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Evidence Report:

Risk of Adverse Health Effects Due to Host-Microorganism Interactions

Human Research Program
Human Health Countermeasures (HHC) Element

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Table of Contents

I. PRD Risk Title: Risk of Adverse Health Effects Due to Host-Microorganism Interactions .. 1
II. Executive Summary 1
III. Introduction 1
A. Identifying the need for investigation. 1
B. Flight experiments used to study host-microbe interactions. 2
C. Earth based cell culture systems used to study host-microbe interactions. 2
D. The need for human surrogate models. 3
IV. Evidence 4
A. Spaceflight Evidence 5
B. Ground based Evidence 13
V. Computer-Based Modeling and Simulation 16
VI. Risk in context of Exploration Mission Operational Scenarios 16
VII. Knowledge Gaps 16
VIII. Conclusion 17
IX. References 18
X. Team 26
XI. List of Acronyms 26
APPENDIX A 28
APPENDIX B 30

I. PRD Risk Title: Risk of Adverse Health Effects Due to Host-Microorganism Interactions

II. Executive Summary

While preventive measures limit the presence of many medically significant microorganisms during spaceflight missions, microbial infection of crewmembers cannot be completely prevented. Spaceflight experiments over the past 50 years have demonstrated a unique microbial response to spaceflight culture, although the mechanisms behind those responses and their operational relevance were unclear. In 2007, the operational importance of these microbial responses was emphasized as the results of an experiment aboard STS-115 demonstrated that the enteric pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) increased in virulence in a murine model of infection. The experiment was reproduced in 2008 aboard STS-123 confirming this finding. In response to these findings, the Institute of Medicine of the National Academies recommended that NASA investigate this risk and its potential impact on the health of the crew during spaceflight. NASA assigned this risk to the Human Research Program. To better understand this risk, evidence has been collected and reported from both spaceflight analog systems and actual spaceflight including Mir, Space Shuttle, and ISS missions. Although the performance of virulence studies during spaceflight are challenging and often impractical, additional information has been and continues to be collected to better understand the risk to crew health. Still, the uncertainty concerning the extent and severity of these alterations in host-microorganism interactions is very large and requires more investigation as the focus of human spaceflight shifts to longer-duration exploration class missions.

III. Introduction

Transfer of microorganisms from person to person are common in closed habitats such as spacecraft (1, 2)[1], including the spread of opportunistic organisms impacting the overall risk to astronaut health during spaceflight missions of extended duration. Current spaceflight data clearly demonstrates alterations in aspects of the crew immune system during spaceflight (3, 4). Latent viral reactivation has been used as a biomarker for reduced immunity during ground-based and spaceflight research activities and represents an additional route of infection (5-12). In addition, bacteria and fungi have been demonstrated to increase virulence and/or virulence characteristics during Space Shuttle and ISS spaceflight experiments (6, 10, 13-17). In this review, we identify evidence of molecular-genetic and phenotypic alterations in microorganisms during spaceflight and ground-based spaceflight analog models. The background information will be presented that outlines the recommendations for investigation, overview of spaceflight and ground-based research including animal models.

A. **Identifying the need for investigation**. In 2008 the Institute of Medicine (IOM) of the National Academies reviewed the Human Research Program Evidence Book of the "Risk

[1] http://ntrs.nasa.gov/search.jsp?R=20050217259

of Crew Adverse Health Event Due to Altered Immune Response."[2] The IOM cited research from a flight experiment by Nickerson and colleagues aboard STS-115, which indicated that the enteric pathogen, *S.* Typhimurium had become more virulent when cultured during spaceflight. The IOM recommended NASA "Develop evidence books on additional risks, including alterations in microbe and host interactions…" In November 2008, a risk entitled, "Risk of Adverse Health Effects Due to Alterations in Host-Microorganism Interactions," was added to the Human Research Program's Integrated Research Plan to determine the likelihood and consequences of alterations in microbial interactions with the crew and their environment that could impact their health and performance.

B. **Flight experiments used to study host-microbe interactions.** While several experiments have been performed in spaceflight to assess the effects of this unique environment on microbes, there are several factors that complicate the evaluation and comparison of the resulting data. Key findings of microbial spaceflight studies that impact our understanding of medically significant microorganisms are listed in Appendix A. Some of these confounding elements include (a) the wide variety of organisms that have been studied including motile versus non-motile bacteria; (b) the different spaceflight parameters that have been used (e.g., differences in lengths of missions, sample handling – fixed or frozen, in-flight centrifuged 1*g* controls versus ground 1*g* controls); and (c) differences in growth media used (e.g., minimal versus rich media or liquid versus solid media). These factors will be discussed in this Evidence Report where appropriate. It is also clear that in spite of these differences, the space environment affects microbes differently than traditionally observed in the Earth environment, and these changes must be understood to ensure the safety of humans during long-duration space missions.

C. **Earth-based cell culture systems used to study host-microbe interactions.** While spaceflight is the ultimate platform for performing experiments to determine alterations in microbial responses and host-pathogen interactions, spaceflight research is constrained by high costs, inconsistent flight availability, minimal in-flight analytical equipment, as well as limitations in power usage, payload weight and volume, and crew time. Thus, ground-based analogs (relevant findings summarized in **Appendix B**) have been developed to evaluate alterations in microbial responses to these conditions (18). These analogs do not remove gravity from the system, but instead develop an environment that reflects many of the secondary effects observed in microgravity (decreased mass transfer, lower fluid shear, etc.). Most all of these analogs rely on the continuous sedimentation of microbial cultures in a growth medium. The simplest system is the clinostat, which is a cylindrical tube completely filled with media (no bubbles, i.e., "zero headspace"), that is rotated perpendicular to the gravitational force vector (19). Likewise, a more complex system designed by NASA, called the rotating wall vessel (RWV), has been used extensively since the mid-1990s (Figure 1). The RWV is also an optimized form of suspension culture and consists of a hollow disk or cylinder that is completely filled with medium and rotates on

[2] http://humanresearchroadmap.nasa.gov/evidence/reports/Immune_2015-05.pdf?rnd=0.22291305066222

an axis perpendicular to the gravitational force vector. Under these culture conditions, the cells are maintained in suspension as the RWV is rotated and a sustained low-shear environment for cell growth is achieved (18). Exchange of nutrients and localized "mixing" of the microenvironment is facilitated by the constant falling of the cells through the local fluid environment and the gentle rotation of the culture medium. Unlike the clinostat, a gas-permeable membrane on one side of the RWV allows constant air exchange during growth. Data from previous research on *S.* Typhimurium indicated that the enhanced virulence observed during spaceflight was also observed at a similar trend and magnitude to virulence changes imparted by culture in the RWV (15, 16, 20). Similar trends in gene expression and regulation were also observed (15, 21).

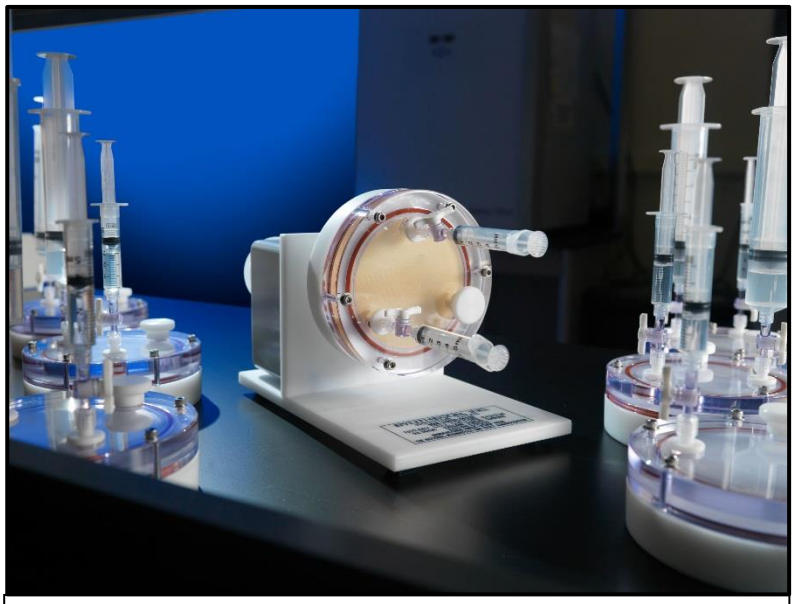

Figure 1. Rotating wall vessel (RWV) developed by NASA and used during ground-based microbiology experiments. *Image: NASA*

Other microbial culture spaceflight analogs have been reported, such as the random positioning machine (RPM) and the use of diamagnetic levitation (22). The RPM also suspends microorganisms in growth media; however, this suspension is maintained by randomly adjusting the movement of the bioreactor. Diamagnetic levitation relies on a strong magnetic field to levitate microbial cultures, and thus reproduce aspects of microgravity. As with all spaceflight analogs, the fidelity of these and other culture devices to reproduce culture during spaceflight is not completely known as the mechanisms driving the alterations in microbial response are unclear.

D. **The need for human surrogate models.** The need for having animal models of microbial infection is based on the necessity of having an experimental species whose inflammatory and pathological response closely resembles the human host. In addition, animal models that can be manipulated genetically provide a tremendous advantage to dissect out the underlying molecular mechanisms. Additional requirements of an excellent animal model are reproducibility of the pathological response and availability of a wide range of molecular/biological targets that can be used to thwart or aggravate the response or design effective countermeasures. Depending on the infection and type of study, mammalian animal models have proven to be useful in terrestrial experiments. Much of our present knowledge about the immune system in space comes from studies conducted on space-

flown mice (23-27). Moreover, to test the pathological potential of spaceflight conditions, murine models have been used to evaluate bacteria grown in space (15, 16). Such studies have looked at survival, local and systemic inflammation, and pathophysiology of organs. This topic is discussed in detail later in this report. Hind-limb unloading is a widely used ground-based model of simulated microgravity in mice and has been used to investigate some of the effects of spaceflight on microbial infection (28, 29).

Some evidence on potential changes in the host response during infection was obtained by challenging *Drosophila melanogaster* (fruit fly) after return from a 12-day spaceflight mission on STS 121 with *Escherichia coli (E. coli)* (30). The study reported that adult flies were able to clear *E. coli* infection postflight but showed differences in the kinetics and levels of antimicrobial peptide (AMP) gene expression when compared to the matched ground-control flies.

Spaceflight infection studies where the host and pathogen are both in microgravity during spaceflight are difficult to perform and virulence data has not been reported to date. Even though mice are relatively small, the number of mice that could be infected during spaceflight is extremely limited due to space and upmass constraints. As such, other models enabling a greater sample size are being investigated. For example, virulence studies using the nematode, *Caenorhabditis elegans*, as a human surrogate model of infection with *S.* Typhimurium have recently been completed aboard the ISS. The results of the experiment, designated as *Micro-5*, are being tracked for future inclusion in this report.

While animal models provide excellent insight into the infection process, reductionist tissue culture models are also commonly used to study the infection process. Accordingly, human tissue culture models have also been investigated for use as infection models during spaceflight. In 2010, the flight experiment designated "Space Tissue Loss, IMMUNE" flew aboard STS-131 and was the first infection of human tissue culture cells by a pathogen to occur on orbit. The potential of this model is intriguing as mammalian cells cultured during spaceflight have been demonstrated to develop a three dimensional architecture that reproduces many *in vivo* characteristics (31). Indeed, these models have been demonstrated to reproduce *in vivo* characteristics that have not been observed using traditional two dimensional, monolayer culture (32).

IV. Evidence

Alterations in microbial responses to spaceflight culture have been well-documented over the past 50 years (18, 33-35). An overview of key findings can be found in Appendices A and B. This Evidence Report will focus only on those responses that substantially impact this HRP risk. The *Risk of Adverse Health Effects Due to Host-Microorganism Interactions* works with other disciplines to gather information and determine the impact to the human as a whole. For example, a large body of evidence indicates dysfunction of aspects of the crewmember's immune system during spaceflight missions. This evidence is described in the HRP evidence report addressing "Risk of Crew Adverse Health Event Due to Altered Immune Response"[3]. Collaborations with the

[3] http://humanresearchroadmap.nasa.gov/evidence/reports/Immune_2015-05.pdf?rnd=0.22291305066222

Immunology discipline are critical to understand the impact the alterations in microbial virulence have on the crewmembers and how to mitigate their effects. In addition, work continues with food science and nutrition to prevent food spoilage microorganisms, and to incorporate beneficial organisms into the food system. Future collaboration efforts have been identified with the Pharmacology discipline to understand the impact of spaceflight on medications and efficacy against microorganisms. The expertise in the radiation health group are used to understand the impact of radiation on microorganisms in the environment and in the human system. Microbial identification and evaluation technology continues to evolve and is monitored for spaceflight applicability in collaboration with the spaceflight medical capabilities group. The current evidence, collaborations and future planned research utilize the ISS as a platform to determine the risk and mitigations required for longer-duration exploration class missions.

A. Spaceflight Evidence

1. *Micro 1: We need to determine the efficacy of current countermeasures and the need for countermeasure development based on changes in microbial populations and characteristics.*

   The primary post-infection countermeasure during spaceflight is the use of antibiotics; however, several spaceflight experiments have provided evidence suggesting alterations in antibiotic resistance when microorganisms are cultured during spaceflight. During the Cytos 2 experiment aboard Salyut 7 in 1982, the minimum inhibitory concentration of oxacillin, chloramphenicol, and erythromycin for *Staphylococcus aureus (S. aureus)* and of colistin and kanamycin for *E. coli* were compared to those of ground controls (36). These early results indicated an increased resistance of both *S. aureus* and *E. coli* to all antibiotics used in this experiment (36). However, the observed alterations in microbial antibiotic resistance during spaceflight may be transient and lost when the microbe has returned to Earth, as attempts to reproduce these changes after return to Earth have been unsuccessful (37). Spaceflight experiments culturing *E. coli* during STS-69 and STS-73 suggested gentamicin on agar slants that were flown was as effective as and possibly more effective than the antibiotic on ground-based control cultures (38). In 1999, Juegensmeyer et al. observed both increased sensitivity and resistance by cultures of *S. aureus*, *Pseudomonas aeruginosa (P. aeruginosa)*, *Bacillus subtilis (B. subtilis)*, and *E. coli* that had been re-grown after having been on the Mir space station for 4 months (39). While these experiments suggest spaceflight-associated changes in microbial response to antibiotics, the information is not adequate to be predictive about reproducibility with the selected microorganisms, the impact of antibiotics on other microorganisms, or the actual microbial response during exposure in a human host.

   Countermeasures directed at minimizing the impact of viral pathogens, such as vaccinations, are being evaluated. For example, preflight vaccination against the varicella-zoster virus (VZV) prevents VZV reactivation and shedding of live, infectious virus into the ISS environment. Even though there are no vaccines currently for the other herpes viruses, countermeasures focused on stress reduction have shown promising results (40, 41).

The current research plan includes foundational research to understand the need for countermeasure development. After the foundational studies have been completed, future areas of study include:

- Evaluation of in-flight efficacy of preventive agents and countermeasures such as disinfectants and antibiotics
- Impact of spaceflight-related alterations in the crew microbiome on antimicrobial efficacy.

2. *Micro 2: We need to determine if spaceflight induces changes in diversity, concentration, and/or characteristics of medically significant microorganisms associated with the crew and environment aboard the ISS that could affect crew health.*

Stringent microbiological monitoring of spacecraft (Figure 2) has been performed operationally aboard NASA spacecraft throughout the human spaceflight program (33, 42). Additional spaceflight experiments have also provided greater detailed information by investigating specific niches aboard spacecraft or using alternative methodologies beyond the culture-based isolation historically used (43). Generally, the data indicate that the potable water, air, and surfaces to which the crew are exposed are free of obligate pathogens; however, opportunistic pathogens such as *P. aeruginosa*, *Stenotrophomonas maltophilia*, and *S. aureus* are not uncommon (42, 44). In addition, identification of microorganisms collected from free-floating water behind panels indicated several potentially medically significant organisms not commonly isolated during standard operational monitoring, including *Legionella* species, and *Serratia marcescens* (*S. Marcescens*), and *E. coli* (45). Further microscopic examination of these samples revealed the presence of amoeba resembling *Acanthamoeba* or *Hartmanella* species and ciliated protozoa resembling *Stylonychia* species (45).

Figure 2. Astronaut performing routine microbiology monitoring during spaceflight. *Image: NASA*

Spaceflight food (Figure 3) is currently provided for missions in a shelf stable form for storage at ambient temperature (46). As such, microbiological contamination control, including stringent microbial monitoring, is maintained. While the incidence of contamination is low, preflight analyses of food samples have indicated the presence of organisms such as *S.* Typhimurium, *S. aureus*, *Enterobacter cloacae* and *Enterobacter sakazakii* (unpublished data). Contaminated lots are removed before shipment for flight; however, these findings suggest a potential route of infection to the crew. Future spaceflight missions may also provide food with potentially high levels of microorganisms, such as freshly grown crops or foods with probiotic organisms to promote astronaut health.

Figure 3. Examples of spaceflight food. *Image: NASA*

The production and monitoring requirements of these foods are only beginning to be evaluated; initial findings can be found in the HRP report, *Development of Spaceflight Foods with High Microbial Concentrations*[4].

For spaceflight missions, the primary source of microorganisms is the crew. Selected preflight microbiological monitoring is performed prior to launch, with testing based on the mission design. One key aspect of preflight operations is NASA's Flight Crew Health Stabilization Program, which was established during the Apollo Program in response to problems with incidences of infectious illness (47). The focus of the program involves reducing the exposure of flight crews to groups and individuals that are at high risk of harboring infectious disease (e.g., large crowds, small children) beginning approximately 10 days before launch.

The microbiome is an important part of the crew health and current spaceflight investigations to understand the alterations in the microbiome are in progress. Previous evaluations of *Bifidobacterium* in cosmonauts by Goncharova noted preflight decreases in bifidobacteria and alterations in acid formation during flight (48).

Astronauts shed Epstein-Barr virus (EBV) in saliva before, during, and after spaceflight. Frequency of shedding in astronauts was several times higher than control subjects, but shedding during flight was approximately 10 times higher than before or after flight. Surprisingly, even though astronauts did occasionally present with cold sores, occurrence of herpes simplex (HSV-1) in saliva was not common. VZV was not present in the saliva of astronauts before flight or in matching ground-control subjects. However, VZV did shed in ~50% of crewmembers during flight and continued up to ~5 days after landing. Aboard the ISS, approximately 60% of astronauts shed VZV during the flight phase and some can shed the virus at least 30 days after flight. A few cases of zoster have occurred either before, during, or after spaceflight. Mehta and

[4] http://www.nasa.gov/centers/johnson/slsd/about/divisions/hefd/about/publications.html

Pierson showed that 47% of Space Shuttle astronauts shed cytomegalovirus (CMV) in urine during spaceflight and continued for 2 weeks after flight. Whereas, less than 1% of control subjects shed CMV (10). Follow-up studies showed that 73% of ISS astronauts shed CMV and shedding continued for 30 days after landing. In one study of 71 astronauts, 77% were seropositive.

Routine microbial monitoring activities are performed operationally to evaluate air, surface and water supplies during spaceflight operations. In addition, cargo and supplies are sampled to minimize the risk of microbial contamination. There is an ongoing effort to evaluate the data collected during routine microbial monitoring and reported incidence of crewmember medical issues.

The current research plan includes foundational research to understand the microbes present in the spaceflight environment. After the foundational studies have been completed, future areas of study include:

- Spaceflight alterations of fungal diversity
- Impact of spaceflight radiation exposure on crew microbiome. Note: Ground-based radiation experiments should use similar exposure methods and simulate the spaceflight environment (such as low earth orbit or deep space) as closely as possible.

3. *Micro 3: We need to determine which medically significant microorganisms display changes in the dose-response profiles in response to the spaceflight environment that could affect crew health.*

S. Typhimurium is an obligate enteric pathogen with a potential to infect the crew during a spaceflight mission through the spaceflight food system. Extensive ground-based studies of the response of *S.* Typhimurium to the spaceflight analog environment in the RWV indicated an increase in microbial virulence using a murine model of infection (20). The microorganisms also displayed altered stress responses, gene expression, and survival in macrophage cells (20, 21). Building upon this information, the MICROBE flight experiment was performed in 2006 aboard the STS-115 mission. In this experiment, *S.* Typhimurium was grown during flight and compared to identically cultured ground controls (15). The cultures were either placed in an RNA fixative during flight or returned as live cultures for virulence testing. The cultures grown aboard the Space Shuttle displayed an extracellular matrix that was not seen in the ground controls. Evaluation of the gene expression indicated 167 genes and 73 proteins were differentially regulated compared to ground controls, with the conserved RNA-binding protein Hfq identified as a likely global regulator involved in the response to this environment. Subsequent experiments using the RWV bioreactor

supported the necessity of Hfq in the spaceflight/spaceflight-analog response (15). In addition, cultures grown in a Lennox Broth medium during flight caused a reduced time-to-death, increased percent mortality, and displayed a 2.7 fold lower LD_{50} (lethal dose required to kill 50% of the mice) in a murine infection model when compared to inoculation with ground-control cultures. This experiment produced several key findings including: (1) the experiment clearly indicated alterations in the expected dose-response curves with implications for the microbial risk assessment of infection potential for the crew during a mission; (2) the experiment provided the first insight into a molecular mechanism behind the alterations of microorganisms during spaceflight culture; and (3) the virulence and gene expression results from the spaceflight experiment paralleled the trends observed with the RWV spaceflight analog (20), supporting this bioreactor as an indicator of potential microbial alterations during spaceflight.

Figure 4. Astronaut Dominic Gorie manually activates the Group Activation Pack (GAP) hardware containing the MDRV spaceflight experiment aboard STS-123 to better understand bacterial responses to the spaceflight environment. *Image: NASA*

In 2008, Nickerson and her colleagues reproduced the evaluation of virulence changes using *S.* Typhimurium cultured aboard STS-123 (16). Figure 4 shows a crewmember performing spaceflight operations. Cultures grown in a Lennox Broth medium during flight displayed a 6.9 fold lower LD_{50} in a murine model when compared to inoculation with ground-control cultures.

During the MICROBE experiment, the global transcriptional responses of *P. aeruginosa* to spaceflight culture were also investigated (14). *P. aeruginosa* responded to spaceflight conditions through differential regulation of 167 genes and 28 proteins, with Hfq as a global transcriptional regulator. Key virulence-related genes that were differentially regulated included the lectin genes, *lecA* and *lecB*, and the gene for rhamnosyltransferase (*rhlA*), which is involved in rhamnolipid production. As with *S.* Typhimurium, the transcriptional response of spaceflight-grown *P. aeruginosa* displayed many similarities to trends observed during culture of *P. aeruginosa* in the RWV bioreactor (49, 50).

In a separate set of spaceflight experiments, Kim et al. investigated biofilm formation of *P. aeruginosa* during spaceflight (51). This research team found that the biofilm architecture was substantially different compared to Earth-grown controls. While the medical implications of this finding are unclear, it is an excellent example of one of many ways in which microorganisms can be altered during spaceflight.

In addition, Pierson and Mehta (5, 6) have studied latent herpes viruses in astronauts for nearly 20 years in spacecraft (Space Shuttle, Soyuz, Mir, and ISS). They found that EBV, VZV, and CMV reactivate and are shed in saliva (EBV, VZV) or urine (CMV) at levels that far exceed control subjects (9, 10). Figure 5 shows an image of saliva being collected during spaceflight saliva collection. The viruses remain latent until the immune system, specifically T-cell function, decreases to levels that can no longer control reactivation of the latent viruses.

Figure 5. Astronaut Doug Wheelock collecting saliva sample during spaceflight operations. *Image: NASA*

The current research plan includes foundational research to understand which medically significant microorganisms display virulence changes during spaceflight. After the foundational studies have been completed, future areas of study include:

- Defining the impact of radiation on microbial virulence
- Does spaceflight-associated virulence change when organisms are evaluated as co-cultures?
- Does spaceflight alter virulence in medically significant fungi?

4. *<u>Micro 4</u>: We need to determine how physical stimuli specific to the spaceflight environment, such as microgravity, induce unique changes in the dose-response profiles of expected medically significant microorganisms.*

The stimulus/stimuli during spaceflight culture that initiate a change in bacterial and fungal response and the molecular-genetic and biochemical processes that result during this response have not been identified, although some evidence is available. Kacena et al. found that growth on semisolid agar negated changes in enhanced microbial growth noted in liquid cultures, suggesting that a physical artifact from the agar influenced the bacterial response (52). Wilson et al. found that the change in *S.* Typhimurium virulence identified when cultures were grown in Lennox Broth was not observed when spaceflight cultures were grown in a simple salt, M9 medium or in Lennox Broth supplemented with 5 key inorganic salts used in the M9 formulation (16). As mechanosensitive ion channels that trigger ion transport exist in bacteria (53), mass transfer during spaceflight or alterations in ion permeability at the cell membrane are also potential factors that could impact the spaceflight-associated response. Notably, both the Kacena and Wilson studies provide evidence that microgravity alone does not stimulate unique bacterial and fungal responses. Rather, secondary effects of decreased gravity (eg, changes in mass transfer or fluidic shear), are likely responsible for the microbial response.

Another key piece of evidence in understanding the bacterial and fungal response to spaceflight culture is the observation by Wilson et al. of the Hfq regulation of a large number of differentially regulated genes in spaceflight-cultured *S.* Typhimurium (15).

This report suggests that the microbial responses that are being documented are aligned with known regulatory pathways (as opposed to random dysfunction of the organism). How the organism uses such a response on Earth is unclear. Importantly, this regulatory protein also substantially impacted spaceflight induced differential gene expression in *P. aeruginosa* (14).

An additional consideration in regard to the cause(s) behind microbial alterations during spaceflight culture was provided by Kim et al. in a spaceflight study investigating *P. aeruginosa,* which displayed higher final bacterial concentrations in spaceflight culture compared to ground controls (54). Previous articles proposed that motility may play a large role in the unique responses of microorganisms to spaceflight culture (55). To test this hypothesis, Kim et al. compared final cell concentrations of a wild-type *P. aeruginosa* and a mutant deficient in swimming motility to their respective ground controls. Similar increases in final cell concentrations of both organisms were observed compared to their respective controls, suggesting motility did not play an important role in the response (54).

Also notable in the discussion about stimulus and response to spaceflight culture is that the data from current spaceflight experiments does not inherently suggest that the alterations observed in spaceflight-cultured microorganisms are transient or represent heritable changes. The environmental conditions during spaceflight missions, especially those beyond low-Earth orbit, could impact the selective pressure to increase and stabilize heritable mutations in the microbial genomes. These environmental conditions include changes in the intensity and type of radiation as well as gravity compared to terrestrial conditions. Spaceflight studies exploring this possibility have been limited in part due to the resources necessary to perform long-duration growth experiments. However, some evidence suggests a change in the normally expected mutation rate may occur. Ciferri et al. evaluated changes in conjugation, transduction, and transformation using *E. coli* cultures (56). While the rate of pairing did not appear to be affected during conjugation in spaceflight cultures, they did note that the pairs were being held longer, which they attributed to the absence of external disruptive forces. No differences were reported for transduction, and the results for transformation were inconclusive. The extent of heritable changes in the microbial genome that are induced by spaceflight radiation and microgravity is unclear. While several spaceflight experiments have investigated aspects of this topic (57-59), no general trend or mechanism has been defined based on current findings.

To fully understand the impact of stimulus/stimuli on microorganisms and their implications on crew health, an understanding of the biochemical responses may enable insight into which organisms may be altered and how the alteration will be manifested in each organism. Alterations in the biochemical pathways of microorganisms have been investigated in multiple spaceflight studies. For example, alterations in the production of the secondary metabolite, Actinomycin D, were measured by Benoit et al. from *Streptomyces plicatus* grown in gas-permeable culture bags aboard the ISS (60). Unfortunately, all cell concentrations over time were not available, and the authors speculated that these changes may have been the result of differences in growth

profiles of spaceflight and ground-based cultures that had been previously reported by Mennigmann et al. in previous studies (61).

Research documenting spaceflight-associated latent virus reactivation in herpes viruses began with EBV evaluation in Space Shuttle astronauts (5). Glaser (62-65) demonstrated decreased cellular immunity and increased antibodies to EBV in chronically stressed individuals. Studies have linked psychological stress with onset and severity of infectious mononucleosis (66). Studies (6) demonstrated increased inflammatory cytokines in astronauts shedding latent viruses.

The current research plan includes foundational research to understand the mechanism of alterations in microbial virulence during spaceflight. After the foundational studies have been completed, future areas of study include:

- How does spaceflight impact the risk for fungal disease?
- How does partial or fractional gravity impact virulence?
- Do changes or differences in the host such as immune function, fluid shift, microbiome, sex/gender, or prior infection impact the risk of host-pathogen interaction?
- Further characterization of genetic and resulting gene expression and phenotypic changes of microorganisms during spaceflight.

5. *Micro 5: Current microbial standards identifying microbial risk limits need to be updated and microbial requirements need to be developed to include new technologies and future mission scenarios*.

Future exploration class missions will require the use of advanced microbial identification technologies. Currently, microbial enumeration of environmental samples is performed during space flight operations and samples are returned to the ground for microbial identification (67). The specifications developed for microbial testing of space foods are in compliance with the United States Food and Drug Administration (FDA) hazard analysis and critical control points (HACCP) requirements. Specifications are maintained in accordance of International Food Standards and updated as required. The current microbial requirements were refined based on a series of forums with input from experts from industry, government and academia (68). The requirements are reviewed regularly to determine applicability to current and future planned spaceflight missions. A continuous effort to identify and understand new technology continues to determine the best methods for microbial identification during spaceflight operations including exploration class missions that will require greater autonomy due to communications delays and limited resupply.

The current research plan includes foundational research to develop future microbial requirements and hardware. After the foundational studies have been completed, future areas of study include microbial risk assessment and clinical relevance.

B. Ground-based Evidence

1. *Micro 1: We need to determine the efficacy of current countermeasures and the need for countermeasure development based on changes in microbial populations and characteristics.*

   The impact of spaceflight on countermeasures, such as antibiotics, and the resulting changes in efficacy is a concern for long-duration spaceflight. The Human Research Program supported a pilot investigation to determine initial characterization of alterations in effectiveness of selected antibiotics using the RWV[5]. This study identified potential alterations in efficacy and the results suggest the best approach for applied forward work is evaluating an in vivo system during spaceflight, including human and rodent studies.

2. *Micro 2: We need to determine if spaceflight induces changes in diversity, concentration, and/or characteristics of medically significant microorganisms associated with the crew and environment aboard the International Space Station that could affect crew health.*

   While the identification, enumeration, and distribution of medically significant microorganisms in spacecraft has been extensively monitored since the Apollo Program, data from closed chamber analogs, such as the Russian Mars-500 mission (69) or Antarctic habitation (70, 71), have also been collected to supplement these findings. One example of a well-controlled system was the Lunar-Mars Life Support Test Project (LMLSTP) consisting of 4 tests of individuals living in an environmentally-closed chamber for up to 91 days (72). Microbiological monitoring results during the LMLSTP displayed microbiota commonly isolated from many terrestrial habitats, with microorganisms in the chamber environment reflecting the human and/or plant inhabitants.

3. *Micro 3: We need to determine which medically significant microorganisms display changes in the dose-response profiles in response to the spaceflight environment that could affect crew health.*

   As mentioned previously, the first pathogenic microorganism to be extensively studied when grown in the spaceflight analog environment of the RWV was *S.* Typhimurium. These early studies indicated that *S.* Typhimurium grown in the RWV were more virulent and were recovered in higher numbers from the murine spleen and liver following oral infection of a murine model compared to organisms grown under a normal gravity control (20). *S.* Typhimurium grown in the RWV also displayed altered stress responses and survival in macrophage cells (20, 21). A comparison of microarray data from the RWV and control cultures indicated 163 differentially expressed genes distributed throughout the chromosome, representing functionally diverse groups including transcriptional regulators, virulence factors, lipopolysaccharide biosynthetic enzymes, iron-utilization enzymes, and proteins of unknown function (21). These studies with *S.* Typhimurium prompted other investigators to study the impact of RWV culture on a variety of microorganisms.

[5] https://taskbook.nasaprs.com/publication/index.cfm?action=public_query_taskbook_content&TASKID=9315

Numerous strains of *E. coli* have been cultured in the RWV. Investigations with *E. coli* MG1655 cultured in Luria Broth displayed decreased growth, the down-regulation of 14 genes, and no discernable changes to environmental stressors, such as resistance to acid and osmotic stress when compared to controls (73). When this same strain was cultured in a minimal salts media, no difference in growth was observed and 35 genes were differentially expressed (73). Conversely, culture of *E. coli* AMS6 in minimal media demonstrated an increased resistance to acid and osmotic stress in response to the low-shear conditions (74). Interestingly, culture of this strain in the RWV displayed significantly higher biofilm production on glass microcarrier beads placed in the reactor (75). Investigation of the response of adherent-invasive *E. coli* O83:H1 to culture in the RWV indicated this organism did not change growth, acid or osmotic resistance; however, it did display an increased resistance to thermal and oxidative stress in minimal media (76). Interestingly, low-shear-cultured *E. coli* O83:H1 displayed increased adherence to epithelial cells although invasion rates were unchanged as compared to controls (76).

P. aeruginosa cultured in the RWV displayed distinct changes in its biofilm architecture compared to controls (49), which could impact its virulence and antibiotic resistance. In addition, RWV culture of *P. aeruginosa* appears to influence the *rhl N*-butanoyl-L-homoserine lactone (C4-HSL) directed quorum sensing (QS) system, increasing the production of rhamnolipids, and potentially having an impact on the virulence of the organism (49). Analysis of gene expression data also identified a role for the global regulatory protein, Hfq, as seen in *S*. Typhimurium (50).

Other organisms beyond gram-negative pathogens have been evaluated using the RWV. The response of *S. aureus* to RWV culture has been the most thoroughly studied among Gram-positive microorganisms. Interestingly, while gene expression appears to be regulated by Hfq (77), as seen with *S*. Typhimurium and *P. aeruginosa*, virulence characteristics, such as staphyloxanthin production and hemolytic activity appear to be repressed (77, 78). Culture of *Streptococcus pneumoniae* in the RWV has also been studied as 41 genes were reported to be differentially regulated (79). The pathogenic yeast *Candida albicans* displayed random budding patterns and enhanced filamentous growth when cultured in the RWV, suggesting a more pathogenic phenotype (80).

4. *<u>Micro 4</u>: We need to determine how physical stimuli specific to the spaceflight environment, such as microgravity, induce unique changes in the dose-response profiles of expected medically significant microorganisms.*

   As mentioned above, after gene expression data from spaceflight culture of *S*. Typhimurium indicated an association of the differentially expressed genes with the global regulatory protein, Hfq, these investigators used the RWV system to show corroborating evidence by comparing the stress response and macrophage survival of a wild type and an *hfq* mutant strain (15). A similar approach with the RWV was used to corroborate the impact of high inorganic ion concentrations on the spaceflight culture response of *S.* Typhimurium, even to the point of suggesting inorganic phosphate as a potential candidate as the causative agent (16). The finding by Wilson et al. is not

completely surprising as earlier work in the RWV indicated that the ferric uptake regulator gene (Fur) is involved in the *S.* Typhimurium acid stress resistance that is induced by space analog culture (21). Thus, the use of the RWV as both an indicator of spaceflight trends in microbial response as well as a tool to understand possible mechanisms has been accepted in the scientific community.

One stimulus that could impact spaceflight culture of microorganisms is the physical impact of fluid dynamics, specifically fluid shear. The potential of a fluid shear response was supported by spaceflight-analog studies of *S.* Typhimurium cultured in the RWV (81). In these experiments, a correlation was observed between the progressive addition of shear into the system and a decrease in microbial responses associated with culture in the RWV. The potential of a spaceflight-associated mechanotransductive response, which is the product of changes in physical forces on the cell membrane would not be without precedence, as shear forces have been demonstrated to impact microbial responses (82, 83). Indeed, a number of bacterial cytoskeletal structures, such as MreB (actin homolog) and FtsZ (tubulin homolog) have been identified (84). Taken together, this evidence suggests the responses, such as altered growth, observed with microorganisms resulting from spaceflight culture may be the result of the secondary effects found in liquid culture during spaceflight, such as very low fluid shear.

An alternative stimulus that has been proposed was based upon differential gene expression data of both *P. aeruginosa* (50) and *S. aureus* (77). In both organisms evidence of low oxygen levels was detected that could have impacted the response of the microorganisms.

As with spaceflight, understanding the biochemical responses of microorganisms to this environment provides insight into both the stimulus/stimuli and implications for crew health. In early studies, Fang et al. reported that culture in the RWV resulted in the reduction in production of β-lactam antibiotics by *Streptomyces clavuligerus* (85), reduction of microcin B17 (MccB17) production by *E. coli* (86), but no change in Gramicidin S production by *Bacillus brevis* (87). These findings suggest a possible difference in membrane structure, biochemical production of these compounds, or an alteration in the transport mechanism.

5. *Micro 5: Current microbial standards identifying microbial risk limits need to be updated and microbial requirements need to be developed to include new technologies and future mission scenarios*.

   As mission scenarios are defined, the microbial requirements will continue to be reviewed and updated to ensure crew health and safety. Technology advancements will be monitored and evaluated for applicability.

   Spaceflight technology developed to study viral reactivation in astronauts has translated to Earth for use in medicine. Some physicians use this polymerase chain reaction (PCR) based technology to analyze for herpes viruses in saliva and other body

fluids (88). This technology is non-invasive, rapid, and highly accurate and has been shown to assist in the diagnosis of difficult cases and prevent misdiagnosis.

V. Computer-Based Modeling and Simulation

Computer-based modeling and simulations are not included in this risk.

VI. Risk in context of Exploration Mission Operational Scenarios

Current medical operations do not incorporate potential alterations in host-microorganism interactions, per se; however, the risk of infection is greatly minimized through current vehicle design and operational requirements. Vehicles and their systems are designed to maintain microbial concentrations at very conservative levels (eg, potable water below 50 CFU per mL). Operational activities are also designed to limit crew exposure, including preflight crew quarantine and stringent preflight/in-flight monitoring.

As the risk of infectious disease is a function of the presence and characteristics of the agents, the dose-response of those agents, and the crew exposure to those agents, the risk of infectious disease during different mission scenarios varies depending on several potential factors, including mission duration, design of the environmental life support system, and continued/repetitive use of the facility. Any change in the risk of infectious disease attributed to spaceflight would have corresponding change in the vehicle design or operational activities. For example, if spaceflight induces changes in the concentration or virulence of opportunistic pathogens during a mission, appropriate adjustments in allowable microbial concentrations, housekeeping, or antibiotic provision may need to occur.

VII. Knowledge Gaps

The Human Research Program has aligned the Knowledge Gaps of this risk to correspond with federal interagency guidelines for microbiological risk assessment outlined in USDA/FSIS/2012-001 and EPA/100/J12/001[6].

These include:

- Micro 1: We need to determine the efficacy of current countermeasures and the need for countermeasure development based on changes in microbial populations and characteristics.
- Micro 2: We need to determine if spaceflight induces changes in diversity, concentration, and/or characteristics of medically significant microorganisms associated with the crew and environment aboard the International Space Station (ISS) that could affect crew health.

[6] http://www.fsis.usda.gov/wps/wcm/connect/d79eaa29-c53a-451e-ba1c-36a76a6c6434/Microbial_Risk_Assessment_Guideline_2012-001.pdf?MOD=AJPERES

- Micro 3: We need to determine which medically significant microorganisms display changes in the dose-response profiles in response to the spaceflight environment that could affect crew health.
- Micro 4: We need to determine how physical stimuli specific to the spaceflight environment, such as microgravity, induce unique changes in the dose-response profiles of expected medically significant microorganisms.
- Micro 5: Current microbial standards identifying microbial risk limits need to be updated and microbial requirements need to be developed to include new technologies and future mission scenarios.

VIII. Conclusion

Numerous spaceflight experiments have been conducted to investigate alterations in microbial responses resulting from culture during spaceflight and spaceflight-analogs. However, recent studies investigating spaceflight-associated alterations in microbial virulence have initiated the review and production of evidence to better understand the impact these alterations would have on the incidence of infectious disease during a spaceflight exploration mission. The preponderance of evidence indicates that alterations in microbial gene expression and phenotype (including virulence) are occurring; however, the clinical implications of such changes are still unclear. Greater knowledge is required including a better understanding of the mechanism behind unique spaceflight-associated microbial responses to determine how this environmental stimulus impacts various microorganisms, their diversity and concentration in the spacecraft and crew microbiome, their impact on the vehicle and crew, and their resistance to current mitigation and antibiotic regimens. This knowledge will enable us to determine requirements, guidelines, and processes for design and monitoring of the next generation vehicles.

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XI. List of Acronyms

AMP – Antimicrobial Peptide
CFU – Colony Forming Units
CMV – Cytomegalovirus
EBV – Epstein - Barr Virus
IOM – Institute of Medicine
ISS – International Space Station
FDA – Food and Drug Administration
HACCP – Hazard Analysis and Critical Control Points
HRP – Human Research Program
HSL – Homoserine Lactone
HSV-1 – Herpes Simplex
LMLSTP – Lunar-Mars Life Support Test Project
NASA – National Aeronautics and Space Administration
PCR – Polymerase Chain Reaction
PRD – Program Requirements Document
QS – Quorum Sensing
RNA – Ribonucleic Acid
RPM – Random Positioning Machine
RWV – Rotating Wall Vessel
STS – Space Transportation System
VZV – Varicella-Zoster Virus

APPENDIX A

Microbial Responses Documented during Spaceflight

Microorganism(s) / Flight	Response to Spaceflight	Reference
Escherichia coli, *Aerobacter aerogenes*, and *Staphylococcus* Unmanned Satellite, 1960	• Bacterial viability was unaffected by spaceflight conditions	Zhukov-Verezhnikov, 1962[89]
Escherichia coli Vostok 2, 1961	• Variant colony type was noted and was determined to be the result of spaceflight factors	Klemparskaya, 1964[90]
Escherichia coli Vostok 5 and 6, 1963	• Increase in the levels of phage induction correlating with the duration of time spent in microgravity was noted	Zhukov-Verezhnikov, 1965; 1966[91, 92]
Escherichia coli and *Salmonella enterica* serovar Typhimurium Biosatellite 2, 1967	• Increased population density for both microorganisms	Mattoni, 1968; 1971[93, 94]
Bacillus subtilis Apollo 16 and 17, 1972	• Developmental process of spore formation was unaffected by spaceflight conditions	Bucker, 1975[95]
Bacillus subtilis Apollo-Soyuz Test Project, 1975	• Colony forming ability of spores was found to be reduced among spaceflight samples	Facius, 1978[96]
Escherichia coli and *Staphylococcus aureus* Salyut 6, 1977 – Salyut 7, 1982	• Both organisms displayed increased resistance to multiple antibiotics • Thickening of the cell wall in *S. aureus*	Tixador, 1983; Tixador, 1985a; Tixador, 1985b; Lapchine, 1987[97-100]
Escherichia coli and *Bacillus subtilis* STS-61-A, Challenger, 1985	• Increased conjugation (*E. coli*) • Increased growth kinetics (*B. subtilis*)	Ciferi, 1988; Mennigmann, 1986[101, 102]
Escherichia coli and *Bacillus subtilis* STS-63, Discovery, 1995	• Decreased lag growth phases • Increased exponential growth phases • Increased cell population	Kacena, 1999 [38]
Pseudomonas aeruginosa *STS-95, Discovery, 1998*	• Documented biofilm formation in microgravity	McLean, 2001[103]
Salmonella enterica serovar Typhimurium STS-115, Atlantis, 2006	• Increased virulence; in a murine infection model, spaceflight cultured organisms caused a reduced time-to-death, increased percent mortality, and decreased lethal dose required to kill 50% of the mice (LD_{50}) as compared to ground control cultures • Differential gene and protein expression • Hfq identified as a possible regulator of the microgravity response	Wilson, 2007 [15]

Microorganism(s) / Flight	Response to Spaceflight	Reference
Pseudomonas aeruginosa STS-115, Atlantis, 2006	• Differential gene and protein expression • Involvement of Hfq in the microgravity response	Crabbe, 2011 [14]
Candida albicans STS-115, Atlantis, 2006	• Differential gene expression • Increased cell-aggregation genes and phenotype • No increase in virulence observed in a murine infection model	Crabbe, 2013[13]
Salmonella enterica serovar Typhimurium STS-123, Endeavor, 2008	• Increased virulence findings confirmed • Media ion concentration influences the spaceflight-related virulence response; when cultured in a modified growth medium, the spaceflight imparted increase in virulence was reduced to the level of ground controls • Differential gene and protein expression • Confirmation of Hfq as a potential regulator of the spaceflight response	Wilson, 2008 [16]
Pseudomonas aeruginosa STS-132, Atlantis, 2010 STS-135, Atlantis, 2011	• Increased number of viable cells • Increased biofilm biomass and thickness • Unique biofilm architecture not previously observed on Earth • Unique biofilm formation was dependent on flagella-drive motility	Kim, 2013 [51, 54]

APPENDIX B

Microbial Responses to Modeled Microgravity

Microorganism	Response to modeled microgravity within the RWV bioreactor	Reference
Salmonella enterica serovar Typhimurium χ^{3339}	• Increased: virulence in a mouse model; resistance to acid, thermal, and osmotic stress; macrophage survival • Decreased: LPS production; resistance to oxidative stress; Hfq expression • Differential gene expression	Nickerson, 2000 [20] Wilson, 2002 [21] Wilson, 2002 [104] Wilson, 2007[15] Pacello, 2012[105]
Salmonella enterica serovar Typhimurium 14028	• Increased: virulence in a mouse model and cellular invasion • Differential gene expression	Chopra, 2006[106]
Escherichia coli AMS6	• Increased biofilm formation and resistance to osmotic, ethanol and antibiotic stress	Lynch, 2006[75]
Escherichia coli E2348/69	• Increased intimin production	Carvalho, 2005[107]
Escherichia coli MG1655	• Decreased growth • Differential gene expression	Tucker, 2007 [73]
Escherichia coli K12	• Differential gene expression	Vukanti, 2008[108]
Escherichia coli 083:H1	• Increased resistance to thermal and oxidative stress and adhesion to epithelial cells	Allen, 2008 [76]
Pseudomonas aeruginosa PA01	• Increased: biofilm formation; elastase production, and rhamnolipid production; alginate production; resistance to oxidative and thermal stress; Hfq expression • Differential gene expression	Crabbe, 2008 [49] Crabbe, 2010 [50]
Streptococcus pneumoniae TIGR4	• Differential gene expression	Allen, 2006 [109]
Staphylococcus aureus N315	• Increased: biofilm formation; susceptibility to whole blood • Decreased: growth; carotenoid production; resistance to oxidative stress; Hfq expression	Castro, 2011 [77]
Staphylococcus aureus RF1, RF6, RF11	• Decreased: carotenoid production; hemolytic activity • Differential gene expression	Rosado, 2010 [78]
Staphylococcus aureus 25923	• Increased: growth and membrane integrity	Vukanti, 2012 [110]
Yersina Pestis KIMD27	• Decreased: Hela cell rounding	Lawal, 2010 [111]

Microorganism	Response to modeled microgravity within the RWV bioreactor	Reference
Haloferax mediterranei DSM 1411	• Increased: antibiotic resistance • Differential pigment production and protein expression	Dornmayr-Pfaffenhuemer, 2011[112]
Halococcus dombrowskii DSM 14522	• Decreased: cell aggregations • Differential pigment production and protein expression	Dornmayr-Pfaffenhuemer, 2011[112]
Saccharomyces cerevisiae BY4743	• Increased: aberrant budding • Differential gene expression	Purevdorj-Gage, 2006 [113]
Candida albicans SC5314	• Increased: filamentous growth; biofilm formation; antimicrobial resistance • Differential gene expression	Altenburg, 2008 [80] Searles, 2011 [114]
Enterobacter cloacae ATCC23355	• Decreased: resistance to acid and oxidative stress • Differential gene expression	Soni, 2014 [115]
Citrobacter freundii ATCC8090	• Decreased: resistance to oxidative stress • Differential gene expression; Hfq expression	Soni, 2014 [115]
Serratia marcescens ATCC14041	• Increased: resistance to acid stress	Soni, 2014 [115]
Streptococcus pyogenes	• Decreased: growth; antibiotic resistance • Differential gene expression	Kalpana, 2015 [116]
Staphylococcus epidermidis ATCC12228	• Increased: growth	Fajardo-Cavazos, 2014 [117]
Bacillus subtilis WN1532	• Increased: growth; antibiotic resistance	Fajardo-Cavazos, 2014 [117]
Rhinovius	• Increased: virus (free and cell-associated)	Long, 1998[118]
Epstein-Barr virus	• Decreased: viral protein expression (immunofluorescence) of host cells	Long, 1999[119]
Epstein-Barr virus	• Microgravity alone decreased: apoptosis, cell death and DNA repair of host cells • Microgravity and radiation exposure increased: DNA damage and reactive oxygen species of host cells	Brinley, 2013[120]
Vibrio fischeri (symbiosis with host squid)	• Increased: bacteria-induced apoptosis • Decreased: host innate immune response	Foster, 2013[121]