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Microbiology of human spaceflight: microbial responses to mechanical forces that impact health and habitat sustainability

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SUMMARY Understanding the dynamic adaptive plasticity of microorganisms has been advanced by studying their responses to extreme environments. Spaceflight research platforms provide a unique opportunity to study microbial characteristics in new extreme adaptational modes, including sustained exposure to reduced forces

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of gravity and associated low fluid shear force conditions. Under these conditions, unexpected microbial responses occur, including alterations in virulence, antibiotic and stress resistance, biofilm formation, metabolism, motility, and gene expression, which are not observed using conventional experimental approaches. Here, we review biological and physical mechanisms that regulate microbial responses to spaceflight and spaceflight analog environments from both the microbe and host-microbe perspective that are relevant to human health and habitat sustainability. We highlight instrumentation and technology used in spaceflight microbiology experiments, their limitations, and advances necessary to enable next-generation research. As spaceflight experiments are relatively rare, we discuss ground-based analogs that mimic aspects of microbial responses to reduced gravity in spaceflight, including those that reduce mechanical forces of fluid flow over cell surfaces which also simulate conditions encountered by microorganisms during their terrestrial lifecycles. As spaceflight mission durations increase with traditional astronauts and commercial space programs send civilian crews with underlying health conditions, microorganisms will continue to play increasingly critical roles in health and habitat sustainability, thus defining a new dimension of occupational health. The ability of microorganisms to adapt, survive, and evolve in the spaceflight environment is important for future human space endeavors and provides opportunities for innovative biological and technological advances to benefit life on Earth.

KEYWORDS spaceflight, mechanotransduction, mechanobiology, microgravity, habitat, astronaut, spacecraft, fluid shear

INTRODUCTION

Overview of spaceflight microbiology for human exploration

Regardless of environmental conditions, microorganisms accompany humans wherever they travel, including on space missions. Accordingly, microorganisms are as essential to support human health and habitat sustainability during space travel (whether in deep space or on planetary or lunar surfaces) as they are on Earth, including disease prevention, clean air and potable water, reduced power consumption, conservation of resources, and *in situ* resource utilization (Fig. 1). As microorganisms are highly adaptable and responsive to extreme environments, understanding the impact of the spaceflight environment on microbial function, adaptation, and diversity is critical to successfully support and sustain human activities in space. This is especially pertinent given the myriad of unexpected microbial molecular and phenotypic responses that have been reported under both spaceflight and spaceflight analog conditions (1–3).

While microorganisms experience a variety of environmental and potentially synergistic stressors during spaceflight—including elevated radiation, increased CO_2, reduced atmospheric pressure, altered circadian rhythms, and confined spacecraft habitats with regenerative life support systems—the hallmark condition during spaceflight missions is sustained exposure to the reduced force of gravity. As life evolved on Earth, a multiplicity of changes in physical and chemical factors invoked adaptations and participated in the complicated selection process. However, the force of gravity on all terrestrial life has been constant for 4.8 billion years and has shaped the architecture and function of all biological systems on our planet. Therefore, there is little or no genetic memory of life responding to force changes in the low gravity range.

Microorganisms cultured in the reduced gravity environment in low Earth orbit (LEO) space habitats [e.g., Space Shuttle, International Space Station (ISS)] have often shown novel and unexpected responses as compared to when studied using conventional culture conditions in terrestrial laboratories, where the force of gravity can mask subtle but important microbial responses. These responses include alterations in virulence and host-pathogen interactions (4–8), biofilm formation (9, 10), antibiotic resistance (11, 12), growth kinetics (13), cell morphology (14–16), motility (17, 18), and global

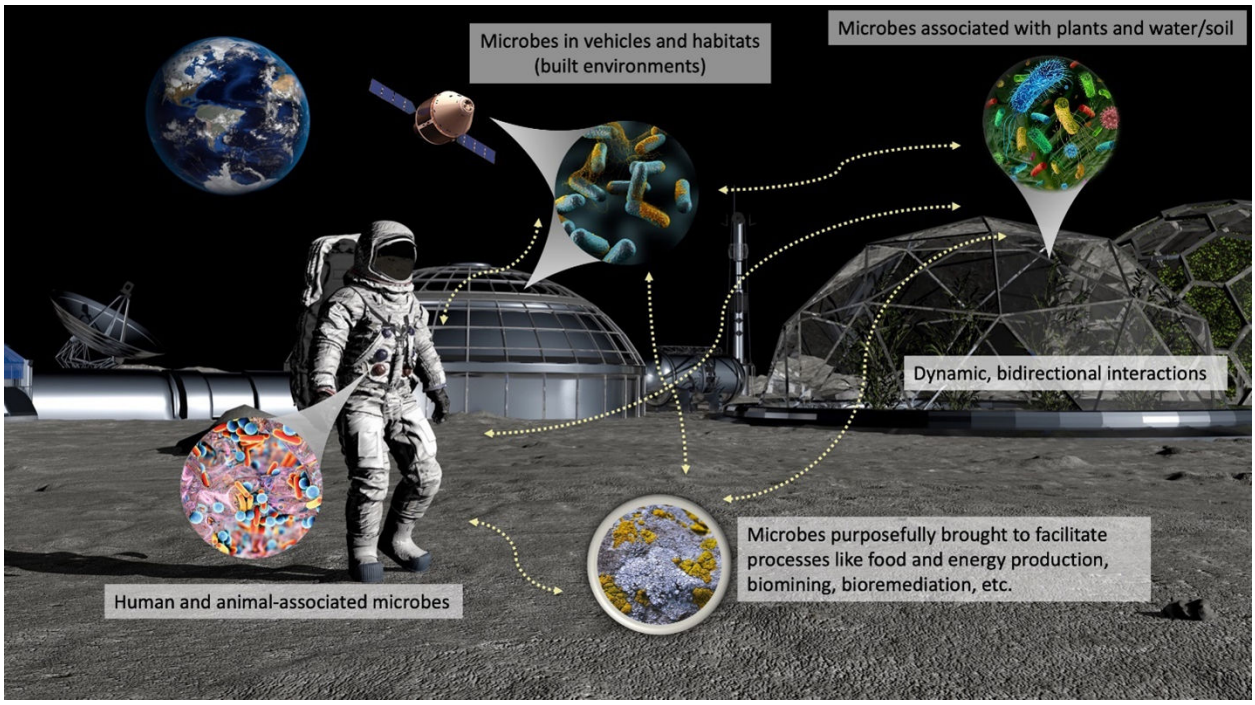

FIG 1 Microbial interactions during human spaceflight missions. When humans, animals, plants, and their associated microbes travel to the moon, they will encounter unique combinations of stressors, including lunar regolith and reduced gravity. The dynamic interactions that take place between these living organisms and non-living surfaces will alter core biological characteristics that could profoundly alter cellular survival, stress responses, and/or disease risks.

reprogramming of gene expression (5, 8, 14). In addition, alterations in the microbiome of astronauts (19, 20) and reactivation of latent viruses have been reported (21–26), which has sparked questions into the potential health implications of these changes (22, 24, 25, 27, 28). Indeed, in combination with evidence of immune dysfunction of astronauts during spaceflight (29–33), spaceflight-induced alterations in microbial characteristics suggest an increase in the risk of infectious disease and other microbial health risks during spaceflight missions. As discussed later in this review, the microbiome of astronauts has additional implications for the success of exploration missions, due to its influence on the spacecraft environmental microbiome composition, including regenerative life support systems (34–36).

Throughout the history of spaceflight microbiology research, compelling evidence has repeatedly demonstrated that the spaceflight environment alters a variety of key microbial properties (Table 1). As the number of important spaceflight and spaceflight analog microbial studies has increased dramatically in both quantity and diversity over the past 25 years, this review will focus solely on key microbial studies from these platforms that have direct implications for human health and habitat sustainability (Tables 1 and 2). For spaceflight-induced alterations in other microbial areas, including astrobiology and planetary protection, the reader is referred to several excellent review articles that have been written on these topics (37–41).

Central role of microbiology throughout the history of human space exploration

Present-day spaceflight microbiology research and discoveries have resulted from a series of progressive advances over the past six decades. To understand the chronology and context of current spaceflight microbiological research and its experimental design, it is important to understand early findings that served as the foundation to stimulate subsequent studies. Our understanding of high-altitude, spaceflight microbiology began as early as 1935 with ballooning experiments designed to evaluate the ability of

TABLE 1 Notable microbiological spaceflight events and research

Year	Description	Reference
1970[a]	An Apollo 13 astronaut suffered a severe *Pseudomonas aeruginosa* urinary tract infection during this deep space mission, demonstrating risk to crew health and importance of understanding infectious disease during spaceflight	(48)
1977[a]	Enhanced inflammatory responses observed in animal lesions after ground-based infection with *S. cerevisiae* which had been exposed to deep space irradiation during Apollo 16 as compared to ground control cultures	(49)
1982	Spaceflight-cultured *Staphylococcus aureus* and *Escherichia coli* exhibited increased resistance to antibiotics compared to ground control cultures	(50, 51)
1997	Evaluation of *E. coli* growth kinetics from multiple spaceflight experiments indicated alterations in lag and exponential growth phases, and final cell density as compared to ground control cultures.	(13)
1997	Enhanced colonization of plants in spaceflight by the opportunistic fungal pathogen, *Neotyphodium*, as compared to ground control cultures	(52)
1999	Investigations of *Bacillus subtilis and E. coli* using liquid and semi-solid growth media suggested differences in microbial responses observed during spaceflight may be due to external physical forces, e.g., fluid dynamics and/or extracellular transport	(53)
2001	First reports of experimental microbial biofilm formation in spaceflight	(10, 54)
2001	Spaceflight enhanced the ability of the obligate fungal pathogen *Phytophthora sojae* to colonize and cause disease symptoms in soybeans as compared to ground control cultures	(55)
2005	Enhanced latent herpes viral reactivation and shedding by astronauts during spaceflight	(25)
2007	First report that the microgravity environment of spaceflight altered the virulence and global gene expression of a microbial pathogen, *S.* Typhimurium, as compared to ground control cultures, confirming previous observations with this pathogen in the rotating wall vessel (RWV) and the transcriptomic and proteomic response of a bacterium to spaceflight. This study also identified the RNA chaperone protein, Hfq, as a master regulator of the spaceflight response	(5)
2008	Increased *S.* Typhimurium virulence in response to spaceflight culture was independently validated on a separate Space Shuttle mission. This study also showed altered virulence observed in spaceflight was dependent upon media ion concentration and that phosphate ion regulated key stress responses	(6)
2011	Similar to *S. Typhimurium, P. aeruginosa* cultured in spaceflight exhibited global alterations in gene expression, including Hfq and its regulon, as compared to ground control cultures, and confirmed that Hfq is a spaceflight-induced regulator acting across bacterial species.	(8)
2013	Spaceflight-cultured *Candida albicans* exhibited global changes in gene expression, random budding, and enhanced cellular aggregation as compared to ground control cultures	(14)
2013	Unique "column and canopy" biofilm architecture observed in *P. aeruginosa* cultured in spaceflight. Role for flagella demonstrated in the biofilm architecture formed in spaceflight-cultured bacteria	(9)
2019	Use of ISS spaceflight microbial isolates from astronauts to characterize the impact of long-duration spaceflight missions on skin, saliva, nostril, and fecal microbiomes	(19)
2019	Genomic and phenotypic characterization of virulence properties using *Fusarium oxysporum* isolates from the ISS	(56)
2019	A multidimensional analytical comparison between the microbiome of an astronaut during a year-long spaceflight mission and his identical twin on Earth	(57)
2020	Second report of a pathogen, *Serratia marcescens,* that displayed increased virulence during spaceflight	(7)
2020	Extensive characterization of six bacterial species collected from the ISS potable water system to understand multispecies interactions and contributions of each microorganism to biofilm development and stability. Loss of community biofilm formation was dependent on dual-species removal, suggesting synergistic interactions between species is necessary for robust biofilm formation	(58)
2020	Genomic and phenotypic characterization of *Burkholderia* isolates from ISS potable water indicated their pathogenic properties may be similar to the same species on Earth. Studies included antibiotic resistance, biofilm formation, hemolysis, and macrophage survival	(59)
2021	First infection of human cells in spaceflight and use of dual RNA-Seq to profile both the host (3-D intestinal cell cultures) and pathogen (*S.* Typhimurium) transcriptomic responses when both were simultaneously exposed to the spaceflight environment	(4)
2021	Longitudinal phenotypic characterization of 16 bacterial species isolated from ISS potable water over multiple years indicated bacterial adaptation over time in this ecosystem. Studies included antimicrobial resistance, biofilm structure and composition, hemolysis, and carbon utilization	(60)

[a]Deep space missions correspond to those beyond LEO and have different types of radiation, fractional gravity, and other environmental factors which can impact biological responses.

TABLE 2 Notable microbial spaceflight analog research

Year	Description	Reference
1997	First use of RWV bioreactor to study microbial responses to the spaceflight analog environment and the first evidence that bacterial responses to this environment are related to fluid shear in the medium. These studies used a variety of environmental bacterial isolates	(61–63)
2000	First report that physical forces are an environmental signal that regulates microbial virulence. Specifically, low fluid shear culture in the RWV bioreactor altered *S*. Typhimurium virulence, stress resistance, and gene expression	(64)
2002	First transcriptomic analysis of a spaceflight analog-cultured microorganism. RWV-cultured *S*. Typhimurium exhibited global reprogramming of gene expression compared to control conditions. Phenotypic validation of transcriptomic results confirmed reduced LPS biosynthesis and a role for the iron transport protein, Fur, in the LSMMG response	(65)
2006	First study of bacterial biofilms in the RWV. Thicker *E. coli* biofilms and increased antibiotic resistance observed in LSMMG as compared to control cultures	(66)
2007	Computational modeling of fluid shear levels in the RWV demonstrated a progressive relationship between applied fluid shear and bacterial gene expression and pathogenic stress responses. This study also demonstrated a correlation between low fluid shear levels experienced by pathogens in both the quiescent microgravity environment of spaceflight and the RWV with those naturally encountered in the infected host	(67)
2008	*P. aeruginosa* cultured in the RWV affected biofilm formation, rhamnolipid production, and quorum sensing as compared to control conditions	(68)
2011	LSMMG culture of *S. aureus* in the RWV altered transcriptomic profiles and pathogenic phenotypes, including biofilm formation, antibiotic resistance, and carotenoid production. This study also indicated a role for Hfq as a master regulator of the LSMMG response, suggesting a common regulatory motif between Gram-positive and previously observed Gram-negative responses	(69)
2013	RWV culture alters host-commensal microbe interactions in the squid-*Vibrio fischeri* model system as evidenced by altered innate immune responses and light organ development	(70)
2013	*Rhodospirillum rubrum,* a key organism proposed for use in future spacecraft regenerative life support systems, exhibited widespread transcriptomic, proteomic, and metabolomic alterations when cultured in the RWV, including increased levels of quorum sensing molecules and pigment production	(71)
2016	Closely related *S*. Typhimurium pathovars exhibited different virulence profiles in response to LSMMG culture in the RWV	(72)
2022	In response to 100-day RWV culture, *S. mutans* developed variants with altered heritable phenotypes such as adhesion and acid tolerance	(73)
2022	LSMMG cultures of *S*. Typhimurium display enhanced colonization (adherence, invasion, intracellular survival) in a 3-D co-culture model of human colonic epithelium containing macrophages. Global transcriptomic responses of host and pathogen aligned with infection phenotypes	(74)
2023	RWV culture altered *S. aureus* membrane lipid profiles and increased its sensitivity to membrane-disrupting antimicrobial compounds	(75)

microorganisms to survive decreased pressure and increased radiation (42–44). In the 1950s, our knowledge continued to expand with almost 30 ballooning and sounding rocket experiments (43). During the late 1950s and early 1960s, the USSR employed Sputnik satellites at higher altitudes to perform microbiological experiments primarily to help determine the safety of spaceflight for humans (43, 44). The National Aeronautics and Space Administration (NASA) not only performed microbiological research on satellites, such as Discoverer XVII and Biosatellite 2, but also incorporated crewed spacecraft, during the Gemini and Apollo Programs, which permitted greater experimental interaction and complexity (43, 44). From these early missions, the ability to perform increasingly complex experiments (42) continued to increase with the construction and launch of more sophisticated spacecraft both with the Soviet, and subsequent Russian, efforts through the Salyut and Mir space stations (43) and the NASA Skylab and Space Shuttle programs (43, 45–47). These efforts have culminated in the development and operation of the single largest space research platform to date, the ISS, a collaborative research platform in LEO between NASA, the European Space Agency (ESA), the Russian Space Agency (Roscosmos), the Canadian Space Agency, and the Japan Aerospace Exploration Agency (JAXA).

Over the past two decades, the number and complexity of spaceflight experiments have been dramatically increased with the assembly of the ISS, with nearly 3,000 experiments having been performed (76, 77). Having operated continuously for over

20 years, the ISS has enabled the most sophisticated and complex spaceflight microbiology research to date. Indeed, the completion of the ISS research platform has enabled a dramatic increase in spaceflight microbiology research publications over the past decade. This increase in microbiology research is expected to grow exponentially as (i) sovereign nations launch new government-operated space stations in LEO (e.g., China), as well as in deep space orbiting the moon (e.g., United States and international partners) that will be inhabited by professional astronauts, and (ii) the efforts of the rapidly emerging commercial spaceflight community (e.g., SpaceX, Blue Origin, Axiom Space, Nanoracks, Northrup Grumman, and Lockheed Martin) that will send civilians into space on a more frequent basis. The broad array of microbiology research capabilities and capacity is expected to further increase, as new commercial human-rated spaceflight platforms are deployed (2). Notably, the vast majority of spaceflight microbiology experiments have been short duration in length and performed inside the pressurized habitable volume of the spacecraft. However, as inflight research capabilities and infrastructure mature from the limitations associated with the current spaceflight biological research paradigm, it will be important to understand long-duration responses of the spaceflight environment on microbial adaptation and survival.

In this review, we focus on key spaceflight and spaceflight analog microbiological research, as well as the technology platforms that enabled these studies, which directly impact human health and space habitat sustainability. The results of these investigations are helping to define a new dimension of occupational health for traditional astronauts during exploration, professional astronauts in military and commercial activities, and civilian astronauts who are expected to become an increasing portion of the space-traveling community.

Mechanobiology of microorganisms and relevance to spaceflight

Mechanobiology is a multidisciplinary field that combines biology, physics, and engineering to study how prokaryotic and eukaryotic cells sense and respond to mechanical/physical forces (e.g., fluid shear, compression, stretch) in their microenvironment. The ability of cells to sense and respond to their physical force environment is an evolutionarily conserved and universal process in all life forms that allows cells to dynamically adapt and respond to changing conditions by converting mechanical force cues into biological responses and is a major regulator of cellular physiology, including the transition from normal homeostasis to disease pathologies (1, 78–83). The concept that physical forces and cellular mechanics play a central role in the regulation of biological form and function is often attributed to the pioneering work of Scottish mathematical biologist D'Arcy Thompson, whose classic 1917 treatise "On Growth and Form" was a foundational contribution to the field (84). Despite this realization over a century ago, the ability of microbial cells to sense and respond to their physical force environment remains a relatively unexplored and emerging frontier. Recently, however, the American Society for Microbiology and the National Academies of Science have identified the fields of microbial mechanobiology and host-microbial mechanobiology and associated phenotypic plasticity in terrestrial and space-based settings as one of the top fields that will define and advance the future of microbial sciences for the next decade (85, 86).

Mechanical forces in prokaryotic and/or eukaryotic microbial cells can be sensed by a variety of mechanosensitive structures, especially those on the cell surface (e.g., flagella, fimbriae, outer membrane proteins, extracellular polymeric compounds like capsules, and biofilms) (1, 80, 87–91), which are then transmitted to the DNA through the interconnected cytoskeletal network within the cell, as well as through the opening and closing of mechanosensitive stretch-activated ion channel proteins (92). Several of these mechanosensing structures have analogs in mammalian cells. This process causes mechanical forces to be transduced to initiate signal transduction and biochemical cascades which alter gene expression and functional phenotypes (collectively referred to as mechanotransduction) that regulate the transition between normal homeostasis

and disease or other abnormal states (1, 78–83). For example, microorganisms experience wide fluctuations in fluid shear levels in their natural environments, including their respective animal and plant hosts, water systems, and industrial settings. Indeed, the mechanical force of fluid shear is known to regulate a broad range of microbial phenotypes, including virulence (5, 64, 72), stress responses (1, 3, 5, 64, 66–69, 72, 93–103), antibiotic resistance (66, 69), metabolism (61, 62, 80, 93, 103, 104), biofilm formation (105–110), motility (18, 111, 112), and colonization of host cells and tissues (74, 102, 113).

While mechanical forces have long been recognized as vital in regulating the dynamic plasticity critical for both cell and tissue function in mammalian cells [e.g., morphology, proliferation, survival, metabolism, migration, differentiation, disease pathologies; we refer the reader to several excellent reviews on this topic (81, 82, 114–118)], only recently has the importance of how physical forces shape microbial behavior and physiology begun to be more widely appreciated by the microbiology community. Just as for other environmental stressors, physical forces like the flow of fluid over the surface of microbial cells (fluid shear) are used as a barometer by cells to provide cues to their environmental location (*in vivo* or *ex vivo*) which leads to corresponding adaptive molecular and phenotypic changes to enhance fitness (1, 3, 64, 109, 119, 120). While progress has been made in understanding the mechanosensory response of microbial cells to physical forces (1, 3, 5, 6, 67, 72, 74, 92, 99, 121–123), the mechanisms underlying how these forces regulate functional plasticity of microbial cells remains incomplete.

The concept that microbial cells actively sense, respond, and adapt their gene expression and physiology to their mechanical force environment is not new, and microbial mechanobiology has been well documented for several decades (1, 3, 67, 93, 121, 124). Indeed, microbial cells experience a variety of physical forces, including fluid shear, in their natural settings in both biological and abiotic environments. For historical context, several notable examples are briefly discussed. The study of biofilms was one of the earliest examples of the microbiology community appreciating the role of physical forces on bacterial behavior. Many publications have long documented the relationship between hydrodynamic fluid shear forces and microbial biofilm properties on inert surfaces, including motility, surface attachment, growth, metabolism, communication, morphology, and gene expression (80, 105, 107–109, 119, 123, 125, 126). Recently, studies using *in vitro* mammalian cell cultures have indicated that mechanical forces of fluid flow can also regulate bacterial adhesion to biological surfaces (74, 99, 127). Much progress has also been made in understanding how microbial cells use mechanosensitive stretch-activated ion channel (Msc) membrane proteins to respond to abrupt changes in osmotic (turgor) pressure. In response to hypo-osmotic shock, increased tension is placed on the lipid cell membrane which causes Msc channels to transition from closed to open confirmation, thereby allowing the release of ions and other cellular solutes to prevent bacterial cell lysis (91). In addition, foundational contributions to the "mechanobiology of infectious disease" were made in 2000 and 2007 with the finding that fluid shear forces relevant to those encountered in the infected host, as well as in the microgravity environment of spaceflight, altered the virulence of the enteric pathogen, *Salmonella* Typhimurium in unexpected ways that were not observed when the organism was cultured under conventional lab conditions. These were the first reports that a physical force could alter microbial virulence (5, 64).

Microbial cells are constantly subjected to changes in their environmental conditions. Their survival requires the ability to mount swift responses to adapt to these dynamic conditions, including alterations in pH, temperature, osmotic and oxidative stress, nutrient availability, and physical forces, regardless of whether they are swimming/floating freely in fluid suspension (planktonic) or adhered to surfaces (sessile). Each of these environmental conditions (alone or in combination) represents ecosystem niches for adaptation and evolution of microbial species and communities (128). While microbial responses to many of these environmental stressors have been well studied, how microorganisms sense and respond to physical force stimuli remains understudied and incompletely understood. Recently, there has been rapidly expanding interest and

activity in microbial mechanobiology, including the role of physical forces like fluid shear in regulating microbial pathogen responses, host-pathogen interactions, and infectious disease mechanisms.

Pertinent to this review, numerous studies have documented that microbial cells dynamically sense and respond to the low fluid shear culture conditions encountered in ground-based spaceflight analogs as well as in the true microgravity environment of spaceflight (3, 5, 6, 18, 65, 122). Importantly, responses exhibited by microorganisms cultured in these unconventional environments have unveiled novel insight into how microbes interact with their environment, their hosts, and each other that are not observed when these same organisms are grown traditionally in shake or static flasks, the latter of which are unable to recapitulate these physiologically relevant biomechanical force cues. It is pertinent to note that fluid shear conditions encountered by microbial cells during culture in the microgravity of spaceflight and in ground-based microgravity analog systems are relevant to environmental ecosystem niches naturally encountered by these organisms in terrestrial settings. For example, the low fluid shear stress, low turbulence environment of microgravity and microgravity analog culture is similar to conditions experienced by microorganisms in certain areas of the host. This includes low fluid shear environments relevant to those of microgravity, and analog culture systems are found between the brush border microvilli of epithelial cells and *in utero* with levels less than 1 dyne/cm^2 (129–133). The former environment is relevant to that encountered by numerous microbial pathogens and commensals (microbiome) during their natural life cycles in the gastrointestinal (GI), respiratory, and urogenital tracts, as well as environmental niches. The parallels between fluid shear forces in microgravity, microgravity analogs, and certain terrestrial ecosystem niches occupied by microorganisms (both in the environment and infected host) will continue to advance our mechanistic understanding of how microbial responses are modulated by these environmental signals and provide clues into microbial mechanobiology and the nuances of microbial responses to physiological environmental niches in space and on Earth.

Low fluid shear forces associated with microgravity and other reduced gravity environments

Most biological research has been performed under the conditions of terrestrial gravity on Earth. The term "microgravity" has evolved to generically describe the decreased gravitational force experienced in LEO, where many spaceflight platforms like the ISS and Space Shuttle have flown and most of the spaceflight research with microorganisms has taken place (134). However, plans to return humans to the Moon and eventually to Mars highlight the importance of understanding how biological systems respond to partial gravity conditions, which at present has minimal data. The moon has 1/6 and Mars has 3/8 of the gravitational force of the Earth. The simplest relationship of microbial responses as a function of gravity would be a linear relationship, where alterations in biological responses directly correspond to the amount of gravitational force. However, if this relationship is similar to many terrestrial dose-responses, the relationship may more sigmoidal in nature, suggesting the biological response may be much larger (or smaller) than expected from a linear model (135). Regardless, this relationship, which will likely need to be determined empirically, will be important to understand as humans colonize the Moon and Mars.

Even with an extensive number of microbiological spaceflight experiments over the past 55 years, no clear evidence is currently available that microorganisms directly respond to decreases in gravity. Instead, microbial cells cultured in this environment have been proposed to respond to "indirect" effects of microgravity, such as low fluid shear, lack of sedimentation, mass transfer, convective forces, and hydrostatic pressure gradients (135, 136). As mass diffusion in liquid cultures in the absence of motile organisms is suspected to be limited in the spaceflight environment, one hypothesis to explain alterations in the responses of non-motile microbial cells is the formation of "depletion zones" around these cells due to limited nutrient flow (13, 137). Modeling

of the potential formation of depletion zones around these cell types suggests that a decrease in nutrient availability is possible and may be influenced by the overlapping of these zones when multiple non-motile microbes are close to each other (138). However, this hypothesis does not account for the spaceflight responses of motile microorganisms (18, 139), where motility could greatly diminish the impact of potential nutrient depletion zones, suggesting that other factors regulate microbial responses in the spaceflight environment. An alternative hypothesis for observed alterations in microbial characteristics in the reduced gravity environment of spaceflight is based on mechanotransductive responses (1, 2) due to the significant reduction of fluid shear forces in the quiescent environment of spaceflight (136, 140). Indeed, validation of this hypothesis has been documented using dynamic fluid shear spaceflight analog bioreactors to correlate incremental changes in fluid shear levels with corresponding alterations in microbial responses (67, 99). However, the cellular and physical mechanism/s that translate this decreased fluid shear force into molecular (e.g., transcriptomic, proteomic) and phenotypic responses of microorganisms cultured under spaceflight conditions, as compared to Earth-based controls, has not been fully elucidated.

One of the earliest (if not the earliest) reports to suggest the possibility of mechanotransductive responses to gravity-driven mechanical forces like fluid shear was proposed for mammalian cells in a 1997 report by an expert scientific panel led by Mina Bissell (141). This report entitled "Modeling Human Risk: Cell and Molecular Biology in Context" was commissioned by the NASA Space Biology Program and postulated that the reduced gravity environment of spaceflight might impact tissue structure and function. Specifically, the panel suggested that *the three-dimensional microenvironments of both the inside and outside of the cell may be distorted from their normal configurations by microgravity* (141). A report from a joint NASA and European Space Agency workshop followed up on this concept for mammalian cellular responses to microgravity and proposed that the concept of cellular tensegrity (how cells sense mechanical forces through the balance of compression and tension) might explain microgravity-induced alterations in cytoskeletal architecture and corresponding phenotypic and molecular changes (142). Subsequent research has since shown that like mammalian cells, bacteria also have the ability to actively sense and respond to physical force changes in their microenvironment and possess several analogous mechanosensitive structures, including cytoskeletal proteins (1, 87, 92).

SPACEFLIGHT AND SPACEFLIGHT ANALOG PLATFORMS USED TO STUDY MICROBIAL RESPONSES

Spaceflight platforms and flight-based analogs

Spaceflight research platforms like the ISS provide exceptional resources for life sciences studies, as these facilities offer a broad spectrum of environmental factors and associated complexities that can impact biological systems, including reduced gravity, increased radiation, increased CO_2 levels, and altered circadian cycles. However, spaceflight microbiology experiments are still relatively infrequent when compared to the many thousands of terrestrial experiments that researchers perform annually worldwide. This lower frequency is due to factors related to opportunity, cost, and the complexities of conducting microbiological experiments in space (2). Accordingly, ground-based spaceflight analog systems which mimic key aspects of the spaceflight environment are valuable alternatives to validate and predict potential experimental outcomes. Researchers use a variety of technologies as platforms upon which to explore the effects of spaceflight or, in the case of spaceflight analogs, space-like stressors [e.g., radiation, simulated microgravity (SMG), planetary regolith], on biological systems. There are a number of considerations in choosing the most appropriate platform and experimental hardware for a particular investigation, including the type of scientific question, experiment duration, cost, technical skill, and need for specialized equipment. The following sections discuss both spaceflight and spaceflight analog research platforms,

with discussion on both the advantages and challenges associated with using these platforms and technologies.

Flight-based facilities available for biological research can be broadly divided into non-orbital (e.g., high-altitude balloons, parabolic flights), suborbital (e.g., sounding rockets), and orbital/deep space (e.g., orbiting vehicles, space stations, and vehicles traveling beyond LEO). Non-orbital and suborbital platforms are used as analogs of LEO and deep space missions to test hardware prototypes, perform pre-flight optimization, predict experimental outcomes, and perform post-flight validation and follow-up studies. Depending on the type of platform selected, researchers can investigate microbial and/or microbial-host responses to different types, dosages, and combinations of environmental stressors. Generally, experiments performed aboard high-altitude balloons, parabolic flights on modified aircrafts, and rocket-powered sub-orbital vehicles tend to be substantially cheaper and enable more rapid design and implementation relative to orbital payloads such as those performed aboard the ISS. These approaches can also provide more consistent access for experiment repetition. Challenges for these research payloads can include short experiment exposure times and/or a lack of the full spectrum of environmental conditions present during orbital and deep space missions. Conversely, although experiments conducted in LEO and in deep space are more expensive and complex to perform relative to flight analogs, organisms are exposed to the full range of environmental stressors encountered during human spaceflight missions, including a range of gravitational forces (e.g., microgravity, partial gravity, and hypergravity during launch/landing) and other space-associated stressors (e.g., radiation, regolith, extreme temperatures).

The exact boundary where "space" begins depends on the international standards set in accordance with the Fédération Aéronautique Internationale (143), which defines the edge of space at 62 miles (~100 km), at a boundary known as the Kármán line. Operating at closer proximity to the Earth's surface (~6–10 km above sea level) are parabolic flights executed aboard specially modified aircraft that are used as an analog for microgravity encountered in orbital and deep space missions (144). Parabolic aircraft generate a series of successive upward and downward arcs during which the plane alternatively dives and soars, wherein organisms are exposed to controlled periods of weightlessness interspersed with alternating periods of increased gravitational force. At the injection phase, the plane climbs at a 45° angle, creating a force of about 1.8 g. Once the top of the trajectory is reached, thrust is reduced, and the aircraft follows a free-fall ballistic trajectory. The period of weightlessness lasts for ~10–20 s. This parabolic flight maneuver is repeated 30–40 times in an individual flight (145). Modified parabolic flight paths are also available to simulate lunar and Martian gravities (0.16 g and 0.38 g, respectively (146). High-altitude balloon flights (~28–48 km) provide another avenue of low-cost, near-space access for researchers. Although organisms are not exposed to a microgravity environment using this approach, they can be challenged with harsh, space-like environmental stressors such as extreme cold, low atmospheric pressure, low relative humidity, and increased UV and cosmic radiation (147). For scientific payloads aiming to evaluate responses to slightly longer periods of weightlessness than possible on parabolic flights, suborbital rocket-powered vehicles provide another alternative. Like the aforementioned aircraft, these rockets also take a parabolic trajectory, but reach much higher altitudes to the edge of space (~48–145 km), providing uninterrupted exposure times ranging from ~3 to 20 min, depending on the vehicle.

The next class of biological research platforms operate in LEO and include several types of orbiting vehicles and space stations. In LEO, biological organisms are exposed to a synergistic combination of stressors, including microgravity and increased levels of radiation. The vast majority of microbial experiments performed aboard LEO spaceflight platforms have been short-duration studies due to existing limitations in power, volume, hardware capabilities, and crew time. However, there is much anticipation that the rapid development of multiple LEO commercial platforms combined with advances in technology will result in urgently needed opportunities to support long-duration

space biosciences studies. Beyond LEO, biological systems are outside of the protective shield of the Earth's magnetic field and are, thus, exposed to the full range of cosmic radiation (galactic and solar) in addition to microgravity (148, 149). At present, there are two fully operational space stations in LEO, the ISS and the Tiangong Space Station, which operate at ~250 miles (~400 km) above the Earth's surface. In addition, there are a number of commercial and government space stations that are expected to be developed and operational in LEO within the next decade, including Axiom Station, Starlab Space Station, Orbital Reef, and the Russian Orbital Service Station, among others (150). These stations are being built using the lessons learned from the construction and operation of ISS and its predecessors, such as SkyLab, Salyut, and Mir. A diverse array of government and commercial vehicles have played a critical role in transporting microbiology experimental payloads to space. Notably, prior to its retirement the Space Shuttle served as a workhorse over the formative years for space life sciences and biomedical experiments.

Beyond LEO, the Artemis program is currently underway to return humans to the moon for the first time since 1972 with a focus on establishing a long-term lunar outpost. The first Artemis lunar missions will use the Space Launch System super heavy-lift rocket to launch the Orion spacecraft and their crew. A key piece of the current Artemis program involves the planned construction of Gateway, which will be the first space station in lunar orbit that will serve as an outpost to support human exploration of both the lunar surface and deep space missions. The Artemis program involves collaboration across multiple government agencies and commercial partners.

In addition to the aforementioned flight platforms, CubeSats have carried biological payloads in LEO and in deep space aboard Artemis I (151–156). A CubeSat is a miniaturized satellite of standardized size (10 cm cube) that can be deployed from various launch vehicles as well as from space stations. The small, standardized size of CubeSats can be beneficial in saving costs and the incorporation of off-the shelf components. Additionally, they have the capability of being deployed into regions of space that are not yet human-occupied. Conversely, this small size can also restrict the types of research payloads that are compatible with this platform and can present challenges for the internal hardware design for environmental support and operations of biomedical payloads (155).

To this end, researchers face a variety of challenges that increase in difficulty depending on the platform selected, the experimental complexity, and the distance from the planet. For example, for flight analogs with short experiment exposure times (e.g., parabolic flight), a key consideration is the functional timescales of different biological processes to predict whether exposure times of the microorganism to an environmental stimulus will be sufficient for the phenotype of interest to manifest. For example, during very brief exposures (<1 min), changes in transcription and translation may not be easily observed due to the necessary timing for those cellular processes to occur. For example, it takes ~1 min to transcribe an average-sized bacterial gene (1 kb) in response to a stimulus (157). This is in addition to the time required for the cell to sense and transduce the appropriate signals. Thus, there may exist a bias in the reliability of measuring expression changes in shorter vs longer sequences (e.g., non-coding RNAs vs longer mRNAs). Similar constraints exist for other cellular characteristics such as growth, signaling, translation, and epigenetic modifications. Conversely, alterations in other processes that occur more rapidly (e.g., macromolecular degradation, transport, conformational changes, motility over short distances) could potentially be measured within a shorter timescale. Along these lines, sample processing time should be considered if samples are to be preserved during or immediately following exposure.

As experiments transition from flight analogs into LEO and deep space missions, experiment difficulty increases. Even for simple microbiological payloads such as the growth and preservation of microbial cultures for post-flight analyses, there is extensive coordination required between multiple teams (e.g., science teams, hardware integrators, government, and commercial partners) to ensure the experiment is safely and

properly executed. For this reason, pre-flight optimization of experiments in terrestrial laboratories using the hardware and environmental configuration which closely mirrors the conditions of the spaceflight mission is critical for all space life science studies. During this critical planning phase, investigators refine the key experimental parameters for the study within the context of the flight hardware, which can include cell concentrations, experimental treatments, incubation temperatures (before, during, and after the experiment), standard operating procedures for on-orbit operations by the crew, and fixation conditions. This phase can often take months to years, depending on the experiment complexity and required hardware modifications. Astronaut time and safety are two key considerations for any microbiology experiment. In particular, microbial pathogens add additional levels of complexity, requiring special procedural considerations and flight hardware designs that incorporate multiple levels of containment in case of unanticipated leaks. The crew also receive specialized training for these situations which are planned and practiced months on the ground before the flight experiment. Operations using biosafety level 2 (BSL2) pathogens often require the use of facilities such as in-flight safety cabinets (e.g., glove box or glove bag) and/or hardware with multiple levels of containment to prevent leaks into the vehicle or station environment which would pose safety risks to the crew. BSL3 pathogens (e.g., SARS-CoV-2, *Mycobacterium tuberculosis, Yersinia pestis*) are not allowed aboard any NASA spacecraft.

Another challenge is the need for more predictive modeling approaches, including those associated with artificial intelligence (AI) and machine learning that incorporate data from system-level omics sequencing with functional phenotypic profiling. To facilitate this effort for spaceflight and spaceflight analog biological studies, NASA has established several open database repositories and archives that are searchable, including GeneLab, the Ames Life Sciences Data Archive (ALSDA), and others, several of which have been integrated into a single portal known as the NASA Open Science Data Repository [OSDR; osdr.nasa.gov/bio (158)]. GeneLab and ALSDA have created analysis working groups comprised of scientists in the community to maximize the utility of this repository and facilitate higher-level integrated analyses of the omics and phenotypic data deposited into OSDR (159, 160). These resources were developed with the intention to better understand biological responses to both short- and long-duration space and spaceflight analog studies in the context of their biological, chemical, and physical force microenvironments. Such tools would ideally be useful in developing more predictive models to effectively delineate between primary and secondary microgravity effects on microbial systems, as well as artifacts that may have occurred due to experimental design and spaceflight hardware. This becomes even more important given the relatively rare opportunity for experimental replication of spaceflight biological results.

As experimental complexity begins to approach that of most terrestrial laboratories, researchers may encounter limits as to the amount and type of science that can be successfully completed due to factors such as limitations in hardware capabilities, crew time, safety, and cold stowage availability (among others). Factors that increase experimental complexity widely vary but can include co-culture of different cell types (e.g., polymicrobial or host-microbe), infection of a multicellular host organism (see below), use of different media types, specific atmospheric conditions (i.e., temperature, gases), serial passaging between cultures (e.g., during multigenerational experiments), countermeasure screening (e.g., antibiotics or disinfectants at different concentrations), monitoring of biofilm growth and analysis, and the inclusion of multiple sampling time points and/or fixation conditions to accommodate different post-flight analyses. Microbiologists new to the field may also be surprised that even routine procedures such as serial dilutions and plating for viable cell counts are not easily performed on orbit (outside of environmental monitoring operations). Handling and transferring liquids in microgravity can present logistical and/or safety challenges, the latter due to a lack of settling of pathogen-containing droplets that can be released during basic operations. For the former, bubbles and lack of mixing in primary culture chambers as well as in tubing lines connecting to secondary chambers have presented major challenges when

trying to transfer homogeneous solutions. These considerations become more impactful as volume sizes decrease (e.g., when using microfluidic devices such as lab-on-a-chip or organ-on-a-chip). To circumvent some of these or other challenges, some researchers have used solid or semi-solid media (16, 53, 104, 161).

Another important spaceflight limitation involves virulence profiling of host-pathogen interactions. During a virulence study, the survival of an infected host organism is monitored from start to finish to acquire a median lethal dose (LD_{50}) or median time-to-death (TD_{50}), depending on the model host organism used. At present, vertebrate models (e.g., rodents) cannot be infected on orbit due to restrictions associated with safety and animal capacity—the latter due to the wide range of pathogen doses required to calculate an LD_{50} using the Reed and Muench method (162). As an alternative approach, in-flight growth of microbial cultures followed by post-flight recovery of these cultures for virulence profiling in rodents or invertebrates has been used successfully (5–7). Additionally, in-flight, real-time monitoring of virulence has also been performed using the invertebrate model organism *C. elegans* (163).

As biological questions continue to grow in complexity, there is a pressing need for the scientific and engineering communities to work together to design a series of modular spaceflight hardware systems that take these requirements into account. The hardware, designed to be automated, manual, or a hybrid of the two (allowing for crew interactions as needed), should be able to interface with other facility instruments used for downstream processing and analysis, such as centrifuges, microscopes, sequencers, and flow cytometers. This will become an especially important consideration in the establishment of life sciences facilities on the lunar or Martian surfaces. To this end, there is no automated or semi-automated hardware available that can conduct complex microbiological experiments with the broad range of capabilities, precision, and accuracy comparable to what is achievable in most terrestrial laboratories (2). This becomes an even more critical consideration as science missions extend beyond LEO into deep space and will, thus, rely more heavily on automation and *in situ* analysis. In particular, assays that require extensive procedural steps still present a challenge for full automation with minimal-to-no crew intervention. For example, in most studies, researchers require the ability to reproducibly transfer small (microliter), well-mixed solutions between different containers. In addition, many experiments require multiple treatment types at several kinetic time points, such as washing, drug treatments, lysis steps, and preservation in different types of fixatives. Likewise, biological samples tend to require a range of different storage temperatures throughout a mission, often ranging from a deep freeze (−80°C) up to body temperature (37°C) or even higher. To our knowledge, outside of basic incubators, there is no automated hardware available capable of handling the temperature extremes needed for this temperature range.

It has also been suggested that, in addition to terrestrial ground control cultures, some studies conducted in the space environment may also benefit from the inclusion of an on-board centrifuge control to run in parallel with flight samples to artificially re-introduce gravity (145, 164). This study design aims to delineate the biological and physical changes occurring due to microgravity (directly or indirectly) vs other factors associated with spaceflight, such as radiation or launch vibrations. This approach also allows investigators to conduct their experimental and control samples under even more tightly regulated environmental conditions (within different chambers of the same hardware) and timing (i.e., no lag time, as is the case for terrestrial controls) (165). However, this approach is not without potential pitfalls. Depending on the size and power requirements of the instrument, it may not be feasible to install and/or operate a centrifuge on smaller vehicles due to space and weight restrictions. Furthermore, depending on the centrifuge design and the type of biological sample being tested, the application of a centrifugal force might introduce artifacts to the flight $1 \times g$ control samples as a result of unintended gravity gradients and inertial shear forces that could ultimately alter experimental outcome and interpretation (165, 166). In particular, adherent vs non-adherent cells can be differentially affected. Adherent cells will

experience shape deformation in a radius-dependent manner, while non-adherent cells will accumulate at the point of highest acceleration (166). Depending on the centrifuge configuration (i.e., centrifuge diameter and distance), it is estimated that inertial forces may account for up to 99% of the total force applied to the cells (165). While centrifuges have not yet been widely adopted in many spaceflight studies, the introduction of artificial gravity using a centrifuge has shown promising results in partially or fully reversing negative spaceflight-associated physiological phenotypes in mice (167–169) and fruit flies (170). Centrifuges have also been used to study the effect of artificially induced Mars gravity on the final cell densities of *Sphingomonas*, *Bacillus*, and *Cupriavidus* bacterial species (171). No differences were observed in the final cell counts between microgravity cultured samples compared to those grown under true Earth gravity or in simulated Mars gravity conditions.

These types of artifacts are not limited to the use of centrifuges. As more space life science investigations report findings using biological organisms cultured in different types of hardware, large-scale data analysis will help identify experimental results that are hardware-driven from those due to spaceflight-specific factors. This approach was recently applied in plants, wherein meta-analysis of 15 transcriptomic data sets of space-flown *Arabidopsis thaliana* separated out the confounding, hardware-related effects in the data from differences specifically due to the microgravity environment (172). The meta-data-based analyses grouped experiments of similar experimental design, including environmental conditions, and hardware systems, which allowed this novel study to address this issue in such a unique way. As these types of trends continue to emerge, it will be critical for scientists and engineers to collaborate from the onset in the design (or re-design) of next-generation hardware systems that are better adapted to function in the microgravity environment while providing optimal support for each biological organism under study.

Ground-based spaceflight analog platforms

Due to the inherent challenges associated with performing research in space, researchers often use spaceflight analog systems designed to recapitulate specific aspects of the spaceflight environment. These approaches, which replicate one or more aspects of orbital spaceflight, tend to be less expensive than conducting spaceflight experiments and also enable more routine access for iterative testing. Spaceflight analogs are used both for predicting in-flight responses and for post-flight validation of results. There are different types of analogs that can be used to model different types of environmental factors encountered during space travel (e.g., alterations in gravitational forces, radiation, regolith, atmospheric conditions). To predict cellular responses to microgravity using ground-based platforms, investigators routinely use experimental approaches such as clinorotation, diamagnetic levitation, and drop towers. Radiation facilities, such as the NASA Space Radiation Laboratory and an international STARLIFE consortium involving investigators from the German Aerospace Consortium (DLR) and JAXA (173), are used to simulate aspects of the complex radiation environment encountered during space travel. Simulants approximating the size and chemical compositions of regolith present in different regions of the moon and Mars are being studied to better understand their impact on biological systems and study their potential use for *in situ* resource utilization. Other aspects of the space environment can also be approximated in specialized chambers (e.g., vacuum chambers) to simulate atmospheric conditions. In this section, we focus on select ground-based spaceflight analogs that are routinely used for simulating reduced gravity environments.

There are several popular approaches that have been used for decades by researchers to simulate reduced gravity environments, including simple 2-D clinostats, the rotating wall vessel (RWV bioreactor, and the 3-D clinostat/random positioning machine (RPM) (Fig. 2). Depending on the technique used, bacterial cultures are rotated about either one axis (simple 2-D clinostat and the RWV bioreactor) or two independent axes (3-D clinostat/RPM) to maintain cells in suspension. These techniques use rotation to decrease

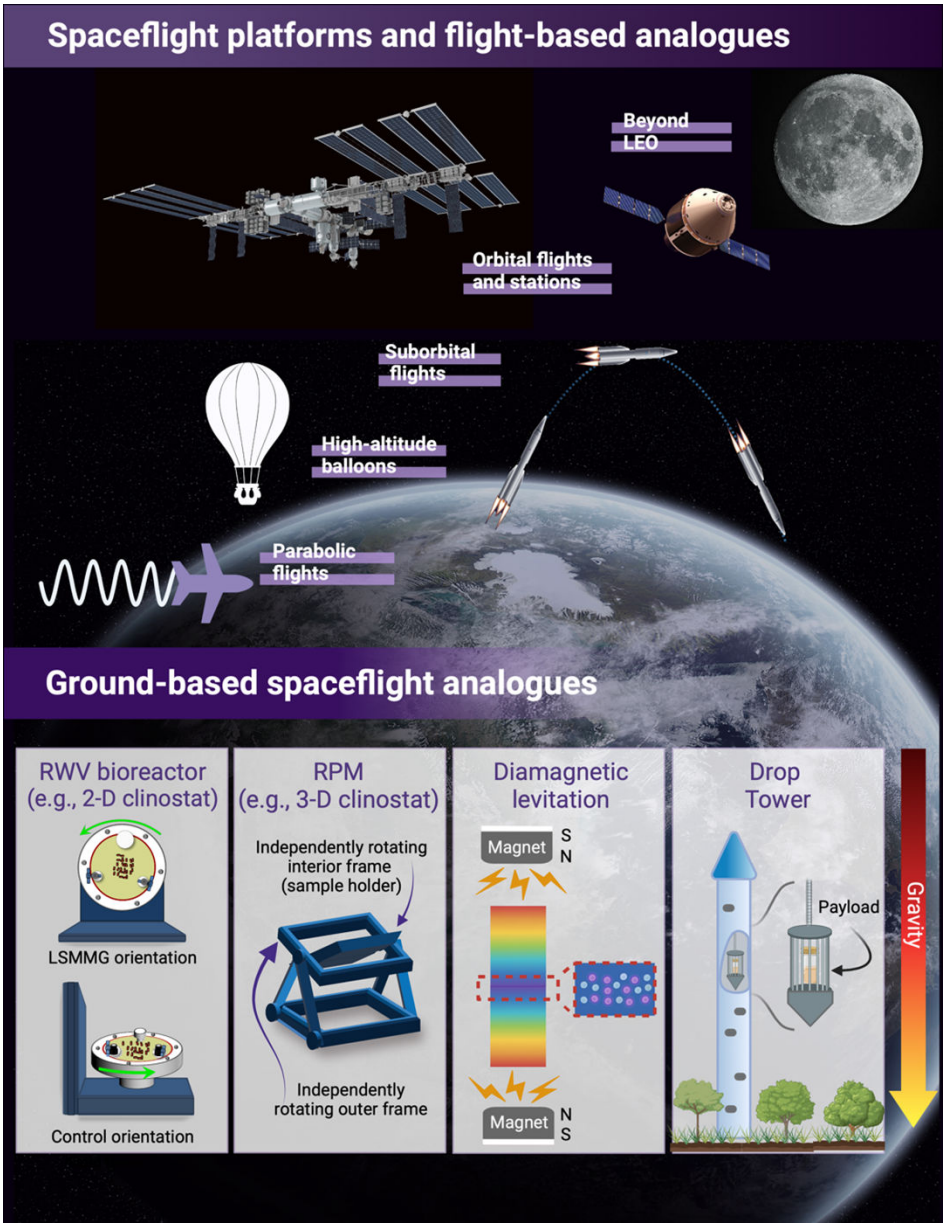

FIG 2 Spaceflight platforms, flight-based analogs, and ground-based spaceflight analogs. Researchers use several types of spaceflight and spaceflight analog platforms to study the impact these environments on microbial physiology, gene expression, and host-pathogen interactions. Spaceflight platforms and flight-based analogs are shown in order of increasing distance from Earth. Ground-based spaceflight analogs including the RWV bioreactor, an example of a 2-D clinostat. The RWV bioreactor has also been referred to as a high aspect ratio vessel/high aspect rotating vessel (HARV) and rotary cell culture system (RCCS). The environmental conditions provided by these analogs have been described by a variety of terms including low shear modeled microgravity (LSMMG/primarily used to describe the RWV environment), SMG, and modeled microgravity (MMG). The RPM is an example of a 3-D clinostat. In diamagnetic levitation, cells are repelled away from the strongest regions of the magnet (red color) toward a region where the gradient approaches zero (blue color), resulting in stable levitation. Drop towers can provide between 2 and 10 s of freefall, depending on the tower. Created in part with BioRender.com.

fluid shear forces over the surface of the cells and prevent sedimentation (1, 174). As there are many excellent reviews that provide excellent detail on the operational principles of each of these systems (1, 140, 174–179), we provide a brief summary of each approach below.

The simplest 2-D clinostat design consists of small tubes, cuvettes, or chambers that are completely filled with the culture medium and rotated about a horizontal axis (perpendicular to the force of gravity). These devices are rotated at rates that depend on the size of the biological organism under study. Sometimes referred to as fast-rotating clinostats (174), it is common to rotate these culture systems at a high speed (~60 rpm) to prevent sedimentation. For temperature control, clinostats are maintained in a temperature-controlled incubator. Although liquid cultures are the most common type of samples used with clinostat rotation, some researchers have used a similar approach to rotate microorganisms grown on solid media (180).

The RCCS is another type of 2-D clinostat, with a slightly more complex design (Fig. 2). Culture vessels called RWV bioreactors attach to the RCCS and are rotated about a horizontal [for modeled microgravity (MMG) cultures] or vertical axis (for control cultures). There are different RWV bioreactor designs, including the high aspect ratio vessel/high aspect rotating vessel (HARV) and the slow turning lateral vessel (STLV). HARVs are pancake-shaped and have a silicone rubber membrane for oxygenation spanning the entire back of the vessel. These vessels are commonly used for both microbial and mammalian cell culture. The shape of STLVs resembles that of a soup can, with the silicone oxygenation membrane running down the center core of the vessel. STLVs are more commonly used for 3-D tissue culture applications, though can also be used for microbial culture if desired.

The RCCS/RWV culture system was designed by the NASA Biotechnology group at the NASA Johnson Space Center in an effort to model the low fluid shear, low-turbulence environment that cells experience in true microgravity (1, 95, 181). To generate an optimized form of suspension culture, the bioreactor is filled completely with the culture medium such that there are no bubbles (i.e., zero headspace) and rotated about a horizontal axis. These features are critical to establishing solid-body rotation of the medium in the RWV that generates a low fluid shear environment for cell growth, which was previously designated as LSMMG (1). For control cultures, bioreactors can be reoriented by 90° to rotate about their vertical axis (Fig. 2), which allows cells to sediment toward the bottom of the vessel. Unlike simpler 2-D clinostats, the RCCS actively provides oxygen to the culture across a gas-permeable membrane on the back of the HARV (or through the central core of the vessel for STLV) bioreactors and is typically operated using a much slower rotation speed (~20–25 rpm for most cultures). These culture systems are small enough to be housed in standard incubators for temperature control and have been used to successfully grow and evaluate the responses of a wide range of microbes and have also been used for the development and testing of advanced 3-D models of human tissue (81, 182). It is also important to note that the low fluid shear conditions during LSMMG culture have been mathematically modeled and found to be physiologically relevant to certain low fluid shear niches encountered in the human body (e.g., *in utero*, between epithelial brush border microvilli) (67, 129–133).

Another approach to simulating microgravity involves the use of 3-D clinostats or the RPM (Fig. 2), which simulates microgravity by rotating cultures about two orthogonal axes. Different types of culture vessels can be attached to these instruments (e.g., flasks, plates, tubes, RWV bioreactors). The earliest versions of these technologies were developed by researchers studying the effects of MMG on plants (183–185). These systems are designed such that an interior rotating frame containing the attached sample is linked to an independently rotating outer frame (100). Experiments can be performed using different modes of operation, including a classic 2-D clinostat mode (i.e., rotating around one axis), 3-D clinostat mode (rotates about two axes, using constant speeds and directions), and 3-D random positioning mode (rotates about two axes, using random speeds and directions) (174, 175). When operating in 3-D clinostat mode, it has been demonstrated that the inner and outer frames should be rotated at different constant speeds since equal rotation speed was found to be inadequate in simulating the microgravity environment (175, 186). This was confirmed in a microbiological study assessing alterations in biofilm formation, transcriptional profiles, and

antibiotic resistance of *Mycobacterium marinum* using both a commercially available RPM (RPM 2.0) and a recently developed more cost-effective 3-D printed 3-D clinostat (186). While some researchers use the term "3-D clinostat" and "RPM" interchangeably, not all 3-D clinostats are operated in a random mode with respect to speed and direction, and thus, it may be more accurate to specifically refer to the operational mode(s) used during experimentation (174).

To minimize the possibility of centripetal acceleration on cultures within the RPM, samples are placed as close to the center of rotation as possible although this becomes more challenging as larger sample volumes or more replicates are tested simultaneously (175). Like 2-D clinostats, culture chambers must be completely filled with the culture medium (no bubbles) during operation to minimize turbulence. Temperature control can also be achieved by maintaining the instrument in incubators. The RPM has been routinely used for assessing responses of plants and mammalian cultures to MMG and, to a lesser extent, to evaluate microbial responses (relative to the RCCS) (187). In a comparative study using HARV bioreactors attached to either the RCCS or the RPM bases, divergent molecular responses were observed for *Pseudomonas aeruginosa* between SMG conditions using these two different technologies (100). Injection of a small droplet of crystal violet into HARV cultures revealed rapid levels of fluid mixing in the cultures attached to the RPM relative to those attached to the RCCS, with LSMMG culture on the RCCS displaying the lowest levels of mixing relative to the RPM cultures and the RCCS reoriented control cultures, which may explain the observed molecular differences (100). This is an important consideration since mixing is anticipated to be low in the quiescent environment of spaceflight. Additional publications also reinforce the concept that fluid shear levels in RCCS cultures are lower than those grown on the RPM (18, 67).

Diamagnetic levitation has also been used to expose microorganisms to a SMG environment. Biological organisms possess diamagnetic properties (largely due to their high water content) that enable their levitation when placed into a strong magnetic field, like those produced by superconducting magnets (188, 189). Within the vertical gradient produced by the external magnetic field, biological systems are repelled away from the strongest regions of the magnet toward a region where the gradient approaches zero, resulting in stable levitation of the organism (190). In a notable early demonstration of this approach, Geim and colleagues successfully levitated a living frog (191). While a wide range of organisms have been successfully exposed to diamagnetic levitation without negatively impacting their viability, an important consideration in this approach is to ascertain whether the strength of the magnetic field can exert negative effects on the organism of interest. Should this be the case, the magnitude of the magnetic field can be reduced by using paramagnetic solutions or ferrofluids to increase the magnetic susceptibility of the medium (190). It is also important to consider whether the biological effects observed are due to weightlessness vs the high magnetic field itself, especially considering the differing magnetic susceptibilities of cellular constituents (192, 193) as well as indirect effects of the magnetic field, such as differences in mixing. For example, Dijkstra et al. found that the application of a magnetic field led to increased growth rates of *Escherichia coli* and *Staphylococcus epidermidis* (188). Upon additional experimentation, the authors discovered magnetically induced convection occurred in the SMG samples, thereby increasing oxygen availability to the bacteria within those cultures (188).

Drop towers are another type of ground-based platform that can be used to study the effects of near weightlessness on biological systems. Drop towers are shafts typically built in excess of 100 m in height where the air can be evacuated from the chamber to reduce aerodynamic drag. There are several drop towers worldwide, including the Zero Gravity Research Facility at the NASA Glenn Research Center, the Dryden Drop tower at Portland State University, the Bremen Drop Tower, the Microgravity Laboratory of Japan (MGLAB), Queensland University of Technology Drop Tower facility, and the Beijing Drop Tower. Most of these drop towers provide between ~2–5 s of free fall for experimental payloads. The Breman Drop Tower in Germany extends this time by catapulting payloads

from its base to the top of the chamber and back down for ~10 s of freefall. In this design, during the acceleration and deceleration phases of the experiment, payloads are briefly exposed (~200 ms) to high *g*-forces (~25–50 × *g*).

As with flight-based platforms, there are a variety of challenges that researchers face depending on the analog platform selected and the complexity of the experimental question. With ground-based technologies, the goal is usually either to predict responses that could occur in the true microgravity environment or to validate spaceflight data. As it is difficult to recreate the complex combination of environmental stimuli in space using a single technology, there is no "one-size-fits-all" approach. Depending on the organism and the phenotype of interest, researchers may find some techniques to be more predictive than others. The combining of different technologies or experimental approaches may help facilitate the dissection of different factors (e.g., fluid shear, oxygen, nutrient distribution, sedimentation, radiation) responsible for spaceflight-associated phenotypes. One challenge facing researchers is the development of novel technologies to accurately model the continuum of gravity levels present in space, such as those present on the moon or Mars. To begin to address this challenge, new software algorithms have been developed for the RPM in an effort to simulate a range of partial gravity environments (194). While this approach has been used to model osteoblast behavior under different simulated partial gravities, it will be interesting to assess the fidelity of this approach in predicting microbial behavior in true partial gravity environments.

SPACEFLIGHT AND SPACEFLIGHT ANALOG ENVIRONMENTS ALTER MICROBIAL RESPONSES IN UNEXPECTED WAYS

Throughout the history of spaceflight microbiology research, compelling evidence has repeatedly demonstrated that spaceflight alters a variety of microbial characteristics. While different microorganisms respond differently to the spaceflight environment, multiple reports have also documented the use of common mechanisms as shared microbial response strategies to both spaceflight and spaceflight analog culture, including both Gram-negative and Gram-positive bacteria (3, 5, 6, 8, 65, 69, 100, 122, 195). As the number of important spaceflight and spaceflight analog studies has increased dramatically over the past 20 years, this review will focus on key microbial studies that have direct implications for human health and habitat sustainability.

Predictably, there has been an increased use of -omics technology (genomics, transcriptomics, proteomics) to study microbial responses to spaceflight and spaceflight analog conditions, which have significantly advanced insight into molecular mechanisms that might be underlying these responses. These approaches have provided a wealth of valuable information that can be openly searched in public databases [like NASA's GeneLab (196)]. However, focusing solely on *-omics* as predictive of cellular function is inadequate, as the presence of a gene does not guarantee its expression and the expression of a gene does not guarantee its function. Rather, these processes are context-specific and driven by the dynamics of the cellular microenvironment that collectively governs cell behavior and function, often in non-intuitive ways, which is commonly observed when microbial cells are studied in the context of their natural fluid shear force environments (1, 5, 64, 65, 67, 68, 72, 74, 88, 99, 107, 108, 119, 120, 127, 197, 198).

This fundamental concept reinforces the importance of combining functional phenotypic studies with multi-omics approaches to accurately predict how different environments, including spaceflight and spaceflight analog conditions, alter microbial physiology in unexpected ways (1–3, 5, 6, 18, 64, 65, 67, 68, 74, 94, 96, 99, 122). This is a critical consideration, as the physical basis of microbial behavior is rarely considered when predicting responses to their complex and dynamic microenvironmental niches, especially in the context of biologically realistic mechanical forces, like fluid shear. Pertinent to this review, it is known that fluid shear forces associated with spaceflight and spaceflight analog conditions can drive emergent microbial phenotypes in ways

that are not intuitive and, thus, cannot be accurately predicted by sequencing alone. Accordingly, the integration of sequencing and functional phenotypic profiling studies is essential for the development of more predictive modeling approaches to generate and test hypotheses to advance basic science and translational applications.

Given the dynamic plasticity of microbial cellular responses to their environment, it should not be surprising that findings from microbiology spaceflight and spaceflight analog studies are both context-specific and organism-specific, and not universally true for all microorganisms, including between closely related pathovars (72, 98). For example, while some microorganisms have demonstrated changes in final cell population densities, antibiotic resistance, morphology, biofilm formation, and virulence in response to spaceflight and spaceflight analog culture, other species have not shown these same phenotypes (1, 5, 6, 8, 12, 14, 15, 64, 69, 199–202). In addition, the lack of proper controls to draw conclusions for some spaceflight and spaceflight analog experiments, along with the use of different flight hardware technologies and different culture conditions, has complicated interpretations and, thus, is not included in this review.

Salmonella pathovars

The model Gram-negative foodborne pathogen, *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium), is the best-characterized microbial pathogen in response to both spaceflight and spaceflight analog culture conditions (4–6, 44, 64, 65, 67, 72, 74, 94, 96, 98, 203–206). Due to its route of access through spaceflight food, *Salmonella* has clear implications for astronaut health. Accordingly, NASA specifically tests for *Salmonella* prior to flight and has disqualified food destined for the ISS based on its isolation (2). Moreover, despite pre-flight screening and quarantine procedures, *Salmonella* spp. have been recovered from crew refuse (207) and ISS surfaces (208). The earliest documented spaceflight study using *Salmonella* was a 1968 report of *S*. Typhimurium cultures grown on the NASA satellite mission, Biosatellite II, which found that cultures grown in space had greater population densities as compared to Earth-grown controls of the same organism (44, 203).

In addition to operational concerns for spaceflight missions, the interest in *S*. Typhimurium as a model organism to mechanistically understand how the microgravity environment impacts microbial behavior began with early seminal studies over two decades ago. In these studies, *S*. Typhimurium cultured to log phase in LSMMG conditions in the RWV bioreactor exhibited a significant increase in virulence (decreased time-to-death, decreased LD_{50}) and increased ability to colonize the deep tissues of liver and spleen in mice following oral infection, as compared to the reoriented control (64). Consistent with the enhanced virulence phenotype observed in animals, LSMMG culture also increased the ability of log phase *S*. Typhimurium to survive challenge with pathogenesis-related stressors, including acid, osmotic and thermal stress, and increased intracellular survival in cultured macrophages as compared to control conditions (64). Furthermore, the LSMMG and reoriented control cultures exhibited the same log phase growth profiles and final cell densities. Transcriptomic profiling showed that LSMMG culture directed global reprogramming of gene expression throughout the *S*. Typhimurium chromosome as compared to control conditions, including changes in a broad range of functionally diverse groups, e.g., transcriptional regulators, virulence factors, lipopolysaccharide (LPS), biosynthetic enzymes, iron-utilization enzymes, metabolism, and proteins of unknown function (65). In agreement with transcriptomic results, functional phenotypic profiling demonstrated (i) significantly less LPS production in LSMMG cultures and (ii) a functional role for ferric uptake regulator in regulating the LSMMG acid stress response (65). Unexpectedly, there was no upregulation of known virulence genes that could explain the increased virulence of *Salmonella* observed in response to LSMMG. Specifically, known virulence genes were either downregulated or showed no differential expression between LSMMG and the reoriented control. This included the *hfq* gene, which encodes the evolutionarily conserved RNA

binding chaperone protein, Hfq, that is now known to regulate the post-transcriptional expression of a large number of genes in *Salmonella* (209), although at the time of the Wilson et al. 2002 publication (65), the role of Hfq as a global regulator of *Salmonella* gene expression and phenotypic properties (including virulence) had not yet been identified.

These early studies using *S.* Typhimurium cultured in the low fluid shear forces of the RWV spaceflight analog bioreactor not only provided important information for astronaut health risk assessments but were also the first reports that a mechanical force could regulate microbial virulence and global gene expression (transcriptomic and proteomic) (64, 65). These studies also demonstrated that fluid shear levels relevant to those encountered by *Salmonella* in the microgravity environment of spaceflight as well as in the intestinal tract of the infected host, acted as a novel environmental signal that regulated virulence, stress resistance, and gene expression in a microbial pathogen (1, 3, 64, 65, 67). In addition, mathematical modeling and computational studies demonstrated a progressive relationship between incrementally increasing, quantified levels of physiological fluid shear in the RWV and gene expression and pathogenesis-related stress responses in a classic GI disease-causing strain of *S.* Typhimurium (67). Collectively, these findings showed that this specific strain of classic GI *S.* Typhimurium (the same strain used in the previous RWV studies) was able to sense and respond to different levels of fluid shear relevant to those encountered during the natural course of infection.

The LSMMG-induced regulation of *S.* Typhimurium virulence, stress response, and gene expression observed in log phase cultures was reminiscent of regulation by the master stress response regulator, RpoS, which regulates these same responses when this pathogen is grown in conventional shake or static flask conditions (210–213). Given that RpoS is operative in both the log and stationary phases and is also regulated by Hfq, it was hypothesized that RpoS was a likely candidate in LSMMG signal transmission in *S.* Typhimurium. However, the LSMMG log phase stress response (acid, osmotic, thermal, oxidative stress, and macrophage survival) and transcriptional response were shown to be independent of RpoS (94). Unexpectedly, stationary phase resistance to acid and bile salts stressors, as well as adherence to and survival within intestinal epithelial cells, did not require RpoS when *S.* Typhimurium was cultured under LSMMG (96). The finding that a functional *rpoS* allele was dispensable for phenotypic responses of stationary phase LSMMG cultures of *S.* Typhimurium was further unexpected considering that LSMMG was shown to differentially regulate multiple genes in the RpoS regulon in stationary phase cultures of this same strain (74).

Consistent with LSMMG cultured classic GI *S.* Typhimurium being more virulent in mice (64), a recent study showed that LSMMG stationary phase cultures of this same strain exhibited enhanced ability to adhere, invade, and survive in 3-D biomimetic human intestinal tissue co-culture models containing immune cells and induced the expression of bacterial genes involved in pathogenesis (e.g., invasion, motility, and chemotaxis) (74). In addition, infection of 3-D intestinal co-culture models with LSMMG cultured *S.* Typhimurium enhanced host cell gene expression profiles reflective of heightened responses to infection (e.g., inflammation, tissue remodeling, and wound healing) relative to control-infected cultures (74). Surprisingly, these LSMMG phenotypic and molecular trends were further upregulated in an *hfq* mutant as compared to wild-type *S.* Typhimurium. The finding that LSMMG culture enhanced the host-pathogen interaction during the earliest stages of infection in an Hfq-independent manner was unexpected given that these pathogenesis-related phenotypes and host responses to infection are profoundly impacted when the *hfq* mutant is grown under traditional culture conditions (209). Collectively, these findings add to the growing body of literature demonstrating the importance of incorporating physical force considerations and advanced biomimetic 3-D tissue culture models into *in vitro* infectious disease studies. These findings further reinforce the critical role of mechanotransductive forces as microenvironmental signals in reprogramming *S.* Typhimurium.

Subsequent studies have since confirmed that when grown in spaceflight analog conditions (RWV), *Salmonella* pathovars that cause different disease pathologies with diverse host tropisms responded differently to alterations in fluid shear (72, 98). A notable example is the LSMMG response of the *S*. Typhimurium pathovar, multidrug-resistant ST313 strain D23580, which causes life-threatening systemic bloodstream infections in humans but does not often cause GI symptoms (214). Unlike classic GI *S*. Typhimurium, a culture of D23580 in higher fluid shear levels increased the virulence and pathogenesis-related stress responses of this pathovar, as compared to LSMMG conditions (72). These findings align with the different fluid shear infection niches exploited by these two closely related pathovars during the infection process, which suggests that D23580 may have adapted to high fluid shear niches in the bloodstream, while classic GI *S*. Typhimurium acclimated to low fluid shear conditions found between/near brush border microvilli in the intestine (67). This possibility is not unprecedented, as interactions between microorganisms and their microenvironmental niches have been postulated to be important for species diversity, selection, and evolution (128). Recently, extensive transcriptomic and functional phenotypic profiling of mechanotransductive pathogenic phenotypes and associated molecular regulatory mechanisms in D23580 expanded the characterization of this pathogen when grown in a broader range of quantitated physiological fluid shear levels in the RWV than previously tested (99). The results of this study confirmed that a progressive series of quantified fluid shear levels altered pathogenesis-related stress resistance (e.g., acid stress, macrophage survival), colonization of 3-D human intestinal tissue models (adherence, invasion, intracellular survival), and transcriptomic responses of this pathogen in a progressive fashion (99).

The increased virulence phenotype and identification of Hfq as a key regulator of the LSMMG response in *S*. Typhimurium were subsequently validated in two independent spaceflight experiments flown aboard NASA Space Shuttle missions, STS-115 and STS-123 (5, 6). These spaceflight experiments cultured *S*. Typhimurium in the quiescent low fluid shear conditions of true microgravity using the same GI strain previously used in the RWV (5, 6) and were the first demonstrations that spaceflight culture increased the virulence of a microorganism. As observed in LSMMG cultures, spaceflight culture conditions in both studies significantly increased the virulence of *S*. Typhimurium in an orally infected mouse model (5, 6) as compared to synchronous ground control cultures. Since NASA does not allow in-flight infection of vertebrate animals, the mice were infected with the spaceflight-grown *Salmonella* immediately after the Space Shuttles landed at the Kennedy Space Center and compared to identical ground controls that were maintained under the same conditions as the flight samples, except they did not fly in the microgravity environment. Global transcriptomic and proteomic profiling of the samples identified the *S*. Typhimurium "spaceflight stimulon" and showed regulation of diverse genes (the majority of which were downregulated as compared to ground-based controls), including those regulating host-pathogen interactions, virulence, stress responses, biofilm formation, flagellar-based motility and chemotaxis, iron utilization/storage, metabolism, horizontal gene transfer, outer membrane proteins associated with the periplasmic stress response, regulatory proteins, ribosome structure, and small non-coding RNAs. Importantly, many of these genes (including small non-coding RNAs) were either members of the Hfq regulon or genes involved in interacting with and/or regulating Hfq (5), which was previously identified as differentially regulated during LSMMG culture in this same organism (64). Hfq binds to small ncRNAs thereby facilitating their association with mRNAs; the outcome of which plays a diverse role in regulating gene expression, virulence, stress responses, antibiotic resistance, DNA methylation, and genome stability in this bacterium (209, 215–225). Interestingly, *hfq* expression was downregulated in response to spaceflight culture (5), which was consistent with its expression during LSMMG culture in the RWV (64). Validation of the role for Hfq as a likely global regulator of the *S*. Typhimurium response to the spaceflight environment was investigated using the RWV bioreactor, which demonstrated a role for *hfq* in regulating the LSMMG acid stress response and macrophage survival (5).

Subsequent to the *Salmonella* spaceflight and spaceflight analog studies, other microbial pathogens and commensals have since been reported to use Hfq as a response regulator to low fluid shear forces experienced by microorganisms in both the microgravity environment of spaceflight and on Earth during their natural life cycles, including in the infected host (8, 69, 100, 195). This shared regulation of Hfq by diverse Gram-negative and Gram-positive bacteria (pathogens and commensals) in response to low fluid shear niches encountered in their natural terrestrial environments may serve to pre-adapt some bacteria to be "hardwired" to respond to the microgravity environment of spaceflight. These fluid forces are relevant to those encountered during the normal lifecycles of microbial cells, but they have been widely overlooked as environmental stressors with the potential to dictate the outcome of infection.

To better understand the connection between media nutrient content and the altered *Salmonella* virulence observed during spaceflight culture, the impact of media ion composition on spaceflight-associated changes in both virulence and gene expression was investigated. The premise for this spaceflight experiment was based on previous transcriptomic data showing that *S.* Typhimurium cultured under LSMMG and spaceflight conditions in Lennox broth (LB) differentially regulated a large number of genes encoding ion response pathways (5, 65), as well as phenotypic differences in lag phase and generation times observed during LSMMG culture as compared to control conditions (94). In addition, RWV studies using the closely related enteric bacterium, *E. coli*, showed differences in gene expression profiles during RWV culture in Luria broth vs MOPS minimal media (226). Based on this information, three different media were used in a separate *S.* Typhimurium spaceflight study to investigate the impact of media ion composition on virulence (6). These media were LB (used in both the original spaceflight and spaceflight analog *Salmonella* virulence studies) (5, 64), M9 minimal media (containing high levels of inorganic ions), and a hybrid LB-M9 media supplemented to contain the same concentrations of five inorganic salts/ions found in the M9 medium (phosphate, potassium, magnesium, sulfate, and chloride). In agreement with the first *Salmonella* virulence spaceflight study, *S.* Typhimurium cultures grown in the LB medium again displayed increased virulence in mice as compared to ground controls, which gave the rare opportunity to independently validate spaceflight results. Importantly, spaceflight cultures grown in the M9 medium did not display increased virulence as compared to ground controls, and the addition of the five inorganic salts found in M9 to the LB medium (hybrid LB-M9) reversed the increase in virulence of spaceflight cultures grown in the LB medium alone (6). Intriguingly, while different virulence responses were observed in *Salmonella* spaceflight cultures grown in LB and M9 media, significant similarities in transcriptomic and proteomic profiles indicated the involvement of the Hfq regulon in both media types (6). These genes included those involved in flagellar motility, Suf transporter formation and other ABC transporters, ribosomal structure, iron utilization, hydrogenase formation, and small non-coding regulatory RNA expression/function. Findings from this spaceflight experiment led to follow-up spaceflight analog studies in the RWV which showed that phosphate and magnesium ions were sufficient to alter the acid stress responses when *Salmonella* was cultured in a spaceflight analog model (6).

The increased ability of ground-based spaceflight analog (RWV) cultured *S.* Typhimurium to colonize 3-D human intestinal tissue models (74) suggested the possibility of a heightened infection of human cells in true spaceflight. To test this hypothesis, 3-D human intestinal cell culture models were infected with *S.* Typhimurium when both the host and pathogen were simultaneously exposed to the spaceflight environment on the ISS. This was the first study to evaluate the response of human cells to infection during spaceflight (4). Transcriptomic and proteomic profiles from this study indicated an exacerbated response of spaceflight intestinal cell cultures infected with *Salmonella* (e.g., higher induction of genes involved in response to LPS, oxidative stress, wound healing, apoptosis, and inflammation) as compared to the same cultures infected in synchronous

ground controls (4). These results were consistent with *S.* Typhimurium being more virulent in both spaceflight and spaceflight analog (RWV) culture (5, 64).

It is important to note that in the previous *Salmonella* virulence spaceflight experiments, the bacteria were cultured in spaceflight and infections took place in mice after the Space Shuttle returned to Earth (5). In other words, the host was not exposed to the spaceflight environment as NASA does not allow in-flight infections of vertebrate animals. However, the non-vertebrate nematode *Caenorhabditis elegans*, which has been used extensively in terrestrial studies as a human surrogate model to study host-pathogen interactions (including with *Salmonella*) (227–229), is permitted by NASA for inflight infections. Accordingly, a recent study performed the first real-time virulence profiling using *C. elegans* as an intestinal model system to study the response to infection with *S.* Typhimurium when both the host and pathogen were simultaneously exposed to the spaceflight environment (163). This study profiled time-to-death, transcriptomic responses of both the host and pathogen, and countermeasure testing in order to understand infectious disease mechanisms, the risk for foodborne infections to the crew and efficacy testing of antimicrobial compounds (C.A.N., J.B., J.Y., C.M.O., unpublished data).

It should be noted that unlike the 1968 report of *S.* Typhimurium spaceflight cultures showing higher final cell densities as compared to ground controls, there were no significant differences in final cell densities of *S.* Typhimurium observed between any of the spaceflight and ground control studies described above. The observed differences between the 1968 *Salmonella* studies and those decades later could be due to the use of different *S.* Typhimurium strains, flight hardware, and environmental growth conditions between these spaceflight studies (5, 6, 203).

Serratia marcescens

Recently, it was shown that both spaceflight and spaceflight analog (RWV) cultures increased the virulence of a different enteric pathogen, *S. marcescens*, in an insect model (*Drosophila melanogaster*) (7, 230). Specifically, spaceflight-cultured bacteria were returned to Earth and stored at −80°C until used to infect *D. melanogaster*. The increased virulence observed in *S. marcescens* after spaceflight culture was transient as it did not persist after the initial subculture on the ground, indicating the bacteria were rapidly adapting to their different environmental conditions (7). Interestingly, follow-up studies to characterize a potential link between media nutrient utilization and the increased virulence of *S. marcescens* observed in response to RWV culture showed a direct link between asparagine utilization and the altered virulence phenotype (230).

Escherichia coli

An extensive number of spaceflight and spaceflight analog experiments have investigated *E. coli* responses with a wide array of findings (12, 13, 53, 66, 97, 102, 199, 226, 231–238). From a human health perspective, one of the most notable findings resulted from the Cytos 2 experiment aboard the Salyut 7 space station in 1982 in which the minimum inhibitory concentration of spaceflight-cultured *E. coli* to colistin and kanamycin was evaluated (11, 50). In this study, spaceflight-cultured *E. coli* had a greater resistance to both antibiotics when compared to control cultures grown on Earth (11). The Cytos-2 experiment also demonstrated that *Staphylococcus aureus* developed an increased resistance to oxacillin, chloramphenicol, and erythromycin as compared to ground controls. In comparison, subsequent separate studies, which investigated the antibiotic sensitivity of *E. coli* to gentamicin on agar slants, did not observe differences between spaceflight and ground control culture growth (12). The mechanism/s behind the difference in outcomes are not fully understood, but the authors speculated that the growth of their organism on agar, instead of in liquid culture, may have been a contributing factor. Another factor that could affect microbial responses during spaceflight was demonstrated in an extensive study of multiple *E. coli* growth curves by Klaus et al. performed over multiple missions (13). This study found that *E. coli* grown in space had a

shorter lag phase, longer logarithmic phase, and higher cell density than control cultures on Earth.

To obtain better insight into the mechanisms behind unexpected *E. coli* responses during spaceflight, spaceflight analog investigations using the RWV bioreactor have been extensively employed (226, 239). In one of the earliest studies, Fang et al. reported that the LSMMG culture of *E. coli* inhibited the production of the antibacterial polypeptide microcin B17 and increased the release of the compound into the media (93). This study was also the first to investigate and report that increased fluid shear mitigated this phenotype in the RWV. Additional secondary metabolite studies from this team confirmed the LSMMG culture in the RWV also inhibited β-lactam antibiotic production by *Streptomyces clavuligerus* (61).

Other *E. coli* studies have focused on pathogenic *E. coli* to determine how spaceflight analog culture affects their pathogenesis-related characteristics, including resistance to a variety of stresses, including low pH, osmotic, alcohol, and thermal stress (66, 97, 102, 103), enterotoxin production (206), altered resistance to antibiotics (66, 240), and increased biofilm thickness (66). In a series of elegant RWV studies by Kim et al., enterohemorrhagic *E. coli* O157:H7 (EHEC) was cultured to determine both physiological characteristics and transcriptomic responses to the LSMMG environment (232–234). In these studies, LSMMG-cultured bacteria exhibited increased cell size in a medium-dependent fashion and were less resistant to thermal stress (55°C) relative to reoriented control cultures, possibly due to the downregulation of eight heat stress-related genes (232, 234). Investigation of changes in gene expression resulting from growth in LSMMG indicated an upregulation of genes in nutrient and energy metabolism, including the TCA cycle, glycolysis, and pyruvate metabolism compared to control cultures (233). Interestingly, growth in the LSMMG environment also showed an upregulation of the Shiga toxin 1 and toxin HokB genes (233). In a separate study investigating the adhesive-invasive *E. coli* strain O83:H1 (AIEC), LSMMG-grown bacteria did not display changes in cell density or resistance to acid or osmotic stress as compared to control conditions; however, the LSMMG cultures did display increased resistance to thermal and oxidative stress (102). In this same study, LSMMG culture increased the adhesion of *E. coli* O83:H1 to Caco-2 monolayers, but not invasion, as compared to controls. In addition, an early study by Carvalho et al. (241) showed that EHEC and enteropathogenic *E. coli* (EPEC) pathovars established a productive *in vitro* infection of 3-D intestinal models (as evidenced by loss of microvilli and pedestal formation/EPEC; pedestal formation and effacement/EHEC) when both the host and pathogen were simultaneously cultured in the RWV bioreactor. While the mechanism(s) behind these changes in different pathogenic strains of *E. coli* are not clear, they reinforce a strong association between alterations in fluid shear forces and virulence potential in these bacteria.

Vibrio species

Studies with *Vibrio fischeri* have focused on the ability of the spaceflight analog environment to alter the beneficial interaction between commensal microbes and their hosts. Foster et al. have undertaken a series of studies to investigate the relationship between the bioluminescent symbiont *V. fischeri* and its mutually beneficial interaction with its squid host, *Euprymna scolopes,* when both were grown simultaneously in the spaceflight analog RWV bioreactor (70). As part of their life cycle, *V. fischeri* colonizes the light organ of the squid and becomes luminescent after reaching a critical cell density (242, 243). Colonization of the squid light organ by *V. fischeri* is required for its proper development and function, while the luminescence provided by the bacteria serves as an anti-predator camouflage for its host (244). When cultured under LSMMG conditions, the symbiont *V. fischeri* enhanced the development of the squid light organ, as evidenced by an accelerated onset of bacterial-induced cell death and increased sensitivity of the light organ to LPS from *V. fischeri*, as compared to the reoriented control (70). Follow-up studies demonstrated that apoptotic gene expression profiles and caspase activity were upregulated during LSMMG culture, which is consistent with the accelerated apoptosis

phenotypes previously observed in the squid light organ during culture under these same conditions (245).

Given the link between Hfq as a central regulator of bacterial pathogen responses to both spaceflight and/or LSMMG culture (5, 8, 65, 69), subsequent studies using *V. fischeri* showed that LSMMG culture also differentially regulated the expression of *hfq* in this bacterium and that squid infected with an *hfq* mutant exhibited alterations in their developmental phenotype during LSMMG culture (195). While the latter observation is specific to this particular host-symbiont model system, the findings from *V. fischeri* analog studies suggest that host interactions with beneficial microorganisms which are important for physiological homeostasis could be altered in a spaceflight environment.

Pseudomonas aeruginosa

P. aeruginosa has been investigated in multiple spaceflight and spaceflight analog studies both due to its potential hazard to both astronaut health (48) and spacecraft systems (246). *P. aeruginosa* was responsible for a life-threatening urinary tract infection during the Apollo 13 mission (46, 48) and has been isolated from Apollo crewmembers (48) as well as from the ISS environment (247). Early studies by McLean et al. confirmed the ability of *P. aeruginosa* to form biofilms on polycarbonate membranes in the spaceflight environment (10). More recently, Kim et al. investigated the architecture of *P. aeruginosa* biofilms when grown during spaceflight (9). These important studies showed that spaceflight culture increased viable cell densities and biomass in biofilms. In addition, spaceflight appeared to alter biofilm architecture, resulting in unique column-and-canopy structures that differed significantly from biofilms formed in the ground control. The authors used wild type and motility mutant strains in this study and reported that the unique biofilm structure formed in spaceflight-cultured *P. aeruginosa* was dependent on flagellar, but not type IV pili motility (9, 18).

In another spaceflight study, Crabbé et al. investigated the transcriptomic and proteomic responses of planktonic *P. aeruginosa* as compared to otherwise identical Earth-based controls (8). In this experiment, *P. aeruginosa* differentially regulated 167 genes and 28 proteins. When compared to ground-based controls, the investigators found an upregulation of the genes that encode for the lectins, LecA and LecB, which play a role in the bacterial adhesion process to eukaryotic cells. They also found an upregulation of the virulence-related gene *rhlA*, which encodes rhamnosyltransferase I, an enzyme involved in rhamnolipid surfactant biosynthesis (8). Notably, as shown previously with *S*. Typhimurium, this study also identified Hfq as a key global transcriptional regulator of the spaceflight response, suggesting the first spaceflight-induced regulator acting across different bacterial species (5).

As with *Salmonella* and *E. coli*, *P. aeruginosa* has been extensively studied using spaceflight analog culture systems (68, 100, 248) with findings that have implications for both astronaut health and habitat sustainability. In response to LSMMG culture in the RWV, *P. aeruginosa* formed loose self-aggregating biofilms as compared to the phenotype formed in the reoriented control, wherein biofilms were tightly attached to the gas-permeable membrane (68). In addition, LSMMG upregulated the expression of *P. aeruginosa* genes encoding virulence factors, e.g., rhamnolipids, elastase, and the *rhl* quorum sensing system (68). In a separate RWV study, growth of *P. aeruginosa* in LSMMG conditions elevated alginate production, and also increased heat and oxidative stress resistance, compared to controls. Analysis of these cultures revealed 134 differentially regulated genes, including genes involved in microaerophilic/anaerobic metabolism, stress resistance, and motility. As observed in spaceflight culture, the RNA-binding protein Hfq also appeared to be a key regulator of many of the differentially regulated genes. In addition, LSMMG culture induced an upregulation of AlgU-controlled transcripts, including those encoding stress-related proteins (100). The upregulation of AlgU is notable as it is essential for alginate production and biofilm formation, an important virulence factor in *P. aeruginosa*.

S. aureus and other Gram-positive bacteria

Early research into the response of Gram-positive microorganisms dates back to early spaceflight experiments using model organisms, such as *Bacillus subtilis*, to better understand concepts such as how spaceflight affects bacterial cell division and biomass (249), viability in response to true space radiation (250), and bacterial response to both vacuum and radiation in space (251, 252). Studies with *B. subtilis* continue to provide an excellent model for the effect of spaceflight and spaceflight analogs on spore-forming bacteria (53, 199, 235, 253–255). Gram-positive bacterial studies have often provided insight into astronaut health and vehicle sustainability, by investigating factors such as changes in antibiotic resistance (11, 50, 256) and its response to antimicrobial surface coatings (257).

The response of *S. aureus* to the spaceflight environment has gained a great deal of attention from a human health perspective due to its ubiquitous nature in the environment (34, 50, 258, 259) and its presence and ability to be readily passed between crewmembers (260–262). While certain spaceflight experiments have provided important information, much of our understanding of potential *S. aureus* responses to spaceflight have been acquired using spaceflight analog systems. For example, a study by Rosado et al. identified decreased production of the pigment, and virulence factor, staphyloxanthin in LSMMG-cultured *S. aureus* (263). In addition, the authors observed a decrease in transcription of the *hla*, which encodes the production of alpha toxin. Separate studies by Castro et al. confirmed the decrease in staphyloxanthin and also reported dramatic increases in extracellular polymeric substances, and repressed virulence characteristics, such as increased susceptibility to oxidative stress and decreased survival in whole blood (69). Interestingly, the LSMMG culture of *S. aureus* resulted in a downregulation of the RNA chaperone Hfq, which parallels low-fluid-shear responses of certain Gram-negative organisms (5, 8, 69, 195, 264). Collectively, the findings on LSMMG-grown *S. aureus* suggest a less virulent phenotype, leading to the postulation that microbial responses to the spaceflight and spaceflight analog environment may differ depending on the benefits to each specific microorganism.

Recently, Jang et al. demonstrated that spaceflight analog culture (RWV) significantly altered the membrane lipid profile of *S. aureus* and increased its sensitivity to membrane-disrupting antimicrobial compounds as compared to control conditions (75). Specifically, LSMMG culture resulted in profile shifts of both branched and straight-chain fatty acids in a manner known to increase cell membrane disorder and fluidity (265, 266). Based on this observation, the authors reasoned that LSMMG culture would increase the sensitivity of *S. aureus* to treatment with membrane-disrupting agents, which was demonstrated using daptomycin, sodium dodecyl sulfate, and violacein (75).

Streptococcus pneumoniae is expected to be carried as a commensal part of a healthy astronaut's normal flora but has the potential to cause a wide range of diseases, especially if the immune system is compromised. Diseases caused by *S. pneumoniae* include otitis media, meningitis, pneumococcal pneumonia, and bacteremia (267, 268). Transcriptomic profiling of *S. pneumoniae* compared cultures grown in microgravity to both traditional ground controls and cultures grown in LSMMG (267). The results indicated a clear difference in gene expression across a range of diverse functional groups between spaceflight-grown cultures and the two control conditions (267). The results from this spaceflight study also revealed some similarities between earlier RWV studies profiling the impact of LSMMG culture on *S. pneumoniae*, which also indicated differential expression in genes encoding diverse functional groups as compared to controls (269).

Streptococcus mutans is a primary causative agent of dental caries, and accordingly, its response to changes during spaceflight is of interest to NASA. Studies by Orsini et al. indicated that *S. mutans* cultured in the LSMMG environment was more susceptible to oxidative stress than control cultures (101). Interestingly, this difference was growth phase-dependent, only being observed during the late-stationary phase. This study also investigated transcriptomic profiles and identified 247 differentially regulated genes

(153 upregulated and 94 downregulated), including genes associated with carbohydrate metabolism, translation, and stress responses (101).

The vast majority of microbial spaceflight and spaceflight analog studies have been short duration in length (hours to several days) due to limitations in power, volume, crew time, and hardware capabilities in performing biological spaceflight experiments. Given the relatively short generation time of microorganisms, long-duration, multigeneration studies enable experimental evolution approaches in which mutations and genetic exchange or rearrangements can be coupled with natural selection (270). Given the importance of microbial changes during anticipated prolonged spaceflight and future lunar bases, long-duration studies have been proposed in the 2011 and 2023 NASA Decadal Report (85, 271). Long-duration studies are challenging, and data interpretation and conclusions drawn from them can be difficult due to challenges in preventing microbial contamination and the need for appropriate controls. Only a few studies have examined long-duration alterations in microbial mutation rate and heritable changes resulting from long-duration growth in the spaceflight or spaceflight analog environment (73, 239, 253, 256, 272), including a study sequencing *E. coli* that had been cultured in the RWV bioreactor under LSMMG conditions for over 1,000 generations (239). Sequencing of these LSMMG cultures revealed 16 distinct mutations relative to the zero generation control; however, as no reoriented control was performed, additional studies are needed to determine if any of these mutations are unique to the LSMMG condition (239). In another notable long-duration study, Fernander et al. evaluated the impact of LSMMG culture (as compared to the reoriented control) on *S. mutans* (73). In this study, *S. mutans* was grown in the RWV for 100 days to determine how this microorganism adapts to the LSMMG environment. The authors provide evidence that multiple variants of *S. mutans* were developed in response to growth in the LSMMG environment, influencing heritable phenotypes such as adhesion and acid tolerance. Importantly, none of the unique genetic variants identified in this study were present in all four of the biological replicates (73), reinforcing the need for appropriate controls and multiple biological replicates for future investigations in spaceflight.

Mycobacterium marinum

The response of several other microorganisms that are relevant to human health and habitat sustainability has been investigated during spaceflight. For example, LSMMG studies of the waterborne pathogen, *M. marinum,* which causes infections in fish and humans, indicated a faster transition to the stationary phase and greater sensitivity to oxidative stress during RWV culture as compared to the reoriented control (273). Transcriptomic data suggested a downregulation of metabolism, an increase in lipid degradation, and increased chaperone and mycobactin expression as compared to controls. Mycobactin proteins play an important role in mycobacterial pathogenesis and virulence by serving as siderophores for iron acquisition in the infected host and are essential for growth in macrophages (274, 275). Based on these data, Abshire et al. proposed that nutrient deprivation could be a signal to the cells in the LSMMG environment and that the sigma factor SigH has a role in modulating transcriptional changes.

The LSMMG response of *M. marinum* is important and relevant to human health, as it is an important model for human pathogens, such as *M. tuberculosis* and *M. avium* (273). Notably, molecular analysis of spacecraft indicated the presence of *Mycobacterium* virulence operons on ISS (208) and the presence of *M. avium* in water samples from the Mir Space Station (276), suggesting a presence of mycobacteria not detected using culture-based monitoring. Of additional medical relevance, Clary et al. demonstrated increased survival to rifampicin when grown in the RPM (186).

Candida albicans and other fungal studies

One of the earliest spaceflight investigations of yeast used cultures of the model eukaryotic microbe, *Saccharomyces cerevisiae,* that had been collected from the Microbial

Ecology Evaluation Device (MEED) aboard the Apollo 16 lunar mission (277). In this rare study beyond LEO, *S. cerevisiae* exposed to the deep space environment-induced dermal lesions in mice and recovered from these lesions at higher levels as compared to ground controls (49). It is important to note that these cultures were exposed to natural ultraviolet radiation (278) during the spaceflight mission and were not immediately tested after landing. In addition, *S. cerevisiae* showed increased uptake of phosphate in response to spaceflight (279). Classically, *S. cerevisiae* has not been considered a pathogenic organism; however, these findings suggested the potential of clinical implications associated with microbial growth in the spaceflight environment.

Characterization of the transcriptomic and phenotypic profiles of the opportunistic dimorphic fungal pathogen, *Candida albicans,* in response to spaceflight culture showed global changes in these properties as compared to synchronous ground controls (14). In response to environmental signals, *C. albicans* transitions between the single-cell yeast and filamentous form, a process which contributes to virulence (280). In response to spaceflight culture, a large number of genes in *C. albicans* representing diverse functional categories were differentially regulated, including those involved with biofilm formation, cell adhesion, filamentous growth, cell budding, actin cytoskeleton organization, cell cycle regulation, transport and multi-drug efflux proteins, drug resistance, and oxidative stress. Interestingly, expression profiles of several of these genes suggested the possibility that virulence-related phenotypes in *C. albicans* might be altered during spaceflight culture; however, this functional phenotype has not been documented. Phenotypic validation of cellular morphology using microscopic imaging and flow cytometry was in agreement with the observed spaceflight-induced expression of genes involved in cell aggregation (relevant to biofilm formation), random budding, and cell size. In addition, spaceflight culture of *C. albicans* induced the expression of the gene encoding transcriptional regulator Cap1 and differentially regulated over 30% of the Cap1 transcriptional regulon. This suggests a possible role for Cap1 in regulating the spaceflight response of *C. albicans*, given its alignment with transcriptomic and phenotypic profiles associated with spaceflight culture (14). Relevant to the spaceflight-enhanced expression of *C. albicans* genes encoding resistance to antimicrobial compounds, a subsequent spaceflight experiment by Nielsen et al. demonstrated an increased resistance of *C. albicans* when challenged with the antifungal drug amphotericin B (281).

In agreement with spaceflight results, ground-based analog studies in the RWV bioreactor showed that *C. albicans* displayed random budding and clumping phenotypes in response to LSMMG culture as compared to control conditions (202), which is consistent with phenotypes observed for this same organism during spaceflight (14). Moreover, LSMMG culture increased filamentation and biofilm formation in *C. albicans*, with a concomitant morphological transition from the yeast to filamentous (hyphal) form which coincided with changes in the expression of genes involved in this transition (202). Interestingly, the culture of the model yeast *S. cerevisiae* in LSMMG conditions showed changes in the expression genes associated with budding, cell polarity, and cell separation (201). In addition, *S. cerevisiae* in LSMMG culture increased random budding patterns and tendency to clump/aggregate under these conditions, which supports morphological changes reported for *S. cerevisiae* in response to spaceflight culture (15, 16), as well as *C. albicans* responses to culture in both LSMMG and spaceflight conditions (282).

As with yeast, filamentous fungi have also displayed unexpected responses to spaceflight and spaceflight analog culture. Early investigations were primarily survival studies, including those using *Penicillium roqueforti* during the Gemini Program (283, 284) and *Tricophyton terrestre* and *Chaetomium globosum* in the MEED experiment during the Apollo Program (279, 285). More recent studies have investigated changes in filamentous fungal characteristics, including many comparing strains isolated from ISS with different terrestrial strains of the same species that had not flown (56, 286–288), thus limiting the exact effect of spaceflight on the organisms. However, in 2019,

Romsdahl et al. reported a comparison of *Aspergillus nidulans* grown on ISS with an otherwise identical ground-cultured control (289). The authors observed significant changes in the expression of proteins associated with stress response, carbohydrate metabolic processes, and secondary metabolite biosynthesis, as well as alterations in specific regions of the genome (e.g., insertions, deletions). A separate study by Blachowicz et al. investigated genomic, transcriptomic, and metabolomic alterations in *Aspergillus niger* cultured on ISS compared to ground-cultured controls (290). The authors identified multiple changes in protein expression associated with carbohydrate metabolism, stress response, and cellular amino acid and protein catabolic processes in the strain cultured on the ISS. Interestingly, the heritable production of the antioxidant, pyranonigrin A, was increased in the ISS-cultured strain, compared to the ground control strain (290). Subsequent investigation by the authors provided evidence that the elevated levels of pyranonigrin A resulted from genetic changes, that they propose could have been induced through selection to adapt to elevated radiation levels (291).

Spaceflight analog studies of filamentous fungi have also been investigated (292–295). For example, Jiang et al. observed an increase in organic acids produced by *Aspergillus carbonarius* grown in a clinostat (294). Subsequent studies also demonstrated that this increase in organic acids could increase the corrosion potential of this organism (295). Due to the nature of filamentous fungal growth and corrosion testing, the organisms were grown on agar Petri dishes that were rotated in the clinostat (294, 295). While some evidence suggests solid vs liquid growth conditions may influence microbial responses to the spaceflight environment (53), these articles identify factors, such as alterations in the potential of microbially influenced corrosion, that should be considered and investigated to maintain the stability of space habitats.

Microbial studies investigating spacecraft habitat sustainability

One key benefit of the culture-based approach currently used for environmental monitoring aboard the ISS is that it enables the development of a historical collection of microflora that can be used for subsequent longitudinal investigations of microbial changes over time in the unique spaceflight environment. Notable examples of this approach to use the ISS as a "microbial observatory" are elegant studies that characterized functional phenotypes from NASA-archived bacterial isolates collected over several years from the ISS potable water system (58–60). These independent studies shared the goal of identifying changes in bacterial characteristics over time that could negatively impact astronaut health and life support systems (58–60).

Using a six-member model microbial community of ISS potable water isolates [*Cupriavidus metallidurans*, *Chryseobacterium gleum*, *Ralstonia insidiosa, Ralstonia pickettii, Methylorubrum* (*Methylobacterium*) *populi,* and *Sphingomonas paucimobilis*], Thompson et al. documented the contributions and interactions of individual community members to the robustness and resilience of multispecies microbial biofilms and found that no individual species was solely responsible for polymicrobial biofilm formation (58). Specifically, the deletion of individual members from the consortium had no significant effect on the overall biofilm population although the species distribution was altered. Furthermore, the introduction of species-specific phage or predatory bacteria into pre-formed biofilms did not selectively remove specific bacterial community members under the conditions of this study (58).

In a separate study, Yang et al. performed longitudinal phenotypic characterizations of multiple ISS potable water bacterial isolates collected between 2009 and 2015 (*R. pickettii, S. sanguinis, C. metallidurans*, *B. cepacia, R. insidiosa, Methylobacterium species, Bradyrhizobium species, Mesorhizobium species,* and *S. aureus*). These studies characterized functional phenotypes of the bacteria as pure cultures or as multispecies consortia, including biofilm formation, structure, morphology, and composition (e.g., microbial composition and community stability), metabolism, hemolysis, and susceptibility to antimicrobial compounds (60). An association was observed between microbial adaptation over time to the microgravity environment, as the interactive behaviors of

some bacterial isolates appeared to depend on when they were collected from the ISS in the same year or different years. The authors hypothesized that for these specific bacteria, a shared period of coexistence in the ISS water system might influence their interactions.

A study by O'Rourke et al. sequenced the genomes of 24 *Burkholderia* isolates that had been collected over 4.5 years from the ISS potable water supply, identifying 19 of these isolates as *B. cepacia* and 5 as *B. contaminans,* with evidence that these two groups arose from the same founding populations (59). Phenotypic traits of these isolates were evaluated and compared to terrestrial reference strains. While the *B. cepacia* strains isolated from the ISS did not display fungal inhibition or hemolysis characteristics, the *B. contaminans* ISS isolates exhibited hemolysis and anti-fungal properties to certain degrees. Interestingly, these *B. contaminans* isolates also displayed higher levels of hemolytic activity than their terrestrial control.

These types of observations have provided new insight into how microbial biofilms might develop and persist over time under microgravity conditions and are essential to predict emergent and non-intuitive phenotypes observed in the context of multi-species consortia, which cannot be fully predicted by sequencing studies alone. For example, nutrient levels coupled with microgravity conditions have also been implicated in the growth and biofilm formation capabilities of ISS isolates (296), suggesting possible approaches to address biofilm formation during spaceflight. Along these lines, a new series of spaceflight studies focused on biofilm control have been conducted to investigate how polymicrobial species form biofilms on spacecraft materials, induce corrosion, and alter resistance to disinfectants (2, 35, 297–299).

HUMAN HEALTH DURING SPACEFLIGHT

Risk of infectious disease in space

NASA has historically implemented stringent preventative measures to mitigate the risk of infectious and other microbially associated disease, including preflight microbiological monitoring of spacecraft potable water systems and habitable volume air and surfaces (35, 247, 300–302). In a similar fashion, cargo bags and spaceflight hardware are also tested for potential microbial hazards to the astronauts (303). Non-thermostabilized foods consumed by the crew are also monitored for microbial contamination prior to spaceflight (304). In addition, biosafety assessments are performed for spaceflight experiments that contain potential biological hazards (303).

One of the most effective mitigation approaches to protect astronauts from infectious disease has been the Health Stabilization Program (HSP) first formally implemented in the early 1970s (305) after a series of infectious disease incidents during the Apollo Program, including upper respiratory infections inflight during Apollo 7 (3 crewmembers); infectious gastroenteritis inflight and preflight during Apollo 8 (3 crewmembers); upper respiratory infections preflight during Apollo 9 (3 crewmembers); upper respiratory infections preflight during Apollo 10 (2 crewmembers); and rubella exposure preflight (306). Perhaps, the most visible incidence of infection during spaceflight occurred during Apollo 13 in which a crewmember suffered a severe urinary tract infection caused by *P. aeruginosa* (48). Treatment with antibiotics (furadantin and pyridium) during the mission was ineffective (48). While the hallmark of the HSP has been astronaut quarantine, this program was also enhanced by employee awareness of infectious disease risk and safety precautions taken prior to spaceflight. Once implemented, its success was immediately evident. Before the HSP, 57% of Apollo crews experienced infectious disease during the 3-week period prior to launch (306). After initiation of the HSP, only skin infections were recorded in this 3-week period during the Apollo Program (306).

Even with these stringent precautions, the risk and incidence of the disease has not been eliminated (48, 307–309). While diagnosing infectious disease at remote locations can be challenging, epidemiological evaluations of Space Shuttle Program data for

missions STS-1 through STS-89 indicated that infectious disease accounted for 1.4% of all medical events (not including skin and subcutaneous tissue) (308). Incidents of infectious disease for Space Shuttle Program astronauts included fungal infection, flu-like illness, urinary tract infection, aphthous stomatitis, infectious gastroenteritis, subcutaneous skin infection, and other viral diseases based on post-flight debriefs (310). In a separate evaluation of infectious disease and allergic symptom rates on ISS, the incidence of infectious disease through 2016 was estimated to be 3.4 events/flight year (309). Importantly, these data reflect infectious disease rates and types under the restrictive regulations NASA has for its astronauts. An overlooked but critical contributor to the risk of infectious disease during spaceflight is the "stacking of risks" associated with different aspects of the spaceflight environment. For example, the combination of a dysfunctional immune system, alterations in microbial virulence, physiological and psychological stress associated with spaceflight missions, closed confined spaces, reduced gravity, radiation levels, and exposure to celestial dusts may combine to increase the overall infectious disease risk in ways that are not expected with any one factor.

As human space exploration transitions to more distant locations like the moon and Mars with traditional NASA career astronauts, the number of commercial civilian and military space travelers in LEO with non-traditional astronauts will increase robustly over the coming years. As many of these individuals will have underlying health conditions that would have been identified by NASA for a traditional career astronaut, the inclusion of non-traditional astronauts into space travel will likely increase the rates and types of infectious disease events (311). This change in the type of space traveler will create a new dimension of occupational health for the new civilian astronaut workforce to understand the space environment on human health and habitat sustainability.

Immune system dysfunction in space

Multiple reviews have described the effects of spaceflight on immune function and have consistently documented that the immune response is altered during spaceflight on both short and long mission durations (29, 33, 312). Spaceflight-induced dysregulation of both innate and adaptive immune responses has been observed in humans and animals, and ground-based spaceflight analog models have validated some of these findings (313–318). Likewise, alterations in the immunobiology of cell culture model systems have also been reported in response to both spaceflight and analog conditions (31, 319). Collectively, the negative impact of spaceflight on host immune functions and enhanced alteration of virulence and pathogenesis properties in some microorganisms suggests an increased risk for infectious disease events in spaceflight [reviewed in references (29, 320)].

Documented spaceflight-induced changes to the immune system have included alterations in lymphoid tissue; the number, proliferation, and function of immune cell populations involved in innate immunity (neutrophils, monocytes, macrophages, and NK cells) and adaptive immunity (B and T lymphocytes), as well as the production of cytokines and immunoglobulins. A large body of evidence indicates a decrease in the cell-mediated immune response during spaceflight (29), including altered cytokine profiles and a change in T-cell subsets indicating a shift from Th1 toward a Th2 immune response (321), the latter of which indicates a greater potential risk for infections by intracellular pathogens. Furthermore, spaceflight-induced alterations in astronaut blood samples indicate that neutrophil phagocytic and oxidative functions are diminished, as is the ability of monocytes to phagocytose a non-pathogenic strain of *E. coli* and, subsequently, elicit an oxidative burst and degranulate (313, 314, 322). Natural killer cell cytotoxicity has also been shown to be diminished by spaceflight (29, 322), as has the production of interferon (29, 323).

In addition, there is evidence of chronic low-level inflammation in astronauts during long-duration spaceflight (324). Consistent with a decrease in immune function, reactivation of latent herpes viruses, including Varicella Zoster, Epstein Barr, HSV-1, and cytomegalovirus has been reported in astronauts (25–28, 325–329). In addition,

transcriptomic profiling of the whole blood of astronauts (both male and female) in response to spaceflight showed differential regulation of stress response genes, including those important for oxidative stress, DNA repair, detoxification, and protein folding/degradation, several of which are associated with modulation of the immune system and protection against microbial infection (330). Although the clinical correlation of these immune changes to infectious disease incidences in spaceflight has not been fully established, they are of concern, and the biological importance of the immunological changes induced by spaceflight with regard to resistance to infection remains to be established.

Many studies involving the effects of spaceflight on immunity have been limited by the use of pre- and post-flight crew samples obtained from short duration space missions, with no samples taken in-flight. Accordingly, there are comparatively fewer studies that have utilized samples acquired during spaceflight, creating many inconsistencies within the current scientific literature. This inconsistency is further exacerbated by the small number of data points and different durations of spaceflight. However, recent investigations from crew members of long duration space missions (3 months and longer) have used blood samples drawn in flight (32, 324). Analysis of in-flight samples indicated that immune dysfunction persisted during long-duration missions, with alterations potentially leading to immune hyperactivity (which may result in risks such as hypersensitivities or autoimmunity) and immune hypoactivity (which suggests increased risks for infectious diseases, viral reactivation, and other disorders) (32, 324). Furthermore, elevated levels of inflammatory cytokines observed in ISS astronauts during long-duration missions suggest that multiple physiological adaptations persist during spaceflight, including inflammation and leukocyte recruitment (32). Further investigation is necessary to better understand the mechanisms of these effects on the immune system induced by spaceflight in order to develop countermeasures to reduce infectious disease risks for the crew.

Although the first human traveled to space over 60 years ago, the number of people who have flown in space is still small. At the time of this article, only a few more than 600 people have traveled to space, which is an average of 10 individuals per year. The vast majority of these individuals have been professional career astronauts whose spaceflight missions have almost exclusively been in the microgravity environment of LEO and within the protective magnetic field of LEO, which protects against radiation. Only a handful of astronauts from the Apollo moon missions have traveled to deep space locations that are associated with different partial gravity conditions and outside of the protective radiation zone, and these were short-duration missions with the crew briefly exposed to these conditions. Future deep space exploration and longer missions to the Moon and Mars will expose humans to these stressful conditions for extended periods of time, which is a major health concern. In addition, the rapid transition to commercial civilian spaceflight in LEO and its reliance on individuals often having underlying health conditions which are not in alignment with those of career astronauts increases the risk for microbial health threats.

Crew microbiome and human pathophysiology

The diverse microbial ecosystems associated with the human body are vital for regulating the balance between health and disease. Numerous terrestrial studies have shown that microbiota composition and function can be modulated by a variety of intrinsic and extrinsic factors (e.g., sex, age, nutrition, psychological stress, infection, medication, radiation) (331). As astronauts are subjected to a unique combination of stressors, understanding how both short- and long-term space travel alters the composition and function of astronaut microbiota is important for the design of strategies that ensure optimal physical and mental health.

Since the early days of human spaceflight, astronauts and cosmonauts have been monitored for alterations in their microbial flora (20, 48, 262, 332–335). Excellent reports by Taylor et al. detailed the specific microbial shifts observed during these early

missions, with a focus on the Apollo (48) and Skylab (47) programs. These foundational studies, which used culture-dependent approaches, routinely demonstrated increased total microbial loads, particularly for Gram-positive opportunistic pathogens, such as *Staphylococcus* and *Streptococcus* spp., as well as opportunistic fungal pathogens like *C. albicans* (20, 48). Of additional concern was the decrease in protective commensal flora, including *Lactobacillus* species. Of interest, microbial monitoring was performed not only to assess changes in the presence and abundance of potential pathogens but also to exclude potential contaminants in material returned from the lunar surface (46, 48).

The advancement of culture-independent sequencing technologies allowed for the broader detection of alterations in the crew microbiome. During the NASA Twins Study, twin astronauts were monitored over the course of 1 year for alterations in their gut microbiome composition (along with a wide range of physiological and -omics data), with one twin living in space and the other on Earth (57). This study revealed slight but significant spaceflight-associated shifts in fecal microbiota composition relative to pre-flight and post-flight levels. A higher ratio of *Firmicutes* to *Bacteroides* was observed during spaceflight relative to pre-flight or post-flight levels; an effect not observed in the ground-based subject over the same time period. In addition, in-flight microbial community taxonomic structure and functional gene content was significantly different from pre- and post-flight samples but returned to normal within 6 months. Metabolomic profiling indicated a spaceflight-associated decrease in some microbial metabolites with known anti-inflammatory properties.

During the Astronaut Microbiome Project, Voorhies et al. longitudinally characterized the impact of spaceflight on microbiomes of nine astronauts living aboard the ISS for 6–12 months (19). Skin, saliva, nostrils, and feces were sampled and profiled using 16S analysis. In parallel, astronauts were monitored for alterations in cytokine expression and viral reactivation by sampling of blood and saliva, respectively. Spaceflight-associated alterations in microbiome composition were observed for all sites sampled relative to pre-flight levels. Alterations in intestinal microbiota indicated increased diversity for almost all crew members, which eventually returned to pre-flight levels after return. Spaceflight was also found to alter the abundance of seventeen GI genera. Of interest, there was a greater than fivefold inflight reduction in abundance for some genera with anti-inflammatory properties, including *Akkermansia, Fusicatenibacter*, and *Pseudobutyvibrio*. This correlated with in-flight increases in some pro-inflammatory cytokines in the crew. The GI microbiome beta diversity analysis revealed an increased similarity across crew members during flight, likely due to commonalities in their diets during their missions aboard ISS. A key consideration in microbiome analysis within a closed environment is increased potential for transmission from the habitat to the crew member and vice versa. Previous studies using culture-based approaches have shown that microbes like *S. aureus* were readily passed between crewmembers (260–262). The study by Voorhies et al. and other sequencing investigations have also observed evidence for transmission between crew members and their environment (19, 336–338).

Short-duration spaceflight has also been shown to alter the composition of astronaut gut microbiota (339). These changes are not unexpected, as the human microbiome is quickly altered by environmental factors and can alter the antibiotic resistance profiles (340–343). Metagenomic sequencing of stool samples collected from astronauts following Chinese spaceflight missions revealed increases in the abundance of *Firmicutes* and *Bacteroides* after spaceflight. Post-flight decreases in probiotic taxa, including *Lactobacillus* and *Bifidobacterium*, were also reported. In addition, there were also fluctuations of genes belonging to multiple gene groups, including those involved in virulence, antibiotic resistance, envelope biogenesis, and biofilm formation.

Sequencing analysis of the salivary microbiome of astronauts indicated alterations in *Streptococcus*, *Proteobacteria*, and *Fusobacteria* (344). In general, microbiome diversity and richness were found to increase with spaceflight and return back to normal post-flight. However, the diversity of *Streptococcus* was found to decrease relative to pre- and post-flight levels and did not entirely return to pre-flight levels after return. The

authors also observed a correlation between the salivary microbiome in the presence and absence of viral reactivation in crew members.

Similar to terrestrial studies, person-to-person variation has also been observed for spaceflight microbiome analyses. Morrison et al. collected samples from different body locations (skin, nose, ear, mouth, saliva) from four astronauts over two consecutive missions (345). These samples were analyzed in tandem with metagenomic sequencing and a high-density DNA microarray. When the data for all astronauts were analyzed as a group, the authors observed no significant differences in the number or relative abundance of taxa. However, analysis of individual astronauts revealed flight-associated changes. In salivary samples, two astronauts displayed an increased abundance of certain commensal microbes such as *Prevotella*, with a concomitant decrease in abundance in other commensals. Interestingly, an elfamycin resistance gene and CfxA6 beta-lactam marker were found to increase in abundance in response to spaceflight.

Collectively, studies have generally reported alterations in astronaut microbiomes following both short- and long-duration missions. However, the potential positive or negative health consequences associated with these changes and the use of microbiome data as a diagnostic tool for crew health have yet to be determined.

Current food systems

Another critical aspect of space missions that hold the potential to impact astronaut health in both potentially positive and negative ways is the spaceflight food system (346, 347). From the beginning of human spaceflight programs, microbiological considerations influenced the processing, packaging, and storage of spaceflight foods (348, 349). Notably, the Hazard Analysis Critical Control Point system that is commonly used in the food industry today was developed through a collaboration between NASA, the Pillsbury Company, and the Natick Laboratories of the U. S. Armed Forces to prevent foodborne illness (349). Samples from each lot of non-thermostabilized foods are microbiologically monitored to ensure the quality of food supplied to the astronauts (303, 304). Preflight analyses of food samples have detected potentially pathogenic microorganisms, including *S.* Typhimurium, *S. aureus*, *Enterobacter cloacae,* and *Cronobacter sakazakii* (350).

HABITAT SUSTAINABILITY

The microbiology of the built environment takes on pivotal importance during human spaceflight, as microbial processes that affect the integrity of the spacecraft, life support, or other critical functions within the habitat (e.g., communications) can become life-threatening. Early problems associated with microorganisms identified on human spaceflight vehicles included corrosion and fouling of systems onboard Mir and other spacecraft, as well as life support components of space suits (351). Unwanted moisture accumulation, material design, and housekeeping concerns were identified as risk factors and probable causes of these problems (351). While many mitigation strategies such as condensation reduction, improved housekeeping, and advanced construction materials and surface coatings are now employed, microbial monitoring remains an important task for the evaluation of safety in space habitats. As is also the case on Earth, microorganisms associated with spaceflight have many potential benefits for habitat sustainability. These can include potential uses in life support and applications in future space manufacturing and other industries. In this section, we address several microbial issues related to habitat sustainability in space and identify key future challenges. We focus primarily on the ISS as a model space habitat. While the Chinese space station, Tiangong, is currently operational, no microbiological data on resident flora is available at this time (352).

Function and integrity of spacecraft and onboard life support systems

There are a number of factors that are involved in the design and construction of spacecraft [reviewed in reference (353)]. Aside from the structural and propulsion

components, other key equipment is associated with navigation, communication, and life support spacecraft functions. Spacecraft-associated microbial problems were prominent in the former space station, MIR, which damaged structural materials and caused equipment malfunctions, including fungal growth that interfered with space-to-ground communications (354). Microbial contamination is also present on the ISS, including biofilm-associated fouling in the water recovery system (WRS) (246, 297) and a fungal contaminant of ISS surface material on which exercise clothes were dried (355) (Fig. 3). While housekeeping and other microbial mitigation tasks have been optimized for the ISS, there are a number of practical considerations that will need to be addressed in more distant space mission locations. Included are items such as cleaning and sanitation supplies and the potential for additive manufacturing technologies for the preparation or repair of equipment (356).

Microorganisms in the spacecraft environment: microbial monitoring

The ISS has been continuously inhabited since November 2000 (34). Present in the spacecraft are the microorganisms that were carried to the ISS as part of the crew's natural microbial flora, as well as organisms that were associated with cargo, supplies, and those introduced with each new ISS module. In 2004, Castro et al. (34) first characterized the microbiota of the early ISS habitat by 16S gene sequencing of samples isolated from potable water, air, and surfaces, thus establishing an important baseline against which future microbial characterization of this space habitat could be compared. These researchers found that the microbiome of the ISS closely resembled the microbiomes of both the Mir Space Station and the Space Shuttle (276, 357), as well as closed

FIG 3 Fungal contamination of a noise mitigation panel in the ISS Zarya module where exercise and hygiene clothes were hung to dry. The arrow points to areas discolored by fungus. (Image from NASA, photo iss009E28777.)

environments on Earth (358). Fifteen years later, this finding was reinforced in a new study which used a metagenomic approach to characterize the microbiome of the ISS (354). A study published in 2016, which described the monitoring of the JAXA "Kibo" module on the ISS, found that the microbiome of various surfaces in this module is composed primarily of human-associated bacteria, as well as some non-human associated bacteria, which were likely introduced during ISS resupply missions (359).

In-flight environmental monitoring on the ISS to mitigate risks to crew health relies upon culture-dependent methodologies to enable microbial enumeration, with subsequent identification after samples are returned to Earth, although in flight culture-independent approaches are being investigated (360–364). Microbial monitoring locations of interest such as the potable water life support system, ambient cabin air, and various surfaces in the habitat are sampled and cultured in flight to determine microbial load. Due to the low microbial numbers in potable water and cabin air, specific volumes of water and air are concentrated by filtration (water) and compaction (air) before being cultured. The specific methods and equipment for microbial sampling and processing that are currently used on the ISS have been described in Pierson et al. (365) and reviewed in Yamaguchi et al. (300). In general, spacecraft air and surfaces during a mission reflect the types of microorganisms found in a terrestrial home, such as the bacterial genera *Staphylococcus* and *Bacillus* and the fungal genera, *Aspergillus* and *Penicillium* (365). An overview of several commonly isolated microorganisms identified in various studies performed over the life of ISS is presented in Table S1. One key benefit of the culture-based approach currently used for environmental monitoring aboard the ISS is that it enables the development of an archival collection of culturable microflora that can be used for subsequent longitudinal investigations of microbial changes over time in the unique spaceflight environment (as previously discussed).

With planned human missions beyond LEO, there is an effort to develop and refine culture-independent approaches to monitor microorganisms, particularly those that can be done entirely on spacecraft. Technological advances include PCR thermocyclers and gene sequencers that have been used on the ISS (360, 361, 366). As an important proof of concept that microorganisms could be sequenced in spaceflight, two Gram-positive *Staphylococcus* isolates (*S. capitus* and *S. hominus*) were isolated on culture plates from ISS surface samples and identified via Nanopore sequencing in flight (366). This approach has subsequently been expanded to sequence other common ISS microbial isolates without prior culture on media plates (361). In addition to sequencing, other technological approaches for microbiological monitoring have been investigated, including mass spectrometry, which has been used in robotic space exploration payloads [reviewed in reference (367)] and could be incorporated for human spaceflight to enhance molecular analyses of proteins, metabolites, or novel chemicals on extraterrestrial environments. Other considerations for microbiological monitoring in deep space include the need for stable consumables and software with appropriate databases to facilitate on site interpretation of monitoring data and associated risk assessment, which will also be crucial for establishing microbial monitoring resources beyond LEO.

Biofilm formation and control

Microbial biofilms associated with spacecraft, including life support systems, are a primary safety concern, as they can affect crew health and vehicle system function. Experiences with long-duration space missions in LEO (Skylab, Mir, and most recently ISS) along with microbial monitoring have led to the refinement of housekeeping protocols to control water condensation and other microbial-promoting conditions. However, biofilm control in spacecraft remains an ongoing challenge.

Experimental biofilm formation in microgravity was first described in studies involving *Burkholderia cepacia* (54) and *P. aeruginosa* (10). However, knowledge of biofouling problems in spacecraft was previously reported on Mir and other space habitats, including contamination of piping, within insulation materials, life support equipment and spacesuits, and a navigation window (351). The major issue with biofilms

is one of persistence as this mode of growth results in the development of tolerance to disinfectants and other adverse chemical conditions (368–370). Biofilms are normally polymicrobial communities, and the close proximity of microorganisms within biofilms can facilitate genetic exchange, metabolic interactions, and protection to some microbial community members [e.g., (369, 371)].

Important issues associated with biofilm contamination include those which occur in the ISS WRS (Fig. 4), which is an important component of spacecraft life support. Due to the expense of sending payloads to the ISS, onboard water is recycled through the WRS. Specifically, water collected from crew urine and humidity condensate is distilled to remove salts, treated with an oxidizer, filtered through a number of resins to remove organic compounds, and disinfected through the addition of iodine (372). This level of treatment is necessary as microorganisms present in urine and humidity concentrate represent a potential risk to both crew health and various WRS components. Even with this level of microbial control, biofilms have been encountered in WRS wastewater and have caused at least one major incidence of fouling that required the replacement of a major component (372). Aside from fouling, other potential biofilm-associated risks to the WRS and other spacecraft systems include microbial corrosion of materials, inactivation of onboard equipment, and potential risks to crew health by embedded pathogens. Microbiological monitoring of the wastewater tank in the WRS indicates the presence of a variety of bacterial genera, including *Ralstonia, Burkholderia, Cupriavidus,* and *Sphingomonas* (60, 297). Interestingly, five representative microorganisms (*Burkholderia multivorans, C. metallidurans, R. insidiosa, P. aeruginosa, and Methylobacterium fujisawaense*) isolated from the WRS and the Russian water recycling system on ISS have been standardized as model bacterial communities in disinfection studies for future spaceflight missions (373), particularly those that will go beyond LEO. Aside from bacteria, fungi and archaea have also been described (288, 374).

Environmental engineering approaches for microbial control

Biofilm control is a global concern in many environments including those associated with medical, industrial, and marine importance, as well as in spaceflight. There are several added complications in spaceflight, notably the finite and limited resources of water and other consumables, as well as payload mass and cost considerations; thus, the efficacy of biofilm controlling approaches beyond LEO would need to involve materials that could be regenerated in spaceflight or on a lunar base [addressed in reference (297)]. Chemical

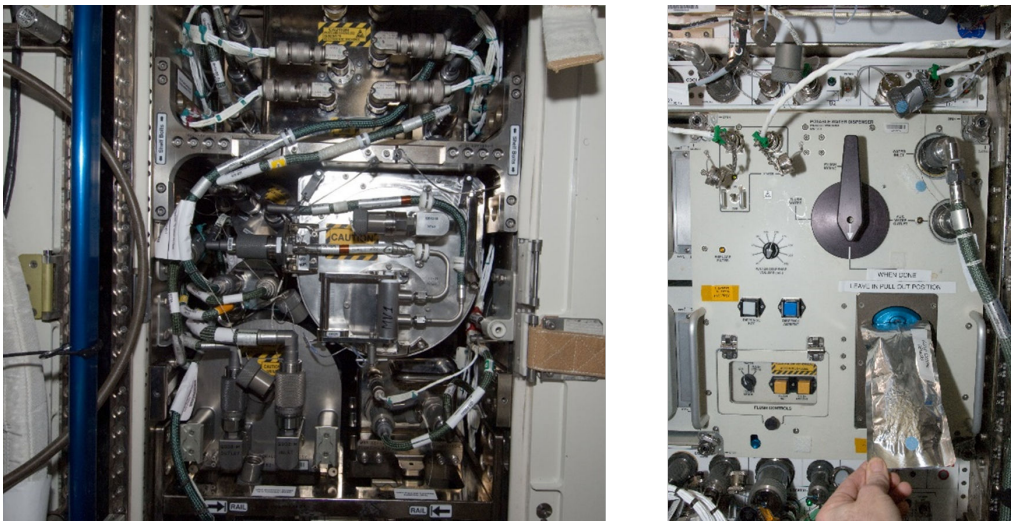

FIG 4 (A) WRS on ISS and (B) potable water dispenser which is used as a source of potable water. The image shows a silver drink bag being filled by a crew member on the ISS with potable water. (Images from NASA, photos iss032e016316 and iss031e012293.)

safety concerns also restrict the use of volatile and highly reactive disinfectants that may negatively impact the crew and equipment. The current disinfectant used for general microbial and biofilm control in potable water is iodine in the U.S. operating segment of the ISS, whereas the Russian water system uses colloidal silver (372). Current biofilm mitigation protocols on spacecraft have been reviewed (297).

As biofilm formation and control continue to be a challenging issue, there is active ongoing research in this field, including developing surfaces that resist colonization, alternative biofilm control chemicals, and improvements in biofilm detection [e.g., (375–377)]. These approaches will be especially important for planned human exploration missions beyond LEO, where it will be necessary to revisit biofilm control strategies in the context of the limited resources available, and the possibility that life support systems in orbiting platforms may be inactive for periods of time when unoccupied. Some strategies being explored include the use of combining treatments with light-activated surfaces or nanoparticles to control biofilms (378) or the removal of some nutrients in the wastewater stream that may promote biofilm growth by ISS microorganisms (296, 379). While there are ground-based studies exploring these strategies, it will be important to test them in microgravity conditions during spaceflight and ultimately partial gravity conditions such as on the Moon or Mars.

THE FUTURE OF SPACEFLIGHT MICROBIOLOGY

As humans travel further from Earth and resupply efforts become impractical and/or impossible, the need for technology to maintain autonomous sustainability increases, including approaches to produce food, replenish water and oxygen, maintain astronaut health, and extend the useful life of spacecraft systems (380, 381). Accordingly, there is keen interest in bioregenerative life support systems in which organisms, including microbial and higher organisms (plants), would be involved in the breakdown of organic waste, while producing oxygen and potentially food for the crew (382, 383). One notable example is the NASA Controlled Ecological Life Support System program operated at the Kennedy Space Center for over 25 years (384–386). Another ongoing example is the Micro-Ecological Life Support System Alternative (MELiSSA), which has been in development since 1989 by the ESA (387). This bioregenerative system involves several compartmentalized bacterial processes and higher organisms. The current version involves several compartments that collectively produce edible products, regeneration of oxygen, and water recovery from wastes (383). A number of tests related to MELiSSA are underway in both ground-based (383) and a limited number of flight studies on the ISS (388). These and previous MELiSSA-related studies hold the potential to not only benefit life support systems for future exploration systems development but also expand our understanding of how environmental microorganisms respond to spaceflight and spaceflight analogs (71, 388–392).

It is important to note the host-pathogen interaction between microorganisms and plants during spaceflight is understudied, and some evidence has been reported that spaceflight may alter pathogenicity factors and the virulence of certain plant pathogens (52, 55, 393, 394). In addition, alterations in plant-microbe interactions during spaceflight could hold the potential to also benefit the host (380, 395). These considerations are important and a major part of future food systems, such as bioregenerative crop growth (363, 396, 397). This concept is important for spaceflight plant growth chambers, such as the Veggie spaceflight hardware, which was the first demonstration of actual open crop food production in the crewed spaceflight environment where humans and crop systems shared microbiome components, thus potentially propagating and spreading foodborne pathogens. In a study by Khodadad et al. of lettuce grown in the Veggie plant growth hardware on ISS, a comparison between the spaceflight and ground-based systems demonstrated changes in microbial concentrations and diversity, but none that would suggest a health risk (396). Bioregenerative food systems have microbiomes which are dependent on nutritient availability, and in the case of plants, a number of important microorganisms have been associated with the rhizosphere and other plant locations

[e.g., (398)]. Certainly, one concern is the potential for microbial-based disease in the plants of a food production system. For example, an opportunistic fungal infection involving *Fusarium oxysporium* was reported on *Zinnea hybrida* plants grown on the ISS (399). From a historical perspective, the devastating Irish potato famine (400) caused by *Phytophthora infestans* provides a cautionary note about reliance on a single food item. Certainly, stability of any bioregenerative life support system including food production must factor in the potential for microbially induced disease.

Future functional studies will assess how spaceflight-associated changes in the astronaut microbiome manifest as physiological alterations in crew health. For example, given the importance of the microbiome in the gut-brain axis (401), mechanistic examination of how changes occurring to astronauts' microbiome and immune function could affect their physical and mental health may facilitate the development of strategies to maintain crew well-being. Similarly, insight into the functional changes occurring to the microbiome during spaceflight may help with the design of new supplements or nutritional strategies. The incorporation of probiotics as food supplements as countermeasures to mitigate health risks is being evaluated to benefit astronaut health and performance (347, 402, 403). While the concept of using probiotics during spaceflight is not new (20, 333), a resurgence in interest into the benefits for humans in space (347, 402, 403) has initiated new studies in this area (404, 405).

Even microbiological concepts that have traditionally been considered problematic could benefit spaceflight missions. For example, biofilms have been associated with a number of beneficial applications, including wastewater treatment (406) and metal extraction (407) and are, thus, being considered for use in spaceflight and space resource utilization. The advantage of incorporating biofilms in spaceflight processes is the ability of the component microorganisms to withstand a variety of chemical and physical stresses. Microbial processes that involve metabolic interactions among different species (i.e., syntrophic metabolism) are enhanced when organisms co-exist in close proximity within biofilms. For example, biofilms are being explored for extraction of oxygen (408) and rare metals such as vanadium (409) from simulated regolith. Accordingly, there is considerable interest in adapting these types of concepts to space travel.

In addition, investigations to further advance our mechanistic understanding of how microorganisms affect crew health and habitat sustainability are critical. For example, while the impact of spaceflight on microbial characteristics is well documented for short-duration culture, the impact of long-duration, multigenerational growth of microorganisms (in pure culture or mixed species consortia) in the chronic stress of microgravity has rarely been investigated—largely due to experimental limitations associated with spaceflight research (2). The implications of this gap in knowledge are tremendous, as the response to short-duration growth in microgravity has been repeatedly shown to alter gene expression, pathogenesis-related stress responses (e.g., biofilm formation and resistance to antibiotics), and virulence in unexpected ways (1, 5, 6, 9, 14, 100, 122, 410, 411).

Thus, in order to effectively mitigate microbial risks to crew health and habitat sustainability, as well as ensure productive use of microorganisms for beneficial purposes in space, it is essential to understand the mechanisms underlying emergent and often non-intuitive microbial behaviors in response to the spaceflight environment. To achieve this goal, a holistic, integrated scientific approach which links omics data with functional phenotypic profiling must be used to understand the role that physical forces play in regulating microbial responses in the context of the multi-faceted stressors associated with the spaceflight environment during both short- and long-duration studies. This is critical for the development of more predictive modeling approaches, including those associated with AI and machine learning, that will successfully support human health and habitat sustainability in space and lead to translational biotechnology and biomedical advances on Earth.

CONCLUSIONS

As humans have worked and lived for longer durations in spaceflight, we have advanced our understanding of how the microgravity environment impacts microbial risks for human health and habitat sustainability. Considering that microorganisms are inextricably connected with all aspects of human spaceflight, changes that occur to microbes could represent a formidable challenge to the successful transition of professional career astronauts and civilians for next generation missions to LEO and deep space. It, thus, follows that successfully expanding our presence and influence beyond the confines of Earth will require that we continue to increase our knowledge of microbial interactions with humans, their environment, and other microorganisms both in space and on the surface of other celestial bodies. Findings from previous spaceflight research indicate that microbial adaptability to the novel environmental niche of spaceflight can result in unexpected alterations in a diverse range of microbial phenotypes and gene expression profiles that are critical for health and habitat sustainability both in space and on Earth (2). The mechanism(s) enabling unexpected microbial alterations to both spaceflight and spaceflight analog conditions are not fully understood. However, a solid body of evidence indicates that mechanotransductive responses to gravity-induced changes (e.g., altered fluid shear) in these environments mimic niches encountered by microbes during their natural terrestrial lifecycles and, thus, play important roles in the observed alterations (1, 87, 92). Accordingly, a thorough mechanistic understanding of these microbial adaptations is critical to successfully support future human spaceflight endeavors. Furthermore, the development of more integrated and realistic models that better mimic ecosystem complexity (e.g., polymicrobial consortia, radiation, celestial dust, altered gravity forces) will be necessary to understand microbial adaptations at the molecular and functional phenotypic levels to fully elucidate and manage microbial risks during human spaceflight. Within these realistic models is the overriding need to study physical forces in the context of the entire biological, chemical, and physical microenvironmental cues to fully translate this knowledge for practical applications.

To successfully achieve these goals, the next generation of spaceflight hardware will require closer interactions between scientists designing and performing the experiment and engineers building the spaceflight technology/hardware to create flexible, easy-to-use formats for future studies with the same accuracy, reproducibility, and modularity associated with those in terrestrial laboratories (2). In addition, the progress of spaceflight experiments can often be hindered due to erratic flight schedules and funding, which can delay or cause loss of experimental opportunities. To engage new microbiologists in spaceflight research and advance the field, the spaceflight platform will need to be simple, affordable, and routinely accessible to enable reproducibility and iterative experimental advances.

It is important to note that the non-intuitive alterations in microbial responses are not restricted to negative outcomes. Indeed, the connection between spaceflight technology and Earth applications continues to expand with the need to solve mutual problems, such as the development of resource-efficient processes (e.g., *in situ* resource utilization, synthetic biology) (412, 413), microbial supplements, vaccines, and therapeutics to benefit human health (e.g., probiotics, pharmaceuticals, nutrient supplements, gut-brain strategies to maintain health) (2, 347), and biologically based life support systems (387, 390). Leveraging possible unexpected spaceflight microbial responses may help enable new technologies that can rapidly benefit future missions.

In conclusion, the spaceflight research platform provides a unique opportunity to see life in a new adaptational mode that has not been seen before and offers a wealth of knowledge and translational opportunities. The rapid expansion of both exploration and commercial spaceflight platforms strongly indicates that the renaissance of spaceflight microbiology will not end after the ISS is decommissioned but instead is only beginning.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Table S1 (MMBR00144-23-s0001.xlsx). Several commonly isolated microorganisms identified in various studies performed over the life of ISS.

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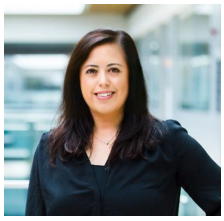
AUTHOR BIOS

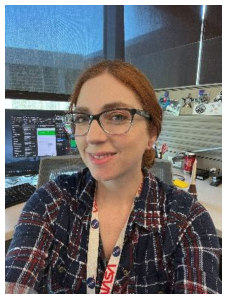
Cheryl A. Nickerson is Professor of Life Sciences in the School of Life Sciences, Center for Fundamental and Applied Microbiomics, in the Biodesign Institute at Arizona State University. Dr. Nickerson's research program blends microbiology, tissue engineering, and physics to mimic the dynamic interactions between microorganisms, their microenvironment, and infected hosts in both terrestrial and spaceflight conditions – with a focus on the connection between cellular biomechanics and host-pathogen systems biology. Her collective body of foundational work in this area helped to establish the field of "mechanobiology of infectious disease" by characterizing the effects of biomechanical forces (physiological fluid shear; microgravity of spaceflight) on microbial pathogens, host-pathogen interactions, and infectious disease outcomes. Her research interests include i) characterization of microbial pathogen responses to physiological fluid shear forces encountered in the infected host and in the microgravity environment of spaceflight, ii) development of 3-D biomimetic tissue models as predictive human surrogates to study host-pathogen and host-commensal interactions, infectious disease mechanisms, and antimicrobial drug efficacy, and iii) characterization of the "Microbiome of the Built Environment" as it pertains to infectious disease risks for astronauts and biofouling of environmental life support systems of spacecraft. Her studies also make extensive use of the spaceflight analogue technology, Rotating Wall Vessel bioreactor. Her research has flown on numerous NASA Shuttle and SpaceX missions to the International Space Station. She is a pioneer in space biomedical research and has received several honors and awards including the Presidential Early Career Award for Scientists and Engineers (PECASE), the NASA Exceptional Scientific Achievement Medal, invited Congressional Testimony on the importance of NASA's spaceflight life sciences and biomedical research for the health of astronauts and the public, Distinguished Lecturer for the American Society for Microbiology (ASM), Founding Editor-in-Chief of Nature journal, npj Microgravity, invited service on numerous NASA, NIH, National Academies, and ASM blue ribbon panels to shape the vision of the future of microbial and host-microbe interaction research, and was selected as a NASA Astronaut Candidate Finalist.

Robert J. C. McLean is a Regents' Professor in the Department of Biology, Texas State University. He graduated with a B.Sc. in Microbiology from the University of Guelph, Canada in 1978 and after working in the food industry for almost 4 years, conducted his Ph.D. at the University of Calgary under JW (Bill) Costerton from 1982-86. After doing a two-year postdoctoral fellowship with TJ Beveridge at the University of Guelph, he began his independent academic career as a Canadian equivalent of a Research Assistant Professor at Queen's University from 1988-93, then moved to Texas State University as an assistant professor in 1993. His research has focused on the biology of pure and mixed culture biofilms in various environments including the urinary tract, aquatic environments, and more recently spaceflight. Dr. McLean is a Fellow of the American Academy of Microbiology and has served on the Editorial Boards of Applied and Environmental Microbiology, Frontiers of Microbiology, the Geomicrobiology Journal, and npj Microgravity. He was also invited to serve on the physical sciences panel for the Decadal Survey on Biological and Physical Sciences Research in Space 2023-2032.

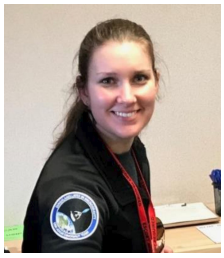


Jennifer Barrila is an Assistant Research Professor in the Biodesign Center for Fundamental and Applied Microbiomics at Arizona State University. She received her B.S. in Biochemistry in 2002 from Syracuse University. In 2008 she received her Ph.D. in Biology from Johns Hopkins University. Dr. Barrila's expertise is in space biomedical research, infectious disease, and 3-D tissue engineering. Her research focuses on understanding the impact of the spaceflight environment on human health and bacterial physiology and using that knowledge to improve human health both in space and on Earth. Her work uses both spaceflight analogues (e.g., the RWV bioreactor, regolith simulants, radiation) and spaceflight platforms (Shuttle, ISS, commercial vehicles) to study the influence of these environments on host-pathogen-microbiome interactions. She has been an investigator on multiple scientific payloads that have launched to the ISS, which notably included the first infection of human cells in space (using Salmonella and a 3-D intestinal epithelial model) and the first full duration virulence study monitored in real time in space. She also uses NASA bioreactor technology to develop advanced 3-D tissue models containing immune cells for the study of disease both in space and on Earth. She has received several honors and awards, including the Thora W. Halstead Young Investigator's Award from the American Society for Gravitational and Space Research and the Presidential Early Career Award for Scientists and Engineers (PECASE) from NASA. She was also selected to serve on the biological sciences panel for the Decadal Survey on Biological and Physical Sciences Research in Space 2023-2032.

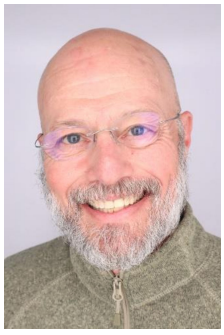
Jiseon Yang is an Assistant Research Professor at the Biodesign Center for Fundamental and Applied Microbiomics at Arizona State University. Specializing in bacteriology and space life science, she has undertaken significant research in bacterial pathogenesis, molecular engineering, biofilms, space biology, vaccine development, and host-pathogen interactions. Yang completed her B.S. and M.S. in Microbiology at Pusan National University, South Korea, and received her Ph.D. at ASU, under the guidance of Dr. Cheryl Nickerson, investigating the mechanosensory responses of multidrug-resistant invasive nontyphoidal Salmonella and their implications for pathogenesis and virulence. Awarded the prestigious Alfred P. Sloan Postdoctoral Fellowship, in partnership with NASA, she delved into the microbiome of the International Space Station and the biofilm formation. Currently, she leads a NASA-funded project to further understand microbial co-adaptation during long-term space travel. Her research interest is to understand how microbial ecological success within a complex microbial community contributes to their pathogenicity, persistence, and species adaptation in terrestrial and extraterrestrial environments.



Starla G. Thornhill is a research microbiologist contracted to work in the microbiology lab at NASA Johnson Space Center supporting the Human Health and Performance Contract. She graduated from Texas State with a Ph.D. in Aquatic Resources and Integrative Biology in 2020. She worked as a Post-Doctoral researcher at Texas State University, and later at Texas A&M – San Antonio before transitioning to her current position at NASA in 2022. Her research interests include microbial responses to spaceflight, control of microorganisms present in manned spacecraft life support systems, and in host-microbe interactions between pathogenic bacteria and astronauts while in space. She also works to support food safety testing for astronaut foods for ISS.



Laura L. Banken is a Ph.D. student in the Microbiology program in the School of Life Sciences at Arizona State University. She graduated with an M.S. from the University of North Dakota in the Space Studies program. At UND she performed research on closed ecological systems of life support using the Inflatable Lunar Mars Analog Habitat (ILMAH) and co-led a flight experiment aboard Blue Origin's New Shepard launch vehicle. She worked in the medical device industry for five years after completing her B.S. in Biochemistry at The College of St. Scholastica in Duluth, MN. Her research interests include microbial responses to microgravity and management of life support systems.



D. Marshall Porterfield is a Professor of Agricultural and Biological Engineering at Purdue, where his research focuses on space biophysics using biosensors, omics, and computational biology approaches. Over the last 30 years he has worked experimentally to study biological systems in the spaceflight environment for biomedical countermeasures and controlled environment space agriculture. This includes bioregenerative life support using omics approaches for personalized medicine, precision agriculture, and microbiome engineering. He served NASA as Division Director for Space Life and Physical Sciences from 2012-2016 (Human Research, Physical Sciences, and Space Biology Programs), focused on science for future human exploration using the International Space Station. During his tenure at NASA he developed a new "open science" paradigm (GeneLab, NASA Twins, Physical Sciences Informatics, Cold Atom LAB) for the utilization of the ISS. He has published 100's of peer reviewed manuscripts, technical publications, patents, and book chapters. National leadership includes service as President of the American Society for Gravitational and Space Research, and the Institute for Biological Engineering. He has received numerous awards including the Halstead Investigator Award, the Purdue University Faculty Scholar Award, and was elected to the College of Fellows for the American Institute for Medical and Biological Engineering.

George Poste is Regents' Professor and Del E. Webb Chair in Health Innovation and Director of The Complex Adaptive Systems Initiative at Arizona State University. This program studies the dysregulation of molecular signaling networks in a variety of diseases by integration of large-scale multi-Omics profiling with machine learning and AI analysis and correlation with patterns of disease risk, progression and clinical outcomes. Several aspects of this research are relevant to this review, including mechanisms of microbial evasion of host immune responses and the role of immune-restriction genotypes in determining disease severity. He has served as a Member of the US Defense Science Board, the National Academy of Medicine Global Forum on Infectious Disease, the NASA (ISS) National Laboratory (ISSNL) and Commercial Low-Earth Orbit (LEO) Development review panel. He is a current ex officio member of the Bipartisan Commission on Biodefense. He is a Fellow of the UK Royal College of Pathologists, the UK Academy of Medicine, the American Institute for Medical and Biological Engineering and a Fellow of the Royal Society.

Neal R. Pellis is retired from NASA and from Universities Space Research Association (USRA), Houston, TX. He received his Ph.D. in Microbiology from Miami University, Oxford, Ohio in 1972 and did a postdoctoral fellowship in Microbiology at Stanford University in Palo Alto, California. Dr. Pellis joined the NASA-Johnson Space Center in October 1994, having previously been on the Faculty of Northwestern University Medical School, the University of Texas Medical School, and directing the Department of Surgical Oncology research laboratory at the University of Texas M. D. Anderson Cancer Center. He has had adjunct appointments at the University of Texas M. D. Anderson Cancer Center, the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences, the Mabee Laboratory of Biochemical and Genetic Engineering, Rice University, and the College of Technology, Department of Engineering Technology, University of Houston, as well as the University of Texas Medical Branch Graduate School of Biomedical Sciences, Galveston, TX. He led the Biotechnology Cell Science Program at NASA from 1994 to 2003, during which time he had 16-month assignment to NASA Headquarters as International Space Station (ISS) Program Scientist from May 2002 through August 2003. Thereafter he was Associate Director of the Biological Sciences and Applications Office working on exploration cell science until January 2005. In 2005, he was appointed Senior Scientist in the Space Life Science Directorate at NASA Johnson Space Center, working with the directorate chief on strategic planning, alliances, and partnerships necessary to acquire basic and applied science and technology for exploration missions and to manage the risks to crew in space and on planetary missions. After retiring from NASA, he joined USRA in October 2010 as the Director of Space Life Science Division. He then retired from USRA in 2016 and remains a volunteer faculty at Baylor College of Medicine's Center for Space Medicine.

C. Mark Ott received his B.S. in chemical engineering from the University of Texas at Austin, his M.B.A. from Louisiana State University, and his Ph.D. in microbiology from Louisiana State University. He has published extensively in the areas of microbial ecology in spacecraft, human and microbial responses to spaceflight, and the development of advanced tissue culture models to investigate infectious disease. For the past 20 years, Dr. Ott has served as a technical lead in the Johnson Space Center Microbiology Laboratory, which is responsible for mitigating infectious disease risk during human spaceflight. His responsibilities include the assessment of microbial risk and development of spaceflight requirements based on vehicle and mission architecture as well as crewmember, food, and environmental monitoring.