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Exposure to elevated relative humidity in laboratory chambers alters fungal gene expression in dust from the International Space Station (ISS)

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Microorganisms are present in all occupied indoor environments, including homes on Earth and within specialized systems like the International Space Station (ISS). Microbes when exposed to excess moisture, such as from an unexpected ventilation system failure, can undergo growth that is associated with material degradation and negative health effects. However, we do not yet understand how exposure of these microbes to excess moisture alters their function. A de novo metatranscriptomic study was performed using dust collected from the US air filtration system of the ISS and incubated in laboratory chambers on Earth at different equilibrium relative humidity (ERH) levels. Changes in fungal function (gene expression) were significantly associated with moisture (adonis2 $p = 0.0001$). Secondary metabolism and fungal growth genes were upregulated (FDR-adjusted $p \leq 0.001$, log2FC ≥ 2) at elevated ERH compared to 50% ERH. Elevated moisture conditions showed upregulation of aflatoxin and fungal allergen genes such as Asp f 4 (log2FC = 26.4, upregulated at 85% ERH compared to 50%) and Alt a 7 (log2FC = 2.98, upregulated at 100% ERH compared to 50%). Our results demonstrate that understanding microbial functional changes in response to elevated moisture will help develop more robust microbial monitoring standards for spacecraft environments to protect astronaut health and spacecraft integrity in low-Earth orbit and beyond.

Keywords Fungi, Microbial function, Microbiome, Moisture, Mold, Spacecraft

Microorganisms are inherently present in human-occupied environments, such as a spacecraft like the ISS[1,2]. Many organisms are inert, but exposure to some has been associated with human health outcomes related to respiratory illnesses, allergies, and infections[3,4]. Health effects may be intensified due to immune deregulation known to occur during space travel[5]. The presence of certain microbes also has implications for spacecraft integrity. Damage to equipment and structural materials from bio-destructive bacteria and fungi was observed on the Russian orbital station *Mir*[6]. Microbial degradation of critical materials aboard spacecraft could result in system failures endangering the crew members. As we strive to achieve long-term settlements beyond Earth, we must also consider planetary protection from microbes. We need to better understand the qualities of a healthy spacecraft microbiome to inform our design decisions and prevent unintended consequences on long duration missions.

About 12,500 different microbial species have been found aboard the ISS[7]. *Aspergillus flavus, Aspergillus sydowii* and *Penicillium chrysogenum* exist on surfaces of the ISS as well as in dust samples collected from the

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ISS[8,9]. These fungal species are commonly associated with house dust and the indoor environment on Earth[10]. The most abundant bacteria on the ISS are human-associated such as *Corynebacterium, Staphylococcus* and *Pseudomonas*[7]. Cultured bacteria from the ISS include *Actinobacteria, Firmicutes*, and *Proteobacteria*, and cultivable fungi include both *Ascomycota* and *Basidiomycota*[11]. Some of these taxa have been identified as opportunistic pathogens[11] and are also present in built environments on Earth[7]. These taxa require special monitoring due to the unique environment in spacecraft.

Microbial growth is the factor most strongly associated with negative human health effects in indoor environments. The presence of water damage, visible mold growth, or moldy odor (indicative of growth) are more strongly associated with health effects than the presence of any specific species or other measured microbial factors[12,13]. Different microbial species may be harmful in different ways, but the occurrence of active growth may be the most crucial factor of all, regardless of taxonomy[14–16]. Microbial growth in the indoor environment is generally limited by moisture availability[17]. Dust is hygroscopic, quickly absorbing water from the environment, and contains enough nutrients to support microbial growth[18]. When ambient relative humidity (RH) levels reach a certain threshold, microbial growth will occur within dust[14,19]. On Earth, fungal growth in house dust will occur at and above 80% ERH and growth rates increase with increasing moisture[14,18]. Microbial growth also likely occurs more slowly at lower relative humidity levels[20].

Microbial growth at elevated relative humidity conditions is not unique to indoor environments on Earth[21]. Microbes have been found to grow both on the ISS and *Mir*. Power supply issues during an event on Mir caused an increase in temperature, moisture and minimal air circulation resulting in a free-floating condensed water ball filled with bacteria. Species of *Escherichia coli*, *Serratia marcescens* and *Legionella* were found within the free-floating condensate[4]. Experience from *Mir* has informed how we design and manage spacecraft, and the ISS has much better control over relative humidity conditions. However, suboptimal conditions have occurred on the ISS in some specific limited events leading to microbial growth. In 2004, a fabric panel on the Russian segment of the ISS was found to be covered in mold due to increased moisture from hanging wet towels[22,23]. Our prior studies have shown microbial compositional changes with increased moisture in dust from the ISS[21], with fungal growth occurring in dust even with short exposures to varying elevated relative humidity[24]. It is also important to understand what growth might occur during unexpected events such as a temporary ventilation system malfunction that could lead to elevated moisture. Moisture is inherent to an occupied spacecraft due to human emissions and must be carefully controlled. Occupants themselves sweat, bathe, and exhale moisture, and the resulting water vapor and condensation occurs on the ISS and will occur on all future human spaceflight missions.

Microbes on Earth growing at elevated ERH conditions are metabolically complex and demonstrate diverse metabolic processes[15,16]. Our previous study[16] showed that increased relative humidity conditions are associated with diverse fungal secondary metabolic processes and upregulation of fungal developmental genes in dust from homes across the United States. Such metabolic processes can result in a greater expression of genes that may potentially impact human health. Fungal communities at 100% ERH upregulate a greater number of genes responsible for allergen, mycotoxin and pathogenicity when compared to fungal communities at 85% ERH or 50% ERH[15,16]. However, it is not known if metabolic processes in fungi occur on spacecraft similar to that observed within indoor environments on Earth.

The goal of this study is to characterize microbial function (gene expression) that occurs when dust from the ISS is exposed to elevated moisture conditions in laboratory chambers. Dust from the ISS was returned to Earth and incubated under various ERH conditions in controlled laboratory chamber experiments. The RNA extracted from the dust was sequenced to investigate the microbial activity occurring at the various ERH conditions. Gene quantification and differential expression analysis investigated metabolic activity at varying moisture conditions. This information can help inform future spacecraft design and identification of situations that might require remediation.

Results

Overview of the metatranscriptomic dataset

A total of 698,885,111 paired-end reads were produced from RNA sequencing. One complete Trinity assembly was constructed to investigate a total of 19 unique samples (Supplementary Table S1). After quality trimming, Trinity assembled a total of 448,821 contigs. The median contig length was 603 base pairs (bp) and 70% of quality trimmed reads mapped back to the full transcriptome.

Differential expression patterns are similar among relative humidity conditions

Microbial gene expression in samples from all bags at 100%, 85% and 50% ERH cluster by ERH condition (adonis2 $p = 0.0001$, $R^2 = 0.39$) (Fig. 1A, Supplementary Table S3), showing clear separation by ERH condition, as seen between the low ERH (50%) and high ERH (85% or 100%) samples, but not by bag (Fig. 1B). This is also indicated by Spearman correlation heatmap of differentially expressed genes (Supplementary Fig. S1). These results indicate significant differences in gene expression as a function of moisture condition.

Higher moisture conditions had a greater number of fungal annotated genes that were upregulated compared to lower moisture conditions. When comparing 100% and 50% ERH, the 100% condition had 2.65 times the number of fungal annotated genes significantly upregulated (FDR-adjusted $p \leq 0.001$, log2FC ≥ 2) compared to the 50% upregulated condition. Comparing elevated ERH conditions (either 100% or 85% ERH) to 50% ERH, both the 100% and 85% upregulated ERH conditions had greater metabolic pathway coverage (Fig. 2A, C) based on the KEGG annotations of the upregulated fungal genes than the 50% upregulated condition (Fig. 2B, D, Supplementary Table S4).

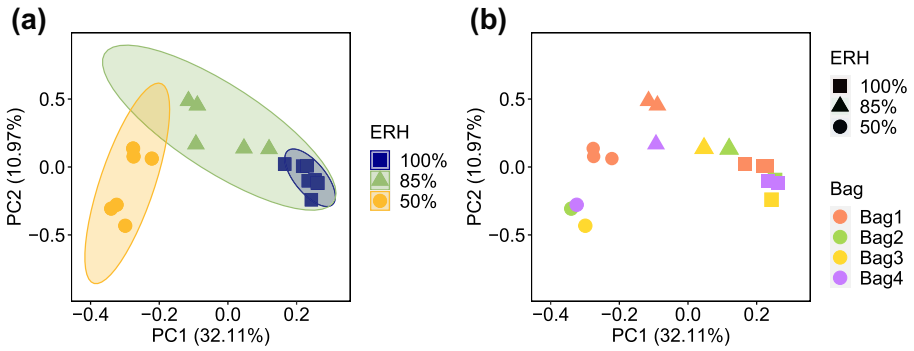

Fig. 1. Principal component analysis (PCA) of samples from all bags grouped by (**a**) ERH and (**b**) bag. Counts per million (CPM) values that were log-transformed and row-mean centered were used. Genes having < 10 total reads across samples were filtered out. Colors represent ERH in (**a**) and Bags in (**b**). Shapes represent ERH condition. Overlap between samples indicate greater similarity based on distance between samples. A 95% confidence ellipse was added for each ERH condition in (**a**).

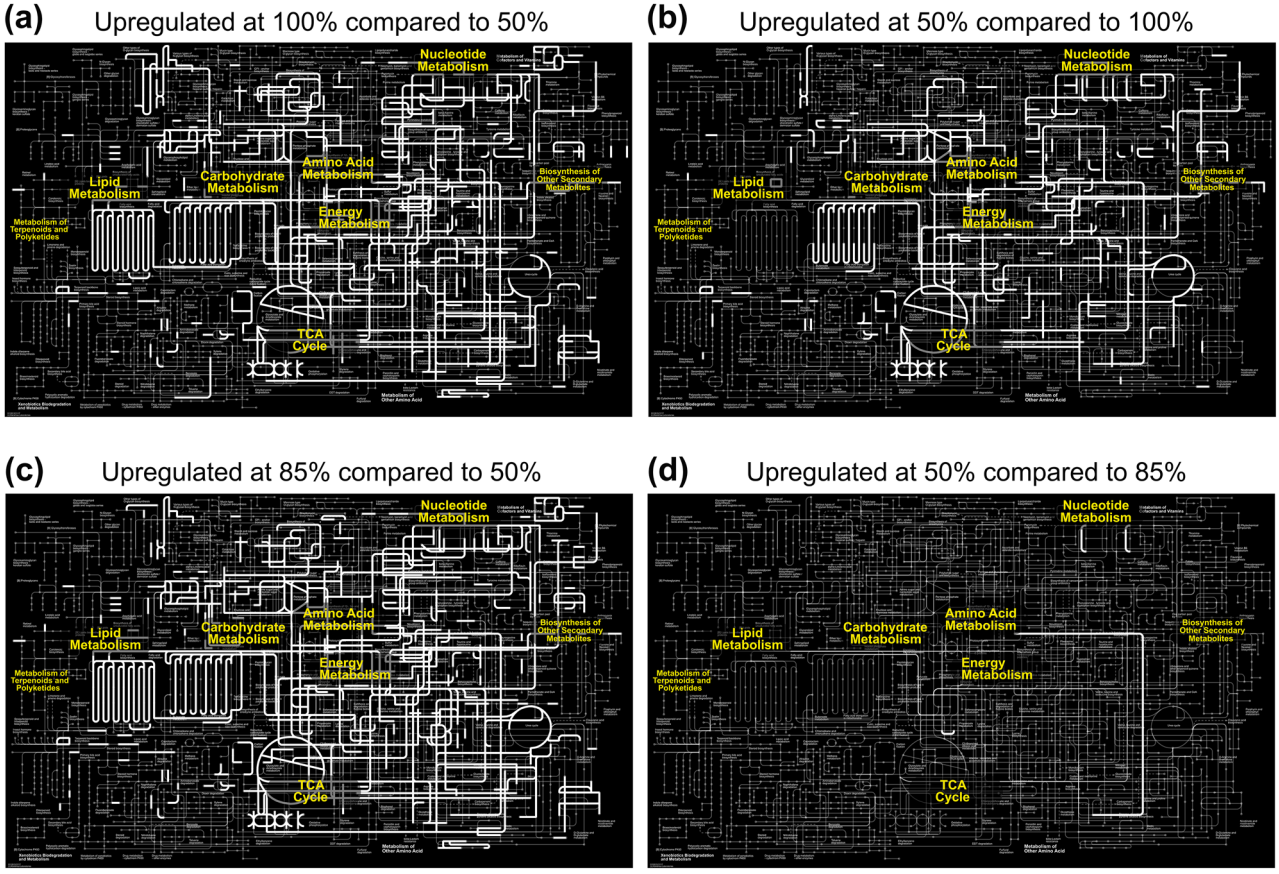

Fig. 2. Fungal metabolic pathways (**a**) upregulated at 100% when compared to 50% ERH, (**b**) upregulated at 50% compared to 100% ERH, (**b**) upregulated at 85% compared to 50% ERH and (**d**) upregulated at 50% compared to 85% ERH. Metabolic pathways that show overlap between maps (**a**) and (**b**) as well as maps (**c**) and (**d**) respectively, could potentially be due to a combination of multiple genes having similar functions at different ERH conditions and limitations in fungal genome annotation databases. All metabolic pathway annotations are additionally subject to fungal genome database limitations.

Secondary metabolic and growth processes are upregulated as moisture conditions increase

Gene expression at each ERH condition shows differences in secondary metabolic processes. When comparing 100% ERH and 85% ERH, the secondary metabolic process GO term i.e., "secondary metabolite biosynthetic process" and "secondary metabolic process" (GO:0044550 and GO:0019748), were enriched at 100% ERH (FDR-

adjusted $p = 0.0003$ and FDR-adjusted $p = 0.002$). All the samples associated with the 100% ERH condition cluster together based on all differentially expressed genes (Fig. 3A). Samples associated with 100% ERH additionally cluster together based on gene expression associated with secondary metabolism (GO:0019748, GO:0044550) (Fig. 3B). Although the term "secondary metabolite catabolic process" was enriched at 85% ERH when compared to 100%, none of the genes had a fungal annotation (either not annotated or from other domains of life); and, hence, was not examined. Out of the secondary metabolism genes upregulated at 100% ERH (compared to 50%), 7 genes were associated with melanin production (Supplementary Table S6), including the genes arp1 and arp2 (log2FC = 5.5 and log2FC = 10.2, respectively).

GO terms associated with fungal growth (n = 10) were enriched at 85% and 100% ERH, and not at the 50% ERH condition (Fig. 4, Supplementary Table S5). Morphological changes that occur during growth, such as "spore-bearing structure development" (GO:0075259, FDR-adjusted $p = 0.014$, enriched at 85% ERH compared to 50%) and "spore wall assembly" (GO:0042244, FDR-adjusted $p = 0.006$, enriched at 100% ERH compared to 85%), were enriched at higher ERH conditions (100% or 85% ERH). The term "reproductive fruiting body development" (GO:0030582, FDR-adjusted $p = 0.014$) was also enriched at 85% ERH compared to 50%, having 24 upregulated genes, where all 24 genes had a fungal annotation. We found mitochondria associated processes (Supplementary Fig. S2) significantly enriched (FDR-adjusted $p < 0.05$) at all ERH conditions. Mitochondrial respiratory processes have potential associations with fungal growth and virulence[25,26] with mitochondria accumulating at the tip of growing hyphae[26]. We found that the 85% ERH condition had 18 times the number of upregulated fungal genes associated with mitochondrial function as the 50% ERH condition (85% vs 50% ERH comparison). Although the exact association between mitochondrial processes and ERH are likely complex, these upregulated genes at 85% ERH (compared to 50%) may be indicative of genes related to mitochondrial processes occurring in growing fungi especially during hyphal growth, at elevated ERH conditions (Supplementary Fig. S2, Supplementary Table S5).The term "primary metabolic process" (GO:0044238, FDR-adjusted $p = 2.45 \times 10^{-84}$) as well as its child GO terms "carbohydrate metabolic process" (GO:0005975, FDR-adjusted $p = 1.34 \times 10^{-07}$) and "alpha amino acid metabolic process" (GO:1901605, FDR-adjusted $p = 5.7 \times 10^{-13}$), were enriched at the 50% ERH condition (50% vs 100% ERH comparisons) (Fig. 4). The term "primary metabolic process" was also enriched at the high 85% ERH (when compared to 100%). Additionally, as previously found secondary metabolic process GO terms were enriched at 100% ERH (compared to 85%). Taken together, this indicates that while primary

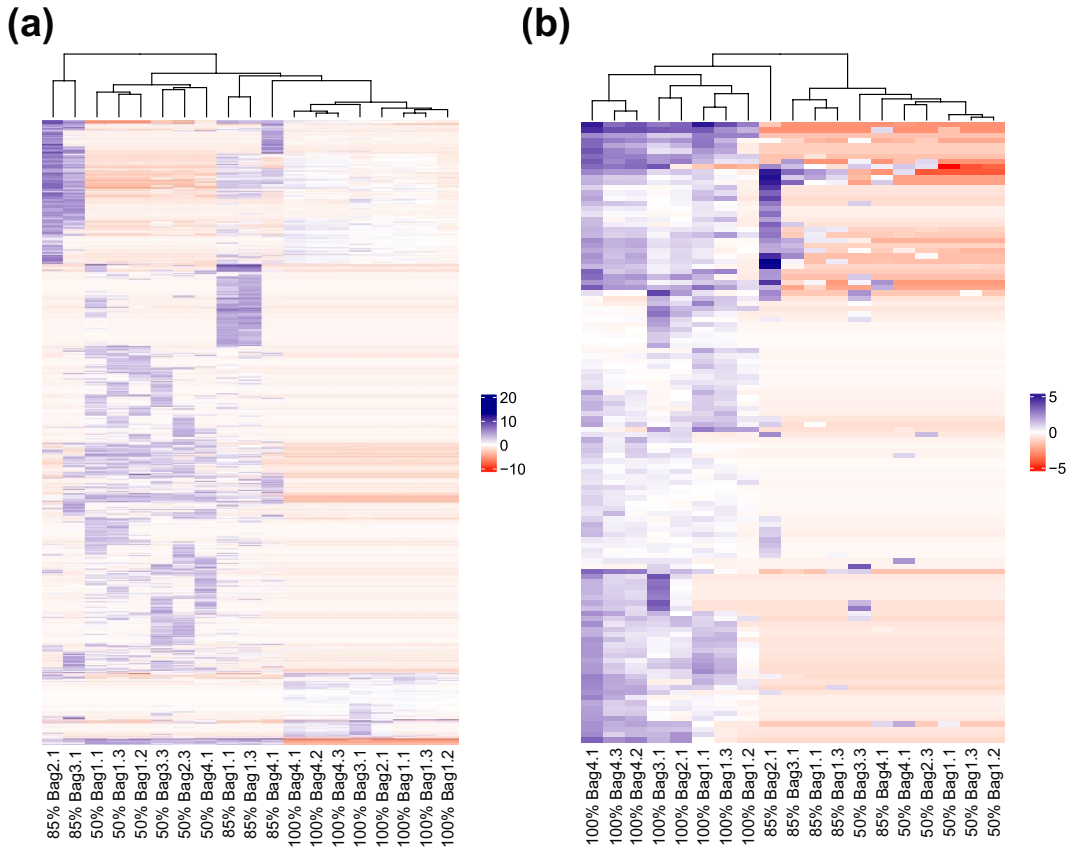

Fig. 3. Expression heat map of (**a**) all differentially expressed genes and (**b**) genes associated with secondary metabolism based on GO annotation (GO:0019748, GO:0044550). Color values are based on the TMM-normalized CPM expression value for each gene, where purple indicates high expression and red indicates low expression. The dendrogram is only plotted for the samples based on Euclidean distances and complete linkage method.

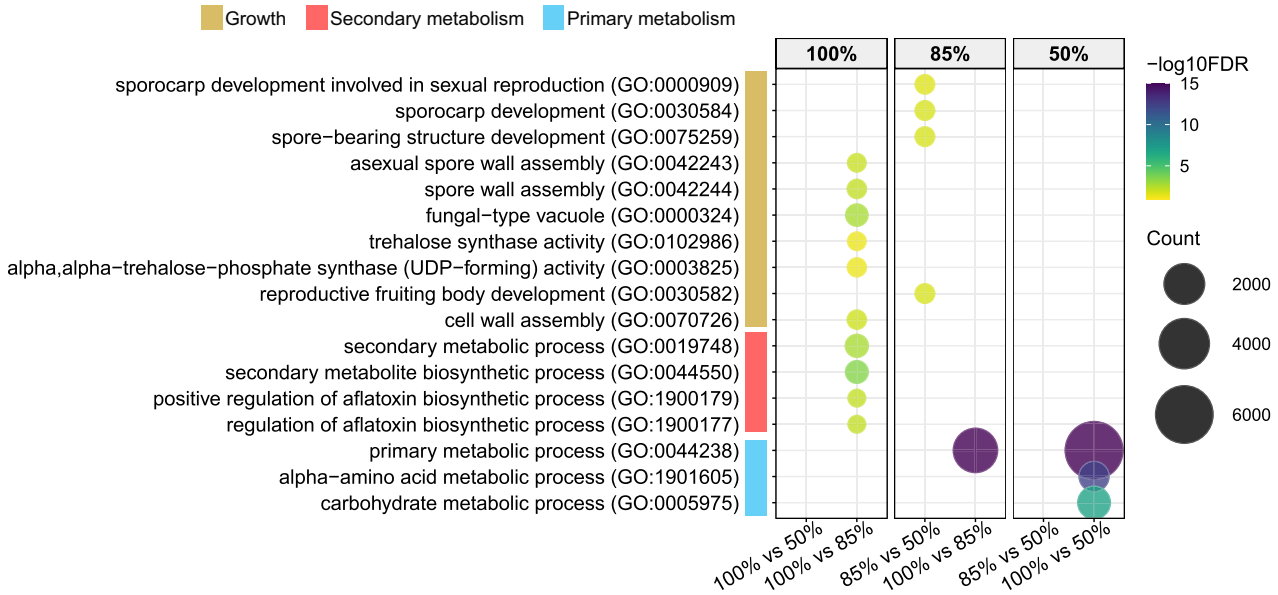

Fig. 4. Bubble plot of representative GO terms associated with Growth, Primary and Secondary metabolic processes enriched at each ERH condition. Growth, Primary and Secondary metabolic processes are represented by the color bars next to GO terms. Bubble sizes represent the number of upregulated genes in that GO category for a specific ERH comparison. Bubble color values are based on the significance (−log10(FDR-adjusted *p*)) of the GO term enrichment. Darker colors of bubbles represent higher significance of the GO term. All enriched GO terms for pairwise ERH comparisons are provided in Supplementary Table S5.

metabolism likely occurs at all moisture conditions, fungal growth and secondary metabolic processes occur in addition to primary metabolism when moisture conditions increase to elevated ERH conditions.

Evidence of increased expression of genes associated with health effects at higher moisture conditions

The gene expression of allergens and toxins were investigated to examine possible effects on human health for future investigations to consider. We found that fungal allergen annotated contigs were differentially expressed based on differences in moisture conditions (Fig. 5, Table 1). Here, samples cluster together by ERH condition, with most fungal allergens upregulated at 85% or 100% ERH compared to 50% ERH (Fig. 5). Several genes that annotated for allergens from the common mold *Aspergillus fumigatus* such as Asp f 4 and the Alt a 7 allergen from *Alternaria alternata* were upregulated at higher moisture conditions (log2FC > 2, Supplementary Table S7). Except for the Mal f 1 gene, the allergen associated with the commensal yeast Malassezia, all fungal allergens including those associated with Aspergillus and Alternaria were upregulated at 85% and 100% ERH and not at the low 50% ERH (Fig. 5). Additionally, genes for several food allergens including olive pollen allergen Ole e 10 and rice allergen Ory s aA_TI were present in the transcriptome but not examined further here.

Several contigs associated with fungal toxins have upregulated expression (log2FC > 2) at the 100% moisture condition (Fig. 6, Table 2, Supplementary Table S8). GO terms related to aflatoxin biosynthetic processes (GO:1900177, GO:1900179) were upregulated at 100% ERH (compared to 85% ERH). Mycotoxin biosynthetic processes such as fumonisin and gliotoxin biosynthesis (GO:1900541 and GO:2001310) were expressed in the transcriptome (Table 2). There were also additional toxin annotations present in the transcriptome that were not differentially expressed.

Discussion

The ISS is home to many different microbial species that interact in a dynamic relationship with astronauts, plants, and systems aboard the spacecraft[27]. This study uniquely studies fungal community gene expression in dust collected from the ISS using metatranscriptomics. Our results improve our knowledge of how microbial function may change under different moisture conditions in spacecraft to impact human health, spacecraft integrity, and planetary protection. Gene expression profiles of microbial communities in dust from the ISS were significantly partitioned by the three ERH conditions. When comparing differential expression patterns, samples consistently grouped according to chamber incubation moisture conditions compared to sampling bag. Additionally, genes associated with potentially negative human health effects are generally expressed at higher moisture conditions, which should be evaluated in future studies. We recognize that in the metatranscriptomes of mixed microbial communities such as in dust, the difference in gene expression with changes in ERH would be due to the combined effects of changes in gene expression of each gene in each microbial cell and the changes in community composition. Even though species composition does not seem to be as influenced by ERH as much as gene expression here, species composition likely influences overall gene expression[24]. Capturing such

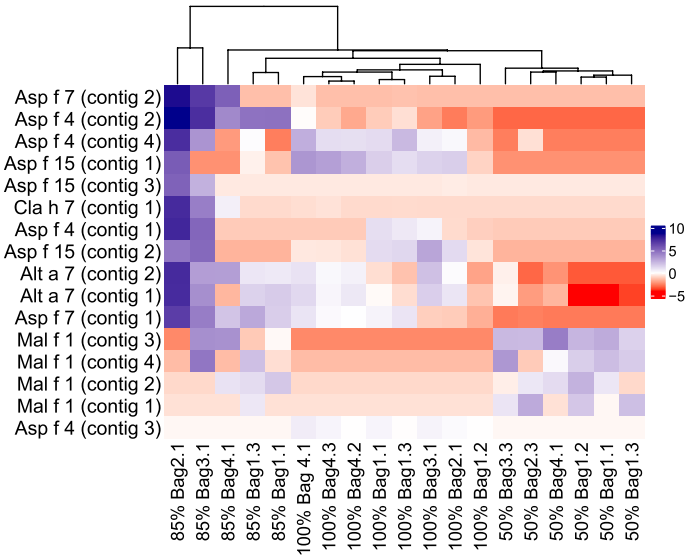

Fig. 5. Heatmap of differentially expressed genes with a fungal allergen annotation. Since multiple contigs had the same allergen annotation, they are shown as numbered separately. Columns represent samples and rows represent genes. Columns and rows are clustered by hierarchical clustering. Color values are based on the mean-centered and log2 transformed TMM-normalized CPM expression counts value for each gene, where purple indicates high expression and red indicates low expression. Gene annotations provided were extracted from BLAST annotations and are provided along with log2FC values in Supplementary Table S7.

Allergen	Organism	Type	Contig	Upregulation at ERH condition
Asp f 7	*Aspergillus fumigatus*	Mold	contig 1	Upregulated at 100% (vs 50%) and 85% (vs 50%)
			contig 2	Upregulated at 85% (vs 100%) and 85% (vs 50%)
Asp f 4	*Aspergillus fumigatus*	Mold	contig 1	Upregulated at 85% (vs 50%)
			contig 2	Upregulated a 85% (vs 50%), 100% (vs 50%) and 85% (vs 100%)
			contig 3	Upregulated at 100% (vs 85%)
			contig 4	Upregulated at 100% (vs 50%)
Asp f 15	*Aspergillus fumigatus*	Mold	contig 1	Upregulated at 100% (vs 50%) and 100% (vs 85%)
			contig 2	Upregulated at 85% (vs 50%) and 100% (vs 50%)
			contig 3	Upregulated at 85% (vs 50%)
Alt a 7	*Alternaria alternata*	Mold	contig 1	Upregulated at 100% (vs 50%)
			contig 2	Upregulated at 100% (vs 50%)
Mal f 1	*Malassezia furfur*	Commensal yeast	contig 1	Upregulated at 50% (vs 100%)
			contig 2	Upregulated at 50% (vs 100%)
			contig 3	Upregulated at 50% (vs 100%)
			contig 4	Upregulated at 50% (vs 100%)
Cla h 7	*Cladosporium herbarum*	Mold	contig 1	Upregulated at 85% (vs 50%)

Table 1. Fungal allergens expressed in the transcriptome and their differential upregulation.

combined system-level functional responses through metatranscriptomics can potentially be a more consistent measure of microbial growth of a mixed and dynamic community such as in house dust[14,16].

NASA uses a robust monitoring program in efforts to eliminate or mitigate the negative effects of microbes in spacecraft[7]. There are acceptable microbial concentration limits in place for food, water, air, surfaces, and crew members[28,29]. Maximum concentrations of bacteria and fungi allowed in potable water, the air, or on surfaces in the living and working areas on the ISS are internationally defined[30]. The standards are scored based on the maximum total number of aerobic and heterotrophic viable cells of bacteria or fungi counted as colony forming units (CFUs) on culture medium[30]. These standards are restricted to measuring only viable cells. Dormant microbial communities can activate after moisture conditions reach a certain threshold[10]. This make it critical to control the moisture levels in spacecraft, which are carefully controlled on the ISS by a combination of ventilation and a temperature-humidity controller (THC)[31–33]. The elevated relative humidity conditions in this study (85% and 100% ERH) are higher than typical conditions would be expected in spacecraft. Thus, the results here mimic an unexpected ventilation system or other moisture event.

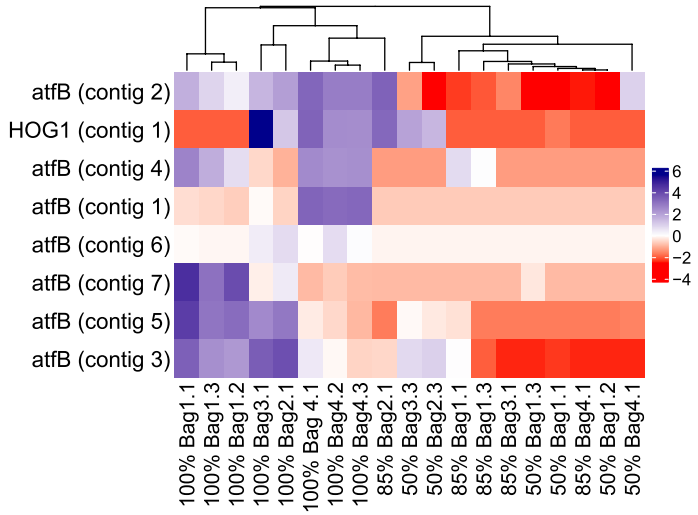

Fig. 6. Heatmap of differentially expressed genes associated with aflatoxin related processes: regulation of aflatoxin biosynthetic process and positive regulation of aflatoxin biosynthetic process. Columns represent samples and rows represent gene annotations. Columns and rows are clustered using hierarchical clustering. Color values are based on the mean-centered and log2 transformed TMM-normalized CPM expression counts value for each gene, where purple indicates high expression and red indicates low expression. The differential upregulation of fungal allergen contigs are also shown in Table 1. Gene annotations provided were extracted from BLAST annotations and are provided along with log2FC values in Supplementary Table S8.

GO ID	GO category	GO term	Expression
GO:1900177	Biological process	Regulation of aflatoxin biosynthetic process	Upregulated at 100% (100% vs 85%)
GO:1900179	Biological process	Positive regulation of aflatoxin biosynthetic process	Upregulated at 100% (100% vs 85%)
GO:0045461	Biological process	Sterigmatocystin biosynthetic process	Expressed in transcriptome
GO:1900541	Biological process	Fumonisin biosynthetic process	Expressed in transcriptome
GO:2001310	Biological process	Gliotoxin biosynthetic process	Expressed in transcriptome
GO:0045122	Biological process	Aflatoxin biosynthetic process	Expressed in transcriptome
GO:0043386	Biological process	Mycotoxin biosynthetic process	Expressed in transcriptome

Table 2. Fungal toxin processes present in the transcriptome and differentially upregulated.

Prevention of microbial problems is preferred over mitigation of problems during flight[34]. Using both molecular-based microbial monitoring technologies[35] and culture-based techniques as complementary methods can provide comprehensive identification of microbial communities while also capturing viability. During the time needed for analysis, microbes may become more difficult to control. Future microbial monitoring standards might incorporate improved predictive modeling of growth from moisture conditions which can be more easily and quickly assessed allowing for crewmembers to rapidly act to prevent microbial growth from occurring[24].

ERH was the more important indicator of gene expression activity compared to vacuum bag, which has important implications for spacecraft monitoring systems. Over approximately a year during which samples were collected for this study, microbial activity remained steady. A spacecraft monitoring system based on microbial activity can establish a baseline level of activity among microbes present in dust. Fluctuations from this baseline could be quickly identified indicating the potential for microbial growth. In a well-confined specialized environment such as a spacecraft cabin, early detection of a microbial growth problem can prevent unwanted exposures for crewmembers and protection of spacecraft materials.

Relative humidity on spacecraft such as the ISS is carefully controlled[32,33], and the results here highlight the need to continue to appropriately control moisture. Microbes found in dust from the ISS, especially at higher relative humidities, express genes implicated in allergens, toxins, and pathogenicity, which demonstrates a potential for increased human health impacts from microbial exposure, especially for those with compromised immune systems. The results are similar to dust from homes on Earth exposed to excess moisture. The expression of allergens and toxins were also expressed in microbes incubated under increased moisture conditions on Earth[15]. Preventing an increase in moisture conditions in spacecraft will reduce the potential for unwanted health effects associated with increased production of allergens and toxins.

Dust is an important source for human microbial exposure on Earth due to resuspension; however, this resuspension does not occur in space the same way it does on Earth. The ISS environment in orbit is a continuous state of microgravity, in which larger particles do not settle out of the air as they would on Earth, with

particles > 100um remaining airborne[36]. Dust can also become liberated when crew members vacuum the debris from the air filters. The increase in airborne particles is short-lived as the filtration system quickly removes particles from the air. Vacuuming on the ISS occurs every week, and sometimes more frequently depending on the location. Particles of varying sizes on the ISS have different fungal and bacterial communities[9] and astronauts may encounter dust particles during activities such as vacuuming or during hardware installation. Exposure to these microbial communities present in dust may impact astronaut health and is an important factor to consider when designing spacecraft life support systems.

When moisture conditions were elevated, secondary metabolic activity occurred in addition to primary metabolic function in dust from the ISS. Secondary metabolic processes involve functions that go beyond processes that are required for growth and maintenance of cells and can involve the production of certain toxins or the utilization of unusual nutrients. Melanin metabolic process was upregulated at 100% ERH compared to 50% ERH. Melanin is a ubiquitous compound found in many microbes and its structure makes it a very stable molecule, resistant to a variety of destructive physicochemical processes such as radiation[37,38]. Spacecraft environments experience increased ionizing radiation levels, but levels observed on the ISS are not fungicidal[37–39]. Instead, melanized fungal species respond to radiation with enhanced growth and become radioresistant when exposed to high doses of ionizing radiation under experimental conditions[40]. The upregulation of genes associated with melanin metabolism that we observed in fungi at increased moisture conditions is not unexpected in our samples. One study found that the viability of melanized yeast post-flight was 50% higher than non-melanized yeasts, while no difference was observed for the Earth samples, suggesting that fungal melanin could potentially increase the lifespan of biological assets in space[41]. This process allows fungi to thrive provided humidity levels are sufficient and many microbes aboard the ISS have been found to be pigmented[38]. This suggests the usefulness of pigments such as melanin found in microbes under extreme conditions such as those on spacecraft.

Limitations

This study has several limitations. It is unknown how transporting the dust back to Earth impacted our gene expression results. Additionally, elevated moisture conditions were simulated on Earth. Growth and microbial functional changes in response to moisture may be impacted in unknown ways when it occurs under spacecraft conditions. We did not have control samples representing indoor environments on Earth to state if gene expression changes were solely due to the effect of moisture or if there were additional effects associated with spacecraft specific conditions such as altered gravity, radiation or other unique spacecraft variables. Future work should also attempt to replicate spacecraft conditions on Earth to better study microbial activity in this specialized environment. Additionally, it is challenging to study fungal metatranscriptomics due to the lack of fully annotated fungal genomes[42] as many contigs can only be identified as the closest homolog present in current databases. Genes are often present in multiple pathways and can be conserved across several taxa. For instance, we noted that for allergen annotated contigs, more than one contig had the same annotation, indicating the lack of accurate and complete fungal genome annotations in addition to potentially insufficient sequencing depth. We also noted that all enriched GO terms in our study may not have been associated with fungal genes. For instance, the term "secondary metabolite catabolic process" that is enriched at 85% ERH compared to 100%, contains no genes with a fungal annotation. In addition, some contigs were not annotated due to no sufficiently similar homologs existing in fungal genome databases. As fungal genomics advances, new insights may be mined from this data, deepening our understanding of how fungal gene expression varies with humidity.

Conclusions

Metabolic activity of microbial communities found in dust on the ISS incubated under elevated relative humidity conditions is impacted more by moisture conditions. Increased potential for negative health effects from the production of allergens and toxins can be avoided by maintaining proper control of moisture on spacecraft, as is already done on the ISS. Understanding how microbes react to increased moisture conditions will help us develop more robust microbial monitoring standards for spacecraft and other specialized indoor environments. Future work will continue to consider specific microbial activity in dust from space environments, including those from the lunar and Martian surface vehicles and habitats. As humans extend their reach farther into space, we will continue to interact with microbes and moisture conditions which are inherent to life itself. Fully understanding microbial activity in these specialized environments will help us design spacecraft prioritizing human health, the integrity of the built environment, and planetary protection.

Methods

Dust collection and incubation chamber experiments

Dust was collected via vacuuming from the HEPA filter screens installed in the air circulation system located within the United States' Segment aboard the ISS. During their regularly scheduled cleaning routine, crewmembers removed the debris from vents and HEPA filter screens throughout the ISS and it was collected in vacuum bags. This study analyzed dust from four different vacuum bags that were sealed and returned to Earth. These bags were returned over a period of 12 months in 2019 and 2020 on SpaceX Commercial Resupply Services (CRS) missions from the ISS. Bag one was returned via the SpaceX CRS-16 payload which splashed down January 13, 2019. Bag two returned via SpaceX CRS-17 on July 7, 2019. Bags three and four returned via SpaceX CRS-19 January 7, 2020. Dust was stored at room temperature under ambient conditions until ready for incubation.

The dust was removed from the vacuum bag in a biosafety cabinet to prevent contamination and 25 mg portions of dust were incubated in sterile glass chambers at 25 °C and either 50%, 85%, or 100% ERH. Different concentrations of salt solutions (NaCl above water activity of 0.76 and $MgCl_2$ below) were used to vary the

relative humidity[13]. Water activity of salt solutions was verified with an Aqualab 4TE water activity meter (Decagon Devices, Pullman, WA, USA). Samples were incubated in the chambers for one week at each ERH condition, after which RNA was immediately extracted to prevent degradation.

Based on the computational capabilities at the time, we estimated that we could process up to 20 samples in our bioinformatics pipeline. We included at least one sample from each ERH condition from each bag. We also included triplicate samples from all of Bag 1 and the 100% ERH condition samples of Bag 4. Samples sequenced from each vacuum bag and their incubated moisture condition are provided in Supplementary Table S1.

RNA extraction and sequencing

RNA was extracted from the dust samples using the Qiagen RNeasy Powermicrobiome extraction kit procedure (Qiagen, Hilden, Germany) modified to use 10 × the recommended beta-mercaptoethanol[15] and 70% ethanol instead of PM4 during the PM3 and PM4 addition to account for RNA degradation per the extraction kit protocol. A Tapestation analysis was conducted on all the samples at The Genomics Shared Resource Center (The Ohio State University Comprehensive Cancer Center Shared Resources, Columbus, OH) using a Eukaryotic total High Sensitivity RNA ScreenTape system (Agilent Technologies, Santa Clara, CA) to check the quality and integrity of samples.

RNA extracts were then sent to the Yale Center for Genomic Analysis (Yale University, New Haven, CT, USA) where they were reverse transcribed and then sequenced on a NovaSeq 6000 (2 × 100 lane) with 25 million reads per sample. RNASeq library preparation was performed using the NEB Next Single Cell/Low Input RNA Library Prep Kit (New England Biolabs, USA) and the NEB Ultra II FS (New England Biolabs, USA) workflow for Illumina. The polyA selection protocol was used to select for eukaryotic mRNA. Sequence data was submitted to NASA's GeneLab database under accession number GLDS-631[43].

After the library preparation step, one of the 85% ERH samples (Bag 1 Sample 2, incubated at 85% ERH, Supplementary Table S1) was excluded from further analysis based on the D1000 ScreenTape results (Agilent Technologies, Santa Clara, CA) of library indexed cDNA. This sample had a lower concentration of indexed cDNA (4.5 ng/ul) compared to the concentrations for the other two 85% ERH sample replicates (15.0 ng/ul and 14.1 ng/ul, respectively), potentially caused due to variability during RNA extractions. This can translate to a lower number of quality reads after sequencing and could potentially confound the results of the differential gene expression analysis. Thus, a total of 19 samples were included in the final analysis.

Initial processing and transcriptome assembly

Processing followed previously established protocols[15,44]. Quality assessment of sequences was determined using FastQC (v0.11.8)[45,46]. Initial processing involved the removal of erroneous k-mers created due to sequencing errors using the tool rCorrector (v1.0.4), a kmer-based error correction method designed for RNA-seq data[47]. rCorrector also identifies reads that contained errors but were deemed unfixable after correction. These unfixable reads were removed using the script FilterUncorrectablePEfastq.py from the TranscriptomeAssemblyTools package[47].

De novo metranscriptome assembly was carried out using the default Trinity (v2.12.0) settings run on the Ohio Super Computer (OSC)[48]. Poor quality reads and all contigs with a length less than 300 base pairs (bp) were removed using Trimmomatic within the Trinity pipeline[49]. One complete assembly was constructed from all sequenced samples. Contigs from the Trinity assembly were clustered using CD-HIT-EST (v4.8.1) which clustered contigs based on 80% sequence similarity[50,51]. CD-HIT-EST identifies representative sequences that meet the set similarity threshold, and these clusters represent all expressed contigs that make up the full transcriptome. A detailed table of parameters used in this pipeline are listed in Supplementary Table S2.

Transcript quantification

The alignment-based quantification method RSEM (v1.3.3) was used to estimate transcript abundance in each sample[52]. The quality trimmed reads from each sample in which both the forward and reverse reads were retained after Trimmomatic were mapped back to the full transcriptome created using CD-HIT-EST. This was performed utilizing Bowtie2 (v2.3.4.3)[53] to determine transcript-level estimates of the count of RNA-Seq fragments that were derived from each transcript. A normalized measure of transcript expression that accounts for transcript length, the number of reads mapped to the transcript and the total number of reads that mapped to any transcript was also derived. Gene-level abundance estimates were used to construct a matrix of counts and a matrix of normalized expression values. Relationships between biological replicates were compared for both assemblies.

Differential expression analysis

Differentially expressed genes and clustering of genes according to expression profiles was performed within the Trinity pipeline leveraging DESeq2 (v1.46.0)[54]. Pairwise comparisons among each ERH condition were performed. Genes that were most differentially expressed based on the most significant False Discovery rate (FDR) adjusted p-values and fold-changes were extracted and clustered according to their patterns of differential expression across samples. Differentially expressed genes that had a FDR-adjusted $p < 0.05$ and $\log2FC \geq 2$, were examined for further analysis.

Functional annotation and GO term enrichment analysis

Contigs from CD-HIT-EST were annotated using Trinotate (v3.2.2)[55], a comprehensive annotation suite designed for automatic functional annotation of de novo assembled transcriptomes. Trinotate depends on inputs from both Trinity as well as TransDecoder (v.5.5.0)[56]. TransDecoder identifies candidate coding regions within transcript sequences utilizing a minimum length open reading frame (ORF) found in a transcript sequence.

All ORFs were scanned for homology to known proteins utilizing a BLAST + [57] search against Swiss-Prot[58], and searching PFAM (HMMER v3.3.2)[59] to identify common protein domains. A Trinotate annotation report was generated utilizing an SQLite database[60] and produced Gene Ontology (GO)[61] terms, KEGG ontology (KO) terms[62]. KEGG assignments for genes were visualized using the iPath3 tool[63] as metabolic pathway maps. BLAST (BlastX or BlastP) annotations were used to identify fungal annotations of genes.

Differentially expressed genes that had an FDR-adjusted $p < 0.05$ and log2FC ≥ 2, in each pairwise ERH comparison were used for GO term enrichment. GO term enrichment finds categories that are overrepresented amongst the differentially expressed genes within a pairwise ERH comparison[64]. Utilizing Goseq (v1.58.0)[64] within Trinity, each of the pairwise differential expression analysis results were analyzed for enriched GO categories for the genes that were upregulated for each pairwise ERH comparison. This meant that there were 6 gene lists that GO enrichment was performed on: genes upregulated at (1) 100% ERH upregulated vs 50%, 100% ERH vs 85% (2) 85% ERH vs 50% (3) 85% ERH vs 100% (4) 50% ERH vs 100% (5) 50% ERH vs 85%. We identified GO terms that were more prevalent under increased moisture conditions and GO terms potentially associated with fungal growth and secondary metabolic processes. In contrast to primary metabolism, which relates to the basic metabolic processes necessary for life, secondary metabolism constitutes the reactions and pathways that are not necessarily required for growth and maintenance of cells[15,65]. Secondary metabolic processes are those that include the production of certain harmful toxins, including mycotoxins and the utilization of unusual nutrients[65,66].

Statistical analysis

The statistical analysis software R (v.4.2.2)[67] was used to perform statistical testing. To compare gene expression profiles based on moisture condition, Principal Component Analysis (PCA) was performed. Gene expression values in Counts Per Million (CPM) that were $\log_2$ transformed and row mean-centered standardization were used prior to analysis to limit bias towards highly expressed transcripts[54,68]. As PCA employs Euclidean distances of the data, the adonis2 function in R using the *vegan* package (v.2.6.4)[69] was used to determine statistical significance of ERH groupings ($p < 0.05$) using the Euclidean distance matrix of the gene expression data. The adonis2 test employed 10,000 permutations and we used the "p.adjust" function with the "BH" option to correct for False Discovery Rate (FDR)[70] to adjust for multiple comparisons. Significance was defined at FDR-adjusted $p < 0.05$[71]. A 95% confidence ellipse using the stat_ellipse function within the *ggplot2* package (v.3.4.3)[72] was created to compare moisture conditions to each other.

The Spearman rank correlation coefficient was calculated using the *cor* function using a Trinity generated R script for differentially expressed genes based on moisture condition. The Spearman rank correlation coefficient determines the strength and direction in the relationship between the data where a value of + 1 indicates the strongest positive correlation and − 1 indicating the highest negative correlation.

Gene expression heatmaps were plotted using the ComplexHeatmap[73] package in R, based on TMM-normalized (Trimmed Mean of M-values normalized) expression values to examine gene expression across samples[74]. Log_2 transformed and row mean-centered standardization were performed prior to analysis to limit bias towards highly expressed transcripts[54,68]. Adobe Illustrator (v.28.3) (http://www.adobe.com/au/products/illustrator.html) and Inkscape (v.1.3) (https://inkscape.org/) were used for finalizing figures.

Data availability

The sequencing data that supports the findings of this study are available in NASA's Open Science Data Repository (OSDR), also known as GeneLab, with the identifier https://doi.org/10.26030/60zg-2n28 (GLDS-631) and at https://osdr.nasa.gov/bio/repo/data/studies/OSD-701. R scripts used for analysis and visualizations, including the figures generated are available in GitHub (https://github.com/n-bsub/Gene-Expr_ISS).

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Author contributions

KCD conceived the idea and oversaw the project. KCD, MEM, and JMH obtained funding. NN collected and processed the samples. NB performed the bioinformatics, data analysis and visualizations on the RNA data. BH helped develop the RNA data processing pipeline. BH and SRH consulted on the bioinformatics analysis. NB, SRH and KCD wrote the initial version of the manuscript. NN, MEM and JMH reviewed and edited the final draft. All authors reviewed, edited, and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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