Microbial Characteristics of ISS Environmental Surfaces

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The microbiome of environmental surfaces from the International Space Station were characterized in order to examine the relationship to crew and hardware maintenance. The Microbial Observatory (ISS-MO) experiment generated a microbial census of ISS environments using advanced molecular microbial community analyses along with traditional culture-based methods. Since the "omics" methodologies generated an extensive microbial census, significant insights into spaceflight-induced changes in the populations of beneficial and/or potentially harmful microbes were gained. Surface samples were collected from several ISS surface locations from three flight opportunities, and were returned to Earth via the Soyuz TMA-14M or the Space X Dragon capsule. In addition to cultivation methods, viable microbial burden, iTag-based sequencing, and metagenome analyses were carried out. The cultivable microbial bioburden differed by location and sampling event. Exploring the ISS environmental microbiome revealed presence of opportunistic pathogens and antibiotic resistant microbes. Genes involved in ATP binding cassette transporters, two component systems, and beta-lactam resistance were among a diverse set of metabolic and genetic information processing pathways. Whole genome sequencing (WGS) of 50 ISS strains exhibiting resistance to various antibiotics was carried out. The antibiotic resistant genes deduced from the WGS were compared with the resistomes generated directly from the gene pool of the environmental samples. Two unique *Aspergillus fumigatus* strains isolated from the ISS were characterized and compared to the experimentally established clinical isolates Af293 and CEA10. A virulence assessment in a neutrophil-deficient larval zebrafish model of invasive aspergillosis indicated that both ISSFT-021 and IF1SW-F4 were significantly more lethal compared to Af293 and CEA10. The findings from this *Environmental "Omics"* project should be exploited to enhance human health and well-being of a closed system. In other words, the ISS-MO research aims to "translate" findings in fundamental research into medical practice (pathogen detection) and meaningful health outcomes (countermeasure development).

Nomenclature

ISS	=	International Space Station
MO	=	Microbial Observatory
WGS	=	Whole Genome Sequencing
NRC	=	National Research Council
MT	=	Microbial Tracking
HRP	=	Human Research Program
IRP	=	Integrated Research Plan
R2A	=	Reasoner's 2A agar

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PBS = *phosphate buffered saline*
PDA = potato dextrose agar
BA = blood agar
DNA = deoxyribo nucleic acid
PMA = propidium monoazide
rRNA = ribosomal ribose nucleic acid
ITS = Internal transcribed spacer

I. Introduction

The International Space Station (ISS) is a closed system that is composed of microorganisms originating from life support systems, Earth based contaminants, and humans. Microorganisms associated with the ISS are exposed to selective pressures induced by an extreme environment, e.g., microgravity and enhanced radiation. These factors can contribute to unpredicted genetic changes that might impact the integrity of the station and/or the health of astronauts. Therefore, understanding the microbiome onboard the ISS may help to develop suitable countermeasures to minimize the risks associated with human spaceflight.

In response to the National Research Council (NRC) Committee's Decadal Survey on Biological and Physical Sciences in Space, which reported that "*microbial species that are uncommon, or that have significantly increased or decreased in number, can be studied in a "microbial observatory" on the ISS,*" the Microbial Tracking (MT-1) flight experiment was carried out. As part of a microbial observatory effort the NRC decadal survey committee recommended that NASA should: "*(a) Capitalize on the technological maturity, low cost, and speed of genomic analyses and the rapid generation time of microbes to monitor the evolution of microbial genomic changes in response to the selective pressures present in the spaceflight environment; (b) Study changes in microbial populations from the skin and feces of the astronauts, plant and plant growth media, and environmental samples taken from surfaces and the atmosphere of the ISS; and (c) Establish an experimental program targeted at understanding the influence of the spaceflight environment on defined microbial populations.*" The ongoing astronaut microbiome work established by NASA [1] partly addresses item *(b).* This MT-1 project allowed us to establish the formation of the ISS-MO, which will lead to long-term, multigenerational studies of microbial population dynamics, addressing *(a)* and *(c)* above, and other NRC recommendations.

The effects of the spaceflight environment on microbial population dynamics are largely unknown and represent both a significant gap in our knowledge and an important opportunity to study the evolution of microbial populations and predict crew health and engineering risks of closed habitation environments during long-term space exploration. The MT-1 project capitalized on both traditional and mature molecular technologies developed by the academia and other institutions including NASA [2]. However, in this communication, we focus our discussion on the microbial burden of cultivable and non-cultivable but viable, bacteria and fungi from eight defined locations on the ISS using three consecutive flights.

This study may provide NASA with the ability to accurately and confidently assess the spectrum of microorganisms associated with the closed habitat environment and crew health maintenance. In addition to microbial profiles that can be cultured, we have identified microbial taxa that may pose threats to crew health and spacecraft systems. The MT-1 study enabled us to address the applicable Human Research Program (HRP) Integrated Research Plan (IRP) risks, to: *(1) determine if changes in the ISS microorganisms are occurring during a spaceflight mission and if so, (2) determine whether any changes noted would create a greater health risk for the crew.*

The objectives of this study were to characterize environmental surfaces from ISS in order to examine the relationship to crew and hardware maintenance. The MT-1 study generated a microbial census of ISS environments and identified them using advanced molecular methods as well as generated whole genome sequences of 22 opportunistic bacterial and fungal pathogens that were collected. Since the "omics" methodologies generated an extensive microbial census, significant insights into spaceflight-induced changes in the populations of beneficial and/or potentially harmful microbes were gained.

II. Materials and Methods

A. Sample collection and processing

Sampling wipes (polyester wipe; 9'' x 9''; ITW Texwipe, Mahwah, NJ) were prepared under aseptic conditions transferred to ISS via sterile zip lock bag [2]. Each sampling kit was sent to the ISS onboard the SpaceX-5, -6, and -8 rockets and returned to the Earth onboard the Russian vehicle (Soyuz TM-14) and Dragon capsules and processed immediately after the arrival to Earth. Eight different locations (1 m^2) were sampled on the ISS summarized in Table1.

Table 1. *Description of various ISS sample locations*

Location	Location description	ISS module
1	Port panel next to cupola	Node 3
2	Waste and hygiene compartment (WHC)	Node 3 "F4"
3	Advanced resistive exercise device (ARED) foot platform	Node 3
4	Dining table	Node 1
5	Overhead 4	Node 1
6	Permanent multipurpose module (PMM) Port 1	PMM
7	Lab 3 overhead	LAB
8	Port crew quarters, bump out exterior aft wall	Node 2

The polyester wipes containing samples were placed in suitable sterile container and 200 mL of sterile phosphate-buffered saline (PBS; pH 7.4) was added and vortexed. The resulting mixture was concentrated 4 mL as per established procedure [3]. Suitable aliquots of samples were placed onto R2A (bacteria; 25˚C; 7 days), blood agar (BA; human commensals; 35˚C; 2 days), and potato dextrose agar (PDA) supplemented with chloramphenicol (fungi; 25˚C; 7 days) for the isolation of various kinds of microorganisms. Furthermore, the appropriate sample sizes were used to treat with PMA to assess viability [4], while the second aliquot was handled similarly but without the addition of PMA. DNA extraction was carried out with the Maxwell 16 automated system (Promega, Madison, WI), in accordance with manufacture instructions [3].

B. Estimation and identification of cultivable microbial population

A minimum of 5 isolates of distinct morphologies were picked and further identified by targeting 16S rRNA gene for bacteria or ITS region for fungal classification. The extracted DNA from bacteria was used for PCR to amplify the 1.5 kb 16S rRNA gene in order to identify bacterial strains as described elsewhere [5, 6]. For fungi, the forward primer ITS1F (5'- TTG GTC ATT TAG AGG AAG TAA-3') [7] and reverse primer Tw13 (5'-GGT CCG TGT TTC AAG ACG-3') [8] were used. The sequencing was performed by Macrogen (Rockville, MD, USA) using 27F and 1492R primers for bacteria, and ITS1F and Tw13 primers for fungi. The sequences were assembled using SeqMan Pro from DNAStar Lasergene Package (DNASTAR Inc., Madison, WI). The bacterial sequences were searched against EzTaxon-e database [9] and the fungal sequences against the UNITE database [10]. The identification was based on the closest percentage similarity (>97%) to previously identified microbial type strains.

C. qPCR assay

Following the DNA extraction with the Maxwell Automated system, the quantitative polymerase chain reaction (qPCR), targeting the 16S rRNA gene (bacteria) or ITS region (fungi), was performed with SmartCycler (Cepheid, CA) to quantify the respective microbial populations [11]. The number of gene copies in our samples were determined with Ribosomal RNA gene standards, spanning 10^8–10^2 gene copies/µl, which were generated by serially diluting 4 nmoles of ULTRAMER® DNA oligos (Integrated DNA Technologies, Coralville, Iowa, USA) .

III. Results and Discussion

A. Cultivable bacteria

Similar bacterial counts were observed between the first two flight sampling sessions, 1 and 2 (Figure 1) and ranged from 4.0 x 10^4 to 1.9 x 10^9 CFU/m^2. However, in flight #3 at least 2-logs more bacterial populations (both R2A and blood agar plate counts) were observed. The lowest two bacterial counts were seen from location #6 (Permanent Multipurpose Module, [PMM]; ISS compartment) and location #3 (foot platform of Advanced Resistive Exercise Device [ARED]) and the highest two being from location #1 (Cupola) and location #5 (Zero G Stowage Rack in Node 1; Figure 1).

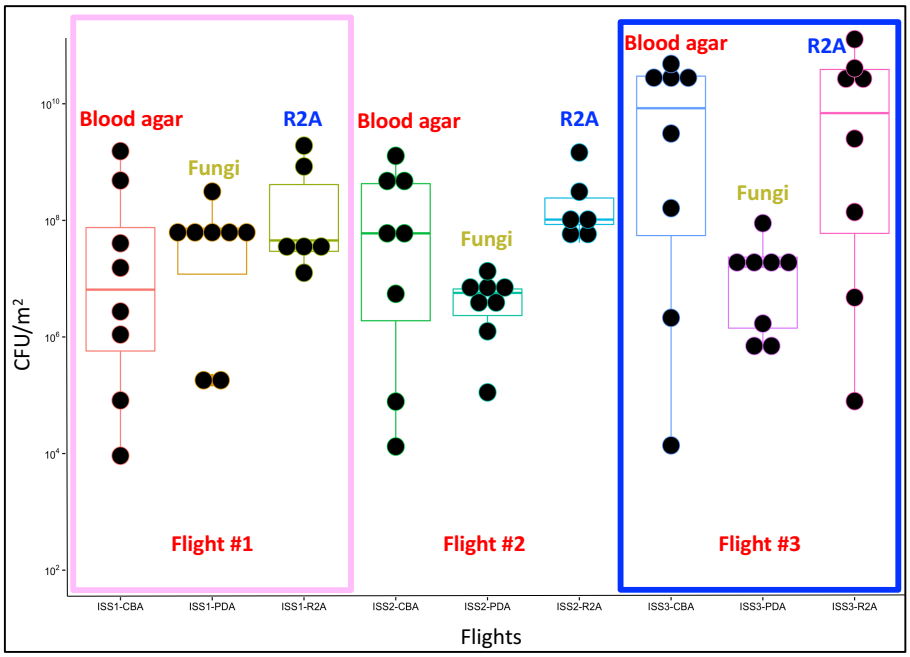

Figure 1. Cultivable microbial burden associated with ISS surfaces. *Box scatter plot of the abundance of bacteria and fungi that could be cultured. Each dot in a column represents a location sampled. Bacteria were isolated from either R2A or Blood agar plates and fungi from PDA plates. Location 6 had no growth on R2A plates from all flight (ISS1, ISS2 and ISS3) and location 8 had no growth on R2A plates from ISS1. No significant differences in abundances were observed among three flight missions and between bacteria and fungi and are not statistically significant (1 way- ANOVA, p>0.05).*

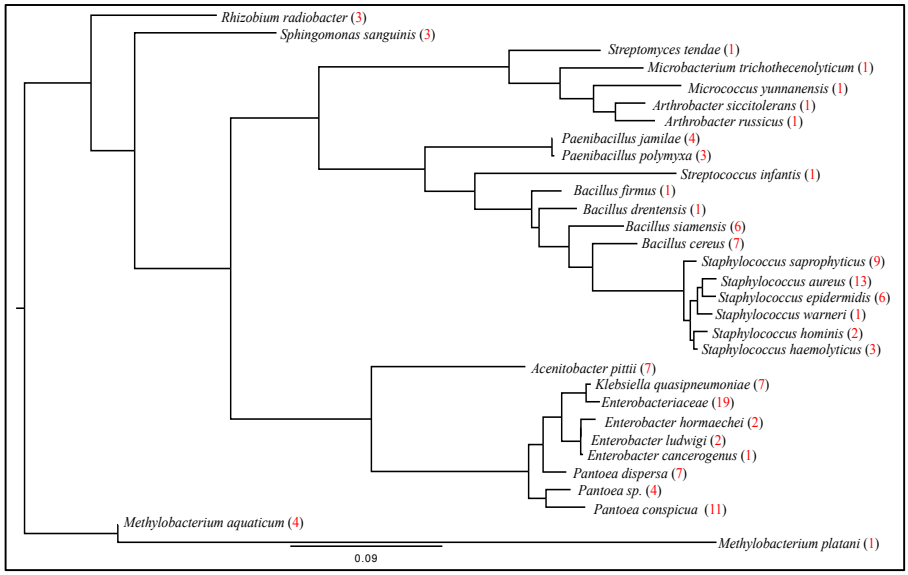

Figure 2. Cultivable bacterial diversity of ISS surfaces identified via 16S rRNA gene sequence method. *The phylogenetic identity of the strain is based on the Eztaxon-e type strain database. The neighbor-joining trees were created using MEGA 7. The phylogeny was inferred by using the Maximum Likelihood method based on the Tamura-Nei model*

The bacterial and fungal isolates (134 bacteria and 64 fungi) were characterized for their phylogenetic affiliations. The bacterial isolates belonged to three phyla: Actinobacteria, Firmicutes and Proteobacteria. At the genus level, the most predominant genera were *Staphylococcus,* (25% of total isolates identified), *Pantoea* (16%), and *Bacillus* (11%). Among 30 bacterial species identified, the most abundant bacterial species were *Staphylococcus* sp. (20%) and *Pantoea* sp. (10%) (Figure 2 and Figure 3). However, several species were identified to the family *Enterobacteriaceae* only.

B. Cultivable fungi

When fungal population density was analyzed, the average fungal concentration was $5.4x10^6$ to $8.1x10^7$ CFU/m^2 and no difference was noticed among samples collected during flight 1 and 3 (Figure 1) whereas the fungal population was at least one log less in samples collected during flight #2. Fungal population ranged from $1.1x10^5$ to $8.9x10^7$ CFU/m^2 with the lowest two fungal counts cultivated from location #3 and #2 (Waste and Hygienic Compartment [WHC]) and the highest from location #8 (Port crew quarters bump-Out exterior aft wall) and #5 (Figure 1). A one-way ANOVA comparing bacterial and fungal counts from the eight locations sampled aboard the ISS, from all three sampling sessions, showed no statistically significant spatial variation in their abundances.

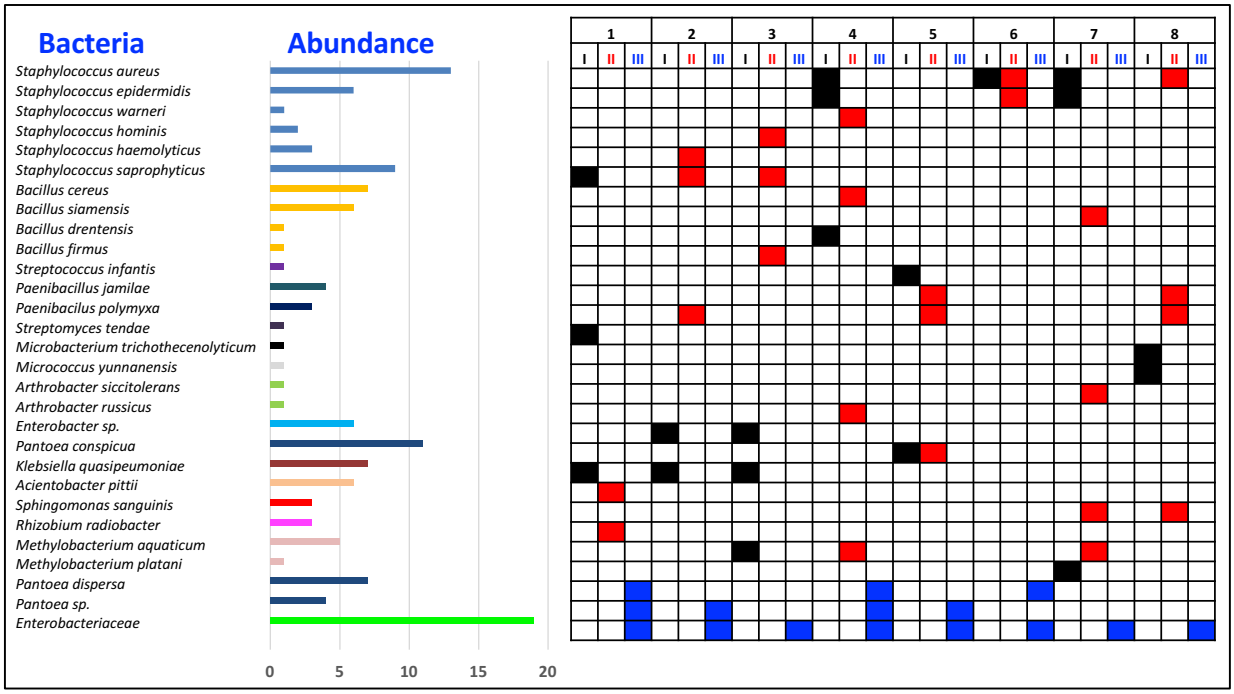

Figure 3. Spatial distribution of cultivable bacterial diversity among various ISS missions and ISS locations. *Total number of isolates associated with each bacterial species is given as bar plot and identical color is given for the species belong to the same genus. The checkboard plot represents the ISS location from where the bacterial species was isolated. Black: Flight 1, Red: Flight 2 and Blue: Flight 3.*

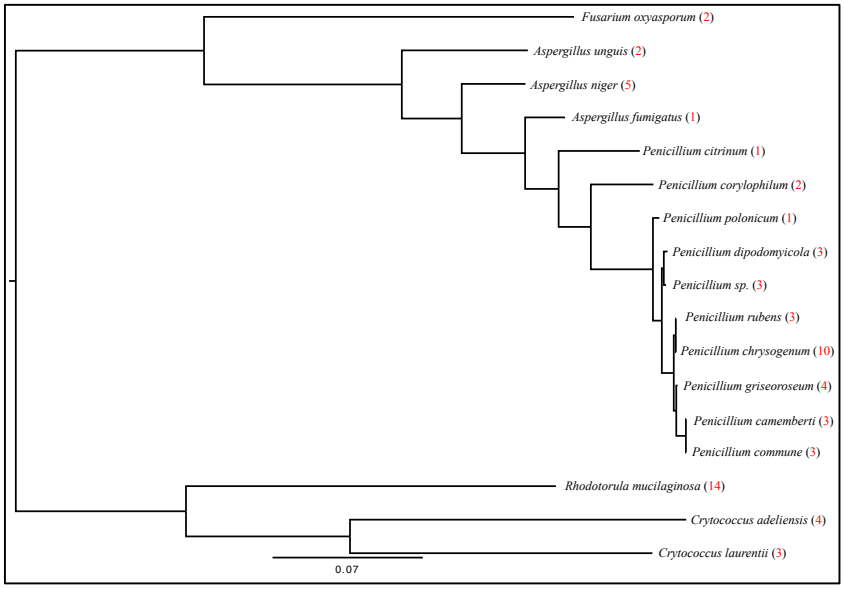

Figure 4. Cultivable fungal diversity of ISS surfaces identified via ITS gene sequence method. *The phylogenetic identity of the strain is based on the UNITE database. The neighbor-joining trees were created using MEGA 7. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model.*

Among 50 fungal strains identified using molecular phylogeny targeting ITS sequences, 5 different genera were observed. *Penicillium* sp. and *Rhodotorula mucilaginosa* were dominant. In addition, though in low numbers, opportunistic human pathogen (*Aspergillus fumigatus*) and plant pathogen (*Fusarium oxysporum*) were also isolated. Among the fungal species *Rhodotorula mucilaginosa* (22 % of 64 fungal isolates examined), and *Penicillium chrysogenum* species (16% of 64 fungal isolates were dominant (Figure 4 and Figure 5). The former was isolated from all samples of flight 1 and 2, with the exception of flight sampling session 1, location #2. However, *R. mucilaginosa* and *P. chrysogenum* were completely absent in flight #3. In addition, isolation of *Cryptococcus* species, yeast, which were reported to be causative agent for human pathogenicity should be noteworthy [12, 13]. *A. fumigatus* is a saprophytic, filamentous fungus that is ubiquitos in outdoors and indoors and can adapt to various environmental

conditions and form airborne conidia that are the inoculum for a variety of diseases in immunocompromised hosts. *A. fumigatus* strains were isolated from a HEPA filter and cupola wall of the ISS [14].

C. Viable microbial population

The total (dead and alive) and viable bacterial and fungal gene copy numbers as determined by PMA-treated (viable) and non-PMA-treated samples were carried out. On average, PMA-treated samples (viable), compared to non-treated samples (total) had an approximately 50% reduction for flight 1 and 75% reduction for flight 2 in bacterial burden, but roughly similar fungal numbers. PMA untreated samples (total) showed the same trends in microbial load at different locations as that of the PMA-treated samples (viable).

The total viable bacterial burden as estimated by the PMA-qPCR assay did not show statistically significant differences in microbial load among three flights (Figure 6). Even though similar copy numbers of bacteria and fungi were noticed in flight 1 and 2 samples, higher viable bacterial (~60%) and lower fungal (~2-logs less) abundance observed in flight 3 samples. The average bacterial 16S gene copy number ranged at 1.0 x 10^9 to 5.9 x 10^9 copies/m^2 and fungal ITS region copy number 9.3 x 10^6 to 1.4 x 10^9 copies/m^2. The lowest 16S rRNA bacterial gene copy number was found at location #6 (PMM) and #3 (ARED) and the highest from location #2 (WHC), #4 (dining table) and #5 (overhead 4). For fungi, the lowest abundance was found at location #2 and #6 and the highest at location #1 (Cupola) and #4.

When the cultivable bacterial density was compared to the viable bacterial population detected by the PMA-qPCR, ~46% of the viable population were cultivated via R2A media. Among 24 data sets collected, only 7 samples exhibited equal number of viable cells and cultivable bacterial population.

D. Opportunistic pathogens and antibiotic resistance profile

Among 30 different bacterial species cultivated, seven species belonged to the biosafety level 2 (BSL-2) bacteria. They were: *Staphylococcus aureus, S. haemolyticus, S. homini, Acinetobacter pittii, Enterobacter piersonii,*

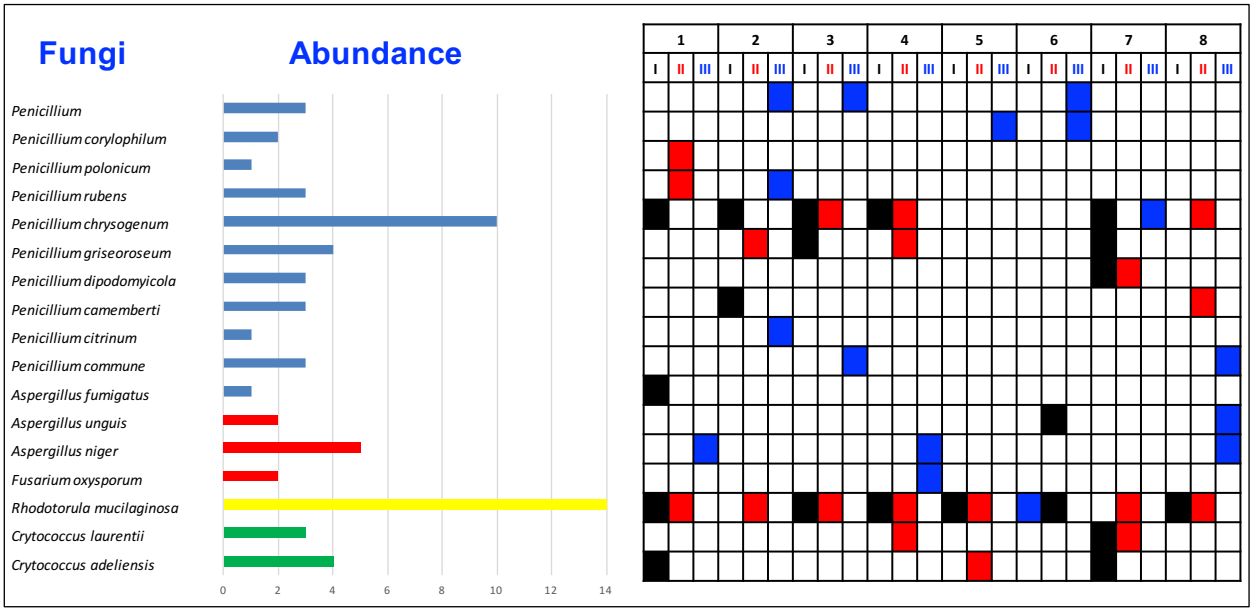

Figure 5. Spatial distribution of cultivable fungal diversity among various ISS missions and ISS locations. *Total number of isolates associated with each fungal species is given as bar plot and identical color is given for the species belong to the same genus. The checkboard plot represents the ISS location from where the bacterial species was isolated. Black: Flight 1, Red: Flight 2 and Blue: Flight 3.*

Klebsiella quasipneumoniae, and *Pantoea conspicua.* Multiple strains of *A. pitti* were isolated from the cupola area (Figure 3) and one strain that was tested for various antimicrobial agent showed resistance to cefazolin, cefoxitin, oxacillin, penicillin, and rifampicin. Two multidrug-resistant *E. piersonii* isolates were found in WHC location. *Enterobacter* species, a member of coliform group of bacteria, were reported to be pathogenic and cause opportunistic infections in immunocompromised hosts and commonly associated with the urinary and respiratory tract infections [15]. *Klebsiella* isolates were retrieved from three locations (cupola, WHC, and ARED). The IF3SW-

P1 showed resistance to all tested antibiotics except tobramycin. *Klebsiella quasipneumoniae* is a blood-borne opportunistic pathogen [16]. *Pantoea conspicua* was the second most prevalent species, and only found in one location during two different flight samplings. *P. conspicua* isolates were resistant to erythromycin, oxacillin, penicillin, and rifampin and was originally isolated from human blood [17].

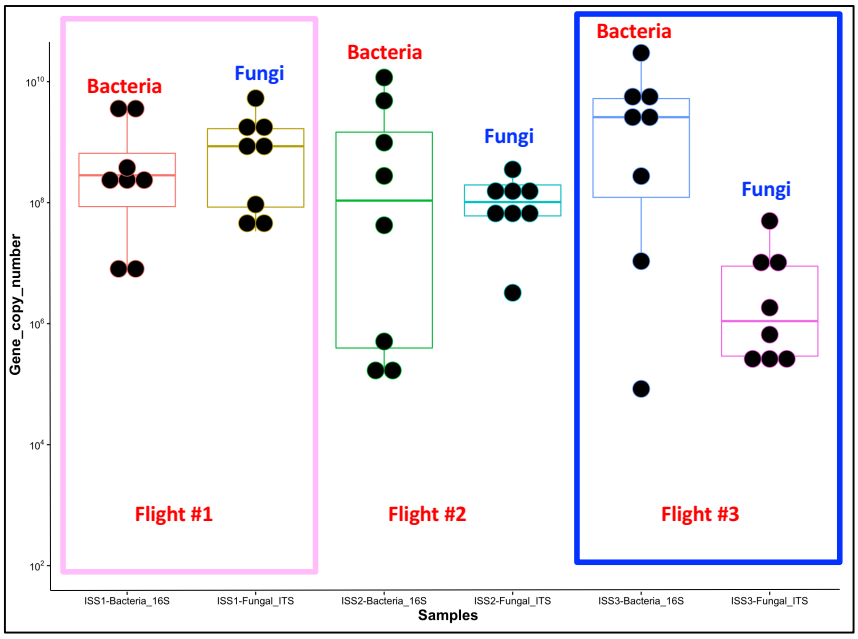

Figure 6. Viable bacterial and fungal burden associated with ISS surfaces as measured by PMA-qPCR method. *Bacterial population was measured targeting 16S rRNA gene for bacteria and ITS region for fungi. Samples collected from all three ISS missions and 8 different locations were subjected to DNA extraction and appropriate gene copies were measured. A 1-way ANOVA showed no statistically significant differences between any of the groups displayed in this figure (p>0.5).*

Staphylococcus isolates were the most prevalent from ISS surfaces. *Staphylococcus aureus* was the most abundant in all ISS locations. Although this species is a common human commensal [18], it causes various types of minor skin infections, bacteremia, or scalded skin syndrome, especially in immunocompromised individuals [19]. *S. aureus* isolates were further tested with the Vitek 2 system (BioMerieux, France) and found that they were not methicillin resistant (i.e. not MRSA strains). However, most of the *S. aureus* isolates exhibited resistance to penicillin, while some of them were resistant to erythromycin, gentamycin and tobramycin. A few isolates acquired rifampicin resistance during the study. *Staphylococcus haemolyticus* and *Staphylococcus hominis* belong to coagulase-negative staphylococci [20, 21]. One *S. hominis* strain tested was resistant to penicillin and erythromycin, but *S. haemolyticus* strain was susceptible to these antibiotics. All three *Staphylococcus* species were reported to be methicillin-resistant by acquiring the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) [22], but the methicillin-resistant phenotype was not observed in strains isolated from ISS surfaces.

Among the fungi, isolation of *A. fumigatus* is noteworthy. As *A. fumigatus* is an opportunistic pathogen causing pathologies ranging from allergic asthma to invasive aspergillosis, several pathogenic characteristics of the ISS isolates in comparison to two experimentally established clinical isolates were assessed [23, 24]. Virulence assessment in a larval zebrafish model of invasive aspergillosis revealed both ISS strains were as significantly more lethal compared to clinical isolates [25]. The omics of these ISS isolates might reveal the molecular mechanisms of the increased virulence. Subsequently, if the enhanced virulence is attributed to the microgravity, NASA potentially should consider developing countermeasures to protect astronaut health, whose immune system is reported to be compromised under microgravity [26]. Similarly, infections caused by cryptococci have been increasingly recognized and *Cryptococcus laurentii* was previously considered as a saprophyte and thought to be non-pathogenic to humans. However, in favorable circumstances like diminished immunity, it seems to be an important pathogen [12]. Furthermore, a case of meningitis caused by *C. adeliensis* in a patient with acute myeloid leukemia undergoing allogeneic peripheral blood stem cell transplantation was reported [13].

E. Whole genome sequencing of ISS cultures

The whole genomes of 20 BSL-2 bacterial [27] and two fungal [28] strains isolated from the ISS were sequenced and now are publicly available. The WGS sequencing of bacterial isolates was performed on an Illumina NextSeq instrument with a paired-end module. The A5 assembly pipeline version 20150522 was used to generate draft

assemblies applying the default parameter settings [29] and annotated with the Rapid Annotations using Subsystems Technology (RAST) [30]. Number of contigs, total genome size, N50 size, median coverage, G+C percentage, error corrected reads used for assembly and number of coding sequences were published [27]. The raw reads were in the range of 24 to 82 Mbp per genome. The G+C content was in the range of 31.5 to 38.7% for *Staphylococcus* species and *A. pittii*; for other strains the G+C contents were 52.1 to 55.8%. Phylogenomics was carried out to describe one of the novel species, *E. piersonii*. Multiple copies of multi-drug resistance genes highly homologous to several pathogens were identified in *E. piersonii* genome and *in silico* analyses showed that these strains had >77% probability to be a human pathogen. However, pathogenic properties of ISS strains should be carried out *in vivo* and compared with clinical strains isolated from patients before making any assumptions that microgravity might have influenced the pathogenicity during spaceflight.

The whole genome of one of the *Aspergillus fumigatus* strains that was isolated from Flight 1, location #1 (Cupola) was recently sequenced and its virulence characterized [25, 28, 31]. The draft genome sequences of two *A. fumigatus* strains (ISSFT-021 and IF1SW-F4) were documented. Briefly, a total of 25,515,334 bp (~230x coverage) for ISSFT-021 strain and 8,466,430 bp (~75x coverage) for IF1SW-F4 strain, high-quality vector-filtered reads were used for assembly with MaSuRCA [32] genome assembler (kmer size = 70). The final assembly of the strain ISSFT-021 contains 301 scaffolds with a total size of 28,526,023 bp and an N_{50} contig length of 275.468 kb; the largest contigs assembled measures 813.103 kb and the GC% was 49.41. Similarly, the final assembly of IF1SW-F4 strain contains 208 scaffolds with a total size of 28,240,437 bp and an N_{50} contig length of 367.421 kb; the largest contigs assembled measures 900.278 kb and the guanine-cytosine content% was 49.45 [28].

IV. Conclusion

Exploring the ISS environmental microbiome revealed a diverse population of viable and cultivable bacteria and fungi, some of which are opportunistic pathogens. Continuous monitoring of the ISS and further studies addressing the functional properties of these organisms are important to maintain crew health and structural integrity aboard the ISS. The findings from this *Environmental "Omics"* project should be exploited to enhance human health and well-being of a closed system. In other words, the ISS-MO research aims to "translate" findings in fundamental research such as isolation of *A. fumiagtus* and BSL-2 bacteria into medical practice (pathogen diagnosis) and meaningful health outcomes (countermeasure development).

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