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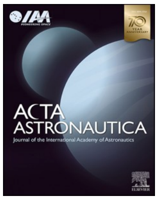

Sowing in space: Seed film enables crew-planted crops on the International Space Station

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ABSTRACT

As efforts continue toward long duration space missions, it is vital that crew members that are a part of these missions have access to whole food nutrition, dietary variety, and psychological stimulation. Space crop production research has shown that fresh vegetables could provide these properties, and, historically, this research has consisted of ground sewn seeds attached to flight hardware. The single use nature of this approach has made it apparent that crew-mediated seed sowing is necessary for sustainable food production. Scientists at NASA'S Kennedy Space Center (KSC) developed a food-safe water-soluble polymer known as seed film into which seeds can be embedded for storage and planting. The reported properties of several polymers were evaluated, and a pullulan formulation was developed, tested, and selected, with the methodology refined to produce a functional approach. This technology was utilized within the Veggie hardware for the VEG-03 J technology demonstration. 'Outredgeous' red romaine lettuce seeds were embedded in the seed film on the ground before being planted by the crew aboard the International Space Station in tandem with ground-grown plants. The mature plants were harvested and returned for molecular and microbiological analyses at KSC via next generation sequencing and plate count data, respectively, for both bacteria and fungi. The data from this experiment did not indicate any negative effects from the introduction of the seed film material, while demonstrating the potential to benefit both germination time and percent viability when compared to data from previous Veggie experiments. The technology itself is flexible in application and merits further study of its capabilities for implementation in a space crop production system.

1. Introduction

Food and nutrition are critical to health and performance and, therefore, to the success of human space exploration. As missions become longer and farther from Earth, bioregenerative foods are expected to become a larger part of the astronaut diet, provide whole food nutrition, increase menu variety, improve behavioral health, and allow human explorers to be more independent from Earth. Microgreens are one of the crop types that hold promise to produce significant quantities of food in relatively short periods of time with diverse colors, flavors, textures, and nutrition. One of the primary challenges of implementing microgreens in space crop production systems is the act of sowing the hundreds of seeds required for an ample crop yield. This challenge was addressed by the crop production and Veggie management teams at NASA's Kennedy Space Center (KSC).

A system was envisioned based on the concept of the popular "fruit roll-up" snack item, in which seeds could be embedded and stored but would dissolve upon deployment to a wet surface such as a wick or growth substrate. Numerous existing food-safe, water-soluble polymers could be used to create a seed-delivery system that would enable seed storage, astronaut seed handling, seed deployment, and film dissolution into the water system, leaving the seeds in place to germinate. Seed films and tapes are not new; the technology to create tapes and sheets is over

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Abbreviations

KSC	Kennedy Space Center
ISS	International Space Station
SVT	Science Verification Test
DAI	Days after initiation
PBS	Phosphate Buffered Saline
TSA	Trypticase Soy Agar
IMA	Inhibitory Mold Agar
PCR	Polymerase Chain Reaction
CFU	Colony Forming Units

Table 2

Processing instructions for early top candidate materials for seed film.

Polymer	Solution technique
Pullulan [3]	1 Add pullulan to stirring water. 2 Stir overnight until clear (>6 h).
Methylcellulose [4]	1 Heat about 1/3 of the required volume of water to 90°C. 2 Add METHOCEL powder to heated water with agitation. 3 Agitate until particles are thoroughly wetted and dispersed. 4 Add remainder of water as cold water or ice. 5 Continue agitation for ≥30 min
Hydroxypropyl methylcellulose [4]	Same as Methylcellulose

100 years old. A German patent dating back to 1895 describes sticking seeds to paper for uniform planting [1], and a US patent from 1915 describes placing seeds and fertilizers on a tape to initiate and sustain plant growth [2].

Materials were down-selected by focusing on polymers (e.g., pullulan, methylcellulose, and hydroxypropyl methylcellulose) that are used to coat pharmaceuticals, medicines, and foods [3,4]. Relevant properties of candidate materials are illustrated in Table 1. The polymers have comparable mechanical properties, and those properties can be altered with additives. The cellulose-based polymers recommended temperature requirements and wetting steps that pullulan did not. A distinct advantage of pullulan is its limited oxygen transmission which could provide an advantage over other food-safe, water-soluble coatings in a seed storage scenario.

Dissolving, casting, and changing material properties with food-safe additives also appeared simpler using pullulan, compared to the cellulose materials [3–6]. Thus, pullulan was chosen as the material for seed films with the goal of enabling off-planet sowing. Table 2 shows the recommended processing steps from the manufacturer of the cellulose polymers as well as an initial procedure developed internally at KSC for creating a pullulan solution. Some papers report heat treatments [7] to the pullulan solution, but those treatments did not seem necessary as a starting point for a simple solution to cast a film.

Having chosen the material, the need for food-safe additives, optimal solution viscosity for film casting, and necessary film thicknesses to hold various seeds and enable planting were determined. Several solutions were made at different pullulan: water weight ratios to assess film casting and film formation characteristics. For creating a film about the thickness of a transparency slide (0.1 - 0.2 mm) with no bubbles, 10% w/w pullulan: deionized water solution was found to produce reasonable films, but the films would tear when handled and were brittle when bent. Plasticizer was deemed necessary, and roughly half a dozen films with increasing amounts of glycerol were added to the pullulan solutions to assess the resulting dry film handling characteristics. The resultant clear films were dry to the touch, workable without cracking or tearing, thick enough to hold seeds in place, rigid enough to push into wick materials without folding or buckling, and thin enough to easily slip in between wicks. These properties were satisfactory for achieving the desired outcome.

Improvements to the film processing were made by Padgett et al. [8]. Methods were developed to create film with consistent seed distribution and introduce sterilization steps to reduce risk of microbial contamination. Films continued to be produced with the same recipe but with an additional autoclaving step which preceded film casting. Autoclaved and non-autoclaved film samples were taken for direct microbiological comparison. The sterilization of the solution, coupled with the surface sanitization of the embedded seeds, resulted in a cleaner product that was more reasonably capable of producing fresh, high-quality produce with a lower risk of contamination. While this technology was originally envisioned for microgreens, the team recognized the utility of using this approach to store NASA's seed library on orbit for a variety of candidate space crops. A library of possible seeds to grow would allow astronauts to choose their crops as desired, but seed handling in microgravity would be challenging and potentially exasperating. The Veggie growth chamber was chosen for the VEG-03 J technology demonstration, as it has been used multiple times to grow edible crops for astronauts [9]. 'Outredgeous' red romaine lettuce (*Lactuca sativa* L.) was selected for VEG-03 J to compare the effects of seed film against previous technology demonstrations in Veggie.

To test the efficacy of seed films in space, empty plant pillows and 'Outredgeous' seed film coupons were prepared separately for astronauts to plant on orbit, unlike previous experiments where seeds were affixed in the plant pillows prior to launch. This paper describes the VEG-03 J flight and ground control testing, as well as comparisons to previous Veggie technology demonstrations that utilized ground-based seed sowing, to demonstrate the function and safety of seed film as a means of handling and planting seeds in microgravity.

2. Materials and methods

2.1. Seed film preparation

Seed films were created by combining 9 g pullulan (TCI America, Portland, OR), 1 g food-grade glycerol, and 90 g deionized water and stirring the solution overnight to allow the polymer to dissolve completely. The homogenous solution was autoclaved at 121°C for

Table 1

Early candidate seed film material properties. Pullulan has comparable mechanical properties of cellulose materials with much lower O_2 transmission, as reported by the manufacturer. The asterisks show the temperature and relative humidity conditions reported in the manufacturer reports. Dashes indicate data not readily available due to variability in polymer length.

Polymer	Specific gravity	Moisture vapor	O_2 transmission	Tensile strength	Elongation
	$g\ cm^{-3}$	$g\ m^{-2}\ 24\ h^{-1}\ \mu m^{-1}$	$cm^3\ m^{-2}\ 24\ h^{-1}\ \mu m^{-1}$	MPa	%
Pullulan [3]	—	—	0.02**	41.5**	2.4**
Methylcellulose [4]	1.39	41.2*	15.3*	62*	5-15*
Hydroxypropyl methylcellulose [4]	1.29	39.7*	42.7*	69*	5-15*
		*(38°C, 50%)	*(24°C)	*(24°C, 50%)	*(24°C, 50%)
			**(25°C)	**(25°C, 75%)	**(25°C, 75%)

30 min and allowed to cool completely. Once cooled, the solution was poured into a 25 cm × 25 cm square Petri dish inside a laminar flow hood, taking care to ensure the solution was evenly distributed. The solution was air-dried for 12-24 h until the film surface was dry. Films were stored with desiccant until use.

‘Outredgeous’ seeds (Johnny's Selected Seeds, Winslow, ME) were surface sanitized for 60 min using chlorine gas-fuming, as described by Massa et al. [9] and Hummerick et al. [10]. The combination of hydrochloric acid and bleach to produce chlorine gas has been shown historically to be effective at surface sanitizing seeds. Seed germination and microbial plate tests confirmed seed viability and sanitization, respectively.

Individual, sanitized ‘Outredgeous’ seeds were placed on the film surface in groupings of three with 2.54 cm of separation between the edge of each grouping. Following placement, 10 μL aliquots of sterile, deionized water were placed on each seed before allowing the droplets to fully dry, resulting in total encapsulation of the seeds within the seed film. The grouped, encapsulated seeds were then cut into 6.45 cm^2 (1 in^2) coupons, and a small, triangular notch was cut into the bottom edge of each coupon to indicate the planting orientation (Fig. 1). Coupons were prepared on January 15, 2020, at which point they were stored in labeled, sterile, sealable bags with desiccant packets until payload preparation on January 21, 2020.

2.2. Preflight verification testing

A 28-day Science Verification Test (SVT) was conducted at KSC in a Veggie ground test unit under ISS environmental conditions. Results were positive with all seed-film plants germinating and growing within acceptable ranges during the test. Testing analysis was in-line with previous red romaine grow-outs in Veggie, and seed film was declared ready for flight.

2.3. Payload preparation

Twelve pillows (6 for flight, 6 for ground) were prepared following methods as described by Massa et al. [9]. Pillow substrate was a 1:1 mix of 600 μm-1 mm and 1-2 mm porous ceramic (Profile Products LLC Greensgrade and Turface Pro-League, respectively). Fertilizer was added into the substrate in the following amounts: Nutricote 18-6-8 T^{70} fertilizer: 1.000-1.0049 g per pillow; Nutricote 18-6-8 $T^{18}0$ fertilizer: 1.2500-1.2549 g (Florikan ESA, Sarasota, FL) per pillow for a total rate of 9 g fertilizer per liter substrate. Wicking material was cut and sterilized with ethylene oxide utilizing a 12-h exposure cycle before being allowed to off-gas in a fume hood for 7 days; biological indicators were used during the sterilization activities to confirm effectiveness. The other Veggie pillow components and root mats were treated with ethylene oxide and off-gassed. Seed film coupons were placed in labeled, sealable plastic bags. The payload was prepared on January 21, 2020, and stored until it was launched on NG-13 on February 15, 2020.

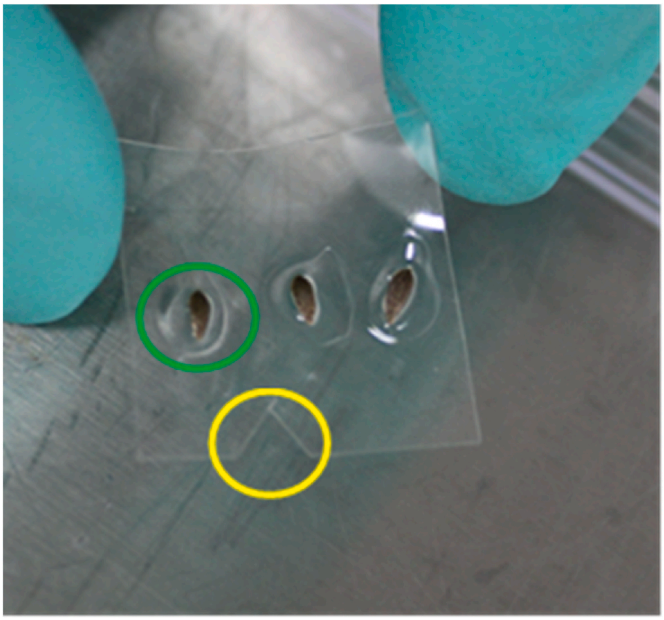

Fig. 1. Seed film coupon containing three 'Outredgeous' red romaine lettuce seeds oriented for downward radicle emergence (green circle). Planting orientation notch (yellow circle) indicates planting direction.

2.4. Flight and ground operations

The ‘Outredgeous’ plants for VEG-03 J were grown for 29 days with the flight component initiated on January 4, 2021, and harvested on February 2, 2021. All flight activities were performed according to a prepared crew instruction document. The ground control was conducted from January 6 until February 4, 2021, at KSC in a controlled environment chamber simulating ISS conditions. All ground control tasks were conducted 48 h after the corresponding flight tasks to allow for replication of on-orbit activities. The real-time ambient temperature, relative humidity, and CO_2 measurements taken inside the Columbus module of the ISS were used as setpoints for the environmental walk-in chamber in which the ground control was conducted. The actual environmental conditions inside the walk-in chamber were also tracked. In flight, one HOBO® data logger (Onset Computer Corporation, Bourne, MA) was secured at the height of the plant pillows to measure the temperature and relative humidity inside the Veggie unit. The ISS environment readings, actual ground control chamber readings, and datalogger measurements from flight are presented in Table 3. Lighting set points in Veggie were the same as those utilized during VEG-01 A [11].

Table 3
Temperature (°C), relative humidity (%), and CO_2 concentration (ppm) values from flight and ground. Arithmetic means are presented with standard deviations in parentheses. Flight ambient values are readings from the Columbus module, which were used as targeted setpoints for the ground control walk-in growth chamber. Ground ambient values are the actual measured conditions inside the growth chamber. The flight component also included records from a HOBO® datalogger, which are shown as the Veggie values. Day and night measurements have been grouped into the first half (1-14 days after initiation [DAI]; $n_{Day} = 9587$; $n_{Night} = 2970$) and the latter half (15-28 DAI; $n_{Day} = 9660$; $n_{Night} = 2939$) of the study to distinguish how plants can affect environmental conditions during their early, slower-growth stages and later, faster-growth stages.

	Flight		Ground	
	1–14 DAI	**15–28 DAI**	**1–14 DAI**	**15–28 DAI**
Temperature				
Ambient				
Day	22.9 (0.003)	23.0 (0.003)	22.6 (0.005)	22.8 (0.004)
Night	22.8 (0.005)	22.8 (0.003)	22.6 (0.006)	22.7 (0.005)
Veggie				
Day	27.6 (0.02)	24.2 (0.05)	-	-
Night	22.8 (0.02)	21.7 (0.05)	-	-
Humidity				
Ambient				
Day	36.4 (0.01)	40.1 (0.03)	36.5 (0.03)	40.1 (0.04)
Night	36.4 (0.01)	39.8 (0.04)	36.4 (0.05)	39.8 (0.05)
Veggie				
Day	74.8 (0.07)	88.5 (0.18)	-	-
Night	80.3 (0.12)	89.6 (0.31)	-	-
CO_2				
Ambient				
Day	1768 (2.7)	1914 (3.4)	1856 (3.0)	2011 (3.7)
Night	1526 (3.8)	1670 (4.6)	1598 (4.0)	1746 (4.6)

Environmental data have been analyzed using day and night groupings, following the same methodology as Bunchek et al. [12].

During planting, wicks were spread open and seed film coupons were inserted with the notch toward the bottom of the pillow allowing the top of the coupon to remain just above the top edge of the pillow gasket. Following coupon placement, wicks were returned to the closed position, and pillows were initiated with 150 mL water from the ISS Potable Water Dispenser. Three days after initiation (DAI), astronauts used forceps to spread wicks apart to assist with seedling emergence. At 7 DAI, seedlings were selectively thinned from the pillows by cutting so that each pillow contained one viable seedling. Throughout crop growth, plant pillows were watered, and the root mat was filled with 600 mL of water at 16 DAI. Watering amounts can be seen in Table 4. Plant cultivation continued until 29 DAI, at which point the plants were harvested. Harvested tissue was wrapped in pre-weighed, sterile foil sheets and placed into sterile, resealable plastic storage bags before being frozen and stored at −80°C until return to Earth.

2.5. Microbiological sample processing

Upon return, samples were collected and placed into −80°C storage at KSC. One at a time, sample bags were placed into a sanitized biological safety cabinet where foils were removed, and a subsample was collected using autoclaved instruments before rewrapping the foil. The subsample was placed into a pre-weighed 50-mL centrifuge tube containing enough sterile 3 mm glass beads to fill the conical tip portion of the tube and 30 mL of phosphate buffered saline (PBS). The tubes were reweighed after subsample collection to measure the sampled mass. This value was added to the measured net mass of the remaining frozen sample to determine the total harvested mass. The tubes containing the subsample were placed into a Bead Ruptor (Omni International, Kennesaw, GA) and shaken at 5 m s^{-1} for three 30-s intervals with 30-s rest periods between each interval. After shaking, samples were serially diluted and 100 μL of the resultant dilutions were plated in duplicate on Trypticase Soy Agar (TSA; Hardy Diagnostics, Santa Maria, CA) and Inhibitory Mold Agar (IMA; Hardy Diagnostics, Santa Maria, CA). All agar plates were incubated at 30°C for 48 h and 120 h for TSA and IMA, respectively. Following incubation, plates were removed, and their colonies were enumerated. Additionally, 1 mL of effluent from the shaken plant samples was plated on coliform and Staph Express Petrifilm Count Plates (Neogen, Lansing, MI). These selective media were utilized to test for the presence of *Escherichia coli* and *Staphylococcus aureus* which are among the most prevalent causes of foodborne illness. The results of these plates were recorded and plotted using a $\log_{10}$ transformation of the raw plate count data. The ground plant samples were frozen for the same length of time and processed via the same procedures as the flight plants.

Table 4
Water added to the Veggie pillows and root mat in Flight on each DAI. Watering protocol was determined by pre-flight testing and on-orbit imagery. Pillow values represent the average amount of water (mL) added to each pillow (n = 6), with standard deviations (mL) in parentheses. The root mat value represents the total amount of water (mL) added to the Veggie root mat (n = 1) on that DAI.

DAI	Pillow (mL pillow^{-1})	Root mat (mL)
0	150 (0)	
3	30 (0)	
5	40 (0)	
7	45 (0)	
9	30 (0)	
11	45 (0)	
13	20 (0)	
15	30 (0)	
16	0	600
18	35 (0)	
20	47 (9)	
22	50 (0)	
25	100 (0)	400
26	100 (0)	
27	100 (0)	
28	95 (11)	
29	100 (0)	
Total	**1017 (17)**	**1000**

Following the plant harvest, individual plant pillows for both the flight and ground control experiments were also placed into sterile, resealable plastic storage bags before being flash-frozen and stored at −80°C. Once the flight pillows were returned to KSC, all pillows were individually removed from the storage bags for photographs before being cut open to access to the wicks, roots, and substrate. Each of these commodities were collected and processed using similar procedures as the associated plants with the same Bead Ruptor cycle settings; however, the 50-milliter centrifuge tubes instead were filled with 30 mL of Buffered Peptone Water and 3 mm glass beads. The shaken samples were plated and incubated in the same way as the previously mentioned plant samples.

2.6. Molecular methods for sample processing

All samples were obtained post-microbiological sampling. Samples from flight and ground were processed similarly. Swab samples were received for molecular analysis in approximately 7 mL of PBS. Approximately 1.5 mL of each sample was centrifuged at 13,000 rpm for 5 min until the sample was processed in its entirety. Once the sample was pelleted, 1 mL of RNALater® was added, and the samples were frozen at −20°C. Plant leaf samples contained in 50-mL Falcon tubes with PBS were placed in an Omni Bead Ruptor at 5 m s^{-1} for two 30-s cycles with a 20-s dwell time (OMNI International, Inc. Kennesaw, GA). Material was filtered in a sterile 70-μm strainer basket to collect all the fluid. Recovered fluid was centrifuged at 13,000 x g for 10 min. Each resulting pellet was resuspended in RNALater® as described above. The plant root tissue and wick material recovered from each returned Veggie pillow were suspended directly in RNALater® and stored at −80°C until processing could be completed.

DNA was isolated from each sample using the Qiagen DNEasy Microbial Cell Kit (Qiagen, Germantown, MD) per the manufacturer's protocol and eluted in 50 μL buffer without EDTA. DNA was quantified using the QUBIT ds high sensitivity DNA assay or the Promega QUANT-IT Kit if below QUBIT detection limits. Polymerase chain reaction (PCR) was completed in duplicate using 1 ng of DNA per reaction and dual unique barcoded 16S rRNA gene primers for bacteria [11]. Enzymatic cleanup of the PCR reactions was completed using the Qiagen MinElute Kit (Qiagen, Germantown, MD). A normalized library was created and sequenced on an Illumina MiSeq instrument with a V2 500 cycle Illumina sequencing kit. To isolate and identify fungi, a 10-ng aliquot of DNA from each sample was processed using the ZYMO Quick ITS library preparation kit following the manufacturer's protocol. The resulting library was sequenced on the Illumina MiSeq sequencer using a V3 600 cycle sequencing kit.

2.7. Statistical analysis

Environmental, crop growth, and watering data were analyzed for arithmetic means and standard deviations in R version 4.4.2. Microbiological data for both heterotrophic plate counts and yeast and mold plate counts were analyzed with a one-way ANOVA with Tukey's multiple comparison tests utilizing Prism version 10.3.1 (GraphPad Software, Boston, MA). Reported mean values were displayed along with standard deviations. Values were assigned significance based on their p-values: $^{*}p < 0.1$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

FASTQ sequences were trimmed and processed using SILVA and UNITE databases to identify and enumerate reads for bacteria or fungi, respectively. The 16S rRNA bacterial amplicon sequences were retrieved from QIIME 2 V. 2024.5.0 through Conda V. 25.3.1 with taxa classification achieved through Greengenes2 V. 2022.10 [13,14]. Percent stacked bar charts for relative abundance were produced through ggplot2 package V. 3.5.1 in addition to the dplyr V 1.1.4 package with resulting plot layouts customized through the patchwork package V 1.3.0 [15–17]. Relative abundance of taxa was presented as the percentage of each taxon within each sample type. Taxa displayed in the stacked bar charts were limited to the top 20 most abundant microbial genera, allowing a visual representation of the dominant taxa. The percent stacked bar chart for 16S sequences excluded two undefined microbial taxa, JC017 and Kingdom Bacteria, from the top 20 to ensure their high abundance did not interfere with representation of the other taxa present. ITS sequences were analyzed via Kraken 2 [18]. A custom database was created with ITS specific DNA sequences retrieved from UNITE database (UNITE general FASTA release for Fungi 2; [19] to map with Kraken 2. Percent stacked bar plots for relative abundance were created for ITS sequences using the same R script and methods as described for 16S sequences. Visualization of both 16S and ITS relative abundance plots was performed in R version 4.1.2.

As sufficient sample size for alpha and beta diversity was lacking, further diversity analyses were limited. To assess mass variability of harvest samples, the coefficient of variation was applied. Coefficient of Variation (CV) was calculated as the standard deviation divided by the sample mean and multiplied by 100. Results were interpreted as low (<15%), moderate (15-25%), or high (>30%) variability.

3. Results

3.1. Crop growth

Crop performance parameters from VEG-03 J indicate that 'Outredgeous' grown in seed film performs well when compared to previous studies (Table 5a). As seed film was a new technology, three seeds were inserted into each Veggie pillow, instead of the former seeding rate of two per pillow, to increase chances that each pillow would ultimately have at least one successfully germinated and established plant. Germination on the ISS was highly successful (94%) with only one out of 18 seeds failing to germinate. Ground germination was comparatively lower (67%), potentially due to water behavior issues; future testing is anticipated to clarify this. All pillows in both flight (total harvested plants [n_2] = 6) and ground ($n_2 = 6$) had successful plant growth for the entire test (Fig. 2), which had been a challenge in the past. Uniformity between plants was higher in later studies, and flight and ground plants both showed similar averages and variability, with flight plants producing slightly higher fresh masses (Table 5b). VEG-03 A ground produced more overall fresh mass than VEG-03 J ground due to longer growth period and multiple harvests. Nonetheless, adjusted values show that 'Outredgeous' had a higher growth rate in VEG-03 J ground (VEG-03 A Ground: 0.51 g plant^{-1} day^{-1}; VEG-03 J Ground: 0.93 g plant^{-1} day^{-1}). External factors like the experience gained from each study have contributed towards this improved success, but seed film additionally caused no adverse effects on 'Outredgeous'.

Table 5a

'Outredgeous' lettuce growth during ground control and flight testing in Veggie. Sample size at germination (n_1), germination (%), days of growth, sample size at final harvest (n_2), and average harvested fresh mass per plant (g) with population standard deviation are presented. Flight data were not gathered for VEG-01 B and VEG-03 A due to planned consumption of crops by the crew. Number of seeds planted (n_1) was either 2 (12 total) or 3 (18 total) per pillow. Number of plants (n_2) at a rate of 1 plant per Veggie pillow are shown at the time of harvest (Days). Germination (%) is based on n_1, and mass (g plant^{-1}) is based on n_2. *Water could not enter one of the pillows, so only 5 pillows were viable. **Sum of 4 harvests over 64 days of growth.

Study	n_1	Germination (%)	Days	n_2	Mass (g plant^{-1})
VEG-01 A Flight	12	75	33	3	20.61 ± 9.52
VEG-01 B Flight	10*	70	33	5	n/a
VEG-03 A Flight	12	100	64	6	n/a
VEG-03 J Flight	18	94	29	6	28.25 ± 6.48
VEG-01 A Ground	12	83	33	5	15.29 ± 8.59
VEG-01 B Ground	12	75	33	4	9.16 ± 1.59
VEG-03 A Ground	12	92	64	5	32.44 ± 8.54**
VEG-03 J Ground	18	67	29	6	26.94 ± 4.43

3.2. Microbiological analysis

3.2.1. Quantified comparison of microbes between Veggie Technology Demonstrations

The culturable microbial load of all returned lettuce samples can be seen in Fig. 3 with statistical significance indicated. Countable TSA plates of the processed flight samples yielded bacterial data for flight and ground, representing a total heterotrophic plate count. VEG-01 A-grown lettuce had a significantly lower bacterial count than samples from VEG-03 J ($p < 0.01$). The bacterial load of flight samples from VEG-01 B was not significantly different than either VEG-01 A or VEG-03 J ($p > 0.05$). Each set of flight samples was significantly different from their ground counterpart with p-values of 0.0073, <0.0001, and <0.0001 for VEG-01 A, VEG-01 B, and VEG-03 J, respectively. VEG-01 A was the only sample set with a lower mean heterotrophic plate count in flight, but this could potentially be attributed to the limited sample size of the flight data set. The VEG-01 B ground samples were also found to be significantly different from the ground samples of VEG-03 J ($p = 0.0476$). Despite having a higher mean heterotrophic plate count value than other Veggie technology demonstrations, the VEG-03 J samples returned negative results on all performed pathogen-specific screening tests. These results were corroborated by the absence of pathogenic organisms in the 16S rRNA data.

The samples also returned countable IMA plates to provide yeast and mold data for flight and ground. None of the sample sets differed significantly from each other between technology demonstrations or between flight and ground experiments ($p > 0.05$). All the ground samples, including those collected in VEG-03 J, were found to have a mean culturable fungal load lower than the current NASA safety standards for non-thermostabilized foods: 1×10^3 Colony Forming Units (CFU) g^{-1} [20]. The only flight samples below this threshold value were from VEG-01 A ($\mu = 5.6 \times 10^2$ CFU g^{-1}). At present, these are the closest approximate standards for the evaluation of food safety of fresh grown produce in a flight scenario. Other standards for the evaluation of food safety of these commodities in situ are in development.

3.3. Molecular biology analysis

3.3.1. Percent relative abundance of bacteria between Veggie Technology Demonstrations

The bacterial communities from the lettuce leaves of the three technology demonstrations – VEG-01 A, VEG-01 B, and VEG-03 J – were similar. *Acinetobacter* and *Burkholderia* were present in both flight and ground samples including both leaf and root tissues. Bacterial taxa present in both flight and ground root included *Burkholderia, Bradyrhizobium, Sphingomonadaceae*, and *Ralstonia*. The roots, wick, and substrates shared several bacteria in high relative abundance. For example, *Burkholderia, Ralstonia, Sphingomonas, Mesorhizobium,* and *Bradyrhizobium* appear in most root, substrate, and wick samples (Fig. 4). Since these materials were near or in contact with each other, this is not unexpected.

3.3.2. Percent relative abundance of fungi between Veggie Technology Demonstrations

The top 20 fungi from the three Veggie technology demonstrations indicated that VEG-01 A and VEG-01 B were similar: *Microidium* dominated the flight and ground leaf tissue, as well as the ground root tissue, but with lower relative abundance in the flight root tissue (Fig. 5). VEG-03 J flight tissue was dominated by *Aureobasidium*, *Rhodotorula*, and *Fusarium*. Across all three technology demonstrations, Veggie pillow components such as the substrates and wicks contained many of the

Fig. 2. Flight (left) and ground control (right) ‘Outredgeous’ at 28 DAI. Flash was used on the camera to take the flight photo.

Table 5b
‘Outredgeous’ lettuce growth during ground control and flight testing in Veggie. Data show average harvested fresh mass per plant (g) with population standard deviation, Coefficient of Variation (CV), and the interpretation of that Coefficient of Variation. CV was interpreted as low (<15%), moderate (15-25%, and high (>30%). Flight data were not gathered for VEG-01 B and VEG-03 A due to planned consumption of crops by the crew. *Sum of 4 harvests over 64 days of growth.

Study	Mass (g plant^{-1})	Coefficient of Variation (%)	CV Interpretation
VEG-01 A Flight	20.61 ± 9.52	46.19	High
VEG-01 B Flight	n/a	n/a	n/a
VEG-03 A Flight	n/a	n/a	n/a
VEG-03 J Flight	28.25 ± 6.48	22.94	Moderate
VEG-01 A Ground	15.29 ± 8.59	56.18	High
VEG-01 B Ground	9.16 ± 1.59	17.36	Moderate
VEG-03 A Ground	32.44 ± 8.54*	26.33	Moderate
VEG-03 J Ground	26.94 ± 4.43	16.44	Moderate

same genera of fungi seen in the leaf and root with similar distribution. All three Veggie flight substrates contained *Alternaria, Rhodotorula,* and *Fusarium,* while *Purpureocillium* was only detected in VEG-03 J samples (Fig. 5). However, VEG-03 J also contained a 30-50% higher relative abundance of *Rhodotorula* in root, substrate, and wick samples when compared to samples from VEG-01 A and VEG-01 B (Fig. 5). The *Purpureocillium* unique to VEG-03 J was found to have <10% relative abundance in all flight samples as well as the ground leaf, root, and wick samples, but the ground substrate sample had a significantly larger presence of *Purpureocillium* with a relative abundance of ~50%.

4. Discussion

The VEG-03 J technology demonstration validated the use of seed film to cultivate spaceflight-grown crops. There were multiple goals of this study: to confirm seed film as a viable space seeding approach, to assess crop growth, to confirm microbiological food safety of the crops grown utilizing seed film as compared to prior technology demonstrations which did not utilize it, and to understand the microbial community impact that resulted from the use of seed film. This study confirmed that astronauts can utilize seed film technology to directly plant seeds in a microgravity environment, furthering NASA's goals to create a seed library from which crews can choose desired crops. Evidence of this

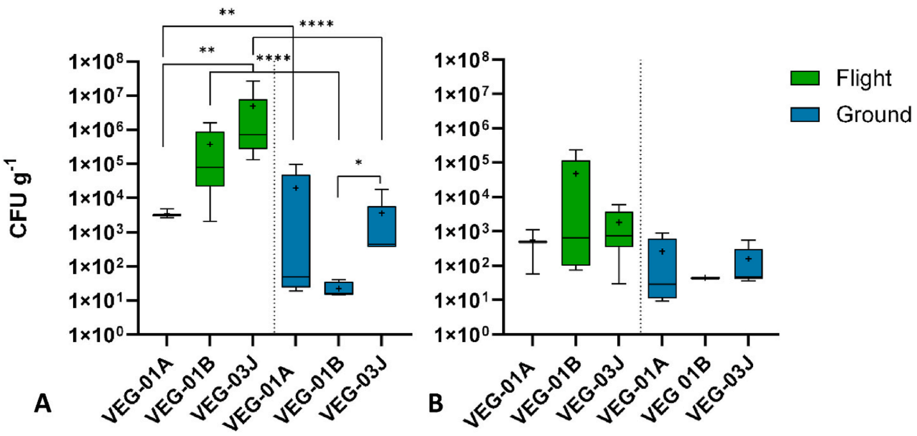

Fig. 3. Culturable microbiological data. Each graph represents either Heterotrophic Plate Count (A) or Yeast and Mold Plate Count (B). Bars represent the minimum and maximum values of a given data set. Statistical significance relationships are represented by pairing lines with p-values represented as follows: *p < 0.1, **p < 0.01, ****p < 0.0001 Note: no statistical differences were found between any yeast and mold counts (p > 0.1).

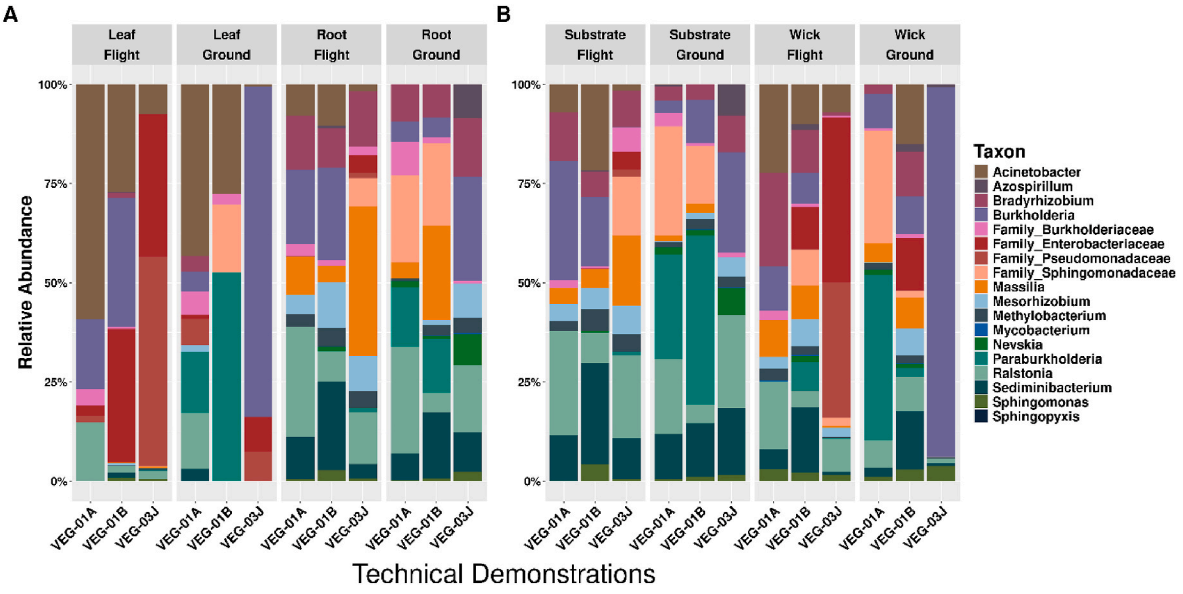

Fig. 4. Percent relative abundance stacked bar chart of the top 20 bacteria isolated from flight and ground leaf and root plant tissue from ‘Outredgeous’ lettuce (A) and the top 20 bacteria isolated from flight and ground Veggie pillow wick and substrate (B). 18 of the top 20 taxa are displayed due to exclusion of undefined taxa JC017 and Kingdom Bacteria whose high abundance interfered with the representation of the remaining bacterial taxa. The bar chart illustrates a comparison with other Veggie technology demonstrations (VEG-01 A and VEG-01 B) completed on the ISS.

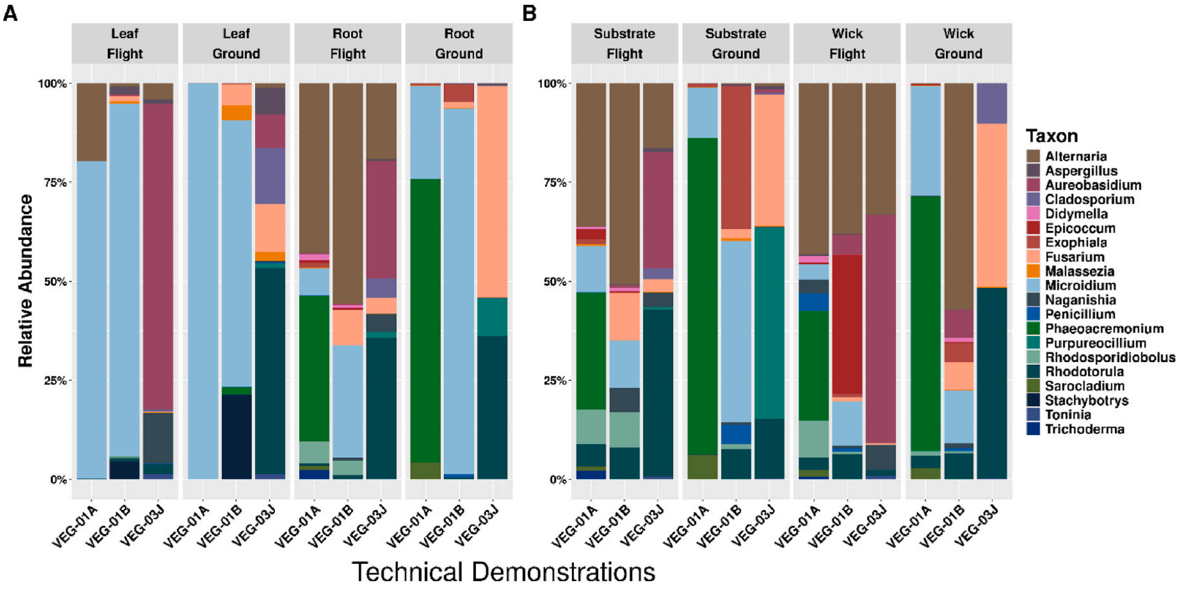

Fig. 5. Percent relative abundance stacked bar chart of the top 20 fungi isolated from flight and ground leaf and root plant tissue from ‘Outredgeous’ lettuce (A) and the top 20 fungi isolated from flight and ground Veggie pillow wicks and substrates (B). The bar chart illustrates a comparison with other Veggie technology demonstrations (VEG-01 A and VEG-01 B) completed on the ISS.

utilization can be seen in the supplementary materials. Ensuring food safety warranted a deeper investigation to determine any effects this method might have on the plant microbiome or crops during cultivation.

Crop growth with seed film was excellent, and the increased uniformity seen on the ISS indicates that seed film may have produced a nurturing environment for germinating seeds in the altered fluid physics of microgravity. Encapsulating seeds in a high-humidity membrane possibly helped retain moisture to aid germination and establishment, similar to seeds that naturally produce polysaccharide mucilage, notably those in the *Salvia* (e.g., chia) and *Ocimum* (e.g., basil) genera within Lamiaceae [21,22]. Seed pelleting or treatments using surfactants may generate a comparable impact by improving water distribution to the seeds. Indoor crop production facilities on Earth often use mist or humidity chambers for seedling nurseries. In the low-humidity environment of the ISS, the seed film may replicate this function. Plant growth after germination was successful as well, with some plants even exhibiting mild tip burn, which is indicative of rapid growth [23]. Whether or not these benefits remain and the impact of the seed film on embedded seed viability would need to be studied in future work.

In terms of food safety, despite the mean value for total culturable heterotrophic bacteria being higher in VEG-03 J than either VEG-01 A or VEG-01 B, the value was well within reason, as other studies have reported a range of total aerobic bacteria between 4 and 10 $\log_{10}$ CFU g^{-1} [24–28]. The absence of pathogenic microbes suggested that properly handled seed film would not be a likely source of these organisms. Seed film's potential impact on the proliferation of microbes should be further studied, but, for the purposes of this experiment, it did not appear to impact the produce from a food safety perspective. The primary impact was the increased proliferation of bacteria which could potentially be attributed to the pullulan as an additional carbon source.

The 16S relative abundance plot revealed that bacterial communities across VEG-01 A, VEG-01 B, and VEG-03 J were well-conserved. Across leaf, root, substrate, and wick samples from flight, the bacterial genera *Acinetobacter* and *Ralstonia* were consistently present. While *Ralstonia* displayed similar relative abundances between the technology demonstrations, lower relative abundances of *Acinetobacter* were exhibited in VEG-03 J. *Acinetobacter* is a well-known plant growth promoter that is commonly associated with rhizosphere microbial communities [29,30]. *Ralstonia* is a waterborne microorganism that has been retrieved from the ISS Water Processor Assembly (WPA) [31]. The *Ralstonia species R. solanacearum* has been identified to cause bacterial wilt disease in tomato [32,33]. However, *Mesorhizobium,* family *Pseudomonadaceae,* and *Sphingomonas* genera, which were abundant across flight samples between VEG-03 J and one or both prior technology demonstrations, were correlated to healthy plant microbiomes resistant to *R. solanacearum* infection [32,33].

For root, substrate and wick flight samples, bacterial genera *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Massilia*, *Sediminibacterium* and *Sphingomonas* were consistently present across the three technology demonstrations. The family *Burkholderiaceae* was shared between VEG-03 J and one or both VEG-01 A and VEG-01 B in these same samples. Family *Enterobacteriaceae* was shared across all three technology demonstrations for leaf flight samples but was unique to VEG-03 J for root and substrate flight samples. These microorganisms are members of the typical plant microbiome. *Bradyrhizobium* and *Mesorhizobium* are nitrogen-fixing bacteria belonging to the plant rhizobia, and *Methylobacterium* has been found to dominate the microbial communities of plant shoots [34]. Additionally, *Massilia* and *Sphingomonas* are plant beneficial microorganisms that promote plant flowering and plant growth and drought resistance, respectively [35,36].

Bacterial communities between flight and ground conditions were highly similar across the three technology demonstrations with minimal differences. *Ralstonia,* while present in VEG-03 J flight leaf samples, was not present in the leaf ground samples of VEG-03 J and so may indicate its introduction through the ISS water, as mentioned previously. The bacterial communities between flight and ground VEG-03 J root and substrate remained consistent, except for the absence of the family *Sphingomonadaceae* and *Massilia* from the top 20 taxa in the ground conditions. Both VEG-03 J wicks and leaves were dominated by *Burkholderia* in the ground samples but otherwise displayed similar bacterial communities to their flight counterparts.

A notable difference was the absence of *Burkholderia* from the top 20 taxa of the VEG-03 J flight samples, although present in the top 20 taxa in VEG-01 A and VEG-01 B. *Burkholderia* dominated the VEG-03 J ground leaf, root, substrate, and wick samples. For flight leaf and wick samples, the absence of *Burkholderia* in VEG-03 J appeared to be substituted by a dominance of the families *Enterobacteriaceae* and *Pseudomonadaceae,* while *Sphingomonadaceae* and *Massilia* appeared to dominate in place of *Burkholderia* in the flight root and substrate samples. This is an interesting observation, as *Burkholderia* would be expected to be prominent in the flight microbial communities due to its inclusion in the five most isolated microorganisms from the ISS WPA (i.e., *Burkholderia, Ralstonia, Methylobacterium, Cupriavidus,* and *Pseudomonas*) [31]. *Pseudomonadaceae* includes species that are responsible for response to *Fusarium* infection in the phyllosphere and resistance to wilt disease, and *Enterobacteriaceae* includes rhizosphere plant growth-promoting species [37,38]. The presence of *Enterobacteriaceae* in the leaf and wick flight samples of VEG-03 J is not unique, as *Enterobacteriaceae* has been reported as a dominant member of the core phyllosphere microbiome of another leafy green crop: arugula (*Eruca sativa* Mill.) [39]. Its dominance in the flight leaf samples rather than the root or substrate samples may indicate its suitability for that environment more so than a disruptive impact on the microbial community. The dominance of *Sphingomonadaceae,* along with the presence of *Massilia*, in the root and substrate flight samples follows their characterization as plant growth-promoting and infection-preventing microorganisms that inhabit the plant rhizosphere [40,41]. In place of *Burkholderia* then, these two bacterial taxa appear to dominate the microbial community as natural inhabitants of the plant root and surrounding substrate.

Similarly, the fungal communities as observed by the top 20 taxa between VEG-01 A and VEG-01 B to that of VEG-03 J are generally well conserved. *Alternaria, Aspergillus,* and *Fusarium* were present across all three technology demonstrations in leaf and root flight samples with a more even distribution of relative abundance in the root. While *Alternaria* is generally recognized as an opportunistic plant pathogen, it is also a prevalent member of plant microbiomes globally [42,43]. Additionally, any concern for potential pathogenic effects of *Alternaria* was mitigated in the VEG-03 J microbial community, as VEG-03 J consistently expressed reduced relative abundance for it across all flight samples as compared to VEG-01 A and VEG-01 B.

The fungal genera observed between the three technology demonstrations are commonly associated with plant microbiomes. The recent work of Chau et al. [44] identified the fungi *Cladosporium, Alternaria,* and *Aureobasidium* as co-abundant members dominant in the microbiomes of flowering plant species according to relative abundance. *Aureobasidium* is a plant beneficial microorganism that is used as a means of biocontrol [45]. These three fungal genera were identified in high relative abundance across the flight samples of VEG-03 J and exhibited particularly higher relative abundances of *Aspergillus* and *Aureobasidium* than in VEG-01 A and VEG-01 B, which may indicate a well-proportioned microbiome in respect to these three genera. Many of these fungal genera are associated with the human oral mycobiome, including *Cladosporium*, *Aspergillus*, *Fusarium*, *Penicillium,* and *Alternaria* [46]. This may indicate the plant microbiome influences not only from the spaceflight environment but that of the crew aboard the ISS.

Fungal communities between flight and ground conditions were consistent for each technology demonstration with few differences. For all three technology demonstrations, *Alternaria* was absent from the top 20 taxa for the root and substrate ground samples, possibly indicating that *Alternaria* was introduced or favored by the spaceflight environment. A higher relative abundance of *Fusarium* was present for the VEG-03 J leaf, root, substrate, and wick ground samples than in the corresponding flight samples; however, the overall community profiles between these VEG-03 J flight and ground samples were similar.

Differences in fungal communities between experiments were primarily observed by the unique presence of *Rhodotorula, Aureobasidium,* and *Purpureocillium* in VEG-03 J as well as by the absence of *Microidium* and *Didymella* genera that were present in both VEG-01 A and VEG-01 B. *Microidium* was highly dominant in the flight and ground leaf samples of VEG-01 A and VEG-01 B. Its absence in VEG-03 J was likely beneficial, as *Microidium* overgrowth can contribute to a powdery mildew disease that inhibits photosynthesis, stunts growth, and causes premature leaf loss [47,48]. Additionally, *Didymella* was also absent from VEG-03 J but was present in the root and substrate flight samples of VEG-01 A and VEG-01 B at low relative abundances. Huang et al. [49] correlated lower relative abundances of *Didymella* to a healthier plant microbiome, as *Didymella* is an opportunistic pathogen responsible for tobacco leaf spot disease. For all VEG-03 J samples, a higher relative abundance of *Rhodotorula* was observed. *Rhodotorula* is a common environmental and human microbiome associated yeast, and a species of *Rhodotorula* (*R. graminis)* has been found to be a plant-growth promoting endophyte [50,51]. *Purpureocillium* presence in the top 20 taxa was unique to VEG-03 J flight and ground leaf, root, and substrate samples. *Purpureocillium* is an entomopathogenic fungus that has been used for biocontrol, with recent studies identifying its role in plant growth promotion due to modulation of the plant microbiome and enhanced nutrient absorption [52]. In spaceflight, *Purpureocillium* demonstrates increased virulence against the spider mite *Tetranychus cinnabarinus*, a significant crop pest [53].

The data here suggest that the bacterial and fungal communities of technology demonstrations VEG-01 A, VEG-01 B, and VEG-03 J share characteristics typical of healthy plant microbiomes. Minor differences in VEG-03 J compared to VEG-01 A and VEG-01 B are not of concern and may even present a more favorable microbiome. No food safety concerns or presence of human pathogens are noted for the bacterial or fungal community of VEG-03 J.

5. Conclusions

While seed film as a technology is not a new concept and has existed for many years, prior to this implementation, no formulation had existed that was specifically intended for spaceflight applications or the challenges that those applications can represent. The VEG-03 J technology demonstration proved that seed film could successfully provide a planting method for the crew. This is especially important when considering the potential negative outcomes associated with failed or contaminated crops during future missions where these commodities will be an important food source. While it warrants further study, the initial impression of seed film is positive with no negative crop growth effects noted in microgravity, and it may have even yielded improved germination, growth uniformity, and overall harvest yield. On average, the additional carbon from the seed films likely led to a higher aerobic bacterial load but did not introduce any causative organisms of food safety concern. Specific community changes may be due to changes in the environmental microbiome of the ISS rather than being directly caused by the seed film. As mentioned previously, further study is warranted into the full impact of seed film on crop growth as well as its long duration efficacy as a storage medium, but initial results from this technology demonstration are promising. NASA recently launched more seed film to the ISS on Crew-11 with 18 plant pillows [54]. VEG-03MNO is an opportunity for on-orbit crews to grow and eat preference crops and for further evaluation of seed film performance.

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CRediT authorship contribution statement

Aaron Curry: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Jess Bunchek:** Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Cristiana Morales:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Christina Khodadad:** Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Mary Hummerick:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Cory Spern:** Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Gioia Massa:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Trent Smith:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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