and thiazine dyes (excitation 514 nm, filter BP 530–600 nm). A microscopic examination of sample fluorescence was performed with confocal scanning laser microscope AXIOSKOP-2 ZEISS equipped by the LSM 510 PASCAL (CarlZeiss, Germany) software.

2.9.2. Scanning electron microscopy/energy-dispersive X-ray spectrometry (SEM/EDXS) microanalysis. The Tescan Mira 3 LMU (Tescan s.r.o., Czech Republic) scanning electron microscope, which was equipped with an energy dispersive spectrometer (EDS/EDX), Oxford X-max 80 mm (Oxford Instrument, UK) controlled by Inca Energy analysis software, was used to provide chemical elemental analysis. Samples of the studied biofilm (5×5 mm) were placed on a specimen mount and dried in the microscope under low pressure.

2.10. Fourier transform infrared (FT-IR) spectroscopy

Infrared Fourier spectroscopy was used for the assessment of structural differences in untreated and treated encrusted cellulose-based matrices. Each cellulose sample was air-dried on a glass slide in the form of a thin film. The film thickness was 0.025–0.03 mm. The IR absorption analysis was carried out with a Bruker-113v Fourier transform spectrometer. The measurements were performed at room temperature in the range of 50–4000 cm^{-1} with a spectral resolution of 1.0 cm^{-1}.

2.11. Test facilities and exposure conditions

2.11.1. Experiment Verification Tests procedure. Two ground-based preflight EVTs, designed to simulate spaceflight and, in part, martian conditions on the EXPOSE-R2 platform outside the ISS (EVT-1 and EVT-2), were performed by using the Planetary and Space Simulation facility (PSI) 2 at the Institute of Aerospace Medicine (DLR, Cologne, Germany). Biomineral samples were exposed to the following conditions: vacuum (10^{-5} Pa, as expected to prevail during the space flight, 7 days), temperature fluctuations (-25/+60°C), temperature cycling (66 cycles 8 h each, 2 h at -10°C ± 1°C, 2 h at +45°C ± 1°C, 2 h each for cooling and heating). The temperature was monitored with a sensor attached to the inner side of the sample carrier. The SOL2000 was used without optical filters to emit polychromatic UV rays (installed at DLR, Cologne, Germany). In EVT-1, the irradiation with monochromatic UVC (254 nm) was applied up to the final dose of 10 kJ/m^2. Within EVT-2, samples responded to enhanced polychromatic UV radiation. For the polychromatic irradiation experiment, unexposed ''dark'' samples served as the control for the irradiation tests. A simulated martian atmosphere (CO_2 gas composition, pressure 10^3 Pa) was used in the EVT and SVT-1, 2. All samples were used in triplicate. The exposure to simulated conditions in EVT-1 and EVT-2 was carried out by placing the samples in the carrier at appropriate positions. EVT-1 and EVT-2 were performed twice.

2.11.2. Science Verification Test hardware. In SVT, samples were arranged in three layers of flight-identical ground carriers (four specimens in each one): in the top of the UV-exposed layer, and dark positions in the medium and bottom layers of tray 2. The tray was attached to PSI 2 for the addition of Mars gas at 10^3 Pa (Fig. 1).

The SVT exposure period started on December 10, 2013, and lasted until January 14, 2014. A total UV fluence of 5.5×10^5 J/m^2 for the biologically active wavelength range of 200–400 nm was applied, which simulated a 1-year mission exposure. Irradiation was performed discontinuously to allow a constant monitoring of the temperature and the cryostat function to avoid heating of the sample during the irradiation. Temperature was controlled by sensors attached to tray 2. The temperature of the tray structure never

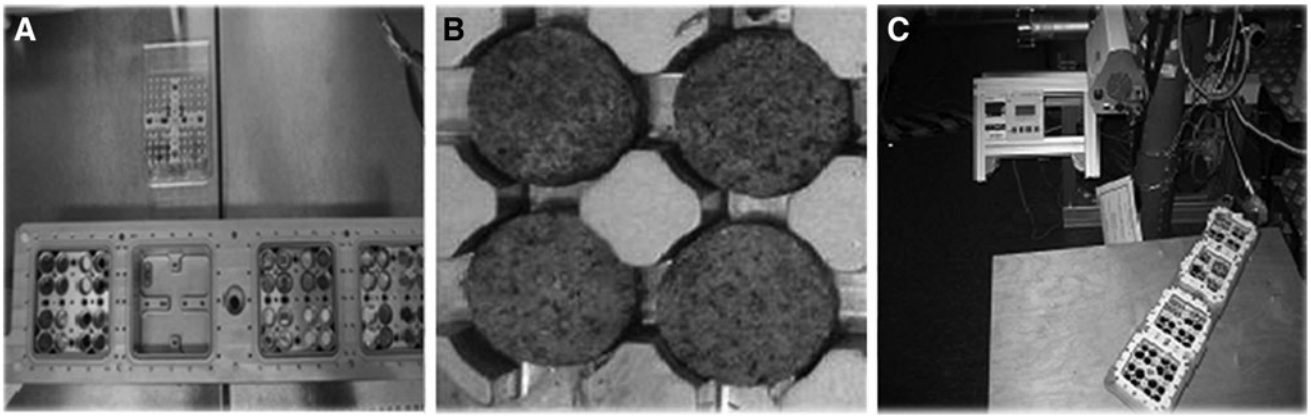

FIG. 1. Tray 2 integration of sample carriers and filter frames. Biomineral samples in a lower sample carrier (**A**); macroscopic image of samples (**B**); tray 2 attached to the PSI (**C**). Gas, simulating the martian atmosphere, was added to a final pressure of 10^3 Pa. (Credit: DLR)

exceeded 10°C during irradiation. Between irradiations, samples were cooled to −25°C. Tray 2 was connected to the vacuum facility PSI 2 (Fig. 1C) and evacuated to 1.3×10^{-3} Pa. The PSI 2 recipient with the attached tray 2 was flooded with Mars-like gas composed of 95.55% CO_2, 2.70% N_2, 1.60% Ar, 0.15% O_2, ~ 370 ppm H_2O, provided by Praxair Deutschland GmbH, to a final pressure of 10^3 Pa. The tray was disconnected from PSI 2 and accommodated on the temperature control interface.

Samples were glued as for the flight (space-approved non-outgassing glue Wacker-silicone RTV-S 691 A + B, prepared from the two components according to the manufacturer's manual) and integrated under sterile conditions into the appropriate sample carriers at their positions.

2.12. Statistical tests

The significance of differences between means from three samples was based on the Student's t test ($p < 0.05$).

3. Results

3.1. Preflight tests: influence of spaceflight and Mars-like factors

3.1.1. Microbial community survival and biofilm formation. For EVT-1, biomineral samples were developed in the form of tablets under aseptic conditions by using pristine kombucha pellicle fragments and cell precipitates mixed with sterile anorthosite powder. For EVT-2, mineralized pellicle was used instead of the pristine one. Supplementary Table S1 shows data on survival and biofilm formation by kombucha multimicrobial culture after irradiation in the frame of the EVT-1 and EVT-2 (run 1) tests after a week- and month-long period of cultivation of treated and control specimens.

In the first preflight, the EVT-1 experiment was performed at a pressure that simulated the martian atmosphere and in vacuum (10^{-5} Pa); biofilm-producing microbes survived and produced thin cellulose-based pellicles in contrast to the laboratory control culture, which produced a more rigid pellicle (Fig. 2A). Short-wavelength UVC radiation was used in the preflight test programs. Four fluences (8,000–10,000 J/m^2) of monochromatic UVC were provided for the evaluation of the dose-effect response of the biomineral samples. After the UV doses of 1,000–10,000 J/m^2, irradiated KMC variants exhibited lysis, which is in contrast to laboratory and transportation controls (Fig. 2B). Nevertheless, the lysed cultures recovered within a week and produced new pellicle. In EVT-1, run 2, mineralized biofilm fragments were used, and no culture lysis occurred. However, a delayed recovery of cellulose-forming bacterial strains was reported. The polychromatic irradiation used simulated the terrestrial UV spectrum without the ozone layer but with the absorption of the terrestrial atmosphere. It was applied as the most deleterious space factor; its influence is lethal to most biological samples. At this condition, KMC retained its cellulose-producing capacity up to 14,000 J/m^2 during 3 h, although the production of the cellulose decreased under the impact of this stressor. Under two higher irradiations with fluences that simulated possible mission durations of 12 months with 5×10^5 J/m^2 and 18 months with 8×10^5 J/m^2, bacterial and fungal members of the cellulose-forming community did not germinate within 30 days. However, *Bacillus* sp. was recovered from the samples irradiated with these high UV doses.

3.2. Preflight EXPOSE-R2 SVT tests: the cumulative effect of simulated stressful factors

For the SVT exposure, biomineral samples were modified as mentioned above and prepared according to the new protocol. In addition to survival and biofilm formation tests, structural integrity of both bacterial cellulose (BC) and the total community DNA after exposure to the stressful factors was tested.

After the flight transportation from Kyiv to Cologne, the specimens were in good condition and were glued into the sample wells of all three layers of tray 2. Once situated in the tray, the samples were exposed to Mars-like conditions, that is, a Mars gas mixture at reduced pressure conditions of 10^{-3} Pa and UV wavelengths >200 nm as would be expected on Mars.

3.2.1. Microbial organism survival and biofilm forming. After the SVT exposure, specimens from the top layer did not exhibit visible bacterial growth in nutrient media after 1

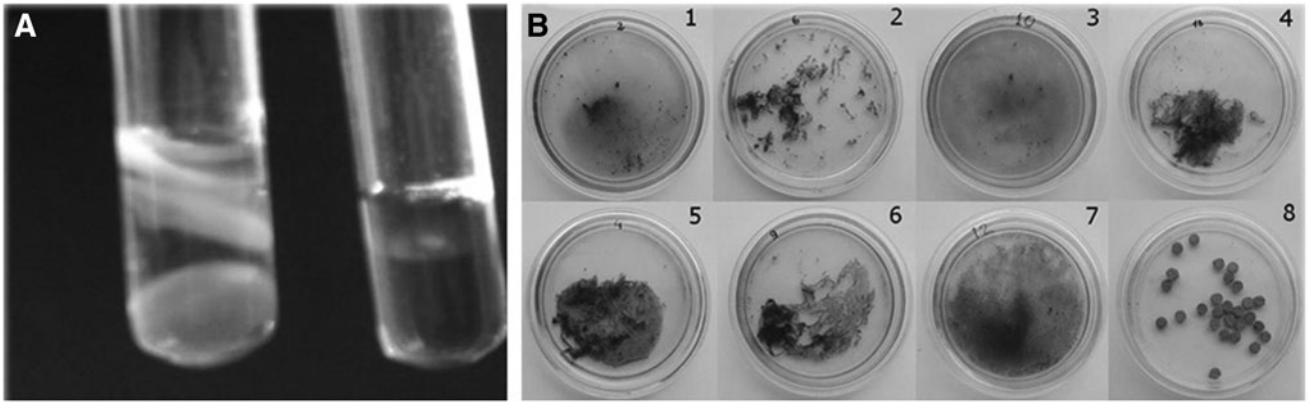

FIG. 2. Post-treatment experiments on recovery of kombucha microbial culture from biomineral samples. (**A**) Biofilm-producing microbes that survived in the simulated martian atmosphere; after revival, kombucha microbial culture retained its cellulose-producing capacity, although the production of cellulose decreased (left: a control, untreated culture; right: the tested kombucha culture, in vials). (**B**) A view of a 21-day kombucha microbial culture grown in Petri dishes after a treatment with different spacelike factors (1: a Mars-like atmosphere; 2, 4–7: UV irradiation of doses 10,000, 1,500, 1,000, 100, and 8 J/m^2) during the preflight EVT-1 as compared to a laboratory control culture (3). Biomineral samples, consisting of anorthosite and the pristine kombucha biofilm fragments (8), were tested, using the Planetary and Space Simulation facilities at the Institute of Aerospace Medicine (DLR, Cologne, Germany).

month of incubation. However, analogous specimens (from the middle and bottom layers), which were protected from UV radiation by the top layer, showed that in a Mars-like atmosphere and pressure, the minimal components of a kombucha community had survived—*Komagataeibacter* spp., *Pichia* sp., *Zigosaccharomyces bailii*. *Dekkera anomala* and *Gluconobacter oxydans,* however, despite the fact that they are among major KMC members at normal conditions, were not observed. In the samples from the bottom and medium layers, the cellulose-forming bacteria exhibited the ability to produce biofilm after revival within 5 weeks. In contrast, the laboratory and transportation control samples produced the film within 7 days.

3.2.2. SEM/EDXS microanalysis of the BC-based membranes after SVT. In our previous study, we showed that pristine pellicle BC-based membranes included several chemical elements that originated from tap water and were also extracted from tea leaves and sugar used in the nutrition medium composition (Zaets *et al.,* 2014). In the presence of anorthosite rock, KMC members bioleached inorganic ions and accumulated them to a larger extent on the bottom side of the pellicle film than would occur with KMC grown without anorthosite. In the present study, a number of detected elements in films accumulated by bacteria that survived after SVT-related stressors within tray 2 (middle and bottom layers) were unexpectedly low compared to native film (Fig. 3A). With the exception of organogenic C and O elements, Ca and K were detected, while the energy-dispersive X-ray spectra of films produced by untreated KMC uncovered Ca, Al, Si, K, Cl (Fig. 3B).

3.2.3. Molecular characterization of total KMC DNA by the RAPD fingerprinting method. The DNA specimen isolated from the dry laboratory control sample reproduced the same RAPD/PCR DNA band patterns as were observed for the sample that was isolated from live KMC and originated from the control sample. However, visible differences were detected between RAPD/PCR patterns of DNA bands from laboratory dry sample and the samples from the middle-layer and top carriers for both bacterial and yeast populations (Fig. 4).

3.2.4. CSLM analysis of biofilms. The revival kombucha culture (from the middle-layer tablets) produced mineral grains (ø 0.09–0.025 mm), which were observed in the bottom side of the encrusted cellulose-based film (Fig. 5A, 5B). Parental culture also produced mineral grains in the presence of anorthosite (Fig. 5C); however, the average size of formed grains was smaller (ø 0.02–0.01 mm). Control cellulose-based pellicle grown in the absence of anorthosite was without visible mineral depositions (Fig. 5D).

On the bottom side of the pellicle produced by KMC after SVT (the middle-layer pills), the microbial landscape differed from the latter in control pellicles by morphology and abundance of kombucha populations (Fig. 5A, 5D). This may serve as additional evidence that some changes occurred in the structure of KMC after the impact of SVT factors.

3.2.5. FT-IR spectroscopy of mineralized BC after SVT. The IR spectra of the cellulose biofilm matrix samples were taken to detect changes that could be attributed to the impact of simulated martian conditions on the BC structure. Figure 6a shows the absorption spectra of BC-based matrix produced by the kombucha culture restored after SVT. By FT-IR spectroscopy, the BC from biomineral samples (the middle-layer tablets) was found to be indistinguishable from the cellulose produced in the presence of anorthosite (Fig. 6b). The treated KMC produced cellulose, which preserved spectral feature characteristics (*e.g.,* 960–730 cm^{-1}, the fingerprint region of anomeric carbons, wherein a band at 897 cm^{-1} confirmed the presence of β-1,4-linkages specific for cellulose polymer). However, several spectral differences were observed between mineralized (Fig. 6b) and native cellulose (Fig. 6c), which resulted most likely from an accumulation of metals bioleached from the rock in the cellulose matrix (Zaets *et al.,* 2014).

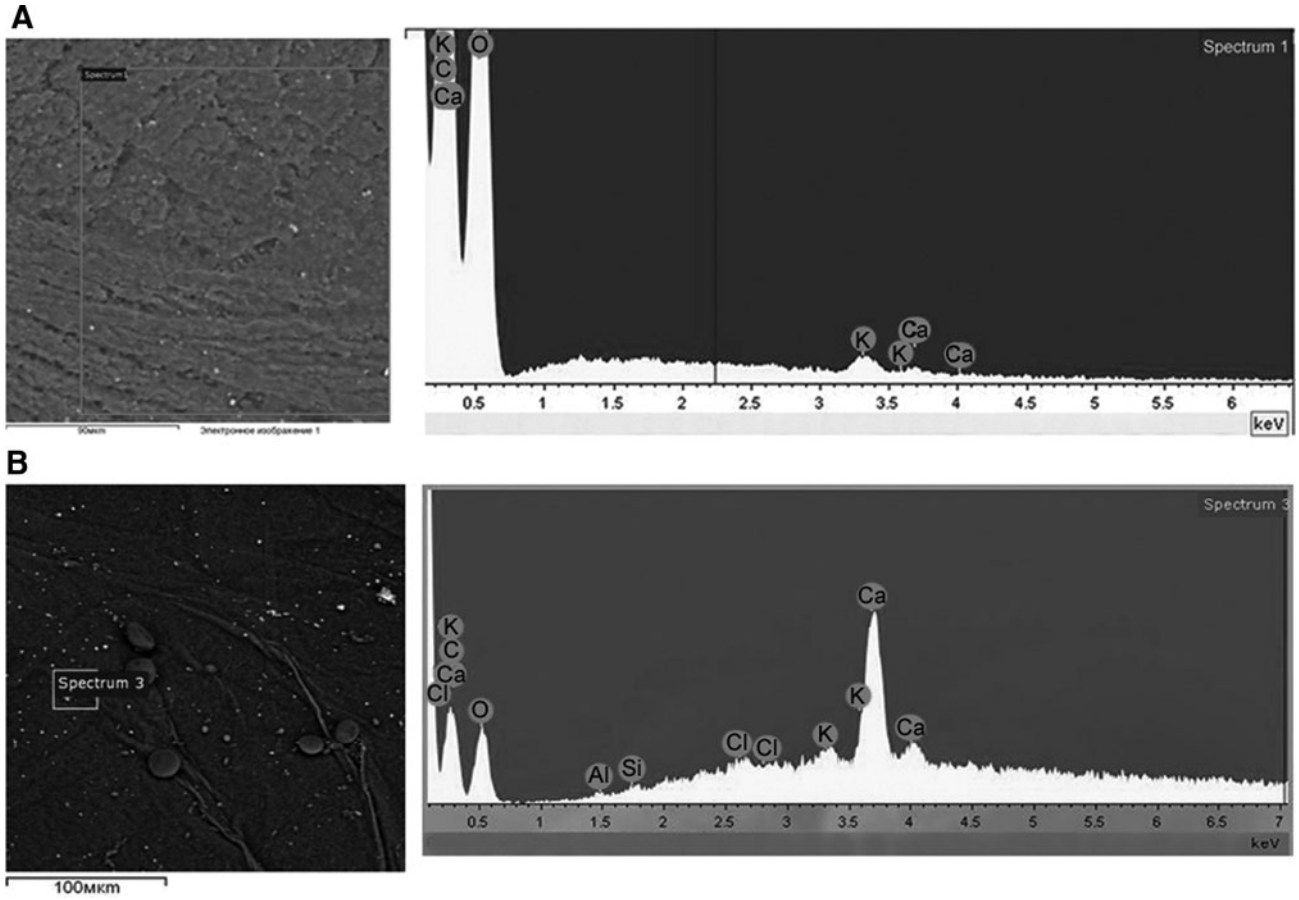

FIG. 3. Scanning electron micrographs (left) and energy-dispersive X-ray spectra (right) of the cellulose-based pellicle film produced by survived cellulose-forming bacteria after the impact of space- and Mars-related stressors in SVT (total UV fluence was 5.5×10^5 J/m^2 for 200–400 nm; Mars gas at 10^3 Pa) (**A**) and by pristine kombucha culture (**B**).

4. Discussion

The focus of this study was to prove the idea of stability of BC as a putative biosignature under preflight simulated conditions. This mini-project consisted of a few successive phases, from the original conception (Kukharenko *et al.,* 2012) and laboratory preparatory research (Zaets *et al.,* 2014; Kharina *et al.,* 2015; Reva *et al.,* 2015; Podolich *et al.,* 2016) to the multistep simulation experiments in which PSI 2 was used (DLR, Cologne, Germany). For the preflight ground experiments, the multimicrobial cellulose-based KMC pellicle films were embedded in the rock material to test, at the outset, the integrity of BC polymer in a Mars-like CO_2 atmosphere under solar radiation that mimics the solar spectrum at the surface of Mars. While the structural integrity of cellulose polymer examined with FT-IR spectroscopy was not disturbed by experimental conditions, the survival capacity of the KMC members depended on exposure conditions within the experimental tray, as well as on the method of specimen preparation.

4.1. Links between stress responses and cellulose structural integrity

The mineralization of pellicle biofilm was recorded in changed cellulose IR spectra; for instance, a bell-like peak in the region that corresponds to –OH stretching could indicate that -OH groups were engaged in BC interaction with inorganic ions and in the formation of complexes with metals. However, the BC molecular identity could be confirmed by IR spectroscopy. After a treatment of biomineral samples, IR spectra were found to be the same as those obtained from the cellulose produced in presence of anorthosite. Also the treated KMC produced cellulose, which preserved spectral feature characteristics, for example, the 960–730 cm^{-1} fingerprint region of anomeric carbons, wherein a band at 897 cm^{-1} confirmed the presence of β-1,4-linkages specific to cellulose polymer. These data demonstrate high stability of the cellulose molecule and prove our assumption that this polymer is a biomarker of live matter.

4.2. Links between stress responses and the structure of KMC

More diverse communities are less affected by perturbations than less diverse ones (Zaetz, 2009; Awasthi *et al.,* 2014); this was a leading criterion for decision-making regarding selection of the KMC model as an alternative model based on a biofilm-producing monoculture. In the preparatory study, the information on the KMC structure was obtained by using cultural and culture-independent (DNA-metabarcoding) approaches. It was shown that KMC consisted of several bacterial and yeast species, some of which

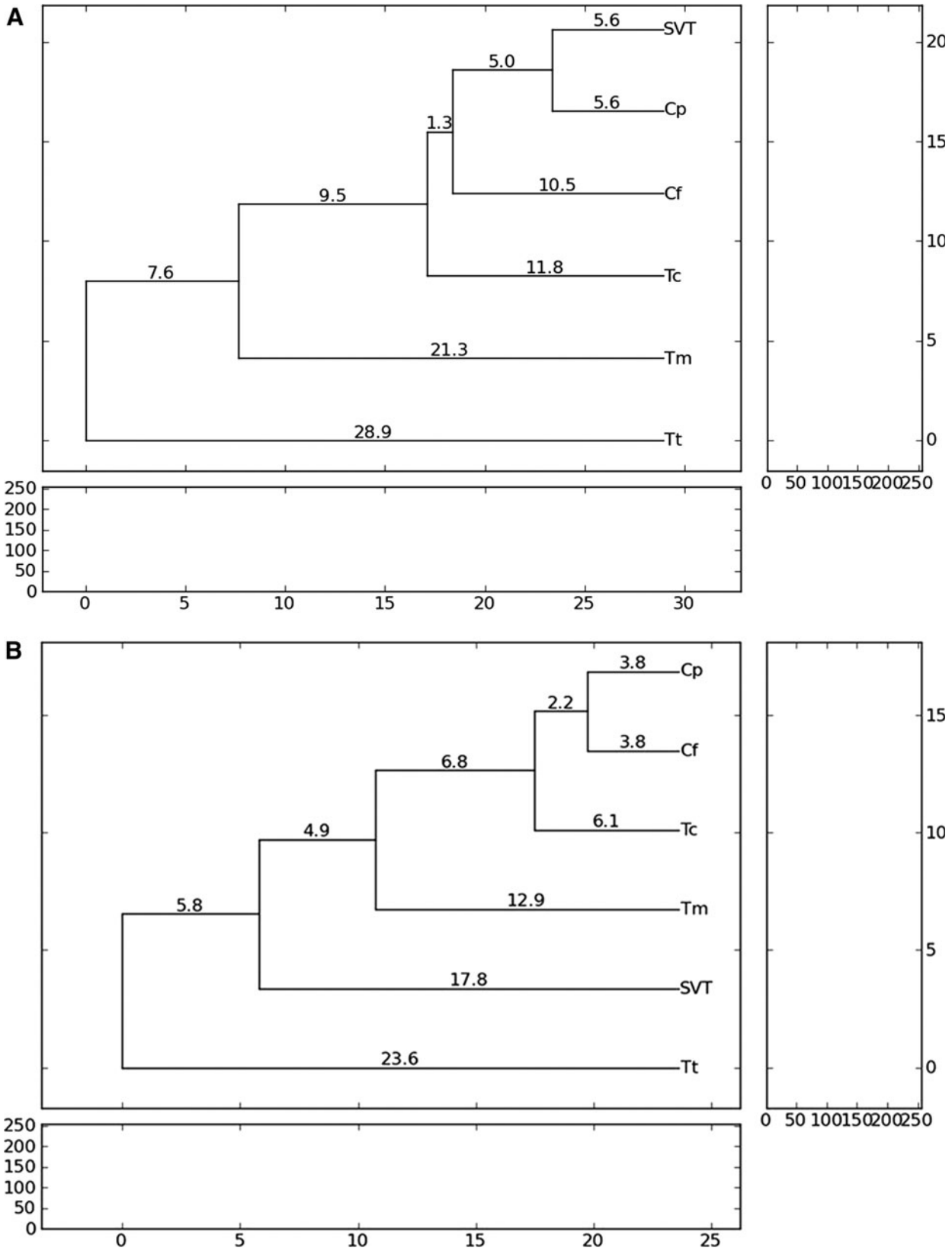

FIG. 4. Cluster analysis of the proteobacterial (**A**) and yeast (**B**) communities' RAPD/PCR band patterns, using the UPGMA clustering method. Control samples: Tc, a dry control sample; Cf, a revived control (biofilm); Cp, a revived control (precipitate). SVT samples: Tt, a dry sample from a top layer; Tm, a dry sample from a medium layer; SVT, a revived sample that originated from a bottom carrier (biofilm).

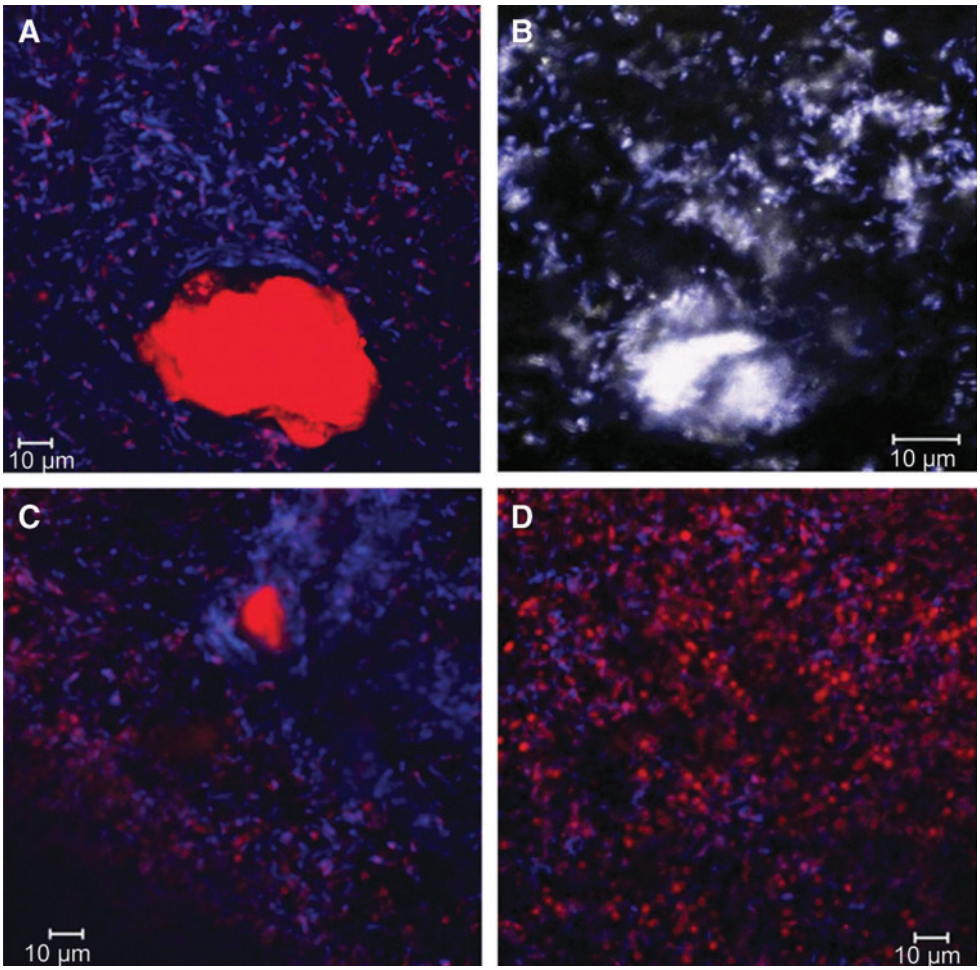

FIG. 5. Confocal scanning laser micrographs of the mineralized cellulose biofilm produced by kombucha culture in the presence of anorthosite. (**A**) Micrograph of the bottom side of encrusted cellulose biofilm produced by the kombucha culture, being restored after SVT (total UV fluence was 5.5×10^5 J/m^2 for 200–400 nm; Mars gas at 10^3 Pa). The source of anorthosite was a rock material that served as a carrier for biomaterial. Nucleic acids stained with ethidium bromide (red signal), cellulose stained with calcofluor with blue excitation. (**B**) Micrograph of the bottom side of encrusted cellulose biofilm, produced by the kombucha culture restored after SVT. Nucleic acids stained with Hoechst (blue signal). (**C**) Micrograph of the bottom side of encrusted cellulose biofilm, produced by the typical kombucha culture in the presence of anorthosite in the medium under normal conditions. In (A)–(C), mineral grains were heavily colonized by microbial organisms. (**D**) Control cellulose-based bottom side pellicle without visible mineral depositions. Cellulose stained with calcofluor with blue excitation, nucleic acids stained with ethidium bromide (red signal). Scale bars equal to 10 μm. (Color images available at www.liebertonline.com/ast)

were uncultivable and unknown (Ovcharenko *et al.,* 2013; Reva *et al.,* 2015). Moreover, it was shown that the naturally selected core kombucha culture composition was stable under different growth conditions and could represent the population under non-optimal conditions (Reva *et al.,* 2015; Podolich *et al.,* 2016). In this study, the minor limiting factor for the community members was anorthosite, more specifically, the excess of biomobilized from anorthosite inorganic ions, which could be a reason for the observed reduction of the cultivable community members. In simulations that mimic a Mars-like atmosphere and pressure, the cellulose-producing bacteria *Komagataeibacter* spp. survived these conditions and produced cellulose-based pellicles after recultivation. Earlier, Kato *et al.* (2007) showed that *Komagataeibacter* sp. possessed a barotolerant feature—the ability to survive at 100 MPa pressure, which corresponded to the deep sea at 10,000 m.

After the impact of different simulated spaceflight and martian factors on KMC, changes in the KMC structure were seen as a consequence of their synergistic effect influenced samples within the bottom and middle carriers in the SVT program. Yeast species *D. anomala* and bacterial species *G. oxydans* were not found in the treated biomineral specimens. Microbiological data were proven by RAPD/PCR and provided evidence that KMC underwent adaptive changes in response to simulated spaceflight and martian factors, as well as dysregulated mineral metabolism.

A set of fluences used in the SVT treatments was harmful for specimens located in the top layer. Microbiological and molecular analysis showed that microbial organisms exposed

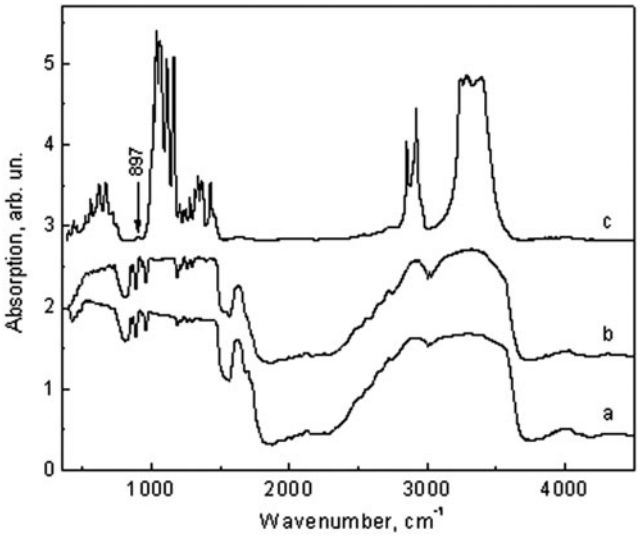

FIG. 6. The IR absorption spectra of a biofilm produced by kombucha culture that was restored after the SVT exposure (a total UV fluence of 5.5×10^5 J/m^2 for 200–400 nm; Mars gas at 10^3 Pa) (a), a biofilm formed in the presence of anorthosite (b), and a BC-based natural biofilm (c) in the range of 400–4000 cm^{-1}. The spectra are shifted on the vertical axis for clarity. The band at 897 cm^{-1} confirms the presence of β-1,4-linkages in the molecule of cellulose.

in the top samples more likely were killed or entered into a viable but nonculturable (VBNC) state, as there was no growth on nutrition medium and their DNA was partially degraded. The major limiting factor for the community members' survival was the UV irradiation that damaged cells and destroyed the community. In analogous tests, Baqué *et al.* (2016) showed that biofilms of desert cyanobacterium *Chroococcidiopsis* tolerated UV polychromatic radiation combined with simulated space vacuum or martian atmosphere in EVT, and Meeßen *et al.* (2015) revealed that specimens of lichen *Buellia frigida* were capable of surviving the conditions tested in EVT and SVT, in the frame of the BIOMEX preflight tests.

The tests on the high-dose-UV irradiation, in which the PSI in DLR (Cologne, Germany) was used, showed that neither the dehydrated cellulose matrix nor the layer of anorthosite above the pellicle in organo-mineral samples could shield and protect cellulose-forming bacteria from UV doses higher than 10 kJ/m^2. However, several KMC members, although dormant and uncultivable, managed to survive these harsh conditions, for example, Gram-positive *Bacillus* sp., which tolerated up to a 10 kJ/m^2 dose. Metabarcoding of KMC showed the presence of *Bacillus* sp. even though they had never been isolated in pure culture (Reva *et al.,* 2015). The ability of *Bacillus* to withstand extreme conditions is well known. For example, the soil isolate *B. pumilus* has shown resistance to a 100 kJ/m^2 UV dose (Gabani *et al.,* 2012). Selection under the influence of the stressors could activate an unculturable population of *Bacillus* sp. in post-irradiation experiments. Here, we once more showed that the structure of polymicrobial kombucha culture depended on exposure conditions, and non-optimal ones either eliminated some of the community members or resuscitated the uncultivable latent forms.

4.3. Link between the structure of KMC and accumulation of elements within the cellulose-based film

The changes of the KMC structure in specimens from the middle and bottom layers correlated with data of the elemental analyses of cellulose-based films produced by surviving bacteria and other inhabiting community members in post-treatment laboratory experiments. In particular, a range of detected elements in those films was unexpectedly narrow, as compared to native film. This may be explained by the reduced richness of community populations after the impact of stressors and appropriate reduction of total biomobilizing activity in KMC. In our previous study, the biomobilization of elements by KMC members and accumulation of them in pellicle films were shown in the KMC biofilm-anorthosite system, where anorthosite was separated from the cellulose-based film with cultural liquid (Zaets *et al.,* 2014). In spite of anorthosite- and biofilm-phase separation, the film accumulated inorganic ions (mobilized from the rock by planktonic microbial cells) and became gray, in contrast to native biofilm produced by KMC without anorthosite. In this study, after preflight simulations, surviving KMC members also exhibited a biomobilization and accumulation capacity; however, this was restricted to a few elements as compared to parental untreated KMC.

5. Conclusion

Dehydrated cellulose-based pellicle protects bacterial and yeast cells from adverse factors such as low-dose UV radiation (<1000 J/m^2), temperature fluctuations, and vacuum or Mars-like conditions. Under stressful conditions due to a higher-dose of UV irradiation (1,000–10,000 J/m^2), the KMC structure is unstable; however, at least minimal community composition—the key players of KMC—recovers its capability to grow under optimal conditions both as planktonic cultures and within the cellulose web. Taking into account the slow revival of the community, low-dose UV is defined as a limiting factor, but it is not critical factor. What is critical for the kombucha culture survival is exposure to a high dose (>10,000 J/m^2) of UV irradiation. Preconditioned kombucha culture, previously grown in the presence of anorthosite, exhibits a better survival/revival capacity of the cellulose-forming bacteria. The mineralization of pellicle biofilm changes the cellulose molecule; however, the BC preserves molecular identity, as confirmed by IR spectroscopy.

Acknowledgments

This study was supported by the National Academy of Sciences of Ukraine (grant 47/2012-15). The preflight programs EVTs and SVTs for the EXPOSE-R2 mission were supported by the European Space Agency.

Author Disclosure Statement

No competing financial interests existed.

References

Aerts, J.W, Röling, W.F.M., Elsaesser, A., and Ehrenfreund, P. (2014) Biota and biomolecules in extreme environments on

Earth: implications for life detection on Mars. *Life* 4:535–565.

Arrhenius, S. (1903) Die Verbreitung des Lebens im Weltraum. *Die Umschau* 7:481–485.

Awasthi, A., Singh, M., Soni, S.K, Singh, R., and Kalra, A. (2014) Biodiversity acts as insurance of productivity of bacterial communities under abiotic perturbations. *ISME J* 8:2445–2452.

Baqué, M., Verseux, C., Böttger, U., Rabbow, E., de Vera J.-P., and Billi, D. (2016) Preservation of biomarkers from cyanobacteria mixed with Mars-like regolith under simulated martian atmosphere and UV flux. *Orig Life Evol Biosph* 46:289–310.

Brandt, A., de Vera, J.-P., Onofri, S., and Ott, S. (2015) Viability of the lichen *Xanthoria elegans* and its symbionts after 18 months of space exposure and simulated Mars conditions on the ISS. *International Journal of Astrobiology* 14:411–425.

Chen, I.A. and Walde, P. (2010) From self-assembled vesicles to protocells. *Cold Spring Harb Perspect Biol* 2, doi:10.1101/cshperspect.a002170.

Cockell, C.S., Rettberg, P., Rabbow, E., and Olsson-Francis, K. (2011) Exposure of phototrophs to 548 days in low Earth orbit: microbial selection pressures in outer space and on early Earth. *ISME J* 5:1671–1682.

Cohn, F. (1876) Untersuchungenüber bacterien. IV. Beiträgezur biologie der Bacillen. *Beiträgezur Biologie der Pflanzen* 7:249–267.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284:1318–1322.

de Gregorio, B.T., Stroud, R.M., Nittler, L.R., Alexander, C.M.O'D., Bassim, N.D., Cody, G.D., Kilcoyne, A.L.D., Sandford, S.A., Milam, S.N., Nuevo, M., and Zega, T.J. (2013) Isotopic and chemical variation of organic nanoglobules in primitive meteorites. *Meteorit Planet Sci* 48:904–928.

de Vera, J.-P., Boettger, U., Schmitz, N., Lange, C., Hübers, H.-W., Jaumann, R., and Spohn, T. (2012) Co-I team of BIOMEX supporting Mars exploration: BIOMEX in low Earth orbit and further astrobiological studies on the Moon using Raman and PanCam technology. *Planet Space Sci* 74:103–110.

Echeverrigaray, S., Paese-Toresan, S., and Carrau, J.L. (2000) RAPD marker polymorphism among commercial winery yeast strains. *World J Microbiol Biotechnol* 16:143–146.

Floss, C., Guillou, C.L., and Brearley, A. (2014) Coordinated NanoSIMS and FIB-TEM analyses of organic matter and associated matrix materials in CR3 chondrites. *Geochim Cosmochim Acta* 139:1–25.

Gabani, P., Copeland, E., Chandel, A.K., and Singh, O.V. (2012) Ultraviolet-radiation-resistant isolates revealed cellulose-degrading species of *Cellulosimicrobium cellulans* (UVP1) and *Bacillus pumilus* (UVP4). *Biotechnol Appl Biochem* 59:395–404.

Georgiou, C.D. and Deamer, D.W. (2014) Lipids as universal biomarkers of extraterrestrial life. *Astrobiology* 14:541–549.

Gill, S. and Forterre, P. (2016) Origin of life: LUCA and extracellular membrane vesicles (EMVs). *International Journal of Astrobiology* 15:7–15.

Hestrin, S. and Schramm, M. (1954) Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. *Biochem J* 58: 345–352.

Hu, L., Grim, C.J., Franco, A.A., Jarvis, K.G., Sathyamoorthy, V., Kothary, M.H., McCardell, B.A., and Tall, B.D. (2015) *Cronobacter* species: prevalence among species and their roles in biofilm formation and cell-cell aggregation. *Food Microbiol* 52:97–105.

Kato, N., Sato, T., Kato, C., Yajima, M., Sugiyama, J., Kanda, T., Mizuno, M., Nozaki, K., Yamanaka, S., and Amano, Y. (2007) Viability and cellulose synthesizing ability of *Gluconacetobacter xylinus* cells under high-hydrostatic pressure. *Extremophiles* 11:693–698.

Kharina, A., Podolich, O., Faidiuk, I., Zaika, S., Haidak, A., Kukharenko, O., Zaets, I., Tovkach, F., Reva, O., Kremenskoy, M., and Kozyrovska, N. (2015) Temperate bacteriophages collected by outer membrane vesicles in *Komagataeibacter intermedius*. *J Basic Microbiol* 55:509–513.

Kukharenko, O., Podolich, O., Rybitska, A., Reshetnyak, G., Burlak, L., Ovcharenko, L., Voznyuk, T., Moshynets, O., Rogutskyi, I., Zaets, I., Yaneva, O., Pidgorskiy, V., Rabbow, E., de Vera, J.-P., and Kozyrovska, N. (2012) Robust symbiotic microbial communities in space research. In *Space Research in Ukraine (2010–2011),* Report to COSPAR, edited by O.P. Fedorov, Academ Periodyka, Kyiv, Ukraine, pp 102–105.

Lee, L.-H., Cheah, Y.-K., Nurul Syakima, A.M., Shiran, M.S., Tang, Y.-L., Lin H.-P., and Hong, K. (2012) Analysis of Antarctic proteobacteria by PCR fingerprinting and screening for antimicrobial secondary metabolites. *Genet Mol Res* 11:1627–1641.

Lovelock, J.E. (1965) A physical basis for life detection experiments. *Nature* 207:568–570.

Meeßen, J., Wuthenow, P., Schille, P., Rabbow, E., de Vera, J.-P., and Ott, S. (2015) Resistance of the lichen *Buellia frigida* to simulated space conditions during the preflight tests for BIOMEX—viability assay and morphological stability. *Astrobiology* 15:601–615.

Miller, J.H. (1972) *Experiments in Molecular Genetics,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mytrokhyn, O.V., Bogdanova, S.V., and Shumlyanskyy, L.V. (2003) Anorthosite rocks of Fedorivskyy suite (Korosten Pluton, Ukrainian Shield). In *Current Problems in Geology,* Kyiv National University, Kyiv, pp 53–57.

Nobles, D.R., Romanovicz, D.K., and Brown R.M., Jr. (2001) Cellulose in cyanobacteria. Origin of vascular plant cellulose synthase? *Plant Physiol* 127:529–542.

Ovcharenko, L.P., Reva, O.N., Zaets, I.E., Kukharenko, O., Burlak, O.P., Podolich, O.V., de Vera, J.-P., and Kozyrovska, N.O. (2013) DNA metabarcoding of complex microbial communities promising for space research. In *Abstract Book 13th Ukrainian Conference on Space Research (Yevpatoria, September 2–7, 2013),* Kyiv, Ukraine, p 84.

Podolich, O., Zaets, I., Kukharenko, O., Orlovska, I., Reva, O., Khirunenko, L., Sosnin, M., Hayidak, A., Shpylova, S., Rohutskyy, I., Kharina, A., Skoryk, M., Kremenskoy, M., Klymchuk, D., Demets, R., de Vera, J.-P., and Kozyrovska, N. (2016) The first space-related study of a kombucha multimicrobial cellulose-forming community: preparatory laboratory experiments. *Orig Life Evol Biosph,* doi:10.1007/s11084-016-9483-4.

Rabbow, E., Rettberg, P., Barczyk, S., Bohmeier, M., Parpart, A., Panitz, C., Horneck, G., von Heise-Rotenburg, R., Hoppenbrouwers, T., Willnecker, R., Baglioni, P., Demets, R., Dettmann, J., and Reitz, G. (2012) EXPOSE-E: an ESA as-

trobiology mission 1.5 years in space. *Astrobiology* 12:374–386.

Rabbow, E., Rettberg, P., Barczyk, S., Bohmeier, M., Parpart, A., Panitz, C., Horneck, G., Burfeindt, J., Molter, F., Jaramillo, E., Pereira, C., Weiß, P., Willnecker, R., Demets, R., Dettmann, J., and Reitz, G. (2015) The astrobiological mission EXPOSE-R on board of the International Space Station. *International Journal of Astrobiology* 14:3–16.

Reva, O.N., Zaets, I.E., Ovcharenko, L.P., Kukharenko, O., Shpylova, S., Podolich, O.V., de Vera, J.P., and Kozyrovska, N.O. (2015) Metabarcoding of the kombucha microbial community grown in different microenvironments. *AMB Express* 5:35–42.

Romling, U. and Galperin, M.Y. (2015) Bacterial cellulose biosynthesis: diversity of operons, subunits, products, and functions. *Trends Microbiol* 23:545–557.

Ross, P., Mayer, R., and Benziman, M. (1991) Cellulose biosynthesis and function in bacteria. *Microbiol Rev* 55:35–58.

Saha, R., Pohorille, A., and Chen, I.A. (2014) Molecular crowding and early evolution. *Orig Life Evol Biosph* 44:319–324.

Zaets, I., Podolich, O., Kukharenko, O., Reshetnyak, G., Shpylova, S., Sosnin, M., Khirunenko, L., Kozyrovska, N., and de Vera, J.-P. (2014) Bacterial cellulose may provide the microbial-life biosignature in the rock records. *Adv Space Res* 53:828–835.

Zaetz, I.E. (2009) Modification of heavy metal accumulation by plants with bacterial consortium. Thesis for candidate's degree in Biological Sciences by specialty 03.00.20–Biotechnology. Institute of Molecular Biology and Genetics of National Academy of Sciences of Ukraine, Kyiv, Ukraine.

Address correspondence to:
Olga Podolich
Institute of Molecular Biology and Genetics of NASU
Acad. Zabolotnoho str., 150
03680 Kyiv
Ukraine

E-mail: podololga@ukr.net

Submitted 18 February 2016
Accepted 7 December 2016

Abbreviations Used

BC = bacterial cellulose
BIOMEX = BIOlogy and Mars Experiment
BTS = filter-sterilized black tea (*Camellia sinensis*) (Lipton, 1.2%, w/v) with white sugar (3%, w/v)
CSLM = confocal scanning laser microscopy
DLR = German Aerospace Center
EDXS = energy-dispersive X-ray spectrometry
EVT = Experiment Verification Test
FT-IR = Fourier transform infrared
ISS = International Space Station
KMC = kombucha microbial community
PSI = Planetary and Space Simulation facility
RAPD = Randomly Amplified Polymorphic DNA
SEM = scanning electron microscopy
SVT = Scientific Verification Test
UPGMA = unweighted pair-group method with arithmetic average