

Life beyond earth: microbial survival and immune health in space

Tommaso Zaccaria



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Radboud Dissertation Series

ISSN: 2950-2772 (Online); 2950-2780 (Print)

Published by RADBOUD UNIVERSITY PRESS

Postbus 9100, 6500 HA Nijmegen, The Netherlands

www.radbouduniversitypress.nl

Design: Proefschrift AIO | Guus Gijben

Cover: Proefschrift AIO | [Ninja]

Printing: DPN Rikken/Pumbo

ISBN: 9789465152288

DOI: 10.54195/9789465152288

Free download at: <https://doi.org/10.54195/9789465152288>

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Life beyond earth: microbial survival and immune health in space

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.M. Sanders,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

vrijdag 19 juni 2026
om 10.30 uur precies

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Life beyond earth: microbial survival and immune health in space

Dissertation to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.M. Sanders,
according to the decision of the Doctorate Board
to be defended in public on

Friday, June 19, 2026
at 10:30 am

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Chapter 1

General introduction and outline of the thesis

Space travel has been one of humanity's most remarkable achievements which has allowed unprecedented technological innovation and scientific research. Since the Apollo missions, the exploration of planetary bodies within our Solar System has steadily progressed and is expected to extend in the coming decades to Mars and eventually to the moons of the giant planets. However, these ambitious goals set forth in the human and robotic exploration of Mars and the icy moons come with many challenges. They include the potential contamination of these celestial bodies with terrestrial microorganisms and the risks to astronaut health arising from altered immune system function during long-duration missions. Addressing these issues requires a thorough understanding of both the changes in human immune responses under spaceflight conditions and the implications of microbial persistence for planetary protection.

Although rigorous bioburden reduction protocols exist, complete sterilization of Earth-originating biological material remains unachievable (1, 2). This underscores the critical importance of effective decontamination measures, particularly when exploring sites of astrobiological interest. Consequently, understanding microbial survival, their metabolism and virulence under space conditions is essential. This thesis explores the implications of space missions in the context of bacterial and yeast survival. The first part investigates the human immune response to space-exposed microorganisms, while the second part explores survival limits and mechanisms of environmental microorganisms that pose potential risks to planetary protection.

Brief Overview of Space Research

Human health is influenced by the natural microbiome and by interactions with environmental microorganisms. The integrity of the body's defenses together with the adaptive capacity of microbes, determines how the immune system responds to physiological and environmental changes. The initial concerns of human-led space travel were focused on preserving living humans from the adverse conditions of space (3). However, given the ubiquitous presence of microorganisms, understanding how microbes grow and interact with humans within the confined environments of a space station became a critical area of investigation (4).

Space microbiology research has investigated numerous microorganisms and groups that constitute the human microbiome. Despite these advances, a comprehensive understanding of the behavior and impact of human pathogens in space remains

limited. To assess how microorganisms respond to space conditions, it is first necessary to characterize this unique environment and its key differences from Earth.

Although Earth-based observations have provided extensive data on the conditions of planetary bodies within the Solar System, *in situ* investigations with the use of unmanned probes have been vital for obtaining detailed information about their physical and chemical characteristics. Past and present robotic missions have investigated celestial bodies by performing flybys (close spacecraft approaches to without achieving orbit), orbital measurements, and surface landings. The data from these missions, which have been collected and analyzed have proven essential for the development of technologies that support human life in space. Despite major advances in technical capabilities, the economic cost and physiological challenges associated with human health continue to limit human exploration of deep space, the region beyond the Moon's orbit, which remains the domain of robotic missions. To date, Earth's Moon is the only other celestial body visited by humans, however, rovers and landers have landed successfully on a number of other Solar System bodies.

Since the 1960s, there have been 61 robotic missions to Mars, although only a fraction has successfully completed their objectives (5). The focus on Mars can be attributed to several factors: evidence that it may have once had an environment similar to Earth, its location within the Sun's habitable zone, and the possibility that it could still support microbial life under certain conditions and during certain seasonal periods. Uncrewed flyby missions have also targeted other locations of astrobiology interest: the icy moons of Jupiter and Saturn, Europa and Enceladus respectively. Europa has been visited seven times by spacecraft through flybys and orbital investigations (6). It is scheduled to be further explored by the end of the decade with the JUICE (Jupiter icy moons explorer) and Europa Clipper missions. Enceladus, in contrast, has been visited three times since the 1970s with the Cassini-Huygens mission providing detailed insights into its environment (7). These moons are of particular interest because flyby data have revealed unique characteristics that support the hypothesis that their subsurface oceans could harbor life (8).

The drive to explore space has also been motivated by socio-economic factors. Scientific and technological research in space has contributed to the development or improvement of numerous biomedical and technological innovations (e.g. GPS, space telecommunication, biometric monitoring, computed tomography scans, LED lighting, water purification systems, freeze-dried food, infant formula), thereby improving human life and health on Earth (9, 10). At the same time, the rise of private

space ventures aimed at advancing technologies such as telecommunication satellites or supporting cargo and human transportation, has led to substantial increase in human activity in space. This began in 1961 and has been uninterrupted since the year 2000. Understanding how the human body and microorganisms respond to space conditions is essential for safeguarding astronaut health, expanding research opportunities, and elucidating the limits of life.

Impact of space conditions on immune health – mission types and research status

Due to the high cost and technical challenges of reaching deep space, the vast majority of biology and human health experiments have been performed in low Earth orbit (LEO), the most accessible region of space, on human-rated inhabited space stations (11). Despite being relatively close to Earth, LEO exposes organisms to environmental conditions that share similarities with other regions of interest in the solar system (**Table 1**) providing valuable data to inform human health strategies for future long-duration space missions. Additionally, Earth-based simulation studies offer a cost-effective and convenient means to investigate how human and microbial physiology change and interact under space-like conditions.

Table 1: Environmental conditions of different locations in the Solar system, references in brackets.

Environmental condition	Earth	LEO	Moon	Mars	Europa	Enceladus
Gravity (m/s ²)	9.807	8.69 (12)	1.62	3.73	1.315	0.311
X-ray (μGy/day)	6 (13)	2880 (14)	316.8 (15)	233 (16)	no <i>in situ</i> measurement	no <i>in situ</i> measurement
UV flux (W/m ²) at 200-400 nm	116.16 (17)	116.16 (4)	116.16 (17)	49.95 (17) 26.9-42.3 (18)	4.3 (17)	1.29 (17)
Atmospheric pressure (hPa)	1013	1 x 10 ⁻⁹	1.33 x 10 ⁻¹¹ (19)	6 (20)	1 x 10 ⁻⁹ (21)	0.002 (22)

Radiation and gravitational conditions on the International Space Station (ISS) in LEO are comparable to those encountered in other regions of the Solar System, making it possible to study the effects of space conditions on the human body. Space stations in LEO experience microgravity similarly to other regions of space. Despite differences on the surface of different planets, the isotropic nature of space radiation (energetic particles confined in the magnetic field) will result in human exposure even in LEO, regardless of mission type (23). Exposure to these space conditions can lead to loss of

homeostasis in multiple human physiological systems. Extensive studies have shown how the human immune system is dysregulated in space (24), leading to increased core body temperature, changes in the leukocyte transcriptome, altered cytokine release, gene expression dysregulation and reductions in lymphocyte and red blood cell counts. Other biological effects of space travel include radiation-induced carcinogenesis, cardiovascular issues and acute effects on the central nervous system (23, 25-27). Additionally, it has been shown how radiation-induced DNA damage appears to be less effectively repaired under microgravity (23). In contrast, many microbial species appear more resilient and less affected by the challenges of the space environment than humans (28-30). This differential response raises concerns, as pathogenic microbes could pose a significant threat to astronauts with compromised immune systems, potentially jeopardizing mission success. The importance of this risk is underscored by the detection of several pathogenic bacteria and fungi in the ISS during microbial isolation campaigns (31-34).

Furthermore, space conditions can have wide-ranging effects on the human body, including acute effects during a mission and chronic effects which might persist after the end of mission (25). Mission-specific environmental stressors can further exacerbate dysregulation of the human immune system. These stressors primarily impact psychological rather than physiological health. Examples include the confined environment of a spacecraft, as can be seen in **Figure 1**, a limited diet, reduced social interactions, and disruption of the circadian rhythm, all of which can impair the cognitive health and the immune system performance (35).



Figure 1: ESA astronaut Samantha Cristoforetti inside the full-scale mockup of the Soyuz capsule, at the Gagarin Cosmonaut Training Centre, on 14 October 2014. Credit: ESA–S. Corvaja, 2014.

The human body, under homeostatic control, is capable of efficient wound and tissue repair. However, the unique conditions of space travel have been shown to impair this process. Reduced skin hydration and compromised barrier function contribute to a loss of wound healing efficiency (36). Additionally, delays in fibroblast migration will also lead to a slower wound healing process, and thereby increase the risks of infections (37). The same has also been observed in the gut. A healthy skin and mucosal microbiome can reduce the risk of infections by maintaining symbiotic populations of bacterial species and fungi. However, in space astronauts exhibit changes in skin microbiome, including reduced microbial diversity and increased loss of keratinocytes (38). Consequently, the risk of wound infection by pathogenic bacteria is elevated, potentially leading to wound control and delayed recovery (39).

The gastrointestinal (GI) tract is particularly susceptible to space-induced dysregulation. This area of the human body plays a key role in nutrient uptake and immune function, with its activity closely modulated by interactions between epithelial cells and the gut microbiome. Changes in the GI tract can cause a range of dysfunctions including immune cell activation resulting in systemic inflammation (40). Furthermore, it has been shown that alterations in the gut microbiome composition have also been linked to digestive disturbances and broader health issues (41). Moreover, space conditions have been shown to affect expression of genes involved in gut healing and inflammatory cytokines regulation (42). Therefore, understanding the survival and function of microbes that constitute a healthy gut microbiome is essential for maintaining nutrient uptake, immune homeostasis, and overall crew health during space missions.

Microbial survival to space conditions - current knowledge

To date, much of the space-related microbiological research has focused on microbial survival as a whole. This work has been conducted for a variety of reasons: ensuring food safety, maintaining industrial manufacturing processes, managing waste disposal, and investigating the origin of life (43, 44). The majority of these studies have involved extremophilic microorganisms, which are found across all domains of life, including bacterial species (e.g. *Deinococcus radiodurans*, *Buttiauxella* sp. MASE-IM-9, *Ignicoccus hospitalis*, *Pyrococcus furiosus*), and eukaryotes (e.g. *Rhodotorula* species, *Aspergillus niger*, *Penicillium simplicissimum*). Extremophiles have the unique ability to tolerate conditions that are extreme from a human perspective, such as very high or very low temperatures (growth $>50^{\circ}\text{C}$ or $<10^{\circ}\text{C}$), dry or desiccating environments, high salinity, extreme pH, and elevated levels of (non-) ionizing radiation. Their ability to survive such environments

makes extremophiles ideal model organisms for investigating survival under space conditions.

Space microbiology research has shown that bacterial and fungal candidates such as *Aspergillus niger*, *Salinisphaera shabanensis*, *Staphylococcus capitis* and *Buttiauxella sp.* MASE-IM-9, can survive radiation doses comparable to those found on Mars or even on certain exoplanets (45, 46). Additionally, studies have shown that microorganisms are capable of surviving for prolonged periods of time on the exterior of the ISS (47, 48), as well as under Mars-simulated conditions (49). Investigating the effects of microgravity has also provided insight into how microorganisms adapt to space conditions. Despite their high resilience to a range of extreme conditions, the potential inhibitory effects of microgravity still warrant investigation. Interestingly, microgravity appears to support the growth of certain microorganisms (50, 51). Bacterial conjugation and plasmid transfer also seem to be facilitated by microgravity, although conflicting findings in the literature indicate that further research is needed (52, 53). Given these microbial capabilities, investigations have also explored the use of microorganisms for *in situ* production of raw materials in space, aiming to enable sustainable resource generation without the need for transport from Earth (54).

Since the start of the 21st century, numerous space microbiology studies have evaluated the survival and physiological responses of microorganisms under Mars-like conditions. This growing interest can be attributed to several factors, including political and intrinsic motivations for human exploration, the rise in robotic missions, and increasing evidence that microbial life could potentially persist on Mars. For example, the 2007 European Space Agency (ESA) ROME (Response of Organisms to the Martian Environment) report summarized contemporary research on the survival and metabolic potential of microorganisms in simulated Martian environments (55). Key findings included the survival of Antarctic rock fungi (*Cryomyces spp.*) under Mars-like conditions, the response of *Bacillus subtilis* to UV-C radiation, the photosynthetic potential of *Chroococcidiopsis* under Martian UV flux, and the response of methanogenic archaea to Martian thermal regimes. Similar studies conducted by other space agencies have since corroborated and expanded upon these findings.

Interest in microbial survival under space conditions has also been driven by data acquired from missions targeting the icy moons of the Solar System, which suggest that these environments may be capable of supporting life (56). Icy moons are celestial bodies which have a thick layer of ice (several kilometers) surrounding a subsurface ocean. Missions such as Cassini-Huygens have been instrumental in

collecting such data. Of particular interest to these studies are psychrophilic or psychrotolerant microorganisms which can grow at temperatures below 0°C and are typically isolated from Arctic-like environments. Several yeasts and bacterial species have shown growth at subzero temperatures such as *R. frigidicola* (formerly JG-1b) and *P. halocryophilus* (57, 58). The capability of such organisms to survive and grow at low temperatures has implications both for food preservation and for the potential contamination of icy moons.

Non-extremophilic, often more fastidious microorganisms have also been investigated in space research, however, these studies have primarily focused on investigating microbial virulence, pathogenicity and drug resistance rather than survival (59-61). Such fastidious, non-extremophiles, are generally unsuitable for Mars survival studies due to their specific and demanding growth requirements. Nonetheless, understanding how pathogenic microorganisms might persist on the Martian surface remains essential to complement existing human health LEO data and to assess virulence and pathogenicity, thereby guiding *in situ* treatment strategies during future space missions.

Planetary protection of Earth, the Moon, Mars and the icy moons: requirements, concerns and current knowledge gaps

Despite the United Nations guaranteeing safe access to space for all nations, guidelines must be established to preserve scientific research, support the search for life and ensure the containment of threats from extraterrestrial samples. These measures aim to mitigate the risks of both forward contamination (transfer of biological material from Earth to other celestial bodies) and backward contamination (transfer of biological material from other celestial bodies to Earth). The Committee on Space Research (COSPAR) plays a key role by providing international standards based on scientific research and evidence-based findings, as well as serving as a forum for discussion (62). Even though the committee's standards are not legally binding, they are internationally endorsed and they support the implementation of guidelines to comply with the United Nation's Outer Space Treaty Article IX of 1967. The COSPAR Panel on Planetary Protection (PPP), has established bioburden requirements and mission categorization depending on the target celestial body. **Table 2** summarizes these for the locations relevant to this thesis.

Although planetary protection requirements have been clearly established, past missions were not conducted in full compliance with these standards. Early Mars landers including the Soviet Union's Mars 3 lander and NASA's Viking 1 missions implemented bioburden reduction measures only to a limited extent. For example,

the Mars 3 lander was not heat sterilized, although other sterilization techniques may have been applied (65, 66). In contrast heat sterilization was applied to certain components of the Viking 1 lander (67), however this does not guarantee compliance with current Category IV requirements. Therefore, contamination risk assessment and the interpretation of localized biosignatures, must take these earlier missions into account.

Table 2: Requirements by the COSPAR panel on planetary protection for sites of interest to this thesis, summarized from Coustenis, Doran (63) and Kminek, Fellous (64).

Location of mission	Mission type	Mission category	Mission requirements
Earth return	From solar system bodies deemed to have no indigenous life	V (unrestricted)	Same as category IV requirements with no additions (i.e. samples from Venus, the Moon, etc.)
	From all other locations	V (restricted)	Unless samples are subjected to accepted and approved sterilization process, sample-holding canister must be closed with verified process, and samples must be contained throughout all mission phases until transport to a receiving facility where they can be opened under containment
			From Europa or Enceladus, the mission must have a method to break chain of contact with icy moons
			Review and approval of flight continuation at three phases: prior to launch from Earth, after sample collection at icy moons and prior to Earth trajectory maneuver, prior to Earth re-entry maneuver commitment
			If samples unsterilized, life detection and biohazard program must be performed or proven sterilization process undertaken before any (portion) of sample is distributed on Earth for research
Moon	Flyby and Orbiter	II	No sterilization or bioburden required but missions should only have a minimal likelihood that spacecraft contamination could compromise future investigations
	Lander	IIa	Requirements to all Moon locations relaxed if organic products are only from propulsion system exhaust
		IIb	Full organic inventory required if travelling to Permanently Shadow Regions (PSR) and lunar poles of the Moon

Table 2: Continued

Location of mission	Mission type	Mission category	Mission requirements
Mars	Flyby and Orbiter	III	Probability of inadvertent Mars contamination within 20 years from launch has to be $\leq 1 \times 10^{-2}$ Probability of impact with Mars from 20 to 50 years from launch is $\leq 5 \times 10^{-2}$
	Lander	IV	Same as category III
	Lander not investigating for life	IVa	Surface of lander must have $\leq 3 \times 10^5$ spores in total or ≤ 300 spores per m^2
	Lander investigating for life	IVb	Maximum surface of lander spore bioburden of ≤ 30 Subsystems involved in interaction with life detection must prevent re-contamination of subsystem as well as contamination of sample
	Lander to Mars special regions* even if not investigating for life	IVc	If landing site within special region, entire lander is restricted to surface bioburden of ≤ 30 spores If special region is accessed only by subsystem, either whole lander restricted to ≤ 30 spores or only the subsystem
Icy Moons (Enceladus, Europa)	Flyby and Orbiter	III	Any mission has to reduce the probability of inadvertent contamination to $< 1 \times 10^{-4}$ per mission. This requirement is extended to any mission phase including beyond end-of-mission
	Lander	IV	

*Special regions on Mars are defined as areas with high likelihood of permitting the replication of terrestrial organisms or with a significant potential for hosting extant Martian life, as described by Kminek, Fellous (64).

Priorities in planetary protection knowledge gaps (KGs) are regularly revisited in order to guide scientific research in the field and to support mission compliance. One such example is the COSPAR Panel on Planetary Protection's (PPP) identification of KGs related to crewed missions to Mars (68). The panel highlights a continued lack of knowledge regarding:

- human-associated pathogens,
- microbial monitoring within spacecrafts,
- astronaut microbiome characterization, and
- microbial growth on spacecraft surfaces.

These KGs show how important astronaut safety is during space missions. For the same aim, chapters 2 and 3 of this thesis initiate an assessment of the role of human

pathogens in space. The PPP also emphasizes the need for appropriate procedures addressing planetary safety which include:

- microbial and organic releases during Mars extravehicular activities (EVAs),
- decontamination procedures, and
- waste management strategies.

These KGs try to ensure that during a human residence on Mars or other celestial body, the contamination of the surrounding environment is maintained at the lowest levels. Additional critical KGs which are not explicitly identified by the planetary protection panel, but of relevance to this thesis in relation to human immune health during Mars and other long-duration deep-space missions, include:

- the effect of extended and deep-space exposure,
- the impact of solar flares (intense and localized emission of electromagnetic radiation from the Sun), and
- the response of the immune system to known pathogens following long-term space exposure.

Such KGs should ensure research is targeted to ensure human health especially in relation to long-term missions. Chapters 2, 3 and 4 already present a rationale and preliminary data to support this research direction.

Given ongoing scientific advancements in understanding microbial growth at low temperatures and tolerance to space-like conditions, planetary protection requirements for celestial bodies are subject to change. For example, in the 1970s after the Apollo missions, Earth's moon was assigned Category I, having been considered too dry to support life. However, after the discovery of ice deposits, in 2008 it was re-categorized to Category II (69). Similarly, once the potential for life-supporting environments was identified on Enceladus and Titan, it was decided in September 2017 to direct the Cassini-Huygens probe into Saturn's atmosphere at the end of its mission to prevent contamination of these moons (7). Comparable end-of-mission strategies are planned for the current JUICE and Europa clipper missions, which are currently enroute to the Jovian system.

Investigating the survival of microorganisms under simulated space conditions helps to assess the risks associated with forward and backward contamination. Such research aligns with the objectives of the COSPAR PPP, which advocates the use of terrestrial analogue missions and investigations to address the existing KGs (68). Analogue environments are defined as locations on Earth that share similar characteristics with extraterrestrial bodies (70). However, some KGs highlighted by

the panel, are more challenging to investigate: for example, those related to the risk of sample-return missions. Undoubtedly, the investigation of space-borne samples on Earth can be much more extensive compared to *in situ* measurements, there remains a risk of contaminating Earth with extraterrestrial material, as emphasized by the panel. Consequently, approved sterilization procedures based on terrestrial biology are being developed for upcoming sample-return missions (64). To date, all sample-return missions have originated from bodies not considered capable of supporting life, including Earth's moon, comets and asteroids (71-73). Nevertheless, plans are underway to retrieve samples from Mars using equipment already present on its surface as well as new hardware (74).

AIMS, OUTLINE AND SCOPE OF THE THESIS

The majority of microbiology space research has focused on non-pathogenic extremophilic or extremotolerant organisms. Recognizing a knowledge gap in pathogen research in space, assessing the survival and virulence potential of pathogens exposed to simulated space conditions is a critical research approach. Understanding pathogen survival traits and virulence in humans can help guide future crewed mission planning. Equally important to this thesis is the focus on planetary protection knowledge gaps highlighted by the COSPAR PPP. By investigating the survival and metabolism of psychrotolerant microorganisms we also aimed to provide data for the planetary protection community to guide bioburden reduction and identification strategies, particularly in the context of forward contamination and the search for life at astrobiologically significant sites in the Solar System (**Figure 2**).

For these reasons, **part I** of this thesis explores the impact of Mars conditions on the physiology of microorganisms, their pathogenic potential after exposure to simulated Mars conditions, microbiome changes, as well as inhalation risks for astronauts.

In **Chapter 2** we investigated the limits of survival of four non-fastidious opportunistic bacterial pathogens exposed to Mars simulated conditions. We evaluated their survival based on prior evidence demonstrating their capability to grow in minimal media supplemented with a single carbon source.

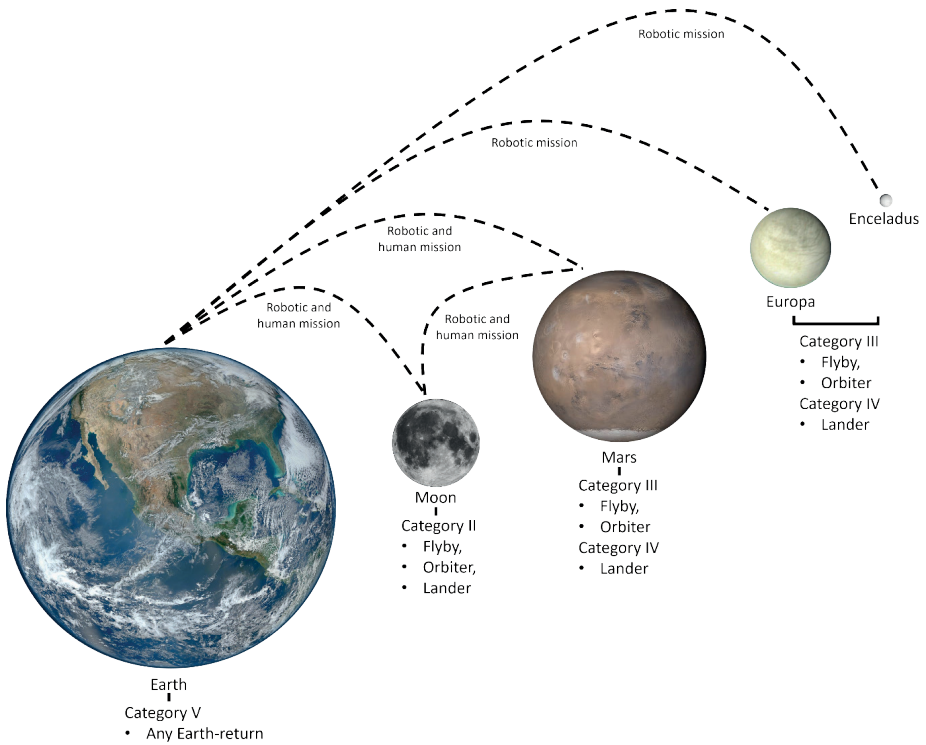


Figure 2: Schematic overview of locations in the solar system of interest to this thesis. Mission types as guide to the immune health and planetary protection investigations performed in the thesis. Additional Planetary Protection mission categories according to mission type for each planet are indicated. Planet sizes are to scale. Credit: NASA, 2025, modified.

To assess the potential risks to astronauts from exposure to Mars-exposed bacterial species, we evaluated the immune response of PBMCs stimulated with *Klebsiella pneumoniae* and *Serratia marcescens* that survived simulated Martian conditions as described in **Chapter 3**.

Risks associated to Lunar and Martian regolith inhalation were first highlighted by astronauts who landed on the Moon during the Apollo missions. Therefore **Chapter 4** focused on evaluating the potential damage caused by Lunar and Martian regolith simulants to the respiratory tract.

Part II of this thesis focuses on the survival limits of environmental microorganisms, the intrinsic traits they retain, and the metabolic strategies they employ to endure extreme conditions.

Having learned how the icy moons Europa and Enceladus could potentially support life, in **Chapter 5** we investigated the risks for Earth eukaryotic and prokaryotic microorganisms, travelling on spacecrafts, contaminating these moons.

To assess the capacity of microorganisms from all domains of life to tolerate space-like ionizing radiation, we investigated whether the intracellular Mn-Fe ratio influences radiation survival, as described in **Chapter 6**.

Finally, the results from Chapter 6 indicated that yeast species are among the most radiation-tolerant microorganisms. Consequently, in **Chapter 7** we investigated the transcriptome of *Rhodotorula frigidicola* exposed to icy-moon-like conditions. This research enabled us to identify metabolic pathways that are differentially expressed under these conditions, thereby advancing our understanding of microbial metabolism under extreme conditions.

The knowledge gained in this thesis, summarized and discussed in **Chapter 8**, provides foundational insights into immune health and planetary protection in preparation for future crewed and robotic missions. The concepts explored in this thesis carry significant implications for guiding future research on immune health, microbial monitoring and planetary protection of Earth, the Moon, Mars, Enceladus and Europa.

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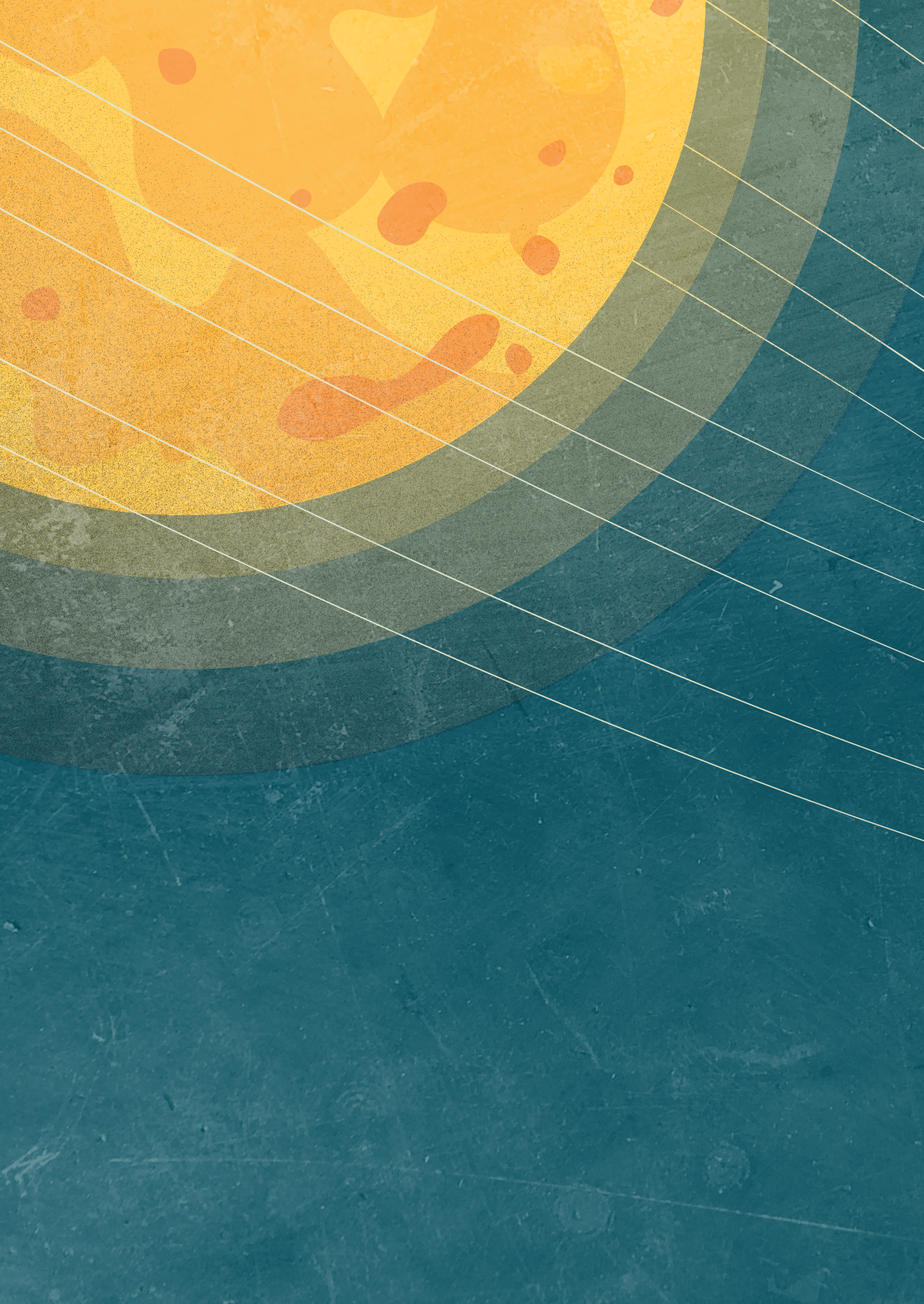
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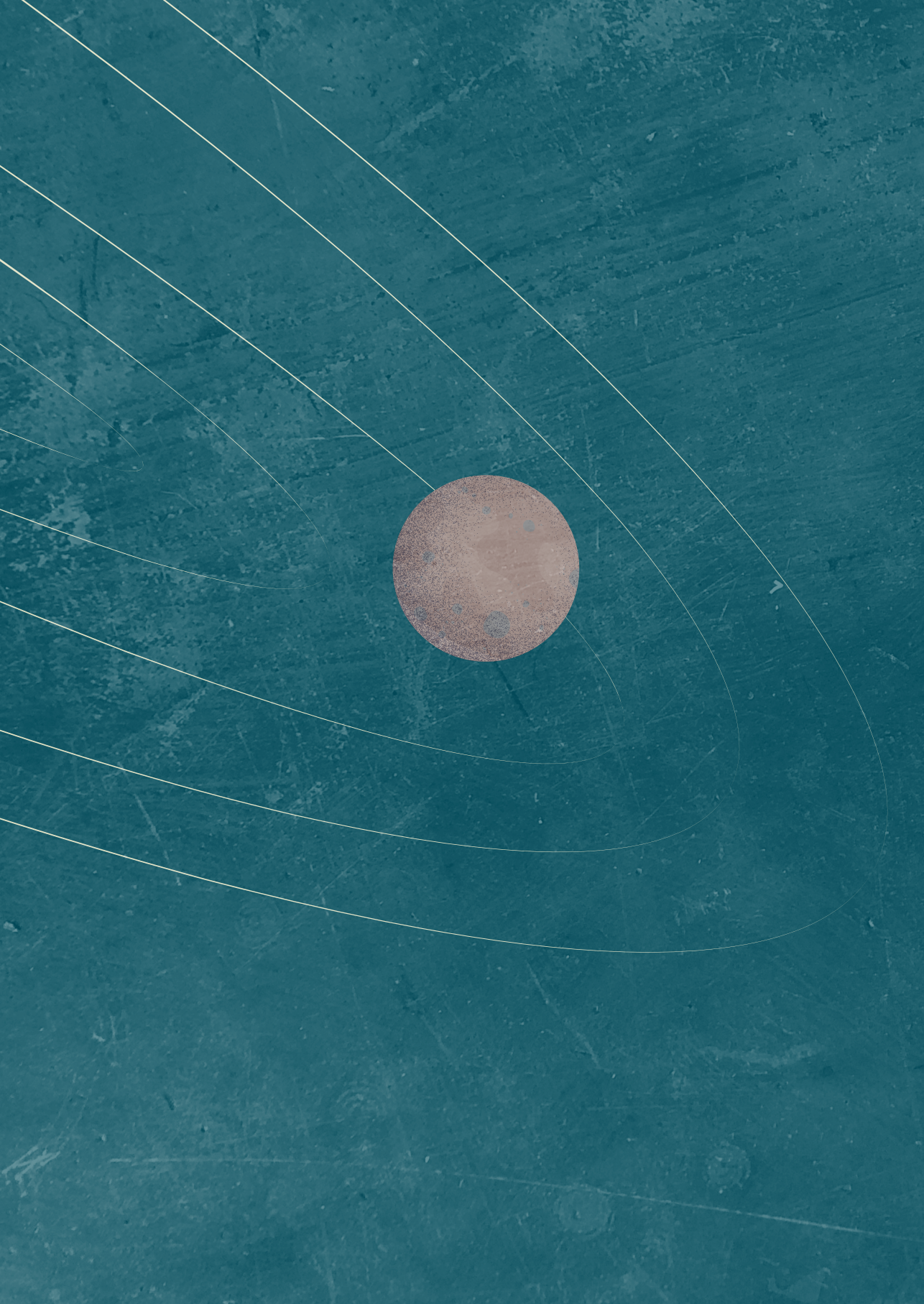
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Part 1

Impact of Mars conditions





Survival of Environment-Derived Opportunistic Bacterial Pathogens to Martian Conditions: Is There a Concern for Human Missions to Mars?

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Abstract

The health of astronauts during space travel to new celestial bodies in the Solar System, is a critical factor in the planning of a mission. Despite cleaning and decontamination protocols, microorganisms from Earth have been and will be identified on spacecraft. This raises concerns for both human safety and for planetary protection, especially if these microorganisms can evolve and adapt to the new environment. In this study, we examined the tolerance of clinically relevant non-fastidious bacterial species that originate from environmental sources (*Burkholderia cepacia*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Serratia marcescens*) to simulated Martian conditions. Our research showed changes in growth and survival of these species in the presence of perchlorates, under desiccating conditions, exposure to UV radiation, and to Martian atmospheric composition and pressure. In addition, our results demonstrate that growth was enhanced by the addition of a Martian regolith simulant to the growth media. Additional future research is warranted to examine potential changes in the infectivity, pathogenicity and virulence of these species exposed to Martian conditions.

Introduction

The survival of microorganisms in a simulated Mars environment has been investigated by a number of recent studies (1-4). These laboratory-based simulations have shown that certain terrestrial microorganisms are capable of surviving under simulated Mars conditions. However, the challenges underlying this premise lie in the accurate representation of the Martian environment: UV and ionizing radiation, dry and cold atmosphere, low water activity and accessibility, and salt/perchlorate rich regolith (5). Even though Mars is in the habitable zone of our Sun and relatively close to Earth, the environmental conditions on its surface differ greatly from our planet.

The global mean annual surface pressure on Mars is 6 hPa with a composition of 95.32% CO₂, 2.7% N₂, 1.6% 40Ar, 0.1% O₂, 0.07% CO, 0.03% (variable) H₂O, 2.5 ppm Ne, 0.3 ppm Kr, 0.08 ppm Xe, 0.04-0.2 ppm (variable) O₃, compared to the 1013 hPa at sea level on Earth (6). The surface of Mars also receives very harmful and deadly radiation. Orbital measurements have shown a total polychromatic UV flux (200-400 nm) of 49.95 W/m² (7) and an ionizing radiation total dose rate of 233 ± 12 µGy/d (8), which is much higher compared to approximately 0.575 µGy/d on Earth (9).

Furthermore, if liquid water is available, it would most likely resemble a brine made up of different salts including perchlorates (10). It is important to investigate the effects of all these factors and try to mimic Mars conditions as accurately as possible.

Most of the research that aimed to understand which microorganisms can survive on Mars has focused on extremophilic microorganisms: halophiles, thermophiles, psychrophiles, xerophiles and radioresistant organisms (2, 3, 11-14). Such experiments rendered valuable information about the mechanisms of survival, the regulation of metabolism and the regulation of different and/or shared genes (4). However, we know much less about the survivability of human pathogens that are derived from environmental sources to the conditions on Mars. This is of crucial importance as these microorganisms have been shown to be highly adaptable and therefore have a high chance to contaminate the surface of Mars particularly with crewed missions. Additionally, these organisms are opportunistic pathogens and therefore have the potential to cause (re-)infection of astronauts during space travel.

Understanding what type of microorganisms are in spacecraft assembly facilities (SAFs) and on the International Space Station (ISS) is of critical importance both for human health and planetary protection, this is why the microbiome of these

locations is continually studied (15-18). In these settings, despite the application of stringent decontamination approaches bacteria and fungi have been identified, including human associated as well as biosafety level 2 ones, an example is *K. pneumoniae* investigated in this study (19, 20). Furthermore, these measures may facilitate the unwanted selection of disinfectant resistant microbial species such as *Bacillus pumilus*, which has been isolated in SAFs several times (16, 21).

For this reason, threshold levels have been implemented on the ISS for the maximum acceptable bioburden for bacteria and fungi (22-24). Therefore, we can assume that if these microorganisms have been identified in SAFs and on the ISS, they can make their way to Mars during a human mission. Furthermore, when on Mars, the distribution of these, however in small amounts, will be unavoidable due to spacesuit and/or spacecraft leakages (25, 26). Additional sources of contamination, could be through waste disposal. Especially when human fecal matter will be processed, which is another source from which the organisms analyzed in this study can be found (27-30).

During the Apollo missions, the waste disposal system was a source of contamination as the inner and outer fecal disposal plastic bags, despite containing germicides (a mixture of sodium orthophenylphenol and sodium chlorophenylphenol) leaked inside the capsule (31). If the waste disposal on Mars will be carried out in a similar way the risks of astronauts coming into contact with these and other organisms is likely. Furthermore, the health risks to the astronauts coming into contact with the bacterial species investigated in this study are to be analyzed further, since the human immune system will be highly dysregulated during a long duration space mission to Mars (32, 33).

Therefore, gaining new insights on the impact of Mars simulated conditions on clinically relevant non-fastidious pathogens is essential. In this study, we focused on the following clinically relevant non-fastidious pathogens: *B. cepacia*, *K. pneumoniae*, *P. aeruginosa* and *S. marcescens*. We decided to study these 4 bacterial species as previous research has shown that these species are capable of utilizing and growing in minimal media with compounds identified in carbonaceous meteorites as sole carbon sources (34). Exposing these bacterial species to Mars-like conditions will help our understanding of how non-extremophiles respond to such an environment. The investigation of heterotrophs has been highlighted as a very important aspect also in regards to habitability constraints (35). We therefore strongly believe this research to be a starting point to understand the infectivity, pathogenicity and virulence of these species upon exposure to a human host on Mars.

In addition, essential elements to consider are not only human safety but planetary protection as well. Here we define planetary protection as the responsibility to avoid the unwanted contamination of habitable sites of interest with terrestrial microorganisms also known as forward contamination, as well as the contamination of Earth with dangerous material, known as backward contamination (36).

Therefore, the assessment of Mars conditions on microorganisms relevant for human health is particularly important in the new age of space exploration, which could enable humans to explore “nearby” planetary bodies such as the Moon and Mars. With current spacecraft technologies, a trip to the Moon would last 3 days and to Mars approximately 6 months. Studies have shown that despite the different approaches to sterilization of spacecraft surfaces, biological contaminants can make their way beyond Earth (37). Undoubtedly, when humans take part in space travel, microbial contamination of the explored site or planetary body is very likely due to the ubiquitous number of microbes harboring in and on the human body, i.e. the microbiome, which is an essential part of human health.

Furthermore, research into the antibiotic effectiveness and drug resistance of microbial species in human-supporting space stations can be traced to the 1980s (16, 38). Previous studies have shown that the human immune system is dysregulated in space due to a variety of factors, including the disruption of the circadian rhythm, differences in gravity, radiation, confinement, psychological stress, restricted diet and limited sanitary measures (39-41). These factors, coupled to a lowered antibiotic efficacy and changes to the human microbiome, could have severe consequences on the health of astronauts, especially in the event of an infection with microorganisms potentially modified by the extraterrestrial planetary conditions (38, 42).

We want to highlight how our study is a starting point to evaluate the survival of human associated pathogens to Mars-like conditions. Key questions this study investigates are: will terrestrial microorganisms be able to survive the Martian environment, especially that in the proximity of human habitats, which will be likely more favorable for microorganism survival? To what extent do they survive? Are there specific Mars conditions/characteristics which improve the survival of the bacteria? If they do, are they modified by the conditions on Mars and what will be the risks for human health upon re-exposure?

Materials and Methods

Growth in minimal media to select the optimal (single) carbon source

Bacterial species were ordered from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, and are the following: *B. cepacia* (DSM 7288), *K. pneumoniae* (DSM 30104), *S. marcescens* (DSM 30121) and *P. aeruginosa* (DSM 22644). All species were grown on nutrient-broth (Difco), NB agar (15 g/L), at 35°C overnight in biological triplicates. After incubation, the colonies were resuspended in M9-complete minimal salts media containing: 47.8 mM Na_2HPO_4 , 22.0 mM KH_2PO_4 , 8.6 mM NaCl, 3.7 mM NH_4Cl , 2 mM MgSO_4 , 0.1 mM CaCl_2 , 0.02 mM Fe(III)Cl_3 , without carbon source as previously described by Domínguez-Andrés, Eleveld (34). The optical density at 600 nm (OD_{600}), of the bacterial culture, was measured after dilution in the M9-complete media with 0.2% (w/v) of a carbon source.

The carbon sources used were DL-glyceric acid 20% in water ($\text{C}_3\text{H}_6\text{O}_4$), D-gluconic acid sodium salt ($\text{C}_6\text{H}_{11}\text{NaO}_7$), both purchased from Fluorochem, and D-glucose monohydrate ($\text{C}_6\text{H}_{12}\text{O}_6$), purchased from Merck. Both, Nutrient Broth and M9 with D-glucose were used as positive controls and M9-complete without carbon source was included as a negative control. Growth was determined by measuring the OD_{600} every 10 minutes with 10 sec of shaking time before each measurement at 35°C for 24 h in 96 well plates using the Elx808 reader, (BioTek® Instruments Inc. Vermont, USA). To determine total cell counts, a Thoma cell counting chamber (volume: 0.02 mm x 0.0025 mm², Brand GmbH, Wertheim, Germany) was used, with a Zeiss Primostar light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a 40x magnification objective.

Starting cell numbers were the following: 2.85×10^6 cells of *P. aeruginosa*, 6.91×10^6 cells of *B. cepacia*, 1.93×10^6 cells of *S. marcescens* and 6.05×10^6 cells of *K. pneumoniae*. Each well contained 190 µl of media with an additional 10 µl of cells grown in NB. In parallel, bacterial cultures were diluted in series with PBS, plated on NB and incubated overnight to determine colony forming units (CFU) counts.

Growth in the presence of sodium perchlorate at Mars-like concentrations

The bacteria were inoculated in M9 complete with D-gluconic (from here on referred to as M9-gluconic) and M9-glucose with Mars relevant sodium perchlorate ($\text{NaClO}_4 \times \text{H}_2\text{O}$) concentrations, 35 and 70 mM. Cells were grown in 96 well plates at 35°C for 15 h in the Elx808 reader to monitor the OD_{600} measurements. A second

similarly-conducted experiment was performed with increased final concentrations of perchlorate of 150 and 300 mM.

Growth with supplementation of MGS-1 to the growth media

In order to evaluate the survival and/or growth of the different bacterial species in the presence of Mars regolith, the cells were diluted in M9-gluconic with the addition of MGS-1 (Mars Global Simulant 1, Exolith Lab, Florida, USA, see Cannon, Britt (43) for regolith composition and supplementary table 1 and 2), at different concentrations (% w/v) in 250 ml Erlenmeyer flasks and incubated overnight with 125 rpm orbital shaking at 35°C. Electron microscopy pictures were taken with the Hitachi TM3000 (Hitachi, Tokyo, Japan) at x100, x400 and x1000 magnification following the manufacturer’s protocol, see supplementary figure 2. The MGS-1 was pre-sterilized by autoclaving prior to addition to the growth media. The concentration of MGS-1 was evaluated from 0.3% (w/v) to 12% (w/v) to determine whether there would be a toxic effect to these bacteria. Monitoring cell survival and/or growth with OD₆₀₀ measurements and microscopy cell counts was not possible due to the turbidity caused by the regolith simulant. Therefore, cell counts were determined by dilution plating on NB agar plates that were incubated overnight at 35°C.

Survival after desiccation treatment

The bacteria were grown in M9-gluconic overnight to have them in late-exponential phase. Aliquots of 200 µl were placed on sterile 1 cm Ø glass disks and left to dry for 24h at room temperature under sterile conditions with a relative humidity of ca. 30% ±10% in a working clean bench. The surface area of each glass disk is 0.7854 cm², based on this value and the average surface area of a cell of each species, we calculated that the number of cells we have on each disk fit, we therefore assume they are in a monolayer. Table 1 summarizes the calculations performed.

Table 1. Calculated Number of Cells on Glass Disks Used in the Desiccation Experiments

Species	Total number of cells that fit on glass disk based on cell surface area	Total number of cells on glass disk during the experiment
<i>Burkholderia cepacia</i>	$1.57 \times 10^7 - 2.61 \times 10^7$	9.14×10^5
<i>Serratia marcescens</i>	$4.91 \times 10^7 - 1.75 \times 10^8$	3.27×10^7
<i>Pseudomonas aeruginosa</i>	$1.57 \times 10^7 - 1.57 \times 10^8$	4.25×10^6
<i>Klebsiella pneumoniae</i>	7.85×10^7	9.77×10^6

To determine the survival rate, each glass disk with desiccated cells was placed in a 5 mL tube filled with 1 mL of PBS and lightly vortexed. A dilution series with PBS was performed to determine the survival rate based on CFU counts. The same experiment was performed with cells grown in M9-gluconic supplemented with 5% (w/v) MGS-1.

Survival to UV radiation

To understand how the bacteria survive to UV-C (254 nm), irradiation was performed by growing them in M9-gluconic overnight. A volume of 2700 μL of each bacterial culture was placed in closed sterile UV transmissible quartz cuvettes (0.5 cm path-length, Hellma GmbH & Co. KG, Muelheim, Germany) and placed vertically at 32.5 cm from the UV source described below. Each cuvette contained a small magnetic stirrer which allowed the bacterial cultures to be stirred while being radiated, in order to have a uniform exposure of most of the bacterial cells. UV-C irradiation was produced by a low-pressure Hg lamp (Model NN 8/15 Heraeus, Berlin, Germany) which was allowed to warm up for >30 minutes prior to exposure (supplementary figure 1). To measure the fluence of the lamp at the sample site, a regularly calibrated UVX radiometer with a UV sensor (UV-25) for 254 nm (both UVP Ultra-Violet Products, Cambridge, UK) was used.

The UV-C fluence rates were 170 $\mu\text{W}/\text{cm}^2$ and to reach the maximum dose of 250 J/m^2 at 32 cm distance from the light source it took 147 seconds. The same experiment was performed with polychromatic Mars-like UV (200-400 nm) radiation using the SOL2 polychromatic lamp, equipped with a UV 500S irradiation source (Dr. Hoenle AG, UV-Technologie, Germany) at the German Aerospace Center in Cologne, Germany (44). The fluence of the lamp was determined by using the Bentham DMc150 transportable spectroradiometer (Bentham Instruments Ltd., Reading, UK), with optics inside the simulation chamber as per Rabbow, Rettberg (45). The polychromatic UV fluence rates were 10.35 W/m^2 at 112 cm distance from the light source and to reach the maximum dose of 1200 J/m^2 , it took 116 seconds.

Survival was determined after exposure to UV-C and polychromatic-UV by dilution and plating of the bacterial cultures on NB agar plates and incubation overnight at 35°C. Prior to dilution plating, during total irradiation times, samples were briefly stored in the dark on ice (at 4°C). Media absorbance measurements were performed with the use of the Thermo Scientific™ NanoDrop™ 2000c, in a UV-transparent 1 mL cuvette. Media absorbance measurements were also taken in order to determine possible absorption effects.

Survival to polychromatic UV radiation in growth medium with and without supplementation of 5% (w/v) MGS-1

The same experimental procedure was performed with cells grown in M9-gluconic and with supplementation of 5% (w/v) MGS-1.

Survival after exposure to 1 day of desiccation and to polychromatic UV radiation

To determine the survival of the bacteria to the combined effect of two Mars-like conditions consecutively, late exponential cell cultures were exposed to desiccating conditions as described above. After 24 h of desiccating conditions, the glass disks with the dried cells were exposed to polychromatic UV irradiation. The disks were exposed to the desired UV fluences. Survival was determined by CFU counts.

Survival in growth medium supplemented with 5% (w/v) MGS-1 after desiccation and exposure to Mars atmosphere and pressure in combination with polychromatic UV radiation

The survival of the species to Martian atmosphere and pressure was evaluated by first desiccating the cells in combination with 5% (w/v) MGS-1, as described in 2.6. The second step was to store the desiccated cells on glass disks in the adapted TREX (Transport and Exposure)-box, as described by (46, 47). The TREX-box was connected to a gas supply of Mars-like atmosphere (2.7% N₂, 1.6% Ar, 0.15% O₂ in CO₂ vol/vol), and a vacuum pump (Rotary vane pump DUO 035 and HiPace 700, Pfeiffer Vacuum GmbH, Germany) to apply the desired pressure (6 hPa), and monitored constantly during the exposure (TPG 262 Full Range Gauge, Pfeiffer Vacuum GmbH, Germany). Samples were exposed to Mars-like atmosphere and pressure (6 hPa) for 24 h in the gas-tight TREX-box. The box, through the use of a suprasil window, enabled full UV-VIS exposure of the samples during polychromatic UV radiation while under desiccating conditions and Mars-like pressure and atmospheric composition. Similar Mars-like exposures with the use of the TREX box were performed by Cortesão, Koch (47). All figures were created with the use of the data visualization library Seaborn version 0.12.1 (48), an interface of Matplotlib (49).

Results

Assessment of the growth on single extraterrestrial carbon sources

The study described by Domínguez-Andrés *et al.* (2020) showed that the selected nonfastidious bacterial species (*B. cepacia*, *K. pneumoniae*, *P. aeruginosa*, and *S. marcescens*) were able to tolerate and grow under minimal media conditions

supplemented with carbon sources that are also present in carbonaceous meteorites. For the survival assays of this study, we first evaluated which carbon source (dl-glyceric acid or d-gluconic acid) showed the greatest OD₆₀₀ increase after incubation. We used glucose and NB as positive controls (Fig. 1). In all cases, the positive controls, as well as d-gluconic acid, showed successful growth during the culture period of 24 h. In the case of dl-glyceric acid, only a minimal increase in OD₆₀₀ was detected during the incubation period. Interestingly, the bacteria did not require an adaptation period or repeated inoculations to show successful growth in M9-complete.

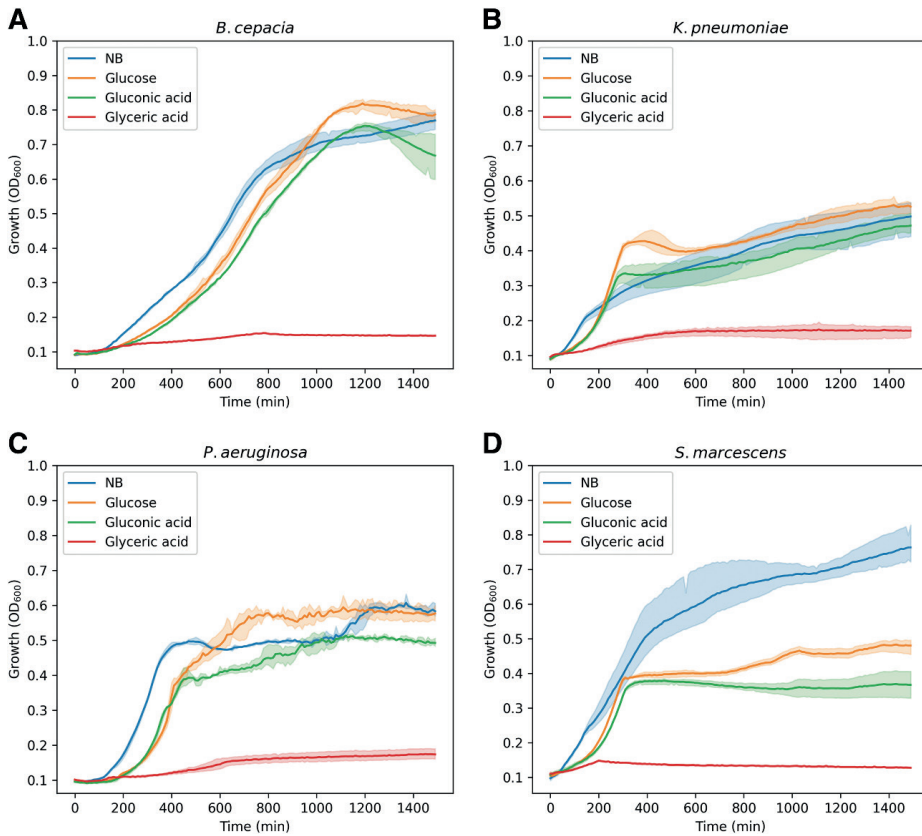


FIG. 1. Nonfastidious bacteria are capable of growing in M9 supplemented with a single carbon source. Growth curves of the nonfastidious bacteria in M9 supplemented with a single carbon source. The graph shows the growth curves in nutrient broth (blue), M9-glucose (orange), M9-gluconic (green), and M9-glyceric (red). The curves represent the OD₆₀₀ measurements taken over a period of 24 h at 35°C. The color shading represents the standard deviation of the OD₆₀₀ values for the three biological triplicates. *Burkholderia cepacia* (A), *Klebsiella pneumoniae* (B), *Pseudomonas aeruginosa* (C), and *Serratia marcescens* (D). OD₆₀₀ = optical density at 600 nm.

Evaluating the survival in the presence of sodium perchlorate

To understand whether the bacteria would be capable of growing in M9 medium, with a single carbon source, in the presence of Mars-relevant sodium perchlorate concentrations of 35 mM and 70 mM (10), we inoculated M9-gluconic and M9-glucose (positive control) with 35 and 70 mM sodium perchlorate. Despite these challenging growth conditions, all species showed growth, however a longer lag time prior to the exponential phase was observed (Figure 2). Furthermore, none of the bacteria achieved the same maximum OD₆₀₀ values as in the controls. When analyzing the results in more detail, one can observe that *B. cepacia* was able to grow in the presence of sodium perchlorate only when glucose was provided as a carbon source.

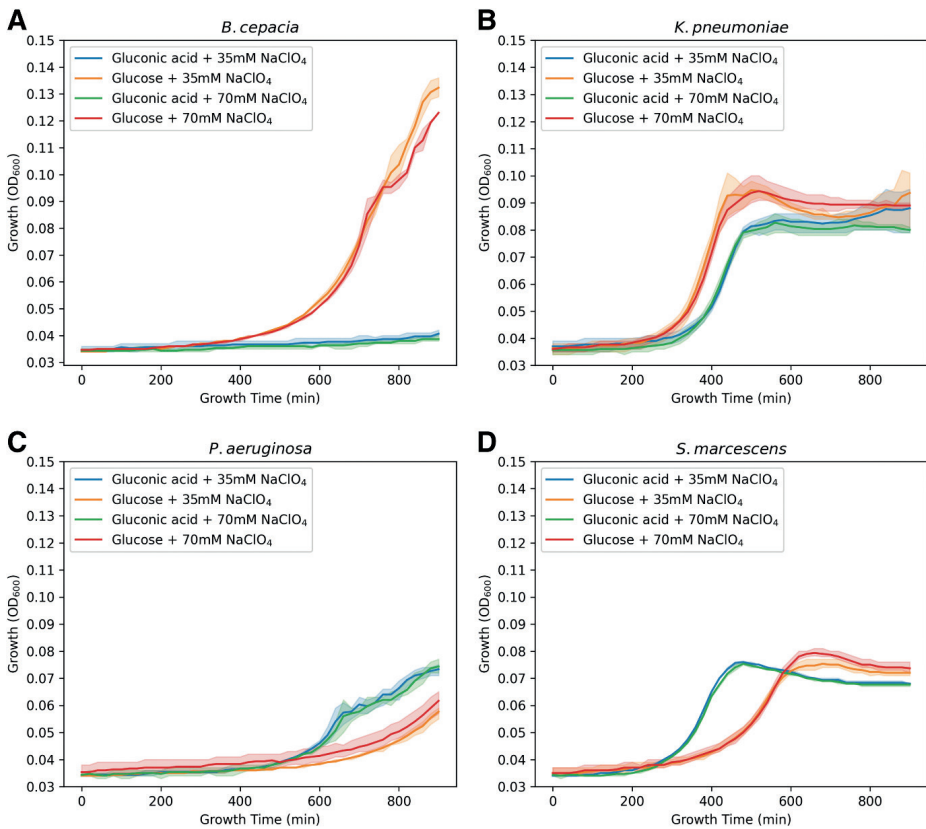


FIG. 2. The bacteria are capable of showing growth with 35 and 70 mM of sodium perchlorate in M9. Growth curves of the bacteria with Mars-relevant concentrations of sodium perchlorate. Growth curves measured by OD₆₀₀ of the bacteria inoculated in M9 with a single carbon source and the addition of sodium perchlorate inoculated over a period of 15 h at 35°C, *Burkholderia cepacia* (A), *Klebsiella pneumoniae* (B), *Pseudomonas aeruginosa* (C), and *Serratia marcescens* (D). The line represents M9-gluconic with 35 mM NaClO₄ (blue), M9-glucose with 35 mM NaClO₄ (orange), M9-gluconic with 70 mM NaClO₄ (green), and M9-glucose with 70 mM NaClO₄ (red). The color shading of the growth curves represents the standard deviation of the three biological triplicates.

Growth of *K. pneumoniae* was hardly affected by the presence of sodium perchlorate; only an increase in the growth was observed in the media with glucose, as was also found for the other species (*P. aeruginosa* and *S. marcescens*). In general, growth of *P. aeruginosa* in all media was limited. Interestingly, in the case of *S. marcescens*, the cells were able to reach the stationary phase more rapidly in M9-gluconic acid, than in the media supplemented with glucose (Fig. 2). Similar growth rates were seen when the bacteria were incubated with increased sodium perchlorate concentrations, except for *B. cepacia* where hardly any growth was measured (Fig. 3). However, this oxidizing stressor caused an even greater reduction in growth speed and concentration.

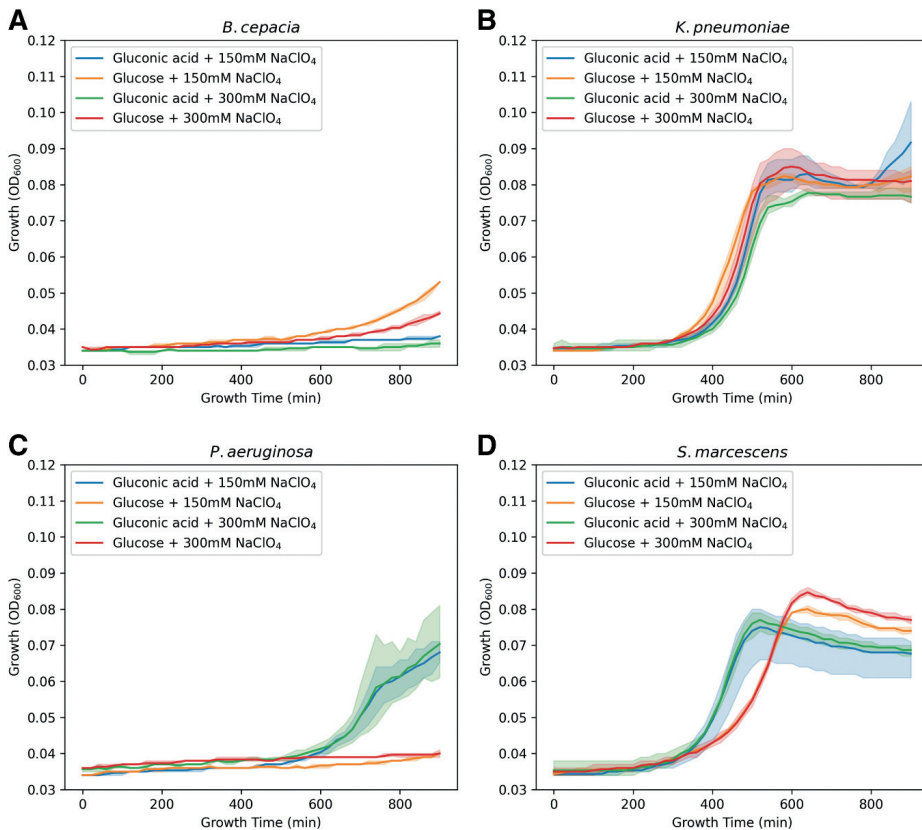


FIG. 3. Evaluating the growth in the presence of higher perchlorate concentrations. Growth curves of the bacteria with increased concentrations of sodium perchlorate. Growth curves measured by OD₆₀₀ of the bacteria inoculated in M9 with a single carbon source and the addition of sodium perchlorate inoculated over a period of 15 h at 35°C, *Burkholderia cepacia* (A), *Klebsiella pneumoniae* (B), *Pseudomonas aeruginosa* (C), and *Serratia marcescens* (D). The line represents M9-gluconic with 150mM NaClO₄ (blue), M9-glucose with 150mM NaClO₄ (orange), M9-gluconic with 350mM NaClO₄ (green), and M9-glucose with 350mM NaClO₄ (red). The color shading of the growth curves represents the standard deviation of the three biological triplicates.

Evaluating the survival to simulated Mars regolith

To understand whether the bacteria could grow in the presence of simulated Mars regolith, as found in Martian brine or ponds, we incubated the species in M9-gluconic acid in the presence of different concentrations (0.3–12% w/v) of MGS-1 (Fig. 4). The control culture was not supplemented with MGS-1. It was found that most concentrations of MGS-1 stimulated rather than inhibited growth. In 1% (w/v) MGS-1, all species showed a higher colony count than the control. In the case of *P. aeruginosa*, the difference in CFU was almost four orders of magnitude higher. Concentrations of MGS-1 in the media were tested up to 12% (w/v) to understand whether the simulated regolith would be limiting for growth; however, this was not observed.

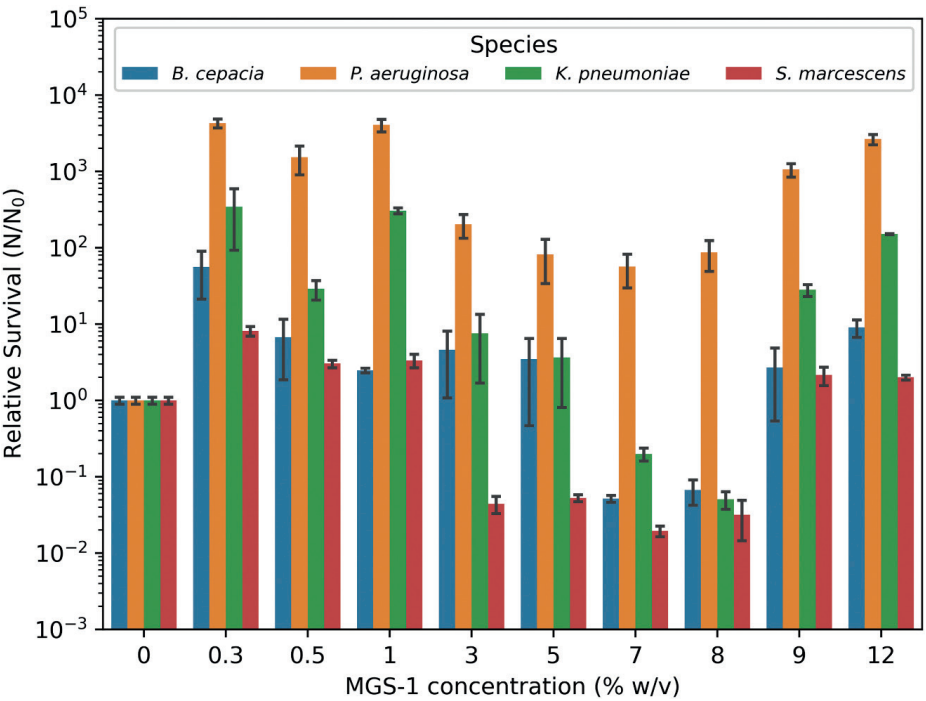


FIG. 4. Evaluating the growth in the presence of MGS-1. Growth in the presence of MGS-1. The regolith simulant concentration was tested from 0.3% to a maximum of 12% (w/v) in liquid M9-gluconic media. The colored bars represent the colony counts of the bacteria inoculated with MGS-1 and incubated overnight at 35°C. MGS-1 = Mars Global Simulant 1.

For all the subsequent exposure experiments, an MGS-1 concentration of 5% (w/v) was chosen as this was the optimal concentration for making dilution series for CFU counting, enabling higher throughput experiments. A concentration of 5% (w/v)

is considered relevant in terms of exposure of the bacterial species to the regolith components, based on which predictive conclusions can be drawn.

Evaluating the survival to desiccation

To understand how bacterial species from Earth would survive under such dry conditions, we assessed the desiccation of the different species. Before desiccation, cells were grown in M9-gluconic acid medium to late exponential phase, at room temperature ($22^{\circ}\text{C} \pm 4^{\circ}\text{C}$). The only species that survived desiccation and showed survival up to a maximum of 16 days were *K. pneumoniae* and *S. marcescens* (Fig. 5). When bacteria were grown in combination with 5% (w/v) MGS-1, all species survived at least 13 days of desiccation. This is a substantial increase in survival especially for *B. cepacia* and *P. aeruginosa*, which previously did not survive any desiccation time frame. Furthermore, the maximum survival for both *K. pneumoniae* and *S. marcescens* increased from 16 to 22 days.

Evaluating the survival to UV exposure

To measure the resistance of bacteria against UV irradiation, we exposed the different species to UV-C (254 nm) and polychromatic UV (200–400 nm) radiation at different fluences. We found that *P. aeruginosa* and *S. marcescens* showed the highest resistance against both UV-C and polychromatic UV (Fig. 6). The calculated F_{10} fluences (fluence to which 10% of the population survives) support this finding. The calculated values were 146 and 102 J/m^2 for UV-C and 736 and 737 J/m^2 for polychromatic UV, for each species, respectively. The two other species, *B. cepacia* and *K. pneumoniae*, had a much lower survival limit, and the calculated F_{10} values were 23 and 45 J/m^2 for UV-C and 69 and 35 J/m^2 for polychromatic UV, respectively.

Evaluating the survival to a combination of Mars-like conditions

The bacteria were exposed to a combination of Mars-like conditions to closely replicate all the conditions present on Mars. The first experiment was conducted with *K. pneumoniae* and *S. marcescens*, the only species that showed survival to desiccation to a combination of desiccation with polychromatic UV radiation. Only *S. marcescens* was able to survive under these conditions (Fig. 7B). Comparing the F_{10} of the species exposed to polychromatic UV grown with and without MGS-1, there is a clear increase in survival when the media are supplemented with MGS-1 (Fig. 7A).

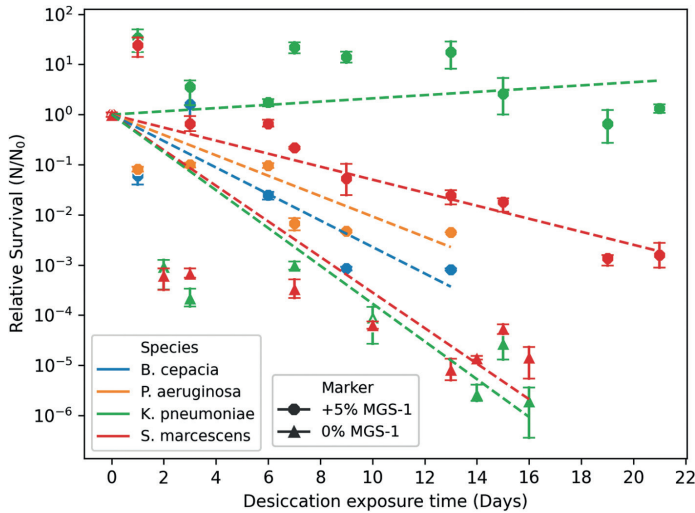


FIG. 5. Desiccation survival of the bacteria in the presence of 5% (w/v) MGS-1. Relative survival of the bacteria when exposed to desiccating conditions. All the bacteria were able to survive this condition when supplemented with 5% (w/v) MGS-1. When grown in M9-gluconic, *Klebsiella pneumoniae* (green triangle) and *Serratia marcescens* (red triangle) were the only species surviving, while *Burkholderia cepacia* and *Pseudomonas aeruginosa* did not survive, points not shown. The survival with MGS-1 can be seen for all species, *K. pneumoniae* (green circle), *S. marcescens* (red circle), *B. cepacia* (blue circle), and *P. aeruginosa* (orange circle). The survival of all the species under all conditions was evaluated until 21 days. The displayed survival shows the limit to which it was detected.

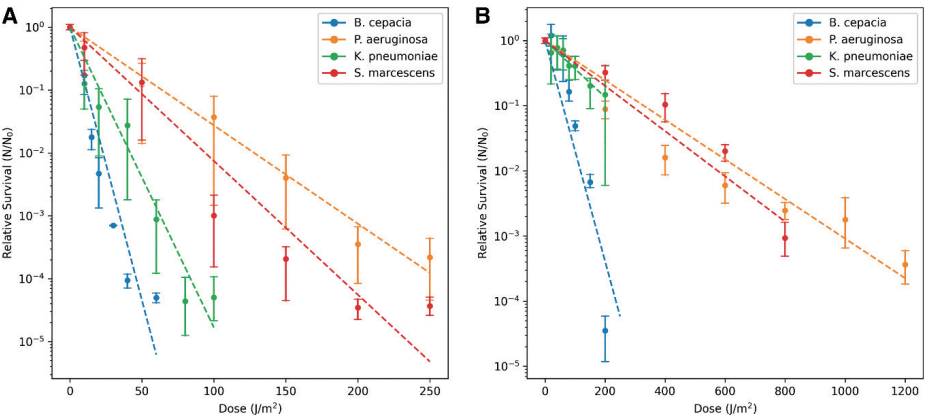


FIG. 6. Relative survival of all the bacteria to UV radiation. Maximum radiation doses for UV-C (254 nm) was 250 J/m^2 (A) and 1200 J/m^2 of polychromatic UV (200–400 nm) (B). Both (A) and (B) plots show the limit of survival for all the species to UV-C and polychromatic UV irradiation. Dilution plating was evaluated at higher doses as well, up to 1500 J/m^2 , however, no survival was detected. UV = ultraviolet.

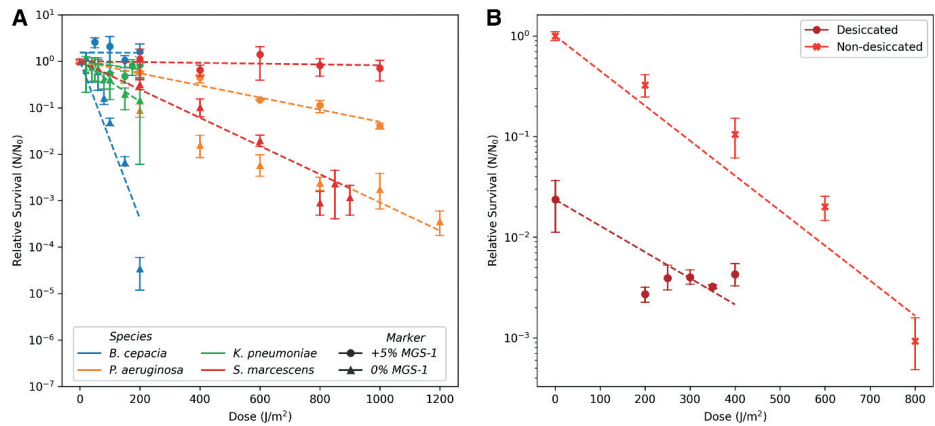


FIG. 7. Survival of the bacteria to combined conditions. Relative survival of the bacteria to polychromatic UV radiation with or without MGS-1 **(A)** and to polychromatic UV and desiccation **(B)**. The figure on the left compares the survival of the bacteria to polychromatic UV radiation after having been grown overnight at 35°C in M9-gluconic (triangles) or in M9-gluconic with the addition of 5% (w/v) MGS-1 (circles). The figure on the right compares the survival of *Serratia marcescens* to polychromatic UV radiation after being grown in M9-gluconic (light red) and after being desiccated for 24 h (dark red). The control (N_0) for the survival to polychromatic UV in the graph on the left is the survival after 1 day of desiccation. The species were exposed up to 1500 J/m^2 , the data in both **(A)** and **(B)** of the figure show the limit to which survival was detected.

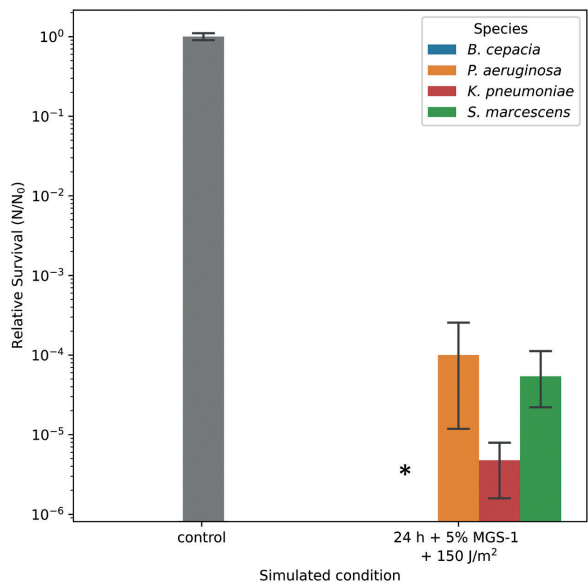


FIG. 8. Survival of the bacteria to combined Mars conditions. All the bacteria were grown in M9-gluconic in combination with 5% (w/v) MGS-1, desiccated o/n, and exposed to Martian atmosphere and pressure for 24 h. All the bacterial species were able to survive these combined conditions with the exception of *Burkholderia cepacia* (*).

Furthermore, we evaluated cell survival following exposure to the combined Martian conditions. This included growth in combination with MGS-1, desiccation, UV irradiation, as well as Martian atmosphere and pressure. Under these conditions the cells were desiccated overnight after being grown in combination with 5% (w/v) MGS-1, placed in a TREX-box with Mars-like atmosphere and pressure and irradiated with 150 J/m² of polychromatic Mars-like UV radiation. This dose would be equivalent to approximately 12 seconds (12 sec x 12.49 W/m²) on the Martian surface assuming the Mars solar constant is 49.95 W/m², no absorption by the atmosphere, and that only half of the time 1 hemisphere of the planet is irradiated with polychromatic UV (200-400 nm) radiation (7). In Figure 8, the survival of the species to these conditions is presented.

All species, except *B. cepacia*, were able to survive these conditions, with *P. aeruginosa* and *S. marcescens* showing the highest degree of survival (one order of magnitude higher than *K. pneumoniae*). These results show that all bacterial species that we have analyzed are able to survive, to a certain extent, under Martian simulated conditions. However, the amount of time the bacteria can survive in the simulated environment varies and is generally limited.

Discussion

The Earth harbors many non-fastidious bacteria in the environment, a number of which can cause opportunistic infections. To protect the crews of future Mars missions, it is essential to assess the capabilities of these potential pathogens to survive the Martian environment. Understanding their resistance to these extreme conditions can help with the selection of targeted disinfectants. Moreover, understanding the survival and likely changes that will be undergone by these bacteria will be very important to understand their future interaction with humans and the capacity to cause disease. Past literature has investigated the survival of different human-related bacteria to simulated Mars and interplanetary spacecraft conditions, including the food contaminant *Bacillus subtilis* (50, 51), the pathogen *Enterococcus faecalis* (52) as well as *Serratia liquefacens* (53).

In the present study, we show that non-fastidious human pathogens can survive for days to weeks under Mars-like conditions. Importantly, it seems that the presence of Mars regolith improves the survival of these microorganisms.

We investigated the survival of the Gram-negative bacteria *B. cepacia*, *P. aeruginosa*, *K. pneumoniae* and *S. marcescens* to Mars-like conditions. Previous work with these species has shown that they are able to grow in different types of oligotrophic media with a range of singularly provided carbon sources (34). These findings are particularly remarkable since these bacterial species are typically grown in rich media. However, being pathogenic they could also be well suited to grow or survive in adverse environments such as the human body. Our study reports concurrent findings for cells grown in M9-complete minimal media supplemented with DL-glyceric acid and D-glucose. The use of NB and glucose highlighted how glyceric acid did not support an increase in growth (measured by OD₆₀₀).

The effect or lack thereof, can be explained due to glyceric acid having a lower amount of carbon atoms (3) than gluconic acid (6). Experiments with adjusted concentrations of carbon atoms as gluconic acid showed the same growth rate as the original concentration. It may be suggested that the bacteria would show a higher growth rate with gluconic acid due to its closer molecular similarity to glucose.

Data has shown that most of the liquid water which would be available on Mars would be in the form of perchlorate brines, within hydrated minerals or as ice frozen beneath the surface (54-56). Moreover, it is likely that the presence of water and temperature changes will change Mars conditions in the proximity of future human habitats on Mars. Therefore, it was important for us to first understand if the selected species would be capable of surviving and/or growing in these environments. Analyzing the growth curves of the different species in simple salts medium (M9) supplemented with Mars relevant sodium perchlorate concentrations and in oligotrophic media with only one C source, we can see how all the species differently tolerate this condition. Both *K. pneumoniae* and *S. marcescens* showed growth in media supplemented with glucose and gluconic acid.

However, *B. cepacia* showed growth only in glucose-supplemented media, while *P. aeruginosa* growth was greatly limited with both sugars. Genes responsible for perchlorate-resistance have been identified in the Atacama Desert, a Mars analogue site on Earth, and research has shown that they can be expressed in bacteria such as *Escherichia coli* (57). A strain of *S. marcescens* NIIST5, isolated from a laboratory-scale fed-batch bioreactor, contained genes involved in the perchlorate reduction pathway, such as perchlorate reductase and chlorite dismutase (58). However, this strain is different from the one used in this study. Furthermore, we are not aware of other perchlorate reducing genes in the additional three investigated

bacterial species. Nonetheless, this could suggest that a similar mechanism of perchlorate reduction could take place on Mars, for example through the process of gene transfer.

Consequently, the transfer of perchlorate reducing genes could support even better growth of the bacteria in perchlorate brines. This is of evermore greater importance, as we have shown that the bacteria can tolerate higher concentrations of sodium perchlorate (Figure 3). Earlier studies have investigated the resistance of *S. marcescens* to perchlorate (58), but this research did not simulate Mars-relevant concentrations. Further investigation is required into the growth potential of the species with other Mars-identified perchlorates.

Having shown that the species are capable of growing in M9-gluconic and, to an extent, in the presence of sodium perchlorate, understanding how the species might interact with the regolith was the next step in replicating Martian conditions. Interestingly, all the bacteria incubated up to 1% (w/v) MGS-1 showed increased cell proliferation compared to M9-gluconic acid alone. Research has shown that some bacteria can make use of rocks and minerals to obtain energy through redox reactions (59, 60). Increasing the MGS-1 concentration in the growth media past 1% (w/v) did not necessarily result in higher cell yields for all species studied. In our experiments, *P. aeruginosa* is the only species which showed a greater cell number after incubation in all MGS-1 concentrations, compared to the control.

In all further experiments where MGS-1 was supplemented, a concentration of 5% (w/v) MGS-1 was used to more closely represent the conditions on Mars, and to have more efficient control over the volumes. Due to the particular composition of the regolith simulant and the grain size distribution of <0.04 to 1000 μm , we speculate that the regolith could shield the bacteria from a wide range of adverse conditions. In addition, on Mars the bacteria would be desiccated in a mixture with regolith. Under this assumption we speculate that the structural composition of the MGS-1 could allow the retention of traces of water which would enable the bacteria to have an increased survival (see supplementary figure 2). Undoubtedly, further investigations into the interaction of these species with the regolith are warranted.

Evaluating the survival of bacteria to desiccating conditions is an area of research which has been extensively investigated, considering its importance for the Mars environmental characteristics. Our results are particularly interesting as they show how two bacterial species, *K. pneumoniae* and *S. marcescens*, were able to survive desiccation for up to 16 days when grown in minimal media, M9-gluconic.

The survival of these two species in this study is particularly remarkable when compared to other bacteria which can only survive these conditions if grown in nutritionally rich media – *E. coli* O15:H7, for 8 days (61) and *Mycobacterium tuberculosis*, 1 week (62). Other extremophilic microorganisms such as the (hyper-) thermophiles *Hydrogenothermus marinus* and *Aquifex aeolicus*, can survive to the same desiccating conditions for 28 days, when cultivated in species-specific standard conditions (63).

The survival of the two species analyzed is interesting when considering the cultivation conditions are in minimal media. When desiccation is combined with polychromatic UV exposure, the only species which survived was *S. marcescens*. The effects of desiccation alone can lead to changes to the cell structures, protein denaturation and DNA strand breaks (64). The combined effects of polychromatic UV radiation and desiccation led to a maximum dry-state survival of 1 day. Due to the inability of the cells to be metabolically active in a dry state, the mechanisms of DNA repair most probably could not be activated and shielding of *S. marcescens* could only be provided with the available intracellular pigment.

Of further interest is the survival of all the species when cultivated in M9-gluconic supplemented with 5% (w/v) MGS-1. All the bacteria were able to survive at least up to 13 days. Furthermore, the bacteria which were able to survive desiccation without MGS-1, had an increase in their tolerance from 16 to 21 days. Other studies have also shown how archaea, used as model organisms for life on Mars, were able to survive more extensively to desiccation when supplemented with regolith simulants (65, 66). We can speculate that the regolith might enable the retention of water, which could support the survival of the bacteria for longer periods of time.

Despite Mars having a lower solar constant (49.95 W/m^2) compared to Earth (116.16 W/m^2), for total UV irradiance (200-400 nm), the thinner and differently composed atmosphere allows more of the short-wavelength solar radiation to reach the Martian surface (67, 68). In order to understand how the bacteria can survive these conditions, we exposed them to UV-C (254 nm) and polychromatic Mars-like UV (200-400 nm) radiation. The UV spectrum of our SOL2 lamp (see supplementary figure 1) is not a replication of what can be seen on the surface of Mars as can be seen by Delgado-Bonal, Zorzano (69), Vicente-Retortillo, Valero (70), however it shows us the effect of simulated UV irradiation on the survival of the bacterial species.

Both *S. marcescens* and *P. aeruginosa* have repair mechanisms that we wanted to limit in this study. These include *S. marcescens*' photolyase enzyme, Nucleotide Excision Repair (NER) (71); and *P. aeruginosa*'s DNA polymerase IV mechanism (72). To avoid the activation of these repair mechanisms, as much as possible, samples were briefly stored in the dark on ice, before dilution plating during total irradiation times. Our results have shown that the two most UV tolerant species, *P. aeruginosa* and *S. marcescens*, are also capable of repairing their DNA with enzymes activated with certain wavelengths of light, such as with photolyase.

The increased survival of these two species compared to the other bacteria in this study could be attributed to the red prodigiosin pigment of *S. marcescens* (73) and the several pigments produced by *P. aeruginosa*, such as pyoverdine, pyocyanin, pyorubin and pyomelanin (74). The pigments, coupled with the NER and polymerase IV DNA repair mechanisms, can give the two bacteria a great advantage when exposed to UV radiation. This relationship can especially be seen when analyzing the F_{10} values of the organisms when exposed to UV-C and polychromatic UV. Some model organisms have been shown to have similar F_{10} UV-C doses such as *Salinisphaera shabanensis* (56 J/m²), *Buttiauxella* sp. MASE-IM-9 (42 J/m²). However, others have demonstrated higher F_{10} values such as *Deinococcus radiodurans*, 660 J/m² (3).

This study has shown that supplementing growth media with MGS-1 can increase the tolerance of certain species to several Mars simulated conditions. This effect is also present when bacteria are grown in 5% (w/v) MGS-1 and exposed to UV-C and polychromatic UV radiation. We suspect that the regolith grains provide partial protection to the microorganisms. Literature has investigated the minimum thickness or depth on Mars which can attenuate the biocidal effect of polychromatic UV radiation (75, 76). Furthermore, Schuerger, Golden (77) reports that a 0.5 mm layer of crushed basalt can attenuate >99% of UV penetration (200-800 nm). Despite the differences in the experimental setup, similar survival characteristics can be seen in our results with the exposed *P. aeruginosa* and *S. marcescens* when grown in 5% (w/v) MGS-1.

Combining the exposure of the strains to desiccation and Mars atmosphere could closely replicate the conditions to which the bacteria would be exposed to on the surface of Mars. The effect of exposing the cells to 24 h of Mars atmosphere and 150 J/m² of polychromatic UV radiation following an overnight exposure to desiccation can be greatly deleterious to the cells. However, only *B. cepacia* could not survive to these cumulative conditions, although the number of viable cells was strongly decreased for the other species as well. Despite 24h being a limited

period of time we wanted to describe this result as it can still be a relevant time frame for astronauts re-encountering the bacterial species during routine waste disposal tasks in a possible Mars colony.

The Gram-negative *Acinetobacter baumannii* is also an opportunistic pathogen which can survive 26 days of desiccation in a mineral medium with one carbon source, a growth condition similar to the one used in this study (78). Investigations into food safety standards with *Salmonella enterica* (79), and nosocomial safety standards with *Staphylococcus capitis* (80), have shown how these two species can also survive desiccation. We believe that the data from such studies could prove useful, not only in identifying possible similarities in survival mechanisms, such as with compatible solutes, but also speculate on their potential survival to Mars-like conditions. Furthermore, our research could be a relevant starting point for investigating the long-term effects of deep space on the human immune system.

We also acknowledge a number of limitations of our study. First, practical aspects precluded us to study the impact of the factors studied for long periods of time, situation likely to be incurred in practice. Second, we have investigated in the present study only the impact of each of the various factors at one time, while these conditions will be likely encountered in combination on the surface of Mars. Finally, these factors may argue that even harsher conditions will be encountered by these microorganisms on Mars. However, we should also point out that the Mars conditions in the proximity of future human habitats are likely to be milder and more conducting for bacterial survival, and this also needs to be considered. All these considerations argue that future studies are needed to extend this field of investigation, some which are currently planned by our group.

In conclusion, identifying the extent to which human pathogens can survive to Mars simulated conditions is the first step to determine possible health threats to astronauts. The potential of the bacteria to grow in minimal media makes them a concern for human safety and planetary protection. As illustrated here, several species can survive a range of Mars-like environments, especially when their growth is supported by the regolith simulant, MGS-1. We highlight how among the species analyzed, *P. aeruginosa* and *S. marcescens* have the highest potential of surviving the combined stressors. Bacteria were exposed to desiccation repeatedly throughout the experiments. This condition is important as the exposure to Mars atmospheric conditions will likely bring desiccating conditions (6). It can be suggested that under specific conditions, the bacteria would be very protected from exposure to UV radiation. For example, when grown with MGS-1 or under a thin layer of regolith.

Undoubtedly, further investigations into the mechanisms of survival to desiccation of the species and other Gram-negative human pathogens is warranted. The identification of potential shared desiccation resistance mechanisms could aid in the targeted development of biocides and drugs for use on Mars. Moreover, this research can also aid in updating sterilization techniques for space missions, as well as identifying treatment techniques for diseases. In this study we only investigated a relatively limited number of bacterial species for their resistance to Mars conditions. One could envisage the selection of more resistant microorganisms when considering the thousands of species that make up the human microbiome. Understanding the selection and evolutionary process of human microbiome in Mars conditions is of urgent importance in light of the planned human missions to Mars.

In addition, studies to investigate the interactions of the potentially-modified microorganisms and the host immune system will be of crucial importance to determine whether the investigated bacteria could pose a greater human health risk after exposure to Mars-like conditions. This is of even higher importance to investigate as the human immune system is highly dysregulated during space travel. The probability of disease-causing organisms making their way to Mars is highly likely when human travelers are involved. While transporting humans to Mars will be a difficult challenge, transporting the microorganisms of the human microbiome will be inevitable. Further studies that investigate how these non-extremophile organisms will respond and change once exposed to the Martian environment are essential to maintaining astronaut health, preventing microbial contamination of Mars, and ultimately ensuring successful missions to Mars.

Acknowledgments

We are grateful to Vincent Andrew Moore and Laura Dominika Los for their assistance with the English language editing of this article. We also thank Andre Parpart for the help with the Mars atmosphere and pressure experiment setup.

Authors' contributions

T.Z., P.R., and K.B.-V. designed the experiments. T.Z. performed the experiments, analyzed the data, and wrote the first draft, with all the authors contributing by providing feedback. M.I.d.J., J.D.-A., and M.G.N. provided advice, guidance, and conceived ideas.

Author disclosure statement

No competing financial interests exist.

Funding information

The study was supported by DLR internal funds, and this project was supported by The Netherlands Organization for Scientific Research (Off Road Grant 04510012010022 to Jorge Domínguez-Andrés).

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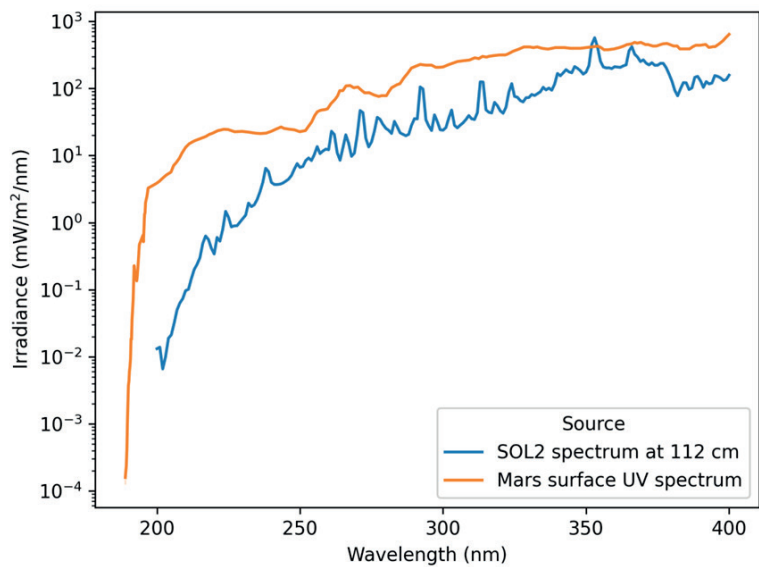
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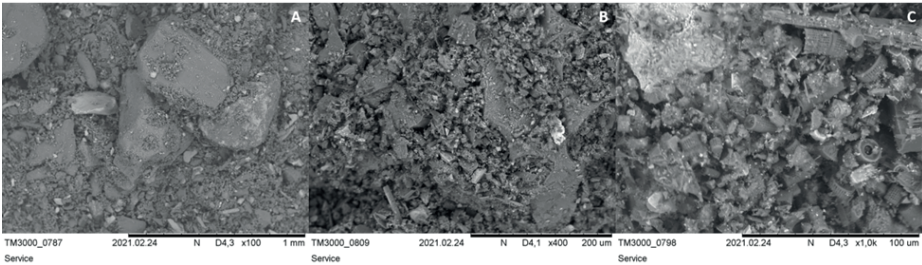
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SUPPLEMENTARY FIGURES



Supplementary figure 1: Polychromatic UV spectrum (200 - 400 nm) comparison of the SOL2 lamp simulator used for the irradiation of the bacteria with the simulated Mars surface UV spectrum adapted from Patel et al. (2004). Plotted are the fluence rates (irradiance in mW m⁻² nm⁻¹) at every wavelength from 200 to 400 nm. The fluence rates for the SOL2 polychromatic were measured at 112 cm distance from the light source.



Supplementary figure 2: SEM pictures of the MGS-1 regolith simulant taken with the Hitachi TM3000 at (A) x100, (B) x400 and (C) x1000 magnification showing the regolith simulant structures.

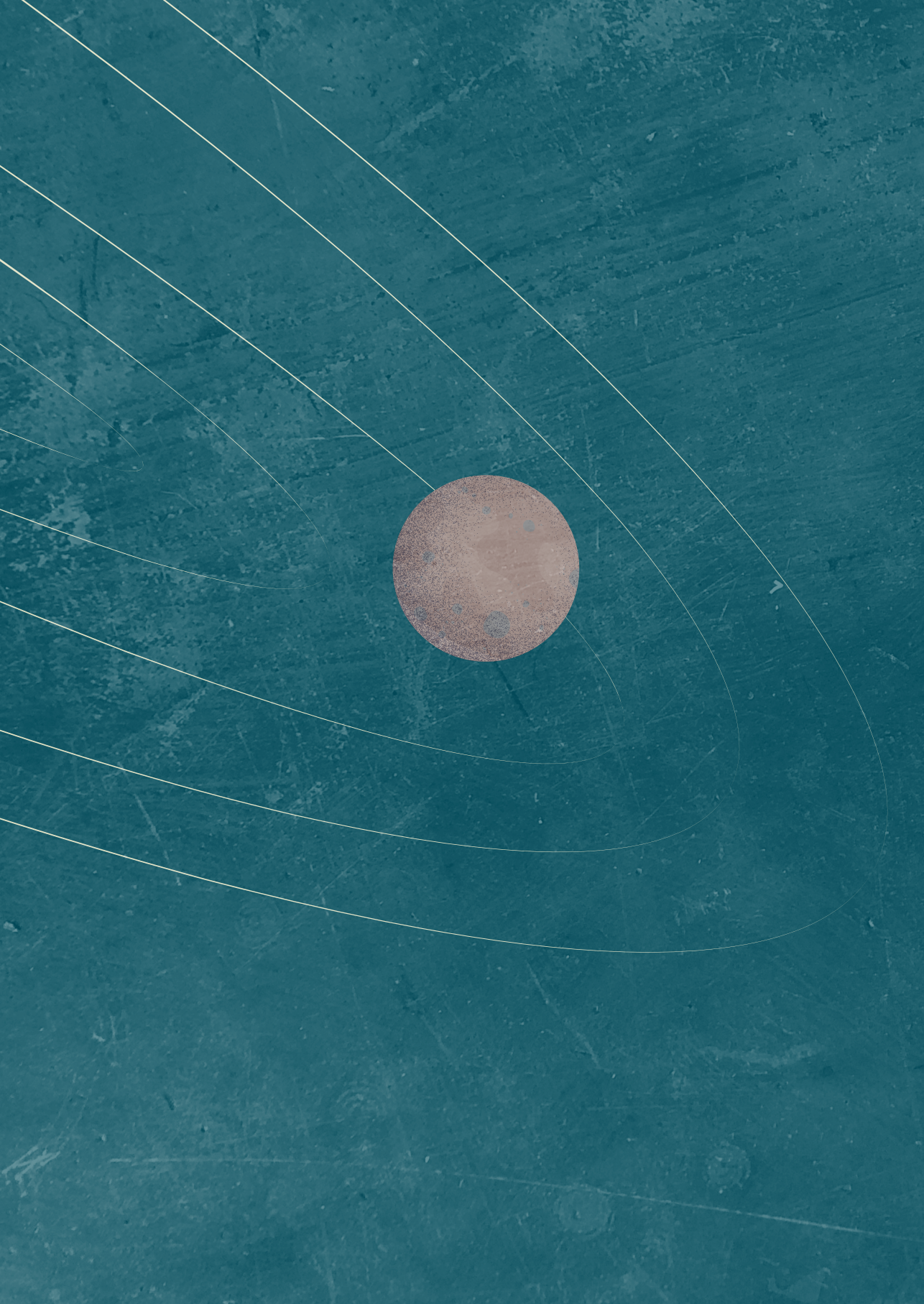
SUPPLEMENTARY TABLES

Supplementary table 1: Mineralogy of the MGS-1 regolith simulant used in this study, from Exolith lab.

Mineralogy	
Mineral	Wt. %
Plagioclase	27.1
Glass-rich basalt	22.9
Pyroxene	20.3
Olivine	13.7
Mg-sulfate	4
Ferrihydrite	3.5
Hydrated silica	3
Magnetite	1.9
Anhydrite	1.7
Fe-carbonate	1.4
Hematite	0.5

Supplementary table 2: Bulk chemistry composition of the MGS-1 regolith simulant used in this study, from Exolith lab.

Bulk Chemistry	
Oxide	Wt. %
SiO ₂	42.9
TiO ₂	0.6
Al ₂ O ₃	12.8
FeO	11.2
MnO	0.1
MgO	14.6
CaO	7.4
Na ₂ O	1.5
K ₂ O	0.6
P ₂ O ₅	0.1
Loss on ignition	5.3
Total	97.1



Effects of simulated Martian environmental stressors on specific human pathogen–immune system interactions

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ABSTRACT

The identification of health risks associated with long-term crewed missions to Mars is critical for mission planning and crew safety. Human-associated pathogens can be part of the microbiome and are likely to be transported during these missions. This study examines the immunological responses of human immune cells stimulated with non-fastidious bacterial species that cause opportunistic infections, i.e., *Klebsiella pneumoniae* and *Serratia marcescens*, after exposure to simulated Martian conditions, including ultraviolet (UV) radiation, desiccation, and atmospheric pressure. We observed that exposure of the bacteria to these conditions altered cytokine secretion, reactive oxygen species (ROS) production, and phagocytic activity in human peripheral blood mononuclear cells. Specifically, exposure to desiccation reduced cytokines and ROS production, indicating impaired innate immune recognition and stimulation. Notably, the altered immune response was partially restored when desiccated bacteria were regrown in standard media. Flow cytometry revealed decreased bacterial size and complexity of both species post-exposure. These findings indicate that Martian conditions induce bacterial morphological and physiological changes, which could impair immune recognition and response. Expanding these studies to *in vivo* models and a broader range of potentially pathogenic microorganisms is essential to estimate infection risks during Mars missions, which is vital for developing strategies to mitigate infection risks and maintain astronaut health during long-term space travel.

IMPORTANCE

Since Yuri Gagarin's 1961 flight, human space exploration has expanded, unintentionally transporting microorganisms, including pathogens, into space environments. Our previous studies demonstrated that opportunistic pathogens like *Klebsiella pneumoniae* and *Serratia marcescens* can survive simulated Martian conditions. With upcoming Mars missions, it is crucial to understand how such conditions influence these pathogens and their interaction with the human immune system. This research evaluates immune responses to bacteria pathogens exposed to Martian stressors such as UV radiation and desiccation, revealing significant changes in the immune responses to the exposed bacteria. These findings provide essential insights into the health risks that astronauts may face if infected with Mars-adapted pathogens. Understanding these interactions will help to develop preventive strategies and therapeutic measures, ensuring the safety and health of crew members during long-term missions. Ultimately, this work contributes to the broader objective of safe human exploration and colonization of Mars.

INTRODUCTION

Robotic exploration of Mars has driven extensive engineering and scientific research, including plans for future human missions to the red planet. Most space biology research to date has been conducted in low Earth orbits (LEOs), particularly on the International Space Station (ISS) and CubeSats (1). Research has shown that microorganisms isolated on the ISS primarily originate from human colonizing flora (2), indicating that similar microorganisms would likely be transported during Mars missions.

The evaluation of microorganisms in spacecraft has been an ongoing focus over the years (3–5) and continues routinely on the ISS (6). Since a significant percentage of space station microbes originate from the crew microbiome (2), it is expected that human microbiota will similarly be transported to Mars. Interestingly, certain human pathogens become more virulent in space conditions (7) and show increased resistance to antibiotics (8). As observed on the ISS, pathogenic bacteria species are expected to be present in future lunar and Martian habitats (9). Thus, the risk of infections could be heightened by both altered bacterial virulence factors (10) and changes in the human immune system, such as immunosuppression during spaceflight (11).

Our previous research demonstrated that *Burkholderia cepacia*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, bacterial species that survive and proliferate in a broad range of environmental conditions on Earth, can survive Mars-simulated conditions, even when grown in minimal media with a single carbon source (12, 13). In this study, we investigated the response of human immune cells to *K. pneumoniae* and *S. marcescens* exposed to Mars-like conditions. These species have been selected for their unique survival traits. We aimed to evaluate whether Mars-simulated conditions affect immune recognition and response to these bacteria. We observed changes in cytokine production, reactive oxygen species (ROS) levels, and phagocytic capacity in response to Mars-exposed *K. pneumoniae* and *S. marcescens*. We also identified morphological changes in these bacteria post-exposure, which may impact their interactions with the immune cells. Our findings enhance the understanding of immune responses to Mars-adapted bacteria, informing strategies to manage infections during long-term missions. These insights are essential for developing targeted therapies and prevention strategies, contributing to preserving astronaut health during space missions.

RESULTS

We investigated responses of human immune cells to *K. pneumoniae* and *S. marcescens* after exposure to Mars-like conditions, including desiccation, ultraviolet (UV) radiation, and Martian atmosphere. Our previous work demonstrated that *Burkholderia cepacia*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* survive in minimal media with a single carbon source and endure Mars-simulated conditions (12, 13). Based on these results and their pathogenic potential, we selected *K. pneumoniae* and *S. marcescens* for further investigation (14, 15). UV doses differed for the two bacterial species, based on the increased resistance to UV irradiation of *S. marcescens* compared to *K. pneumoniae*, which we have highlighted in our previous study (13).

Mars-like conditions decrease the capacity of bacteria to trigger innate and adaptive cytokine production

We first quantified innate and adaptive cytokine release from peripheral blood mononuclear cells (PBMCs) stimulated with Mars-exposed bacteria. *K. pneumoniae* exposed to simulated Martian conditions led to significantly reduced secretion of proinflammatory cytokines (interleukin [IL]-1 β , IL-6, and tumor necrosis factor alpha [TNF α]) and anti-inflammatory cytokines (IL-1RA and IL-10) compared to controls grown in minimal media (Fig. 1A). After 7-day stimulation, Martian condition-exposed *K. pneumoniae* also triggered less T-cell-derived cytokines, IL-17 and IL-10, compared to controls (Fig. 1B). However, interferon gamma (IFN γ) and IL-22 production remained unchanged across conditions (Fig. 1B). All simulated Martian conditions triggered a statistically significant decrease in the release of TNF α and IL-10, while desiccation did not alter IL-1 β , IL-6, or IL-1RA release significantly.

Cytokine release in response to *S. marcescens* exposed to simulated Martian conditions differed from *K. pneumoniae*, with reduced IL-1 β and TNF α upon desiccation alone or combined with Martian atmosphere or UV radiation (Fig. 2A). For all exposure regimens, the release of IL-6 and IL-10 did not show differences compared to the unexposed controls. Notably, unlike *K. pneumoniae*, IL-1RA release increased significantly when *S. marcescens* was exposed to desiccation. The production of T-cell-derived cytokines IFN γ and IL-10 was lower upon stimulation with *S. marcescens* exposed to desiccation alone and desiccation combined with UV radiation when compared with the standard-grown microorganism, while IL-22 secretion was not altered and IL-17 production could not be detected (Fig. 2B). Exposures of *S. marcescens* to UV radiation (400 J/m²) did not alter cytokine induction in human immune cells compared to control bacteria.

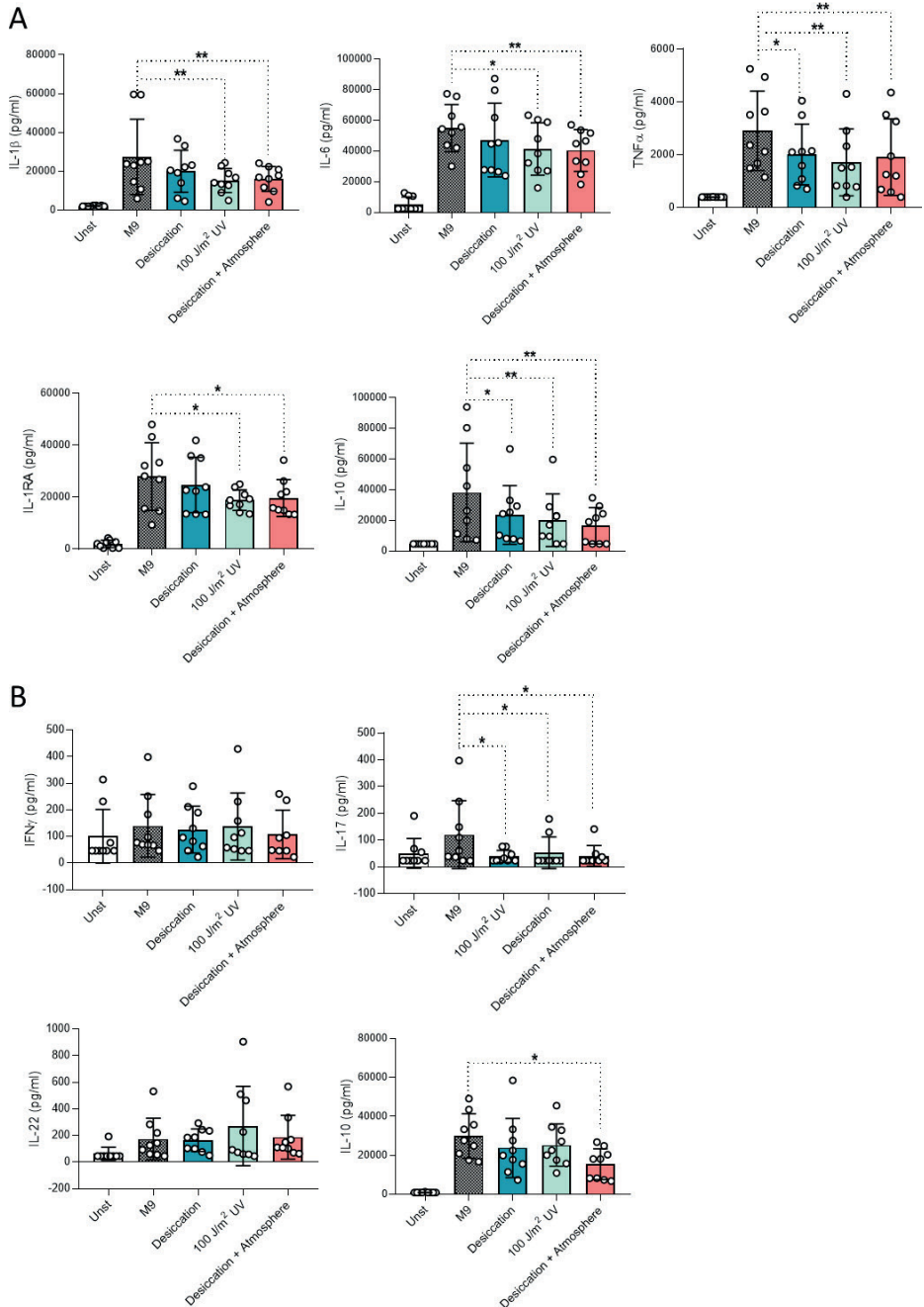


Fig 1. Cytokine production of PBMCs incubated with *K. pneumoniae* previously exposed to Mars-like conditions. PBMC innate (**A**) and adaptive (**B**) cytokine responses. $n = 9$ biological replicates pooled from three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$. Friedman test followed by Dunn's multiple comparison test. M9, minimal media; Unst, unstimulated; UV, ultraviolet.

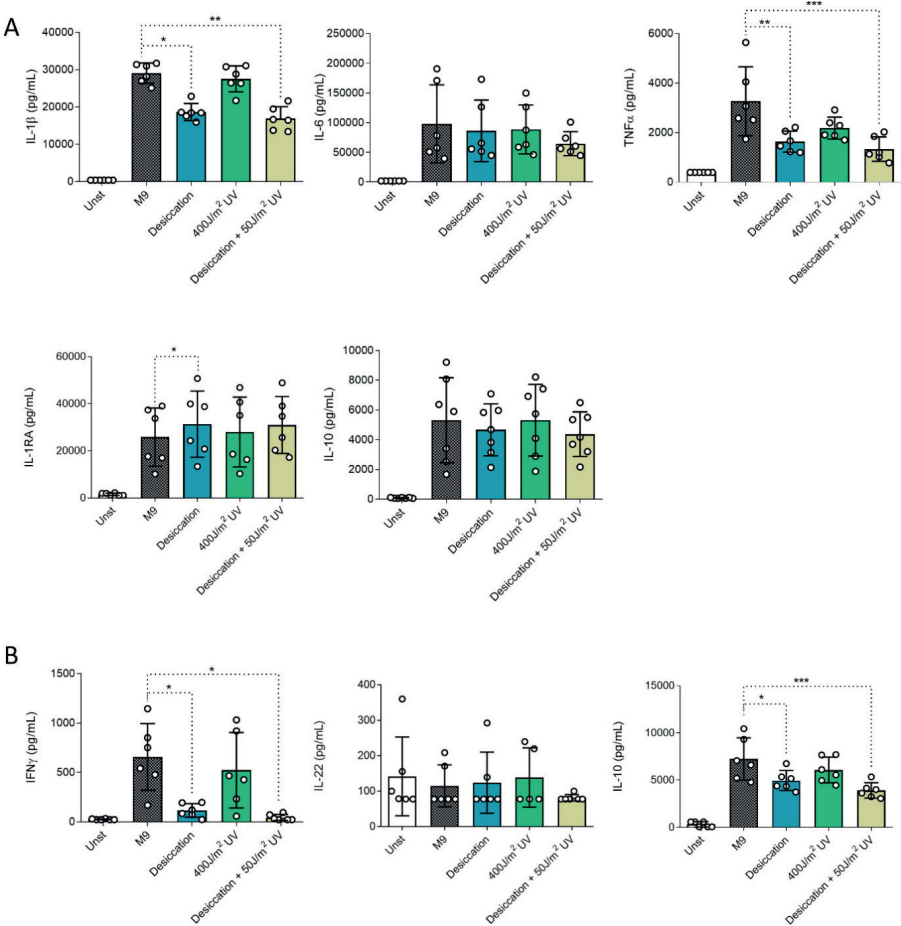


Fig 2. Cytokine production of PBMCs incubated with *S. marcescens* previously exposed to Mars-like conditions. PBMC innate (**A**) and adaptive (**B**) cytokine responses. n = 6 biological replicates from two independent experiments. *P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001. Friedman test followed by Dunn's multiple comparison test. M9, minimal media; Unst, unstimulated; UV, ultraviolet.

Taken together, our results show that the cytokine release profile triggered by the bacterial species exposed to Mars-like conditions differs between the two species tested. However, the exposure to the desiccation condition altered the cytokine response (TNF α , IL-10, and IL-17 for *K. pneumoniae* and IL-1 β , TNF α , IL-1RA, IFN γ , and IL-10 for *S. marcescens*) for both bacterial species. In contrast, the effects of exposure to polychromatic UV radiation were not shared, with a reduction in the cytokine response (IL-1 β , IL-6, TNF α , IL-1RA, IL-10, and IL-17) only seen for *K. pneumoniae*, despite the lower dose of UV exposure.

Bacterial species exposed to Mars-like conditions showed altered capacity to induce reactive oxygen species and phagocytosis

To more broadly understand the innate immune responses to simulated Martian condition-exposed *K. pneumoniae* and *S. marcescens*, we quantified the production of ROS by PBMCs incubated with the bacteria (Fig. 3A and B). Desiccation reduced ROS production induced by both species compared to standard-grown microorganisms, while UV exposure showed no effect. Similarly, a significant reduction in ROS production by PBMCs was seen for *K. pneumoniae* exposed to desiccation and pressure in Mars atmosphere and in *S. marcescens* exposed to desiccation coupled to UV radiation. These reductions in ROS and cytokine production indicate impaired immune recognition of bacterial species adapted to Mars-like conditions. To further elucidate innate responses upon a potential infection on Mars, the phagocytic capacity of CD11b+ cells was evaluated with flow cytometry. Although surface-bound and internalized bacteria cannot be distinguished with this method, it is a widely used and validated method to assess phagocytic capacity (16, 17). All the exposure conditions tested altered phagocytic capacity compared to the control for both bacterial species. All simulated Martian conditions led to enhanced *K. pneumoniae* phagocytosis by human CD11b+ immune cells (Fig. 3C and D). In contrast, the phagocytosis of *S. marcescens* was significantly reduced upon exposure to desiccation or to UV radiation (Fig. 3E and F). These differences in phagocytic capacity indicate that *K. pneumoniae* and *S. marcescens* adapt distinctively to Mars-like conditions, suggesting divergent phenotypic changes between the two species.

Desiccation-induced changes are partially reversible upon bacterial regrowth

Bacterial species with pathogenic potential exposed to Mars-like conditions may pose a health risk not only during human visitation to the planet but also upon return to Earth. Therefore, we evaluated whether desiccated bacteria retain their altered phenotype after regrowth in minimal media. Regrowth of desiccated *K. pneumoniae* restored IL-10 production, while TNF α release remained unchanged (Fig. 4A). Notably, exposure to desiccated *K. pneumoniae* did not significantly reduce TNF α secretion in this set of experiments in contrast to Fig. 1. Regrowth also reversed ROS reduction and restored phagocytosis of *K. pneumoniae* (Fig. 4C and D). Similarly, regrowth of *S. marcescens* restored IL-1 β and IFN γ production to control levels (Fig. 4E and F). However, TNF α and IL-1RA production stimulated by *S. marcescens* remained altered after regrowth, indicating partial reversibility of the immune response upon regrowth. *S. marcescens* also showed recovery in ROS production and phagocytosis upon regrowth (Fig. 4G and H). Taken together, these results suggest that desiccation induces partially reversible

phenotypic changes in both *K. pneumoniae* and *S. marcescens* and highlight the bacteria's ability to rapidly adapt after exposure.

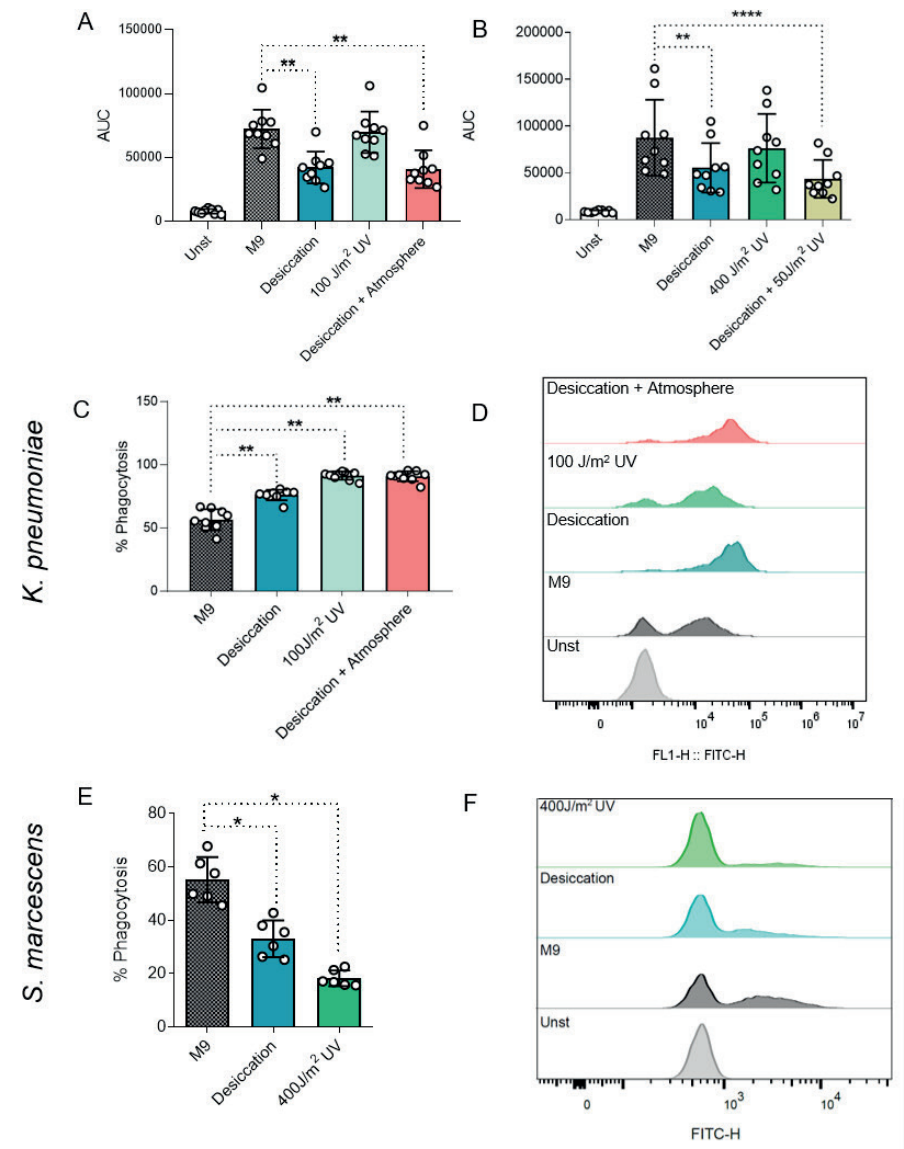


Fig 3. Effector functions of PBMCs against bacteria exposed to Mars-like conditions. Reactive oxygen species (ROS) production by PBMCs stimulated with *K. pneumoniae* (A) or *S. marcescens* (B). Percentage of CD11b+ cells positive for DTAF (FITC)-labeled bacteria (C and E) and representative histograms (D and F) for *K. pneumoniae* and *S. marcescens*. n = 6–9 biological replicates from two to three independent experiments, *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001 Friedman test followed by Dunn's multiple comparison test. AUC, area under curve as a ratio of ROS production over time; M9, minimal media; Unst, unstimulated; UV, ultraviolet.

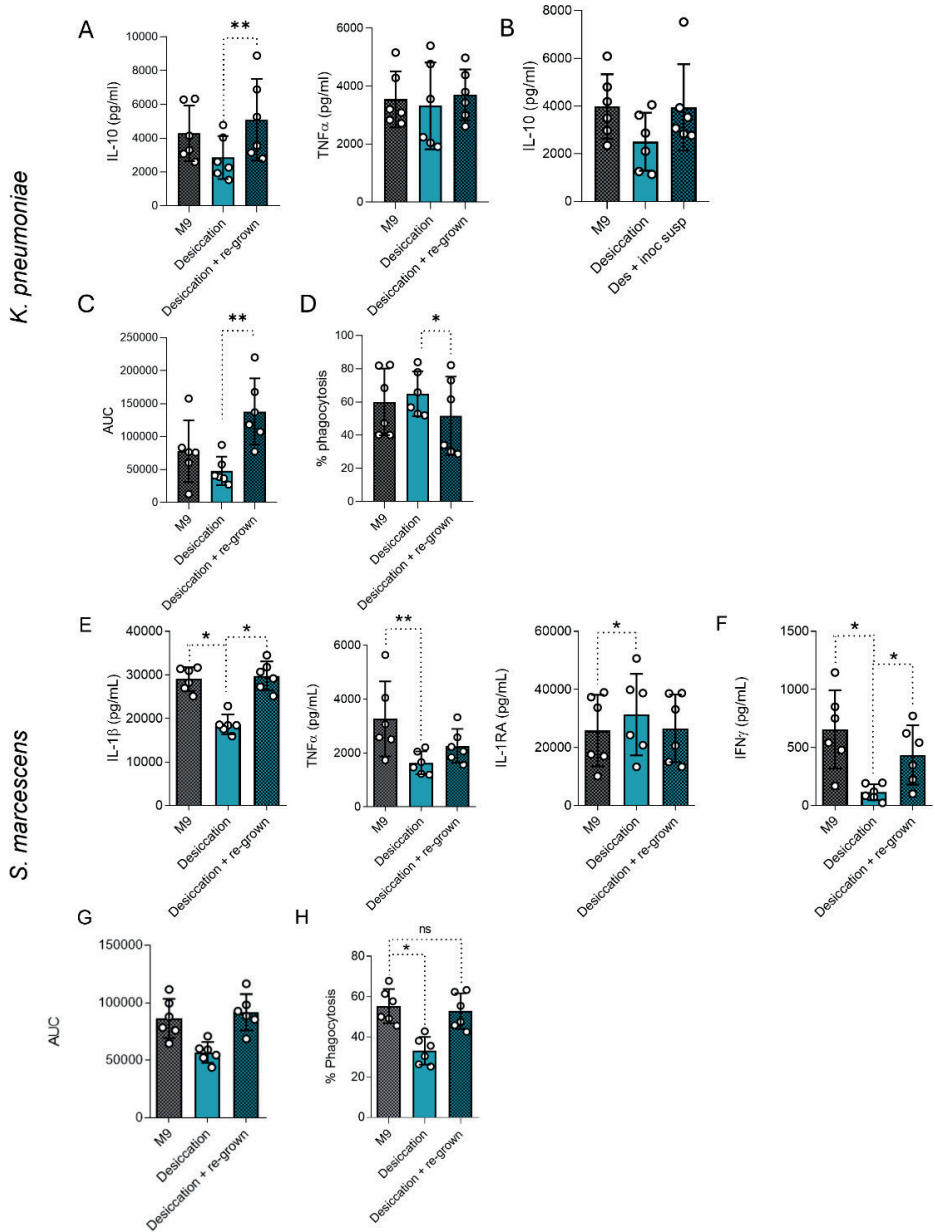


Fig 4. Regrowth after desiccation partially rescues the effects of desiccation. Cytokine production following stimulation of PBMCs for 24 h (**A** and **E**) and 7 days (**B** and **F**) with *K. pneumoniae* (**A** and **B**) or *S. marcescens* (**E** and **F**). Reactive oxygen species production when stimulated with *K. pneumoniae* (**C**) or *S. marcescens* (**G**). Phagocytosis of *K. pneumoniae* (**D**) and *S. marcescens* (**H**) by CD11b $^{+}$ cells. $n = 6$ biological replicates from two independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$. Friedman test with Dunn's multiple comparison test. M9, minimal media.

Desiccation reduced bacterial size and complexity

Morphological changes in bacteria exposed to Martian conditions likely influence immune responses. We investigated the cell morphology of *K. pneumoniae* and *S. marcescens* after exposure to desiccation, UV radiation, desiccation and Mars atmosphere, and desiccation and regrowth using transmission electron microscopy (TEM) (Fig. 5A and C). TEM revealed distinct morphological changes in both bacterial species, indicating cell envelope stress as well as a large population of cells with reduced electron density. *K. pneumoniae* cells remained rod shaped post-desiccation but had an irregular and shriveled appearance after UV exposure or combined conditions (desiccation and atmosphere and desiccation and regrowth). In contrast, *S. marcescens* generally retained its shape, showing irregularities resembling membrane blebbing only after desiccation and regrowth.

To further characterize bacterial morphology, we quantified the bacterial size (forward scatter [FSC]) and complexity or granularity (side scatter [SSC]) by flow cytometry. UV radiation had no impact on size or complexity, but both bacterial species showed size and complexity variation after desiccation alone or combined with other Mars-like conditions. Notably, the reduced size and complexity of desiccated *K. pneumoniae* and *S. marcescens* persisted after regrowth. These findings suggest that desiccation induces persistent morphological changes that might affect the recognition and response by immune cells.

DISCUSSION

Understanding how Earth-born pathogens exposed to Martian conditions interact with the human immune system is vital for assessing risks to crewed Mars missions. Our findings show that Mars-like conditions alter the morphology of the *K. pneumoniae* and *S. marcescens* cells and subsequently the interaction with immune cells, affecting cytokine release, phagocytosis, and ROS production, all important antimicrobial mechanisms. The immune response differences are likely driven by the specific stressors applied to the bacterial cells. Cell inactivation by Mars-like stressors, as shown in our previous research (13), may also impact cytokine responses. Research in LEO has demonstrated changes in bacterial growth rates, drug interactions, and cell structure (18, 19), similar to the alterations observed in our study. Our findings provide a valuable foundation for understanding the potential infection risks during crewed Mars missions, emphasizing the need for further investigation.

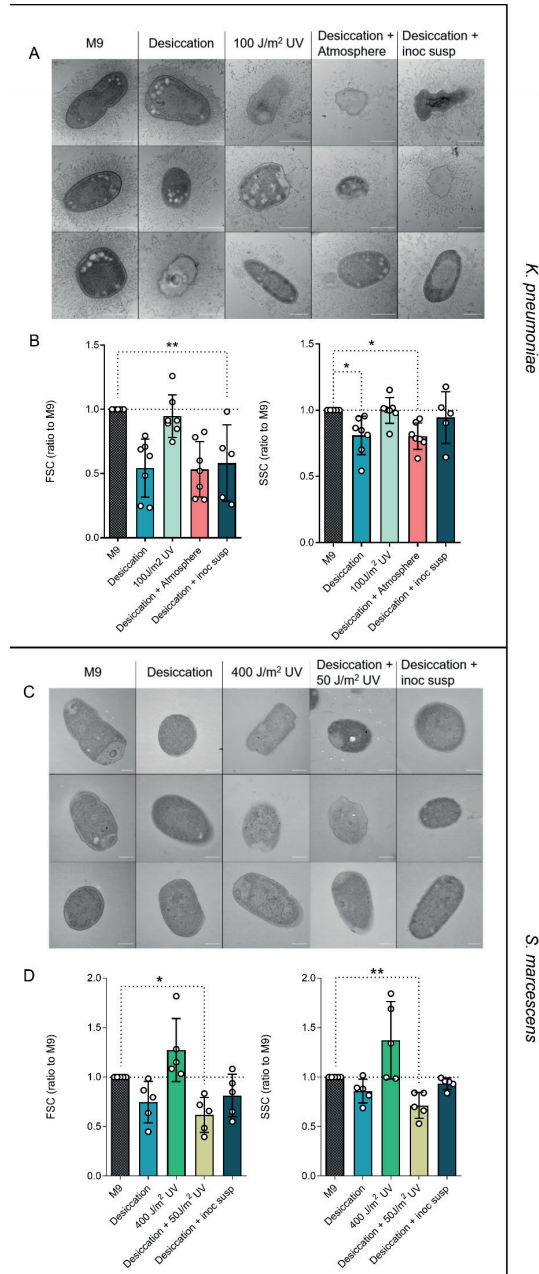


Fig 5. The morphology of bacterial cells is altered following exposure to simulated Martian conditions. TEM visualization of *K. pneumoniae* (A) and *S. marcescens* (C), size (FSC) and complexity (SSC) quantification by flow cytometry of *K. pneumoniae* (B) and *S. marcescens* (D). Scale bars are 500 nm in panel A and 250 nm in panel C. n = 5–7 biological replicates from two independent experiments. *P ≤ 0.05, **P ≤ 0.01. Friedman test followed by Dunn's multiple comparison test. FSC, forward scatter; M9, minimal media; SSC, side scatter; UV, ultraviolet.

Martian conditions may alter molecules on the surface of bacteria and the structure of the bacterial cell walls and other cell surface structures, impacting their interactions with immune cells (20). Epithelial and immune cells express pattern recognition receptors that, upon detecting foreign material, trigger cytokine release and further activation of the immune system. Variations in cytokine and chemokine responses can lead to the altered activation of immune cells (21). Human immune cells are also affected by space conditions (e.g., low gravity); however, this interaction was mostly studied in the context of the adaptive immune system (22). Concerning the innate immune cells, we know that the number of neutrophils increased at landing compared to pre-flight levels, while phagocytosis and oxidative burst capacities were significantly lower than control mean values after 9- to 11-day missions (23). Also, changes in monocytes have been studied in astronauts participating in spaceflights, showing that monocytes exhibited reductions in the ability to engulf *Escherichia coli*, to elicit an oxidative burst, and to degranulate (24). Furthermore, when challenged with endotoxin (lipopolysaccharide), the spaceflight crew member's monocytes collected at different time points produced lower amounts of IL-6 and IL-1 β and higher levels of IL-1RA and IL-8 compared to those of control subjects (25). Immune dysregulation during spaceflight, combined with microbial presence in spacecraft (11, 26–28) and microbiome changes (29), may pose significant health risks and treatment challenges. Earlier studies have shown that during *K. pneumoniae* lung infection, the concentrations of IL-1 β , IL-6, and TNF α are induced, while TNF α neutralization increased mice mortality (30). Therefore, the observed reductions in cytokine release may indicate bacterial evasion strategies to escape immune detection. This suggests that the exposure to the different environmental Martian conditions might alter bacterial pathogenicity.

The altered cytokine and ROS profiles indicate that the immune system may have impaired the ability to recognize Mars-adapted bacteria. Defective cytokine stimulation could also decrease other immune processes, including phagocytosis and bacterial killing (31, 32). Our results indicated that PBMCs were more efficient in eliminating the exposed *K. pneumoniae* but less efficient with *S. marcescens*. Literature has shown how there is a direct link between the phagocytosis rate by macrophages of clinical *K. pneumoniae* isolates and the extent of pulmonary clearance (33). To assess whether the cytokine changes induced by the Mars-like conditions would persist, we assessed PBMC reactions to desiccated and subsequently regrown bacterial cells. Our findings show that regrowth of desiccated bacteria partially restores the immune system's ability to recognize them. Further studies are needed to assess immune responses to bacteria regrown after exposure to other Mars-like conditions or multiple desiccation cycles, including

in vivo infection models, providing valuable insights for long-term Mars missions. Additionally, the desiccation exposure experiments did not incorporate the diurnal temperature and humidity conditions that occur on Mars. However, we believe that combining desiccation with a simulated Martian atmosphere at a pressure of 6 hPa approximates these conditions as closely as possible within the constraints of our experimental setup.

Although Martian conditions were simulated in this study, they also provide valuable insights applicable to Earth-based scenarios. Desiccation, known to cause significant cellular damage, is particularly relevant. Many microbial species exhibit desiccation survival, which facilitates pathogen transmission (34). This survival adaptation enables pathogens to spread and increase host infection risks (35, 36). Thus, desiccation is significant not only for astrobiology but also for public health, particularly in understanding bacterial spread in hospitals and public transport. Despite its importance, studies on immune recognition of desiccated bacteria remain limited. Our study demonstrated that desiccation decreased bacterial size and complexity, aligning with previous findings that oxygen deprivation reduces cell size (37). Under Mars-like low-pressure conditions, further size reduction in *K. pneumoniae* was not observed, likely due to the already strong effect of desiccation. Transmission electron microscopy revealed heterogeneity among treatment groups. Understanding these morphological changes is crucial for developing sterilization techniques for both hospitals and spacecraft (38).

This study revealed that Mars adaptation of bacteria significantly alters immune responses, offering fundamental insights into potential health risks for future crewed Mars missions. While our findings are derived from healthy donor samples on Earth, evaluating immune reactions of immune cells isolated from astronauts during or after spaceflight would provide a more precise understanding of the pathogenic threats posed by these adapted microorganisms. While this study characterized the impact of individual Martian conditions, more complete exposure regimens should be investigated to adequately reflect the real conditions on the planet. Furthermore, future research should investigate *in vivo* infection models in order to provide a clear understanding of the physiological consequences of the immune alterations observed in this study. Overall, these results highlight the urgent need for proactive risk prevention strategies, both for crew safety and planetary protection. Comprehensive immunological measures are essential throughout all mission phases: departure, travel, and habitation on Mars. Implementing rigorous protocols to control bacterial spread on both internal and external surfaces of habitats must be a top priority. As we prepare for the complexities of long-duration

space missions, early identification and mitigation of these health risks are crucial to ensure mission success and the well-being of both crew members and the humans who will come into contact with them upon their return to Earth.

MATERIALS AND METHODS

Bacterial strains and growth media

The bacterial species *K. pneumoniae* 298-53 (DSM 30104) and *S. marcescens* BS303 (DSM 30121) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH. Following growth in nutrient broth (NB, Difco) and on NB agar (15 g/L), at 35°C overnight, colonies from each species were incubated in M9-complete minimal salts media (47.8 mM Na_2HPO_4 , 22.0 mM KH_2PO_4 , 8.6 mM NaCl, 3.7 mM NH_4Cl , 2 mM MgSO_4 , 0.1 mM CaCl_2 , and 0.02 mM Fe(III)Cl_3 without carbon source) as described previously by Domínguez-Andrés et al. (12). Growth curves were plotted by measuring the optical density at 600 nm of the bacterial culture following the dilution of 0.2% (wt/vol) D-gluconic acid sodium salt ($\text{C}_6\text{H}_{11}\text{NaO}_7$) (Fluorochem) and D-glucose monohydrate ($\text{C}_6\text{H}_{12}\text{O}_6$) (Merck) in the M9-complete media. Liquid NB and M9-complete media with D-glucose were used as positive controls, while M9-complete without carbon source was used as a negative control. The Mars-like exposure regimens were selected based on Mars-simulated conditions which affected the survival of the bacteria while retaining viability of the cultures as described in Zaccaria et al. (13).

Exposure to desiccation

To understand the effect of desiccation on the immune response to the bacteria, late exponential phase cultures of *K. pneumoniae* and *S. marcescens* were grown in M9-gluconic medium. For each species, aliquots of 200 μL were placed on sterile 1 cm diameter glass disks and left to dry in a running clean bench at room temperature for 24 h, under sterile conditions with a relative humidity of ca. $30\% \pm 10\%$. For processing, the cells were resuspended in 5 mL Eppendorf tubes containing 1 mL of phosphate-buffered saline (PBS), lightly vortexed, and allowed to soak for 1 h at room temperature.

Exposure to polychromatic UV radiation

The effect of UV radiation was evaluated by growing *K. pneumoniae* and *S. marcescens* in M9-gluconic to late exponential phase. In closed sterile UV transmissible quartz cuvettes (0.5 cm path length; Hellma GmbH & Co. KG, Muelheim, Germany), a volume of 3 mL of each bacterial culture was placed. The

cuvettes contained a magnetic stirrer allowing all the cells to be stirred while being irradiated. The cuvettes were placed vertically at 112 cm from the light source. Each sample was irradiated with polychromatic UV (200–400 nm) with fluence of 10.35 W/m² using a SOL2 polychromatic UV lamp, equipped with a UV 500S irradiation source (Dr. Hoenle AG, UV Technologies, Germany) at the German Aerospace Center in Cologne, Germany. The fluence of the lamp was determined with the Bentham DMc150 transportable spectroradiometer (Bentham Instruments Ltd., Reading, UK), with optics inside the simulation chamber as per Rabbow et al. (39). Following irradiation, liquid cultures were visually evaluated under a light microscope (Zeiss Primostar; Carl Zeiss Microscopy GmbH, Jena, Germany) to identify a potentially altered morphology.

Exposure to Mars atmosphere and pressure

The species were exposed to a Mars-like atmosphere composed of a Mars-like gas mix (2.7% N₂, 1.6% Ar, and 0.15% O₂ in CO₂ vol/vol) and Mars-relevant pressure of 6 hPa using a vacuum pump (Rotary Vane Pump DUO 035 and HiPace 700; Pfeiffer Vacuum GmbH, Germany) monitored constantly during the exposure (TPG 262 Full Range Gage, Pfeiffer Vacuum GmbH). The samples were exposed to Mars atmosphere following desiccation as described in Exposure to desiccation. The desiccated glass disks were placed in the gastight transport and exposure box (40), and the Mars atmosphere at Mars-relevant pressure was applied.

The bacterial species were exposed to the described conditions in order to replicate Mars-relevant conditions and at the same time to have enough cell survival to potentially determine infection of the human body and to assess the immune response.

Inactivation of the bacteria

Immunological evaluations were performed at RadboudUMC, Nijmegen, the Netherlands. The bacteria were shipped inactivated using β-propiolactone (BPL) as follows. A citrate buffer was prepared with 125 mM sodium citrate (C₆H₅Na₃O₇ × 2H₂O, Sigma-Aldrich) and 150 mM sodium chloride (NaCl, Merck). A 10% (vol/vol) stock solution of 98% BPL (Acros Organics) in citrate buffer was prepared. The 10% stock solution was added to the exposed bacteria to a final concentration of 0.1% (vol/vol) of BPL. The bacterial samples were stored at 4°C until processing at Radboud UMC. The samples were then incubated at 37°C for 4 h to inactivate the BPL.

Isolation of human PBMCs

Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, Netherlands). Samples were anonymized to safeguard donor privacy. PBMC isolation was performed by differential density centrifugation over Ficoll-Paque (GE Healthcare). Cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Dutch modified Invitrogen) supplemented with 5 µg/mL gentamicin (Centrafarm), 2 mM Glutamax (Gibco), and 1 mM pyruvate (Gibco).

PBMC culture stimulation

PBMCs (0.5×10^6) per well were seeded in round-bottomed 96-well plates and stimulated with 10^6 CFU/mL *K. pneumoniae* or *S. marcescens* for 24 h or 7 days in RPMI medium supplemented with 10% human pooled serum. All conditions were tested for cytotoxicity by measuring lactate dehydrogenase in the conditioned media after 24 h incubation according to the manufacturer's instructions (CytoTox96, Promega).

Cytokine quantification

Secreted cytokines were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits for IL-1β, IL-6, TNFα, IL-10, IL-1Ra, IL-17, IL-22, and IFNγ (R&D Systems) following the instructions of the manufacturer. Concentrations lower than the detection limit of the ELISA were replaced with the lowest detectable concentration for statistical analyses.

ROS quantification

Superoxide anion levels were evaluated using luminol-enhanced chemiluminescence and determined in a luminometer (Biotek Synergy HT). A total of 0.5×10^6 PBMCs per well were incubated with serum opsonized 10^6 CFU/mL *K. pneumoniae* or 10^7 CFU/mL *S. marcescens*. Luminol (Sigma) was added to each well to start the chemiluminescence reaction. Each measurement was carried out in quadruplicate. Chemiluminescence was determined every 145 seconds at 37°C for 1 h. Luminescence was expressed as relative light units per second and as area under the curve.

Phagocytosis assay

Bacteria were stained overnight with 100 µM dichlorotriazinyl aminofluorescein (DTAF) (Thermo Fisher). After washing with PBS three times, stained bacteria were opsonized for 1 h at 37°C in RPMI with 10% pooled human serum. PBMCs (0.5×10^6) per well were plated in round-bottomed 96-well plates and incubated with *K. pneumoniae* or *S. marcescens* (multiplicity of infection 2:1) for 15 minutes.

Prior to flow cytometry analysis, cells were stained with anti-CD11b-BV785 (BioLegend) for 20 minutes at 4°C. Measurements were done using CytoFLEX (Beckman Coulter). Data were presented as percentage of DTAF-positive events within the CD11b+ population. Analysis was performed using FlowJo v.10.9.

TEM

K. pneumoniae and *S. marcescens* were grown overnight at 35°C in M9-gluconic medium and exposed to desiccation, polychromatic UV radiation, or Mars atmosphere and pressure as described previously. The cell number of the bacteria for TEM was determined by CFU plating on NB agar (8 g/L). The cells were then fixed in a 4% solution of paraformaldehyde (PFA) in 0.1 M PIPES, HEPES, EGTA, and magnesium sulfate (PHEM) buffer. An equal volume of PFA in PHEM buffer solution was added to unwashed cells following exposure to the listed conditions. The cells were fixed at room temperature for 15 minutes with gentle agitation. The fixed cells were pelleted at $1,500 \times g$, and the fixative was removed and replaced with fresh fixative. The cells were resuspended and fixed for 40 minutes at room temperature with gentle agitation. The cells were then washed three times with PBS and stored in 0.1% PFA in PHEM at 4°C until processed for TEM.

Pre-fixed cells were gently pelleted (600-RCF 5 min), after which the supernatant was removed and the pellet was resuspended in the remaining medium. Next, samples with a thickness of 100 μm were high-pressure frozen using the 3 mm platelet system in an HPM100 high-pressure freezer (Leica Microsystems). Samples were freeze-substituted in anhydrous acetone containing 2% OsO_4 , 1% H_2O , and 0.2% uranyl acetate and subsequently embedded in Epoxy resin (41). Sixty nanometer sections were prepared on a Reichert–Jung Ultracut ultramicrotome and collected on 100 mesh copper grids with a carbon-coated formvar support film. Grids were post-stained with 0.5% uranyl acetate and Reynolds lead citrate before analysis in the JEOL-1400 Flash TEM operating at 120 kV.

Bacterial size and complexity quantification by flow cytometry

Bacteria were stained with Syto40 (Thermo Fisher) to stain nucleic acids. Size and complexity are presented as geometric mean of FSC and SSC, respectively, as a ratio to M9 control bacteria of Syto40+ events. Measurements were done using CytoFLEX (Beckman Coulter), and analysis was performed using FlowJo v.10.9.

Acknowledgments

We thank Andre Parpart for help and guidance in setting up the atmosphere exposure experiment and Hetty Manenschijn for maintaining the transmission

electron microscope at the Department of General Instrumentation (Faculty of Science, Radboud University).

This work was supported by an Off Road ZonMw Grant awarded to J.D.-A (04510012010022). M.G.N. was supported by an ERC Advanced Grant (833247) and a Spinoza Grant of the Netherlands Organization for Scientific Research.

No competing financial interests exist related to the work presented in this study. M.G.N. is scientific founder of Biotrip, TTxG, Lemba and Salvina.

Data availability

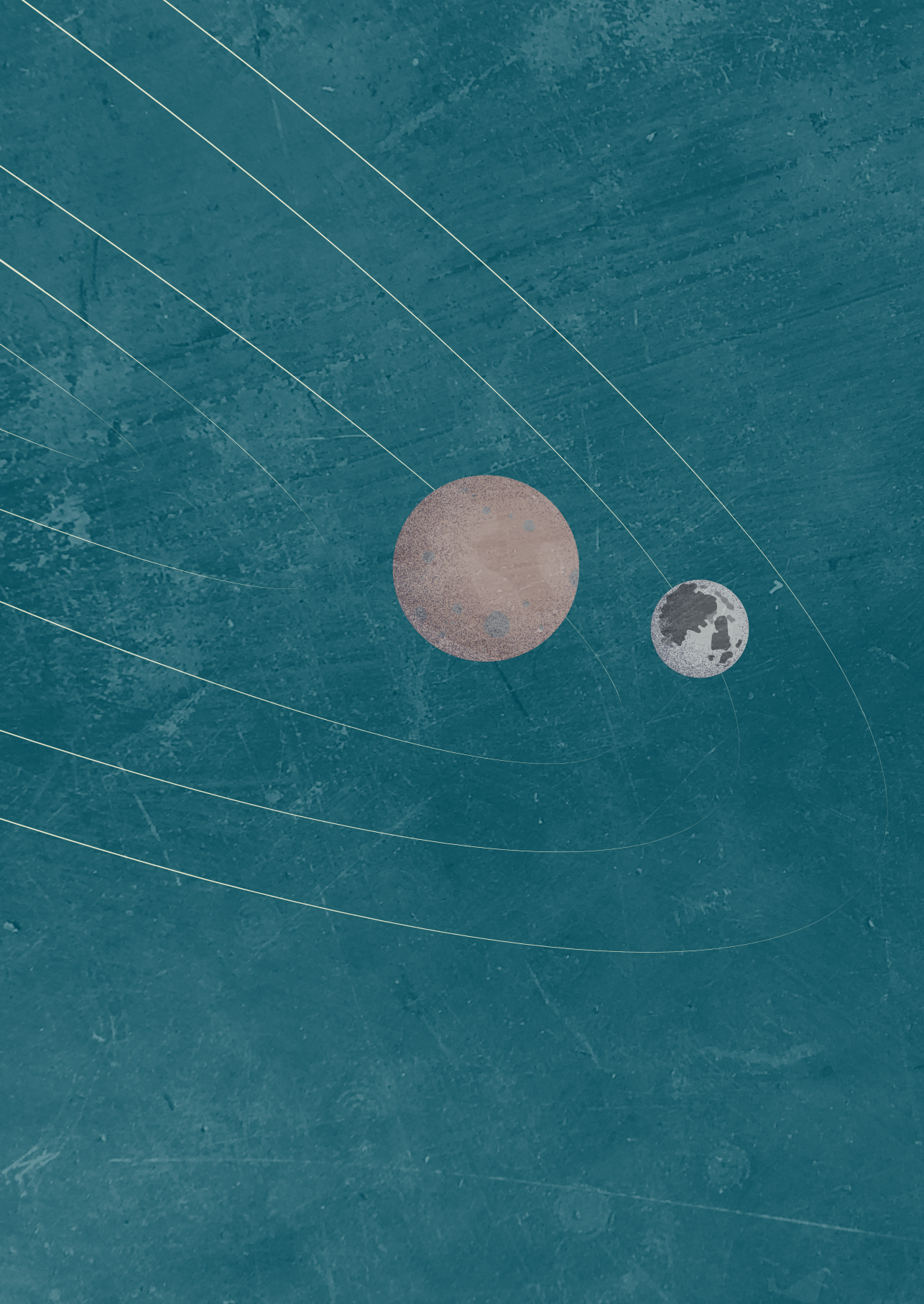
Cytokine quantification, reactive oxygen species quantification, phagocytosis assay, bacterial size, and complexity quantification by flow cytometry data are available online and can be accessed with the following DOI: [10.17632/g5xmvmc4nb.1](https://doi.org/10.17632/g5xmvmc4nb.1).

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Lunar and Martian Regolith Simulants Compromise Airway Barrier Function and Trigger Inflammation

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ABSTRACT

Little is known on the health risks for the astronauts exposed to space-derived materials such as regolith, the mixture of dust and rock particles covering the surfaces of Moon and Mars. We combined an in vitro model of exposure of human airway epithelial and peripheral blood mononuclear cells to regolith, with an in vivo murine exposure model, to assess the effects of Martian and Lunar regolith simulants. Exposure to both Moon or Mars regolith caused cellular cytotoxicity and induced strong proinflammatory responses in vitro. In mice, intranasal regolith exposure led to impaired epithelial barrier integrity in the airways, increased pro-fibrotic gene expression, immune cell recruitment, and elevated amounts of inflammatory proteins in bronchoalveolar lavage fluid. Lunar regolith simulant LMS-1 showed the highest barrier-disrupting and pro-inflammatory capacity. These findings highlight regolith as a potent airway irritant driving inflammation and barrier dysfunction, underscoring the need for dust mitigation strategies to protect astronaut health during long-term space missions on Moon or Mars.

INTRODUCTION

As humanity prepares to return to the Moon and extend its reach to Mars, exposure to the fine dust covering the surfaces of these celestial bodies, known as regolith, has emerged as a critical health concern. Upcoming missions, such as NASA's Artemis program, aim to establish a sustainable human presence on the Moon within the next decade. In addition to national space programs, several private enterprises, such as SpaceX, are actively developing plans for human missions to Mars. One very important aspect that needs to be explored is the effect of space travel on the health status of the astronauts, encompassing factors encountered during transit (e.g. microgravity, isolation, space radiation), as well as those present on planetary surfaces, of which exposure to Moon or Mars regolith is one of the most significant risks. Furthermore, the potential return of regolith-containing materials to Earth for scientific or commercial purposes raises additional concerns regarding possible health hazards.

Although space suits and habitat systems are designed to minimize exposure to the extraterrestrial environment, astronauts on the Apollo missions between 1969 and 1972 reported continual exposure to Lunar regolith, reporting symptoms such as nasal congestion, eye irritation, skin burning, and respiratory discomfort despite decontamination efforts (1). As Apollo astronaut Gene Cernan described: "You can be as careful in cleaning up as you want to, but it just inhabits every nook and cranny in the spacecraft and every pore in your skin." In addition to its effects on human health, regolith compromised spacecraft systems, spacesuits, and other equipment, causing abrasion, clogging and mechanical wear. While various mitigation strategies are being developed, further testing and validation are required before they can be implemented in operational missions (2).

Lunar regolith is a heterogeneous mix of jagged, fine rock particles formed through micrometeoroid impacts and prolonged exposure to cosmic radiation (3). It is composed primarily of silicon dioxide (SiO_2 ~50%) with significant proportions of aluminum oxide, calcium oxide, magnesium oxide, titanium dioxide, and iron (4). Martian regolith shares many of these physical features, although it may also contain toxic compounds, such as perchlorates, which can interfere with thyroid function and pose additional health risks (5). Both Lunar and Martian regolith have highly irregular chemically reactive surfaces capable of inducing cellular damage and oxidative stress upon contact with human tissues (6, 7).

On Earth, prolonged exposure to mineral dust such as crystalline silica is a known cause of chronic respiratory diseases, including silicosis, which are characterized by fibrosis, inflammation, and impaired lung function (8). The ultrafine size of most regolith particles ($<2.5\ \mu\text{m}$) allows for deep penetration into the lungs, where clearance mechanisms are slow and inefficient (9). Once inhaled, a fraction of these particles can translocate into the circulation and reach other organs (10). Moreover, microgravity on the Moon or Mars may enhance the persistence of airborne regolith particles, altering their deposition dynamics in the lungs, further impairing clearance (11, 12). Spaceflight conditions such as microgravity and radiation also modulate immune function, potentially amplifying susceptibility to dust-induced injury (13). Consequently, interactions between regolith particles and airway epithelium or immune cells under these conditions may lead to disproportionate or prolonged inflammatory responses, posing significant health risks to crew members during long-duration missions and potentially, to Earth inhabitants who might be exposed to regolith-containing materials returned for potential future exploitation.

Despite the relevance of these concerns, studies investigating the immunological and barrier-disruptive effects of Lunar or Martian regolith remain scarce. Previous *in vitro* studies have focused on the interaction of regolith with macrophages (14, 15) and keratinocytes (3), while its effects on the respiratory epithelium and systemic immune responses has been largely unexplored. In a recent study systemic cytokine concentrations and immune cell populations were measured in rats exposed to Lunar regolith (16), another study evaluated its effects on human peripheral blood mononuclear cells (PBMCs) (17) or airway epithelial cell lines (18). However, a comprehensive approach integrating *in vitro* and *in vivo* models is needed to fully assess local and systemic immune responses and epithelial barrier integrity, in the context of regolith exposure.

To address this gap, we conducted a study using human PBMCs, airway epithelial cell lines, air-liquid interface (ALI) cultures of primary human bronchial epithelial cells, and a murine model of intranasal regolith exposure to Martian or Lunar regolith. We tested well-characterized Martian and Lunar regolith simulants (MGS-1, MGS-1S, and LMS-1) and compared them with crystalline silica (SiO_2) as a terrestrial control to examine their toxicity, immunogenicity, and capacity to impair airway epithelial integrity. Our findings provide critical insights into the acute health risks associated with Lunar and Martian regolith, underscoring the need for effective dust mitigation strategies in future space missions.

METHODS

Regolith simulants

All regolith simulants were purchased from Space Source Technologies, USA (previously The Exolith Lab). Information on the mineralogy and the bulk chemistry of the products were obtained from the manufacturer. Silica quartz (SiO_2), used as the terrestrial control, was purchased from KSL Staubtechnik (Germany). Grinding of all regolith and silica to achieve particle sizes of $\leq 20 \mu\text{m}$ was performed by the milling and sieving method.

Size distribution analysis

Particle size distribution (PSD) measurements were carried out using a Laser Diffraction Particle Size Analyzer (Horiba LA960) equipped with a wet measurement cell. The particle sizes in suspensions were measured via laser light scattering by directing a laser at the sample. As the laser light interacts with the particles, they scatter the light in different directions depending on their size. The system captures this scattered light across a range of angles using a multi-channel detector array. The scattered light intensity is analyzed using Mie scattering theory. A refractive index of 1.460 in water was used for the measurements.

Scanning electron microscopy

Electron microscopy images of the pre-grinding regolith were generated at German Aerospace Center using the TM3000 microscope (Hitachi). The post-grinding imaging was performed at the Museum für Naturkunde, Germany.

Image analyses

To measure the circularity and solidity of the regolith or silica particles from electron microscopy images, the software ImageJ 1.45g was used. The images were first converted to grayscale and the particles were analyzed. Values for Feret angle, solidity and circularity were measured using the shape descriptors function. Averages and standard deviation of the three values were calculated from the values of 3 different images for each regolith type or silica.

Preparation of regolith simulants for use in cell culture

Regolith simulants and silica were autoclaved in a glass container for sterilization. Then, a 20% (weight/volume) suspension was prepared in Dutch-modified RPMI cell culture medium (Gibco) to serve as stock and stored at 4°C . Stocks were further diluted with RPMI for experiments to achieve different working concentrations. For ALI culture experiments, stocks were prepared in LPS-free PBS (Millipore)

and diluted in Opti-MEM medium (Gibco). In all cases, since the regolith particles sediment quickly, the suspensions were vigorously vortexed each time before pipetting. Before every experiment, regolith suspensions and medium-only controls were incubated with 5 µg/ml Polymyxin B sulfate (Sigma-Aldrich) for 1 hour at 37 °C to neutralize possible LPS contamination.

Peripheral blood mononuclear cell (PBMC) isolation and stimulation

Buffy coats from anonymized healthy donors were provided by Sanquin Blood Bank (Nijmegen, Netherlands) after written informed consent. PBMC isolation was performed by differential density centrifugation using Ficoll-Paque (GE Healthcare). Cells were then resuspended in RPMI supplemented with 5 µg/mL gentamicin (Centrafarm), 2 mM GlutaMAX (Gibco), and 1 mM pyruvate (Gibco). 5×10^5 PBMCs/well were seeded in round-bottom 96-well cell culture plates for stimulation. Medium control or varying concentrations of regolith suspension pre-incubated with 5 µg/ml Polymyxin B were added to the cells. The culture medium was supplemented with 10% pooled human serum. After 24 hours, plates were centrifuged, and supernatants were collected and stored at -20 °C until measurement.

Maintenance and stimulation of cell lines

A549 lung epithelial cell line and Detroit 562 pharynx epithelial cell line were cultured in DMEM (Gibco) supplemented with 4.5 g/L glucose, 5 µg/mL gentamicin, 2 mM Glutamax, 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS Xtra, Capricorn Scientific) using T75 culture flasks. The cells were subcultured every 2-3 days when 80-90% confluency was reached. To detach the cells, they were incubated with 0.05 % Trypsin-EDTA (Gibco) at 37 °C for 5 and 8 minutes for A549 and Detroit 562, respectively.

For stimulations to measure cytokine production, 5×10^4 cells/well were seeded in 48-well cell culture plates and incubated for 4-5 hours at 37 °C until they were attached. Then, medium control or regolith suspensions in various concentrations pre-incubated with 5 µg/ml Polymyxin B were added to the cells. Supernatants were collected 24 hours later and stored at -20 °C until cytokine quantification. For stimulations to measure gene expression, 5×10^5 cells/well were seeded in 6-well plates and treated as described above. 6 hours later, cells were washed with warm PBS to remove the regolith, lysed in 500 µl/well Lysis Buffer RA1 (Macherey-Nagel), and transferred to tubes. Lysed samples were stored at -80°C until RNA isolation.

Quantification of cytokines and chemokines

Cytokine and chemokine concentrations in cell culture media were determined using DuoSet® ELISA kits (R&D Systems) and OptEIA™ Human ELISA Set (BD Biosciences) following the protocols of the manufacturers. Values below the detection limit were replaced with the lowest detectable value to be able to perform statistical analyses.

Cytotoxicity assay

Cytotoxicity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), which quantifies lactate dehydrogenase release. Assays were performed following the manufacturer's protocol. Experiments included a non-stimulated control well lysed 30 minutes before the assay using the kit's lysis solution. Relative cytotoxicity in treatment groups was calculated based on this 100% cytotoxicity control.

RNA extraction and qRT-PCR

Cell culture samples stored in Lysis Buffer RA1, as described above, were processed using the NucleoSpin™ RNA kit (Macherey-Nagel) following the manufacturer's protocol. 1 µg of the purified RNA was converted to cDNA with the iScript™ cDNA Synthesis Kit (Bio-Rad). cDNA samples were diluted 5x with nuclease-free water for quantitative real-time PCR and combined with 0.5 µM forward and reverse primers. Reactions were performed using QuantStudio 6 Pro Real-Time PCR System (Applied Biosystems).

For murine lung RNA isolation, a portion of 20-30 mg from the right lung was harvested, snap-frozen in liquid nitrogen, and stored at -80 °C. Afterwards, samples were disrupted in liquid nitrogen with a pestle and homogenized in lysis buffer. RNA from collected samples was isolated with RNeasy Mini Kit (Qiagen), and cDNAs were generated with a PrimeScript RT Reagent Kit (Takara Bio). Finally, qPCR was performed by using FastStart Universal SYBR Green Master (Roche) and reactions were carried out in a QuantStudio 12k Flex (Applied Biosystems) at the Genomics Unit of Complutense University of Madrid. All procedures were performed following the manufacturer's protocols. Gene expressions relative to the housekeeping gene *Eef1a1* were calculated using the ΔCt method.

The sequences of all primers employed for qPCR analysis are listed in Supplementary Table 1.

Differentiation and stimulation of air-liquid interface cultures of human bronchial epithelial cells

Primary human bronchial epithelial cells (HBECs) of healthy donors were purchased from commercial distributors (Epithelix). HBECs in passage 1 were expanded in monolayer cultures with BEBM supplemented with all BEGM SingleQuots (Lonza) in 150 cm² T-flask at 37 °C and 5% CO₂. Once cultures reached 80-90% confluency, HBECs were harvested with 0.05% trypsin-EDTA and seeded onto 6.4-mm-diameter 0.4 µm-pore size polyester membrane transwells (Corning) at a density of 1.5x10⁵ cells per well. From this point, the growth medium was replaced by BEBM supplemented with BEGM SingleQuots, except for retinoic acid and triiodothyronine, and mixed 1:1 with DMEM (Gibco). All-trans retinoic acid (ATRA, Sigma-Aldrich) was freshly supplemented to the differentiation medium at a concentration of 15 ng/mL, and the medium was changed every second day. First, cells were grown submerged for 3-5 days until they reached confluency. Then, the apical compartment medium was removed to allow differentiation in the air-liquid interface (ALI) of the pseudostratified epithelium for the next 21 days.

The fully differentiated ALI cultures were stimulated in Opti-MEM medium (Gibco). To assess the effects of particulate materials in ALI cultures of HBECs, Earth silica or regolith simulants were administered daily into the apical compartment of the cultures in a model of repetitive exposure for three consecutive days. Briefly, fully differentiated cells were apically exposed to the regolith simulants for 24 hours. Then, the apical compartment was emptied and re-filled with fresh regolith-containing medium for an additional 24 hours. The following day, this process was repeated to complete the protocol. All experiments were performed at 37 °C in a 5 % CO₂ humidified incubator.

Transepithelial electrical resistance measurement

Trans-epithelial electrical resistance (TEER) was measured in fully differentiated ALI cultures using Millicell-ERS Volt Ohm Meter (Millipore). The electrode was washed with PBS, sterilized for 15 minutes in 70 % ethanol, and washed again with PBS before use. TER values were measured by placing the long arm of the electrode into the basolateral compartment while the short arm was positioned into the liquid in the apical compartment of the transwells.

Paracellular FITC-Dextran flux measurements

The paracellular flux of ALI cultures of HBECs was assessed 72 hours after the first stimulation of the cultures. Fluorescein isothiocyanate (FITC)-labeled 4-kDa dextran (FITC-dextran, 2 mg/mL) (Sigma-Aldrich) was apically administered, and the fluor-

escence intensity in the basolateral compartment was analyzed after 24 hours using a fluorescence plate reader (BMG LabTech).

Murine model of acute regolith exposure

6-week-old female C57BL/6J mice (Charles River) were randomly assigned into 4 groups. Mice were intranasally instilled for three consecutive days with 50 μ L containing PBS (control), SiO₂ (50 mg/kg), LMS-1 (50 mg/kg), or MGS-1 (50 mg/kg). PBS-containing silica or regolith simulants were vigorously vortexed before each intranasal treatment to achieve homogeneous particle suspensions. All mice were sacrificed 24 hours after the last particulate exposure, and the bronchoalveolar lavage fluid (BALF) and right and left lung samples were collected. BALF samples were used to study epithelial barrier leakiness and inflammatory cell infiltration, 20-30 mg of the right lung were used for gene expression analysis, and left lung samples were used for histological analysis.

BALF collection

BALF samples were collected from each mouse by infusing and extracting 0.7 mL of ice-cold 10 mM EDTA in PBS. The procedure was repeated twice to ensure the optimal collection of BAL cellular infiltration. BALF obtained from the first lavage was centrifuged at 300 $\times$ g for 10 min. Subsequently, cell-free BALF supernatants were collected and stored at -80°C for further analysis. Cell pellets from all lavages of each mouse were pooled and analyzed to assess cell recruitment and composition by flow cytometry. Briefly, BALF samples were washed with 2 mM EDTA 0.5% BSA in PBS and stained in the dark for 15 min at room temperature with the corresponding fluorescence-labeled or isotype control antibodies. Then, cells were washed again and centrifuged at 300 $\times$ g for 5 min at room temperature. Lastly, cells were fixed in 0.1% PFA in PBS before flow cytometry measurements. All reagents and antibodies employed are included in Supplementary Table 2.

Quantification of markers of epithelial barrier status in BALF

Bronchial epithelial leakage to the airway lumen was assessed by analyzing total protein and serum albumin content in BALF. Measurement of total protein concentration in cell-free BALF supernatants was performed using the Micro BCA Kit (Thermo Fisher Scientific). Mouse albumin content in BALF samples was quantified using a competitive ELISA kit (Abcam). All procedures were carried out following the manufacturer's protocols. Epithelial damage was assessed by determining LDH activity in BALF using CyQUANT LDH Cytotoxicity Assay Kit (Invitrogen), following the manufacturer's protocol.

Hematoxylin and eosin staining of peribronchial tissue

Mice lungs were collected and immediately fixed in 4% paraformaldehyde for 48 hours. Then, the lungs were washed with PBS and embedded in paraffin. Paraffin-embedded tissues were cut into 5 μm -thick sections and stained with Hematoxylin and eosin (H&E). Finally, samples were digitalized, and 200x200 μm images of peribronchial areas of the different groups of mice were visualized to evaluate airway inflammation.

Targeted proteomics

Inflammation-related protein concentrations in the BALF and sera of mice were measured with targeted proteomics, using the Olink® Target 48 Mouse panel consisting of 43 markers (Olink Biosciences). This method employs a proximity extension assay and provides relative protein quantification expressed as normalized protein expression (NPX) values on a log2 scale. Proteins with >80% missing frequency were excluded from analyses, and values below the detection limit were replaced with the lowest quantifiable value for statistical analyses.

Statistical analysis

Analyses were performed using Prism 8 (GraphPad) or R and RStudio. The Friedman test, followed by uncorrected Dunn's test, was used for paired comparisons of multiple groups against a control group. For unpaired comparisons, the Kruskal-Wallis test was followed by the uncorrected Dunn's test. Two-way ANOVA followed by Fisher's LSD test was employed to analyze the TEER measurements. A t-test and Benjamini-Hochberg correction were used to compare groups in the Olink proteomics data. Correction for multiple testing was employed using the Benjamini-Hochberg method. Corresponding statistical analyses for each figure are specified in the figure legends.

RESULTS

Physicochemical characterization of the regolith simulants

Before evaluating the biological effects of the regolith, we first performed a physicochemical characterization of the Martian (MGS-1, MGS-1S) and Lunar (LMS-1) regolith simulants, along with silica (SiO_2) as the terrestrial control. Information on the mineral composition and bulk chemical profiles of the simulants was provided by the manufacturer (Figure 1A-B). All simulants contained olivine ($(\text{Mg,Fe})_2\text{SiO}_4$), pyroxene (ABSi_2O_6), and glass-rich basalt. The Martian simulants contained a much broader variety of minerals than the Lunar simulants, in particular LMS-1. SiO_2

was the most abundant compound shared in all simulants, followed by Al_2O_3 , FeO , and CaO .

Due to the heterogeneous nature of the regolith simulants with sizes ranging from $< 0.04 \mu\text{m}$ to $1000 \mu\text{m}$ (Supplementary Figure 1), the material was milled and sieved to achieve a particle size of $\leq 20 \mu\text{m}$ for *in vitro* and *in vivo* experiments. Dynamic light scattering analysis confirmed that grinding effectively reduced the average particle size to $\leq 20 \mu\text{m}$, with small fractions of larger particles up to $80 \mu\text{m}$ (Figure 1C). Scanning electron microscopy revealed that heterogeneity persisted after grinding (Figure 1D). Pre-grinding samples consisted of large, irregular aggregates, whereas post-grinding samples were fragmented but retained angular structures (Supplementary Figure 1). Compared to silica, regolith simulants were more heterogeneous and exhibited rougher jagged morphologies.

To quantify particle morphology, we analyzed circularity, solidity, and Feret angle using the ImageJ software. Circularity, ranging from 0 (irregular) to 1 (perfect circle), improved after grinding for all samples, particularly for MGS-1S and LMS-1, which were highly irregular before grinding (Table 1 and Supplementary Figure 2). Solidity, which indicates edge smoothness, improved slightly after grinding, reaching statistical significance only for silica. Feret angle distributions, reflecting particle elongation, remained broad for all samples, consistent with their heterogeneous shapes.

Overall, these data confirmed that post-grinding samples were morphologically more uniform and more comparable to one another. Together with the limited size distribution, this allows for a more direct and controlled comparison of their biological effects.

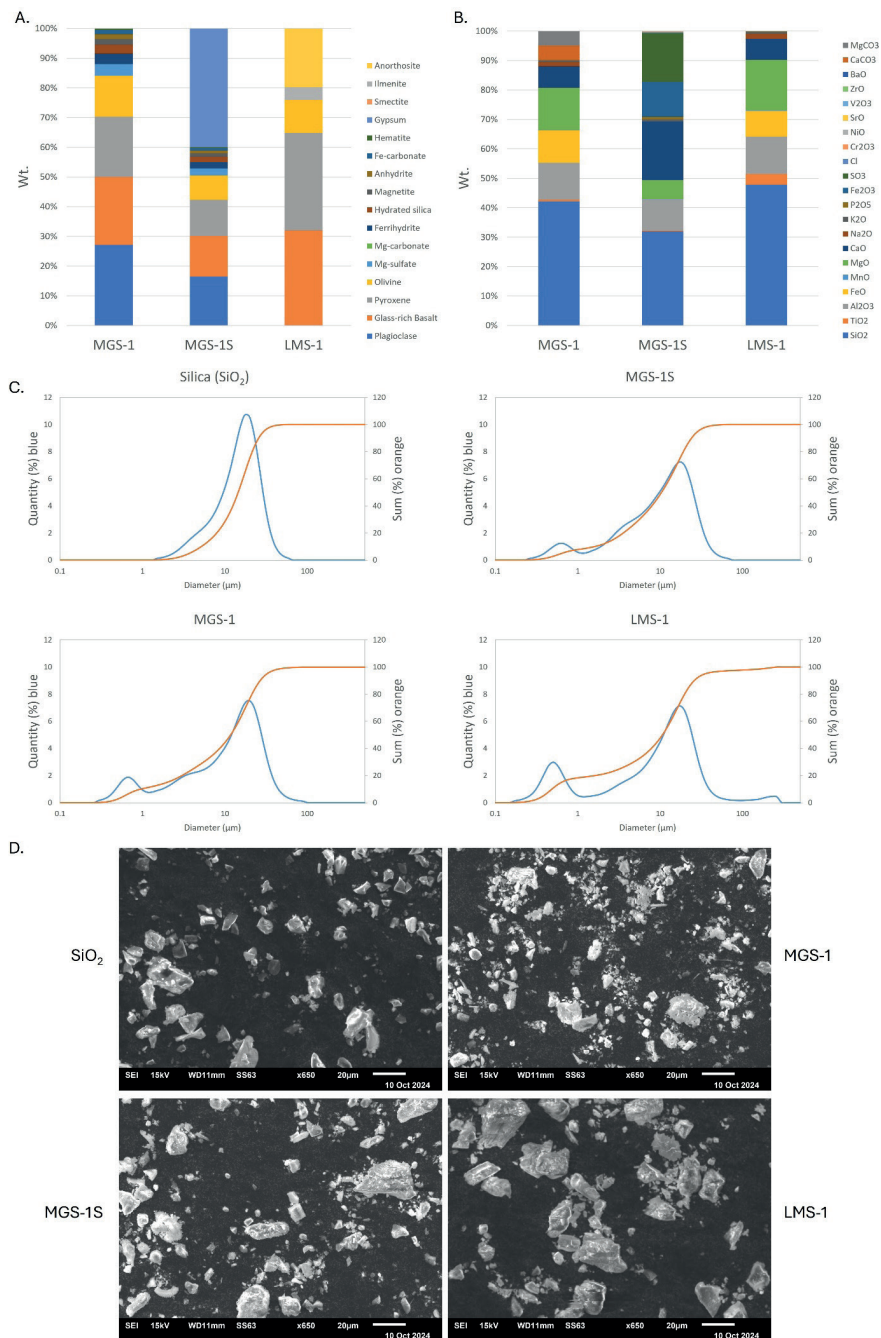


Figure 1. Physicochemical properties of the Martian and Lunar regolith simulants. **A)** Mineralogy and **B)** bulk chemistry of the regolith simulants. **C)** Particle size distribution and **D)** representative scanning electron microscopy images of the silica control and regolith simulants. MGS-1: Mars Global Simulant 1, MGS-1S: MGS enriched in polyhydrated sulfate, LMS-1: Lunar Mare Simulant 1.

Table 1. Summary of the circularity, solidity and Feret angle measurements of the samples pre- and post-grinding.

Grinding	Sample	Particle Count	Circularity (mean $\pm$ SD)	Solidity (mean $\pm$ SD)	Min Feret Angle (mean $\pm$ SD)	Max Feret Angle (mean $\pm$ SD)
Pre	SiO ₂	2033	0.332 $\pm$ 0.047	0.567 $\pm$ 0.056	5.251 $\pm$ 1.318	91.919 $\pm$ 1.709
Post		757	0.692 $\pm$ 0.034	0.851 $\pm$ 0.013	2.588 $\pm$ 0.500	99.057 $\pm$ 7.366
Pre	MGS-1	1096	0.551 $\pm$ 0.220	0.732 $\pm$ 0.063	3.634 $\pm$ 2.891	94.219 $\pm$ 0.100
Post		2132	0.675 $\pm$ 0.003	0.816 $\pm$ 0.002	1.489 $\pm$ 0.088	96.044 $\pm$ 0.602
Pre	MGS-1S	1693	0.008 $\pm$ 0.001	0.776 $\pm$ 0.041	2.337 $\pm$ 0.984	90.003 $\pm$ 0.040
Post		1695	0.661 $\pm$ 0.018	0.819 $\pm$ 0.004	1.636 $\pm$ 0.134	95.180 $\pm$ 1.080
Pre	LMS-1	740	0.007 $\pm$ 0.003	0.814 $\pm$ 0.048	2.851 $\pm$ 0.226	98.310 $\pm$ 6.313
Post		649	0.719 $\pm$ 0.019	0.867 $\pm$ 0.006	2.801 $\pm$ 0.288	101.080 $\pm$ 3.746

SiO₂: silica dioxide quartz, MGS-1: Mars Global Simulant 1, MGS-1S: MGS enriched in polyhydrated sulfate, LMS-1: Lunar Mare Simulant 1. Circularity index: 0-1, 1 indicates a perfect circle. Solidity index: 0-1, lower values indicate rougher edges. Feret angles: 0-180.

Martian and Lunar regolith induce proinflammatory cytokine production from human PBMCs

To explore the immunological effects of regolith, we investigated the response of PBMCs from healthy human donors to Lunar and Martian regolith simulants, using silica (SiO₂) as a control. After 24 hours of stimulation, all regolith types dose-dependently induced the pro-inflammatory cytokines IL-6, TNF, IL-1 α , and IL-1 β (Figure 2A). Both Martian regolith simulants induced similar amounts of IL-6 and TNF compared to SiO₂, while LMS-1 induced significantly lower amounts. MGS-1 strongly promoted IL-1 α and IL-1 β production (3.2-fold and 2-fold higher production than that induced by silica, respectively), followed by MGS-1S. LMS-1 was the least potent inducer of IL-1 α and IL-1 β .

Given the role of anti-inflammatory factors in balancing inflammation, we also measured IL-1RA and IL-10. All stimuli induced these cytokines in a dose-dependent manner (Figure 2B). Regolith simulants caused less IL-1RA release than SiO₂ but similar IL-10 production. Only low-dose MGS-1 led to higher IL-10 production than silica. To assess the balance of pro- and anti-inflammatory responses, we compared the ratio of IL-1 β to its antagonist IL-1RA. SiO₂ and LMS-1 had similar IL-1 β /IL-1RA ratios (Figure 2C), while Martian simulants, especially MGS-1, skewed the balance towards inflammation. The average IL-1 β /IL-1RA ratio after MGS-1 exposure of PBMCs was 3.2 times higher than that after SiO₂ exposure.

Cytotoxicity assays showed that MGS-1, followed by MGS-1S, caused most cell death at high doses, whereas low-dose exposure did not affect viability (Figure 2D).

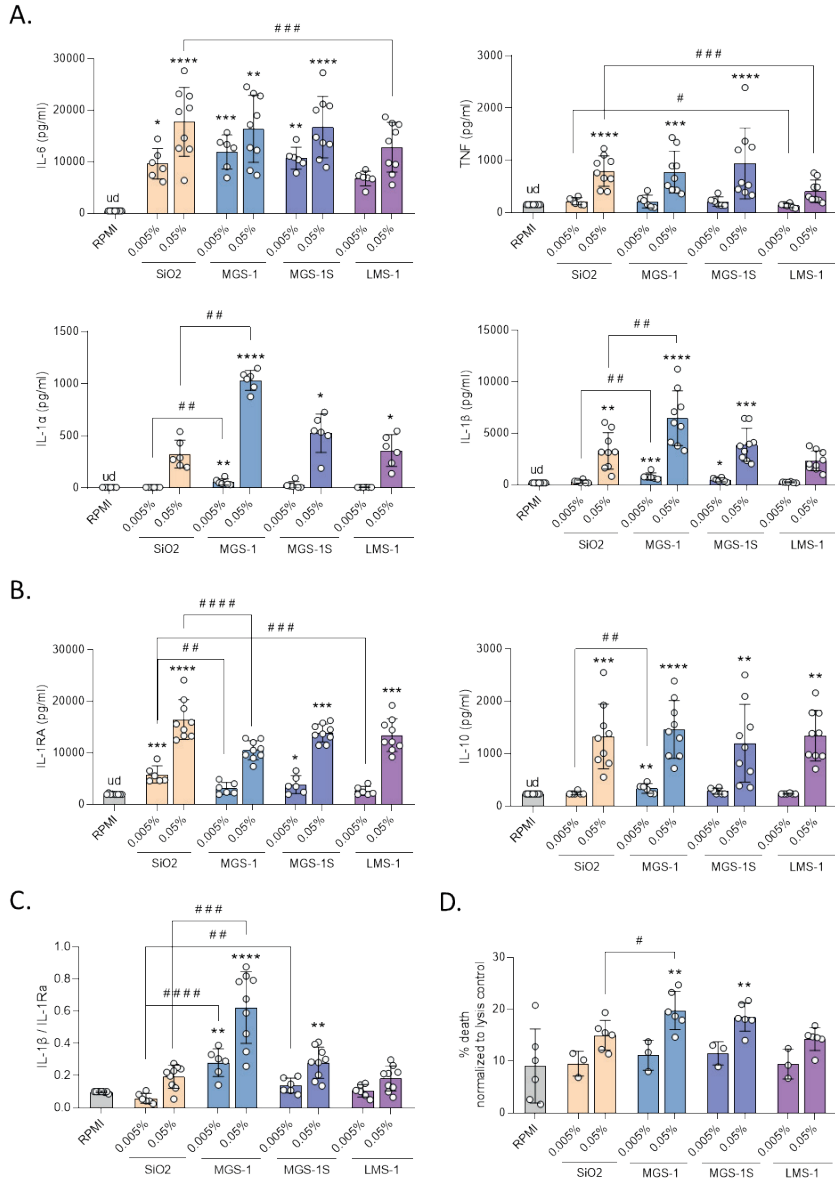


Figure 2. Martian and Lunar regolith induce pro- and anti-inflammatory cytokine production from PBMCs. PBMCs from healthy donors were stimulated with 0.005% or 0.05% w/v SiO_2 , MGS-1, MGS-1S, or LMS-1 for 24h. Particles were pre-incubated with Polymyxin B (5 $\mu\text{g}/\text{mL}$) for 1h before PBMC stimulation. Quantification of **A)** pro-inflammatory and **B)** anti-inflammatory cytokines detected in supernatants. **C)** Ratio of IL-1 β to IL-1Ra. **D)** Cytotoxicity expressed as % of lysis control. Data expressed as mean $\pm$ SD. (A-C) $n=6/9$ pooled from 2/3 experiments, (D) $n=3/6$ from 1/2 experiments. Friedman test followed by uncorrected Dunn's test. * compared to media control (RPMI), # compared to SiO_2 , */# $p<0.05$, **/## $p<0.01$, ***/### $p<0.001$, ****/#### $p<0.0001$. ud: under the detection limit, replaced with the lowest detectable value for statistical analysis.

Taken together, these data suggest that all regolith simulants trigger both pro- and anti-inflammatory cytokine responses by human PBMCs, but Martian simulants skew the balance towards increased inflammation while also causing cytotoxicity.

Regolith simulants induce cytotoxicity and cytokine production in human airway epithelial cell lines

During Mars or Moon missions, the respiratory tract represents a primary site of regolith exposure, along with the skin and eyes. Therefore, we investigated the effects of regolith simulants on two human airway epithelial cell lines: A549 (lung epithelium) and Detroit 562 (pharyngeal epithelium). After 24 hours of stimulation, both concentrations of SiO_2 promoted IL-8 production and release from the epithelial cell lines (Figure 3A). Low-dose LMS-1 significantly increased IL-8 in A549 cells, while high-dose LMS-1 elevated IL-8 in Detroit 562 cells. Interestingly, high-dose MGS-1 decreased IL-8 levels below baseline in A549 cells, reaching statistical significance.

High-dose SiO_2 , MGS-1, and MGS-1S also increased the production of monocyte chemoattractant protein 1 (MCP-1) in A549 cells. Both Martian simulants elevated IL-6 release from Detroit 562 cells, whereas LMS-1 and SiO_2 showed similar but non-significant increases. Cytotoxicity assays revealed dose-dependent effects in A549 cells, with up to 40% cell death induced by LMS-1 and SiO_2 (Figure 3B). No regolith simulant caused cytotoxicity in Detroit 562 cells at any concentration (Figure 3B).

Overall, these data reveal that all regolith simulants possess cytotoxic and immunostimulatory capacities comparable to those of SiO_2 on human airway cell lines.

Regolith exposure impairs epithelial integrity and induces inflammation in air-liquid-interface cultures of human airway epithelial cells

To further understand the potential harmful effects of Martian and Lunar regolith on the respiratory tract, we differentiated primary human bronchial epithelial cells (HBECs) into a pseudostratified bronchial epithelium using an air-liquid-interface (ALI) culture, and established a model of repeated daily exposure to regolith simulants (Figure 4A). Following complete differentiation, the bronchial epithelium was apically exposed to PBS, SiO_2 , or regolith simulants for three consecutive days. Epithelial barrier integrity was assessed by daily measurements of transepithelial electrical resistance (TEER) and by evaluating paracellular flux of apically administered FITC-dextran 24 hours after the final regolith exposure.

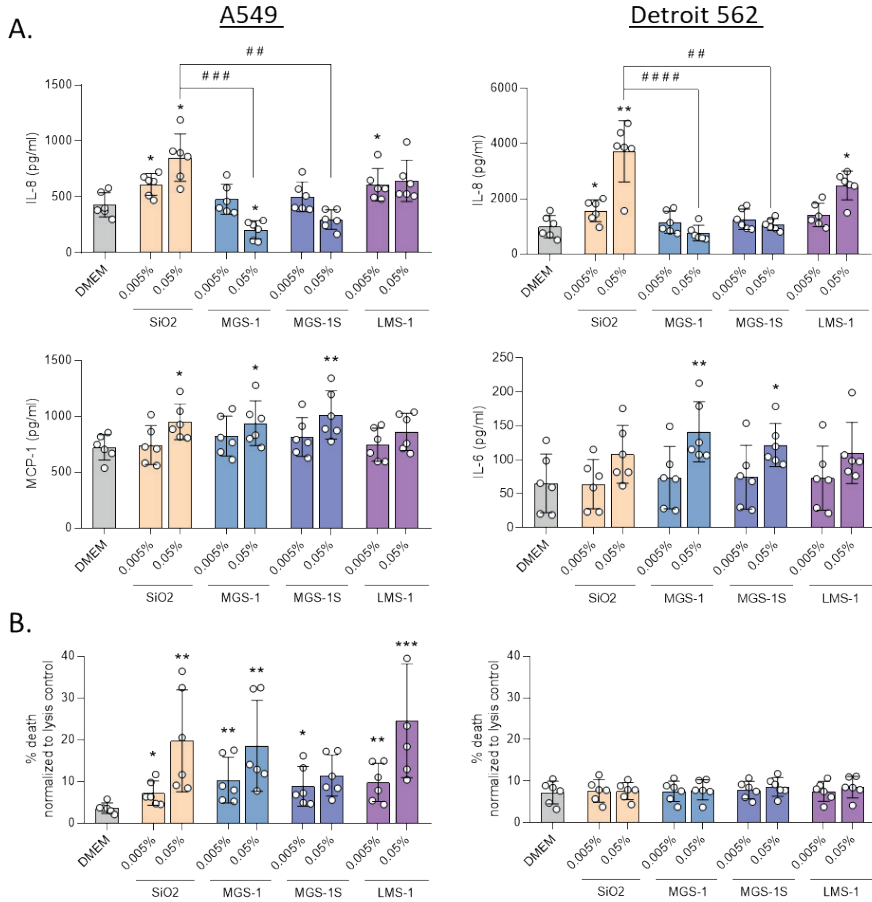


Figure 3. Lunar and Martian regolith induce cytotoxicity and cytokine production in human airway epithelial cell lines. Human airway epithelium cell lines A549 and Detroit562 were stimulated with 0.005% or 0.05% w/v SiO₂, MGS-1, MGS-1S, or LMS-1 for 24h. Particles were pre-incubated with Polymyxin B (5 µg/mL) for 1h before PBMC stimulation. **A)** Cytokine and chemokine production. **B)** Cytotoxicity expressed as % of lysis control. Data expressed as mean ± SD. n=6 independent experiments, each data point showing the average of 3 technical replicates. Kruskal-Wallis test followed by uncorrected Dunn's test. * compared to media control (DMEM), # compared to silica control (SiO₂). * p<0.05, **/## p<0.01, ***/### p<0.001, #### p<0.0001.

Compared to the PBS control, LMS-1 and MGS-1 promoted the earliest significant drop in TEER values 48 hours after the first exposure, which remained significantly below control levels at 72 hours (18.2 and 22.1 % average reduction, respectively) (Figure 4B). MGS-1S induced a significant decline in TEER (17.1 % on average) only after 72 hours. Similarly, ALI cultures exposed to SiO₂ displayed a downward tendency (14.6 % average reduction at 72 hours), although it did not reach

statistical significance. Paracellular flux analysis was consistent with the TEER data, showing that LMS-1 and MGS-1 caused a significant increase in airway permeability compared to control cultures (Figure 4C). A non-significant trend towards higher permeability was also observed following SiO₂ and MGS-1S exposures. These findings suggest that regolith exposure, especially LMS-1 and MGS-1, could be detrimental and potentially more harmful to the airway epithelial barrier than silica.

To explore whether airway epithelial cells initiate inflammatory responses upon regolith exposure, we quantified cytokine concentrations in both the apical and basolateral compartments 24 hours after the final stimulation. In the apical compartment, LMS-1 induced significantly higher amounts of IL-1 β compared to control cultures, but no stimuli led to significant changes in apical IL-6 and IL-8 concentrations (Figure 4D). In the basolateral compartment, SiO₂ and LMS-1 significantly boosted IL-8 production, with a similar trend in cultures exposed to MGS-1S (Figure 4E). LMS-1 also increased IL-6 levels, and a similar but non-significant trend was observed for IL-1 β .

Taken together, these data indicate that regolith simulants, particularly LMS-1 and MGS-1, compromise epithelial barrier integrity and trigger inflammatory responses in an *in vitro* HBEC ALI model following repeated exposure.

Intranasal regolith administration compromises bronchial epithelial function in C57BL/6J mice

To validate our results indicating that Martian and Lunar regolith could be harmful to the airways, we intranasally administered PBS (control), MGS-1, LMS-1, or SiO₂ for three days to C57BL/6J mice (Figure 5A). We chose LMS-1 and MGS-1 for the *in vivo* experiments, as they previously showed the most disruptive potential. 24 hours after the last intranasal administration, analysis of the bronchoalveolar lavage fluid (BALF) from mice showed that LMS-1 caused the highest translocation of serum proteins, determined by increased total protein and serum albumin concentrations, along with higher LDH activity (Figure 5B), suggesting that this regolith displays a potent barrier disruptive capacity *in vivo*. Similar trends were observed in mice exposed to MGS-1, but only the increase in serum albumin content was statistically significant. Intranasal instillation of SiO₂ also led to significantly increased markers of epithelial barrier dysfunction (Figure 5B).

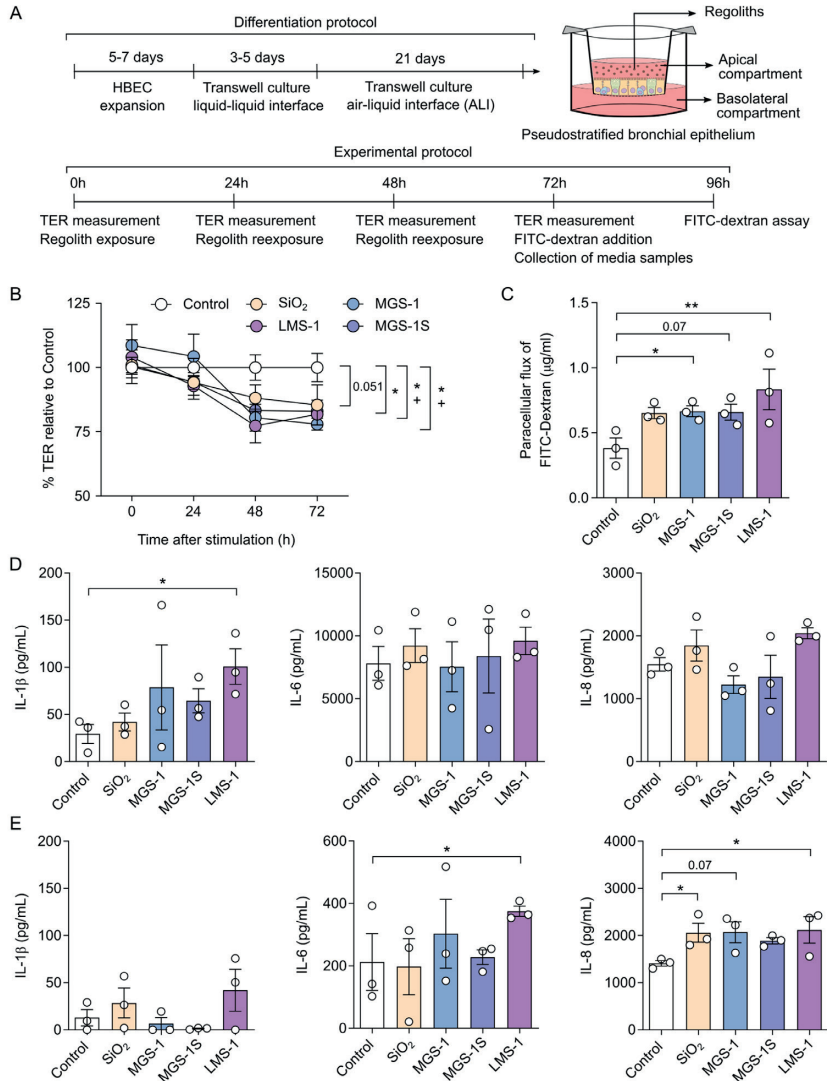


Figure 4. Regolith exposure impairs bronchial epithelial integrity and induces inflammation in a model of differentiated pseudostratified epithelium. **A**) Schematic representation of the differentiation and experimental protocols employed to generate and stimulate air-liquid interface (ALI) cultures of human bronchial epithelial cells (HBECs) with PBS (control) or 0.05% w/v regolith. **B**) Percentage of transepithelial electrical resistance (TER) after stimulation. TER levels are expressed relative to control cultures. + Control vs. indicated treatment 48h after stimulation, * Control vs. indicated treatment 72h after stimulation. **C**) Paracellular flux of apically administered FITC-Dextran to the basolateral compartment of transwell inserts. Cytokine measurements from **D**) the apical and **E**) basolateral compartments. $n=3$ donors, each dot represents the average of 2/3 experimental replicates. All data are represented as mean $\pm$ SEM. Statistical significance was determined by Two-way ANOVA followed by Holm-Sidak's multiple comparisons test (B), or Friedman test followed by Uncorrected Dunn's test (C-E). */+ $p<0.05$ and ** $p<0.01$.

Moreover, SiO_2 significantly reduced lung mRNA expression of the tight junction proteins *Ocln* (Occludin) and *Tjp1* (ZO1), confirming its barrier-disruptive effects, but had minimal impact on the expression of other gene markers (Figure 5C). In contrast, LMS-1 not only downregulated the expression of genes coding for tight junctions but also upregulated the expression of fibrosis-associated markers such as *Col1a1* and *Tgfb*, goblet cell markers including *Muc5ac* and *Gob5*, and the ciliogenesis master transcription factor *Foxj1*, while reducing *Scgb1a1*, a marker of club cells. Similarly, MGS-1 reduced the expression of *Ocln*, *Tjp1*, and *Scgb1a1*, while enhancing fibrosis-related gene expression.

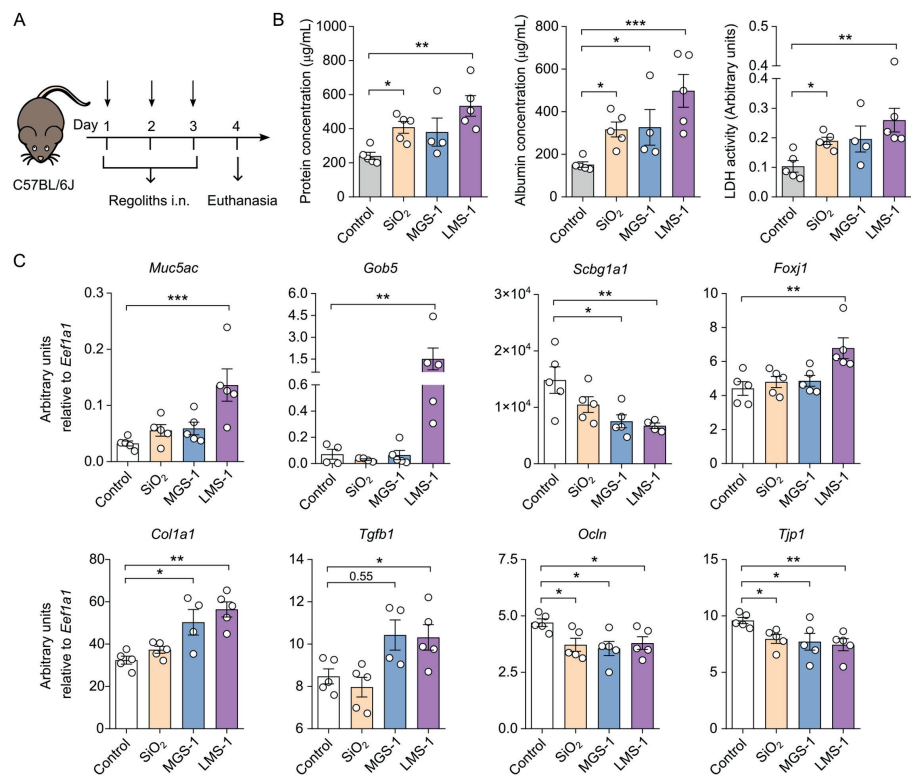


Figure 5. Intranasal regolith administration compromises bronchial epithelial function in C57BL/6J mice. **A**) Schematic representation of the experimental protocol. Mice were intranasally instilled with PBS (control), 50mg/kg of SiO_2 , MGS-1, or LMS-1 for three consecutive days. 24h after the last administration, they were sacrificed, and samples were collected. **B**) Total protein content, albumin concentration, and LDH release in bronchoalveolar lavage fluid (BALF). **C**) Lung mRNA expression of bronchial epithelial cell markers relative to a housekeeping gene (*Eef1a1*). All data are represented as mean $\pm$ SEM (n=4-5). Kruskal-Wallis test followed by uncorrected Dunn's test. * p<0.05, ** p<0.01, *** p<0.001.

Intranasal regolith administration triggers airway inflammation in C57BL/6J mice

Lastly, we assessed inflammation in the airways of mice intranasally exposed to MGS-1, LMS-1, or silica. All stimuli caused a significant recruitment of immune cells into the BALF (Figure 6A). The flow cytometric analysis of BALF cell populations showed that mice intranasally administered with either regolith or silica exhibited marked neutrophilia compared to controls (Figure 6B-C). Additionally, other immune cell populations, including eosinophils, dendritic cells, and T lymphocytes were significantly elevated following exposure to both silica and regolith. Notably, the relative distribution of recruited cell types differed depending on the specific stimulus. No significant changes were observed in the number of alveolar macrophages in any of the conditions (Figure 6C).

We then evaluated the gene expression of key inflammatory markers in the lung tissue. MGS-1 and LMS-1 caused a significant upregulation of the inflammatory mediators *Tnf* and *Il6*, accompanied by the induction of genes such as *Nlrp3* and *Il1b*, indicating active lung inflammation and inflammasome activation (Figure 6D). SiO_2 also enhanced *Tnf*, *Nlrp3*, and *Il1b*, but did not significantly affect *Il6* expression. Consistent with the observed airway neutrophilia, all stimuli significantly increased *Il17f* expression. Furthermore, MGS-1 and LMS-1, but not silica, upregulated *Ifng* and *Il13*, while increased *Il10* expression was observed only after LMS-1 exposure. Histological assessment of peribronchial areas further demonstrated the disrupted epithelial linings and the *in situ* inflammatory cell infiltration in mice exposed to SiO_2 , MGS-1, or LMS-1, compared to controls (Figure 6E).

Finally, we assessed a panel of 43 inflammation-related proteins in the BALF and sera of exposed mice using proximity extension assay (Olink targeted proteomics). In the BALF, exposure to SiO_2 or either type of regolith resulted in significant changes in inflammatory biomarkers compared to PBS controls (Figure 7A). LMS-1 exposure induced the strongest response, significantly increasing the expression of 31 proteins, including many chemokines, cytokines, and proteins involved in tissue remodeling. SiO_2 similarly triggered the upregulation of 28 inflammatory proteins. However, MGS-1 exposure elevated only 6 markers (INFL2, CCL2, CCL4, CCL12, CSF3, and CXCL9), all of which were also elevated by SiO_2 and LMS-1. When directly compared to the silica control, LMS-1 exposed mice exhibited significantly higher concentrations of IL-9 and IL-5, but lower levels of IL-22. To determine whether these local airway responses had systemic consequences, we also analyzed the sera of the same mice (Figure 7B). Only MGS-1 exposure caused significant systemic changes, with elevated circulating

CCL2, CCL12, IL-10, and FGF21 and reduced IL1 β and CXCL2 levels. A similar pattern was observed when MGS-1 was compared to SiO₂.

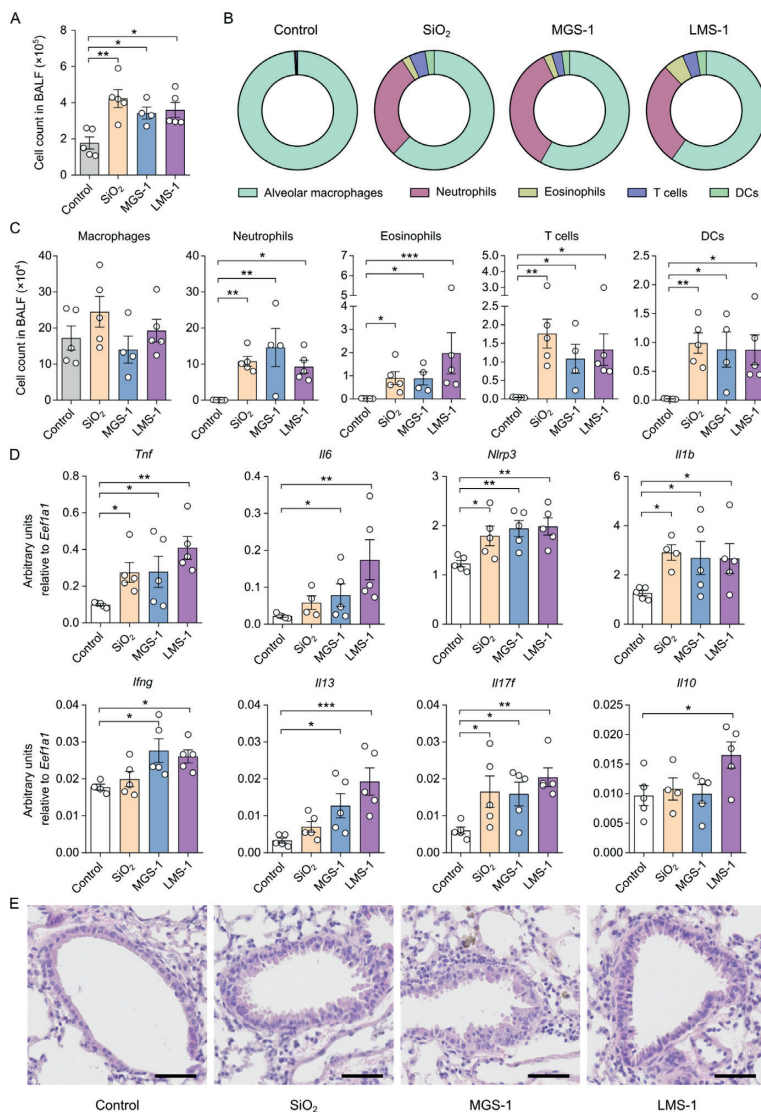
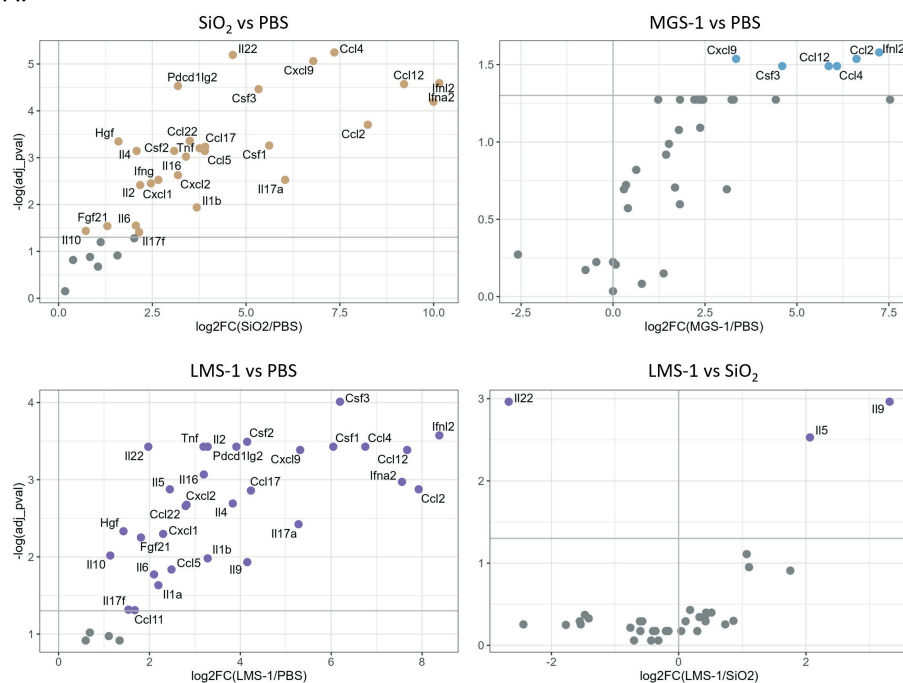


Figure 6. Intranasal regolith administration induces airway inflammation in C57BL/6J mice. **A)** Cell recruitment in the bronchoalveolar lavage fluid (BALF) of mice exposed to PBS (control), SiO₂, MGS-1, or LMS-1. **B)** Percentage and **C)** absolute numbers of the indicated cell populations present in BALF. **D)** Lung mRNA expression of inflammatory markers relative to a housekeeping gene (*Eef1a1*). **E)** Representative images of peribronchial areas after Hematoxylin and Eosin (H&E) staining of lung tissue sections. Scale bars: 50 μ m. All data are represented as mean $\pm$ SEM (n=4-5). Statistical significance was determined by the Kruskal-Wallis test followed by uncorrected Dunn's test * p<0.05, ** p<0.01, *** p<0.001.

Collectively, these data suggest that both Martian (MGS-1) and Lunar (LMS-1) regolith simulants trigger robust airway inflammation *in vivo* at least comparable to or exceeding silica exposure, and may therefore pose a significant respiratory health risk.

A.



B.

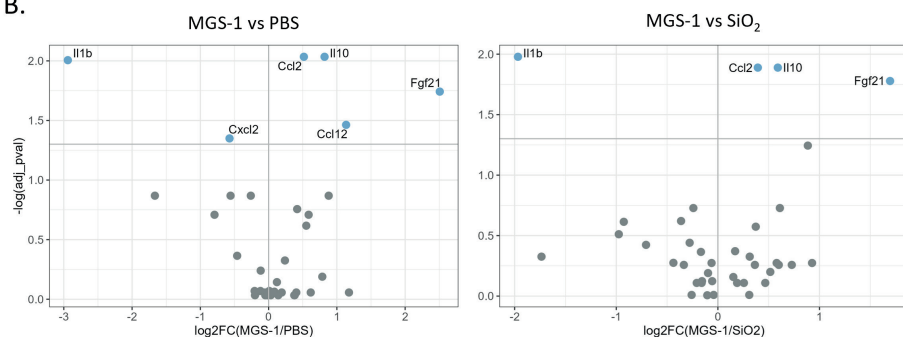


Figure 7. Changes in inflammatory proteins in the BALF and sera of C57BL/6J mice intranasally exposed to regolith. Inflammation-related proteins in A) the BALF and B) sera of mice exposed to PBS (control), SiO₂, MGS-1, or LMS-1 were quantified using the Olink® Target 48 Mouse panel. Statistical significance was determined by a t-test followed by the Benjamini-Hochberg method of multiple testing correction. Only the proteins with an adjusted p-value <0.05 are highlighted and annotated.

DISCUSSION

With the growing interest in long-duration space exploration, potential habitation of planetary bodies of the Solar System, and the exploitation of space-derived materials on Earth, understanding the biological impact of regolith exposure on astronauts and Earth's inhabitants is increasingly urgent. Our study provides a comprehensive *in vitro* and *in vivo* analysis of the cellular effects of Lunar and Martian regolith simulants, demonstrating that both compromise airway barrier integrity and trigger inflammation in human and murine models.

Previous research on regolith's biological effects has largely been limited to isolated observations in specific cell types or animal models (3, 14-19), leaving a gap in comprehensive studies examining barrier damage and immune responses together. In contrast, our work integrates physicochemical characterization of the simulants with immunological and barrier function assays across human and murine models. Notably, the Lunar simulant LMS-1 emerged as the most potent disruptor of airway barrier function and inducer of inflammation, surpassing or matching the effects of crystalline silica, a well-established pulmonary toxicant responsible for over 10,000 deaths annually (20).

Particle size, irregular morphology, and surface reactivity of regoliths are likely key drivers of their biological effects. Even after grinding, regolith samples retained jagged structures and angular shapes capable of damaging cell membranes. Lunar regolith and, to a lesser degree Martian regolith, also contains metallic iron particles on the surface, which can generate hydroxyl radicals that could potentially react with and denature proteins (21). These physicochemical properties likely underlie the observed epithelial barrier dysfunction following regolith exposure, as evidenced by reduced TEER, increased paracellular flux, and elevated albumin and LDH in BALF.

Differences in chemical composition might underlie the distinct biological effects of Martian and Lunar regolith simulants. LMS-1 lacked many minerals present in Martian simulants, but contained a considerable amount of anorthosite, which was shown to induce cytotoxicity and cytokine production in human bronchial epithelial cells and macrophages (22). Consequently, the role of anorthosite in the pro-inflammatory and pro-fibrotic effects *in vivo* with LMS-1 warrants further investigation.

Inhaled particulate matter can trigger macrophage apoptosis, ferroptosis, inflammasome activation, and the release of reactive oxygen and nitrogen species, cytokines, chemokines, and proteolytic enzymes, collectively contributing to inflammation and fibrosis (23-25). Previous studies have shown that Martian and Lunar simulants, other than those used in this study, can induce apoptosis in human alveolar macrophages (14). Particulate matter can also stimulate cytokine production by epithelial cells (26, 27), and a different lunar simulant was shown to cause oxidative stress and apoptosis in human lung epithelial cells (28). Our results extend these findings, positioning regolith exposure as a serious health risk capable of inducing inflammation and compromising epithelial barrier integrity.

Interestingly, our *in vivo* data showed that systemic immune changes were minimal and were detected only after MGS-1 exposure, suggesting either containment of the inflammatory response or insufficient time to generate systemic spillover. Nonetheless, barrier impairment is not merely a consequence of local airway damage; growing evidence indicates that it may serve as a trigger for the development of a wide range of chronic non-communicable diseases (29, 30). Accordingly, regolith exposure may not only cause local deleterious effects but also pose potential long-term risks to overall health. Chronic exposure models with extended follow-up are needed to determine if regolith exposure could lead to systemic effects as well as persistent local damage.

This study is directly relevant not only to astronaut safety but also to Earth's inhabitants who could be exposed to space-derived materials returned for future exploitation. Ultrafine regolith particles can reach penetrate deep into the alveoli, evade macrophage clearance, and potentially migrate into interstitial tissues or the bloodstream (9, 10). In microgravity, impaired mucociliary clearance and altered particle deposition in the lungs may exacerbate these risks (11, 12). Additionally, spaceflight-induced immune dysregulation could increase susceptibility to regolith-induced damage (13). Together, these interactions could have long-term consequences, including chronic inflammation and fibrosis, potentially compounding other microgravity-associated health risks, such as cardiovascular dysfunction (30).

While our findings across multiple models are robust, some limitations remain. The regolith simulants used may not perfectly recapitulate actual Martian or Lunar regolith, and our experiments modeled acute exposure, leaving chronic or cumulative effects untested. Future missions that return extraterrestrial regolith for research purposes will be critical to validate these observations.

In conclusion, our work reveals that regolith exposure, especially that of Moon origin, poses significant risks to airway integrity and immune homeostasis. These findings highlight the need for dust mitigation strategies, including surface coating for electrostatic repulsion, improved filtration, and effective dust removal techniques (31). As we prepare for sustained human presence beyond Earth, addressing the health risks of regolith will be essential for astronaut safety and mission success. Moreover, our findings provide insight into the potential health risks associated with the handling of extraterrestrial materials upon their possible return to Earth for scientific or commercial purposes.

Resource availability

Materials availability: This study did not generate new unique reagents.

Data and code availability: Data used to generate the figures has been deposited at Mendeley Data and is available with the following doi: 10.17632/4992f8pnjg.1.

Declaration of interests

M.G.N. is a scientific founder of Trained Therapeutix Discovery (TTxD), Biotrip, Lemba Therapeutics, and Salvina Therapeutics. O.P. received research grants from MINECO, Ministerio de Ciencia e Innovación, Inmunotek S.L., Novartis, and AstraZeneca and fees for giving scientific lectures or participation in Advisory Boards from: AstraZeneca, Pfizer, GlaxoSmithKline, Inmunotek S.L., Novartis, Sanofi-Genzyme and Regeneron. M.D.J. is advisor for Abera Bioscience AB.

Acknowledgements

This work was supported by an ZonMw Off Road Grant awarded to J.D.A (04510012010022). M.G.N. was supported by an ERC Advanced Grant (833247) and a Spinoza Grant of the Netherlands Organization for Scientific Research. O.P. was supported by grants PID2020-114396RB-I00 and PID2024-156862OB-I00 from Ministerio de Ciencia, Innovación y Universidades, Spain.

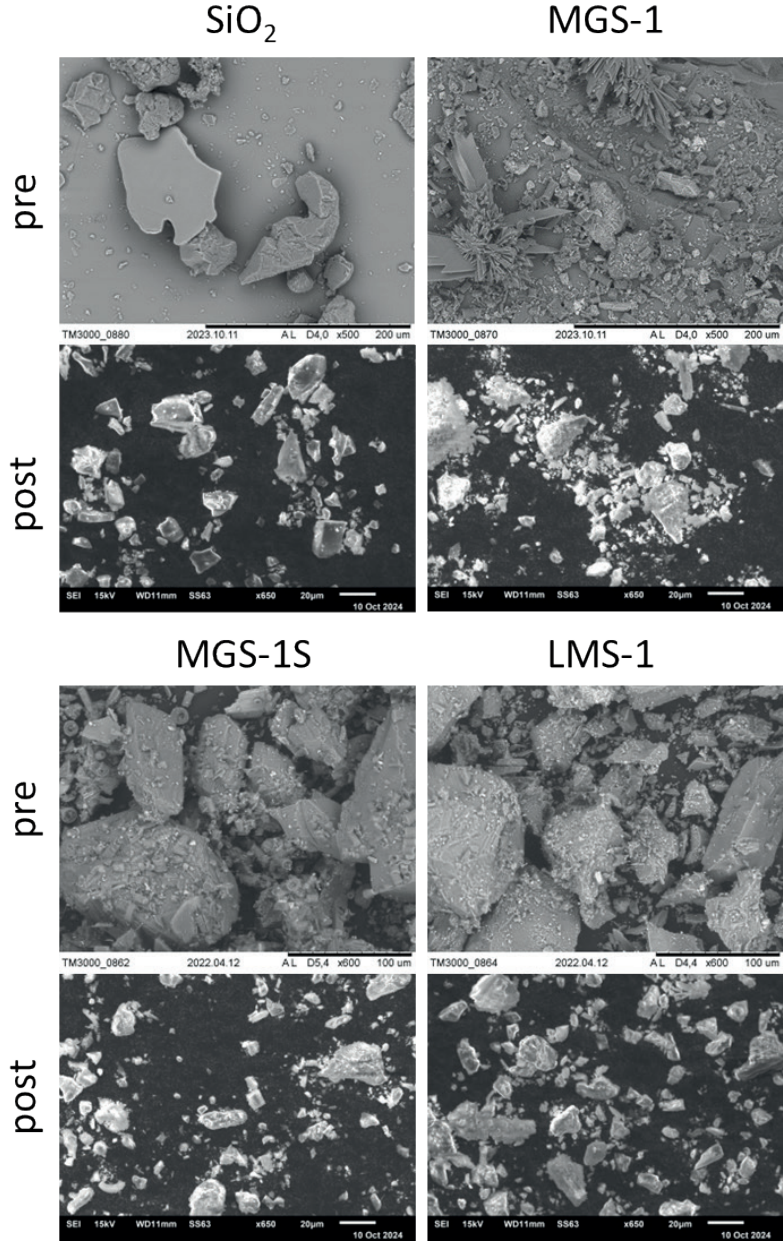
The authors would like to thank Dr. Alessandro Airo from Berlin Museum für Naturkunde for grinding the regolith and silica samples, and the PBMC donors for their contributions.

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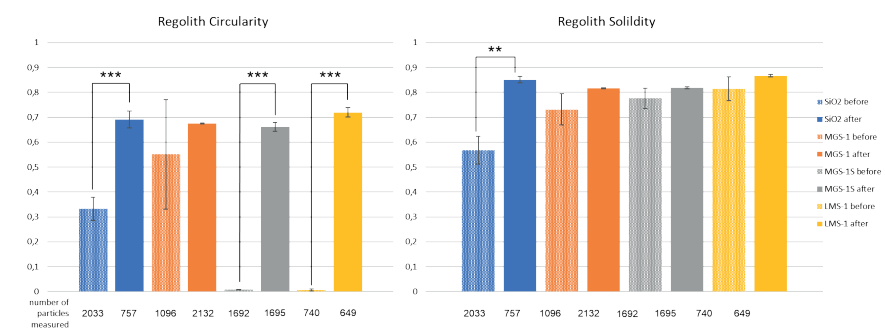
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SUPPLEMENTARY



Supplementary Figure 1: Electron microscopy images of the regolith samples pre- and post-grinding. Representative images of silica (SiO_2), Mars Global Simulant 1 (MGS-1), MGS enriched in polyhydrated sulfates (MGS-1S), and Lunar Mare Simulant 1 (LMS-1) as purchased from the manufacturer (pre) and after grinding to $\leq 20 \mu\text{m}$ particles (post).



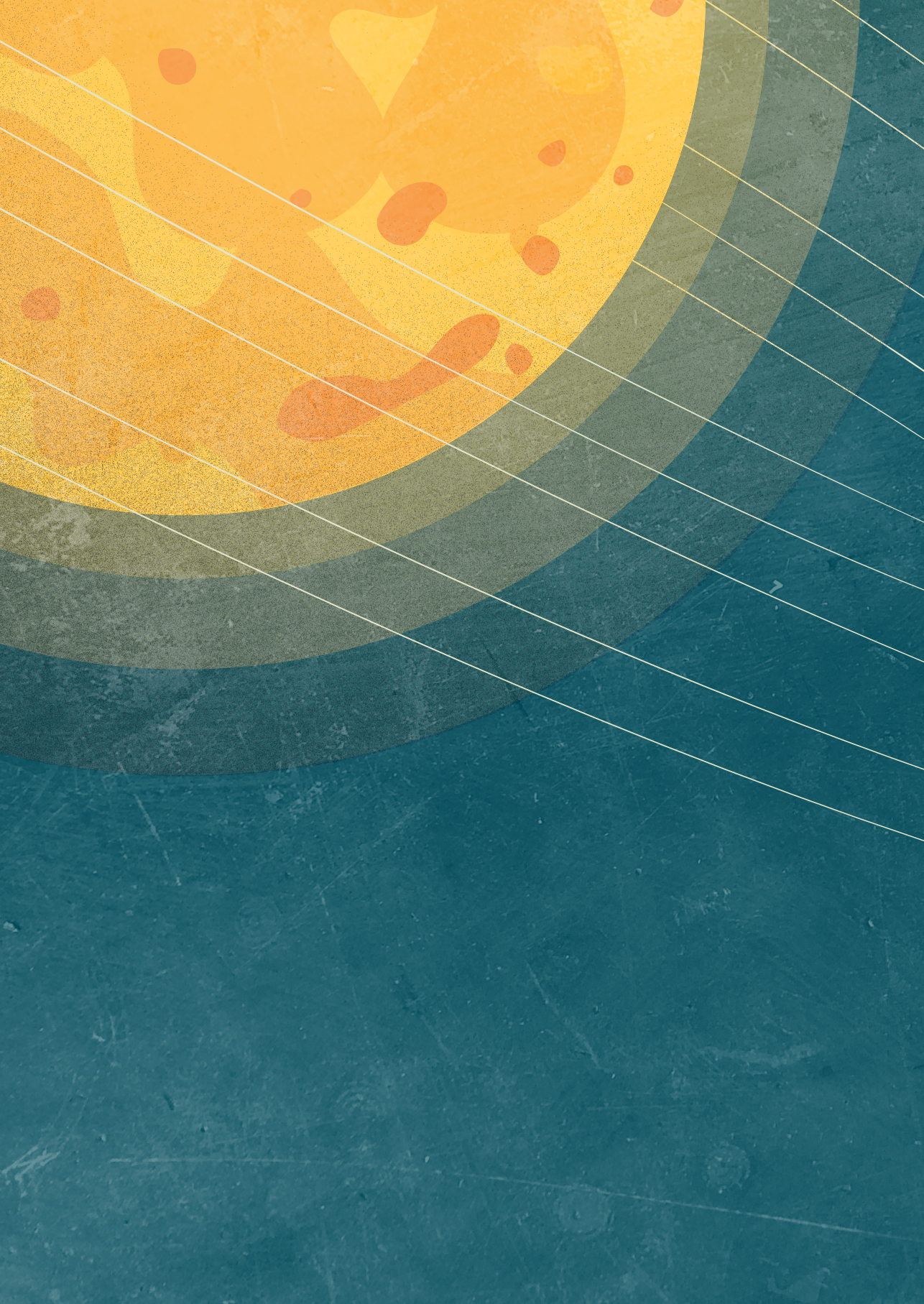
Supplementary Figure 2: Comparison of circularity and solidity of regolith samples pre- and post-grinding. Circularity as a range from 0 to 1 from irregular to perfect circle and solidity as ratio of particle area to convex hull area. Statistical comparison made between pre- and post-grinding silica (SiO₂, blue), Mars Global Simulant 1 (MGS-1, orange), MGS enriched in polyhydrated sulfates (MGS-1S, grey), and Lunar Mare Simulant 1 (LMS-1, yellow) samples. **p<0.01, ***p<0.001 t-test.

Supplementary table 1. List of oligonucleotides used for qPCR.

Gene Target	Forward primer sequence	Reverse primer sequence
Eef1a1	CGGCAGTCGCCTTGACGTT	CGGTGGTTTTCACAACACCTGCGT
Muc5ac	TGCACAGGCCAGGCTCAACA	CCCCTCTGGTATGTCCTGGGT
Clca1 (gob5)	AACGAGCAGGTGCCGATGC	AGTGACTCCTCCCAGAGCCCA
Scgb1a1	TGCTGCAGCTCAGCTTCTTCGG	GCCAGGGTTGAAAGGCTTCAGGG
Foxj1	ACCAAGATCACTCTGTCGGC	GCGGATGGAATTCTGCCAGG
Col1a1	GAACTGGACTGTCCCAACCC	TTGGGTCCTCGACTCTCTAC
Tgfb1	CTTCAATACGTCAGACATTCGGG	GTAACGCCAGGAATTGTTGCTA
Ocln	ACAAGAGAAATTTTGATGCAGGTCT	CATCAGCAGCAGCCATGTACTC
Tjp1 (Zo1)	GGAGATGTTTATGCGGACGG	CCATTGCTGTGCTCTTAGCG
Tnf	CCACCACGCTCTTCTGTCTA	TGATGAGAGGGAGGCCATTG
Il6	GAAACCGCTATGAAGTTCCTCTCTG	GTATCCTCTGTGAAGTCTCTCTCC
Nlrp3	ACCAGCCAGAGTGGAATGAC	ACAAATGGAGATGCGGGAGAG
Il1b	GCTGAAAGCTCTCCACCTCA	CTTGGGATCCACACTCTCCAG
Ifng	CAGCAACAGCAAGGCGAAAAAGG	TTTCCGCTTCTGAGGCTGGAT
Il13	GCTTGCTTGGTGGTCTCGCC	GGGCTACACAGAACCCGCCA
Il17f	ATTCTGAGGGAGGTAGCAGC	TGGAATTACGTGGGACAGA
Il10	GGCCAGAAATCAAGGAGCA	AGGGGAGAAATCGATGACAGC

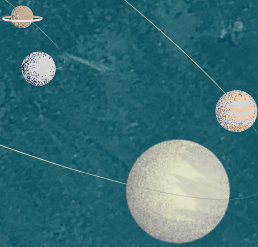
Supplementary table 2. List of antibodies and reagents employed for flow cytometry.

Antigen target / Reagent	Target species	Host species	Conjugate	Clone	Dilution	Supplier
CD16/CD32	Mouse	Rat	-	2.4G2	1:100	BD
CD3	Mouse	Armenian Hamster	FITC	145-2C11	7:100	Biolegend
Siglec F	Mouse	Rat	PE	SI7007L	1:50	Biolegend
CD11b	Mouse	Rat	PerCP-Cy5.5	M1/70	1:50	Biolegend
MHCII	Mouse	Rat	PE-Cy7	M5/114.15.2	1:100	Biolegend
CD11c	Mouse	Armenian Hamster	APC	N418	1:50	Biolegend
Ly6G	Mouse	Rat	BV405	1A8	1:25	Biolegend
ST1/St2	Mouse	Rat	BV605	DIH9	1:25	Biolegend
eFluor780 (Viability probe)	-	-	-	-	1:2000	Invitrogen



Part 2

Icy moon conditions and the impact on terrestrial microorganisms





Survival limits of psychrotolerant microorganisms with relevance for planetary protection of the icy moons

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Summary

Investigating the survival limits of extremophilic microorganisms exposed to simulated space conditions can shed light on the ability of terrestrial microorganisms to survive and propagate on other planetary bodies. Although microbes can be found in all environmental niches on Earth, this study focuses on psychrophilic and psychrotolerant microorganisms (prokaryotes and eukaryotes) which have been isolated from locations of interest such as icy moon analogue environments (e.g. Canadian high arctic, Antarctica) and cleanrooms, which might be relevant for forward planetary protection. Our research aimed to reproduce conditions for microorganisms on spacecraft travelling to the outer solar system which could contaminate the icy moon's subsurface oceans. The microorganisms were grown under oligotrophic conditions in minimal media supplemented with only a single carbon source and exposing them to extreme conditions, in terms of temperature fluctuations, in terms of freeze and thaw cycles, and radiation, as they occur during the space travel to the outer solar system. Our results in combination with future metagenome data and phenotype prediction tools will allow the identification of planetary protection relevant microorganisms in spacecraft assembly cleanrooms and on spacecraft and support the development of a target-oriented planetary protection constraints for missions to the icy moons.

Introduction

Exposing extremophilic and non-extremophilic microorganisms to simulated space conditions has been extensively researched (1-4). This knowledge plays a vital role in understanding the survival limits of a range of microbial species and will give insight into how and if such organisms could survive on other planetary bodies. Assessing the bioburden of a spacecraft prior to launch has been performed since the first robotic mission to Mars in the 1970s (5). Such evaluations were the reasons for which the Viking missions (American space probes which landed on Mars in 1976) were one of the first to include heat sterilization to reduce the bioburden (6). Spacecrafts are assembled in cleanrooms, sterilized through the use of heat, chemicals and/or radiation, in order to reduce the microbial counts to target limits (7, 8). Additionally, cleanrooms are monitored frequently for bioburden in order to guarantee effective measures and to determine which microbial species could potentially contaminate spacecraft, thereby providing knowledge on contamination risk management and microbial survival (9-11).

The Committee on Space Research (COSPAR), and here its panel on planetary protection is tasked with developing a planetary protection policy and providing implementation guidelines for compliance with the UN Outer Space Treaty of 1967 (12), to protect the Earth (backward contamination) and celestial bodies from harmful contamination (forward contamination). In regards to icy moon exploration, the panel has focused on psychrophilic/psychrotolerant microorganisms capable of growing between 20 and -20°C, and therefore posing a risk of microbial contamination for icy moon environments. Having identified microorganisms capable of growing until -20°C and in order to entrust tight guidelines for forward planetary protection of the icy moons, the planetary protection policy has adopted a -28°C temperature threshold for replication (13). Therefore, the assembly and preparation of spacecraft which will travel to icy moons should adhere to the updated policy. This is even more relevant since one of the organisms investigated in this study was isolated in a spacecraft assembly facility (SAF) cleanroom. It is essential to follow planetary protection policies in order to avoid the unwanted contamination of sites of astrobiological interest.

In the context of this paper we define icy moons as celestial bodies orbiting a planet of the outer solar system which have an icy surface with a subsurface liquid ocean. Currently, two missions are being conducted, including ESA's JUICE and NASA's Europa Clipper mission, which at the time of writing are both heading for the Jupiter system. These missions have been prepared based on knowledge

gained by the Cassini-Huygens mission to the Saturn system which has helped to understand the geological characteristics of the icy moons (14, 15). These celestial bodies are of interest due to their peculiar environmental characteristics, which could harbor life (16). On Enceladus, a moon of Saturn, the environmental conditions, potentially suitable for life as we know it on Earth are determined by a subsurface ocean below a 30 to 40 km thick ice layer (17). Furthermore, plume analysis from the Cassini mission has shown how organic material is being continuously ejected from the subsurface (14). This cryovolcanism effect has also been shown to take place on Europa, one of Jupiter's moon, which harbors a subsurface ocean under a thicker ice layer of approximately 100 km (18). Despite difficulties in accessing the subsurface ocean characteristics of the icy moons, the kilometer-thick ice layer would protect microbes against cosmic radiation. Researchers have shown how even a less thick ice layer would protect from ionizing and non-ionizing radiation on Enceladus (19, 20). Simulation studies to investigate microbial survival, have been suggested to be performed in order to close knowledge gaps ahead of future space missions including temperature, desiccation, UV-C and polychromatic UV radiation, low (Mars-relevant) pressure and synergistic effects of biocidal factors (13, 21).

Psychrotolerants are not only of concern for planetary protection, but also for the food industry as they can grow at locations where food is being stored and cause potential harm after food ingestion (22, 23). At the same time, however, these microorganisms have also proven to be very useful to the food industry as they contain enzymes capable of catalyzing reactions at low temperatures which are interesting for biotechnology and food processing applications (24, 25). Based on the aforementioned reasons, this study wanted to investigate at least one organism from each domain of life. The selection criteria of the chosen microorganisms were based on growth temperature (capable of growing at subzero temperatures), growth on solid media, visible growth within 48 hours, and the capability to grow in minimal media supplemented with a single carbon source different than glucose. This is because carbon sources have been identified in carbonaceous chondrites which have arrived on Earth and which could have arrived on the icy moons as well (26). Furthermore, icy moon subsurface ocean composition measurements have suggested a mineralogic composition containing carbon sources (27). Literature research yielded a list of possible microbial candidates, Table 1, including the bacteria *Planococcus halocryophilus* Or1 (DSM24743) (28), *Chromohalobacter sarracensis* LV4 (DSM15547) (29), *Paenisporsarcina antarctica* N-05 (DSM21991) (30), *Psychromonas boydii* 174 (DSM17665) (31), *Cryobacterium flavum* Hh8 (DSM26475) (32) and *Virgibacillus arcticus* Hal1 (DSM19574) (33). The archaea *Halorubrum luteum* CGSA15 (DSM23812) (34) and *Halorhabdus tiamatea* SARL4B (DSM18392) (35) also fulfilled these criteria, as well as

the yeast species *Rhodotorula frigidicola* JG1b (36) and *Rhodotorula mucilaginosa* Toulouse J8, A2- 5 (DSM114276). The experimental methodologies and subsequent results are presented without the inclusion of the archaeal species and the bacterial species *V. arcticus*, *C. flavum* and *P. boydii*, due to their inability to grow in our minimal media and due to their low growth rates.

Given the promising nature of both the (subsurface-)environment of the icy moons to support life and of the microbial candidates isolated from analogue environments on Earth, we developed several research questions to be tackled. These include: Can Earth-based life survive on the icy moons? Can Earth-borne microorganisms survive a trip to the icy moons by hitch-hiking on a spacecraft? What are the risks to planetary protection? We believe the microbial candidates chosen for this research to be suited for answering these research questions. Furthermore, this research would help in gaining knowledge in order to develop strategies to tackle planetary protection concerns related to icy moon exploration.

Methods

Growth in minimal media in order to determine optimal carbon source

The bacteria and yeast species were ordered from DSMZ (Leibniz-Institute DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), with the exception of *R. frigidicola* which was kindly provided by Prof. Dr. Whyte from McGill University, Montreal, Canada. The organisms were first grown on their respective liquid and solid optimal media to determine viability, Table 2. The organisms were then inoculated in M9-complete minimal salts media containing the following: 47.8 mM Na_2HPO_4 , 22.0 mM KH_2PO_4 , 8.6 mM NaCl, 3.7 mM NH_4Cl , 2 mM MgSO_4 , 0.1 mM CaCl_2 , and 0.02 mM Fe(III)Cl_3 , with 0.2% (w/v) of differing carbon sources. This was performed to evaluate the growth, or lack thereof, in M9 and to determine which carbon source would be chosen for the experiments. The carbon source choice was made based on shortest time to reach late-exponential growth phase with a cell concentration of at least 107 cells/ml. The evaluated carbon sources were d-alanine ($\text{C}_3\text{H}_7\text{NO}_2$) purchased from Sigma-Aldrich, d-gluconic acid sodium salt ($\text{C}_6\text{H}_{11}\text{NaO}_7$) purchased from Fluorochem, d-glucose monohydrate ($\text{C}_6\text{H}_{12}\text{O}_6$) purchased from Merck only used as control, l-glutamic acid ($\text{C}_5\text{H}_9\text{NO}_4$) purchased from Sigma-Aldrich, dl-glyceric acid 20% in water ($\text{C}_3\text{H}_6\text{O}_4$) purchased from Fluorochem, and glycerol ($\text{C}_3\text{H}_8\text{O}_3$) purchased from Sigma-Aldrich. All experiments in this study were performed at least in biological

triplicates. Relative survival (N/N_0) described in each figure was determined for each experiment as the number of CFUs of the microorganisms grown in media (N_0) divided by the CFUs of the microorganisms surviving the exposed conditions (N).

Table 1: Characteristics of the microorganisms investigated in the study. The table includes information of the organism type, growth characteristic, isolation location, aerobic use, organic use of the candidate organisms initially screened, as well as relevant characteristics.

Organism	Domain of life	Growth condition	Isolation Location	Gram	Motility
<i>Planococcus halocryophilus</i> Or1 (DSM 24743)	Bacteria	Psychrophilic	High Arctic permafrost	Positive (28)	Positive (28)
<i>Chromohalobacter sarecensis</i> LV4 (DSM 15547)	Bacteria	Mesophilic/ Moderate Halophile	Saline lake Laguna Verde, Bolivia	Negative (29)	Positive (29)
<i>Paenisporosarcina antarctica</i> N-05 (DSM 21991)	Bacteria	Psychrophilic	Antarctic soil	Positive (38)	Negative (38)
<i>Psychromonas boydii</i> 174 (DSM 17665)	Bacteria	Psychrophilic	Sea-ice core, Point Barrow, Alaska, USA	Negative (31)	Negative (31)
<i>Cryobacterium flavum</i> Hh8 (DSM 26475)	Bacteria	Psychrophilic	Permafrost in Tibet	Positive (32)	Negative (32)
<i>Virgibacillus arcticus</i> Hal1 (DSM 19574)	Bacteria	Psychrophilic	Permafrost Canadian high arctic	Positive (33)	Positive (33)
<i>Halorubrum luteum</i> CGSA15 (DSM 23812)	Archaea	Mesophilic/ Halophilic	water of Lake Chagannor, China	Negative (34)	Positive (34)
<i>Halorhabdus tiamatea</i> SARL4B (DSM 18392)	Archaea	Mesophilic/ Halophilic	Shaban deep, Northern Red Sea	Negative (35)	Negative (35)
<i>Rhodotorula frigidicola</i> JG1b	Eukarya	Eurypsychrophilic	Upper-elevation McMurdo Dry Valleys of Antarctica	Not applicable	Negative (36)
<i>Rhodotorula mucilaginosa</i> Toulouse J8, A2-5 (DSM114276)	Eukarya	Mesophilic/ Psychrotolerant	Isolated from a SAF cleanroom	Not applicable	Negative (40)

Spore formation	Aerobic or anaerobic growth	Carbon source utilization	Pigment	Other Characteristics
Negative (28)	Aerobe (28)	Acetate, alanine, arginine, cellobiose, dextrin, fructose, galactose, gelatin, gluconate, glucosamine (28)	Positive (28)	-
Negative (29)	Aerobe (29)	Asparagine, histidine, L-alaninamide, lactose, ribose, trehalose, xylitol (37)	Positive (29)	Heterotroph (29)
Positive (38)	Facultative anaerobe (30)	Glucose (38), alpha-ketobutyric acid (30)	Positive (38)	-
Unknown	Facultative anaerobe (31)	D-mannose, D-xylose (31), acetate, aspartate, cellobiose, citrate, fructose, glucose, glutamate, lactate (38)	Positive (38)	Gas vacuole (31)
Negative (32)	Aerobe (32)	Fructose, ribose, cellobiose, arabinose, mannose, glucose, galactose, glycerol, starch, sodium gluconate (32)	Positive (32)	-
Elliptical endospores (33)	Facultative anaerobe (33)	Glucose, fructose, mannose, melbiose, trehalose, mannitol, casein, gelatin, lactose, xylitol (33)	Positive (33)	-
Unknown	Aerobe (34)	L-aspartic acid, pyruvate, glycerol, dl-lactate, l-malate, fumarate, citrate, glycine, l-alanine, l-glutamate (34)	Positive (34)	-
Negative (35)	Anerobe (35)	Glucose, xylose, fructose, galactose, proline, esculin (35)	Negative (35)	-
Negative (36)	Aerobe (39)	D-glucose, sucrose, raffinose, galactose, trehalose, maltose, melezitose, L-sorbose, L-rhamnose, D-xylose, Dl-arabinose, D-ribose, glycerol, galactitol, D-mannitol, and xylitol (39)	Positive (36)	-
Negative (40)	Aerobe (40)	Unknown	Positive (40)	Resistance to a number of antifungals (40)

Table 2: Growth characteristics of the initial candidate organisms selected to study. Summary of data related to media type, growth time on solid and liquid media, growth temperature and growth in minimal media with preferred carbon source. *not possible to determine NaCl concentration.

Organism	Growth media	Total NaCl concentration in media	Incubation time on solid media	Incubation time in liquid media
<i>Planococcus halocryophilus</i> Or1 (DSM 24743)	Tryptic Soy Broth	*	48 hours	48 hours
<i>Chromohalobacter sarecensis</i> LV4 (DSM 15547)	Marine Broth (6% w/v NaCl)	6% (4.06% added)	24 hours	3-4 days
<i>Paenisporosarcina antarctica</i> N-05 (DSM 21991)	Marine Broth	1.94%	48 hours	4-5 days
<i>Psychromonas boydii</i> 174 (DSM 17665)	Marine Broth	1.94%	10-15 days	4-5 days
<i>Cryobacterium flavum</i> Hh8 (DSM 26475)	Polypeptone media (DSMZ Media 513)	*	7-8 days	20 days
<i>Virgibacillus arcticus</i> Hal1 (DSM 19574)	Marine Broth	1.94%	48 hours	No growth (60 days)
<i>Halorubrum luteum</i> CGSA15 (DSM 23812)	Natronobacteria media (DSMZ Media 371)	20%	3-4 days	20 days
<i>Halorhabdus tiamatea</i> SARL4B (DSM 18392)	Halobacteria media (DSMZ media 372)	20%	30 days	No growth (60 days)
<i>Rhodotorula frigidicola</i> JG1b	Universal media for yeasts (DSMZ media 186)	*	48 hours	48 hours
<i>Rhodotorula mucilaginosa</i> Toulouse J8, A2-5 (DSM114276)	Universal media for yeasts (DSMZ media 186)	*	48 hours	48 hours

Minimal growth temperature (°C)	Optimal growth temperature (°C)	Maximal growth temperature (°C)	Growth in minimal media (carbon source)
-10 (28)	25 (28)	37 (28)	Yes (L-alanine)
0 (29)	30 (29)	45 (29)	50% MB-50% M9 (D-gluconic)
0 (30)	18 (30)	23 (30)	No
0 (31)	6 (31)	10 (31)	No
0 (32)	10 (32)	19 (32)	No
0 (33)	25 (33)	30 (33)	No
17 (34)	35 (34)	41 (34)	No
15 (35)	45 (35)	55 (35)	No
-10 (36)	25 (36)	30 (36)	Yes (L-glutamic)
5	25 (41)	30	Yes (L-glutamic)

Exposure of the organisms to desiccation

The bacteria and the yeast species were grown aerobically to late exponential phase in M9-complete with their respective carbon source, see Table 2. 200 μ l aliquots of each grown culture were placed on sterile 1 cm glass disks. The disks were left to dry at room temperature for 24 hours with a relative humidity of $40\% \pm 10\%$ under sterile conditions in a clean bench. The disks which were exposed to desiccation under anoxic conditions for the same amount of time in an anoxic chamber (Coy Laboratory Products Inc., MI, USA, with $O_2 < 5$ ppm, relative humidity $13 \pm 0.5\%$) containing Forming gas ($5\% H_2$ in N_2). Following drying, the samples which were meant for cold-exposure were transferred to a sterile plastic box and stored in freezers at $-20^\circ C$ or $-80^\circ C$. The samples exposed to cold temperatures and anoxic conditions following drying, were transferred in a Trex box Transport and Exposure Box as per Beblo-Vranesevic, Bohmeier (42), inside the anoxic chamber, which was then sealed and stored at $-20^\circ C$ or $-80^\circ C$ for the desiccation timepoints. Survival was determined by dilution plating for colony counts where the disks were re-suspended in a 5 ml Eppendorf tube containing 1 ml of sterile PBS (4g NaCl, 3g KH_2PO_4 , 7g Na_2HPO_4 in 1000 ml MQ) which was lightly vortexed for ca. 30 seconds before an aliquot was taken.

Exposure to freezing

The bacterial species and yeast species were grown aerobically to late exponential phase in M9 with the respective carbon source. Aliquots of 1 ml were transferred to sterile 1 ml Eppendorf tubes. The tubes were placed in cryo-boxes and stored at $-80^\circ C$ for the selected timepoints. To determine the survival, one tube per replicate was thawed at $25^\circ C$ for 1 hour prior to dilution plating.

Exposure to freezing and thawing

In order to determine the survival of the bacteria and the yeast species to freezing and thawing, 4 ml of late exponential phase cultures, grown in M9, were transferred to sterile 5 ml Eppendorf tubes. The tubes were placed inside a cryo-box and placed in a $-80^\circ C$ freezer for 1 hour. The samples were then thawed for 1 hour at $25^\circ C$ and an aliquot of 100 μ l was used for dilution plating. Following the thawing time, the 5 ml Eppendorf tubes containing the samples were placed again at $-80^\circ C$ for 1 hour. The cycle was repeated until no survival was detected. No survival was then determined to be the lack of CFU identification for 3 cycles in a row.

Exposure to UV radiation

To determine the limits of survival to UV radiation, the organisms grown in M9 were irradiated with UV-C (254 nm) radiation. An aliquot of 2700 μ l from each organism

grown to late exponential phase was placed in a closed sterile UV transmissible quartz cuvette (0.5 cm path-length; Hellma GmbH & Co. KG, Muelheim, Germany). The cuvettes with the samples were placed 32.5 cm below the UV lamp and contained a magnetic stirrer which allowed the culture to be mixed during irradiation in order for all the cells to receive the same homogenous amount of UV fluence. The *C. sarecensis* cultures which were not grown in 100% M9 were washed twice in PBS in order to remove media traces which could potentially absorb UV and could therefore lead to artefactual protection. The washing was performed by placing the grown cultures in 50 ml Falcon tubes, centrifuging the tubes at 2500 x G for 10 minutes, removing the supernatant and filling the tube with the same volume of PBS. The UV-C lamp was a low-pressure Hg lamp (Model NN 8/15; Heraeus, Berlin, Germany). Prior to each irradiation experiment, the lamp was allowed to warm up for at least 30 minutes and the fluence was measured. The fluence was determined by the use of a UVX radiometer with a 254 nm sensor (UVP Ultra-Violet Products, Cambridge, United Kingdom). The exposure time was calculated by dividing the target dose (in J/m²) by the fluence rates reported on the radiometer (in $\mu\text{W}/\text{cm}^2$).

The exposure to polychromatic (200 - 400 nm) Mars-like UV radiation was performed in the same fashion. Irradiation was performed by using the UV 500S irradiation source (Dr. Hoenle AG; UV-Technologie, Germany). The fluence rate of the lamp was determined prior to each experiment, after 30 minutes to allow the lamp to warm up, by using the Bentham DMc150 transportable spectroradiometer (Bentham Instruments Ltd., Reading, United Kingdom) as per Rabbow, Rettberg (1).

Exposure to X-ray radiation

The different bacteria and yeast species were exposed to ionizing radiation by growing the cultures to late exponential phase and placing the samples in the Gulmay RS 225A (Gulmay Medical Ltd.) X-ray source. The output of the X-ray source was set to 200 kV and 15 mA. The samples were placed at a 10 cm distance below the x-ray source and were irradiated with 30 Gy/min $\pm$ 5 Gy/min until the target dose was reached. The irradiation dose in Gy/min was determined with a UNIDOS dosimeter (PTW Freiburg, Germany).

All exposure experiments, with the exception of specific desiccation storage conditions, were performed at room temperature.

The $D_{10}/F_{10}/E_{10}$ (Dose, Fluence, Exposure) values, representing 10% survival, were calculated by plotting the relative survival in logarithmic scale (log natural) in SigmaPlot 13.0 with the following equation:

$$D_{10} = \frac{\ln(0.1) - \ln(\exp(b[0]))}{b[1]}$$

Where $b[0]$ and $b[1]$ are the coefficients of the linear regression curve plotted by SigmaPlot version 14.5. All figures were created with the use of the data visualization library Seaborn version 0.12.1 (43), an interface of Matplotlib (44). When stated in the figure description, the polynomial regression lines were plotted with the following regression function:

```
def polynomial_regression(x, y, degree):
    coeffs = np.polyfit(x, np.log(y), degree)
    coeffs[-1] = 0
    return np.exp(np.polyval(coeffs, x))
    degree = x
```

Results

Having selected the organisms for the experiments and having confirmed their growth in minimal media supplemented with a single carbon source, the organisms were exposed to the described extreme conditions. Due to the extreme length in desiccation survival of the organisms to conditions such as desiccation, values reported in the results reflect the latest data at the time of publication. Despite reporting a defined value we believe specific species investigated could be able to tolerate a further extended extreme condition exposure. Details on species and experiment termination have been reported in the figure captions when relevant.

The summary of the growth media and conditions tested can be seen in Table 2. Microbial species including *P. boydii*, *C. flavum*, *V. arcticus*, *H. luteum* and *H. tiamatea* were excluded from further investigations as they did not satisfy all growth criteria for this study. The maximum limit for survival of the species, selected for investigation, after exposure to the different conditions is summarized in Table 3 and the maximum survival for the species exposed to desiccation is summarized in Table 4. These values refer to the highest exposure dose or exposure time for which viable colony counts were detected. Higher exposure times and doses were performed to confirm no viable cells.

When the bacteria and yeast species were exposed to desiccating conditions, all differences in limits of survival became apparent. The survival limits changed for

each species depending on the availability of oxygen and on the temperature during the desiccation exposure. For example, *R. mucilaginosa* which has a survival limit of 100 days when desiccated at 20°C, but an increased survival of >246 days at -20°C and >156 days at -80°C, Figure 1. Increasing survival with decreasing temperatures can also be seen with *C. sarecensis* which was able to tolerate 13 days of desiccation at 20°C, 19 days at -20°C and 22 days at -80°C. In contrast, both *P. halocryophilus* and *R. frigidicola* showed the same survival limit across the three temperature ranges. Nonetheless, there seems to be a general trend showing increased desiccation survival at lower temperatures. Despite similar survival limits, when *P. halocryophilus* and *R. frigidicola* were exposed to desiccation under anoxic conditions, survival decreased faster when the samples were exposed to desiccation at temperatures above 0°C. Therefore, also under anoxic conditions, a decrease in exposure temperature increases the survival trends of both prokaryotic and eukaryotic microorganisms.

Growing the microorganisms in minimal media and exposing them to freezing conditions at -80°C was conducted to evaluate the survival limits of the microorganisms when grown under nutrient-limiting conditions and in environments with temperatures closer to the ones on the surfaces of Enceladus and Europa. For this reason, after the bacterial species were grown in M9-complete, they were frozen at -80°C and stored until predefined timepoints to evaluate survival through time. The results summarized in Figure 2A highlight how the microorganisms which were the most tolerant to this condition are the two yeast species, *R. mucilaginosa* surviving up to 230 days and *R. frigidicola* up to 728 days. We speculate that since cell reductions for these time points are low, both yeast species would be capable of surviving more extended periods of time. In contrast, the bacterial species *C. sarecensis* and *P. halocryophilus* were only able to tolerate 6 and 30 days of desiccation (under all tested conditions) respectively.

Understanding the survival of the organisms when exposed to freezing and thawing without additional cryoprotectants might not only give an indication to their survival through seasonal changes, but also to spacecraft rotation and position in the solar system and thereby being exposed to the extreme low temperatures of space and the heat of the sun. Our results show how also for this condition, the most tolerant organisms are the yeast species compared to the two bacterial species, Figure 2B. With *C. sarecensis* surviving only 2 cycles of freeze and thaw and *P. halocryophilus* 25 cycles. In comparison *R. frigidicola* was capable of surviving 38 cycles and *R. mucilaginosa* more than 300. Furthermore, based on the survival curve of *R. mucilaginosa* we expect it to survive many additional cycles.

Table 3: Survival characteristics of the organisms selected for study. Summary of maximal survival fluences, doses and exposure times of the selected bacteria and yeast species. The table highlights the maximum extent to which survival was detected when the organisms were exposed to desiccation, UV-C and polychromatic UV radiation, X-ray radiation and freezing and thawing cycles. *Survival values for *P. antarctica* were determined after growth in Marine Broth. #In brackets E_{10} value, exposure time in days to which there is 90% of sample lethality.

Organism	Extent of desiccation survival (days)	UV-C F_{10} fluence (J/m ²)	Maximum UV-C survival fluence (J/m ²)	Polychromatic UV F_{10} fluence (J/m ²)
<i>Planococcus halocryophilus</i> Or1	60	30	150	297
<i>Chromohalobacter sarecensis</i> LV4	13	487	2000	5189
<i>Paenisporosarcina antarctica</i> N-05	nd	nd	nd	nd
<i>Rhodotorula frigidicola</i> JG1b	123	382	1450	5757
<i>Rhodotorula mucilaginosa</i> ESA	>70	429	1500	10478

Table 4: Survival characteristics of the organisms selected for study to desiccation at different temperatures with oxic and anoxic conditions. Rows show maximum exposure time, in days, to which each organism was capable of tolerating desiccating conditions.

Organism	Extent of desiccation survival at room temperature (days)	Extent of desiccation survival at -20°C (days)	Extent of desiccation survival at -80°C (days)
<i>Planococcus halocryophilus</i> Or1	61	61	49
<i>Chromohalobacter sarecensis</i> LV4	13	19	22
<i>Rhodotorula frigidicola</i> JG1b	123	123	123
<i>Rhodotorula mucilaginosa</i> ESA	101	246	156

	Maximum polychromatic UV fluence (J/m ²)	X-ray D ₁₀ dose (Gy)	Maximum X-ray dose (Gy)	Maximum number of freeze and thaw cycles	Maximum amount of days to freezing
	1250	158	1000	20	70 (18 [#])
	12500	249	1000	2	6 (1.3 [#])
	2000*	nd	nd	2*	6*
	17000	770	3000	38	>728 (6769 [#])
	20000	838	2500	>350	>250 (234 [#])
				ongoing	ongoing

	Extent of anoxic desiccation survival at room temperature (days)	Extent of anoxic desiccation survival at -20°C (days)	Extent of anoxic desiccation survival at -80°C (days)
	61	61	61
	nd	nd	nd
	123	123	196
	nd	nd	nd

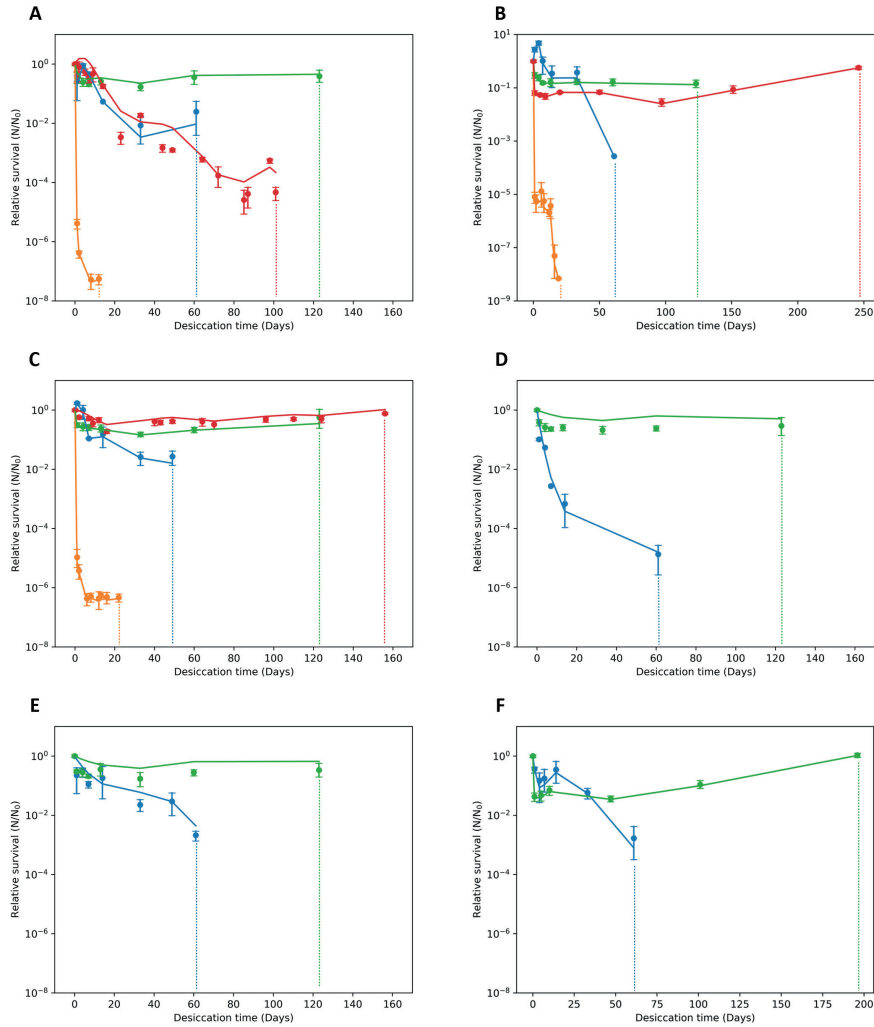


Figure 1: Survival to desiccation increases with lower temperatures. Desiccation survival of *P. halocryophilus* (blue), *C. sarecensis* (orange), *R. frigidicola* (green) and *R. mucilaginosa* (red) under oxic and anoxic conditions and at different temperatures including: oxic at 20°C (A), oxic at -20°C (B), oxic at -80°C (C), anoxic at 20°C (D), anoxic at -20°C (E) and anoxic at -80°C (F). The coloured solid curves represent the polynomial regression for the survival of each plotted organism. Vertical dotted lines represent the end of an experiment due different factors: sample limitations *P. halocryophilus* (A and C) *R. frigidicola* (A), no CFUs *C. sarecensis* (all subfigures), *R. mucilaginosa* (A), *P. halocryophilus* (B, D, E and F), and time limitations *R. mucilaginosa* (B and C), *R. frigidicola* (all subfigures). *C. sarecensis* survival was not detected under any anoxic desiccation condition. *R. mucilaginosa* survival under anoxic desiccation was not performed due to limited time.

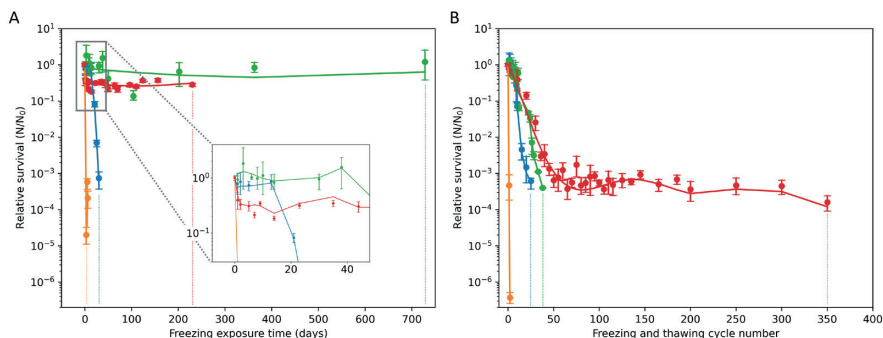


Figure 2: Yeasts are tolerant to freezing and thawing. Survival of *P. halocryophilus* (blue), *C. sarecensis* (orange), *R. frigidicola* (green) and *R. mucilaginosa* (red) to freezing at -80°C (A) and to freezing and thawing (B). The coloured solid curves represent the polynomial regression lines of the survival of each plotted organism. Determining the survival of *R. mucilaginosa* exposed to freezing conditions (A) as well as *R. frigidicola* to freeze and thaw (B) is an ongoing experiment. Vertical dotted lines represent the end of an experiment. In this figure for *C. sarecensis*, *P. halocryophilus* and *R. frigidicola* (B) survival was not detected further. For *R. mucilaginosa* and *R. frigidicola* (A), the dotted lines represent the latest survival data at the time of submission.

An important (and somewhat surprising) observation is that the eukaryotic microorganisms were generally more tolerant to freezing and thawing than the prokaryotes. However, both for UV-C and polychromatic UV radiation, *C. sarecensis* was equally tolerant as both yeast species. When exposed to UV-C radiation, *P. halocryophilus* was able to tolerate up to 150 J/m^2 in media, *C. sarecensis* up to 2000 J/m^2 and *R. frigidicola* up to 1450 J/m^2 and *R. mucilaginosa* up to 1500 J/m^2 . A similar trend can be seen with polychromatic UV light as *P. halocryophilus* was able to tolerate up to 1250 J/m^2 , *C. sarecensis* up to 12500 J/m^2 , *R. frigidicola* up to 17000 J/m^2 and *R. mucilaginosa* up to 20000 J/m^2 . The F_{10}/D_{10} values which is the dose to which there is 90% lethality, shows a similar relationship, see Table 3.

When exposed to ionizing radiation however, a more detrimental effect was seen on the survival of *C. sarecensis*, as this species was possibly not able to tolerate the penetrating effects as the eukaryotic microorganisms. *C. sarecensis* was able to tolerate up to 1250 Gy , *P. halocryophilus* up to 1000 Gy while *R. frigidicola* was able to tolerate up to 3000 Gy and *R. mucilaginosa* up to 2500 Gy .

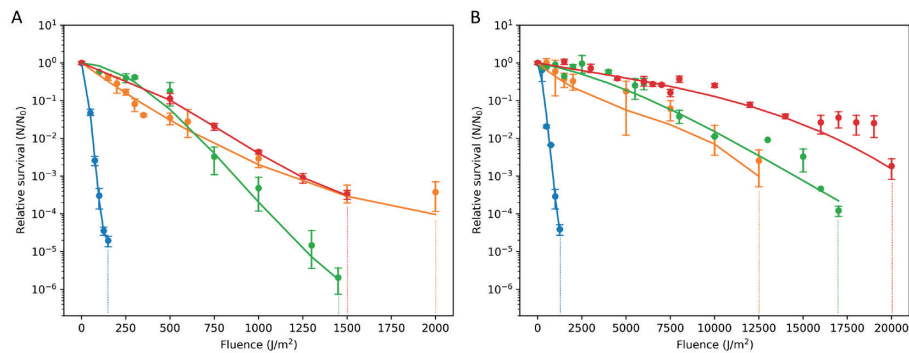


Figure 3: UV wavelength favours different organisms. Survival of *P. halocryophilus* (blue), *C. sarecensis* (orange), *R. frigidalcoholis* (green) and *R. mucilaginous* (red) to UV-C (254 nm) (A) and polychromatic UV (200-400 nm) (B) radiation. All the organisms were exposed to a fluence ranging from 50 to 2000 J/m² for UV-C and from 100 to 20000 J/m² for polychromatic UV. The coloured solid curves represent the polynomial regression for the survival of each plotted organism. Vertical dotted lines represent the end of an experiment where no further survival was detected.

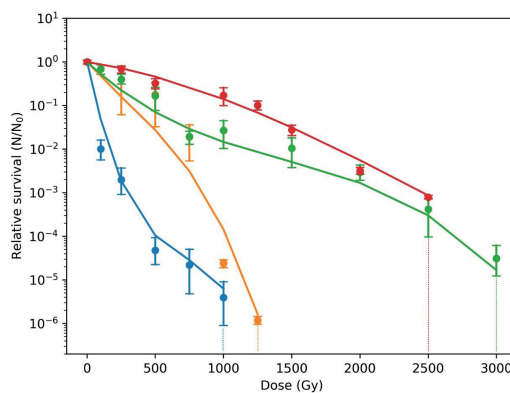


Figure 4: Eukaryotic microorganisms tolerate ionizing radiation more than the prokaryotes. Survival of *P. halocryophilus* (blue), *C. sarecensis* (orange), *R. frigidalcoholis* (green) and *R. mucilaginous* (red) to ionizing radiation from 50 to 3000 Gy. The coloured solid curves represent the polynomial regression for the survival of each plotted organism. Vertical dotted lines represent the end of an experiment where no further survival was detected.

Assumed radiation doses at Europa and Enceladus

Despite the fact that no lander with radiation measurements to date has arrived on the surface of Europa or Enceladus, measurements from orbit and from telescopes can provide useful information concerning the radiation doses. For example the ionizing radiation energy reaching Europa from Jupiter is in between 10 kiloelectron volt (KeV) and 100 megaelectron volt (MeV), instead on Enceladus

galactic cosmic ray energies are >3 gigaelectron volt (GeV) (20). Paulino-Lima, Pilling (45) showed that the Earth solar constant for total UV (200-400 nm) is 116.16 W/m². By using the following equation, we can approximate the solar constant for Europa and Enceladus.

Where *d* is the distance in AU of the target from the Sun. With Jupiter being 5.2 AU and Saturn being 9.5 AU from the Sun. We assume that the two moons are the same distance from the Sun as their respective home planets, we can determine that the UV solar constant for Europa is circa 4.3 W/m² and circa 1.29 W/m² for Enceladus. With these values and our survival data we can estimate how long the organisms would be capable of surviving on the surface of Enceladus or Europa when only exposed to polychromatic UV radiation in the 200-400 nm range, Table 5.

Table 5: Estimated polychromatic UV (200-400 nm) radiation survival timelines for the organisms investigated in this study on the surface of Europa and Enceladus.

Species	Maximum polychromatic UV survival dose (J/m ²)	Survival time on surface of Europa assuming solar constant of 4.3 W/m ²	Survival time on surface of Enceladus assuming solar constant of 1.29 W/m ²
<i>R. mucilaginosa</i>	20000	1.29 hours	4.3 hours
<i>R. frigidicola</i>	17000	1.09 hours	3.66 hours
<i>C. sarecensis</i>	12500	48 minutes	2.69 hours
<i>P. halocryophilus</i>	1250	4.8 minutes	16 minutes

Discussion

Evaluating the survival of the microbial species exposed to simulated space conditions is important in order to evaluate the risk of contamination of sites of interest in the solar system. Our results unexpectedly highlight how the investigated psychrotolerant eukaryotic microorganisms are more tolerant to space-like conditions than psychrotolerant prokaryotes. Nonetheless, yeasts have been already highlighted for their unique tolerances to extreme conditions especially related to the extreme environments in which they have been isolated from (46) and in relation to Mars and icy moons (47). Despite extensive research having focused on yeasts as model organism for investigating the survival to space-like conditions, limited research is linked to yeasts of the *Rhodotorula* species (48-50). Furthermore, we believe our results to be useful in order to identify species of interest when assessing the bioburden of cleanrooms and SAFs and for targeted sterilization techniques. Especially since the strain of *R. mucilaginosa* investigated in this study

was isolated in a SAF (Table 2) and other *Rhodotorula* species have been identified in the ISS which is of concern for planetary protection (51, 52). Studies such as this provide a framework for assessing microbial survival under extreme conditions and informing stakeholders in order to maintain, expand, and update planetary protection measures during missions to sites of astrobiology interest including the surface and subsurface of icy moons.

Growth in minimal media

In order to evaluate the growth on icy moon-like conditions, we used minimal media composition that was previously developed in our group in order to evaluate the growth of the organisms with a single carbon source (53). Despite the exact composition of the icy moon subsurface ocean being unknown, the growth in minimal media would support the results produced by having the microorganisms grown in oligotrophic conditions. Therefore, one of the selection criteria for the organism to be evaluated in this study was the capability to grow in minimal media with a single carbon source. Additionally, we aimed to study microorganisms that can reach the late exponential growth phase within 96 hours to ensure feasibility in the frame of this project. Therefore, from the list highlighted in Table 2 the organisms selected were four including *P. halocryophilus*, *C. sarecensis*, *R. frigidicoholis* and *R. mucilaginosus*, which were capable of growing in our desired conditions. Despite *R. mucilaginosus* not being a psychrophile and not isolated from an analogue site, it was chosen to be investigated due to the fact that it was isolated in the cleanroom of a SAF and it is an organism which can be part of the human microbiome, making it of importance for future long-term crewed missions (40). Earlier research has investigated microbial communities with media simulating the subsurface ocean of Europa and pressure to evaluate growth (54). Importantly, community growth and growth of single microorganisms can be different, whereby communities of different bacterial species can support each other whilst single organism growth do not do this (55).

Desiccation

Evaluating the survival to desiccation in this study was not only important to understand the potential survival of the organisms in SAFs and spacecraft surfaces, but also to environments such as the icy moons. Regular isolation campaigns in cleanrooms and SAFs identify microorganisms on surfaces and in the air (56, 11). Additionally, isolation studies are also performed in spacecraft interiors such as inside the International Space Station (ISS) (57, 58). Therefore, despite strict sterilization techniques, microorganisms make their way to space. Undoubtedly, evaluating the survival of more microbial species to desiccation is needed in order

to guide in updating sterilization techniques, risks for planetary protection and public health (hospitals and public transport). This is especially important when considering that the strain of the yeast *R. mucilaginosa*, used in this study and known to cause disease in immunocompromised patients, was isolated in the cleanroom of a SAF where conditions might be suitable for selection to desiccation (59, 60).

Our results highlighted how yeast species are more tolerant than bacterial species to desiccation, and how the decrease in temperature can aid survival for the organisms tested in this study. Despite challenges in maintaining cell homeostasis at lower temperatures, psychrophilic microorganisms are well adapted to these conditions. Characteristics to which they have adapted is maintaining an adequate membrane fluidity in order to perform the needed metabolic functions at low temperatures (61). It has been suggested that psychrophiles can increase the concentration of unsaturated fatty acids of in order to the phospholipids maintain membrane fluidity at low temperatures (62). Perhaps this suggests the increase in desiccation survival of *R. mucilaginosa* at oxic conditions at -20°C and *R. frigidicola* at anoxic conditions at -80°C. However, research with mesophilic organisms such as *E. coli* have shown that there is an increase in saturated fatty acids during desiccation in order to maintain membrane viscosity at low water activity (63, 64). This suggests that the traits highlighted pertain to mesophiles and psychrophiles only, especially since *C. sarecensis* had poor desiccation tolerance regardless of the temperature. Perhaps traits associated to desiccation survival are linked to environment, and *C. sarecensis* being isolated from the soil where it has not evolutionarily been selected to withstand these conditions (29).

Freezing and thawing

Survival to freezing and thawing conditions is another characteristic important for survival of the organisms on spacecraft surfaces exposed to space conditions. The results we have presented are very meaningful from a planetary protection point of view, as the only organism (*R. mucilaginosa*) which is also associated with the human microbiome is the one capable of surviving the most the freezing and thawing condition. During the journey to the Jupiter system, targeting the icy moons, ESA's JUICE (Jupiter Icy Moons Explorer) spacecraft will be exposed to temperatures ranging from 250°C when performing the Venus flyby, to -230°C when furthest from the Sun (65). Nonetheless, we acknowledge the limitation of this results not having tested the sublimation/re-sublimation process which would take place during freezing and thawing conditions under the vacuum of space. Additionally, the survival of the bacterial species *P. halocryophilus* to freeze and thaw has been evaluated, which also provides interesting results and conclusions in relation to

Mars habitability (66). Despite the study by Heinz et al., 2018 evaluating the survival to freeze and thaw at a comparatively reduced ΔT ($^{\circ}\text{C}$) and with an increased salt concentration, the results provide a valuable comparison of 70 cycles of freeze and thaw as well as can highlight how microorganism survival can differ under different experimental approaches. Understanding if Earth-borne microorganism can tolerate these conditions is critical in evaluating the risk for forward contamination of the icy moons. Our results have shown how all organisms tested in this study are capable of tolerating at least 2 cycles of freezing and thawing.

In standard laboratory practice, storage of microorganisms is performed at very low temperatures such as -80°C with glycerol or other compounds used as cryoprotectants in order to prevent the intracellular ice crystal formation and damage to the cell (67). Cryoprotectants are not available in the natural environment where the investigated organisms were isolated from. Nevertheless, the organisms of this study have the ability to produce cryoprotectants or make use of compatible solutes to avoid damage from freezing (68-70). What must be noted, however, is that despite having these abilities, it is not known whether the microorganisms have the time to produce sufficient cryoprotectants during freezing exposure in our experimental setup, especially since the time taken for the sample tubes to reach 0°C was approximately only 10 minutes per cycle (Figure S1). The high latitude freezing and thawing cycles that take place during seasonal shifts in the natural environment where the organisms were isolated from, differ greatly from our experiment conditions (71). Undoubtedly, freezing the microorganism at -80°C and thawing at 25°C is not what they are exposed to in the natural environment. Nonetheless, given the psychrophilic/psychrotolerant nature of these organisms, it was expected that they would be better adapted compared to other mesophilic microorganisms.

Exposure to freezing conditions at low temperatures without additional cryoprotectants can be a challenge. As shown in our results, we expected *C. sarecensis* and *P. halocryophilus* to tolerate years of storage at -80°C in minimal media such as the yeast species instead of only 6 and 70 days of freezing respectively. Additionally, as research has shown, *E. coli* (a mesophilic organism) was capable of surviving 6 cycles of freezing and thawing, more than the investigated *C. sarecensis* and *P. antarctica* (72). However, in this experiment *E. coli* was exposed to freezing at -18°C and thawing at 4°C , milder conditions compared to our simulation studies. Nonetheless, this provides a comparison of the survival characteristics of psychrophilic/psychrotolerant with mesophilic microorganisms. In addition, our results highlight the extreme conditions to which the selected yeast species are

capable of withstanding compared to the selected bacterial species. At the same time, the results highlight the importance of explorative studies in order to identify such extreme microbial characteristics.

UV radiation and X-ray

Ionizing and non-ionizing radiation are two other extreme aspects of space travel and icy moon environment. Evaluating the tolerances of the microorganisms to these environmental conditions is critical in order to highlight the risks for planetary protection and survival limits. Our results suggest that if the microorganisms evaluated in this study would survive the hitchhike on spacecraft such as JUICE, they would be able to tolerate the polychromatic UV conditions from as little as 5 minutes to 4.3 hours. Despite other extreme environmental conditions coming into play on the surface of the icy moons, the risk of the organism contaminating the surface is not negligible. Furthermore, the presence of fissures or cold-geysers (73, 74) on the surface of the icy moons could increase the risk of subsurface ocean contamination; however, it must be emphasized that the extent and timescale of material exchange between the icy moon's surface and subsurface are unknown and strongly dependent on the specific location on the moon in both (21).

Even though the microorganism were isolated from environments with high UV flux (28, 29, 36), they did not all tolerate the same radiation-exposure conditions. Despite demonstrating sensitivity in the freeze and thaw experiments, *C. sarecensis* was able to tolerate similar UV fluence as the yeast species, when exposed to both UV-C and polychromatic UV radiation. Literature research yielded no information on the presence of pigments in *C. sarecensis*. Instead the genus of *Rhodotorula* was found to be able to produce pigments including torularhodin, torulene, β -carotene and γ -carotene, precursors of carotenoids (75). In particular *R. mucilaginosa* through the use of torularhodin can, to an extent, prevent damage induced by UV radiation by scavenging oxygen and peroxy radicals (76). The importance of carotenoid pigments has been found in other yeast species as well. Previous research has shown that when comparing the survival of albino and wild-type *Sporobolomyces ruberrimus* and *Cystofilobasidium capitatum* yeast against UV-B (280-315 nm) exposure, the albino strains were more sensitive to the radiation (77). Furthermore, research has shown that *R. frigidicola* is capable of overexpressing the genes related to β -carotene precursor when at 0°C. The authors suggest this is the case due to the pigments ability to modulate the cellular membrane fluidity and to protect both from solar irradiation and γ radiation, both sources being present in the isolation environment (39). This ability is also shared by bacterial species such as *P. halocryophilus*, investigated in this study (78).

Conclusions

The microorganisms evaluated in this study have been shown to tolerate a range of extreme environmental conditions, primarily selected to mimic the conditions on the icy moons. However, these extreme conditions can also be found in other locations of the solar system. Understanding the survival limits of such organisms will help to identify species of interest for exploring mechanisms of survival on non-terrestrial bodies, as well as to determine risks for planetary protection. Undoubtedly, experiments such as these require a long time to be conducted: exemplified by the 700-day sampling of *R. frigidicola* to freezing, or the 196-day sampling for exposure to desiccation. Interestingly, the yeast species were found to be much more tolerant to different conditions mimicking icy moons than the bacterial species. These results add valuable information to the planetary protection community as they will help with improving the guidelines for sterilization and identification/characterization of microorganisms. Combining the results of this study with future metagenome data from SAFs could aid in the development of tools allowing the identification of microorganisms relevant for forward planetary protection during spacecraft assembly. Nonetheless, we acknowledge limitations in the experimental approach including: a reduced number of microbial species, exclusion of archaea from further investigations, relatively short growth periods, choice of organisms capable of colony formation, effects of vacuum, lower temperatures during exposure to extreme conditions as well as a combination of these, which despite being desirable were not feasible in the frame of this project. However, we believe these results show the extent Earth-based life can survive in extreme environments such as the ones of the icy moons. Especially in the age of missions such as ESA's JUICE and NASA's Europa Clipper, the maintenance of planetary protection guidelines and approaches as well as identifying risks such as the ones in this study is imperative to avoid the contamination of icy moon environments.

Data accessibility

Raw data used to generate figures are available in open access format on the Dryad database: <https://datadryad.org/dataset/doi:10.5061/dryad.70rxwdc7r> (79). Supplementary material is available online (80).

Declaration of ai use

We have not used AI-assisted technologies in creating this article.

Authors' contributions

T.Z.: formal analysis, investigation, methodology, writing—original draft; K.B.-V.: methodology, resources, supervision, writing—review and editing; M.IdJ.: supervision, writing—review and editing; M.G.N.: supervision, writing—review and editing; P.R.: methodology, supervision, writing—review and editing. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration

We declare we have no competing interests.

Funding

The research of this article was supported by DLR internal funds. M.G.N. was supported by an ERC Advanced Grant (833247) and a Spinoza grant of the Netherlands Organization for Scientific Research.

Acknowledgements

We thank Andre Parpart, technician at DLR, for the set-up of the UV irradiation experiment. Many thanks go to the reviewers, who contributed to a significant improvement of the manuscript with their detailed, highly knowledgeable and competent reviews.

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Elucidating the interplay between intracellular Manganese-Iron-ratio and radiotolerance across all domains of life

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Abstract

Exposure to natural or artificial ionizing radiation induces direct damage to intracellular macromolecules and promotes the formation of reactive oxygen species (ROS), which can further amplify cellular injury. Although many organisms tolerate high radiation doses, the basis of this resilience is not fully understood. A proposed determinant is the intracellular manganese-to-iron ratio (Mn/Fe-ratio), thought to reduce oxidative stress. To evaluate its relevance, Mn and Fe levels were measured in 21 archaeal, bacterial, and eukaryotic species and compared these values with their survivability after ionizing radiation exposure. There was no consistent relationship between Mn/Fe ratios and radiation tolerance across this broad phylogenetic range. Integrating our results with prior studies suggests that while elevated Mn/Fe ratios may contribute to exceptional resistance in certain specialized microorganisms, the ratio is not a reliable, universal predictor of survivability. These findings highlight the multifactorial nature of radiation tolerance and indicate that other processes (e.g. DNA repair mechanisms, antioxidant defenses, and metabolic adaptations) likely play central roles. By clarifying the limitations of Mn/Fe-ratio as a generalizable marker, this study provides a more nuanced understanding of the biochemical determinants of radiation resistance and informs future research into mechanisms of cellular resilience under extreme stress.

Introduction

Astrobiology is the scientific study of life in the universe—its origins, evolution, and potential distribution—and examines how microorganisms use diverse cellular adaptation mechanisms to survive in extreme environments both on Earth and potentially beyond (1). Extremophilic (micro-) organisms have entered an evolutionary path that allows them to thrive under extreme conditions even to the extent that they strictly depend on the extreme parameters in their natural habitat (2), (3). Among these extremophiles, Archaea are thought to be among the earliest cellular life-forms on Earth, representing a very ancient lineage well adapted to primordial extreme environments (4), (5). These cellular adaptation mechanisms, which enable microorganisms to withstand multiple types of environmental stress, are of particular relevance to astrobiology because they help define the limits of life and the conditions under which life might persist beyond Earth. These extreme conditions, both natural and artificial, include a combination of extreme chemical conditions, e.g. low or high pH, high salinity; or physical conditions, such as high pressure, cold or hot temperatures, low water activity, or high radiation levels. Low doses of radiation, here ionizing radiation, can be detected in certain areas with the occurrence of special mineralogical compounds. For example, at beach sands in Southeastern Brazil natural radioactivity has been measured in the range of nano-Gray (45-100 nGy/h; (6)). In contrast, high doses (in the kilo-Gray range) of ionizing radiation occur on Earth only in artificial environments. For example, doses exceeding 20 kGy are used in industrial applications, such as sterilization of food (7), (8), (9).

The key question is, what happens in the microbial cell when it is exposed to lower, non-lethal doses of ionizing radiation with low linear transfer (e.g. X-ray)? Exposure to ionizing radiation causes direct effects on cellular components such as DNA, in addition to indirect effects due to radiolysis of (intracellular) water molecules. Direct effects occur when the energy of ionizing radiation is absorbed by DNA, leading to excitation or direct ionization of the molecule with the possible consequence of DNA single- or double strand breaks, base damage, or base loss, or cross-links within DNA or with neighboring proteins (10). However, damages can also occur if other macromolecules (proteins, lipids) are hit and this leads to the degeneration of both proteins and lipids. The indirect effects arise from the radiation-induced radiolysis of water. Various ions and radicals, such as H_2O^+ , $\text{H}^\cdot$, $\text{OH}^\cdot$, $\text{OH}_2^\cdot$, $\text{O}_2^{\cdot-}$, can be formed through the splitting of H_2O or O_2 molecules. In turn, these ions and radicals, also called reactive oxygen species (ROS), mainly lead to the same damages as the direct effects in macromolecules like DNA, proteins and lipids (11), (12), (13). Since vegetative cells consist of about 80% water and mostly live in an aqueous environment, this plays

a significant role in cell damage processes. Microorganisms, like all other living organisms, have evolved various defense mechanisms to counteract ROS-induced damage and to react on oxidative stress.

The key to surviving exposure to ionizing radiation is to prevent ROS-induced cellular damage. Several strategies are known to prevent or repair ROS-induced damage, e.g. enzymatic antioxidants (14). Bacteria produce antioxidant enzymes such as superoxide dismutase (SOD), catalase, and peroxidases. These enzymes catalyze for example the conversion of ROS to hydrogen-peroxide (H_2O_2) which is then split into less harmful molecules, thus neutralizing the damaging effects of ROS (15). Another strategy is the expression of non-enzymatic antioxidants. Thereby, the cells synthesize or acquire non-enzymatic antioxidants like glutathione, thioredoxin, vitamins (e.g., vitamin C and vitamin E), carotenoids, and flavonoids. These molecules scavenge ROS directly, preventing oxidative damage to cellular components (16). Repair mechanisms/systems are also an option to correct damages caused by ROS. For instance, DNA repair enzymes can correct ROS-induced DNA damage to maintain genomic integrity (17). Alternatively, cells counteract via metal ion homeostasis. Here, the intracellular concentration of free metal ions, (e.g. manganese; Mn and iron; Fe), is upregulated which can counteract oxidative stress reactions (18). This theory is based on the observation that the intracellular Mn/Fe-ratio correlates with viability following irradiation based on some examples of radiation-resistant and radiation-sensitive organisms like e.g. *Deinococcus radiodurans* and *Escherichia coli*, respectively (17), (19). While this theory was based mainly on evidence from experiments with prokaryotic organisms, there have only been limited experiments using Eukarya.

In this study, we investigate the intracellular Mn/Fe-ratio in twenty-one (micro) organisms of all three domains, e.g. Bacteria, Archaea, and Eukaryotes, including eight strict anaerobic and microaerophilic microorganisms never investigated in depth before. The Mn/Fe-ratio was compared to new and already existing data about the tolerance to ionizing radiation of these strains.

Material and Methods

List of investigated microorganisms

The microorganisms used in this study are listed in Table 1. They were either grown in large-scale biofermenters at fermentation facilities (Biotechnikum, Archaea-Center, Regensburg, Germany; indicated as (F) in Table 1) or cultivated in the laboratories of the German Aerospace Center (DLR, Cologne, Germany; indicated

as (L) in Table 1). All strains were grown under their optimal temperature and pH conditions, with supplements added as specified in Table 1.

Cell harvest and sample preparation

Cells were cultivated under the indicated conditions (Table 1). The cells in the large-scale fermentation facilities (up to 300 L) were grown to the stationary growth phase and harvested overnight by centrifugation. The cell pellets were flash-frozen in liquid nitrogen and stored at -150°C . Approximately two grams of frozen samples were delivered to DLR for further investigations and stored at -80°C . To prepare samples from frozen biomass, the samples were thawed at 4°C overnight. Aliquots (300 μL) were transferred into 5 mL of adapted phosphate-buffered saline (PBS; 4.0 g NaCl, 3.0 g KH_2PO_4 , 7.0 g Na_2HPO_4 per liter H_2O , adjusted to pH 7.4), which served as a washing buffer solution. PBS was adapted in terms of salinity (NaCl) and pH to the conditions of the respective strain as indicated in Table 1. To wash the cells, samples were mixed until fully re-suspended, centrifuged, and the supernatant carefully removed.

To prepare samples from fresh cultures, 800 mL of each liquid medium was inoculated and incubated until the stationary growth phase was reached. Cells were harvested by cycles of centrifugation and supernatant removal. The pellets were then resuspended in adapted PBS to wash the biomass. All centrifugation steps were performed at 4500 g for 25 min at 2°C .

From this point onward, all samples collected from fresh and frozen biomass were processed in the same manner. Cell pellets were obtained again by centrifugation and supernatant removal. The wet weight of the biomass samples was recorded, and samples were stored at 4°C . All supernatant fractions from every sample were removed after each centrifugation step and stored for further analysis.

Cell numbers and determination of protein content

To determine reference parameters, cell pellets were resuspended in adapted PBS, and a sub-sample of the cell suspension was taken to determine the total cell number (Thoma cell count chamber; depth: 0.02 mm) and protein content. The protein content was measured after cell lysis using the Bradford assay. For each sample, 200 μL aliquots were taken, and B-PER™ (Bacterial Protein Extraction Reagent; Thermo Fisher Scientific) was added to disrupt the cells according to the manufacturer's protocol, for prokaryotic and archaeal organisms. Eukaryotic cells were lysed by heat shock and bead beating. The protein concentration was then determined using the Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific), following the manufacturer's instructions.

Table 1. Cultivated and investigated strains for this study. T refers to type strain. Strains were cultivated under optimal conditions in terms of temperature, pH, salinity, additives and oxygen availability.

Organism, strain, reference	Culture media / form of cultivation: fermentation (F) or laboratory (L)
Archaea	
<i>Archaeoglobus fulgidus</i> , DSM 4304T, (16)	MGG (17) / (F)
<i>Ignicoccus hospitalis</i> , DSM 18386T, (18)	½ SME (19) / (F)
<i>Metallosphaera sedula</i> , DSM 5348T, (20)	Allen-Media (21, mod. 22) / (F)
<i>Methanocaldococcus jannaschii</i> , DSM 2661T, (23)	MAI (24) / (F)
<i>Methanothermobacter thermoautotrophicus</i> , DSM 1053T, (25)	M (26) / (F)
<i>Pyrococcus furiosus</i> , DSM 3638T, (27)	½ SME (19) / (F)
<i>Sulfuracidifex metallicus</i> , DSM 6482T, (28)	Allen-Media (21, mod. 22) / (F)
<i>Saccharolobus solfataricus</i> P8, (29)	Allen-Media (21, mod. 22) / (F)
<i>Thermoproteus tenax</i> S2, (30)	Allen-Media (21, mod. 22) / (F)
Bacteria	
<i>Aquifex pyrophilus</i> , DSM 6848T, (31)	Modified SME (31) / (F)
<i>Buttiauxella</i> sp. MASE-IM-9, DSM 105071, (32)	Tryptic soy broth No. 2 / (L)
<i>Chromohalobacter sarecensis</i> , DSM 15547, (33)	Marine broth 2216 / (L)
<i>Deinococcus radiodurans</i> , DSM 20539T, (34)	TGY / (L)
<i>Escherichia coli</i> , DSM 18039, (35)	LB / (L)
<i>Hydrogenothermus marinus</i> , DSM 12046T, (36)	VM1 (mod. 37) / (F)
<i>Paenisporosarcina antarctica</i> , DSM 21991, (38, 39)	Marine broth 2216 / (L)
<i>Planococcus halocryophilus</i> , DSM 24743, (40)	Tryptic soy broth No. 2 / (L)
<i>Salinisphaera shabanensis</i> , DSM 14853T, (41)	Marine broth 2216 / (L)
<i>Staphylococcus capitis</i> , DSM 111179, (42)	Tryptic soy broth No. 2 / (L)
Eukarya	
<i>Rhodotorula frigidicola</i> EXF- 10854, formerly JG1b, (43)	DSM medium 186 / (L)
<i>Rhodotorula mucilaginosa</i> , DSM 114276, (44)	DSM medium 186 / (L)

DSM: number of the strain in the catalogue of German Collection of Microorganisms and Cell Cultures GmbH (DSMZ), YE: yeast extract, S°: elemental Sulphur.

Optimal growth temperature (°C)	Additives (% w/v)	Gas phase (pressure; % v/v); O ₂ -metabolism
80	0.1 % lactate 0.05 % YE	H ₂ /CO ₂ (300 kPa; 80:20); anaerobic
90	0.5 % S° 0.1 % YE	H ₂ /CO ₂ (300 kPa; 80:20); anaerobic
70	0.02 % YE	O ₂ /CO ₂ (140 kPa; 95:5); aerobic
85	-	H ₂ /CO ₂ (300 kPa; 80:20); anaerobic
65	-	H ₂ /CO ₂ (300 kPa; 80:20); anaerobic
95	0.1 % starch 0.1 % YE 0.1 % peptone	N ₂ /CO ₂ (300 kPa; 80:20, v/v); anaerobic
65	0.1 % S° 0.02 % YE	O ₂ /CO ₂ (200 kPa; 90:10); aerobic
80	0.1 % S° 0.02 % YE	O ₂ /CO ₂ (200 kPa; 90:10); aerobic
85	-	N ₂ /CO ₂ (300 kPa; 80:20); anaerobic
85	0.5 % S°	N ₂ /CO ₂ /O ₂ (300 kPa; 79.75:19.75:0.5); microaerophilic
30	-	aerobic
30	6 % NaCl	aerobic
30	-	aerobic
37	-	aerobic
65	4 % NaCl	H ₂ /CO ₂ /O ₂ (200 kPa; 78:20:2); microaerophilic
18	-	aerobic
25	-	aerobic
30	10 % NaCl	aerobic
37	-	aerobic
25	-	aerobic
25	-	aerobic

Exposure to ionizing radiation and determination of the survival fraction

To determine D_{10} -values of the organisms, cells were treated with X-rays in-house as described earlier (49). Briefly, liquid cell cultures were irradiated in 2 ml plastic tubes (*Buttiauxella* sp. MASE.IM-9, *C. sarecensis*, *D. radiodurans*, *E. coli*, *P. antarctica*, *P. halocryophilus*, *S. shabanensis*, *S. capitis*, *R. frigidicola*, *R. mucilaginosa*) or in 4 ml glass vessels (all other organisms). Irradiation was carried out with the X-ray source Gulmay RS 225A (Gulmay Medical Ltd.) at 200 kV and 15 mA without a filter. The cells were irradiated at a distance of 19.5 cm below the X-ray source with 20 Gy/min $\pm$ 5 Gy/min. The dose rate was measured with a UNIDOS dosimeter (PTW Freiburg GmbH). All irradiation experiments were performed at room temperature. All anaerobic and microaerophilic organisms were kept always under anoxic conditions, including the ionizing radiation treatment (see O_2 -metabolism in Table 1).

For the determination of the survival rate, aliquots were taken under anoxic or oxic conditions and the survival was determined via the most probable number technique in dilutions series (for organisms not able to grow on solid media: *A. fulgidus*, *I. hospitalis*, *M. sedula*, *M. jannaschii*, *M. thermoautotrophicus*, *P. furiosus*, *S. metallicus*, *S. solfataricus*, *T. tenax*, *A. pyrophilus*, *H. marinus*) or via plating assay on agar plates (all other organisms). The irradiation experiments were conducted at least in triplicates through three independent experiments.

The survival rate (S) was calculated as relative survival after cell damaging treatment (N) compared to the non-treated control (N_0) ($S = N/N_0$). D_{10} -values were determined after irradiation. The D_{10} -values indicate the dose in Gy which reduces the survival rate by one order of magnitude. The values were calculated from the regression lines of the exponential slopes of the survival curves (50), (51).

Analysis of Fe and Mn concentration

Metal contents of the samples, supernatant, adapted PBS buffer, and media were determined at the Elements and Minerals of the Earth Laboratory (ElMiE-Lab) at GFZ Potsdam. Samples were treated with 100 μ L concentrated (69–70 %) nitric acid at 95 °C for 2 h. Hydrogen peroxide was then added to a final concentration of 5 mM, and samples were further incubated for 90 min at 75 °C. The digested samples were diluted using ultra-pure reagents and deionized water prior to analysis. The metal content of each digested sample was determined by High-Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS; Element XR series 2, Thermo Fisher Scientific) or by ICP-OES (5110, Agilent). Concentrations were determined by external calibration for both instruments. For ICP-MS, an internal standard was used to correct for instrumental mass bias.

The measurement of intracellular Mn and Fe concentrations was performed in at least triplicate.

Data treatment and calculations of intracellular Mn/Fe-ratio

The concentrations of Mn and Fe were measured in the pellets, as well as in the supernatant after washing and in the washing buffers as controls. The final amount of Mn and Fe present in the sample, corresponding to the intracellular amount of Mn and Fe, was determined by subtracting the amount found in the supernatant from that in the biomass.

The absolute measured quantities of Mn and Fe were normalized to cell numbers and protein content, respectively and their ratio was determined. This intracellular Mn/Fe-ratio was then correlated with the D_{10} -value. Both the Mn/Fe-ratio and the D_{10} -value were plotted in a double-logarithmic scale to assess potential dependency and a regression curve was plotted. The coefficient of determination (r^2) and regression coefficient ($b[1]$) were determined to describe a possible correlation. Thereby, r^2 gives the proportion of the variance of the variables and the regression coefficient indicates the slope of the regression line. In the case of a maximum dependency, the $b[1]$ would be equal 1.

Results

Mn/Fe-ratio and D_{10} -value of investigated organisms

This study involved quantifying intracellular Mn and Fe levels relative to biomass, total intracellular protein content, and cell number, as presented in Supplementary Tables 1, 2, and 3. Based on these measurements, Mn/Fe-ratios were calculated, and their potential impact on cell survival following exposure to ionizing radiation was evaluated. Irradiation experiments were conducted using plastic tubes or glass vessels. Previous experiments with *Buttiauxella* sp. MASE-IM-9, *D. radiodurans*, *E. coli*, and *S. shabanensis* demonstrated no significant difference in survival outcomes between cells irradiated in plastic versus glass containers (data not shown).

Table 2. Mn/Fe-ratio calculated from the quantitative determination of metals by ICP-MS and D_{10} - values collected from literature and measured in this study (indicated with an asterisk *).

Archaea		
Organism	Mn/Fe-ratio*	D_{10}-value (Gy)
<i>A. fulgidus</i>	0.03296 ± 0.003165	1090 (45)
<i>I. hospitalis</i>	0.00059 ± 0.000505	4700 (48)
<i>M. sedula</i>	0.00007 #	490 (45)
<i>M. jannaschii</i>	0.00275 ± 0.000222	1040 (45)
<i>M. thermoautotrophicus</i>	0.73377 ± 0.040578	2750*
<i>P. furiosus</i>	0.03397 ± 0.013189	1020 (45)
<i>S. solfataricus</i>	0.00001 ± 0.000004	1020 (45)
<i>S. metallicus</i>	0.04686 ± 0.004601	760 (45)
<i>T. tenax</i>	0.00518 ± 0.000249	870 (45)
Bacteria		
<i>A. pyrophilus</i>	0.01129 ± 0.001363	2840 (45)
<i>Buttiauxella</i> sp. MASE-IM-9	0.04902 ± 0.001053	500 (49)
<i>C. sarencensis</i>	0.00048 ± 0.000004	258*
<i>D. radiodurans</i>	0.31408 ± 0.135525	10000 (50)
<i>E. coli</i>	0.05604 ± 0.036069	250 (51)
<i>H. marinus</i>	0.03084 ± 0.001271	750 (45)
<i>P. antarctica</i>	0.00075 ± 0.000007	520*
<i>P. halocryophilus</i>	0.17458 ± 0.026111	510*
<i>S. shabanensis</i>	0.00248 ± 0.000028	1700*
<i>S. capitis</i>	0.31472 ± 0.008334	98 (52)
Eukarya		
<i>R. frigidicola</i>	0.04826 ± 0.003919	640*
<i>R. mucilaginoso</i>	0.03667 ± 0.000667	680*

#No standard deviation is shown because the Mn concentration was below detection limit in two replicates. Detailed and absolute numbers of Mn and Fe related to cell mass, protein content, and cell numbers can be found in Supplementary Tables 1, 2, 3.

Comparison of Mn/Fe-ratio of same organism between measured values and literature data

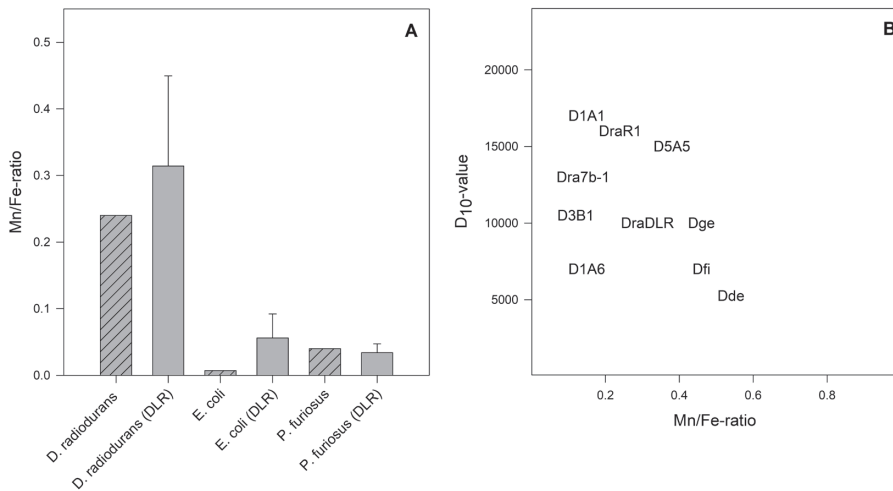


Figure 1. Comparison of measured intracellular Mn/Fe-ratio with literature data. A: Shaded columns correspond to literature data (Table 3). Unshaded columns are values measured in this study in triplicates with standard deviation. B: Comparison of Mn/Fe-ratio and D_{10} -value of different *Deinococcus* species. Dde: *Deinococcus deserti*, Dfi: *Deinococcus ficus*, Dge: *Deinococcus geothermalis*, DraR1: *Deinococcus radiodurans* R1, Dra 7b-1: *Deinococcus radiodurans* 7b-1, D1A1: *Deinococcus* sp. 1A1, D5A5: *Deinococcus* sp. 5A5, D1A6: *Deinococcus* sp. 1A6, D3B1: *Deinococcus* sp. 3B1, DraDLR: *D. radiodurans* R1 DLR (Table 3).

There is a general offset in the Mn/Fe-ratios between the results from this study and those reported in the literature. The data obtained in this study were approximately twofold higher for *D. radiodurans* and *E. coli*, while they were within the same range for *P. furiosus* (Fig. 1A). However, as the differences are not systematic, they are considered not significant. All *Deinococcus* species cluster together, with the measured data of *D. radiodurans* R1 DLR (DraDLR) in the middle, indicating their similarity in measured values (Fig. 1B).

Dependence between Mn/Fe-ratio, D_{10} -value, and domain in measured data and comparison with literature data

The calculated Mn/Fe-ratios with respect to the D_{10} -values are distributed throughout the entire graph in a double logarithmic fashion (Fig. 2A). No distinct dependence on the domain of life the organism under study belongs to can be observed. The two eukaryotic representatives are closely grouped, indicating their close evolutionary relationship, both belonging to *Rhodotorula*. There is no specific range that is solely dominated by Archaea or Bacteria. A closer look at the data

and considering the optimum growth conditions (especially temperature) does not reveal any dependence on these parameters either (Fig. 2A). When examining the literature data (Mn/Fe-ratio and D_{10} -value) using the same representation as in the measured data, a dependence between the Mn/Fe-ratio and the D_{10} -value can be discerned (Fig. 2B). This general difference between measured data and data from literature is supported by the plot regression values (measured: r^2 0.00065, $b[1]$ 0.0091 vs. literature data: r^2 0.4705, $b[1]$ 0.5779).

To better compare the measured data with the literature, the Mn/Fe-ratio and the D_{10} of various literature data are summarized in Table 3.

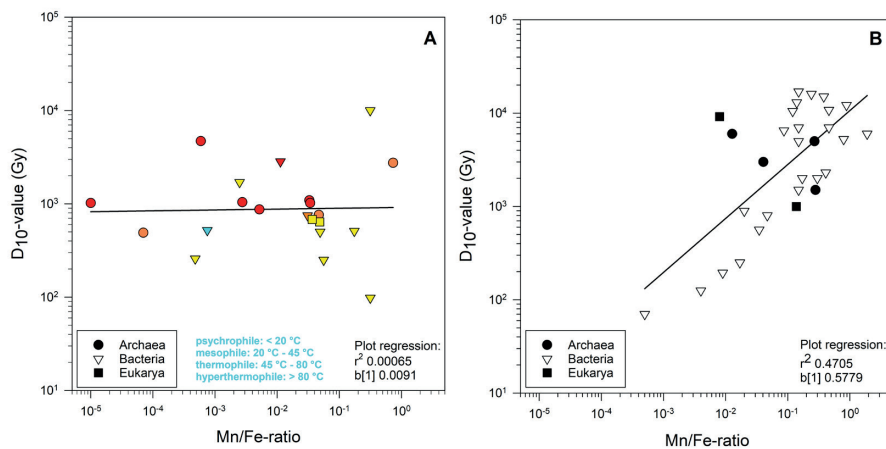


Figure 2. Double logarithmic representation of D_{10} -value and Mn/Fe-ratio. A: Measured Mn/Fe-ratio, D_{10} -value and domain of investigated microorganisms in this study (Table 2). B: Visualization of Mn/Fe ratio and D_{10} -value from literature data (Table 3). Archaea are symbolized as circles, Bacteria as triangles. Eukarya are represented as squares. The color code is giving the organisms' optimal growth temperature (psychrophile blue, mesophile yellow, thermophile orange and hyperthermophile red). Regression lines and regressions analyses are based on all data in plot.

Table 3. Mn/Fe-ratio and D₁₀-value of Archaea, Bacteria and Eukarya retrieved by literature search.

Archaea		
Organism (references)	Mn/Fe-ratio	D₁₀-value (Gy)
<i>Halobacterium salinarum</i> (53)	0.27	5000
<i>Haloferax volcanii</i> (8, 53, 54)	0.28	1500
<i>Pyrococcus furiosus</i> (9)	0.04	3000
<i>Thermococcus gammatolerans</i> (9)	0.01	6000
Bacteria		
<i>Acinetobacter radioresistens</i> (8)	0.15	5000
Alphabroteobacterium 4A6 (50)	0.15	1500
<i>Bacillus subtilis</i> spores (55)	0.3	2000
<i>Bacillus anthracis</i> spores (56, 57)	0.41	2300
<i>Deinococcus deserti</i> (58, 59)	0.54	5250
<i>Deinococcus ficus</i> (8, 60)	0.46	7000
<i>Deinococcus geothermalis</i> (14)	0.46	10000
<i>Deinococcus radiodurans</i> R1 (14)	0.24	16000
<i>Deinococcus radiodurans</i> 7b-1 (50, 61)	0.14	13000
<i>Deinococcus</i> sp. 1A1 (50)	0.15	17000
<i>Deinococcus</i> sp. 5A5 (50)	0.38	15000
<i>Deinococcus</i> sp. 1A6 (50)	0.15	7000
<i>Deinococcus</i> sp. 3B1 (50)	0.12	10500
<i>Enterococcus faecium</i> (14)	0.17	2000
<i>Escherichia coli</i> (14)	0.0072	700
<i>Kineococcus radiotolerans</i> (62, 63)	0.087	6000
<i>Macrococcus caseolyticus</i> (64)	0.027	607
<i>Neisseria gonorrhoeae</i> (50)	0.004	125
<i>Pseudomonas putida</i> (14, 65)	0.017	250
<i>Rubrobacter radiotolerans</i> (9, 66)	0.88	12000
<i>Rubrobacter xylanophilus</i> (9, 66)	1.9	6000
<i>Shewanella oneidensis</i> (14)	0.0005	70
<i>Staphylococcus aureus</i> (64)	0.009	194
<i>Thermus thermophilus</i> (67)	0.047	800
Eukarya		
<i>Chlorella</i> sp. (68, 69)	0.008	9170
<i>Saccharomyces cerevisiae</i> (70, 71)	0.138	1000

Discussion

In addition to the direct radiation effect where radiation has directly impacts on DNA integrity (single and double strand breaks), indirect radiation effect affects interaction with other molecules (e.g. water molecules) in the cell to produce free radicals, among them ROS, which in turn induce cellular damage. Tolerance to ionizing radiation, and consequently the tolerance to ROS, is a common phenomenon across all domains of life (49), (53), (77). However, the reason for these tolerances has not yet been completely understood. It is suggested that these tolerances are a multifactorial trait involving small RNAs, the Mn/Fe-ratio, enzyme-based protection mechanisms (e.g. SODs), the metabolism (O_2), the cultivation temperature, and specialized DNA repair systems (19). These observations align with the emerging consensus that Mn/Fe-ratios are important but not solely determinative, and other molecular and environmental factors substantially influence radiation tolerance (78).

Recent studies indicate that small regulatory RNAs, such as DrsS in *Deinococcus radiodurans*, are induced by ionizing radiation and actively contribute to the detoxification of ROS, complementing classical antioxidant systems (79). Although small regulatory RNAs have been identified in some organisms tested here, it remains unclear whether they perform the same ionizing-radiation-responsive or ROS-detoxifying functions as those characterized in *D. radiodurans* (80).

Mn and Fe ions plays a crucial role in the tolerance to ionizing radiation, as has been demonstrated in some microorganisms, among them the well-known model organism *D. radiodurans*. However, our results do not support a simple or consistent correlation between the tolerance to ionizing radiation and the intracellular Mn/Fe-ratio. ROS are not only produced during exposure to ionizing radiation, but also occur naturally in lower amounts during aerobic and anaerobic metabolism or can even be available in the environmental habitat of the organisms (81), (82). Therefore, all aerobic and anaerobic organisms need to have defense mechanisms against ROS and oxidative stress. One key defense mechanism is the presence of proteins containing a metallic ion. These metals serve as co-factors to the enzymes they are bound to, playing crucial roles in catalyzing biochemical reactions (83). Detoxification examples include SOD alone or in combination with a catalase system. SOD or superoxide reductases recognize ROS such as superoxides (O_2^-) and hydroxyl radicals ($\cdot OH$) and convert them to H_2O_2 . SODs utilize metal cofactors such as manganese, iron, nickel, or copper/zinc combinations, while catalases and hydroperoxide reductases subsequently convert H_2O_2 into water and oxygen (15), (84). Catalase enzymes

often contain iron or manganese at their active sites (85). Other examples include manganese-dependent peroxidases, which require Mn ions for catalytic activity (86). Recent research highlights that the SOD/catalase system remains central for ROS detoxification, but additional protective mechanisms, such as Mn-antioxidant complexes and radiation-induced peptides, contribute significantly to resistance (87), (79), (88). In *Aquifex aeolicus*, and probably also in the investigated close relative *A. pyrophilus*, three genes for SOD are present in the genome, but catalases are absent (89). Nevertheless, several peroxidases, including a cytochrome c peroxidase and a hydroperoxide reductase, have been identified at the genome level, which could take over the removal of H_2O_2 (90). In *A. pyrophilus*, a thermostable Fe-SOD has been described and characterized (91). *A. fulgidus* possesses a catalase/oxidase system (92) and *P. furiosus* a superoxide reductase/oxidase system (93), which was shown to be induced by treatment with ionizing radiation (94) and both organisms react in the same way to ionizing radiation documented by a nearly same D_{10} -value around 1000 Gy (49). Also, *M. thermoautotrophicus* possesses a Fe-SOD (95), and *I. hospitalis* has a superoxide reductase and cytoplasmic peroxiredoxin system (96), (97). Nevertheless, both Archaea have significantly different D_{10} -values (*M. thermoautotrophicus*: 2750 Gy vs. *I. hospitalis*: 4700 Gy (49). In *E. coli*, both Mn- and Fe-SOD have been identified (98). *Rhodotorula* species also possess Fe- and Mn-SODs, providing protection against oxidative stress (99). Cu/Zn-SOD genes are present but often not expressed under normal conditions (100). Independent of SOD systems, Mn ions alone have ROS-scavenging properties, contributing to radiation tolerance (101). However, this mechanism requires higher intracellular Mn levels than those needed for SOD-mediated protection (8); (12). For instance, *D. radiodurans* contains high concentrations of free Mn ions and Mn-antioxidant complexes (Sharma et al. 2017). Interestingly, some organisms with low Mn levels still survive irradiation well (e.g., Sulfolobales strains: *M. sedula*, *S. solfataricus*), showing that Mn/Fe-ratios alone cannot fully explain radiation tolerance.

Under oxic conditions, organisms tend to favor iron in contrast to manganese as a cofactor for aerobic metabolism (101). Free Fe can participate in redox reactions but also catalyze ROS formation via the Fenton reaction (102). In anoxic environments, manganese-dependent enzymes, such as certain SODs, may become more prevalent, potentially increasing the intracellular Mn/Fe-ratio (103). However, our anaerobic organisms do not show a strict correlation between Mn/Fe-ratios and radiation tolerance, suggesting that additional protective factors, such as antioxidants and DNA repair systems, are involved. During irradiation, extracellular and intracellular oxygen can be radiolyzed into ROS, making oxygen

a radiation sensitizer (104). Reduced oxygen in the irradiation medium decreases radiation damage (105). Some of the microorganisms with the highest survival rates in our study (microaerophilic *A. pyrophilus* or strictly anaerobic *I. hospitalis*, *M. thermoautotrophicus*) were irradiated in reducing media, limiting oxygen availability. Reducing agents in the medium (Na_2S , L-cysteine) may further scavenge ROS, enhancing survival.

A correlation between growth temperature and radiation tolerance is possible. High temperatures can increase metabolic ROS production and damage cellular components. Membrane rigidity in hyperthermophiles helps prevent penetration of ROS (106), (107). Indeed, hyperthermophiles exhibit high D_{10} -values (*I. hospitalis*: 4700 Gy, (52); *M. thermoautotrophicus*: 2750 Gy, (49)), although not all thermophiles display uniform responses.

DNA repair efficiency is influenced by growth temperature. In *I. hospitalis*, few known repair gene homologs exist, yet the organism is highly radioresistant. After irradiation with 5 kGy, ~200 DSBs occur in *D. radiodurans* (108). Genomes are expected to be highly fragmented after 20 kGy (52). With a genome size of 1.3 Mbp (97), only about 1600 bp long fragments are present after this irradiation. If it were not for an extremely efficient, yet unidentified repair or protection system, propagation after irradiation would not be possible. This underscores the importance of highly efficient, and possibly previously unknown, repair or protection systems for survival.

Repair systems are critical for tolerance to heat and radiation. Many of the microorganisms investigated in this study encode either RecA or its archaeal homolog RadA, indicating that homologous recombination systems are broadly conserved across diverse lineages (109), (110). RadA/RecA is vital for repairing radiation- and radical-induced damage (111), (112). However, the regulatory dynamics and functional efficiency of these systems vary substantially between species, contributing to the wide range of observed radiation tolerances (113). The presence of RecA/RadA system alone does not predict tolerance, highlighting the complex and multifactorial nature of radioresistance involving metal ions, antioxidant systems, and repair networks (79), (78).

In conclusion, tolerance to ionizing radiation is widely distributed across the tree of life, yet its evolutionary origins remain incompletely understood in many organisms, including model species (114), (115). In this study, we analyzed both prokaryotic organisms and two eukaryotic yeast strains, integrating our own data with recent

advances in the literature to underscore the diversity and specificity of radiation resistance. The Mn/FeSOD family, which is conserved across Bacteria, Archaea, and Eukarya and likely derived from a common ancestor over three billion years ago (116), highlights the fundamental importance of ROS defense systems in evolution. Recent work further emphasizes how radiation tolerance is strainspecific and dynamically regulated: novel non-sporulating bacterial isolates with unexpectedly high radiation tolerance have been identified, with whole-genome sequencing revealing conserved DNA repair systems such as RecA and UvrABC (117), and halophilic Archaea have been shown to exhibit dramatically different Xray sensitivities even within the same lineage (118). Moreover, extracellular vesicles derived from *Deinococcus radiodurans* have been demonstrated to provide radioprotective effects in mammalian tissues (119). Taken together, these findings strongly support the notion that tolerance to ROS — including that elicited by ionizing radiation — is not a monolithic trait but a highly strainspecific, evolutionarily tuned adaptation. A clearer understanding of how these defense mechanisms diversify and function will be valuable for biotechnology, medicine, and astrobiology.

Conflict of interest statement

The authors have no conflicts of interest to declare. DG is co-founder of Microbify GmbH. However, there are no commercial interests by the company, or any financial support granted by Microbify GmbH. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

Acknowledgements

We thank Dipl. Ing. (FH) Thomas Hader, Simon Dechant and the whole team (Konrad Eichinger, Gabi Gmeinwieser, Harald Huber) at the University of Regensburg for technical support in cultivating the organisms in large-scale biofermenters. Additionally, we thank Heike Rothe and Sabine Tonn, both from the GFZ (Potsdam), for their laboratory work with ICP-MS measurements.

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Exploring the habitability of the outer solar system icy moons for the extremotolerant yeast *Rhodotorula frigidicola*

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Abstract

Psychrophilic and psychrotolerant microorganisms have the unique ability to grow below 0°C, which makes them ideal candidates for studying how life could survive on the icy moons of the solar system. The renewed interest, in view of the JUICE and Europe Clipper missions, to explore these locations has pushed for the identification of organisms which could survive on the icy moons. In this study we selected the extremophilic yeast *Rhodotorula frigidicola* given its innate ability to grow between 30°C and -10°C and to survive a range of extreme conditions including X-ray, UV-C and polychromatic UV radiation, desiccation at different temperatures, and freeze - thaw cycles. We report the survival of *R. frigidicola* under conditions analogous to those found on icy moons. Using transcriptomic approaches, we present novel insights into differential gene expression before, during, and after exposure to combined icy moon conditions. We also identified the rapid activation of genes involved in catalytic activity and DNA repair during exposure. Our results contribute to a better understanding of the survival mechanisms of psychrotolerant microorganisms in extreme environments and can inform future life-detection missions on moons such as Enceladus and Europa, also highlighting the need to consider yeasts in planetary protection efforts.

Introduction

Exploring survival mechanisms for microbial life on the icy moons would improve our understanding of how these locations could support life. The renewed interest in solar system locations that may harbor life has been supported by ESA's JUICE and NASA's Europa Clipper missions currently on their way to the Jupiter system (1). Investigations in the potential survival of microorganisms to the conditions of the icy moons can help elucidate risks associated with the unwanted contamination with biological material from Earth. Despite stringent decontamination strategies, forward contaminating risks remain, as elucidated by the COSPAR planetary protection community (2). Extensive astrobiology research has investigated the survival and adaptation of extremophilic microorganisms exposed to simulated planetary conditions. However, the number of studies focusing on yeasts are limited as compared to studies evaluating prokaryotic microorganisms (3). While yeasts have been included in a few spaceflight experiments, most of these have concentrated on *Saccharomyces cerevisiae* (4). Nonetheless, important fungal studies in astrobiology have demonstrated that interest in other yeast and filamentous fungal species is increasing. Examples include the cultivation of *Cladosporium sphaerospermum* aboard the international space station (ISS), the survival of *Rhinochlamydia similes* in Mars-like perchlorate environments, or the exposure of the Antarctic *Cryomyces antarcticus* exposed to different ISS experiments (5-7). The remarkable resilience of fungal microorganisms, compared to other eukaryotic organisms, is well documented in terrestrial extreme environments and under space conditions (8-10). Furthermore, the growing interest in using fungi for in situ resource utilization (ISRU) on the Moon and Mars, further supports their relevance as promising model systems for astrobiology research (11-13). Therefore, investigating other yeast species, particularly psychrotolerant species, which might be better adapted to surviving in extreme environments, is both timely and scientifically warranted.

Icy moons such as Saturn's Enceladus and Jupiter's Europa, are celestial bodies with a frozen surface that overlay subsurface oceans, which may resemble those found on Earth (14). The Cassini-Huygens mission played a crucial role in characterizing these environments by analyzing material ejected from the moon's surface by cold plumes (15). Additional factors contributing to the potential habitability of these moons include a kilometer thick ice shell that shields the subsurface ocean from the radiation-rich external environment as well as internal heating of the ocean (16). The mechanism underlying this heating remains under debate, with two leading hypotheses: tidal heating generated by the moons' orbital interactions with their host planets, and the presence of hydrothermal vents (16, 17), or possibly both.

Given the environmental conditions of icy moons, psychrophilic and psychrotolerant microorganisms are ideal candidates to investigate the potential for life and proliferate in such environments, due to their ability to grow at temperatures below the freezing point of water. Arctic and Antarctic locations, known to harboring diverse microbial communities, have recently been shown to contain high numbers of fungal species, highlighting the adaptability of such microorganisms to extreme conditions (18). Investigating the metabolism of these organisms under simulated icy moon conditions can provide critical insights into their survival strategies and inform organism-specific handling protocols for space missions.

The yeast *Rhodotorula frigidicola* (formerly JG-1b) is a psychrotolerant organism isolated from Antarctic environments, capable of growing at temperatures below 0°C (19, 20). This yeast has demonstrated tolerance to a range of extreme conditions following cultivation in minimal media, including X-ray radiation, UV-C and polychromatic UV radiation, desiccation, and freeze-thaw cycles (21). Our research indicates that *R. frigidicola* exhibits more tolerance to extreme conditions compared to several bacterial species. It has also been hypothesized that carotenoid pigment production, common among *Rhodotorula* species, may contribute to radiation resistance, particularly given the high UV flux in its native Antarctic habitat (22). Furthermore, transcriptomic analysis by Touchette, Altshuler (23) revealed metabolic changes in *R. frigidicola* when grown at 0 versus 23°C. These findings position *R. frigidicola* as a strong model organism for investigating metabolic responses under simulated icy moon conditions. Therefore, the aim of our study is to determine the survival limits of *R. frigidicola* exposed to multifactorial stressors (desiccation, polychromatic UV, X-ray) simulating icy moon conditions and to investigate its transcriptomic response to elucidate strategies for survival.

Experimental Procedures

Sample growth

The yeast strain was kindly provided by Prof. Dr. Whyte from McGill University, Montreal, Canada. The organism was first grown in Universal Media for yeasts (UMY), containing 3.0 g yeast extract, 3.0 g malt extract, 5.0 g peptone, 10.0 g glucose in 1L MQ water, at 25°C with orbital shaking at 125 rpm until late-exponential phase. The organism was then inoculated in M9-complete minimal salts media containing: 47.8 mM Na_2HPO_4 , 22.0 mM KH_2PO_4 , 8.6 mM NaCl , 3.7 mM NH_4Cl , 2 mM MgSO_4 , 0.1 mM CaCl_2 , and 0.02 mM Fe(III)Cl_3 . The media was supplemented with 0.2% (w/v)

of l-glutamic acid ($C_5H_9NO_4$) as carbon source, purchased from Sigma-Aldrich, the media will be mentioned as M9-glutamic from this point.

Sample exposure

Three samples with four biological replicates were used for the exposure to icy moon conditions, and all exposure conditions were performed at room temperature, including desiccation, polychromatic UV and X-ray radiation. This included control (growth in M9-glutamic), exposed (growth in M9 and exposure to the icy moon simulated conditions) and repaired (all the previous with a 4-hour incubation step post-exposure). Determination of the survival of the microorganism to single conditions was performed as per Zaccaria, Beblo-Vranesevic (21). After growth in M9-glutamic, 200 μ l of yeast culture was placed on sterile 1 cm glass disks. The disks were allowed to dry oxically at room temperature (relative humidity of 40% $\pm$ 10%) overnight under sterile conditions. The disks were stored for 7 days under the same and sterile conditions. UV exposure involved placing the disks under the 500S irradiation source (Dr. Hoenle AG; UV-Technologie, Germany) for exposure to polychromatic UV radiation. Prior to sample irradiation, the UV source was allowed to warm up for >30 minutes to allow the lamp to reach the full UV spectrum. UV fluence to reach the desired fluence was measured with the Bentham DMc150 transportable spectroradiometer (Bentham Instruments Ltd., Reading, United Kingdom) and sample exposure time was determined prior to the start of each experiment. The UV fluence during the exposure of the samples was measured at 20.838 W/m² for a 100 cm distance from the light source for all the dose ranges of 500, 1000, 2500, 5000 and 7500 J/m².

Following UV exposure, the disks were transferred to the Gulmay RS 225A (Gulmay Medical Ltd.) X-ray source for exposure to ionizing radiation. The output of the X-ray source was set to 200 kV and 15 mA with the samples at a 10 cm distance below the source. The output of the source was measured with a UNIDOS dosimeter (PTW Freiburg, Germany) which registered 30 Gy/min $\pm$ 5 Gy/min. After desiccation, UV and X-ray exposure, the glass disks with the yeast samples were resuspended by vortexing in a 5 mL Eppendorf tube containing 1 mL of sterile PBS (4 g NaCl, 3 g KH_2PO_4 and 7 g Na_2HPO_4 in 1 L MQ water) for 30 seconds. The resuspended cells were used for dilution plating to determine survival via CFU counts and for the isolation of RNA.

To analyze the RNA and determine the growth kinetics of the yeast post-exposure, cells were resuspended in a sterile Erlenmeyer flask containing 100 mL of M9-glutamic at 25°C. The growth kinetics of the yeast during incubation was

determined by serial dilution plating and CFU counts every hour after incubation. The identification of increased CFU counts, and thus cell duplication, after 5 hours, Figure 1, informed the selection of 4 hours as the appropriate timepoint for RNA isolation in the Repair condition. This was performed in order to critically select a timepoint prior to the onset of cell division to avoid capturing the transcripts of the new cell generation instead of those actively recovering from exposure.

RNA isolation and quality control

RNA was isolated following the phenol-chloroform protocol from Jeffrey and Stuecker (24). Following RNA isolation, electropherogram measurements were performed in order to ensure RNA quality. The Agilent 4200 TapeStation (Agilent Technologies Deutschland GmbH) with the Agilent High Sensitivity RNA ScreenTape was used to measure RNA concentration, eRIN values, and to visually identify the 18S and 28S peaks prior to RNA sequencing.

RNA shipping

Following extraction, the RNA samples were shipped in dry ice overnight to GENEWIZ Germany GmbH, Leipzig, Germany for RNA sequencing and preliminary analysis.

RNA sequencing for pathway assessment/Bioinformatics

Next generation Illumina sequencing with 30 million reads per sample was performed by the GENEWIZ Germany GmbH, Leipzig.

Mapping sequence reads to the reference genome

Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the *Rhodotorula*_JG-1b (GCA_001541205.1_Rhosp1) reference genome available on EnsemblFungi (https://ftp.ensemblgenomes.ebi.ac.uk/pub/fungi/release-61/fasta/fungi_basidiomycota1_collection/rhodotorula_sp_jg_1b_gca_001541205/cds/) using the STAR aligner v.2.5.2b (<https://www.ncbi.nlm.nih.gov/Traces/wgs/LQXB01>).

Extracting gene hit counts

Unique gene hit counts were calculated by using featureCounts from the Subread package v.1.5.2. Only unique reads that fell within exon regions were counted.

Differential Gene Expression Analysis

After extraction of gene hit counts, downstream differential gene expression analysis (DGEA) was performed using DESeq2, which is a comparison of gene expression between the selected groups of samples, summarized in Table 1. The

Wald test was used to generate p-values and $\log_2$ fold changes. Genes with an adjusted p-value <0.05 and absolute $\log_2$ fold change >1 were called as differentially expressed genes (DEGs) for each comparison. Further filtering for known genes was performed in order to exclude unknown or hypothetical proteins.

Table 1 Sample grouping for differential gene expression analysis

Sample label (replicate)	Sample description	Grouping for DGEA
A	Controls	Control (Group E)
B		
C		
D		
IA	Exposed to 7 days of desiccation, 750 Gy and 7500 J/m ²	Exposed (Group F)
IB		
IC		
ID		
RA	Exposed and allowed to repair for 4 hours at 25°C in liquid media	Repaired (Group G)
RB		
RC		
RD		

To visualize global transcriptional patterns, volcano plots were created to visualize the significant (adjusted p-value <0.05) upregulated and downregulated genes for each sample. This was performed by plotting on the y-axis of $\log_{10}$ adjusted p-value and the $\log_2$ fold change on the x-axis. Furthermore, to visualize differences between samples, principal component analysis (PCA) plots were generated. First by standardizing the data in order to normalize the gene expression levels, then by reducing the data to two principal components and plotting the scatterplot.

To understand the distribution of functional genes in clusters of ortholog groups (COG, Table 2), in the genome of *R. frigidicola*, the genes of the reference genome were transformed into COG categories, using the NCBI database (<https://ftp.ncbi.nlm.nih.gov/pub/COG/COG2024/data/>) and plotted as a single bar chart. Furthermore, the DEGs identified by DGEA and grouped according to the samples described in Table 1 were also differentiated by COG categories and plotted in a bar chart to evaluate the distribution of functional genes by ortholog groups.

Table 2 Summary, Label and description of COG (cluster of ortholog groups) categories extracted from Galperin, Vera Alvarez (25)

Label	Description	Sub-groups
J	Translational, ribosomal structure and biogenesis	Information storage and processing
A	RNA processing and modification	
K	Transcription	
L	Replication, recombination and repair	
B	Chromatin Structure and dynamics	
D	Cell cycle control, cell division, chromosome partitioning	Cellular processes and signaling
Y	Nuclear structure	
V	Defense mechanisms	
T	Signal transduction mechanisms	
M	Cell wall/membrane/envelope biogenesis	
N	Cell motility	
Z	Cytoskeleton	
W	Extracellular structures	
U	Intracellular trafficking, secretion, and vesicular transport	
O	Posttranslational modification, protein turnover, chaperones	
C	Energy production and conversion	Metabolism
G	Carbohydrate transport and metabolism	
E	Amino acid transport and metabolism	
F	Nucleotide transport and metabolism	
H	Coenzyme transport and metabolism	
I	Lipid transport and metabolism	
P	Inorganic ion transport and metabolism	
Q	Secondary metabolites biosynthesis, transport and catabolism	
R	General function prediction only	Poorly characterized
S	Function unknown	

To understand the number of shared DEGs between samples, a Venn diagram was generated to reflect these values as well as the number of shared up and down-regulated DEGs. Additionally, the generation of a bar plot representing the COG category distribution of the shared genes was performed to evaluate shared functional metabolism for each sample.

Significant DEGs were analyzed further with the gene set enrichment analysis (GSEA) of FungiFun3 (26). The FungiFun3 tool was used to evaluate genome-wide expression profiles of terms associated with *R. frigidicola* and the DEGs

identified from RNA-seq analysis. The filtered DEGs were tested against the entire set of known genes as a background. Pathways were considered significant with an adjusted p-value threshold (Benjamini-Hochberg correction) <0.05 and were reported by plotting an enrichment score bar plot reporting which pathways have genes over or underrepresented. The reported values are normalized based on the differences in the sizes of the gene set and of the statistical background.

DEGs identified from all three sample comparisons were used to generate a clustered heatmap. To generate the hierarchically clustered heatmaps, the input data (gene expression $\log_2$ fold changes) was first normalized by z-score using the following formula:

$$Z = \frac{X - \mu}{\sigma}$$

where: X is the original value, μ is the mean of the column and σ is the standard deviation. Then the gene expression was compared between the three samples (Control vs Exposed, Control vs Repaired and Exposed vs Repaired). Hierarchical clustering was performed separately on genes and on samples using Euclidian distance and average linkage computed with `scipy.cluster.hierarchy` of Seaborn 0.13.2. Dendrograms were set to represent cluster relationships. The $\log_2$ fold change color scale of the heatmap was set between +8 and -8.

Identification of resistance genes was performed by using the search function on UniProtKB and filtering the results by genes identified in the *R. frigidicola* genome (27). If a gene was identified in the genome and was differentially expressed, it was reported in a table and as a clustered heatmap.

All the figures were generated using the Seaborn data visualization library version 0.13.2 (28) which is an interface of Matplotlib (29). Unfiltered figures can be found in the supplementary material.

Results

Survival to combined conditions

Previous research has shown the remarkable tolerance of *R. frigidicola* to single extreme conditions (21). Building on this, the yeast was subsequently exposed to a combination of stressors. Remarkably, it also survived combined exposure to desiccation, X-ray and polychromatic UV (200-400 nm) radiation. The exposure

regimens were applied in sequence to ensure that damage accumulated across treatments. All samples were exposed to 7 days of desiccation and increasing X-ray and UV radiation regimens. The limit of survival was measured with 7 days of desiccation, 750 Gy of X-ray radiation and 7500 J/m² of polychromatic UV radiation (Figure 1).

To evaluate the metabolic changes required for survival, it was essential to ensure that *R. frigidicola* could resume normal growth following exposure to combined conditions. Therefore, after exposure to the highest doses of combined conditions (7 days of desiccation, 750 Gy of X-ray and 7500 J/m² of polychromatic UV radiation), the desiccated samples were re-suspended in PBS and incubated in minimal media at 25°C. An aliquot of the growing sample was taken every hour for 5 hours, and at 22 hours, to measure cell division by CFU plating (Figure 2). Based on the observed growth kinetics, a 4-hour post-rehydration timepoint was chosen for RNA extraction.

Gene expression under extreme conditions:

To determine the reproducibility within conditions and differences between them, multiple principal component analyses (PCA) were performed. This revealed that the largest differences were found within the Exposed vs Repaired comparison, with the strongest variance in PC1 (69%) between the two conditions (Figure 3C). However, the highest variances in PC2 were observed among the Control replicates, (30%) (Figure 3A), and 34% (Figure 3B).

The total distribution of significant known DEGs (differentially expressed genes) for each comparison, as summarized in Table 3, showed a higher number of DEGs in the Control vs Exposed and Exposed vs Repaired comparisons than in the Control vs Repaired comparison. Furthermore, except for the Exposed vs Repaired, the number of downregulated DEGs exceeded the number of upregulated ones in the other two comparisons.

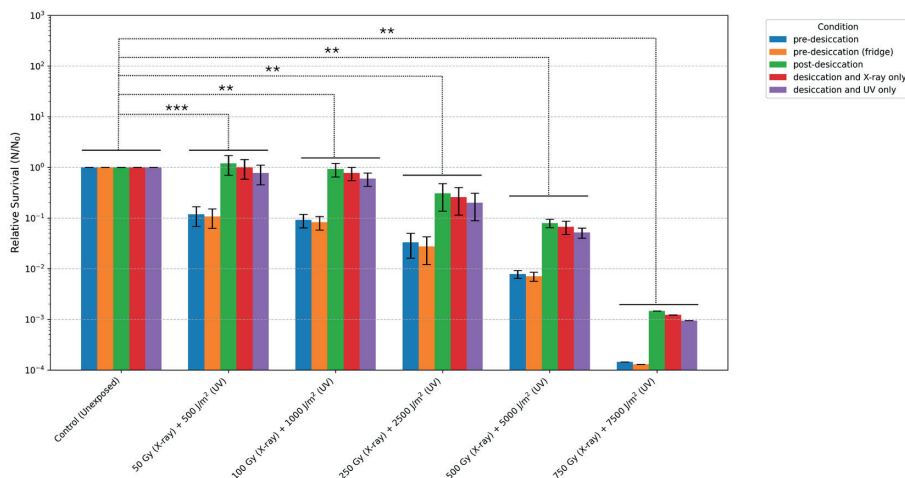


Figure 1 Survival of *R. frigidicola* exposed to combined desiccation, X-ray and polychromatic UV exposure conditions with different control conditions including without desiccation and storage at room temperature for the same period of time as desiccation (blue), with storage at 6°C for the same period of time as the desiccation condition (orange), with desiccation (green), excluding UV radiation (red), and excluding X-ray radiation (purple). $n = 3-6$ biological replicates from three independent experiments, ** $P < 0.01$, *** $P < 0.001$ t-Test: Two-Sample Assuming Unequal Variances.

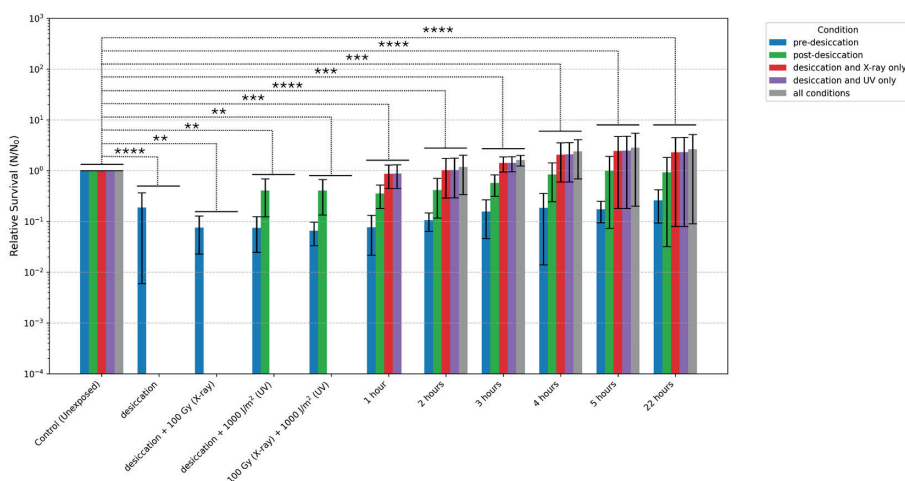


Figure 2 Growth of *R. frigidicola* after exposure to the combined desiccation, X-ray and polychromatic UV exposure conditions with different control conditions including without desiccation (blue), with desiccation (green), excluding UV radiation (red), excluding X-ray radiation (purple) and with all the conditions combined (grey). $n = 3-9$ biological replicates from three independent experiments, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ t-Test: Two-Sample Assuming Unequal Variances.

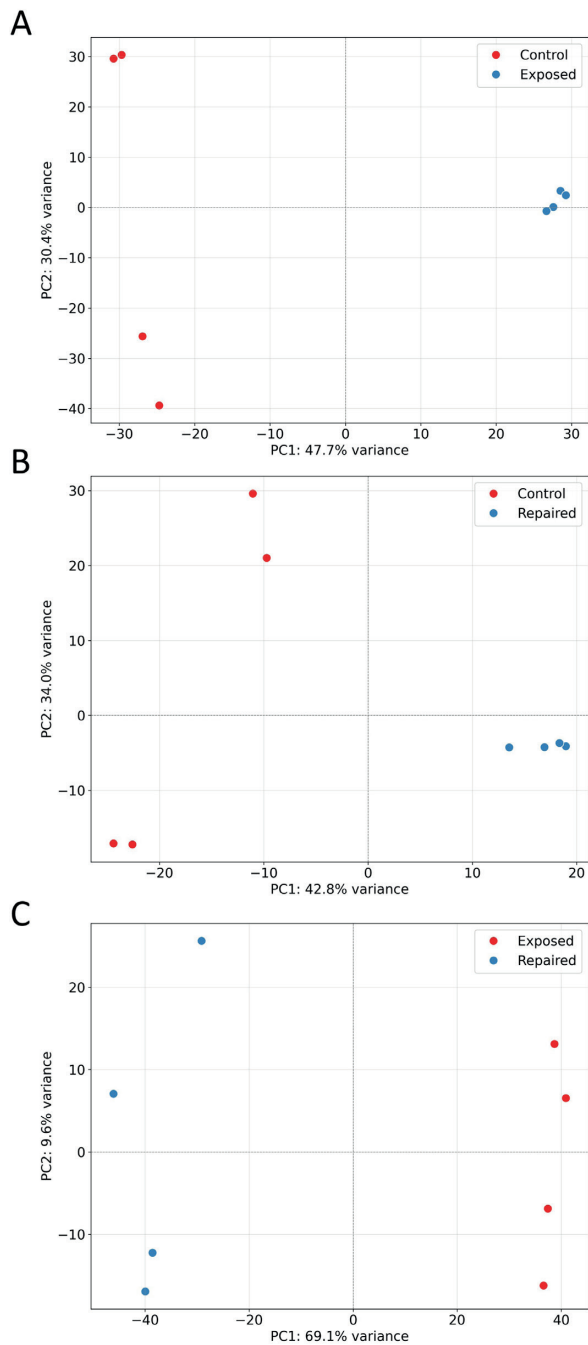


Figure 3 PCA plots showing the reproducibility within conditions and differences between them Control vs Exposed (A), Control vs Repaired (B) and Exposed vs Repaired (C).

Table 3 Total number of upregulated and downregulated DEGs per comparison

Comparison	Total DEGs	Upregulated DEGs	Downregulated DEGs
Control vs Exposed	1653	799	854
Control vs Repaired	726	273	453
Exposed vs Repaired	2164	1119	1045

Although the Exposed vs Repaired comparison display the highest total number of significant DEGs, the volcano plots show a narrower distribution of $\log_2$ fold changes compared to the Control vs Exposed and Control vs Repaired comparisons (Figure 4), indicating a lower diversity in gene expression changes but with a higher statistical significance. Nonetheless, Exposed vs Repaired exhibits the broadest distribution of upregulated and downregulated DEGs, as based on $-\log_{10}$ (adjusted p-value). When applying a ranking metric, it becomes evident that the top five most upregulated and downregulated genes are not consistently shared across all comparisons. However, a gene coding for a Major Facilitator Superfamily (MFS) substrate transporter, responsible for transporting a range of molecules across the cell membrane, consistently ranks among the most highly upregulated DEGs across all comparisons.

The distribution of Cluster of Orthologous Groups (COGs) in the genome of *R. frigidicola* revealed, that a large proportion of genes have an unknown function (Figure 5A). Among the annotated genes, the majority are classified under category K, involved in transcription, followed by J, involved in translation, including ribosomal biogenesis. As expected, a high number of genes are also associated with lipid transport and metabolism (category I) and amino acid transport and metabolism (category E).

When examining the distribution of DEGs across the COG categories for the three pairwise comparisons, the highest number of both upregulated and downregulated genes is associated with transcription (Figure 5B). The relatively low number of DEGs observed in the Control vs Repaired comparison (Table 4) is reflected by the shorter orange bars in the graph.

The distribution trend of up- and downregulated DEGs across COG categories is uneven for each comparison, with very few categories showing balanced number of genes in both directions. For example, the *Control vs Exposed* comparison, more upregulated genes fall within category J, i.e. translation, ribosomal structure and biogenesis, while in the *Exposed vs Repaired* comparison, this trend is reversed, with more genes being downregulated in the same category.

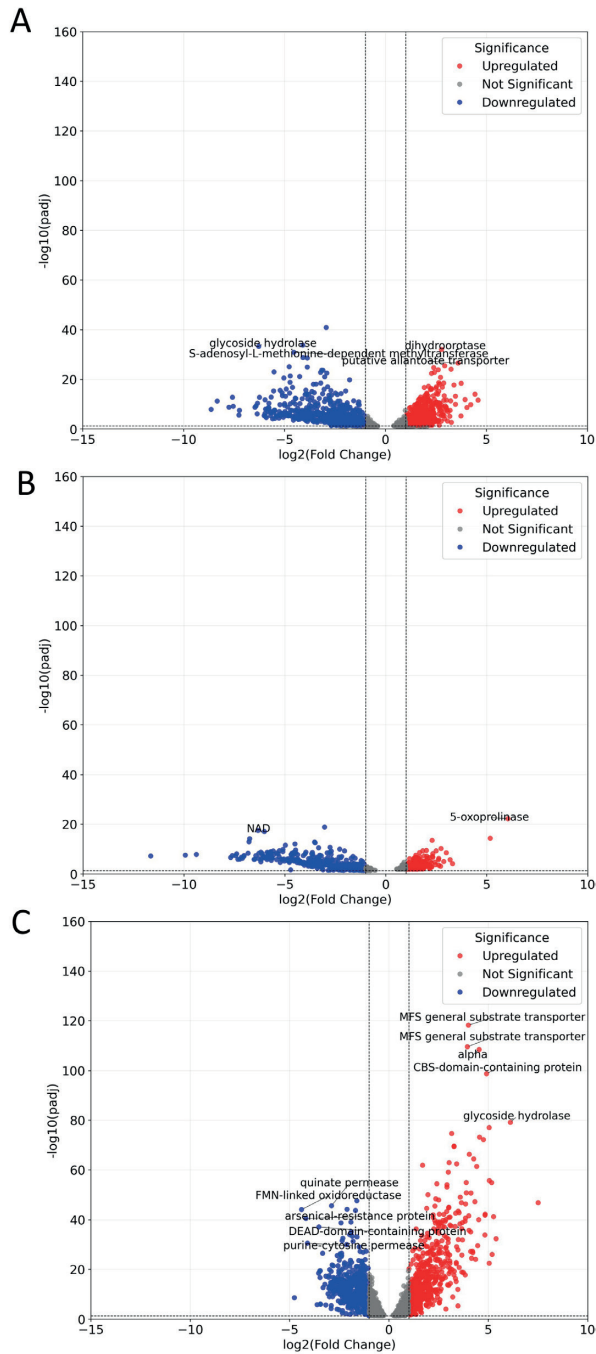


Figure 4 Volcano plots showing the significant distribution of upregulated (red dots) and downregulated (blue dots) DEGs for the samples Control vs Exposed (A), Control vs Repaired (B) and Exposed vs Repaired (C).

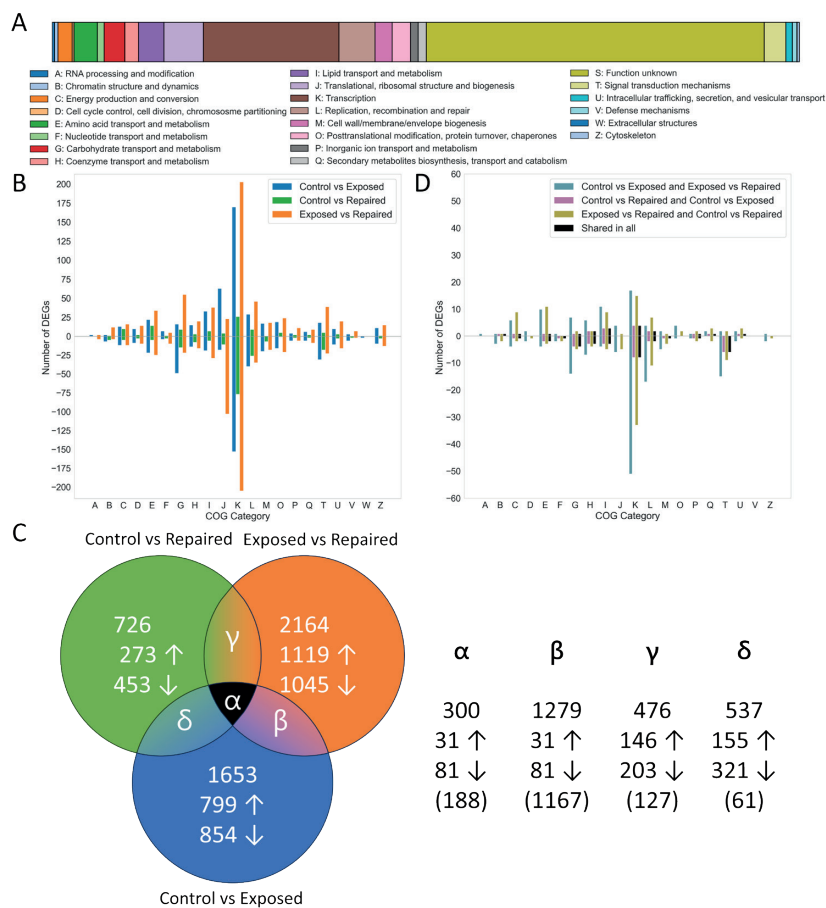


Figure 5 Distribution of COGs in the genome of *R. frigidicola* (**A**). Frequency of upregulated and downregulated DEGs for each COG category under the different conditions. The total number of DEGs in each COG category that showed significant ($p < 0.05$) upregulation ($> 1 \log_2$ fold change) for Control vs Exposed (Blue), Control vs Repaired (Orange) and Exposed vs Repaired (Green) (**B**). Distribution of unique and shared DEGs. Direction of arrows inside Venn diagram represent upregulation or downregulation. Numbers in brackets represent the DEGs which are shared between samples but are not upregulated or downregulated (**C**). COG category distribution for DEGs shared between samples. These include Control vs Exposed and Exposed vs Repaired (pink), Control vs Repaired and Control vs Exposed (cyan), Exposed vs Repaired and Control vs Repaired (yellow) and shared with all samples (black) (**D**).

Interestingly, certain COGs, such as coenzyme transport and metabolism (H), cell wall/membrane biogenesis (M), posttranslational modification, protein turnover, chaperones (O), and intracellular trafficking, secretion, and vesicular transport (U), show a nearly equal distribution of up- and downregulated genes in both *Control vs Exposed* and *Exposed vs Repaired* comparisons. This suggests that the expression of genes in these categories may be consistently regulated during exposure and recovery, highlighting key cellular processes actively engaged by the yeast during stress response.

To identify genes shared across conditions and to identify expression trends, pairwise comparisons of DEGs were performed (Figure 5C). This analysis showed that 300 DEGs are shared across all three comparisons. Among these, 31 genes are consistently upregulated and 81 genes are consistently downregulated, while the remaining 188 genes are shared but show opposing expression patterns. As expected, the comparison between *Exposed vs Repaired* and *Control vs Exposed* shows the highest number of shared genes, reflecting the higher total number of DEGs in these comparisons. Overall, among the shared genes, the majority are downregulated, consistent with the general expression trend observed across individual comparisons except for *Exposed vs Repaired*, where the pattern is reversed.

Although the distribution of COG categories among the shared genes generally follows the overall trend, observed across all DEGs, notable differences are apparent (Figure 5D). As with the full dataset, the highest number of shared genes is still associated transcription (K). However, several COG categories are absent in certain comparisons. Specifically, exposure-related genes not associated with RNA processing and modification (A), cell cycle control, cell division, chromosome partitioning (D), translation, ribosomal structure and biogenesis (J), post-translational modification, protein turnover, chaperones (O), defense mechanisms (V), and cytoskeleton (Z). In contrast to the overall COG distribution across all DEGs, the shared genes exhibit a stronger downregulation trend, as shown in Figure 5D. This pattern indicates that many of the genes common across comparisons, even when not consistently regulated in the same direction, tend to be more frequently downregulated. Despite these differences in COG category frequencies, the functional profile of the shared genes remains largely similar to that of individual comparisons. Most are still associated with key metabolic and regulatory functions, particularly coenzyme transport and metabolism (H), signal transduction mechanisms (T) and carbohydrate transport and metabolism (G).

Gene set enrichment analysis

Gene set Enrichment analysis (GSEA) using the FungiFun3 tool was performed to elucidate the over or underrepresented pathways between samples. The analysis revealed 94 pathways involved in the *Control vs Exposed* comparison, 101 in *Exposed vs Repaired*, Figure 6A and B, and 35 pathways in *Control vs Repaired* Figure S2. The normalized enrichment score (NES) in *Control vs Exposed* ranged between 4.11 and -2.02 and upregulated pathways reflected protein synthesis (ribosome biogenesis, nuclear lumen, nucleolus) while downregulated pathways are related to cell structure components (microtubule cytoskeleton, cell wall organization) and biosynthetic processes (dioxxygenase activity, microtubule motor activity) (Figure 6A). However, the strongly enriched up- and down-regulated pathways had a lower gene count than ones with weaker enrichment (hydrolase activity, cellular process, intracellular non-membrane-bounded organelle, intracellular anatomical structure).

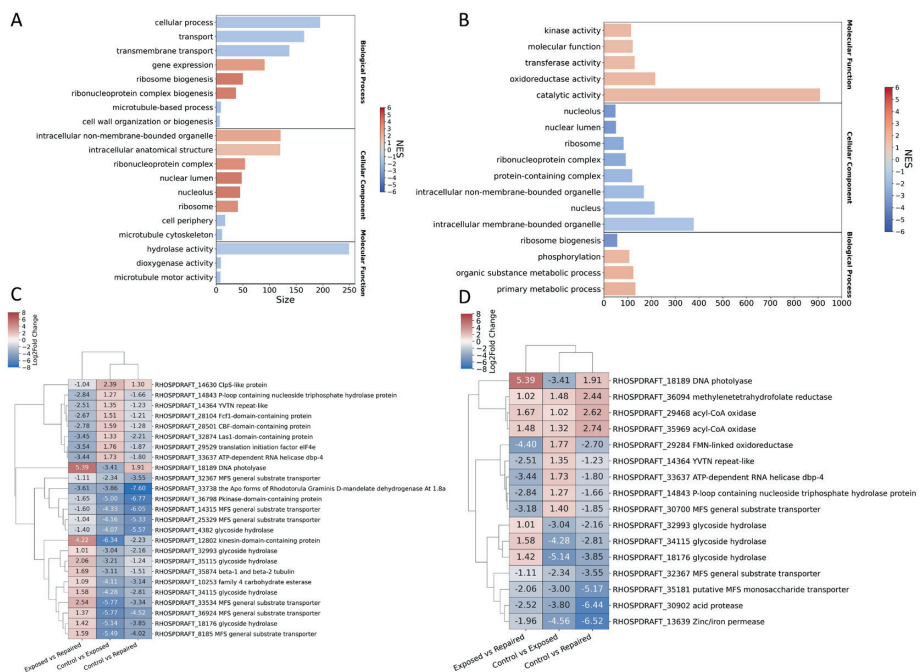


Figure 6 Gene Set Enrichment Analysis (GSEA) of the sample comparisons performed with FungiFun3. The figure summarizes the enrichment of gene set pathways highlighting significant ones ($p_{adj} < 0.05$). The pathways are separated by ontology class (Biological process, Cellular component and Molecular function). Clustered heatmaps show the DEGs of interest for Control vs Exposed (A and C) and Exposed vs Repaired (B and D) comparisons.

In contrast to *Control vs Exposed* the pathways identified in the *Control vs Repaired* comparison, were fewer indicating similar functions being carried out by the yeast under the two conditions (Figure S2). This is also supported by the low number of genes employed in the pathways (anion binding), and the low enrichment score (amino acid synthesis). Nonetheless, *Control vs Repaired* shows an overall positive enrichment, 30 out of 35 pathways being upregulated. The nature of the pathways in the comparison being associated to baseline activity can also be identified by their functions (amino acid biosynthetic process, oxidoreductase activity, anion binding, ligase activity).

The high number of pathways as well as the high number of pathways with a size >100 genes in *Exposed vs Repaired* reflects the difference in activity performed between the two conditions (Figure 6B). The GSEA also highlighted how catalytic activity is the pathway with the highest number of genes involved across all comparisons in this study. Despite a number of pathways identified in *Control vs Exposed* the reversal in NES range for *Exposed vs Repaired* shows the difference in metabolic function between the two comparisons. Under this comparison, ribosome biogenesis and protein synthesis are downregulated (ribonucleoprotein complex, ribosome, ribosomal subunit, nucleolus) while catalysing and phosphorylating mechanisms, including DNA repair, are upregulated (organic substrate metabolic process, primary metabolic process, phosphorylation, transferase activity, kinase activity).

Generation of clustered heatmaps from the genes involved in the pathways highlighted by GSEA support our findings (Figure 6C, D), for further details see supplementary figures S4, S5 and S6. For example, ribosome biogenesis and protein synthesis genes are upregulated in *Control vs Exposed* only (Figure 6C) and the importance DNA repair and hydrolyzing genes such as DNA photolyase and Alpha/beta hydrolase being shared with other species of the *Rhodotorula* genus. Gene clustering also revealed how a number of genes are shared across pathways (acyl-CoA oxidase, glycoside hydrolase), underlining their role.

Desiccation and radiation resistance gene expression patterns

Investigations into the *R. frigidicola* genome using UniProtKB (27) revealed several gene orthologs which might support resistance to a number of extreme conditions. Such genes were identified in other organisms which showed tolerance to desiccation or radiation. The search yielded 72 of these “resistance genes” differentially expressed under all sample conditions, 53 of which are related to radiation and 19 to desiccation (Table 4). Interestingly, despite all the genes eliciting a response, only the *Control vs Exposed* comparison showed higher

$\log_2$ fold changes. Furthermore, under *Control vs Exposed* approximately half of the radiation and half of the desiccation resistance genes are upregulated. However, the DEGs with the highest $\log_2$ fold change can be seen at the top as RHOSPDRAFT_31273 (LPX1), RHOSPDRAFT_31279 Alpha/beta-hydrolase (LPX2), and RHOSPDRAFT_33214 Protection of telomeres protein 1 ssDNA-binding domain-containing protein (POT1), clustered separately from all other DEGs and associated to desiccation tolerance. As a whole these results support the GSEA analysis and further cement the understanding of how *R. frigidicola* is metabolically active under the exposure conditions compared to Control or Repair.

Table 4 Desiccation and radiation resistance ortholog genes found to be differentially expressed under the experimental conditions

Resistance	Mechanism	Gene name	Function
Desiccation	fatty acid oxidation	LPX1	Alpha/beta-hydrolase
		LPX2	Alpha/beta-hydrolase
		POT1	Protection of telomeres protein 1 ssDNA-binding domain-containing protein
	osmolarity	hog1	mitogen-activated protein kinase hog1
		pbs2	MAP kinase
		ssk2	Kinase-like protein
		ssk2	Kinase-like protein
		ssk2	STE/STE11 protein kinase
		ssk2	Pkinase-domain-containing protein
		opy2	Membrane anchor Opy2 N-terminal domain-containing protein
	salinity	nst1	Stress response protein NST1
	trehalose	tps1	Trehalose-6-phosphate synthase component TPS1
		tps2	Family 20 glycosyltransferase
	mediators and repressor	srb8	Mediator of RNA polymerase II transcription subunit 13
		pex10	RING-type E3 ubiquitin transferase
		pex10	RING-type E3 ubiquitin transferase (cysteine targeting)
		ras2	Small G-protein Ras2
Radiation	Replication recombination repair	ire1	3-phosphoinositide-dependent protein kinase activity
		nth1	DNA glycosylase
		rad1	Rad1-domain-containing protein
		rad10	DNA repair protein rad10
		rad18	RING-type domain-containing protein
		rad50	Rad50/SbcC-type AAA domain-containing protein
		rad50	RINT-1 family protein
		rad9	Rad9-domain-containing protein
	trehalose dispensability	rad9	Histone-lysine N-methyltransferase, H3 lysine-79 specific
		mec3	Checkpoint protein

ditions.

Control vs Exposed	Control vs Repaired	Exposed vs Repaired	Reference
-5.59	-7.68	-1.95	Robinson, Ren (30)
-3.55	-3.36	2.01	
-3.49	-1.77	3.35	
0.55	5.67E-6	1.37E-5	Calahan, Dunham (31)
0.79	4.18E-8	1.3E-7	
-5.25	-3.36	2.01	
-4.99	-1.77	3.35	
1.59	3.21E-22	3.98E-21	
-1.09	1.12E-4	3.85E-4	
1.26	1.03E-5	4.58E-5	
1.02	0.01	0.02	
-2.62	1.19E-3	3.21E-3	
-1.93	0.01	0.02	
-2.48	2.8E-6	1.41E-5	
1.31	2.25E-5	9.16E-5	
1.65	2.25E-8	7.97E-8	
-1.43	8.92E-4	2.5E-3	
0.50	4.77E-3	0.01	Ren, Brenner (32)
1.89	1.78E-4	5.9E-4	Tapia, Young (33)
-1.73	3.96E-6	1.94E-5	Bennett, Lewis (34)
-0.78	0.03	0.06	
-1.19	8.13E-12	3.8E-11	
-1.15	0.01	0.02	
0.44	3.32E-3	0.01	
-1.43	1.59E-8	1.43E-7	
-0.73	1.81E-3	3.19E-3	
0.80	3.5E-3	0.01	

Table 4 Continued

Resistance	Mechanism	Gene name	Function
		mec3	Hus1-like protein
		rad17	p-loop containing nucleoside triphosphate hydrolase protein
		rad17	Rad1-domain-containing protein
		ctf8	Transcription factor TFIIIC triple barrel domain-containing protein
		chl1	ATP-dependent DNA helicase CHL1
		chl1	DNA 5'-3' helicase
		ctf4	
		dhh1	RNA helicase
		nup133	Methyltransferase type 11
		nup133	Nucleoporin-domain-containing protein
		HPR1	THO complex subunit 1 transcription elongation factor-domain-containing protein
		bem1	Bud emergence protein 1
		nup84	SEC7 domain-containing protein
		rad17	P-loop containing nucleoside triphosphate hydrolase protein
		rad17	Rad1-domain-containing protein
	Checkpoint	mec3	Hus1-like protein
	Nuclear pore complex	nup133	methyltransferase type 11
		nup133	nucleoporin-domain-containing protein
	Chromatin silencing telomers	ard1	acyl-CoA N-acyltransferase
		dhh1	DEAD-domain-containing protein
	mitotic chromosome transmission	chl1	DNA repair helicase
		chl1	DNA repair helicase
		pat1	Sm-like ribonucleo protein
	Ubiquitin degradation pathway	ubr1	Metallo-hydrolase
		doc1	anaphase-promoting complex
	Transcription/ RNA metabolism	rpb9	DNA-directed RNA polymerase II subunit I
		ccr4	putative Rpc11-DNA-directed RNA polymerase III subunit C11
		ccr4	putative RPA12-13
		ccr4	Not1-domain-containing protein
		ccr4	CAF1-domain-containing protein
		ccr4	Rcd1-like protein
		ccr4	Endonuclease
	Cytokinesis cytoskeleton spindle	rvs161	BAR-domain-containing protein
		sac6	putative SAC6-actin filament bundling protein

Control vs Exposed	Control vs Repaired	Exposed vs Repaired	Reference
0.73	0.01	0.01	
-1.13	4.74E-4	1.42E-3	
-1.73	3.96E-6	1.94E-5	
-0.88	0.01	0.01	
-1.65	1.21E-3	3.27E-3	
-1.45	1.15E-3	3.14E-3	
-1.79	0.01	0.01	
1.28	1.95E-3	4.98E-3	
0.75	1.51E-8	4.92E-8	
0.50	1.11E-5	2.61E-5	
0.80	2.70E-11	1.18E-10	
1.19	9.25E-4	2.58E-3	
0.67	1.07E-8	3.56E-8	
-1.13	4.74E-4	1.42E-3	
-1.73	3.96E-6	1.94E-5	
0.73	0.01	0.01	
0.75	1.51E-8	4.92E-8	
0.50	1.11E-5	2.61E-5	
1.13	0.02	0.04	
1.28	1.95E-3	4.98E-3	
-1.65	1.21E-3	3.27E-3	
-1.45	1.15E-3	3.14E-3	
0.90	0.01	0.02	
0.82	2.31E-4	4.63E-4	
-1.24	0.01	0.02	
1.82	2.99E-4	9.38E-4	
1.41	0.01	0.03	
2.27	8.05E-9	7.91E-8	
0.85	9.57E-6	2.25E-5	
1.13	1.85E-6	9.61E-6	
1.98	1.00E-7	7.47E-7	
-0.75	0.02	0.04	
1.00	9.27E-8	2.77E-7	
0.95	2.40E-6	6.11E-6	

Table 4 Continued

Resistance	Mechanism	Gene name	Function
	cell wall heat shock	pdr13	HSP70-domain-containing protein
		bck1	Pkinase-domain-containing protein
	karyogamy	zuo1	DnaJ-domain-containing protein
		zuo1	DnaJ-domain-containing protein
	sterol metabolism	erg28	Erg28-like protein
	radioresistance	mre11	Rad50/SbcC-type AAA domain-containing protein
		mre11	BRCT domain-containing protein
	pigment	N/A	Ferritin-like domain-containing protein
		N/A	p-loop containing nucleoside triphosphate hydrolase protein
	SOS response	N/A	DUF159-domain-containing protein
	DNA damage sensor	Ku70/80	ATP-dependent DNA helicase II subunit 1
		Ku70/80	ATP-dependent DNA helicase II subunit 2

Control vs Exposed	Control vs Repaired	Exposed vs Repaired	Reference
2.27	6.89E-4	1.98E-3	Wu, Song (35)
-1.09	1.12E-4	3.85E-4	
-2.36	0.01	0.02	
2.30	3.19E-3	0.01	
0.89	1.37E-3	2.47E-3	
-1.15	0.01	0.02	
-1.12	0.03	0.05	
-1.78	0.01	0.02	
-3.49	5.89E-5	2.19E-4	
-1.18	2.36E-3	0.01	
0.71	0.05	0.08	
-1.03	0.05	0.08	

Discussion

Earlier studies have shown the resilience of *R. frigidalcoholis*, particularly they have highlighted its ability to grow at subzero temperatures and to tolerate extreme environmental conditions (19, 23). However, the survival and metabolic activity of this yeast, and other microorganisms, under icy moon-like conditions remain largely unexplored (36). The multifactorial effects of various environmental stressor characteristics of icy moons on microbial survival is still poorly understood. This knowledge gap is particularly important given the upcoming end-of-mission plans for JUICE and Europa Clipper spacecraft, both of which are scheduled to crash on Ganymede, the largest moon of Jupiter and the entire solar system (37). Despite Ganymede being chosen as a crash site for both ESA's and NASA's missions due to the assumption that its subsurface ocean does not interact with the surface, this end-of-mission decision remains subject to change if new evidence suggests otherwise. Therefore, it is crucial to investigate how icy moon conditions affect psychrotolerant microorganisms, which are the most likely terrestrial life forms to survive such environments. The selection of *R. frigidalcoholis* as a model organism is further supported by its intrinsic ability to adapt by for example intracellular accumulation of osmolytes during high osmotic pressures, which occur during freezing of water, and to maintain membrane fluidity (3).

Fungal research in the context of space biology is growing, however, most of the investigations focus on the response of the microorganisms to radiation, largely because fungi are among the most radiation-tolerant eukaryotes (38). Most yeast research studies to date have centred on the well-studied genus *Saccharomyces*, particularly *S. cerevisiae*. For example, *S. cerevisiae* has been reported to tolerate up to 60 Gy of particle galactic cosmic ray (GCR)-like ionizing radiation (39). In contrast, our findings show that *R. frigidalcoholis* demonstrates significantly higher resistance to higher doses of ionizing radiation, suggesting that it may be a more robust candidate for space survival under such conditions. Supporting the importance of testing multiple stressors, earlier research on *Saccharomyces cerevisiae* var. *ellipsoideus* reported survival after exposure to 750 Gy of γ -radiation and brief heat shock at 60°C, highlighting the need to investigate synergistic stress effects (40). Additionally, environmental yeast isolates such as *R. mucilaginosa*, *Dioszegia fristingensis*, and *Cystofilobasidium macerans* have also been studied for their tolerance to UV radiation. However, these investigations did not specifically examine the impact of space-relevant DNA-damaging UV-C radiation (41). While, desiccation tolerance has been investigated in some yeast species, those studies have typically shown limited survival and no growth under nutrient-limited

conditions (32). Nonetheless, other fungal candidates such as the black fungus *Cryomyces antarcticus* and *Rhinoctadiella similis* have been used in studies to evaluate the extraterrestrial habitability of Mars through exposure to Fe particle radiation and interactions with Mars regolith simulants (42, 43). Furthermore, proteomic analyses of the halotolerant yeast *Debaryomyces hansenii* have also shown its resilience to perchlorate stress under Martian brine conditions (44). Taken together, our results demonstrate that *R. frigidalcoholis* is a highly promising (and potentially superior) candidate for space survival compared to other yeast species, due to its exceptional resistance to multiple space-relevant stressors, including radiation, desiccation and nutrient limitation.

The genome of *S. cerevisiae* is circa 12 Mbp in size (45) and studies on the reference strain S288c have shown that only around 6% of its genes remain functionally uncharacterized (46). In contrast, although the genome of *R. frigidalcoholis* is larger in size, it contains 53% of genes with unknown function, underscoring how comparatively understudied this organism is (20). This high number of unknown genes is reflected in our transcriptome analysis where a large fraction of DEGs lack known functional annotations. These unknown DEGs might play important roles in stress responses and survival under the simulated icy moon conditions, highlighting the need for future functional studies.

Among the known DEGs the majority of both up- and downregulated genes are related to transcription and translation. This suggests that significant changes in these core processes occur during exposure and recovery. In particular, the *Exposed vs Repaired* comparison showed strong downregulation of translation-related genes, likely indicating temporary cell cycle arrest. Such an arrest is a known stress response. For instance, *Exophiala dermatitidis* halts cell division after γ -irradiation to allow DNA repair before resuming growth (47). A similar mechanism may occur in *R. frigidalcoholis* as evidenced by downregulation of genes involved in translation, ribosome biogenesis, and DNA replication during recovery, suggesting a coordinated response to preserve genomic integrity.

Concurrently, the upregulation of genes encoding for enzymes with catalytic activity in the *Exposed vs Repaired* condition suggests that *R. frigidalcoholis* activates biochemical repair processes. The enzymes are essential for double-strand break (DSB) repair in yeast and humans (48). Increased expression of genes related to energy production further supports this, indicating that energy-intensive repair processes are prioritized during stress. Similar responses have been observed in industrial yeasts during alcohol fermentation (49).

Plasma membrane damage can also halt cell cycle progression. This includes damage from desiccation, ionizing radiation, DNA damage such as pyrimidine dimers and DSBs, and the production of reactive oxygen species (ROS) triggered by both UV and ionizing radiation (50-53). In *S. cerevisiae* membrane damage has been directly linked to inhibition of DNA replication and activation of preservation mechanisms (54, 55). Given the severity of the stressors applied in our experiments, likely *R. frigidicola* experienced similar membrane disruption. Despite compositional differences, studies have shown that even lower x-ray radiation doses than those used here can damage eukaryotic membranes (52).

Interestingly, despite the presumed need for signalling to initiate repair and arrest growth, we observed a significant downregulation of DEGs associated with signal transduction. This may indicate dysregulation of signalling pathways in *R. frigidicola* under extreme stress. In *S. cerevisiae*, DNA damage checkpoints are known to delay mitosis allowing repair, which could explain our findings (56, 57).

The experimental design aimed to capture activation of DNA repair pathways during recovery. Unexpectedly, several key repair genes were already upregulated during exposure, while the cells were desiccated. These include DNA photolyase, LPX2, POT1, RAD50, MRE11, Ku70, and Ku80. This suggests that the exposure induced both pyrimidine dimers and DSBs, supported by activation of both homologous recombination (RAD50, MRE11) and non-homologous end joining (Ku70, Ku80) pathways (34, 35). The upregulation of DNA photolyase, which specifically repairs cyclobutene pyrimidine dimers, further supports this (58). Increased expression of *POT1*, a gene involved in telomere protection, underscores the cell's effort to maintain genomic stability (59). These mechanisms are critical, as faulty repair can lead to tumorigenesis in higher eukaryotes (60).

DNA photolyase is conserved across all domains of life (61) and is activated by visible light between 300 and 500 nm (58). While Earth's hydrothermal vents primarily emit 750-1050 nm wavelengths, light in 400-600 nm range has also been detected (62, 63). Although insufficient for photolyase activation under Earth conditions, icy moon subsurface ocean chemistry might enable production of suitable light for *in situ* enzyme activity. Such environments, analogous to Earth's hydrothermal systems, could support life and have been proposed as potential sites for life's origin (64, 65). Thus, activation of repair mechanisms, including photolyase, in *R. frigidicola* under extreme conditions supports its candidacy as a model organism for astrobiology and life detection missions. These findings also strengthen the case for the habitability of icy moons by psychrotolerant organisms.

Our study demonstrates that *R. frigidicola* can tolerate icy moon-like conditions and activates DNA repair and metabolic pathways even in a desiccated state. Like other microorganisms it appears to prioritize repair to cell cycle progression. We also emphasize the need for improved genome annotation, as most DEGs have unknown functions. Limitations in our study include conducting experiments at room temperature, not evaluating the growth after freezing the yeast cells, a small number of RNA sampling timepoints, making it unclear whether certain responses occur during desiccation (within 24 hours) or exposure (within 7 days). Additionally, comparisons with other yeasts are limited, as most reference data come from *S. cerevisiae*. We also recognize potential bias in selecting for desiccation- and radiation-resistant genes.

Future studies should determine how rapidly *R. frigidicola* activates DNA repair and whether this occurs under predicted in situ icy moon conditions. Data from upcoming missions like JUICE and Europa Clipper will be key in assessing true habitability. Multifactorial exposure studies, like ours, also offer valuable insights for planetary protection, informing strategies to prevent forward contamination. Ultimately, broader investigations across life domains are needed. Given the ethical and scientific imperative to protect extraterrestrial environments, particularly promising ones like icy moons, stringent contamination control is essential.

List of abbreviations

COG	Cluster of Ortholog Groups
ESA	European Space Agency
DEG	Differentially Expressed Genes
DGEA	Differential Gene Expression Analysis
GSEA	Gene Set Enrichment Analysis
GO	Gene Ontology
ISS	International Space Station
NASA	National Aeronautics and Space Administration
JUICE	Jupiter Icy Moon Explorer
PCA	Principal Component Analysis
STAR	Spliced Transcripts Alignment to a Reference

Acknowledgements

We thank Johanna Piepjohn for the help in preparation of the exposure samples as well as for the RNA isolation.

Conflict information

The authors have no relevant financial or non-financial interests to disclose.

Funding information

The research of this manuscript was supported by DLR internal funds.

Credit author contributions

Conceptualization: Petra Rettberg, Marien I. de Jonge; Methodology: Petra Rettberg, Tommaso Zaccaria, Kristina Beblo-Vranesevic; Formal Analysis and investigation: Tommaso Zaccaria, Xuheui He; Writing – original draft preparation: Tommaso Zaccaria; Writing – review and editing: Petra Rettberg, Kristina Beblo-Vranesevic, Mihai G. Netea, Marien I. de Jonge, Xuehui He; Funding acquisition: Petra Rettberg, Mihai Netea; Resources: Petra Rettberg, Kristina Beblo-Vranesevic; Supervision: Petra Rettberg, Kristina Beblo-Vranesevic, Mihai G. Netea, Marien I. de Jonge.

Data availability

The data underlying this article is available in the Mendeley Data repository, at: <https://data.mendeley.com/datasets/m65mc3vd5w/1>.

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Supplementary figures

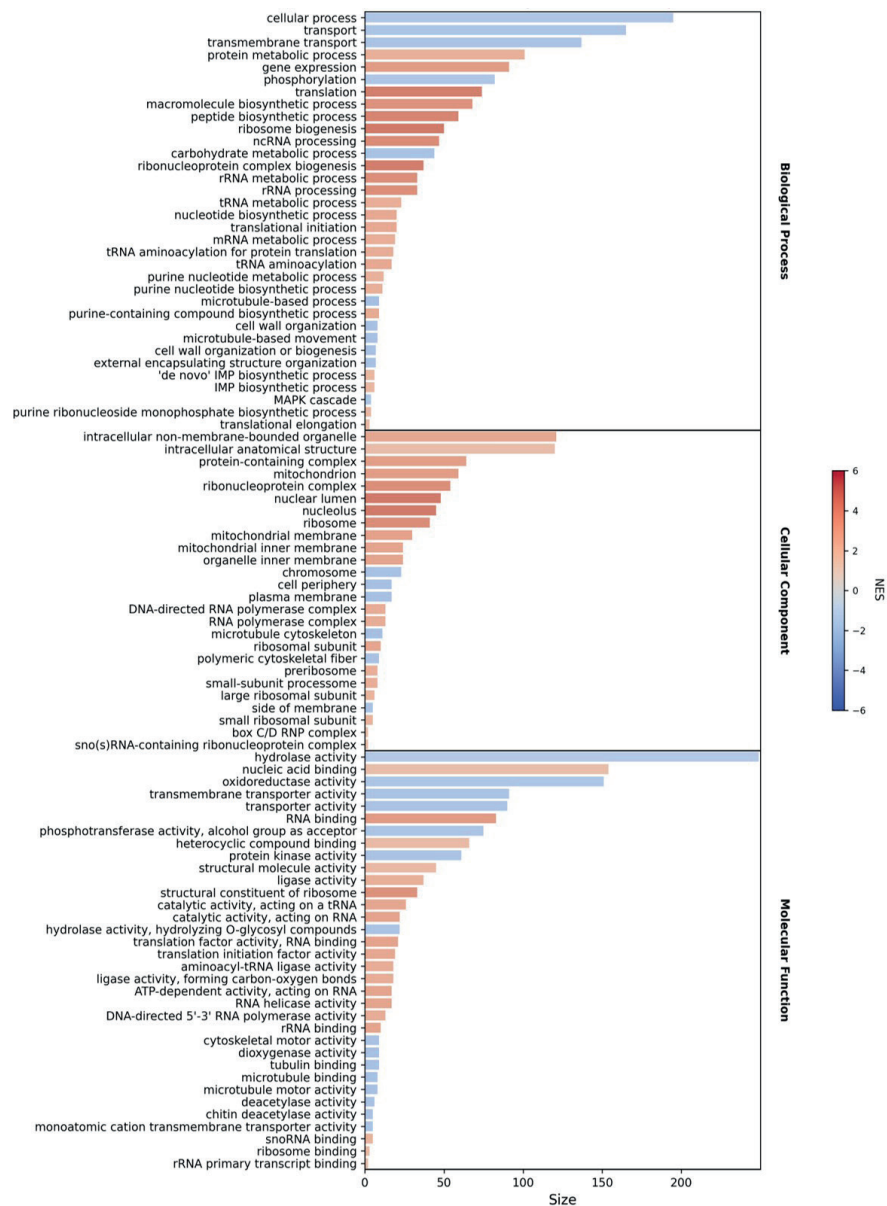


Fig. S1 Shows the GSEA (Gene Set Enrichment Analysis) GO enrichment analysis performed with FungiFun3 for the Control vs Exposed sample. The figure summarizes the enrichment of each gene set and shows the significant ones $p_{adj} < 0.05$. The genes are separated by ontology class: Biological process, Cellular component and Molecular function. The color of each bar represents the normalized enrichment score (NES) with for each pathway

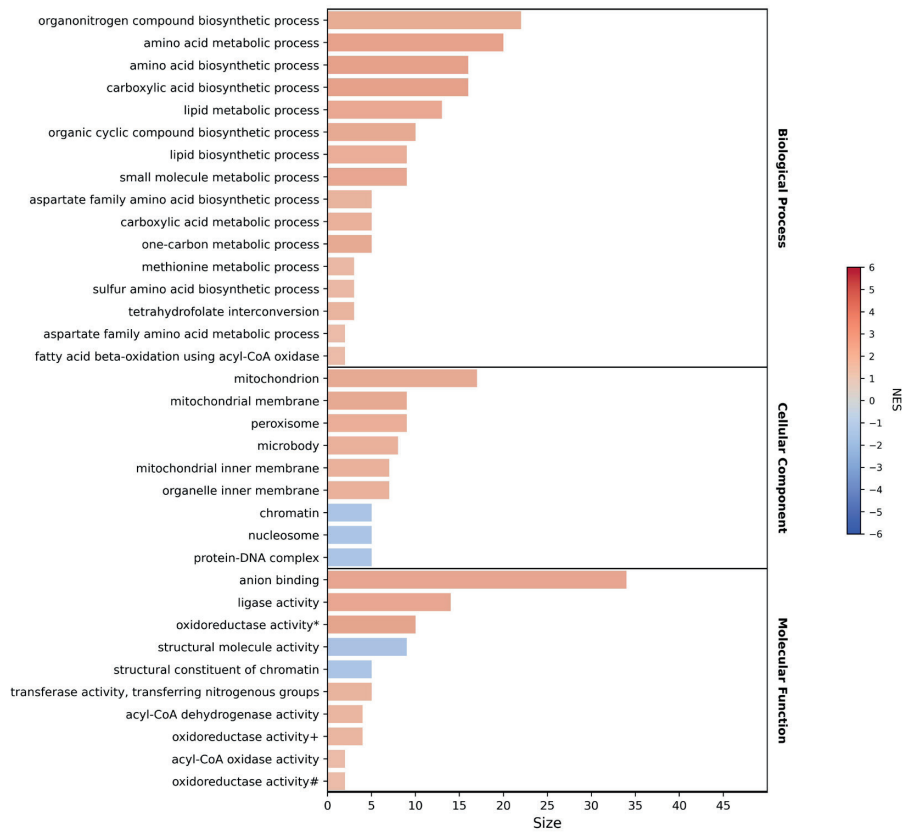


Fig. S2 Shows the GSEA (Gene Set Enrichment Analysis) GO enrichment analysis performed with FungiFun3 for the Control vs Repaired sample. The figure summarizes the enrichment of each gene set and shows the significant ones $p_{adj} < 0.05$. The genes are separated by ontology class: Biological process, Cellular component and Molecular function. The color of each bar represents the normalized enrichment score (NES) with for each pathway. *Oxidoreductase activity, acting on CH-CH group of donors. +Oxidoreductase activity, acting on the CH-CH group of donors, oxygen as acceptor. #Oxidoreductase activity, acting on the CH-CH group of donors, with flavin as acceptor.

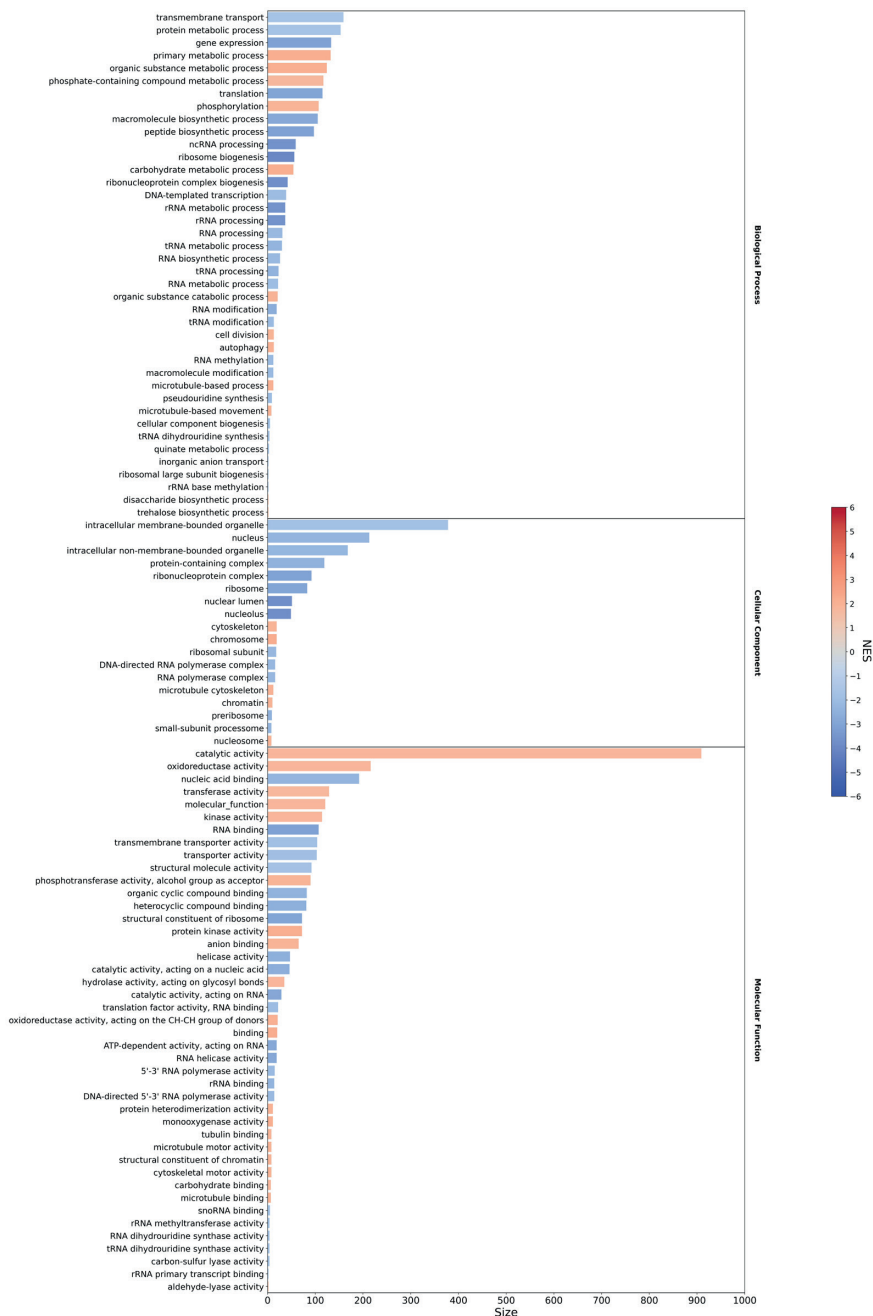


Fig. S3 Shows the GSEA (Gene Set Enrichment Analysis) GO enrichment analysis performed with FungiFun3 for the Exposed vs Repaired sample. The figure summarizes the enrichment of each gene set and shows the significant ones $p_{adj} < 0.05$. The genes are separated by ontology class: Biological process, Cellular component and Molecular function. The color of each bar represents the normalized enrichment score (NES) with for each pathway.

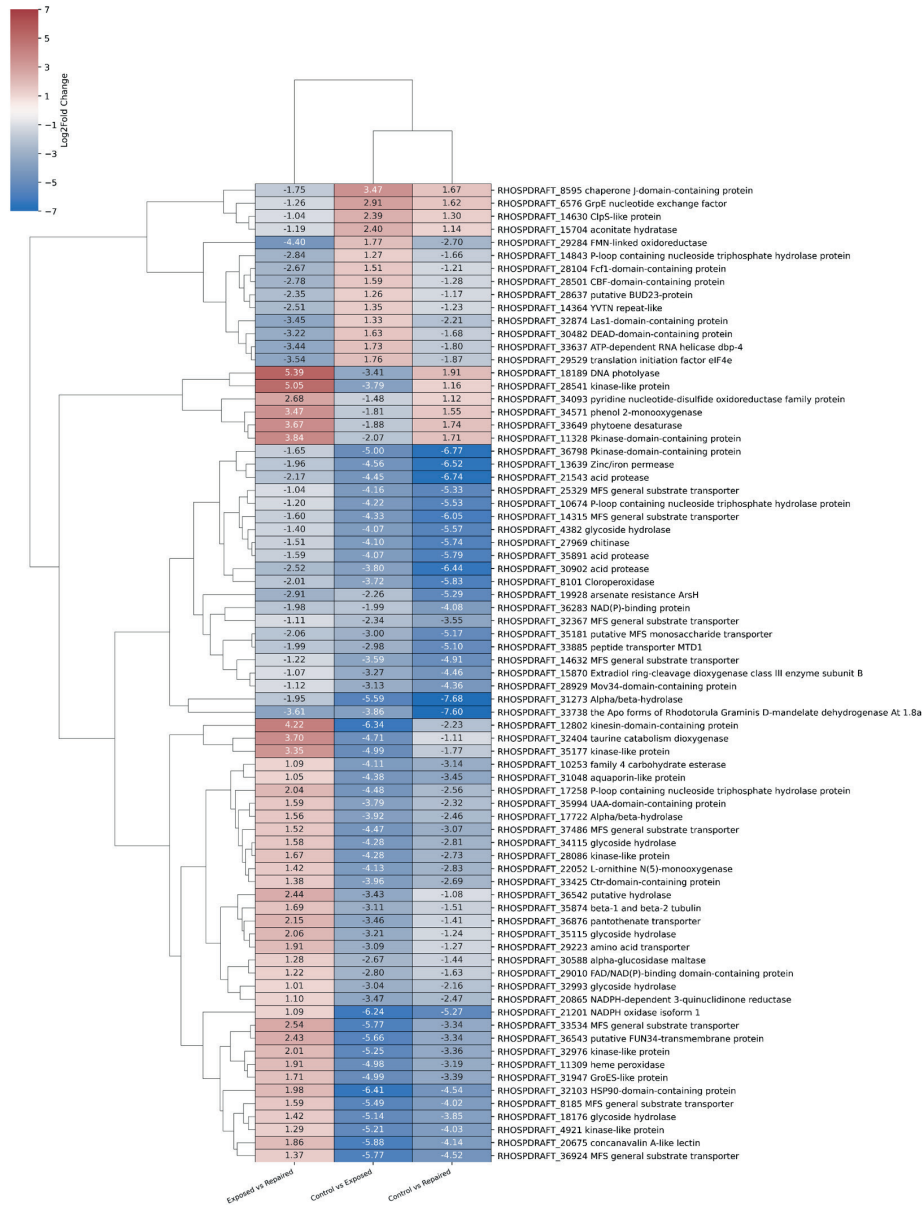


Fig. S4 Clustered heatmap of the Control vs Exposed of GO enrichment of the differentially regulated genes in the analysis by FungiFun3.

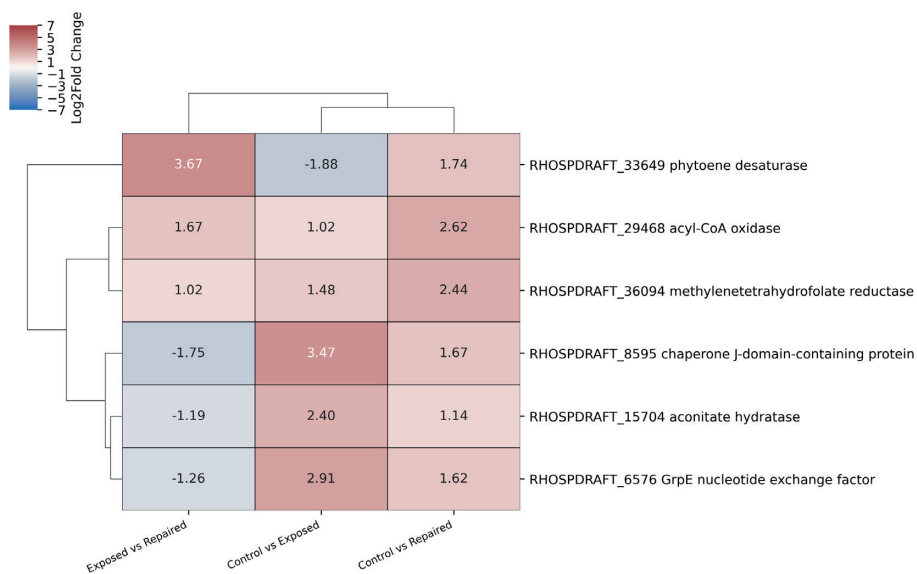


Fig. S5 Clustered heatmap of the Control vs Repaired of GO enrichment of the differentially regulated genes in the analysis by FungiFun3

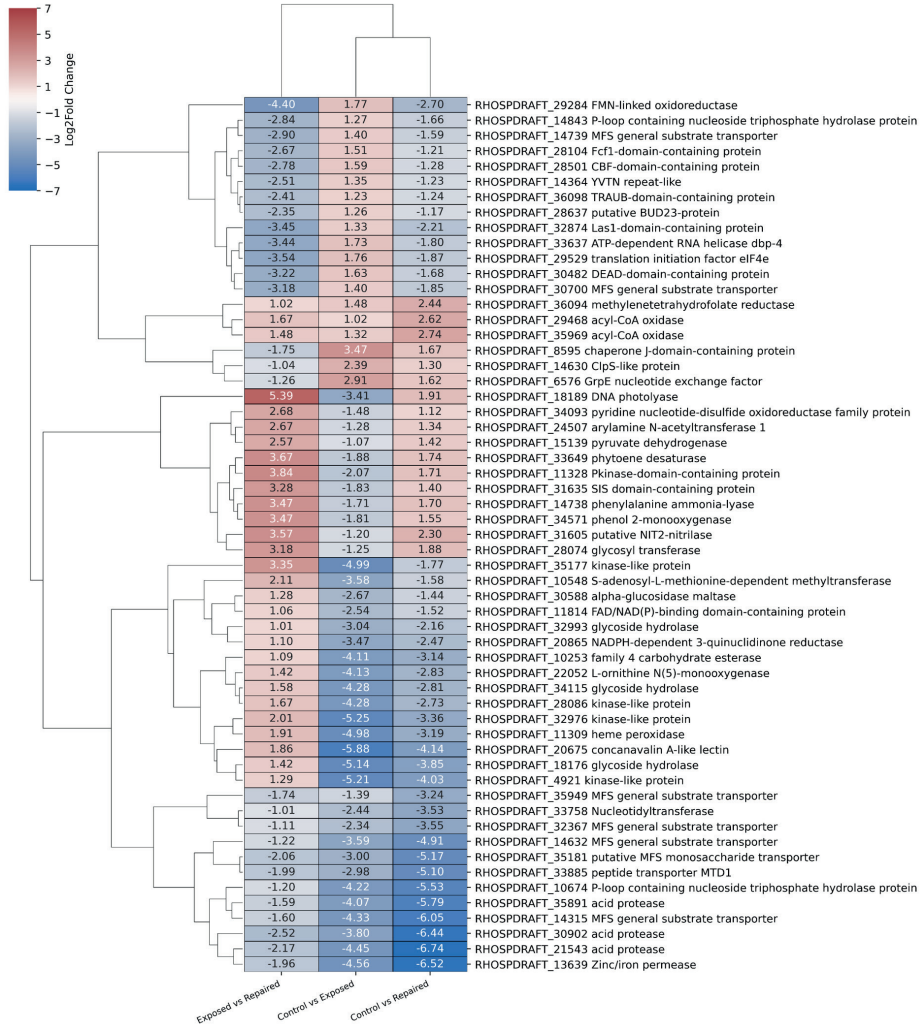


Fig. S6 Clustered heatmap of the Exposed vs Repaired of GO enrichment of the differentially regulated genes in the analysis by FungiFun3

Chapter 8

General Summary and Discussion

The research presented in this thesis provides new insights into microbial survival and behavior in space. Our studies have elucidated how non-fastidious bacteria exposed to Mars-like conditions interact with the human immune system. We also examined the survival of microbial environmental isolates under icy moon conditions, highlighting how the resilient *R. frigidicola* regulates its gene expression to endure extreme environments. The main research questions and the key findings of each chapter are summarized in Table 1. Furthermore, our studies identified areas that require further investigation to ensure crew and planetary safety during future space missions.

Table 1: Summary of the major research questions and key findings of the thesis for each chapter.

Chapter	Research question(s)	Key finding(s)
2	Are human bacterial pathogens capable of surviving Mars-like conditions?	All bacteria investigated tolerate Mars-like conditions, with <i>S. marcescens</i> and <i>K. pneumoniae</i> exhibiting the highest levels of resilience.
3	What is the immune response of Mars-adapted bacterial human pathogens?	1. Simulated Mars conditions influence bacterial morphology and size, impacting immune recognition. 2. Regrowth in media restores immune response after exposure to Mars conditions.
4	What are the dangers of Lunar and Martian regolith inhalation?	Acute regolith exposure impairs bronchial tight junction integrity. Chronic regolith exposure compromises epithelial barrier function and promotes inflammation. Regolith induces more cell death and cell line organization compared to Earth silica.
5	How do psychrotolerant organisms tolerate extreme space conditions?	Overall, the selected yeasts exhibited more tolerance to extreme conditions than the bacterial species tested.
6	Does the intracellular Mn/Fe ratio play a role in ionizing radiation survival in all domains of life?	Intracellular Mn/Fe ratio does not correlate with tolerance to ionizing radiation survival in all microorganisms and species-specific survival strategies vary.
7	What mechanisms of survival does <i>R. frigidicola</i> employ when exposed to simulated icy moon conditions?	<i>R. frigidicola</i> halts cell cycle progression to allow for DNA repair. The findings indicate that a number of repair mechanisms are active when the yeast is desiccated.

Part I – How do human related microorganisms tolerate space conditions and interact with the human immune system?

In **Chapter 2** we evaluated the survival of non-fastidious bacterial pathogens under simulated Mars conditions. Previous studies demonstrated that the investigated bacterial species were capable of growing in minimal media supplemented with

a single carbon source, mimicking oligotrophic extraterrestrial environments (1). Our research extended these findings, showing that these species showed tolerance to Mars-like conditions to a significant extent, thereby underscoring the importance of studying non-extremophilic microbial survival and addressing the human associated pathogens knowledge gap highlighted by the PPP (2). Given the presence of pathogenic microorganisms in the human microbiome, and the associated transport risk during upcoming crewed Mars mission, this investigation was particularly warranted (3, 4). Our study also showed that supplementing growth media with Mars regolith simulant enhanced bacterial tolerance especially to desiccation. This enhanced desiccation tolerance could enable the survival and dissemination of pathogens on Mars during dust storms and wind events (5, 6). Consequently, our research reinforces the need for crewed Mars exploration to be strictly limited to areas with low potential for supporting extant Martian life, such as regions outside polar regions or zones without caves or liquid water, consistent with existing planetary protection guidelines (7, 8).

This study also found that Mars regolith enhanced bacterial survival under UV radiation and simulated Martian atmospheric conditions. Although the investigated pathogens were more sensitive than extremophiles to the Mars-like conditions (9-11), they remained viable for 24 hours, a period sufficient for astronauts to come into contact with the bacteria during EVAs (extravehicular activity) or other activities. Contact with pathogens could occur during waste disposal procedures or astronaut interaction with surfaces on which microorganisms easily persist. Given this remarkable survival, we concluded that additional evaluations of other (bacterial) pathogens are needed, including assessments of immune responses to bacteria exposed to Mars-like conditions.

For this reason, we investigated the immune responses of PBMCs stimulated with Mars-adapted *K. pneumoniae* and *S. marcescens*, as described in **Chapter 3**. Addressing the implications of bacterial survival, highlighted in chapter 2, was essential to understand host-pathogen interactions under putative space conditions. This is particularly relevant as even a brief spaceflight is known to dysregulate human immune function (12). Our data showed that the production of cytokines by PBMCs was altered, including reduced IL-17 and IL-10 induction by *K. pneumoniae*, and decreased IL-1 β and TNF α production by *S. marcescens*. ROS secretion was also affected with reduced ROS in response to desiccated bacteria. In addition, phagocytosis of *S. marcescens* by CD11b $^{+}$ cells was impaired potentially limiting pathogen clearance (13). The chapter emphasizes the importance of

human health and immune response during space missions, one of the knowledge gaps highlighted by the PPP.

Morphological analyses of bacteria exposed to Mars-like conditions showed reduced cell size and altered complexity. Notably, the reduction in bacterial cell size persisted after regrowth, while complexity returned to normal, suggesting that Mars-like conditions induced morphological changes may impair immune recognition and clearance of the bacteria (14). After regrowth in standard medium, bacteria exposed to conditions other than desiccation showed partial restoration in immune responses, including the production of IL-10, TNF α and IL-1RA by PBMCs. An exception was desiccation causing long-term changes in bacterial interactions with immune cells, suggesting that it is the most detrimental condition for immune recognition. Given that bacterial spread on Mars would inevitably involve desiccation, these findings underscore the importance for further studies. Additional bacterial species should be investigated under Mars-like conditions, and these studies should be integrated with *in vitro* and *in vivo* models to comprehensively evaluate host-pathogen interactions.

The Apollo missions highlighted the health risks associated with regolith inhalation. For this reason, in **Chapter 4** we investigated the effects of grinded Lunar and Martian regolith on human airway epithelial cells and PBMCs using both *in vivo* and *in vitro* models. While previous studies addressed some of the effects of regolith on immune cells, a comprehensive evaluation of these effects was missing (15). Our *in vitro* investigation made use of air-liquid interface cultures of human bronchial epithelial cells exposed to regolith simulants, while *in vivo* experiments, mice were subjected to intranasal airway inhalation. The analysis revealed that regolith simulant exposure leads to production of pro- and anti-inflammatory cytokines, and that the Lunar and Mars regolith caused more cytotoxicity compared to Earth-type silica. Additionally, repeated exposure caused more epithelial barrier damage and inflammation, with Martian and Lunar regolith showing the strongest barrier-disruptive effects, particularly Lunar simulant. This suggests that regolith-associated dangers are more pronounced with repeated exposures, as previously suggested by Pohlen, Carroll (16). Furthermore, while exposure to SiO₂ reduced mRNA expression of tight junction proteins, regolith simulants additionally increased fibrosis markers. All regolith types and silica induced neutrophilia and enhanced recruitment of eosinophils, dendritic cells and T-cells in murine models.

The findings from this study underscore the risks that regolith exposure poses to airway integrity and immune health (17). Despite these adverse health effects,

regolith remains a valuable resource for plant growth, food production (18, 19) and *in situ* resource utilization (20-22) when combined with microorganisms in Lunar or Martian settlements, applications that could be useful to reduce resupply demands from Earth. Nonetheless, effective airborne regolith mitigation strategies will be essential on the Moon and Mars to protect the health of the astronauts while enabling safe planetary exploration and resource processing. Such strategies will need to be further tackled to address the knowledge gap with risks associated to sample return missions identified by the PPP.

Part II – How do terrestrial microorganisms survive icy moon conditions?

Part II of this thesis, having focused on the icy moons of Jupiter and Saturn, has shifted the attention from human associated to environmental microorganisms due to the unlikely human presence past Mars in all future planned space missions. Environmental candidates were selected due to their innate predisposition to space survival through tolerance to extreme conditions in comparison with their human associated counterparts. Therefore, in **Chapter 5**, we investigated the survival limits of different psychrotolerant bacteria and yeasts under extreme conditions. These microorganisms play a key role in astrobiology research, as assessing their tolerance to extreme conditions helps to elucidate planetary habitability and planetary protection requirements (23-26). Exposing the microorganisms to desiccation, UV and X-ray radiation as well as freezing and thawing cycles revealed that the yeasts we selected are generally more resilient than the bacteria that were tested. For example, under specific icy moon assumptions, yeasts could withstand UV radiation for a number of hours, whereas the bacterial species tolerate UV only for minutes. Astrobiological studies on yeast have so far only been conducted exclusively with *Saccharomyces cerevisiae* and *Candida albicans*. However, our findings show that other species, such as *R. frigidicola* and *R. mucilaginosa*, offer valuable new insights into the limits of life under simulated planetary conditions.

These results emphasize the need for further investigations on such eukaryotic microorganisms to better define habitability constraints and identify unique survival mechanisms (27-29). Nonetheless, psychrotolerant bacterial and archaeal species with similar growth capabilities should not be excluded as they could also be biological contaminants. Finally, our findings support the targeted identification of yeasts and other psychrotolerant microorganisms in spacecraft assembly facilities (SAFs) to better assess forward contamination risks during missions to the icy moons.

In **Chapter 6** we investigated the role of intracellular manganese (Mn) and iron (Fe) as radiation tolerance mechanisms in microorganisms. Despite extensive research showing the detrimental effects of space ionizing radiation, most early studies focused exclusively on bacterial species as model microbial systems (30, 31). Recent work, has explored the role of intracellular Mn and Fe in microorganisms as innate strategy to support microbial radiation tolerance (32). However, such capabilities have not been explored across all domains of microbial life, rather only select radiation-resistant microorganisms have been studied. Our research aimed to determine whether this survival trait is shared across domains of life or restricted to specific species. Therefore, we analysed 21 microorganisms including bacteria, archaea and yeasts, and quantified the intracellular Mn and Fe concentrations. We then compared these metals concentrations with D_{10} values (X-ray) and found that, although intracellular Mn/Fe ratios reflected trends reported in the literature, high metal concentrations did not consistently correlate with higher D_{10} values across all microorganisms. Our results do not exclude the role of Mn and Fe in radiotolerance, instead they show that this survival mechanism is not universal across domains of life but rather a characteristic of select microbial species. Microorganisms with low intracellular Mn/Fe appear to rely on alternative strategies such as metabolic type (autotrophic vs. non-autotrophic growth), growth temperature adaptations, reactive oxygen species (ROS) scavenging abilities, or enhanced DNA repair systems, to tolerate ionizing radiation (33, 34). Overall, while several survival traits are shared among microorganisms, the environments where microorganisms in which they reside likely exert a stronger influence on their adaptive responses. As such, future studies addressing strain-specific survival strategies are needed to evaluate whether such shared mechanisms are being similarly used.

Finally, after having uncovered *R. frigidicola*'s remarkable survival to extreme conditions in chapter 6, in **Chapter 7** we investigated the transcriptional adaptation of this yeast after exposure to icy moon conditions. Our research exposed *R. frigidicola* to 7 days of desiccation, 750 Gy of X-ray radiation and 7500 J/m² of polychromatic UV radiation. To identify differentially expressed genes associated with survival mechanisms, RNA was isolated prior, during and after exposure and repair to icy moon-like conditions. Studying microbial survival under multifactorial stress conditions is essential to deepen our understanding of forward contamination risks for celestial bodies such as Mars and the icy moons. This is also important to enable an updated understanding of microbial persistence on spacecraft surfaces both before and during flight which addresses the knowledge gap described by the PPP (8, 26, 35).

Our results revealed that the largest difference in gene expression takes place between the *Exposed* and *Repaired* samples, indicating a high level of activity being induced under both conditions. Furthermore, the gene set enrichment analysis highlighted that in the *Exposure* condition the yeast upregulates genes responsible for catalytic and DNA repair to increase survival (36, 37). Additionally, the yeast halts cell cycle progression to prioritize repair functions, a strategy also employed by other organisms under stress (38, 39). The potential role of yeasts and other psychrophilic microorganisms in planetary habitability is particularly noteworthy, as such organisms can inform our understanding of the limits of life in extraterrestrial environments.

Implications for space missions

Overall, the study of human health and microbial survival in space, especially during long-term missions, must be further expanded. This includes ground-based simulation studies combining the effects of space conditions on the immune system and its interaction with (opportunistic) pathogens. While the research presented in this thesis explored how space conditions affect microbial biology and their interaction with immune cells, a broader evaluation of how these conditions influence the gut microbiome, alone or in combination with regolith inhalation, will provide essential insights into the physiological implications of long-duration human residence on other Solar System bodies. In parallel, experimental models should also be developed to assess the long-term effects of exposure to space conditions using both *in vitro* and *in vivo* systems.

Immune system interaction with pathogens and external material in space

Chapters 2 and 3 described the survival and immune response of pathogens exposed to Mars simulated conditions. During future crewed Mars missions, the contamination of Mars with bacteria will be all but ensured, however, the spread of such microorganisms may occur through different pathways depending on the tasks performed by astronauts. The spread of human-originating bacteria on Mars can occur in different ways including through the contact with surfaces, improper disposal of biological waste, or airborne transport (40). In chapter 2, despite having determined the survival of bacteria on Mars, we did not address how their dissemination might occur. Research into the different ways through which bacteria will be exposed to Martian conditions depending on the way they spread might provide valuable mission-specific antimicrobial strategies. This is especially important when increased number of crew members will travel and waste production will increase. Identifying an appropriate manner of waste disposal is a

key challenge for human settlements which will need to be addressed to ensure the safety of the crew and that of planetary protection as identified by the PPP.

First, understanding how microorganisms spread in environments where multiple individuals live for extended periods within confined spaces is of critical importance for space travel (41). This is particularly relevant given that as observed on the ISS, human-associated (opportunistic) pathogens, including *K. pneumoniae*, have been detected (42-44). Ongoing microbial monitoring campaigns in SAFs and aboard the ISS aim to track microbial load and identify novel isolates ensuring the up-to-date status of planetary protection requirements (43, 45). Unlike spacecraft surfaces, humans cannot be sterilized, meaning that bacteria will inevitably accompany crewed missions to Mars.

Second, the immune system is known to be influenced by psychological stressors such as mission-related stress, sleep deprivation, and circadian rhythm disruption (46). Therefore, studying how PBMCs exposed to space-like ionizing radiation respond to bacterial stimulation could provide valuable insights into functional immune responses during space travel and will address the response of the immune system to deep space knowledge gap described by the PPP. Although the effects of radiation on PBMCs have been investigated, most existing studies focus on patients undergoing radiotherapy (47, 48). This research direction combined with the results of this thesis could thus advance our understanding of bacterial-immune system interactions in space and help inform potential countermeasures or treatment strategies.

From the first crewed Moon landing in 1969 with Apollo 11, until the last in 1972 with Apollo 17, Lunar regolith has shown to affect electrical, mechanical, and human biology systems (49, 50). Adverse effects of regolith on any of these systems during future missions, when more frequent EVAs might be needed, could compromise the success of the mission. Although engineering-based regolith mitigation strategies have been developed, the risks to human health remain significant (51, 52). As outlined in chapter 5, exposure to regolith simulants caused epithelial barrier disruption and decreased expression of tight junction proteins in human cell lines, lead to increased inflammation. Mitigating such effects on the Moon will be challenging not only because of the repeated exposure, but also because space conditions contribute to immune dysregulation and impaired wound healing (53). Regolith-induced epithelial damage could compromise the body's first line of host defense, potentially enabling bacterial infections following regolith inhalation, especially since this thesis has shown how pathogen survival is

increased in the presence of regolith. Inhalation of contaminated regolith particles by crew members with damaged epithelial barriers, combined with a dysregulated immune system, could therefore result in more severe infections and inflammation-mediated responses. Of particular concern are infections with *K. pneumoniae* and *S. marcescens*, two bacterial species well adapted to causing lung infections and already identified inside MIR and ISS (54-57). Understanding how such infections might develop through *in vivo* modelling will be essential to devise appropriate treatment and prevention strategies.

Microbial survival and planetary contamination

Transport of microorganisms to space undoubtedly started with the first human-made object to reach space. However, the concern with contamination only started with spacecraft targeting celestial bodies of astrobiological interest. Therefore, the decontamination of spacecraft is now mandated for all missions targeting celestial objects with a chance to harbor life (e.g. Mars or the icy moons). Nonetheless, decontamination efforts at the start of the space age were far less effective than those currently employed. Spacecraft decontamination started with NASA's Ranger program which utilized ethylene oxide and heat (>135°C) to decontaminate the vehicles destined for the Moon. However, current planetary protection requirements for our natural satellite are not as stringent as for other celestial bodies since no indigenous life is present (58, 59). Despite current planetary protection guidelines not requiring severe decontamination of spacecraft set for the Moon, past ineffective techniques might have enabled the transport of a higher biological load than currently accepted. This can also be the case for Mars or other celestial bodies; therefore, it is especially important to investigate newly identified poly-extremotolerant microorganisms to refine and update decontamination techniques for the effective preservation of extraterrestrial locations. This is especially true since maintaining the original environment of another planet is essential to avoid compromising present or future life detection efforts. For these reasons, the research in part two of this thesis has focused on the survival of microorganisms isolated from extreme Earth environments and has investigated the mechanisms they employ in order to survive the extreme conditions of space.

For this aim in chapter 5, we selected six different psychrophilic and psychrotolerant microorganisms isolated in icy moon-analogue environments on Earth to evaluate their limits of life, as they could pose the highest risk to the forward contamination of the icy moons (8). Here we found that the yeasts were more tolerant than the investigated bacteria and that the ubiquitous presence of yeasts of the *Rhodotorula* genus, explains the presence eukaryotic microorganisms in the ISS and SAF (60-64).

Additionally, the opportunistic pathogen role of *R. mucilaginosa* and the plasticity of the species of the *Rhodotorula* genus to industrial and ISRU purposes, makes yeasts research essential for astrobiology (65-72).

Understanding how life survive extreme conditions has long motivated investigations, especially in astrobiology. Such research can expand our knowledge of habitability within the Solar System and beyond, while also aiding the identification of past life on other planets. Experiments in this field are inherently complex not only because of the technical challenges but also due to the high operational costs of simulation facilities. Most microbial survival mechanisms are linked to the environments in which microorganisms naturally reside, and the adaptation they evolved in response. However, certain microbial species are very well adapted to conditions absent from their native habitats. For example, microorganisms that grow at high temperatures ($>45^{\circ}\text{C}$) often display increased resistance to ionizing radiation. This is observed in multiple domains, including archaea, such as the hyperthermophile *Ignicoccus hospitalis* which has a D_{10} value (X-ray) of 4.7 kGy, as well as in bacteria and eukaryotes investigated in this thesis (73-75). Notably, our research also demonstrated that microorganisms adapted to low temperatures, such as *R. frigidalcoholis* can survive, high doses of ionizing radiation highlighting the diversity of microbial survival strategies. These survival characteristics enable the development of models to estimate the relevant bioburden of spacecraft throughout their missions, including during assembly and cruise (76-78). Some of the cruise stage bioburden models do not take into account the effects of ionizing radiation. Nonetheless, the results show the high extent of microbial viability reduction on external spacecraft surfaces. However, microorganisms residing on internal surfaces could remain viable and survive the cruise. Studying these microbial survival mechanisms thus provides insights into the vast differences in resilience across domains of life, genera, species and even individual strains.

Beyond this thesis

The research presented in this thesis advances our understanding of how space travel affects human-relevant microorganisms and their interaction with the host immune system, as well as microbial resistance to extreme conditions relevant for icy moon environments.

First, it identifies microbial candidates capable of surviving space conditions, highlighting those with a high risk of contaminating other Solar System bodies. However, additional microbial candidates should be investigated in order to have an improved understanding of microbial survival to space conditions.

Second, by studying how human pathogens interact with the immune system under simulated space conditions, the work provides insights into potential disease pathophysiology during space travel. Nonetheless, additional pathogens should be investigated and the research should be expanded to *in vivo* models to have a broader overview of disease progression in space.

Third, studying the effects of Lunar and Martian regolith inhalation in animal models offering valuable insights on the acute and chronic effects to astronauts in settlements. Future studies with similar approaches should investigate additional regolith simulants composition to inform damage-prevention measures in different settlement locations on the Moon or Mars.

Finally, this thesis describes that even non-extremophilic microorganisms can survive extraterrestrial environments, it also emphasizes the need to expand astrobiological studies to fungal candidates as well. Such research is critical for preserving human health, investigating planetary habitability, and developing *in situ* resource utilization techniques. Nonetheless, the innate survival mechanisms employed by microorganisms under the exposure conditions of this thesis should be explored further. Microbial dormancy as well as biofilm formation are examples of mechanisms employed by microorganisms to tolerate changes to environmental conditions. Furthermore, the production of pigments both by the candidate microorganisms in the first and second part of this thesis warrant further investigation as it enables the physical shielding against UV and ionizing radiation.

The findings of this thesis are well adapted and tackle directly immune health and planetary protection knowledge gaps highlighted by the COSPAR PPP and that of space agencies. Furthermore, our findings reinforce the importance of microbial analysis and pathophysiology especially in the context of crewed missions. While uncrewed missions will dominate deep space exploration in the near future, human travel to the Moon and Mars is expected to increase in the coming decades. Ensuring astronaut health will remain a top priority for mission success. The research of this thesis has highlighted the importance of studies of microbial survival in extreme environments and their interaction with the human host, paving the way for future investigations into microbe-host dynamics during space travel.

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Appendices

Appendix I Thesis Summaries

NEDERLANDSE SAMENVATTING

Deel I - Hoe overleven ziekteverwekkende micro-organismen de omstandigheden in de ruimte, en hoe reageert ons immuunsysteem op micro-organismen die aan deze extraterrestriale omstandigheden zijn blootgesteld?

In eerste instantie hebben we ons gericht op bacteriële opportunistische pathogenen die zich gemakkelijk aanpassen aan hun omgeving, vaak in het milieu voorkomen en met name in mensen met een verzwakt immuunsysteem ziekte veroorzaken. De implicaties van hun overleving onder gesimuleerde Mars-omstandigheden worden besproken in **Hoofdstuk 2**. Nadat we hadden vastgesteld dat er in het ruimteonderzoek gegevens ontbreken over de overleving van niet-extremofiele menselijke pathogenen, hebben we deze kennislacune onderzocht. Bovendien wordt het immuunsysteem tijdens ruimtereizen ontregeld door de impact van de ruimteomstandigheden, waardoor het nog belangrijker wordt om de overleving van menselijke pathogenen te onderzoeken. Eerder onderzoek toonde aan dat de organismen die in dit hoofdstuk werden onderzocht, *K. pneumoniae*, *S. marcescens*, *B. cepacia* en *P. aeruginosa*, in staat zijn om te groeien in minimale media aangevuld met één enkele koolstofbron. Daarom richtte ons onderzoek zich op de overleving van de organismen die in minimale media waren gekweekt na blootstelling aan perchloraten, Mars-regoliet simulant, uitdroging, UV-straling en Mars atmosfeer. Ons onderzoek toonde aan dat alle bacteriën in staat waren om de omstandigheden van een Mars-simulatie te overleven. Van alle bacteriën waren *S. marcescens* en *K. pneumoniae* echter de meest productieve soorten. We ontdekten ook hoe het aanvullen van de groeimedia met Mars-regoliet simulant de bacteriële groei en overleving onder alle geteste omstandigheden verbeterde. Hoewel de bacteriën gevoeliger zijn voor de extreme omstandigheden dan andere extremofiele organismen, verhoogt hun overleving het risico dat astronauten ermee in contact komen tijdens een ruimtemissie. De overleving van de in dit hoofdstuk onderzochte bacteriële soorten vormde een belangrijke opmaat voor het onderzoek naar de reactie van het immuunsysteem op deze bacteriën, zoals beschreven in het volgende hoofdstuk.

In **Hoofdstuk 3** gebruikten we de overlevingsgegevens uit het vorige hoofdstuk om de immunologische reacties gemeten aan de hand van perifere bloedmononucleaire cellen (PBMCs) op aan Mars aangepaste *K. pneumoniae* en

S. marcescens te identificeren. Dit is essentieel omdat inzicht in deze specifieke interacties tussen gastheer en ziekteverwekker helpt bij het inschatten van de potentiële gevaren voor astronauten. PBMCs die gestimuleerd werden met de aan Mars aangepaste bacteriën vertoonden een veranderde cytokinesecretie, veranderingen in de reactieve zuurstofradicaal (ROS)-productie en in de fagocytosesnelheid. De afscheiding van cytokinen werd veranderd met verlaagde IL-17 en IL-10 niveaus voor *K. pneumoniae* en verlaagde IL-1 β en TNF α niveaus voor *S. marcescens*. Blootstelling van de bacteriën aan uitdroging verminderde ook de ROS-productie door PBMCs voor beide soorten. In plaats daarvan werd fagocytose door CD11b+ cellen verminderd voor *S. marcescens*, maar verhoogd voor *K. pneumoniae*. Met behulp van flowcytometrie en transmissie elektronen microscopie (TEM)-metingen identificeerden we bacteriën met een verminderde celgrootte na blootstelling aan uitdroging en UV. Deze resultaten toonden aan dat de Mars-condities de bacteriële morfologie beïnvloeden en de herkenning en klaring door het aangeboren immuunsysteem belemmeren. Toen we de bacteriën na blootstelling opnieuw in kweekmedia plaatsten, zagen we echter een gedeeltelijk herstel van de immuunrespons. Zo werd IL-10 hersteld tot controlelevel voor *K. pneumoniae*, terwijl TNF α en IL-1RA hersteld werden voor *S. marcescens*. Deze resultaten laten zien hoe de door Mars-condities geïnduceerde veranderingen de bacteriële morfologie en immunologische interactie beïnvloeden.

De Apollo-missies onthulden de gevaren van het inademen van regoliet (het losse 'oppervlaktegrondmateriaal' van een planeet) na 'extravehicular activities' (EVAs) op het maanoppervlak. Daarom onderzochten we in **Hoofdstuk 4** zowel *in vitro* als *in vivo* de gevaren van het inademen van regoliet op de Maan, met behulp van Maan en Mars-regoliet. We gebruikten drie commercieel verkrijgbare regoliet simulanten als monsters en siliciumdioxide als controle. Het *in vitro* onderzoek maakte gebruik van een model van primaire menselijke bronchiale epitheelcellen gekweekt in de lucht-vloeistof fase om het *in vivo* epitheel na te bootsen. Deze epitheelcellen werden blootgesteld aan de regoliet simulanten. In plaats daarvan gebruikten *in vivo* studies muizenmodellen met intranasale inhalatie van regoliet. De *in vitro* modellen toonden aan dat acute blootstelling aan regoliet simulanten de integriteit van de nauwe verbindingen in het bronchiale epitheel aantasten. Ook chronische blootstelling aan regoliet simulanten brengt de barrièrefunctie in gevaar en bevordert ontstekingen. Bovendien leidt blootstelling van keratinocyten aan Mars-regoliet tot een hogere afgifte van pro-inflammatoire cytokinen. We zagen ook dat alle regoliet simulanten celdood induceren in A540 (longepitheel) cellijnen, waarbij Maan-regoliet de hoogste mate van celdood bleek te veroorzaken. Verder ontdekten we hoe regoliet simulanten de normale organisatie van Detroit562

(farynxepitheel) cellijnen verstoren, terwijl silica dat niet doet. De intranasale toediening van regoliet schaadt de functie van bronchiale epitheelcellen en veroorzaakt ontstekingen bij muizen. Het cytotoxische effect van maan-regoliet en de ontstekingsbevorderende werking van Mars-regoliet laten de verwachte gevaren van regoliet inhalatie zien. De gegevens uit dit hoofdstuk, samen met de kennis over het overleven van pathogenen en veranderingen in hun interactie met het immuunsysteem bij contact met de gastheer, ondersteunen verder onderzoek op dit gebied en de voorbereiding op dergelijke risico's voor astronauten.

Deel II - Hoe overleven aardse micro-organismen de ijzige maanomstandigheden?

In **Hoofdstuk 5** onderzochten we de overlevingsgrenzen van psychrotolerante micro-organismen, waaronder bacteriën en gisten, onder extreme omstandigheden om hun relevantie voor de planetaire bescherming van ijzige manen te begrijpen. In deze studie selecteerden we micro-organismen uit analoge omgevingen van ijzige manen op aarde. De geselecteerde organismen waren twee bacteriële soorten en twee gistsoorten. De experimentele opzet omvatte de blootstelling van de micro-organismen aan uitdroging, UV- en röntgenstraling en vries- en dooicycli om hun overlevingslimieten te bepalen. Onze gegevens toonden aan dat de onderzochte gisten toleranter zijn dan de bacteriën voor alle blootstellingsomstandigheden.

In **Hoofdstuk 6** bestudeerden we de mechanismen van stralingstolerantie bij micro-organismen met behulp van intracellulaire concentraties mangaan (Mn) en ijzer (Fe). Uit literatuurgegevens bleek dat sommige micro-organismen met hoge intracellulaire concentraties Mn en Fe een verhoogde tolerantie hebben voor ioniserende straling. Het doel van dit hoofdstuk was om te bepalen of dit voor alle levensdomeinen geldt. Daarom hebben we de intracellulaire Mn- en Fe-concentraties gemeten in 21 micro-organismen, waaronder archaea, bacteriën en gisten met behulp van 'Inductively Coupled Plasma Mass Spectrometry' (ICP-MS). We vergeleken de concentratie van deze metalen met de D_{10} -waarde (röntgenstraling) en ontdekten dat sommige organismen, ondanks het feit dat ze hoge niveaus van ioniserende straling verdragen, lage concentraties Mn en Fe hadden. De conclusies uit dit hoofdstuk, sluiten de Mn/Fe-verhouding niet uit als mechanisme voor stralingstolerantie, maar laten juist zien dat micro-organismen verschillende mechanismen voor stralingstolerantie hebben.

De overleving van de gisten onder extreme omstandigheden in hoofdstuk 5 leidde tot het onderzoek naar differentieel tot expressie komende genen voor overleving. Daarom isoleerden we in **Hoofdstuk 7** het RNA van de gist *R. frigidicola* na

blootstelling aan ijzige maanomstandigheden. 'Icy moons' (ijzige manen) zijn natuurlijke satellieten van planeten waarvan het oppervlak voornamelijk uit ijs bestaat, vaak met een rotsachtige kern eronder. De blootstelling van de gist aan deze omstandigheden bestond uit 7 dagen uitdroging, 750 Gy röntgenstraling en 7500 J/m² polychromatische UV-straling. Om te identificeren welke genen differentieel tot expressie komen tijdens overleving, isoleerden we RNA van de gist op drie verschillende tijdstippen, voor, tijdens en na de blootstelling. Onze resultaten tonen aan hoe de gist ijzige, maanachtige omstandigheden kan verdragen en hoe het de expressie van een groot aantal genen reguleert om de ontstane schade te overleven. Het grootste verschil in pathway-activatie, vastgesteld door middel van genexpressieanalyse, werd waargenomen tussen de blootgestelde monsters en de monsters na herstel. Enkele van de belangrijkste genen die ge-upreguleerde genen die bijdragen aan de overleving van de gist, zijn genen die betrokken zijn bij katalytische activiteit en DNA-herstel. Tegelijkertijd toonde onze analyse ook aan hoe de gisten genen down-reguleren die te maken hebben met celcyclusprogressie, zodat er tijd is om de schade door de extreme omstandigheden te herstellen. De resultaten van deze studie laten zien hoe tolerant gisten zijn voor extreme omstandigheden, hoe relevant ze zijn voor bewoonbaarheidsstudies en voor de planeetbescherming om besmetting van de ijzige manen te voorkomen.

ENGLISH SUMMARY

Part I - How do human-related microorganisms survive space conditions and then interact with the human immune system?

The effects of the survival of bacterial opportunistic, non-fastidious pathogens under simulated Martian conditions are discussed in **Chapter 2**. Having noted that space research lacks data on the survival of non-extremophile human pathogens, we investigated this knowledge gap. Furthermore, the immune system is dysregulated during spaceflight due to the effects of space conditions, further increasing the importance of studying the survival of human pathogens. Previous research has shown that the organisms studied in this chapter - *K. pneumoniae*, *S. marcescens*, *B. cepacia*, and *P. aeruginosa* - were able to grow in minimal media supplemented with a single carbon source. Therefore, our research focused on the survival of organisms grown in minimal media after exposure to perchlorates, Mars regolith simulant, desiccation, UV radiation, and the Martian atmosphere. Our research showed that all bacteria were able to survive the simulated Martian conditions. However, of all the bacteria, *S. marcescens* and *K. pneumoniae* were the most productive species. We also found that supplementing the growth media with Mars regolith simulant improved bacterial growth and survival under all tested conditions. Although the bacteria are more sensitive to the extreme conditions than other extremophilic organisms, their survival increases the risk of astronauts coming into contact with them during a space mission. The survival of the bacteria studied in this chapter supported the immune studies in the following chapter. Furthermore, the data from this chapter and the understanding of human immune responses to bacteria adapted to the Martian environment were the main focus of Part I of this work.

In **Chapter 3**, we used the survival data from the previous chapter to identify the immunological responses of PBMCs to Mars-adapted *K. pneumoniae* and *S. marcescens*. This is important because understanding the host-pathogen interaction in space will help guide treatment strategies during infection. PBMCs stimulated with the Mars-adapted bacteria showed altered cytokine secretion, changes in ROS production, and phagocytosis rates. Cytokine secretion was altered, with reduced IL-17 and IL-10 levels for *K. pneumoniae* and reduced IL-1 β and TNF α levels for *S. marcescens*. Desiccation of the bacteria also reduced ROS production by PBMCs for both species. While phagocytosis by CD11b+ cells was reduced in *S. marcescens*, it was increased in *K. pneumoniae*. Using flow cytometry and TEM measurements, we identified bacteria with reduced cell size and complexity after exposure to desiccation and UV. These results demonstrate that Martian conditions

affect bacterial morphology and impair recognition and clearance by the innate immune system. However, when we cultured the bacteria in media after exposure, we observed a partial recovery of the immune response. For example, IL-10 was restored to control levels for *K. pneumoniae*, and TNF α and IL-1RA were restored for *S. marcescens*. These results demonstrate how the changes induced by Martian conditions affect bacterial morphology and immune interaction. While this chapter underscores the need for further *in vitro* and *in vivo* models, we already highlight the interaction of bacteria with the immune system after Mars exposure.

The Apollo missions demonstrated the dangers of inhaling regolith after EVAs on the lunar surface. For this reason, in **Chapter 4**, we investigated the hazards of inhaling lunar regolith *in vitro* and *in vivo* using lunar and Martian regolith simulants. We used three commercially available regolith simulants as samples and silica as a control. For the *in vitro* investigation, cultures of human bronchial epithelial cells at the air-liquid interface with deposits of regolith simulants were used. Instead, the *in vivo* studies used mouse models with intranasal regolith inhalation. The *in vitro* models showed that acute exposure to regolith simulants compromises the integrity of bronchial epithelial tight junctions. Instead, chronic regolith simulant exposure impairs barrier function and promotes inflammation. Furthermore, exposure of keratinocytes to the Martian regolith leads to an increased release of pro-inflammatory cytokines. We also found that all regolith simulants induce cell death in A540 cell lines (lung epithelium), with lunar regolith being the most potent. Furthermore, we found that regolith simulants disrupt the normal organization of Detroit 562 cell lines (pharyngeal epithelium), whereas silicon dioxide does not. Intranasal administration of regolith impairs the function of the bronchial epithelium and triggers inflammation in mice. The cytotoxic effect of lunar regolith and the pro-inflammatory induction by Martian regolith demonstrate the expected hazards of regolith inhalation. The data obtained in this chapter, along with knowledge of pathogen survival and changes in the immune system's interaction with the host, support further research in this area and the preparation of astronauts for such risks.

Part II- How do terrestrial microorganisms survive icy moon conditions?

In **Chapter 5** we investigated the survival limits of psychrotolerant microorganisms including bacteria and yeasts to extreme conditions to understand their relevance for the planetary protection of the icy moons. In this study we selected microorganisms from icy moon analogue environments on Earth. The organisms selected for study were two bacteria and two yeasts. The experimental set up involved the exposure

of the microorganisms to desiccation, UV and X-ray radiation as well as freezing and thawing cycles to determine their survival limits. Our data showed that the investigated yeasts are more tolerant than the bacteria to all the exposure conditions.

In **Chapter 6** we studied the mechanisms of radiation tolerance in microorganisms using intracellular concentrations of manganese (Mn) and iron (Fe). Literature data showed how some microorganisms with high Mn and Fe intracellular concentrations have an increased tolerance to ionizing radiation. The aim of this chapter was to determine if this is the case for all domains of life. Therefore, we measured the intracellular Mn and Fe concentration by ICPMS in 21 microorganisms including archaea, bacteria and yeasts. We compared the concentration of these metals to the D_{10} value (X-ray) and found that some organisms despite tolerating high levels of ionizing radiation had low concentrations of Mn and Fe. The conclusions we drew from this chapter do not exclude the Mn/Fe ratio as a mechanism for ionizing radiation tolerance, rather we show how microorganisms have different radiation tolerance mechanisms.

The interesting survival of the yeasts to extreme conditions in chapter 5 prompted the investigation of differentially expressed genes for survival. Therefore, in **Chapter 7** we isolated the RNA of the yeast *R. frigidicola* after exposure to icy moon conditions. The exposure of the yeast to these conditions consisted in 7 days of desiccation, 750 Gy of X-ray radiation and 7500 J/m² of polychromatic UV radiation. To identify which genes are differentially expressed during survival, we isolated the RNA from the yeast at three different timepoints, before, during and after exposure. The results from our study showed how the yeast can tolerate icy moon-like conditions and how it regulates the expression of a high number of genes in order to survive the damage received. The largest difference in pathway activation identified by gene expression analysis was seen between the samples exposed and the ones which were repaired. Some of the most important genes which are upregulated and therefore responsible for the survival of the yeast are catalytic activity and DNA repair genes. At the same time our analysis also showed how the yeasts downregulate genes related to cell cycle progression to allow time for the damage by the extreme conditions to be repaired. The results from this study show how tolerant yeasts are to extreme conditions, how relevant they are for habitability studies, and for the focus of planetary protection in order to avoid the forward contamination of the icy moons.



DEUTSCHE ZUSAMMENFASSUNG

Teil I - Wie überleben, mit dem Menschen assoziierte, Mikroorganismen die Bedingungen im Weltraum und wie interagieren sie nach der Exposition mit dem menschlichen Immunsystem?

In **Kapitel 2** werden die Auswirkungen des Überlebens opportunistischer, nicht-fastidialer bakterieller Krankheitserreger unter simulierten Marsbedingungen untersucht. Nachdem festgestellt wurde, dass in der aktuellen Weltraumforschung Daten zum Überleben nicht-extremophiler humaner Krankheitserreger fehlen, adressiert das zugrunde liegende Projekt diese Wissenslücke. Frühere Forschungsergebnisse zeigen zudem, dass das Immunsystem während der Raumfahrt durch die Bedingungen des Weltraums moduliert und teilweise dysreguliert wird, was die Relevanz der Untersuchung des Überlebens menschlicher Krankheitserreger zusätzlich unterstreicht. Frühere Studien haben gezeigt, dass die in diesem Kapitel untersuchten Organismen *Klebsiella pneumoniae*, *Serratia marcescens*, *Burkholderia cepacia* und *Pseudomonas aeruginosa* in Minimalmedien wachsen können, die jeweils nur eine Kohlenstoffquelle enthalten. Das vorgestellte Projekt konzentrierte sich daher auf die Überlebensfähigkeit dieser Spezies unter Exposition gegenüber Perchloraten, Mars-Regolith-Simulanz, Austrocknung, UV-Strahlung und simulierten Marsatmosphärenbedingungen. Alle untersuchten Organismen überlebten die simulierten Marsbedingungen, wobei *S. marcescens* und *K. pneumoniae* die höchste Reproduktionsfähigkeit zeigten. Außerdem wurde beobachtet, dass sowohl Wachstum als auch Überleben verbessert waren, wenn die Organismen in Minimalmedium mit zugesetztem Mars-Regolith-Simulanz kultiviert wurden. Obwohl diese Organismen empfindlicher auf die simulierten Bedingungen reagieren als bekannte Extremophile, erhöht ihr Überleben das Risiko einer Exposition von Astronauten während Weltraummissionen. Die Überlebensdaten bilden die Grundlage für die Immunanalysen in **Kapitel 3**. Zusammenfassend liefert dieses Kapitel kritische Einblicke in die Anpassungsfähigkeit humaner Pathogene an extraterrestrische Umweltbedingungen und ihre potenzielle Bedeutung für die Immunantwort bei Astronauten.

In **Kapitel 3** werden die Überlebensdaten aus dem vorherigen Kapitel genutzt, um die immunologischen Reaktionen peripherer mononukleärer Blutzellen (PBMCs) auf Mars-adaptierte *K. pneumoniae* und *S. marcescens* zu identifizieren und zu analysieren. Dies ist relevant, da das Verständnis der Interaktionen zwischen Wirt und Erreger unter Weltraumbedingungen wesentlich zur Optimierung von Behandlungsstrategien während Raumfahrtmissionen beitragen kann. PBMCs, die den an den Mars angepassten Bakterien ausgesetzt wurden, zeigten eine veränderte

Zytokinsekretion, eine Modifikation der Produktion reaktiver Sauerstoffspezies (ROS) sowie Unterschiede in der Phagozytoserate. Die Veränderungen in der Zytokinsekretion äußerten sich in einer Abnahme von IL-17 und IL-10 bei Kontakt mit *K. pneumoniae* sowie in einer Reduktion von IL-1 β und TNF- α bei Kontakt mit *S. marcescens*. Zudem wurde für den Kontakt mit beiden Spezies eine verringerte ROS-Produktion durch PBMCs beobachtet, wenn die Mikroorganismen zuvor ausgetrocknet worden waren. Während die Phagozytose durch CD11b⁺-Zellen bei *S. marcescens* Kontakt reduziert war, zeigte der Kontakt mit *K. pneumoniae* eine gesteigerte Phagozytoserate. Mittels Durchflusszytometrie und Transmissionselektronenmikroskopie (TEM) wurden Bakterien mit verringerter Zellgröße und reduzierter Komplexität nach Austrocknungs- und UV-Exposition identifiziert. Diese Ergebnisse zeigen, dass die Marsbedingungen die bakterielle Morphologie beeinflussen und die Erkennung sowie Eliminierung durch das angeborene Immunsystem beeinträchtigen. Wurden die Bakterienstämme nach der Exposition erneut in Nährmedien kultiviert, konnte eine teilweise Wiederherstellung der Immunantwort beobachtet werden. So erreichte die IL-10-Antwort auf *K. pneumoniae* wieder das Kontrollniveau, während die TNF- α und IL-1RA Antworten auf *S. marcescens* ebenfalls wiederhergestellt wurden. Diese Ergebnisse verdeutlichen, dass Marsbedingungen sowohl morphologische Veränderungen als auch modifizierte Immunreaktionen induzieren und unterstreichen den Bedarf an erweiterten In-vitro- und In-vivo-Modellen.

Kapitel 4 präsentiert die Ergebnisse von In-vitro- und In-vivo-Untersuchungen zu den Gefahren der Inhalation von Regolith. Diese Risiken sind bereits seit den Apollo-Missionen der 1970er-Jahre bekannt. Die in diesem Kapitel beschriebenen Experimente untersuchten die Effekte von drei kommerziell erhältlichen Regolith-Simulanzien. Für die In-vitro-Analysen wurden Zelllinien humaner Bronchialepithelzellen in eine regolithhaltige Luft-Flüssigkeits-Grenzfläche eingebettet. In den In-vivo-Studien wurden Mäusemodelle mit intranasaler Regolith-Inhalation verwendet. Die In-vitro-Modelle zeigten, dass eine akute Exposition gegenüber Regolith-Simulanzien die Integrität der Tight Junctions der bronchialen Epithelzellen beeinträchtigt. Wiederholte oder dauerhafte Exposition führte zu einer zusätzlichen Störung der Barrierefunktion und förderte entzündliche Prozesse. Auch bei Keratinozyten konnte eine proinflammatorische Reaktion beobachtet werden. Die Exposition gegenüber Marsregolith resultierte in einer erhöhten Freisetzung proinflammatorischer Zytokine. Zudem wurde festgestellt, dass alle Regolith-Simulanzien den Zelltod in Lungenepithelzellen (A540-Zelllinie) auslösten, wobei der stärkste Effekt bei Mondregolith auftrat. Darüber hinaus zeigten die Experimente, dass Regolith-Simulanzien die normale Organisation

von Rachenepithelzellen (Detroit562-Zelllinie) stören, während das als Kontrolle genutzte Siliziumdioxid diesen Effekt nicht hervorrief. Die In-vivo-Studien belegten, dass die intranasale Verabreichung von Regolith die Funktion des Bronchialepithels beeinträchtigt und bei Mäusen Entzündungen induziert. Die zytotoxische Wirkung von Mondregolith sowie die proinflammatorische Induktion durch Marsregolith zeigen die potenziellen Gefahren der Regolith-Inhalation.

Die in den **Kapiteln 2–4** gewonnenen Daten bilden eine solide Grundlage für die Risikobewertung, die Vorbereitung von Astronauten und insbesondere für weiterführende Forschung auf diesem Gebiet.

Teil II - Wie überleben terrestrische Mikroorganismen die Bedingungen auf Eismonden?

In **Kapitel 5** wurden die Überlebensgrenzen psychrotoleranter Mikroorganismen, einschließlich Bakterien und Hefen, unter extremen Bedingungen untersucht, um deren Bedeutung für den planetaren Schutz der Eismonde zu bewerten. Für diese Studie wurden Mikroorganismen ausgewählt, die aus eismondähnlichen terrestrischen Habitaten stammen. Insgesamt wurden zwei Bakterien- und zwei Hefespezies untersucht. Der Versuchsaufbau zur Bestimmung der Überlebensgrenzen umfasste die Exposition der Mikroorganismen gegenüber Austrocknung, UV- und Röntgenstrahlung sowie wiederholten Gefrier- und Auftauzyklen. Die Ergebnisse zeigen, dass die untersuchten Hefen im Vergleich zu den Bakterien eine deutlich höhere Toleranz gegenüber allen getesteten Stressbedingungen aufwiesen.

Kapitel 6 untersucht die Mechanismen der Strahlungstoleranz bei Mikroorganismen auf Grundlage ihrer intrazellulären Konzentrationen von Mangan (Mn) und Eisen (Fe). Frühere Studien deuten darauf hin, dass Mikroorganismen mit erhöhten intrazellulären Mn- und Fe-Gehalten eine gesteigerte Resistenz gegenüber ionisierender Strahlung besitzen. Ziel dieses Kapitels war es, zu überprüfen, ob dieser Zusammenhang universell für alle Domänen des Lebens gilt. Hierfür wurden die intrazellulären Konzentrationen von Mn und Fe in 21 Mikroorganismen, darunter Archaeen, Hefen und Bakterien, mittels ICP-MS bestimmt. Anschließend wurde die Konzentration dieser Metalle mit dem D_{10} -Wert (Röntgenstrahlung) der jeweiligen Organismen korreliert. Die Analysen zeigten, dass einige Mikroorganismen trotz hoher Strahlungstoleranz nur geringe Mn- und Fe-Konzentrationen aufwiesen. Dies lässt den Schluss zu, dass das Mn/Fe-Verhältnis als möglicher, aber nicht alleiniger Mechanismus der Strahlungstoleranz zu betrachten ist. Die Ergebnisse verdeutlichen zudem, dass verschiedene Mikroorganismen unterschiedliche molekulare Strategien zur Strahlungstoleranz entwickelt haben.

Das in **Kapitel 5** beschriebene Überleben der Hefen unter extremen Bedingungen bildet die Grundlage für die in **Kapitel 7** dargestellte Transkriptomanalyse. Hierbei wurde die RNA der Hefe *Rhodotorula frigidicola* untersucht, nachdem sie simulierten Eismondbedingungen ausgesetzt war. Die getesteten Bedingungen umfassten sieben Tage Austrocknung, eine Dosis von 750 Gy Röntgenstrahlung und 7500 J/m² polychromatische UV-Strahlung. Um Veränderungen in der Genexpression im Zusammenhang mit Überlebensstrategien zu identifizieren, wurde RNA zu drei Zeitpunkten isoliert: vor der Exposition, unmittelbar danach und vier Stunden nach der Exposition. Die Ergebnisse zeigen die Aktivierung zahlreicher Gene, die an der Anpassung und dem Überleben unter extremen Umweltbedingungen beteiligt sind.

Die Transkriptomanalyse offenbarte die größten Unterschiede in der Genexpression zwischen den unmittelbar exponierten und den vier Stunden regenerierten Proben. Besonders stark hochreguliert waren Gene, die an katalytischen Prozessen und der DNA-Reparatur beteiligt sind. Gleichzeitig zeigte sich eine Herunterregulierung von Genen, die mit dem Fortschreiten des Zellzyklus assoziiert sind. Diese Anpassung ermöglicht es der Hefe, Zeit für die Reparatur strahleninduzierter Schäden zu gewinnen. Insgesamt verdeutlichen die Ergebnisse die außergewöhnliche Widerstandsfähigkeit von Hefen gegenüber extremen Umweltbedingungen, ihre Relevanz für Studien zur Bewohnbarkeit extraterrestrischer Habitate und ihre potenzielle Bedeutung im Kontext des planetaren Schutzes. Diese Erkenntnisse unterstreichen die Notwendigkeit, bei zukünftigen Explorationsmissionen das Risiko einer biologischen Kontamination der Eismonde gezielt zu minimieren.

RIASSUNTO ITALIANO

Parte I - In che modo i microrganismi legati all'uomo sopravvivono alle condizioni spaziali e poi interagiscono con il sistema immunitario umano?

Questo documento riassume la tesi che esplora i rischi per la salute umana e i rischi di colonizzazione batterica involontaria dei pianeti durante le missioni spaziali.

Gli argomenti principali sono suddivisi in sette capitoli di ricerca:

1. Sopravvivenza di batteri patogeni su Marte
2. Interazione tra batteri Marziani e sistema immunitario
3. Pericoli dell'inalazione di regolite
4. La sopravvivenza di batteri ambientali sulle lune di ghiaccio
5. L'ipotesi di un collegamento tra la concentrazione intracellulare di Mn/Fe contro la sopravvivenza alle radiazioni
6. Il metabolismo di un lievito durante la sopravvivenza sulle lune di ghiaccio

Capitolo 2

La nostra ricerca ha dimostrato che i batteri patogeni opportunisti come *Klebsiella pneumoniae*, *Serratia marcescens*, *Burkholderia cepacia* e *Pseudomonas aeruginosa* possono sopravvivere, in una certa misura, a condizioni marziane simulate. La loro crescita e sopravvivenza sono state potenziate dall'aggiunta di regolite marziana simulata al terreno di coltura. Sebbene la loro sopravvivenza sia inferiore a quella dei batteri estremofili, esiste un rischio potenziale di contaminazione. Il primo aspetto da considerare è che, nonostante i batteri selezionati abbiano avuto una sopravvivenza minore rispetto a batteri estremofili alle condizioni Marziane simulate, il rischio che gli astronauti vengano in contatto con questi o altri patogeni è una evenienza possibile. Il secondo aspetto da considerare sono le implicazioni che la sopravvivenza di batteri patogeni, in condizioni marziane simulate, potrebbero avere per la protezione planetaria durante le missioni spaziali umane.

La ricerca spaziale non dispone di dati relativi alla sopravvivenza di patogeni umani non estremofili; quello che però si conosce riguarda lo scombinamento del sistema immunitario umano durante i viaggi spaziali a causa delle differenti condizioni climatiche. Capire le interazioni tra ospite e patogeni è fondamentale per chiarire possibili insulti batterici durante missioni spaziali. Nel **Capitolo 3** Abbiamo studiato come il sistema immunitario umano risponde a batteri adattati alle condizioni marziane simulate. L'esposizione a tali condizioni altera la morfologia dei batteri, riducendone le dimensioni e la complessità. Questo compromette

il loro riconoscimento da parte del sistema immunitario umano, portando a una ridotta produzione di citochine infiammatorie e antinfiammatorie e a una minore produzione di specie reattive all'ossigeno (ROS). Abbiamo usato i dati relativi alla sopravvivenza dal capitolo 2 per identificare le risposte immunitarie delle cellule mononucleate del sangue periferico (PBMCs) contro *K. pneumoniae* e *S. marcescens*. La stimolazione di PBMCs con *K. pneumoniae* e *S. marcescens* adattati a condizione Marziane ha evidenziato come la produzione di citochine sia alterata; per esempio, con l'essiccazione la produzione di citochine infiammatorie ed antinfiammatorie è ridotta. La stimolazione con *K. pneumoniae* esposta a Marte ha ridotto la produzione di IL-17 e IL-10. La stimolazione con *S. marcescens* esposta a Marte ha ridotto la produzione di IL-1 β e TNF α . Analizzando l'essiccazione si è osservata una riduzione della produzione di specie reattive all'ossigeno (ROS) in entrambe le specie batteriche. Analizzando la fagocitosi da parte delle cellule CD11b+, si è osservato un comportamento opposto tra i due batteri: questa capacità è risultata ridotta in presenza di *S. marcescens*, mentre è apparsa invece aumentata con *K. pneumoniae*. Tuttavia, è stato osservato un ritorno parziale a risposte immunitarie normali quando i batteri, dopo essere stati esposti alle condizioni Marziane, sono ricresciuti nel terreno di coltura precedentemente utilizzato. Per esempio, il rilascio della citochina IL-10 è stato ripristinato a livelli basilari per *K. pneumoniae*, e così pure è stato ripristinato il rilascio di TNF α e IL-1RA per *S. marcescens*. Questo studio ha dimostrato come i cambiamenti indotti dalle condizioni Marziane influenzano la morfologia batterica e l'interazione immunitaria ospite e patogeni; suggeriamo quindi l'implementazione di protocolli di sicurezza per evitare la dispersione di batteri su Marte.

Le missioni Apollo hanno evidenziato i rischi dell'inalazione della regolite lunare dopo le attività extraveicolari (EVA) sulla superficie lunare. Perciò nel **Capitolo 4** abbiamo esaminato i pericoli associati all'inalazione della regolite lunare utilizzando regolite lunare e marziana simulata in modelli *in vitro* e *in vivo*. I test hanno coinvolto tre regoliti simulate disponibili in commercio e la silice come controllo. Sono stati investigati i rischi associati all'inalazione di polvere lunare e marziana (regolite). Esperimenti *in vitro* hanno rivelato che l'esposizione alla regolite simulata compromette la funzione di barriera delle cellule epiteliali bronchiali e promuove l'infiammazione. La regolite lunare simulata ha mostrato la maggiore citotossicità sulle cellule epiteliali polmonari, mentre quella marziana ha indotto un aumento delle citochine pro-infiammatorie nei cheratinociti. Per le indagini *in vitro* abbiamo impiegato culture di interfacce aria-liquido con cellule bronchiali epiteliali umane esposte a regolite simulata. Si è visto che l'esposizione acuta danneggia l'integrità delle giunzioni strette epiteliali, mentre l'esposizione cronica

compromette la funzione di barriera dell'epitelio e promuove l'infiammazione. I cheratinociti esposti alla regolite marziana simulata mostrano un aumento nella produzione di citochine pro-infiammatorie. Abbiamo osservato che tutte le regoliti simulate causano la morte cellulare nelle cellule A540 (epitelio polmonare), con la regolite lunare simulata come la più letale. Inoltre, la regolite simulata altera la normale organizzazione cellulare nelle cellule Detroit562 (epitelio faringeo), contrariamente alla silice. L'inalazione di regolite compromette le funzioni epiteliali bronchiali e stimola l'infiammazione. Gli effetti citotossici della regolite lunare e il rilascio di citochine pro-infiammatorie dalla regolite marziana mettono in luce i rischi associati all'inalazione di regolite.

Parte II – Come sopravvivono i microorganismi terrestri alle condizioni delle lune ghiacciate?

Gli estremofili e i microorganismi psicrotolleranti giocano un ruolo chiave nelle investigazioni astrobiologiche. Nel **Capitolo 5** abbiamo investigato i limiti di sopravvivenza a condizioni estreme di batteri e lieviti psicrotolleranti. Capire come funzionano i loro meccanismi di sopravvivenza è fondamentale per valutare quale rischio di contaminazione rappresentano per le lune ghiacciate. I criteri utilizzati per selezionare i microorganismi da studiare sono stati la loro capacità di crescere: 1. A temperature sotto lo zero, 2. In terreni di coltura minimi, 3. Su terreni di coltura solidi. Le condizioni utilizzate per simulare l'ambiente delle lune ghiacciate sono state: 1. L'essiccazione, 2. Le radiazioni UV e i raggi X, 3. Il congelamento e lo scongelamento delle cellule. I nostri risultati hanno dimostrato che, tra tutti gli organismi selezionati, i lieviti, sono stati quelli con la maggior capacità di sopravvivenza, in special modo a cicli di congelamento e scongelamento. In particolare, in determinate condizioni di simulazione della superficie delle lune di ghiaccio, i lieviti possono tollerare esposizioni a radiazioni UV per diverse ore mentre i batteri possono sopravvivere solo per diversi minuti. Abbiamo pertanto dimostrato quanto sia importante ricercare specificamente questi tipi di microorganismi nelle camere bianche SAF per rispettare i requisiti di protezione planetaria e per evitare la contaminazione delle lune di ghiaccio. Le radiazioni ionizzanti sono una delle condizioni climatiche spaziali più dannosi per la sopravvivenza microbiologica. Dei ricercatori hanno ipotizzato come alcuni microorganismi con concentrazioni intracellulari specifiche di Mn/Fe abbiano una tolleranza innata per le radiazioni.

Seguendo questa ipotesi nel **Capitolo 6** il nostro gruppo ha studiato la relazione tra il rapporto intracellulare del manganese e del ferro (Mn/Fe) con le capacità di tollerare le radiazioni. Per capire se questo fosse vero per tutti i domini della vita, abbiamo utilizzato 21 microorganismi tra cui batteri, archea e lieviti. Dopo

aver esposto i microorganismi alle radiazioni abbiamo estratto e misurato i livelli intracellulari di manganese e ferro per poi compararli alle misurazioni della letteratura. Nonostante i dati della letteratura suggeriscano che ci sia una relazione tra i rapporti Mn/Fe e la tolleranza alle radiazioni, i nostri risultati hanno dimostrato che, per i microorganismi studiati, questi presupposti non sono validi. Questi dati contraddittori dimostrano quanto la ricerca spaziale non disponga di sufficienti informazioni relativamente alla capacità di sopravvivenza dei microorganismi esposti a diverse condizioni combinate (e.g. radiazione, essiccazione, pressione atmosferica, etc.).

Per colmare questa lacuna abbiamo indirizzato le nostre ricerche per studiare se e come microorganismi di origine terrestre possono sopravvivere sulle lune di ghiaccio come Europa ed Encelado nel **Capitolo 7**. Il rinnovato interesse da parte di missioni spaziali come Juice dell'ESA ed Europa Clipper della NASA hanno sostenuto la necessità di effettuare questi esperimenti. I dati relativi alla sopravvivenza utilizzati nel capitolo 6 hanno indicato nel lievito *R. frigidicola* il candidato ideale per questi tipi di esperimenti. Le motivazioni sono dovute alla sua capacità di crescere tra 30°C e -10°C, l'estrema tolleranza alle radiazioni ionizzanti e non ionizzanti nonché all'essiccazione. L'aggiunta di ulteriori esperimenti combinati ha potuto garantire l'identificazione attraverso l'analisi del RNA quali geni fossero sovra regolati o sotto regolati. Le analisi trascrittomiche hanno identificato diversi geni che vengono espressi in maniera diversa prima, durante e dopo l'esposizione alle condizioni combinate. Le nostre analisi hanno dimostrato la rapida attivazione di geni coinvolti in attività catalitiche e di riparazione del DNA durante l'esposizione; ma ancora più interessante è il fatto che il lievito blocca la riproduzione e il progresso del ciclo cellulare per completare attività riparative. Si raccomandano future ricerche per studiare altri microorganismi psicrotolleranti e psicrofilici in modo da identificare quali siano i più rischiosi per la contaminazione delle lune di ghiaccio.

Appendix II Data management

Ethics and privacy

This thesis does not include research involving human participants. Buffy coats from anonymized healthy blood donors were obtained from Sanquin Blood Bank (Nijmegen, Netherlands). All donors provided written informed consent, and sample collection and use were conducted in compliance with institutional and national ethical standards following the principles of the Declaration of Helsinki (1).

Data collection and storage

The investigations performed in this thesis were conducted following the FAIR principles. Data produced at the German Aerospace Center, both raw and processed, is stored in lab journals deposited at the Applied Aerospace Biology department, and on the DLR server of the same department. Paper (hardcopy) data is stored in cabinets on the department. The server is backed up every 24 hours and is only accessible by DLR project members and available upon request. These storage options maintain the safety, integrity, and availability of the data.

Data sharing

The data from this thesis are publicly available under open access. The accession information for each data repository is provided within the relevant chapters and summarized below.

- Chapter 2 is published open access; raw data has been stored on the Data Station Life Sciences (DANS) and can be accessed through the following link: <https://doi.org/10.17026/LS/ID0NTR>.
- Chapter 3 is published open access; processed data has been stored on the Mendeley Data repository and can be accessed through the following link: <https://data.mendeley.com/datasets/g5xmvmc4nb/1>.
- The data used in the unpublished chapter 4 are archived at the department server. Upon publication the data will be published via a repository to be decided with open access.
- Chapter 5 is published open access; raw data in relation to microbial survival can be found on the Dryad database and can be accessed through the following link: <https://doi.org/10.5061/dryad.70rxwdc7r>.
- The data used in the unpublished chapter 6 are archived at the department server. Upon publication the data will be published via a repository to be decided with open access.

- Chapter 7 has been submitted to the journal; primary and secondary data have been stored in the Mendeley Data repository and can be accessed through the following link: <https://data.mendeley.com/datasets/m65mc3vd5w/1>.

References

1. World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA*. 2013 Nov 27;310(20):2191-4. doi: 10.1001/jama.2013.281053.



Appendix III Portfolio

PhD portfolio

Department: **Internal Medicine**

PhD period: **01/06/2021 – 12/11/2025**

PhD Supervisor(s): **Prof. dr. Mihai G. Netea, Prof. dr. Marien I. de Jonge**

PhD Co-supervisor(s): **Dr. Petra Rettberg, Dr. Kristina Beblo-Vranesevic**

Training activities	Hours
Courses	
• 2021 PhD Theme- and Posterworkshop (2021)	4.50
• DLR Graduate program: Science communication - Science outreach (2022)	2.00
• GP-Science Communication - Science Outreach (2022)	2.00
• RIMLS - Introduction course "In the lead of my PhD" (2022)	15.00
• GP-Communication Basics (2022)	16.00
• GP-PhD study - how to master it (2022)	16.00
• GP-Presenting at Scientific Conferences (2022)	17.00
• Radboudumc - Scientific integrity (2023)	20.00
• Meet the Expert session: How to prepare for you PhD defense (2024)	1.00
Seminars	
• Water microbiology in Germany / Seminar talk by Dr. Hügler (2021)	1.50
• VI ENCUESTRO INTERNACIONAL DE LA REDLATINOAMERICANA Y DEL CARIBE DEL ESPACIO (2021)	8.00
• RED'21 Exoplanets & Habitability (2021)	17.00
• Virtual AbGradE (2021)	9.00
• Extremophiles webinar 2021 (2021)	2.50
• 6th Human Physiology Workshop (2021)	8.15
• Updates for Web seminar: Europa Lander Speaker Series Session #5 : " A Reconnaissance Strategy for Landing on Europa " (2022)	2.00
• Life in Extreme environments - R. Amils (2022)	1.00
• Electron Microscopy Seminar: When Light is Not Enough (2022)	1.50
• EAI (European Astrobiology Institute) seminar (2022)	78.00
• Life inside the rocks: microbial diversity and metabolisms in the deep surface (2022)	1.00
• Enrichment and isolation of hyperthermophilic, anaerobic organisms with example of the extraordinary isolate "Mucithermu (2022)	1.00
• GoToWebinar - Are you familiar with pipetting quality control and ISO8655 standards? (2022)	1.00
• Exploring Ocean Worlds Through Analogs: Lab Studies (2022)	1.00
• Isolation and characterization of Nautilia sp. strain PV-1, a piezophilic Epsilonproteobacterium isolated from a deep-s (2023)	1.00
• 66. Helmholtz Open Science Online Seminar: Policy for Open Science (2023)	1.50
• Sustainability Workshop (2023)	1.00
• DABG Icy Moon workshop (2023)	10.00
• Launching Space Biology (2023)	40.00
• The Exploration Company - How to do life-science in space, and why? (2023)	1.00
• Big Bang microbes (2023)	8.00
• DGLRM workshop (2023)	16.00
• Human Physiology workshop (2023)	8.00
• Open University Seminar Talk (2024)	1.00
• Gravimeeting (2024)	20.00

• Irradiating microorganisms and organics in ice to guide the search for life on icy-ocean worlds (2024)	1.00
• IAA day (2024)	8.00
• Your Comprehensive Guide to RNA-Seq Data Analysis (2024)	1.00
• Journal Club (2025)	50.00
• Institute seminar (2025)	12.00
• Doctoral Seminar (2025)	48.00
• Workshop of the VAAM special groups (2025)	8.00
Conferences	
• RIMLS PhD retreat (2022)	25.00
• COSPAR (2022)	40.00
• EANA-EPSC (2022)	40.00
• EANA conference (2023)	40.00
• COSPAR Busan (2024)	56.00
• DGLRM (2024)	12.00
• VAAM 2025 (2025)	23.50
• PhD retreat (2025)	15.00
• FALCON: Frontiers in Astrobiology and Origins of Life Conference (2025)	29.00
• BEACON (2025)	40.00
• EANA conference (2025)	32.00
Other	
• OCelloScope workshop (2021)	1.00
• DLR Doctoral Theme and Poster Workshop (2021)	5.50
• DAbG working group Ocean Worlds and Icy Moons meet-up (2021)	2.00
• Ruhr Universitat Bochum (2023)	2.00
• Space.com interview (2024)	1.00
Teaching activities	
Supervision of internships / other	
• Bachelor student supervision (2022)	40.00
• Master student internship (2022)	40.00
• Master student internship and thesis (2023)	50.00
Total	955.65

Financing

The PhD candidate was financed by German Aerospace Center (DLR) with an internal contract for the first three years and subsequently by a DLR/DAAD (Deutscher Akademischer Austauschdienst) fellowship (Fellowship No. 633).



Appendix IV Acknowledgements

Acknowledgements

This section of my thesis I would like to express my deep appreciation to all who have made this PhD journey possible.

In diesem Abschnitt meiner Dissertation möchte ich meine tiefe Dankbarkeit gegenüber allen zum Ausdruck bringen, die mir diese Promotionsreise ermöglicht haben.

In questa sezione della mia tesi vorrei esprimere il mio profondo apprezzamento a tutti coloro che hanno reso possibile questo percorso di dottorato.

PhD supervisors

Dear **Petra**, since the first day I visited DLR in February 2020 I knew I would be fascinated by astrobiology research. Your precise and careful guidance have shaped me as a scientist and my career as well as my life. You welcomed me in your group and taught me much about astrobiology and planetary protection while at the same time giving me the opportunity to interact with scientists from many different backgrounds at DLR and elsewhere. You also gave me the opportunity to travel to a number of conferences, I especially enjoyed overseas ones (Greece, twice in Spain, USA, Netherlands and South Korea), and to share our research with scientists from all over the world. Such experiences have made me grow both as a scientist and as a person, and I will always treasure them. I wish you all the best for the future.

Dear **Kristina**, thank you for your unwavering support throughout my PhD journey. You have helped me since my shy day one at DLR and you have always been available to answer my many questions. Your guidance has been extremely valuable to develop the experiments I performed and to share the results we produced. I enjoyed the different conferences together in Erlangen, Bochum and Braunschweig as well as the fun Karneval parties and Phantasialand trips. Thank you for rooting for me, always supporting me, and giving me the opportunity to travel to conferences, for experiments and to sampling campaigns, Danke.

Dear **Mihai**, thank you for your constant support and enthusiasm for my research. When I attended the Microbiology masters at Radboud University your friendly and uplifting lecture style sparked my medical microbiology interest and at that point I didn't know I would pursue a PhD nor did I imagine my lecturer would be my

PhD promotor. During these years I always looked forward to our monthly Astro-Immunology meetings where your interest and passion for our research motivated me. Your guidance and feedback have pushed me to improve my work and have shaped me as a scientist. I would also like to thank you for connecting me with your lab and especially with Özlem, Anaïsa and Jorge who have been invaluable for the experiments we conducted and are great people and scientists. Thank you.

Dear **Marien**, when in January 2020, after I had struggled to find a research internship for my masters, you offered me to work at the “German NASA”, I could not believe my ears. I remember immediately calling my family to share the amazing news. I would not have imagined how this opportunity you offered me would shape my career and my life. I am very grateful for your support and your teachings on the importance of knowledge dissemination. Thank you for connecting me to members of your department who have taught me much and helped the research of this thesis. I would also like to thank you for your openness and availability to answer all my questions. Having your support throughout my PhD has been invaluable and will always be important for me.

I would also like to express my thanks to **Stefan**. Your support during the last stages of my PhD has meant a lot to me, and I am grateful you gave me the opportunity to travel and to share my research.

My Moral support AKA my home away from home

I have been extremely lucky in having found an amazing group of friend-colleagues at DLR who have helped and supported me throughout my PhD. Having shared labs, activities, and travels to conferences has been the highlight of these past 4 years and a half. Thank you all.

Johanna, my daily moral support, the person who tries to answer, but also asks all our “interesting” questions we constantly have for each other. Thank you for being such a great friend but also a great supporter too. You have pushed me to take on challenges, to apply for prizes and have made my PhD journey so much easier. Your help with all my experiments has been invaluable, especially when trying to figure out something we both didn’t understand. You have always been available for assistance and I hope I did the same with you. You also helped me with my German skills and I cannot thank you enough. Your organizational and challenge-driven spirit with our PhantasiaLand trips and group activities have made my experience with the people we work with amazing. I have always enjoyed sharing an office with you, having a laugh, interrupting, helping each other and travelling to the

many conferences together. I hope your PhD journey is smooth and wish you all the best, Danke!

Dear **Hector**, you have been the greatest of friends and it has been so exciting having spent the time with you in Germany. You were my first friend in Cologne and you have always been there for me, be it for help with basic math or just for a laugh. Your Mexican cooking is a masterpiece which cannot be rivalled and I miss it dearly. I believe that without your moral support I wouldn't have been able to conduct this PhD. You are an amazing teacher, person and friend, I miss you and wish you all the best Jefe!

Dear **Ina**, the plant mom, who I firstly have to apologize to for taking so long to learn how to use emojis. I hope to have improved my skills by now, and that you understand I am not angry XD. Thank you for taking care of my small number of plants and for pushing my inside plant-dad spirit out. I hope to develop your plant caring skills and to have as many plants as you one day. Thank you for being so amazing throughout my PhD and to have shared so many journeys to DLR with. Your out of character statements are hilarious and always make me giggle when I think about them. I am literally laughing while I remember when we had to park: *"Beep Beep out of my way!"* and when I overflowed the coffee machine: *"There's a button!"*. I hope to experience your funny spirit many times more in the future. Thank you!

Dear **Marta**, thank you for sharing your house and having me over for dinner one so many times. I apologize again for exploding the wine bottle on your kitchen wall. I realized only later how important those experiences were for me especially during Corona zeit. You're a great scientist, listener, and I have to learn a lot of public speaking skills from you! Thank you for helping to keep the Latin spirit alive in Germany and for having me in Porto, Obrigado!

Dear **Stella**, your smile has always lifted my spirit. I have enjoyed the many adventures with you, your housewarming party, celebrating your wedding, and travelling to Iceland and Lisbon. You are an amazing person who is very funny and an amazing scientist. Your support to me but to so many other people has been a beacon for me which I always looked up to. Thank you for listening me complain and guiding me through the PhD one so many times. I also want to thank you for spelling my name wrong I have learned how many options there are.

Dear **Caro**, thank you for being an incredibly funny person who I have enjoyed the many trips to Madrid and Lisbon with. Thank you for having me in Nurnberg and showing me around with Marvin. But the most important thing is that you don't forget to answer: *"why you do a did?"*

The DLR people

A special thanks goes to all the DLR people who have helped me with this journey. These are all the members of the space microbiology group: **Yen, Katha-otic** AKA the "Skyl-influencer", **Franca, Fina, Andrea, Elke, Corinna**. I would also like to thank the interns we had who have also made working in the lab a delight. Thank you **Alessa, Florian** and **Amelie**. Thank you also to all the amazing students we've had who helped me with experiments and who we have travelled with to conferences and workshops.

Radboud UMC

Dear **Özlem, Anaïsa** and **Jorge**, thank you for being such great collaborators. I enjoyed working remotely with you throughout my PhD. The insights I learned throughout our joint research efforts have been invaluable for me. I especially enjoyed travelling to Nijmegen where you showed me how to inflate zebrafish and all the different aspects of the Astro-Immunology projects we performed together. I wish you all the best for your future! Thank you **Özlem** for hosting me during my Astro-Immunology travels and welcoming me to the Radboud UMC community during the PhD retreat back in 2022 and for hosting me during my travels to Nijmegen.

A special thanks also goes to **Margo** for showing me how the zebrafish infection model works.

Thank you **Xuehui** for your support and guidance with the RNA analysis. Your help has been key for the research and has taught me much.

A tutti quelli che fanno parte della mia famiglia

I miei genitori sono stati le persone più importanti, le persone che senza le quali: questa tesi, i suoi risultati, le sue scoperte e le sue conclusioni non esisterebbero. Siete i genitori migliori al mondo, le persone altruistiche a cui voglio bene, che ammiro, e senza le quali non sarei qui, ma tutto questo lo sapete già.



Come dice Edoardo Bennato, Viva **la Mamma**! La persona che ci è sempre stata, ci sarà sempre e che ammiro. Mi hai aiutato con qualsiasi cosa io abbia avuto bisogno. Grazie mamma!

Come dice il saggio narratore della *"natura sociale dell'uomo"* (**il Papà**): *"ogni lasciata è persa"*. Io ne ho lasciate tante nel mio percorso ma quelle che non ho lasciato, grazie anche al tuo consiglio e mente critica, mi hanno portato avanti e fatto crescere. Mi hai insegnato a relazionarmi con gli altri nei momenti più difficili e a essere una brava persona. Il tuo spirito d'avventura mi spinge tutti i giorni. Non conosco altra persona che ha un papà-amico come te.

Non posso credere io possa essere chi sono senza l'aiuto dei miei fratelli **Ettore** e **Bianca**. La vostra presenza simpatica, ironica, difficile, amorevole mi ha sempre aiutato. A discapito dei vostri, e i miei, difetti è anche grazie a voi che questa tesi è stata scritta, grazie.

La persona più simpatica al mondo, **La zia Antonella**. Sono cresciuto con te e la tua famiglia. Mi hai aiutato sempre, ci sei sempre stata nei momenti difficili e in quelli facili. La tua voglia di fare ed esplorare mi ha insegnato molto. Grazie maestra!

Anche se il nonno **Sergio** non c'è più, sei sempre stato lo stimolo della mia immaginazione e sono sicuro che ti sarebbe piaciuto ti raccontassi del mio dottorato e di come la biologia si possa condurre nello spazio. Sono sicuro ti sarebbe piaciuto. Sono cresciuto con te, le tue storie, la tua immaginazione, il tuo spirito ingegneristico e la tua cucina. In questa tesi c'è anche un po' di nonno Sergio.

Nonna **Rosaria** la nonna terrona ma genovese dentro. Il tuo duro carattere, anche se molto difficile mi ha insegnato molto, specialmente che la vita non è facile, grazie.

Zia **Assia**, la zia con cui sono cresciuto, sempre scattante e attenta. Sempre pronta a vedermi e a chiedere dove sono.

Se c'è una persona divertente e simpatica che mi manca è il nonno **Fernando**. Le tue storie ripetitive e il tuo amore per la cucina e per la nostra famiglia è una lanterna per me. Condividiamo un compleanno e il regalo più bello che ricevo ogni anno è essere cresciuto con te. Il nonno terrone, il nonno che cucina, il nonno che ci è sempre venuto a prendere, il nonno che si preoccupa sempre se non abbiamo mangiato e che abbiamo abbastanza soldi, il nonno dei gelati e della pizza, il nonno che tutti invidiano.

Nonna **Loredana**, il tuo amore mi ha aiutato a crescere, a voler bene, e ad aiutare le persone, un po' come mi hai detto faceva nonno Ettore. Mi hai insegnato che tutte le persone sono degne del nostro amore. Ti sei sempre preoccupata che avessi mangiato, che mi fossi divertito e che avessi abbastanza soldi. Se tutto il mondo fosse un po' come te sarebbe un posto più giusto e migliore.

Le mie cuginette, non più tanto cuginette ma cugine ormai, **Alice** e **Lara**. Sono cresciuto con voi e mi mancate.

Zia **Gogo** e Zio **Nino**, anche se siete andati via quando non c'ero, sono diventato la persona che sono anche grazie alla vostra accoglienza, simpatia, e gentilezza, grazie.

There hasn't been a pair of fluffy quadrupeds I have loved more in my life than our dogs **Milady** and **Jack**. The building blocks which have kept this family together in tough and in happy times.

Maxi, I met you during my PhD, at a point in my life when I was scared, confused and ashamed. With your gentle and kind manners, you have taught me how to embrace who I am. You are my rock, my umbrella, and my compass through life. A day hasn't gone by where I haven't missed to see you. Meeting you was the best thing that could ever happen to me.

Auch wenn es schwer war, meine italienische Familie zurückzulassen, war es eine Freude, eine deutsche Familie zu finden. Daher geht ein weiteres Dankeschön an die gesamte **Familie Engelhardt**. Sie haben mich immer mit offenen Armen empfangen, mir immer das Gefühl gegeben, zu Hause zu sein, und immer dafür gesorgt, dass es mir gut geht. Ich hoffe, dass ich in Zukunft noch viele mehrere schöne Tage mit Ihnen verbringen werde.

Appendix V

Curriculum vitae

Education:

July 2021 – Nov 2025	PhD Candidate, Radboud University Medical Centre-German Aerospace Center (DLR), PhD project title: <i>“Microbial survival and immune health: implications for space missions”</i>
Sept 2018 – June 2021	MSc. Biology, Microbiology specialization, Radboud University, Nijmegen, Netherlands
Sept 2020 – Mar 2021	Masters Internship, German Aerospace Center (DLR), Institute of Aerospace Medicine, Radiation Biology Dept., Astrobiology working group. Report: <i>“Assessment of the adaptability of non-fastidious pathogenic bacteria to the Martian environment”</i>
Jan 2019 – June 2019	Masters Internship, Environmental Sciences Dept. Radboud University Nijmegen. Report: <i>“What is the role of bacterial community structures in the degradation of pollutants in wastewater treatment plants (WWTPs) or bioreactors?”</i>
Sept 2015 – June 2018	BSc (Hons) Biomedical Science, Anglia Ruskin University, Cambridge, United Kingdom. Dissertation: <i>“The Role of Wilms Tumor 1 Mutations in Acute Myeloid Leukaemia.”</i>

Volunteering and Work Experience:

26 – 30 June 2017	Director's Assistant to the “City Camp” educational event organized by Educational Linguistic Association (ACLE – Associazione Culturale Linguistica Educational), Genova, Italy
Jan – May 2014	Trainee rescuer at the White Cross, Genova, Italy

Language and IT skills:

28 January 2015	DELE B2 Certificate: Diploma de Español Como Lengua Extranjera
15 October 2013	CAE C1 (Certificate in Advanced English), Cambridge ESOL Examinations
31 July 2013	DELE B1 Certificate, Diploma de Español Como Lengua Extranjera
24 May 2013	ECDL (European Computer Driving License)

Additional Courses:

5 January 2014	Certificate of Immobilization of Trauma patients and Basic Life Support Defibrillation (BLSD), White Cross, Genova, Italy
20 April 2013	Basic Life Support Defibrillation (BLSD), Italian Resuscitation Council (IRC)
5 November 2013	Volunteering as student rescuer, White Cross, Genova, Italy

Awards:

2024	Albrecht-Ludwig-Berblinger-Preis Deutsche Akademie für Flug- und Reisemedizin
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VAAM (Vereinigung für Allgemeine und Angewandte Mikrobiologie – Association for General and Applied Microbiology) member

Appendix VI

List of publications

Zaccaria, T., Beblo-Vranesevic, K., de Jonge, M. I., Netea, M. G., Rettberg, P., (2026) Survival limits of psychrotolerant microorganisms with relevance for planetary protection of the icy moons. *Phil. Trans. R. Soc. A*, 384.

Zaccaria, T., Bulut, Ö., Ferreira, A. V., Dona, M., Langereis, J. D., Mesman, R. J., Wesseling, J., van Niftrik, L., Netea, M. G., Rettberg, P., Beblo-Vranesevic, K., de Jonge, M. I., Dominguez-Andres, J., (2025) Effects of simulated Martian environmental stressors on specific human pathogen-immune system interactions. *mBio*, 16(9).

Zaccaria, T., de Jonge, M.I., Domínguez-Andrés, J., Netea, M.G., Beblo-Vranesevic, K., Rettberg, P., (2024) Survival of Environment-Derived Opportunistic Bacterial Pathogens to Martian Conditions – Is There a Concern for Human Missions to Mars? *Astrobiology*, 24(1).

Author Biography

Tommaso Zaccaria was born in Genova, Italy in 1997. Since an early age he has been fascinated with space, microbiology and human health. During his high school years, he attended Deledda International School in Genova where he already expressed his interest in microbiology. After completing the international baccalaureate (IB) diploma, he studied Biomedical Sciences BSc (Hons) at Anglia Ruskin University in Cambridge, UK. Having achieved his Bachelors in science, in 2018 he moved to Nijmegen the Netherlands to enroll in the Biology (Microbiology specialization) MSc at Radboud University. In 2021, during his master's degree he completed an internship at the German Aerospace Center (DLR), in Cologne, Germany. During this time, Tommaso was able to learn about microbiology research in space and the importance of ground-based experimentation. After completing the Masters in 2021, Tommaso started a PhD in Astrobiology at the DLR supervised by Radboud University Medical Center in Nijmegen. His PhD research focused on microbial limits of survival in space and the implications microbial life has for planetary protection, especially to the forward contamination of celestial bodies. To understand the risks of an infection to astronauts during space travel, Tommaso's research also investigated the survival of human pathogenic bacteria exposed to Mars environmental conditions and how they interact with the human immune system. After his PhD, Tommaso hopes to continue working and performing research in astrobiology.

