## A microbial survey of the International Space Station (ISS)

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Background: Modern advances in sequencing technology have enabled the census of microbial members of many natural ecosystems. Recently, attention is increasingly being paid to the microbial residents of human-made, built ecosystems, both private (homes) and public (subways, office buildings, and hospitals). Here, we report results of the characterization of the microbial ecology of a singular built environment, the International Space Station (ISS). This ISS sampling involved the collection and microbial analysis (via 16S rDNA PCR) of 15 surfaces sampled by swabs onboard the ISS. This sampling was a component of Project MERCCURI (Microbial Ecology Research Combining Citizen and University Researchers on ISS). Learning more about the microbial inhabitants of the "buildings" in which we travel through space will take on increasing importance, as plans for human exploration continue, with the possibility of colonization of other planets and moons.

Results: Sterile swabs were used to sample 15 surfaces onboard the ISS. The sites sampled were designed to be analogous to samples collected for 1) the Wildlife of Our Homes project and 2) a study of cell phones and shoes that were concurrently being collected for another component of Project MERCCURI. Sequencing of the 16S rDNA genes amplified from DNA extracted from each swab was used to produce a census of the microbes present on each surface sampled. We compared the microbes found on the ISS swabs to those from both homes on Earth and data from the Human Microbiome Project.

Conclusions: While significantly different from homes on Earth and the Human Microbiome Project samples analyzed here, the microbial community composition on the ISS was more similar to home surfaces than to the human microbiome samples. The ISS surfaces are species-rich with 1036-4294 operational taxonomic units (OTUs per sample). There was no discernible biogeography of microbes on the 15 ISS surfaces, although this may be a reflection of the small sample size we were able to obtain.

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### **A microbial survey of the International Space Station (ISS)**

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### 20 Abstract

- 21 Background: Modern advances in sequencing technology have enabled the census of
- 22 microbial members of many natural ecosystems. Recently, attention is increasingly being
- 23 paid to the microbial residents of human-made, built ecosystems, both private (homes) and
- 24 public (subways, office buildings, and hospitals). Here, we report results of the
- 25 characterization of the microbial ecology of a singular built environment, the International
- 26 Space Station (ISS). This ISS sampling involved the collection and microbial analysis (via
- 27 16S rDNA PCR) of 15 surfaces sampled by swabs onboard the ISS. This sampling was a
- 28 component of Project MERCCURI (Microbial Ecology Research Combining Citizen and
- 29 University Researchers on ISS). Learning more about the microbial inhabitants of the
- 30 "buildings" in which we travel through space will take on increasing importance, as plans



- 31 for human exploration continue, with the possibility of colonization of other planets and
- 32 moons.
- 33 Results: Sterile swabs were used to sample 15 surfaces onboard the ISS. The sites sampled
- 34 were designed to be analogous to samples collected for 1) the Wildlife of Our Homes
- 35 project and 2) a study of cell phones and shoes that were concurrently being collected for
- 36 another component of Project MERCCURI. Sequencing of the 16S rDNA genes amplified
- 37 from DNA extracted from each swab was used to produce a census of the microbes present
- 38 on each surface sampled. We compared the microbes found on the ISS swabs to those from
- 39 both homes on Earth and data from the Human Microbiome Project.
- 40 Conclusions: While significantly different from homes on Earth and the Human Microbiome
- 41 Project samples analyzed here, the microbial community composition on the ISS was more
- 42 similar to home surfaces than to the human microbiome samples. The ISS surfaces are
- 43 species-rich with 1036-4294 operational taxonomic units (OTUs per sample). There was no
- 44 discernible biogeography of microbes on the 15 ISS surfaces, although this may be a
- 45 reflection of the small sample size we were able to obtain.

#### 46 Introduction

- 47 There is a growing appreciation of the importance of microbial communities found in
- 48 diverse environments from the oceans, to soil, to the insides and outsides of plants and
- 49 animals. Recently, there has been an expanding focus on the microbial ecology of the "built
- 50 environment" human constructed entities like buildings, cars, and trains places where
- 51 humans spend a large fraction of their time. One relatively unexplored type of built
- 52 environment is that found in space. As humans expand their reach into the solar system,
- 53 with renewed interest in space travel, and with the possibility of the colonization of other
- 54 planets and moons, it is of critical importance to understand the microbial ecology of the
- 55 built environments being utilized for such endeavors.
- 56 Interest in the microbial occupants of spacecraft long precedes the launch of the
- 57 International Space Station (ISS) (Trexler 1964)(Silverman 1971). Early work primarily
- 58 focused on ensuring that the surfaces of spacecraft were free of microbial contaminants in
- an effort to avoid inadvertent panspermia (seeding other planets with microbes from
- 60 Earth) (Pierson 2007). Work on human-occupied spacecraft such as Mir, Space Shuttles,
- and Skylab focused more on microbes with possible human health effects. With the launch
- 62 of the ISS, it was understood that this new built environment would be permanently
- 63 housing microbes as well as humans. Calls were made for a better understanding of
- 64 microbial ecology and human-microbe interactions during extended stays in space
- 65 (Pierson 2007) (Roberts, Garland, and Mills 2004) (Ott, Bruce, and Pierson 2004). Efforts
- 66 were made to establish a baseline microbial census. For example, Novikova et al (Novikova
- et al. 2006) obtained more than 500 samples from the air, potable water, and surfaces of
- 68 the ISS, over the course of 6 years.
- 69 These early studies were unavoidably limited by their reliance on culturing to identify
- 70 microbial species. Culture-independent approaches were eventually implemented,

- 71 including some small-scale 16S rDNA PCR surveys (Castro et al. 2004), and the Lab-On-a-
- 72 Chip Application Development Portable Test System (LOCAD-PTS) (Maule et al. 2009),
- 73 which allows astronauts to test surfaces for lipopolysaccharide (LPS a marker for Gram
- 74 negative bacteria). Originally launched in 2006, the capability of the LOCAD-PTS was
- 75 expanded in 2009 to include an assay for fungi (beta-glucan, a fungal cell wall component)
- and Gram positive bacteria (lipoteichoic acid, a component of the cell wall of Gram positive
  bacteria.) The first large-scale, culture-independent 16S rDNA PCR survey was published
- only in 2014 using the Roche 454 platform (pyrosequencing), looking at dust on the ISS
- 79 (Venkateswaran et al. 2014). A more recent study examined several samples collected on
- 80 the Japanese module of the ISS over a period of four years, also sequenced with
- 81 pyrosequencing (Ichijo et al. 2016). We report here on a further effort involving 16S rDNA
- 82 PCR and sequencing, using the Illumina platform, to examine the microbial communities
- 83 found on 15 surfaces inside the ISS. The advantage of Illumina sequencing, relative to
- 84 previous pyrosequencing efforts, is the significant increase in depth of sequencing. This
- 85 increased depth allowed us to analyze 15-20 times as many sequences as these earlier
- 86 studies.
- 87
- 88 The 15 surfaces sampled on the ISS were chosen by the Project MERCCURI team in an effort
- to make them analogous to 1) the surfaces sampled for the "Wildlife of Our Homes" project
- 90 (http://homes.yourwildlife.org) (Dunn et al. 2013) (Barberán et al. 2015), which asked
- 91 citizen scientists to swab nine surfaces in their homes, and 2) cell phone and shoe swab
- 92 samples that were also being collected via Project MERCCURI. The sample matching is
- 93 imperfect, for example doorsills were used in houses because they collect dust but in the
- 94 microgravity of the ISS, dust accumulates in air filters. The motivation for choosing the sites
- 95 in this way was both to increase public awareness of the microbiology of the built
- 96 environment, as well as to begin to compare the microbial ecology of homes on Earth with
- 97 the only current human home in space. We also present a comparison of the ISS swab
- 98 results with data from 13 human body sites sampled via the Human Microbiome Project.
- 99 This comparison was done to assess the potential human contribution to the microbial life
- 100 on the ISS.
- 101 We have also compiled a collection of papers on space microbiology in an online resource
- 102 to provide a more comprehensive historical perspective of this kind of work (see
- 103 http://www.mendeley.com/groups/844031/microbiology-of-the-built-
- 104 environment/papers/added/0/tag/space/).

### 105 Methods

#### 106 Surfaces swabbed:

107 Astronauts were asked to swab 15 surfaces on the International Space Station. Below are

108 their verbatim instructions.

109 1. Audio Terminal Unit (telephone) hand held push-to-talk microphone located in the 110 forward portion of the US Lab Module Audio Terminal Unit (telephone) hand held push-to-talk microphone located in the aft 111 2. 112 portion of the US Lab Module US Lab Robotic Work Station laptop PC keyboard used to control the robotic arm 113 3. 114 4. US Lab Robotic Work Station hand controller used to control the movement of the robotic arm 115 116 US Lab Robotic Work Station foothold, left side 5. 117 US Lab Robotic Work Station foothold, right side 6. One of the main laptop keyboards in the US Lab used to control science experiments 118 7. and the systems of the space station 119 120 8. One of the vertical handrails on the equipment racks inside the US Lab 121 9. Air vent in the front portion of the US Lab 122 10. Air vent in the aft portion of the US Lab 123 11. Air vent located on the right crew sleep compartment 124 12. Tab used to open, close, and secure the Nomex privacy panel located on the starboard crew sleep compartment 125 126 13. Air vent located on the port crew sleep compartment 14. Tab used to open, close, and secure the Nomex privacy panel located on the port crew 127 128 sleep compartment 129 15. Crew Choice Surface: Audio Terminal Unit (telephone) hand held push-to-talk microphone located in the starboard portion of the Harmony module (Node 2). 130 Swabbing instructions as given to astronauts: 131 Setup Node-2 Camcorder to capture NanoRacks surface swab Ops throughout the US 132 1. 133 LAB. 134 2. Retrieve a clean NanoRacks Swab Kit, Move to ISS location listed on NanoRacks Swab 135 Kit label. Remove cotton swab from NanoRacks Swab Kit, being careful not to touch the cotton 136 3. 137 swab tip to avoid contamination. Rub cotton swab vigorously against designated surface. Spin and turn the swab to 138 4. 139 ensure maximum sample collection.



140 5. Return cotton swab to NanoRacks Swab Kit and press to close (squeeze excess air out

- 141 of bag before sealing). Circle number of location swabbed and label with GMT
- 142 (dd/hh:mm). If swab is contaminated by touching a surface other than the designated
- 143 location on the label, Label NanoRacks Swab Kit with a large, "X" and move on to the
- 144 next location. Notify POIC of NanoRacks Swab Kit S/N that was contaminated
- 145 6. Repeat step 2 to step 6 for all 15 locations listed on the NanoRacks Swab Kit label.
- 146 NOTE: An additional large Ziplock Bag is provided (stowed inside the same bag as the
- 147 NanoRacks Swab Kits) to use per crew preference to separate the used NanoRacks Swab
- 148 Kits from the clean (unused) NanoRacks Swab Kits for crew efficiency during sampling.

#### 149 ISS Crew

- 150 Swabbing was conducted during Expedition 39
- 151 (http://www.nasa.gov/mission\_pages/station/expeditions/expedition39/index.html). The
- 152 crew included NASA astronauts Steve Swanson and Rick Mastracchio and Russian
- 153 cosmonauts Oleg Artemyev, Alexander Skvortsov, and Mikhail Tyurin. Japan Aerospace
- 154 Exploration Agency (JAXA) astronaut Koichi Wakata was the commander for this
- 155 expedition, and is the astronaut who performed the swabbing.

#### 156 Sampling site choice

- 157 These surfaces were chosen in an attempt to sample surfaces analogous to those sampled
- 158 in the pilot study for the Wildlife of Our Homes project (Dunn et al. 2013). For this study,
- 159 involving 40 homes, volunteers swabbed nine surfaces in their homes: kitchen cutting
- 160 board, kitchen counter, a shelf inside a refrigerator, toilet seat, pillowcase, exterior handle
- 161 of the main door into the house, television screen, the upper door trim on the outside
- 162 surface of an exterior door, and the upper door trim on an interior door. We were not
- 163 granted access to all corresponding surfaces aboard the ISS. The kitchen surfaces aboard
- the ISS are in the Russian module, which we did not have permission to access, swabbing
- 165 the toilet seat was deemed inappropriate due to biosafety concerns, and the exterior 166 surfaces are accessible only via an "Extra-vehicular Activity" (space walk), which was not
- 167 requested for this experiment. We also sought to collect samples that would be analogous
- 168 to the cell phone and shoe samples that were being obtained from thousands of Citizen
- 169 Scientists across the country in a different component of Project MERCCURI. A final
- 170 constraint was the limitation of only 15 swabs that was imposed by NASA, severely limiting
- 171 the number of replicates we could collect. See Table 1 for a list of the ISS sampling sites and
- to which Earth samples they were intended to be analogous.
- 173 Upon successful completion of the swabbing on May 9, 2014,
- 174 http://blogs.nasa.gov/stationreport/2014/05/09/iss-daily-summary-report-050914/, all
- 175 swabs were stored at -80 °C in the Minus Eighty-degree Laboratory Freezer for ISS (MELFI)
- 176 freezer onboard the ISS, until transfer to the SpaceX Dragon spacecraft. In the Dragon, the
- 177 swabs were stored at -80 °C in the General Laboratory Active Cryogenic ISS Experiment
- 178 Refrigerator (GLACIER), that runs off of Dragon's batteries until it is plugged in (either to
- the ISS or on the ground.) The Dragon re-entered the Earth's atmosphere and splashed

- 180 down in the Pacific Ocean at 12:05 pm PT on May 18, 2014. Samples were transferred to a
- 181 cooler with dry ice, and shipped to the Earth Microbiome Project (EMP) lab
- 182 (http://earthmicrobiome.org)(Gilbert et al. 2011).

#### **183 DNA Extraction and Library Preparation**

184 All samples were prepared using a modified version of the Mo BIO UltraClean®-htp 96

185 Well Swab DNA Kit (MO BIO). Samples were purified using the Zymo ZR-96 DNA Cleanup

- and Concentrator<sup>™</sup>-5 kit according to Zymo Protocol (Zymo). DNA was then amplified
- 187 using the EMP barcoded primer set, adapted for the Illumina HiSeq2000 and MiSeq by
- adding nine extra bases in the adapter region of the forward amplification primer that
- support paired-end sequencing. The V4 region of the 16S rRNA gene (515F-806R) was
   amplified with region-specific primers that included the Illumina flowcell adapter
- 190 amplified with region-specific primers that included the mumina howcen adapter 191 sequences and a twelve-base barcode sequence. Each 25 ul PCR reaction contained the
- following: 12 ul of PCR water certified DNA-free (MO BIO), 10 ul of 1x 5 Prime
- HotMasterMix (5 Prime), 1 ul of Forward Primer (5 uM concentration, 200 pM final), 1 ul of
- Golay Barcode Tagged Reverse Primer (5 uM concentration, 200 pM final), and 1 ul of
- 195 template DNA. The conditions for PCR were as follows: 94°C for 3 minutes to denature the
- 196 DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, with a final
- 197 extension of 10 min at 72 °C to ensure complete amplification. Amplicons were quantified
- 198 using PicoGreen (Invitrogen) and a plate reader. Once quantified, different volumes of each
- 199 of the products were pooled into a single tube so that each amplicon was represented
- 200 equally. This pool was then cleaned up using UltraClean® PCR Clean-Up Kit (MO BIO), and
- 201 then quantified using Qubit (Invitrogen). Sequencing of the prepared library was
- 202 performed on the Illumina MiSeq platform, using the sequencing primers and procedures
- 203 described in the supplementary methods of (Caporaso 2012).

### 204 **Bioinformatic Analysis**

- 205 Unless otherwise noted, all microbial community analyses were conducted using the QIIME
- 206 workflow version 1.8 or R (R-project 2014). All python scripts referred to are components
- 207 of QIIME (Caporaso et al. 2010).
- 208 Demultiplex and QC: An in-house script was used to assign sequences to samples, using
- 209 dual-index barcoding. This script is available on github
- 210 (https://github.com/gjospin/Demul\_trim\_prep). This script allows for 1 base pair
- 211 difference per barcode. The paired reads were then aligned and a consensus was computed
- using FLASH (Magoc and Salzberg 2011) with maximum overlap of 120 and a minimum
- 213 overlap of 70 (other parameters were left as default). The custom script automatically
- demultiplexes the data into fastq files, executes FLASH, and parses its results to reformat
- 215 the sequences with appropriate naming conventions for QIIME v. 1.8.0 in fasta format.
- 216 OTU assignment and QC: Chimeric sequences were identified using usearch61 as
- 217 implemented in the identify\_chimeric\_seqs.py script, resulting in the removal of 8760
- 218 sequences. The pick\_open\_reference\_otus.py script was used to cluster sequences at 97%
- 219 similarity to generate OTUs (Operational Taxonomic Units, a proxy for species). Taxonomy
- 220 was assigned to each OTU by comparing a representative sequence from each cluster to the

- 221 gg\_13\_8\_otus reference taxonomy provided by the Greengenes Database Consortium
- 222 (http://greengenes.secondgenome.com) (McDonald et al. 2011). OTUs that were classified
- as chloroplasts or mitochondria were removed from further analysis. The number of high-
- 224 quality sequences remaining per sample ranged from 26831 to 77843 (see Table 1). All
- 225 subsequent beta diversity analyses (comparisons across samples) were performed with all
- 226 samples rarefied to 26830 sequences.

#### 227 Comparison of ISS surfaces to analogous surfaces in homes on Earth and to the

- 228 Human Microbiome Project
- 229 The sequences and associated metadata from a 40-home pilot study for the Wildlife of Our
- 230 Homes Project are available for download from Figshare (Menninger; 2013). We also
- obtained 100 samples from each of 13 body sites from the HMP Data Portal
- 232 (http://hmpdacc.org/HM16STR/)(Huttenhower et al. 2012)(Gevers et al. 2012). These two
- additional datasets were used in a combined analysis with the ISS sequences presented
- here. Because the sequences from the three projects are not all the same lengths, each
- 235 dataset was independently analyzed using a closed-reference OTU-picking approach, with a
- 236 97% similarity cutoff, and the resultant biom tables were merged with the
- 237 merge\_otu\_tables.py script. While the closed-reference approach will miss any novel taxa,
- this was required since both of our comparison datasets were analyzed this way. To
- account for uneven sampling depth, all samples in the combined analysis were rarefied to
- 240 1000 sequences. Shannon diversity, as well as non-metric multidimensional scaling
- 241 (NMDS) based on Bray-Curtis (Bray and Curtis 1957) and Unweighted Unifrac (Lozupone
- and Knight 2005) distances were computed and plotted using Phyloseq (McMurdie and
- Holmes 2013) and the ggplot2 (Wilkinson 2011) packages in R (R-project 2014).

### 244 Comparison to rooms with mechanical ventilation or open windows.

- 245 We obtained a list of human pathogens, compiled by Kembel et al, 2012 from the author.
- 246 We then used BLAST (Altschul et al. 1990) to search a representative sequence from each
- of the ISS OTUs against the NCBI Reference Sequence (RefSeq) database (Pruitt 2004).
- 248 OTUs with 97% similarity to an organism that was on the list of known pathogens were
- flagged as "related to a known human pathogen". The phylogenetic diversity (Faith's PD)
- 250 was calculated using the alpha\_diversity.py script, with samples rarefied to 700 sequences.

### 251 Results and Discussion

## **Overall taxonomic diversity of ISS surfaces and comparison to**

### 253 previous high-throughput 16S rDNA study

- 254 After filtering chimeric and eukaryotic sequences from the data, the number of sequences
- 255 per surface sampled ranged from 26,221 76,656. Open-reference clustering at 97%
- similarity resulted in 12,554 OTUs (OTU is a proxy for microbial "species".) This exceeds
- the number of species observed by Venkateswaran *et al.* 2014, and Ichijo et al. 2016 which
- 258 is not surprising, given the increased sampling depth in this study (~1 million versus ~

259 50,000-71,000 high-quality sequences.) Our study also had four notable, qualitative 260 differences from these earlier studies. In Venkateswaran et al. 2014, more than 90% of all sequences were assigned to 4 bacterial genera (Corynebacterium, Propionibacterium, 261 262 *Staphylococcus*, and *Streptococcus*), while in the study here, they comprised only 24% of the 263 data (9.6%, 0.05%, 10.7%, and 3.6%, respectively). Ichijo et al. 2016 didn't report genus 264 level taxonomy but the phyla containing these groups (Firmicutes and Actinobacteria) 265 were highly abundant in all samples. Second, Venkateswaran et al. found no evidence of 266 archaea in their samples, even when interrogating with archaeal-specific primers, but we 267 did find evidence for a very low-abundance archaeal presence (2335 sequences, from three archaeal phyla). No archaeal results were reported by Ichijo et al. 2016. Next, despite the 268 269 fact that Venkateswaran et al. were able to culture many spore-forming organisms from 270 their samples, they observed no sequence data from putative spore-forming organisms. 271 However, a large percentage of sequences in our study are from spore-forming genera: 272 20.9% *Bacillus* and 9.6% *Clostridium*. These differences are potentially due to differences in 273 PCR primers and/or DNA extraction method, both of which have known taxonomic biases 274 (Brooks et al. 2015). Lastly Ichijo et al. 2016 noted a significant amount of both 275 Legionellaceae and Neiseriaceae which are both of potential concerns as families 276 containing may pathogenic members. However, our study observed no OTUs for these 277 groups which is most likely due to sampling site differences or PCR primer differences as

278 noted above.

279 The 19 most abundant orders found in our study represent 93.8% of the data (Figure 1).

280 Within each of these 19 orders, the most abundant genus found in our samples tends to be

human-associated (Table 2). This is not surprising, as the only source of microbial influx is

via occasional crew and cargo deliveries aboard spacecraft that have been stringently

cleaned to avoid microbial contamination. It should be noted, as with all 16S rDNA gene

- surveys, that nothing can be said about the viability of these bacteria. Typically much of the
- bacterial DNA on a surface is from dead or non-viable organisms. In built environments on
- earth this DNA is assumed to come from many sources including outdoor air, soil, and the
- 287 passage of people and animals. On the ISS all of these taxa, viable or not, represent
- organisms that have managed to survive the various protocols designed to limit them, the
- 289 most likely passage being on the crew themselves.

290 There were no apparent biogeographical patterns on the ISS surfaces. That is, there were 291 no significant differences between samples obtained from the different modules (crew vs 292 lab) or different surface types (keyboards, vents, or handheld mics). This can be visualized 293 in Figure 2, in which each point represents one of the 15 samples, and the distance between 294 samples indicates the overall difference in community composition. In Panel A, the metric 295 used to calculate the distance between samples is the Bray-Curtis dissimilarity, and in 296 Panel B, an alternative distance metric (Unifrac) is used, which takes into account the 297 phylogenetic distance between the OTUs in samples. For the most part, all 15 samples form 298 a tight cluster on the NMDS plots, but there is one sample, the starboard crew vent, that 299 appears distinct from all of the other samples in Panel A. In Panel B, that same sample, as 300 well as the aft lab vent sample appear separate from the others. In order to visualize which 301 OTUs are contributing the most to the uniqueness of those samples, we looked at the 302 overall distribution of the most abundant bacterial families in those samples. The three

- 303 most abundant families in the starboard crew vent sample are Bacteroidaceae,
- Ruminococcaceae, and Verrumicrobiaceae (comprising 60.1% of all sequences); and the
- 305 three most abundant families in the aft lab vent sample are Rikenellaceae, Bacteroidales
- 306 S24-7, and Lactobacillaceae (comprising 60% of all sequences). In Figure 3, the relative
- abundance of these six families in all 15 samples from the ISS provides a clear indication
- 308 that they are driving the distinctiveness of those two samples.
- 309 The massive increase in environmental 16S rDNA gene surveys over the last several years
- has seen a greater understanding of the caveats and limitations with this kind of data, in
- 311 parallel with their unambiguous utility in understanding microbial communities. When this
- 312 experiment was designed in 2012, negative kit controls were not common but now they are
- 313 considered standard for good reason (Salter et al. 2014). Lacking a kit control, we cannot
- 314 say for certain which low-level taxa may have come from the swabs or reagents used
- 315 themselves.

#### 316 Comparison to the microbial communities of homes on Earth and

#### 317 from the Human Microbiome Project

- 318 To put the microbial communities that we found on ISS surfaces in the context of homes on
- Earth, we compared them to the communities found by citizen scientists when they
- 320 swabbed nine surfaces throughout 40 homes, as part of the "Wildlife of Our Homes" project
- 321 (Dunn et al. 2013). We found that the ISS and homes on Earth were significantly different
- from each other, both based on the Bray-Curtis dissimilarity (adonis, R<sup>2</sup>=0.0666, P=0.001)
- and the Unifrac distance (adonis,  $R^2$ =0.04189, P=0.001). These differences can be
- 324 visualized in the ordination plots in Figure 4 A and B.
- It is perhaps not surprising that the insular environment of the ISS would be unlike homes on Earth. Unlike the ISS, homes on Earth are exposed to a variety of sources of microbes, including the outdoor air, tracked-in soil, plants, pets, and human inhabitants (Barberán et al. 2015) (Barberán et al. 2015). The dominant source of microbes on the ISS is presumably the human microbiome. All spacecraft and cargo undergo rigorous decontamination procedures before launch to rendezvous with the ISS. Therefore, we hypothesized that the
- microbial communities of the ISS surfaces might be more similar to human-associated
   microbial communities than Earth home surfaces. To test this hypothesis, we obtained 16S
- 332 rDNA sequence data for 100 random samples from each of 13 body sites from the HMP
- 334 Data Portal (http://hmpdacc.org/HM16STR/)(Huttenhower et al. 2012) (Gevers et al.
- 335 2012). The microbial communities associated with the ISS, homes on Earth, and the HMP
- samples were significantly different from each other (adonis,  $R^2 = 0.08$ , P < 0.001) (Also see
- Figure 5). We note that as with any meta-analysis, this difference could be also be partly
- due to differences in sample collection/preparation. However, the ISS communities are
- 339 significantly more similar to the Earth home samples than the HMP samples (Student's t-
- test, p< 0.00001). This combined analysis also indicates that the starboard crew vent
- 341 sample, which appears quite distinct from the rest of the ISS samples in Figure 2A, is more
- 342 similar to the human gastrointestinal HMP samples, which is corroborated by the
- 343 dominance of animal gut-related OTUs found in that sample (see Figure 3, and Table 2.)

- 344 Finally, because the ISS is designed only to house six crew members, for a stay of six
- months each, only 220 individuals have visited the ISS since the year 2000. We
- 346 hypothesized that there might be a relatively low microbial diversity on the ISS, either due
- to having a few total number of species, or due to the dominance of a very few species. In
- 348 Figure 6, we note that Shannon diversity (which takes into account both the number of
- 349 species present, and how evenly our sequences are distributed throughout those species) is
- actually relatively high on the ISS.

### 351 Comparison to rooms with mechanical ventilation or open windows.

- 352 Kembel et al., 2012, showed that rooms in a health-care facility that were primarily
- 353 ventilated via an open window had greater phylogenetic diversity and lower proportion of
- 354 OTUs closely related to known human pathogens than rooms that were mechanically
- ventilated. The only window on the ISS is never opened, and the doors are opened only
- 356 briefly, every few months. Therefore, we hypothesized that for the samples from the ISS,
- 357 the phylogenetic diversity would be lower and the proportion of OTUs closely related to
- known human pathogens would be higher than that seen for mechanically ventilated
- 359 rooms. To test this hypothesis, we obtained the list of known human pathogens compiled
- by Kembel *et al.*, 2012, and followed their procedure to identify the proportion of OTUs in
- the ISS samples that were closely related to them (see Methods for details). Surprisingly,
- but reassuringly, we found that the ISS samples are similar in both phylogenetic diversity
- and the proportion of OTUs closely related to known human pathogens as compared to the
- 364 mechanically ventilated rooms in the health-care facility (Figure 7). As with the studies
- above, some observed variance may be due to differences in sample
- 366 collection/preparation.
- 367

### 368 Conclusion

- 369 This is the first time that the ISS has been analyzed in the broader context of the
- 370 "microbiology of the built environment", and is the most in-depth comparison of the
- 371 microbial communities found on the ISS to those found either in buildings or in the human
- 372 microbiome. Perhaps surprisingly, given the extreme rarity of exchange with any external
- 373 microbes, we found the ISS to be species-rich, and more similar to the surfaces of human
- homes on Earth than it is to human bodies. We found that the ISS is home to at least 12,554
- 375 distinct microbial species, including Archea in very low abundance, and that the proportion
- 376 of species that are closely related to known human pathogens is on par with similar built
- 377 environments on Earth. Given the low number of samples in this study, no viability
- 378 assessment, as well as the lack of sample preparation control we view these results as
- 379 simply a starting place for more detailed future studies.
- 380 As outlined in the 2010 U.S. National Space Policy and in the bipartisan NASA Authorization
- 381 Act of 2010, NASA is targeting the 2030s for a manned spaceflight to Mars, with one
- 382 ultimate goal of having people live and work on the Martian surface (see
- 383 www.nasa.gov/exploration and www.nasa.gov/mars). We know that the microbial



- 384 communities found in our terrestrial built environments play an important role in human
- 385 health. Therefore it's crucial to characterize and understand the microbial population of the
- 386 only environment in which people are currently living and working in space. This study is
- 387 one small step in that direction.

#### 388 Data Accessions

- 389 Sequencing data has been deposited at NCBI under BioProject PRJNA376404. All data and
- analysis files are available on FigShare https://doi.org/10.6084/m9.figshare.4244123.v3
- 391
- 392

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530

# Figure 1

Relative abundances of the most common bacterial families found on surfaces of the ISS.

Pie chart showing the relative abundances of the most common bacterial families found on the 15 surfaces of the International Space Station. This graph was produced using METAGENassist [26].



## Figure 2(on next page)

Non-metric multidimensional scaling (NMDS) ordination plots of 15 ISS surface samples

Non-metric multidimensional scaling (NMDS) ordination plots, based on Bray-Curtis (Panel A) or Unweighted Unifrac (Panel B) distances between the samples obtained from the International Space Station. In these plots, points that are closer together have more similar microbial communities. Note, there is a (starboard) crew vent sample that does not cluster with the other ISS samples in Panel A, and in Panel B, a second sample (aft lab vent) appears closer to it. This graph was produced using the Phyloseq package [18] in R [13].



## Figure 3(on next page)

Most abundant bacterial families found in each of the two "outlier" samples on the ISS.

Bar chart showing the distribution across all samples of the 3 most abun- dant bacterial families found in each of the two samples (starboard crew vent and aft lab vent) that do not cluster with the others in Figure 2. All six of these families are known to be found in association with human (or animal) gastrointestinal tract.



bacterial Family



### Figure 4(on next page)

Non-metric multidimensional scaling (NMDS) ordination plots of ISS surface samples.

Non-metric multidimensional scaling (NMDS) ordination plots, based on Bray-Curtis (Panel A) or Unweighted Unifrac (Panel B) distances between samples obtained from the International Space Station and samples obtained from homes on Earth. In these plots, points that are closer together have more similar microbial communities. We found that the ISS samples and Earth home samples were significantly different from each other, both based on the Bray-Curtis dissimilarity (adonis, R<sup>2</sup> =0.0666, P=0.001) and the Unifrac distance (adonis, R<sup>2</sup> =0.04189, P=0.001). Note, the crew and lab vent samples that are distinct from the other ISS samples Figure 2, do not cluster with any of the Earth home surfaces. This graph was produced using the Phyloseq package [18] in R [13].



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# Figure 5

NMDS plots showing clustering of ISS, Earth homes, and Human Microbiome Project body sites.

Non-metric multidimensional scaling (NMDS) ordination plots, based on Bray-Curtis (Panels A and C) or Unweighted Unifrac (Panels B and D) distances between samples obtained from the International Space Station, from homes on Earth, and from 13 body site from the Human Microbiome Project. The plots in panels A and B show identical data, as do the plots in panels C and D. The points in A vs. B and C vs. D are colored differently as an aid for visualization. In these plots, points that are closer together have more similar microbial communities. The microbial communities associated with the ISS, homes on Earth, and the HMP samples were significantly different from each other (adonis,  $R^2 = 0.08$ , P < 0.001). Note, the crew and lab vent samples that are distinct from the other ISS samples in Figure 2 are more similar to the human gastrointestinal tract samples from the HMP. This graph was produced using the Phyloseq package [18] in R [13].

## Manuscript to be reviewed



### Figure 6(on next page)

Comparison of Shannon diversity among the ISS, Earth homes, and HMP body sites.

Shannon diversity, a measure of how many species there are as well as how evenly the counts of individuals are distributed across species is plotted for every sample. There is wide variation among the HMP samples, with the oral (blue) and gastrointestinal (green) samples typically having more diversity than the skin (pink) or airway (coral) samples. Surfaces on the International Space Station have relatively high Shannon diversity, on par with that of the most diverse HMP samples, and the average home sample. This graph was produced using the Phyloseq package [18] in R [13].



### Surface

- vent (ISS)
- nomex (ISS)
- mic (ISS)
- Node2 mic (ISS)
- handrail (ISS)
- foothold for RWS (ISS)
- joystick for RWS (ISS)
- keyboard (ISS)
- cutting board (Homes)
- door handle (Homes)
- exterior door trim (Homes)

- interior door trim (Homes)
- pillowcase (Homes)
- refrigerator (Homes)
- television (Homes)
- toilet seat (Homes)
- kitchen counter (Homes)
- gastrointestinal tract (HMP)
- airways (HMP)
- oral (HMP)
- skin (HMP)



# Figure 7

Proportion of OTUs found in the ISS samples that were closely related (97% sequence similarity) to human pathogens versus the phylogenetic diversity of those samples.

This figure was modified from Figure 4a. of [25]. The pink star represents the ISS samples. The plot shows the proportion of OTUs that were closely related (97% sequence similarity) to human pathogens versus the phylogenetic diversity of those samples.



Environment ISS Proportion bacterial sequences Indoor - Mechanical closely related to pathogens Indoor - Window 0.6 Outdoor 0.4 0.2 0.0 10 15 20 25 30 Phylogenetic diversity

(Faith's PD per 700 sequences)

## Table 1(on next page)

ISS sample surface descriptions and sequence statistics.

#### Table 1:

Sample	Earth analog	Number of sequences obtained	Number of Species Observed (OTUs at 97% similarity)
forward lab mic	cell phone	45902	1744
lab robotic workstation keyboard aft lab mic	none cell phone	31612 63958	1320 2457
lab robotic workstation joystick	door handle	76198	2820
lab robotic workstation left foothold	shoe	77843	1995
lab robotic workstation right	shoe	74023	2129
aft lab vent	interior door trim	64782	1456
starboard crew vent	interior door trim	63280	4294
starboard sleep quarters nomex port crew vent	pillow interior door trim	26831 50418	1036 1757
port sleep quarters nomex	pillow	61306	1349
node2 mic	cell phone	50416	1429
lab handrail	door handle	02307 70418	1078 2904
forward lab vent	interior door trim	57715	2380

1

### Table 2(on next page)

The most abundant organisms on the ISS are human-associated.

From each of the 19 orders shown in Figure 1, we selected the most abundant genus and conducted a literature review to identify whether or not it is known to occur in association with the human microbiome.

## Manuscript to be reviewed

#### Table 2:

Order	% abundance	dominant Genus	common habitat	reference
Actinomycetales	18.3	Corynebacterium	human skin, oral cavity	(Grice 2009),(Zaura 2009)
Bacillales	14	Staphylococcus	human skin, oral cavity	(Grice 2009),(Zaura 2009)
Bacteroidales	12.8	unclassified Rikenellaceae/S24- 7	animal gut	(Langille 2014),(Krych 2015)
Lastabasillalas	11 1	Ctroptococcuc	human oral	(4.5.5, 2005)
Lactobacillales	11.1	Streptococcus		(Aas 2005)
Clostrialaies		Finegolala	human skin	
Pseudomonadales	6.1	Pseudomonas	numan skin	(Cogen 2008)
<b>B</b> 11 11 11	5.6	unclassified		
Burkholderlales	5.6	Comamonadaceae	environmental	(Willems 2014)
Neisseriales	2.3	Neisseria	human mucous membranes	(Liu 2015)
Fusobacteriales	2.2	Fusobacterium	human oral cavity	(Schwarzberg 2014)
Pasteurellales	1.7	Haemophilus	human respiratory tract	(Murphy 2007)
Verrucomicrobiales	1.6	Akkermansia	human gut human oral	(Belzer 2012)
Flavobacteriales	1.1	Capnocytophaga	cavity human oral	(Zaura 2009)
Selenomonadales	1	Selenomonas	cavity	(Ribeiro 2011)
Sphingomonadales	0.9	Sphingomonas	environmental	(Seifried 2015)
Sphingobacteriales	0.8	Sphingobacteriales	environmental	(Steyn 1998)
Enterobacteriales	0.8	Enterobacteraceae	animal gut	(Linton 1988)
Rhizobiales	0.6	Methylobacterium	environmental	(Knief 2010)
Campylobacterales	0.6	Campylobacter	animal gut	(Young 2007)

1