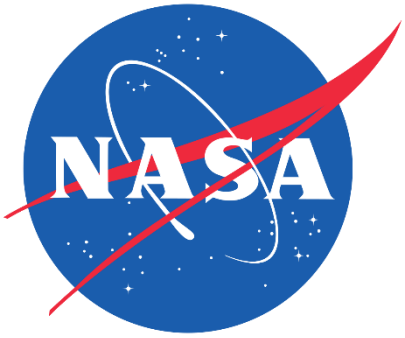
A Microbial Analysis of Space-Grown Produce

Eric L. Barash
NASA Kennedy Space Center
NIFS Internship Program Spring Session
April 12th 2018



A Microbial Analysis of Space-Grown Produce

Eric L. Barash[1]
Wake Forest University School of Medicine, Winston-Salem, NC, 27101

Christina Khodadad[2] and Mary Hummerick[3]
NASA Kennedy Space Center, Space Biology Division, Merritt Island FL, 32899

Abstract: Before space crops become a permanent staple of the astronaut diet, we must first understand how plants and pathogenic microbes interact in microgravity. Crops such as red romaine lettuce and Chinese cabbage were grown on the International Space Station and sent back to Kennedy Space Center for microbial analysis. Aerobic plate counts and metagenomic sequencing were used to characterize bacterial and fungal communities for plants and their respective "pillows." These data will be used to create new guidelines for the microbial safety of space-grown produce, and will help us better protect astronauts from food-borne pathogens such as *E.coli*, *Staphylococcus*, and *Salmonella*.

I. Introduction

Sustainable agriculture is integral to future long-term space exploration. In addition to supplementing key calories and nutrients to the astronaut diet, space produce can aid in atmospheric regeneration and water purification. Since food, oxygen, and clean water are limited resources in space travel, plants are essential interplanetary companions. Furthermore, space plants provide an often overlooked psychological benefit by giving astronauts a (figurative and literal) taste of life back on Earth. Even so, farming in microgravity is no trivial task; plants must be grown in environments with controlled water, light, nutrients, convection, and temperature: all of which pose challenges on the International Space Station (ISS). The design of the Veggie Food Production Chamber (ORBITECH: Madison, WI) helps tackle some of these challenges (Figure 1). Veggie contains a RGB LED lighting array and "pillows" filled with sterilized arcillite clay and time-release fertilizer. Seeds are planted between two wicks in the pillows (Figure 2) which draw up water via capillary action. The roots grow down into the pillow while the shoots grow up towards the LED panel. The chamber is enclosed in plastic bellows which help to regulate humidity and temperature, while a fan helps to circulate air. Since its launch to the ISS in 2014, the Veggie unit has successfully grown red romaine lettuce, zinnia flowers, mizuna, and Chinese cabbage.

Our team is responsible for the molecular and microbial analysis of Veggie flight (ISS) and ground samples. This includes instrument swabs, plant tissue samples, and pillow hardware. Since astronaut health is a top priority, we must ensure that the crops meet microbial safety guidelines and are void of pathogenic bacteria like *E.coli* and *Salmonella*. To begin to assess the microbial composition of space produce, we must first identify the culturable and unculturable species.

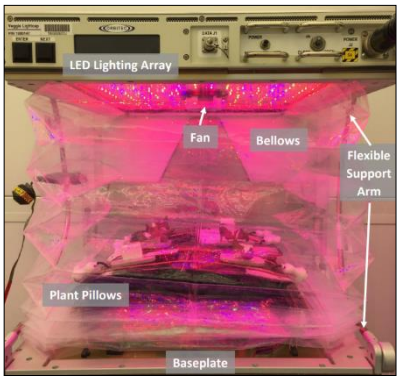


Figure 1. Veggie Growth Hardware Unit. Photo courtesy of NASA.and Massa et. al *Open Agriculture*. 2017.

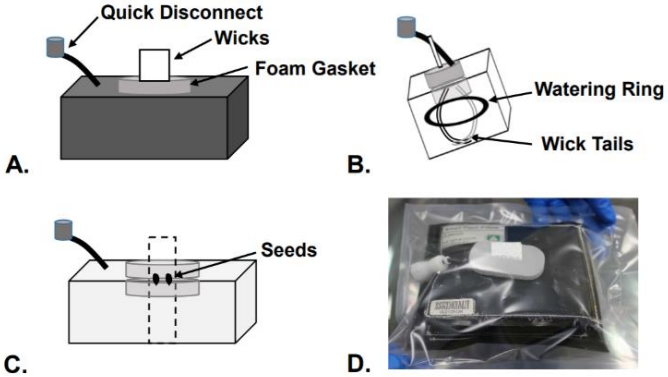


Figure 2. A. Schematic side view of a plant pillow. B. Schematic end cross sectional view of plant pillow. C. Schematic cut away side view of plant pillow showing seed placement. D. Photo of a planted pillow prepared for flight. Source: Massa et al. *Gravitational and Space Research*. 2017.

[1] NIFS Intern, Space Life Sciences Group, KSC, and Wake Forest University School of Medicine.
[2,3] Molecular Biology and Microbiology Departments, NASA, KSC.

II. Veggie: Quantifying, Isolating, and Identifying Culturable Aerobic Bacteria and Fungi

Determining Microbial Loads

Our team spends significant time processing Veggie flight and ground samples. The goal is to isolate, quantify, and identify microbes from swabs, plants, and pillows. The frozen samples are first thawed and suspended in 1x Phosphate Buffered Saline (PBS). They are then thoroughly shaken to make a homogenous mixture and plated at various dilution factors on Tryptic Soy Agar (TSA) or Inhibitory Mold Agar (IMA) plates. The TSA selects for bacteria, while the chloramphenicol-containing IMA selects for yeast and fungi. Plate colonies are counted to determine the approximate number of Colony Forming Units (CFU) for each sample. This allows us to calculate the total aerobic microbial load on Veggie swabs, plants, and pillows.

The preliminary data below show Veggie pillow aerobic plate counts (APC) for flight and ground control samples. Pillow samples were subdivided into three sections including wick, root, and soil. Three soil samples were taken from different regions of the pillow, and the CFU counts were averaged. All other root and wick CFU values represent the average of duplicate plates, so no standard deviation is shown. The VEG03-A experiment grew 'Outredgeous' red romaine lettuce, while VEG03-C grew Chinese cabbage that was harvested in a cut-and-come-again fashion. All ground control experiments were conducted in replica Veggie units that mimicked the ISS in humidity, temperature, light intensity, and carbon dioxide concentration.

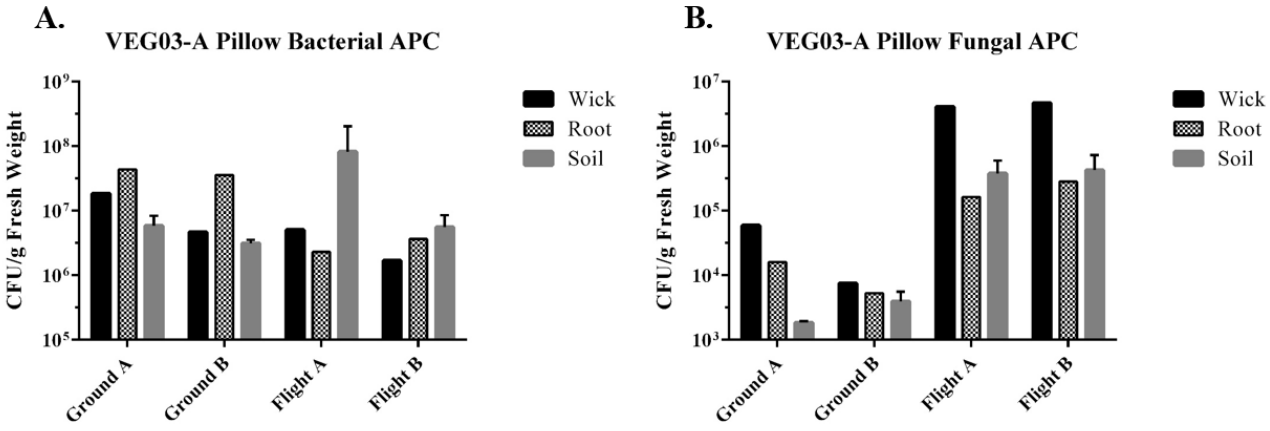


Figure 3. VEG03-A Pillow Ground and Flight Aerobic Plate Counts for Wick Root and Soil. Ground A and B as well as Flight A and B represent two individual plant pillows. A. VEG03-A pillow bacterial APC. B. VEG03-A pillow fungal APC. Standard deviation not shown for wick and root samples because only two measurements were taken per sample. Soil data are the average of three measurements, so the standard deviation is shown. VEG03-A grew red romaine lettuce.

Figure 3A shows that Outredgeous ground samples tended to have more bacteria on the roots compared to soil or wick. This is not observed for flight samples, where Outredgeous samples tended to have more bacteria in the soil compared to the root or wick. Overall, in VEG03-A, ground root samples had significantly more bacteria than flight root samples, possibly because bacteria diffuse into the soil more easily in microgravity. Figure 3B shows that VEG03-A flight samples had significantly more fungi than ground samples for wick, root, and soil. Unlike Figure 3A, there seemed to be more microbes on the flight wick compared to any other area sampled. This makes sense given that the flight wicks were visibly dirtier than ground control wicks, which were usually pristine white condition. Overall, VEG03-A fungi levels were lower than bacteria levels for both flight and ground samples.

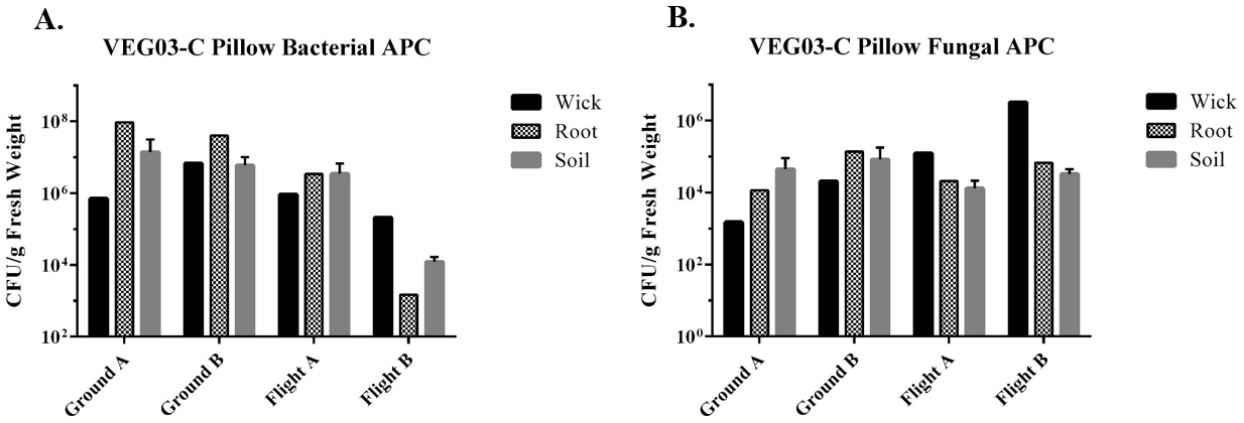


Figure 4. VEG03-C Pillow Ground and Flight Aerobic Plate Counts for Wick Root and Soil. Ground A and B as well as Flight A and B represent two individual plant pillows. A. VEG03-C pillow bacterial APC. B. VEG03-C pillow fungal APC. Standard deviation not shown for wick and root samples because only two measurements were taken per sample. Soil data are the average of three measurements, so the standard deviation is shown. VEG03-C grew Chinese cabbage.

VEG03-C Chinese cabbage pillows showed less distinct trends. Figure 4A shows that ground samples tended to have more bacteria than flight samples for root, wick, and soil. Given the small sample size, it is difficult to say which part of the pillow had the greatest bacterial load. In Ground A, the roots had significantly more bacteria than the wick, but these trends were not seen in Ground B. Likewise, in Flight B the wick had significantly more bacteria than the root, but this trend was not replicated in Flight A. Overall, Figure 4A suggests that VEG03-C pillows had slightly elevated bacterial loads compared to flight samples. In respect to VEG03-C fungal loads, Figure 4B may suggest that flight wicks more fungi than the root or soil, but this trend was only significant in Flight B samples. Overall, ground and flight samples have roughly 10^4 CFU/g fungi for wick, root, and soil.

These data help us estimate the concentration and distribution of microbes in Veggie flight and ground pillows.

Isolating and Identifying Phenotypically Unique Microbes

TSA and IMA plates were also used to isolate and identify phenotypically distinct colonies. These unique colonies were carefully restreaked on fresh plates, and the resulting growth was analyzed with a BIOLOG assay. Colonies were resuspended in the BIOLOG buffer and were added to a 96-well Gen3 BIOLOG plate. Each well in the Gen 3 plate contains a unique growth medium that undergoes a colorimetric reaction in response to the metabolic byproducts of a microbial sample. After 24-48hrs of incubation, the BIOLOG computer software analyzes the unique 96-well colorimetric fingerprint and matches the data to one of the 2,900 species of bacteria or fungi in their database.

However, BIOLOG has limitations and may fail to identify a subset of bacteria and a majority of fungi species. For gene-based identification, the unidentified samples were analyzed using MicroSeq sequencing technology. To begin, DNA was extracted from bacteria and fungi samples. The Applied Biosystems PrepMan Ultra Kit (Cat. No. 4318930) was used to extract bacterial DNA, while the Qiagen DNeasy PowerSoil Kit (Cat. No. 12855-100) was used to extract fungal DNA. Next, the DNA was quantified using the ThermoFisher Qubit Fluorometric Quantifier. To proceed with sequencing, each DNA sample was diluted to a concentration of less than or equal to 1ng/μL. Dilutions were made using sterile nanopure water, and the samples were prepped for a Polymerase Chain Reaction (PCR). In addition to the enzyme-containing master mix, forward and reverse 16S primers were added to the bacterial samples, while D2 LSU primers were added to the fungal samples. The DNA mixture was inserted into the thermocycler and went through a series of melting steps at 95°C which denatured the double stranded DNA and annealing steps at 64°C which enabled the polymerase to bind the primers. A final extension step at 72°C allowed the enzyme to add new nucleotides. Ultimately, PCR yields billions of copies of 16S and D2 LSU DNA fragments. These ~500 base pair sequences are highly conserved among bacteria and fungi respectively, and are an accurate way to distinguish species.

The PCR products were verified using gel electrophoresis. DNA and loading dye were added to the wells of a 1% agarose gel containing Sybr Safe DNA gel stain. To prepare for the sequencing reaction, a ThermoFisher ExoSAP-IT kit (Cat. No. 78201.1.ML) was used to remove excess dNTPs and primers. Then, the cleaned samples and sequencing reagents were added to a 96-well plate. These reagents included fluorescently tagged dideoxynucleotides which

fragmented the DNA into hundreds of variably-sized fluorescent amplicons during the sequencing reaction. A Qiagen DyeEx 2.0 Spin Kit (Cat. No. 63204) was used to remove excess fluorescent dyes and primers from the sequencing products, and the samples were then loaded onto an Applied Biosystems 3130 Genetic Analyzer. The instrument uses automated capillary electrophoresis to separate the DNA amplicons by size, and a fluorescence meter reads the color (red, blue, green, or yellow) that represents the terminal nucleotide (A,T,C, or G) in the DNA fragment. These reads are combined to generate the full sequence for the 16S or D2 LSU fragment, and the data can be analyzed by software like MicroSeq or BLAST to identify the organism.

Table 1. Species Identification from Veggie Unit Swabs. All fungal data derived from Microseq, while bacterial samples were processed using both Microseq and BIOLOG. G=ground, F=flight and A, B, and C represent the corresponding VEG03 A, B, or C experiment.

	LOCATION	IDENTIFICATION
BACTERIA	Fan screen (GA)	*Bacillus pumilus*
	Bellows right (GA)	*Staphylococcus capitis*
	Bellows left (FA)	*Microbacterium resistans*
	Pillow surface (FA)	*Microbacterium marytipicum*
	Bellows (GB)	*Micrococcus luteus*
	Pillow surface (GB)	*Brevibacterium casei*
	Bellows right (GB)	*Staphylococcus epidermidis*
	Pillow surface (GB)	*Micrococcus luteus*
	Pillow surface (GC)	*Bacillus megaterium*
	Pillow surface (FC)	*Micrococcus luteus*
	Pillow surface (FC)	*Microbacterium marytipicum*
	Bellows left (FC)	*Paenibacillus pabuli*
	Bellows middle (FC)	*Paenibacillus pabuli*
FUNGI	Pillow surface (GC)	*Penicillium oxalicum*
	Bellows right (GA)	*Aspergillus niger*
	Pillow surface (GA)	*Penicillium solitum*
	Bellows middle (GA)	*Trichoderma longibrachiatum*
	Fan screen (GA)	*Penicillium solitum*
	Fan screen (GA)	*Penicillium oxalicum*
	Fan screen (FA)	*Penicillium chrysogenum*
	Fan screen (FA)	*Emericella parvathecia*
	Fan screen (FA)	*Aspergillus ustus*
	Pillow surface (GB)	*Penicillium olsonii*
	Pillow surface (FB)	*Penicillium citrinum*
	Pillow surface (GC)	*Penicillium islandicum*

The data in Table 1 show culturable aerobic organisms found on Veggie swabs taken from both flight and ground Veggie units. These swabs represent three Veggie experiments (A, B, and C) which grew red romaine lettuce, Chinese cabbage, and Chinese cabbage respectively. Veggie units were swabbed on their right and left bellows, fan screen, and pillow surface. Swabs taken on the ISS were frozen at -80°C before being sent back to Earth.

All of the fungal samples were identified using MicroSeq, while the bacterial samples were identified using MicroSeq and BIOLOG. To our knowledge, none of the species listed pose threats to healthy humans. Overall, the microbial community inhabiting the Veggie units appears to be free of pathogenic microbes. Many of these species including *Staphylococcus epidermidis, Micrococcus luteus,* and *Trichoderma longibrachiatum* were also found on Veggie A, B, and C plant samples.

III. Veggie: Identifying, Isolating, and Quantifying Unculturable Bacteria and Fungi

Unfortunately, plating and culturing bacteria only provides a narrow view of a community's microbial constituents. In fact, some scientists claim that up to 99% of microorganisms cannot be cultured in a lab (Reisenfeld: 2004). Therefore, metagenomic sequencing is a superior option to fully understand the true diversity of microbial communities. Metagenomic 16S sequencing (for bacteria) and ITS sequencing (for fungi) can identify entire populations of microbes in a single sample, including the species that cannot be cultured.

To begin the Illumina Metagenomic MiSeq, DNA from each sample was isolated quantified, diluted, and amplified by PCR. When preparing the PCR reaction, every sample had to be barcoded; a barcode is nucleotide sequence contained within specific forward and reverse primers which allows the sequencer to match DNA to its original sample. For example, simultaneously analyzing 96 plant samples required eight distinct forward barcodes and twelve

distinct reverse barcodes, giving each sample a unique "zip code." After using PCR to amplify the 16S or ITS regions, the concentration of DNA had to be normalized. Next, a DNA library was created by adding equimolar concentrations of each sample into a common tube. This library was further dulited to 4nM, and NaOH was used to denature the DNA. Ultimately, the DNA library reached a final concentration of 10pM and was combined with a PhiX control. The PhiX control contains viral DNA and adds to the fluorescent diversity of the run. Greater sequence diversity yields higher quality results. Finally, the diluted DNA library and the PhiX control were added to an Illumina MiSeq Cassette. This cassette comes pre-loaded with all of the sequencing reagents and fits directly into the sequencer. After roughly 48 hours of analysis, the sequencer identified more than 7 million different DNA reads and assigned each read to a barcoded sample. The instrument can identify most organisms down to the genus and species taxon level and can also determine the relative abundance of each species. Overall, metagenomic sequencing gives a useful summary of a sample's microbial diversity.

Below are recently generated 16S metagenomic data for VEG03-A and VEG03-C flight and ground plant samples.

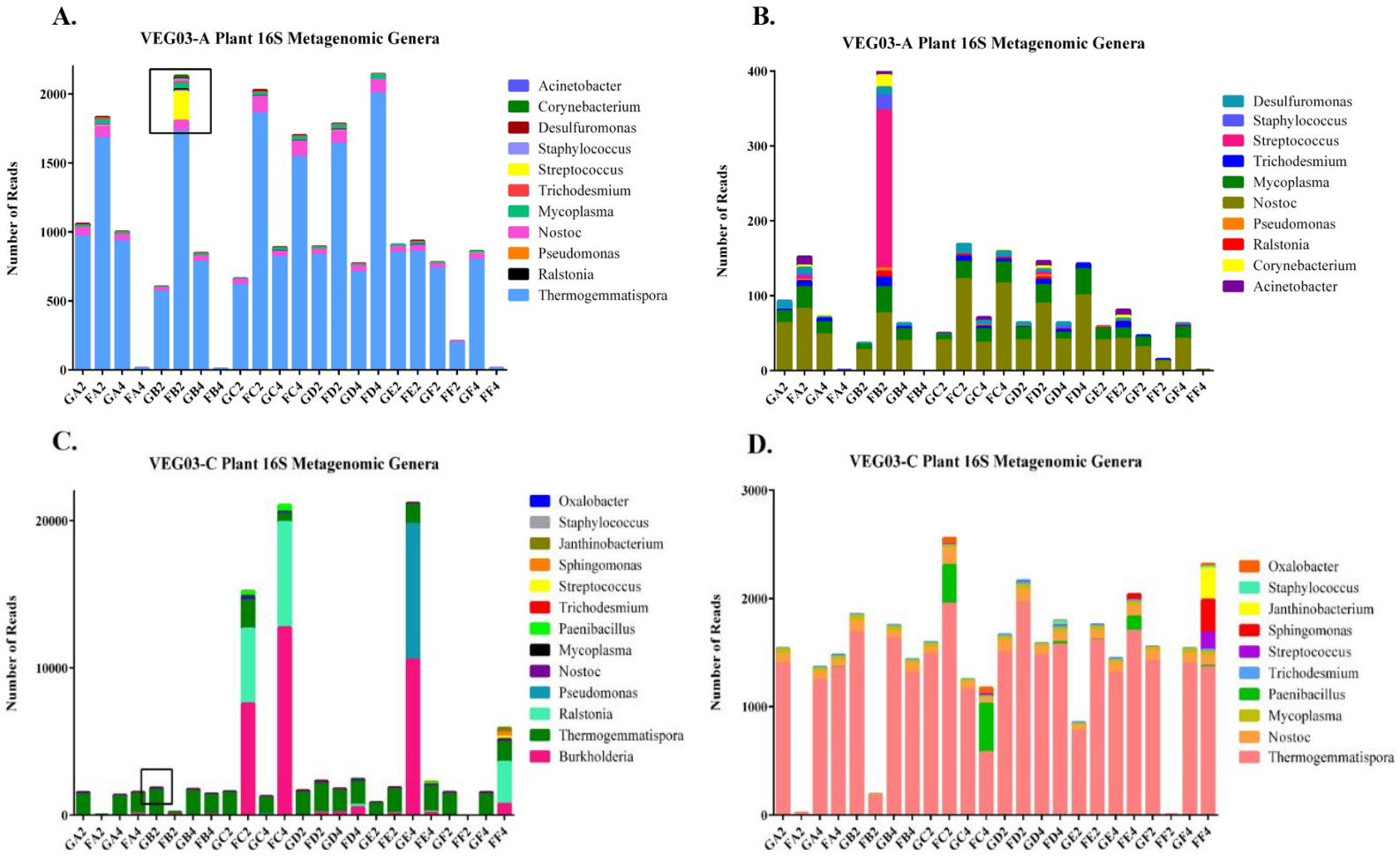


Figure 5. 16S Metagenomic Bacterial Genera for VEG03A and C. The dominant genus *Calothrix* has been omitted from all graphs to see the nuances in genus diversity. B and D are magnified data from the black squares present in A and C. The genus *Thermogemmatispora* was omitted from B, and *Burkholderia, Ralstonia,* and *Pseudomonas* were omitted from D. The x-axis shows ground or flight samples (G/F) and is followed by the plant letter (A-F) and harvest number. Only harvests 2 and 4 are shown here. Note that for VEG03-A no data could be retrieved from samples GE4 and FE4.

These results are unlike any data we have seen in previous Veggie experiments. Although not shown in Figure 5, both VEG03-A and C were heavily dominated by the genus *Calothrix*. In fact, *Calothrix* made up on average over 91% of the reads per sample. These organisms are water-dwelling filamentous cyanobacteria that pose no known hazards to healthy humans (Shivonen: 2007). Perhaps the *Calothrix* species originated from the water supply. Regardless, the second most abundent genus in all VEG03-A and most VEG03-C samples was *Thermogemmatispora*.

Species in this genus are Gram-positive, spore-forming thermophiles and are typically found on leaves (Komaki: 2016). Looking at Figure 5B, *Nostoc* was an abundant genus in VEG03-A, except for sample FB2 where *Streptococcus* was prevalent. In VEG03-C (Figure 5C), *Thermogemmatispora* dominated most samples except for FC2, FC4, and GE4 which were dominated by *Burkholderia*, *Ralstonia*, and *Pseudomonas*. These three genera are typically abundant in Veggie experiments, and it is puzzling why they hardly appeared in any VEG03 A or C samples. Figure 5D again emphasizes the dominance of *Thermogemmatispora* and highlights the absence of many genera including *Methylobacterium*, and *Mesorhizobium* that have routinely occurred in large numbers in previous experiments.

In light of these unexpected results dominated by *Calothrix* and *Thermogemmatispora*, we took a second look at the quality of our 16S DNA library. Using an Agilent DNA chip, we generated an electropherogram showing the quality of our DNA library. As shown in Figure 6, our 3ng/µL DNA library had one distinct peak, meaning that the DNA was intact and had not suffered from tangling or shearing. However, the electropherogram also shows that the relative intensity of our DNA peak is low compared to the two standard markers. This implies that our library had a low concentration of amplicon. Perhaps, in future experiments we will use a more concentrated DNA library. In addition, we recently learned that libraries with many samples tend to have a PCR bias for certain species. Given that our sequncing library consisted of amplicons from 92 different samples, it is possible that the sample diversity led to a bias towards *Calothrix*. Therefore, in future runs we may use a more concentrated DNA library that contains fewer samples.

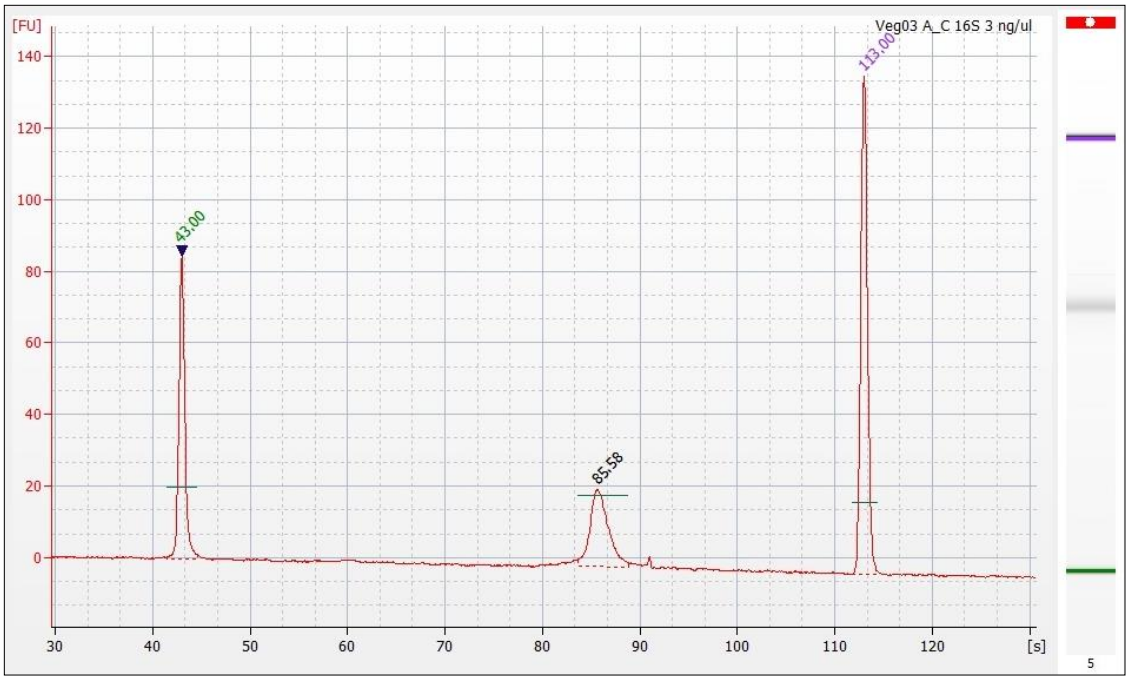


Figure 6. Electropherogram for VEG03 A, B, and C 16S Library. This 3ng/µL DNA library contains 92 barcoded plant samples. Graph shows Fluorescent Units on the y-axis and time on the x-axis. The middle peak is the DNA library and the two outer peaks are the DNA standard markers used with the Agilent DNA Chip Kit. The gel on the far right shows the 16S library (gray) in between the two markers (green and purple).

IV. Using Microbial Data to Recommend Safety Guidelines for Space Agriculture

For the first time in August 2015, NASA allowed astronauts Scott Kelly, and Kjell Lindgren to consume space-grown produce. Figure 7 shows the two NASA astronauts and Kimiya Yiu (JAXA) happily eating red romaine lettuce. After, Scott Kelly tweeted: "It was one small bite for man, one giant leap for #NASAVEGGIE and our #JourneytoMars." However, before this snack, NASA scientists followed a rigorous health and safety protocol that included microbial ground testing and pre-consumption leaf sanitization. The astronauts sanitized the lettuce by blotting the leaves with antimicrobial ProSan wipes. So far, analysis of ISS-grown crops has never shown the

presence of human pathogens including *E. coli*, *S. aureus*, and *Salmonella* (although Veggie-grown Zinnia flowers suffered from a *Fusarium* fungal infection which killed most of the plants). Many of the common species identified on the plants and pillows fall within the "fab five" genera including: *Burkholderia*, *Methylobacterium*, *Cupriavidus*, *Pseudomonas*, and *Sphingomonas*. But again, none of the species identified in these taxa pose harm to healthy astronauts. Regardless, sanitary precautions are still important because the human immune system weakens in microgravity (Borchers: 2002).

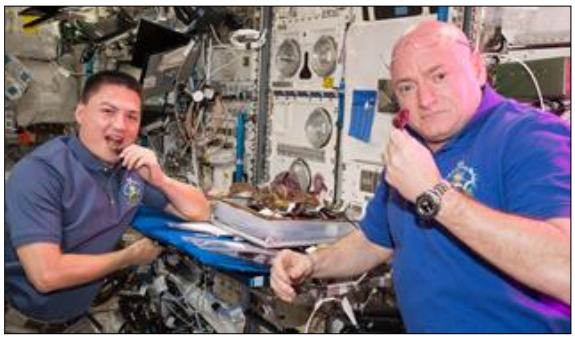


Figure 7. Astronauts Scott Kelly (right), Kjell Lindgren (left) eat space-grown red romaine lettuce from the Veggie plant habitat. Photo courtesy of NASA.

Figure 8. Astronaut Scott Kelly harvesting Zinnia flowers from a Veggie unit on Valentine's Day. Photo courtesy of NASA.

But the question still remains: what is the standard of microbial safety for space-grown produce? Currently, NASA only has strict microbial regulations for non-thermostabilized food which applies to prepackaged, low moisture, and freeze-dried foods. In anticipation of an increased consumption of space crops, our team aims to determine microbial guidelines for ISS-grown fresh produce. We are currently comparing microbial loads of crops grown in station-like conditions to microbial loads of store-bought vegetables. We will harvest five crops including mizuna, pepper, lettuce, radish, and tomato which represent the broad categories of leafy greens, roots, and fruits. These vegetables will be planted in controlled growth chambers that mimic the space station's humidity (45% RH), carbon dioxide concentration (3000 ppm), temperature (23°C), and fluorescent light intensity (300 μmol $m^{-2}s^{-1}$). We will focus on isolating three common human pathogens: *Enterobacteria (E.coli)*, *Salmonella*, and *Aspergillus flavus*. In addition, we will measure the total number of culturable aerobic organisms including bacteria, yeast, and mold. Overall, we hypothesize that the crops grown in a sanitary controlled environment will have fewer microbes than store-bought vegetables. These findings will be used to recommend a microbial safety standard for space-grown produce. Currently, this experiment is near its end, and final data are expected within a few months.

Hummerick et. al. (2018) conducted a preliminary study analyzing the microbial composition of tomatoes and lettuce sampled from three different markets, and no *E. coli*, *Salmonella*, or *Aspergillus flavus* was found. Although lettuce total microbial counts were above NASA's current standards for thermostabilized food, these levels were consistent with levels described in other peer reviewed studies (Figure 9).

Table 2. Microbial loads from crops grown in controlled environment chambers. Hummerick et al. Poster Presentation. 2018.

Crop	APC CFU/gfw	Yeast and Mold CFU/gfw
Tomato	10^4	10^3
Rom./Red Leaf	10^3	10^2-10^3
Mizuna	10^3-10^6	10^2-10^3
Radish	10^6-10^8	10^2-10^6
Peppers	10^4	10^3-10^4

CFU = Colony Forming Units, APC = Aerobic Plate Count, gfw = grams of Fresh Weight

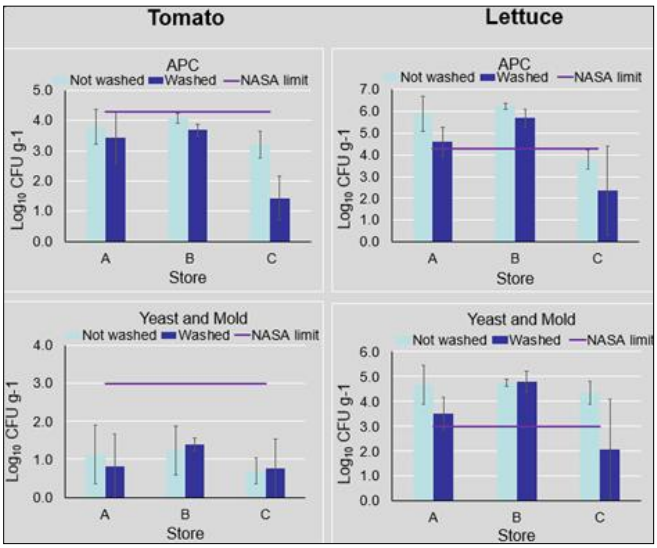


Figure 9. Bacterial and fungal APC from store-bought washed and unwashed tomato and lettuce. NASA limit shown is for non-thermostabilized food. Hummerick et al. Poster Presentation 2018.

V. Conclusion

The budding field of plant astrobiology promises to push the limits of bioregenerative life support. In fact, it is difficult to imagine a Martian settlement without crops providing a significant portion of oxygen, food, and clean water. To ensure the health of plants and the astronauts who consume them, we must first characterize crop microbial communities and determine how they change in microgravity. New microbial techniques including metagenomic 16S and ITS sequencing make this easier by allowing us to identify unculturable bacteria and fungi. As we continue to study the phyllosphere of space-grown crops, we promote the extraterrestrial symbiosis between humans, plants, and the microbes in between.

Figure 9. Artist rendition of agriculture on Mars. Image courtesy of Pat Rawlings and NASA.

VI. References

1. Massa, Gioia D., et al. "VEG-01: veggie hardware validation testing on the International space station." *Open Agriculture* 2.1 (2017): 33-41.
2. Massa, Gioia D., et al. "Plant Pillow Preparation for the Veggie Plant Growth System on the International Space Station." *Gravitational and Space Research* 5.1 (2017).
3. Sihvonen, Leila M., et al. "Strains of the cyanobacterial genera Calothrix and Rivularia isolated from the Baltic Sea display cryptic diversity and are distantly related to Gloeotrichia and Tolypothrix." *FEMS microbiology ecology* 61.1 (2007): 74-84.
4. Komaki, Hisayuki, et al. "Draft genome sequence of Thermogemmatispora onikobensis NBRC 111776T, an aerial mycelium- and spore-forming thermophilic bacterium belonging to the class Ktedonobacteria." *Genome announcements* 4.5 (2016): e01156-16.
5. Riesenfeld, Christian S., Patrick D. Schloss, and Jo Handelsman. "Metagenomics: genomic analysis of microbial communities." *Annu. Rev. Genet.* 38 (2004): 525-552.
6. Class *Ktedonobacteria*." *Genome Announcements* 4.5 (2016): e01156–16. *PMC*. Web. 11 Apr. 2018.
7. Borchers, Andrea T., Carl L. Keen, and M. Eric Gershwin. "Microgravity and immune responsiveness: implications for space travel." *Nutrition* 18.10 (2002): 889-898.
8. M.E. Hummerick, R.M. Wheeler, G.D. Massa, C.M. Johnson , L. Spencer, "Baseline Microbial Assessment of Fresh Produce" Poster Presentation. 2018.
9. Wheeler R., Massa G., Hummerick M., "Baseline Microbial Assessment of Fresh Produce" (Project Proposal) 2017.
10. M. Mickens, "Growing Plants in Space" Presentation. 2018
11. Christina L. Khodadad, Mary E. Hummerick, Lashelle E. Spencer, John A. Catechis, Jessica E. Scotten, Trent M. Smith, Nicole F. Dufour, Raymond M. Wheeler, Gioia D. Massa. ASGSR Presentation. "Microbial Community Analysis to Assess Food Safety of Crops Grown in the Veggie Plant Chambers on ISS." 2017.

Thank you so much to NASA NIFS Program and the Space Life Sciences Program for giving me the opportunity to research at KSC.