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# Pick-and-eat space crop production flight testing on the International Space Station

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**ABSTRACT**

Fresh, nutritious, palatable produce for crew consumption on long-duration spaceflight missions may provide health-promoting, bioavailable nutrients and enhance the dietary experience. VEG-04A and VEG-04B explored growing leafy greens on the International Space Station using the Veggie Vegetable Production System. Two flight tests with ground controls were conducted in 2019 growing mizuna mustard, where Veggie chambers were set to different red-to-blue-to-green light formulations. Light quality affects plant growth, nutrition, microbiology, and organoleptic characteristics on Earth, and we examined how these vary in microgravity and under different harvest scenarios. Astronauts harvested and weighed mizuna and completed organoleptic evaluations. Flight samples were returned to Earth for nutritional quality and microbial food safety analyses. Yield and chemistry differed between ground and flight samples and light treatments, and bacterial and fungal counts were lower in ground than in flight samples. This research helps increase our understanding of the requirements for growing high-quality crops in spaceflight.

**Policy highlights**

- The Veggie system can provide astronauts with nutritious, safe-to-eat produce that they enjoy eating, which can help crews stay healthy during long-duration space missions.
- The duration and method of growing and harvesting crops can influence the yield, organoleptic acceptability, microbial load and food safety, nutritional content, and resources required. A decision on optimum methods will likely involve trade-offs and needs to be weighed against mission objectives.
- The light spectrum used to grow plants impacts the growth and nutritional content of leafy green crops.
- Continued research in this area is recommended to test additional crops and to increase research sample sizes.

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Food safety; International Space Station; nutrition; plant growth; space-crop production; Veggie

## Introduction

Salad crop production is one approach that NASA is researching with potential to help support a nutritious and acceptable food system on long-duration missions beyond low Earth orbit (LEO). The packaged space diet, while diverse, nutritious, and safe, decreases over time in quality aspects and vitamin content, such as Vitamin C, under ambient storage conditions that are relevant for space missions (Cooper et al. 2017). Additional options besides vitamin supplements to the packaged diet are needed, as supplements can become toxic at high levels, do not support the psychological aspects of food, do not support caloric and macronutrient intake, degrade with storage, and lack synergistic benefits from the phytochemicals in whole-food delivery (Lane and Schoeller 2000; Liu 2003; Basu and Imrhan 2007; Zwart et al. 2009; Polivkova et al. 2010).

Alternatively, growing and adding fresh, pick-and-eat produce to the space diet may supplement nutrition and reduce menu fatigue by adding variety to the food system (Perchonok et al. 2012). Growing and consuming fresh produce may also support astronaut behavioral health and performance, especially as mission duration and the distance from Earth increase and astronauts can no longer receive fresh produce in resupply, as they do limitedly on the International Space Station (ISS). NASA has developed crop assessment methodology to screen salad crops for supplementing the packaged diet, assign weighted scores, and evaluate crop growth, nutritional composition, and organoleptic acceptability (Massa et al. 2015; Spencer et al. 2019). This approach aligns with NASA's proposed Crop Readiness Levels for screening different crop types (Romeyn et al. 2019).

NASA's Vegetable Production System, or 'Veggie,' has been in operation on the ISS since May 2014, with a second chamber added in 2017. Veggie is a simple low-power, low-mass plant growth system with adjustable red, blue, and green LED lights, a controllable fan, and transparent,

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flexible bellows to draw the ISS atmosphere through the plant canopy (Morrow et al. 2005; Morrow and Remiker 2009; Massa et al. 2016). Plants can be grown in experiment-unique hardware, but most larger crop plants are grown in plant 'pillows' – small flexible containers filled with a porous ceramic substrate and controlled-release, polymer-coated fertilizer (Massa et al. 2017b) that passively interact with a root mat reservoir. A number of leafy green vegetable crops have been grown in the Veggie units and consumed by astronauts, with additional samples returned to Earth for food safety analyses (Massa et al. 2017a; Khodadad et al. 2020; Hummerick et al. 2021). Veggie is the primary plant growth system that NASA has used to test candidate spaceflight crops and horticultural practices for future spaceflight dietary inclusion.

The goals for VEG-04A and VEG-04B were to advance salad crop production in space by assessing whether yield, nutrition, and microbial quality of a leafy green crop varied when grown under different lighting conditions in Veggie. The leafy green crop initially selected was 'Tokyo Bekana' Chinese cabbage based on prior crop selection experiments (Massa et al. 2015). While 'Tokyo Bekana' had excellent yield and organoleptic acceptability, as well as satisfactory nutrition, testing revealed that this crop exhibited stress responses under spaceflight-relevant, elevated $CO_2$ (Burgner et al. 2019, 2020). Therefore, VEG-04 testing proceeded with mizuna, a variety of mustard that also demonstrated an excellent combination of growth, nutritional composition, and organoleptic acceptability in prior tests (Massa et al. 2015), but did not show similar stress responses (Massa 2017). Both Chinese cabbage and mizuna are brassicaceous crops with known health-promoting secondary metabolites (Neugart et al. 2018). Mizuna has a significant spaceflight heritage: in Veggie during the VEG-03 missions (Hummerick et al. 2021) and in Russian spaceflight hardware on both the space station MIR (Sychev et al. 2001; Ivanova 2002) and the ISS (Bingham et al. 2003; Sugimoto et al. 2014; Morrow et al. 2017).

Responses to light quality have been studied since the earliest eras of plant biology and crop research; however, studies to better understand these impacts were limited by the tools available at that time (Graham et al. 2019). In the past 30 years, LEDs have become a tool for both better understanding unique plant responses and for controlled environment agriculture (CEA) crop production (Bula et al. 1991; Barta et al. 1992; Mitchell 2022). LEDs are the best available electric lighting option for space crop production due to their solid-state nature and durability, long lifetime, small size, cool surface temperature, and wide range of selectable wavelengths (Massa et al. 2006, 2008; Bourget 2008). Light spectral quality impacts on crop yield and composition have been shown to vary, often with conflicting results observed between different crops and crop cultivars, growth conditions, and the stage of plant growth examined (e.g. Sergejeva et al. 2018). Effects also vary by the desired output. Blue wavelengths in the lighting recipe can contribute to accumulation of health-promoting secondary metabolites, such as bioprotective compounds in red lettuce (Stutte et al. 2009) and glucosinolates and mineral elements in microgreen and baby green brassica crops (Kopsell et al. 2015). Crop-specific custom lighting protocols are being developed to stimulate characteristics of interest in fresh produce grown with sole-source or supplemental LED lighting in terrestrial agricultural systems, but lighting protocols for crops are not well understood in spaceflight, where adaptation to microgravity and altered environmental conditions may modify desired outcomes.

Light spectrum impacts on microbiological safety are of utmost importance in spaceflight, where cleaning, sanitation, testing capabilities, and access to medical support are confined and limited. Outbreaks or recalls of crops like leafy greens due to the presence of human pathogens *Salmonella spp.*, *E. coli*, and *Listeria spp.* highlight the significance of this risk (Takkinen et al. 2005; Taylor et al. 2013; Tataryn et al. 2014). While CEA systems are perceived to be at a lower risk of external contamination from foodborne pathogens, this depends on many factors such as hygienic practices, irrigation water quality, and pest control (Steele and Odumera 2004; Taylor et al. 2013; Wadamori et al. 2017), and opportunities for contamination, especially through irrigation water, are still present (United States Food and Drug Administration [FDA] 2022). Crops grown on the ISS have stringent levels of microbial contamination control. However, there are scenarios unlike terrestrial agriculture that cannot be controlled, such as microgravity, ISS environmental sources of contamination, and spaceflight-induced water stress in crops (Porterfield 2002). Since the first Veggie study in 2014 with red romaine lettuce, microbiological testing has been performed on plants and surfaces of the Veggie facility on the ISS to understand potential risks associated with growing edible crops (Massa et al. 2017a; Khodadad et al. 2020; Hummerick et al. 2021). The resident microbiota on the ISS and environmental conditions such as temperature, elevated $CO_2$, and humidity may play a role in determining colonization by microbes on crops grown in Veggie (Khodadad et al. 2020; Hummerick et al. 2021). A review by Mogren et al. (2018) on the subject of controlling food safety risks in a system applied to leafy green production addresses the effects of irradiation, including the visible spectrum on phyllosphere microbiota. Depending on the presence of bacterial or fungal photoreceptors, different light wavelengths can influence growth patterns and gene regulation of virulence genes, as well as the production of plant-growth-promoting metabolites (Gharaie et al. 2017; Losi and Gärtner 2021). Blue wavelengths can have biocidal effects on bacteria *in vitro* such as *Staphyloccocus aureus, E. coli*, and *P. aeruginosa* (Mckenzie et al. 2014; Bache et al. 2018; Haridas and Atreya 2022). This benefit could potentially translate to the plant growth environment.

We report here on tests with mizuna plants grown on the ISS under different red: blue light ratios and how the spaceflight environment affected organoleptic acceptability, growth, nutrition, and microbial food safety when compared to ground-grown crops. A second research question asked if the duration of growth and/or the cut-and-come-again repetitive harvest approach impacted nutrition or food safety. This question helps address important aspects of crop scheduling, materials and inputs, and sustained productivity.

## Materials and methods

### *Preflight preparations*

#### *VEG-04A and VEG-04B test overview*

Two experiments were conducted on the ISS, with ground controls conducted at a slight offset to prevent scheduling

overlap between flight and ground operations. While both experiments grew the same crops under the same light conditions, VEG-04A was designed to be a short-growth test with a single terminal harvest of mature leafy greens, while VEG-04B focused more on longer-term sustainability with multiple harvests and regrowth from the same plants and growth resources. VEG-04A ran on the ISS 6 June–11 July 2019. VEG-04B ran 3 October–30 November 2019.

### Preflight verification testing

Mizuna was first grown in 28-day tests at the Kennedy Space Center (KSC), Purdue University, and Sierra Space using ISS conditions of temperature, relative humidity, and $CO_2$, with lighting similar to that found on the ISS Veggie units (Massa et al. 2016), to down select the most effective red and blue light treatments for producing plants with desirable growth and organoleptic qualities. From this testing it was determined that 90%R:10%B and 50%R:50%B resulted in better fresh mass production than in other treatments (Massa 2018). Hence, these were the two light levels selected for the VEG-04A and VEG-04B flight experimentation, with 90%R:10%B as the 'red-rich' treatment and 50%R:50%B as the 'blue-rich' treatment. Additional ground tests were conducted to optimize fertilizer levels, determine the recovery and regrowth period for repetitive harvesting, and plan water usage.

Ground testing determined that the light-spectral treatments caused differential heating and water usage in the plant pillows. The black material of the pillows had a relatively high light absorbance and emissivity. The blue-rich treatment added approximately 15% more energy than the red-rich light treatment at the same photosynthetic photon flux (PPF), which translated to a greater heating effect that resulted in higher root zone temperatures prior to canopy closure. To mitigate this, white Beta Cloth (Dunmore Aerospace, Bristol, PA) pillow shades were developed to reflect light, blocking 62% of the light hitting the surface of the plant pillows and significantly reducing the thermal difference.

### Seed preparation

Mizuna (*Brassica rapa* var. Japonica; also reported as *Brassica rapa* var. nipposinica) seeds (Johnny's Selected Seeds, Winslow, ME) were surface sanitized using a chlorine gas-fuming method as described by Massa et al. (2017b) and Hummerick et al. (2021). Seed germination tests confirmed seed viability and consistency across batches.

### Plant pillow assembly

Veggie plant pillows were assembled under clean laboratory conditions at KSC using the procedure described by Massa et al. (2017b). Each pillow contained 250 mL autoclaved, porous ceramic substrate (Turface Proleague Profile Porous Ceramics, LLC) sifted to 600 µm–1 mm and 1–2 mm, and mixed in proportions of 1:1. Substrate was then mixed with controlled-release, polymer-coated fertilizer. For VEG-04A, the fertilizer used was 7.5 g $L^{-1}$ T70 + 5 g $L^{-1}$ T180 Nutricote 18-6-8 (Florikan ESA, Sarasota, FL), selected based on ground testing in analog hardware to be used in an ~1-month growth test. Unfortunately, the Veggie system performed differently from the ground analog, as this fertilizer concentration was found to be too strong for the plants in VEG-04A. After salt stress and plant death were observed in VEG-04A, additional ground testing in flight-like hardware was conducted, and the concentration of the fast-release formulation (T70) was reduced by 3.5 g $L^{-1}$, or 47%, to 4 g $L^{-1}$ T70 + 5 g $L^{-1}$ T180 Nutricote 18-6-8 for VEG-04B. For each pillow, two surface-sanitized seeds were attached to germination wicks with guar gum as reported by Massa et al. (2017b). Pillows were individually sealed inside Tedlar® gas-impermeable bags (165 mm × 203 mm Tedlar® bags, SKC Inc., Eighty Four, PA), weighed, and photographed for quality and consistency.

### Sanitizing wipe preparation

Sanitizing wipes for this study were prepared by cutting Kimtech™ Pure W4 (30.5 cm × 30.5 cm) dry wipes into quarters and steam-sterilizing the quarters in an autoclavable tray with foil cover for a 15-min dry autoclave cycle. Microcide® ProSan® was diluted to a 1% solution with deionized (DI) water, autoclaved for 15 min, and confirmed at pH 2.9 (target pH: 2.5–3.0). Under clean laboratory conditions, the sterile ProSan® solution was poured into the autoclaved tray containing the wipes. Batches of 10 sanitized wipes were placed in a Ziploc® bag, and the bag was positioned vertically overnight to allow excess liquid to collect in the bottom of the bag. Excess solution was removed with a sterile 10-mL pipette.

### VEG-04A & VEG-04B operations

The impact of red-rich and blue-rich lighting on crop growth was analyzed for biomass yield, leaf nutritional composition, and root, substrate, wick, and surface microbial levels. Ground control studies were conducted in parallel using similar conditions in controlled environment chambers at KSC. Real-time temperature, relative humidity, and $CO_2$ readings from the ISS were used as setpoints for the ground control chambers, and two HOBO® (Onset Computer Corporation, Bourne, MA) data loggers collected temperature and relative humidity readings inside each Veggie unit (Table 1).

The experiment timeline is in Table 2.

At initiation for each experiment, the crew installed plant pillows (12 total, 6 per light treatment) and HOBO® data loggers in the Veggie baseplates, added water to the dry pillows, and programed the Veggie lights and fans. At 3 days after initiation (DAI), the crew opened the pillow wicks to help mizuna seedlings emerge. Each pillow had two seeds to improve chances of successful plant establishment; additional seedlings were thinned to one seedling per pillow. The removed seedling was cut at the stem base, instead of pulled out, to avoid disturbing the remaining seedling. Watering activities increased in frequency throughout the studies to support increasing plant growth rate, and the crew photographed plant pillows at each activity. The Veggie science team used the expedited, downlinked photos to determine the next day's water recommendations for the crew, provided via Execution Notes. The team also monitored plant growth and health status, providing feedback in daily and weekly notes to the astronauts.

Plant, pillow, and surface swab samples were collected from flight and ground experiments. The crew harvested whole mizuna plants from VEG-04A at 35 DAI, while for VEG-04B, outer leaves were harvested at 29 and 43 DAI with removal of the whole plant with the final harvest at 58 DAI (Figure 1). Approximately half of each plant's harvest

**Table 1.** Ambient environmental conditions inside the ISS and ground control chambers, as well as inside the Veggie system, with observation arithmetic means and standard errors displayed.

| | VEG-04A | | | | VEG-04B | | | |
|---|---|---|---|---|---|---|---|---|
| | Flight | | Ground | | Flight | | Ground | |
| *Temperature* | | | | | | | | |
| *Ambient* | | | | | | | | |
| Day | 22.4 (0.001) | | 22.4 (0.002) | | 22.4 (0.002) | | 22.3 (0.002) | |
| Night | 22.3 (0.002) | | 22.3 (0.003) | | 22.2 (0.002) | | 22.1 (0.002) | |
| *Veggie* | **Red** | **Blue** | **Red** | **Blue** | **Red** | **Blue** | **Red** | **Blue** |
| Day | 23.1 (0.02) | 23.4 (0.02) | 22.1 (0.02) | 22.0 (0.02) | 22.4 (0.02) | 23.0 (0.01) | 21.3 (0.02) | 21.5 (0.02) |
| Night | 20.3 (0.02) | 20.5 (0.02) | 20.0 (0.02) | 19.8 (0.02) | 20.0 (0.01) | 20.7 (0.01) | 19.4 (0.02) | 19.5 (0.02) |
| *Humidity* | | | | | | | | |
| *Ambient* | | | | | | | | |
| Day | 40.4 (0.01) | | 40.4 (0.02) | | 41.7 (0.01) | | 41.7 (0.02) | |
| Night | 40.6 (0.01) | | 40.6 (0.03) | | 41.7 (0.01) | | 41.7 (0.02) | |
| *Veggie* | **Red** | **Blue** | **Red** | **Blue** | **Red** | **Blue** | **Red** | **Blue** |
| Day | 78.9 (0.1) | 81.7 (0.1) | 73.9 (0.1) | 70.4 (0.1) | 83.4 (0.1) | 89.8 (0.1) | 77.2 (0.1) | 76.7 (0.1) |
| Night | 82.6 (0.2) | 84.9 (0.1) | 79.2 (0.1) | 76.6 (0.1) | 86.5 (0.2) | 91.9 (0.1) | 83.3 (0.2) | 82.3 (0.1) |
| $CO_2$ | | | | | | | | |
| *Ambient* | | | | | | | | |
| Day | 2009 (5) | | 2139 (4) | | 2605 (2) | | 2673 (2) | |
| Night | 2143 (8) | | 2189 (8) | | 2940 (2) | | 3011 (2) | |

Note: Parameters are grouped across day (lights on) and night (lights off) cycles with a large number (n) of data points analyzed for both ISS ambient conditions ($n_{Ambient}$) and conditions inside Veggie ($n_{Veggie}$). VEG-04A day ($n_{Ambient}$ = 25,583; $n_{Veggie}$ = 2,618) and night ($n_{Ambient}$ = 8,323; $n_{Veggie}$ = 884); VEG-04B day ($n_{Ambient}$ = 35,926; $n_{Veggie}$ = 4,180) and night ($n_{Ambient}$ = 11,797; $n_{Veggie}$ = 1,375). Initiation and harvest dates are omitted due to elevated crew presence. Data logger readings following day/night and night/day transitions until condition restabilization have been excluded. Ambient ground data reflect actual readings, not chamber setpoints.

was wrapped in aluminum foil and stowed in the minus eighty-degree laboratory freezer for ISS (MELFI) at −80°C. After harvest, the crew collected surface-swab samples using self-contained sterile swabs (Becton Dickinson, Franklin Lakes, NJ,USA) from the two Veggie facilities including three different plant pillows and bungee cords securing the pillows in place (Samples labeled 1, 2, and 3; Supplemental Figure 1), three areas on the interior bellows surface (Samples labeled 4: top of the bellows, 5: middle bellows, and 6: bottom bellows; Supplemental Figure 2), and two areas of the ventilation hardware (Samples labeled 7: the interior fan screen, and 8: air outlet vent; Supplemental Figure 3). Two plant pillows from each Veggie chamber were removed and stored for analysis. All ground control samples were immediately placed into a −80° C freezer at KSC, while ISS samples were stored in MELFI until return to KSC. Frozen samples were maintained between −80°C and −100°C. After return, samples from VEG-04A and VEG-04B were maintained in a −80°C freezer until analysis. Frozen science samples and the HOBO®s were returned to KSC for microbiological, chemical, and environmental analyses.

The remaining half of harvested mizuna was weighed in the Mass Measurement Device (MMD) and then manually sanitized prior to consumption with ProSan® wipes by the astronauts. Produce sanitizing consisted of pressing leaves between wipes for 30 s. Upon consumption, crew members completed an organoleptic analysis of the fresh produce.

**Table 2.** VEG-04A and VEG-04B flight experiment operations timeline.

| Activity | VEG-04A | VEG-04B |
|---|---|---|
| Initiation | 0 DAI | 0 DAI |
| Wick Opening | 3 DAI | 3 DAI |
| Thinning | 7 DAI | 13 DAI |
| Harvest | 35 DAI | 29, 43, 58 DAI |
| Pillow Watering | At Thinning; every 2 days after 10 DAI; daily after 16 DAI | At 7 DAI; every 2 days after 7 DAI; daily after 15 DAI but skipped the day after each harvest |
| Photographs | With every crew activity | With every crew activity |
| Video | Initiation and harvest | Initiation and each harvest |

Note: Initiation is regarded as 0 days after initiation (DAI).

Water use is provided in Supplemental Table 1. VEG-0A used considerably more water on average over time than VEG-04B. Testing conducted between these experiments enabled better planning and less excess water in VEG-04B, which led to better plant responses, both for flight and ground plants. For the 35-day test, VEG-04A used ∼9 L for five plants in the red-rich treatment and ∼6 L for three plants in the blue-rich treatment, where the daily average water use per pillow was 51 mL for the red-rich and 58 mL for the blue-rich treatments. VEG-04B, in contrast, used ∼12 L for the red-rich treatment and ∼11 L for the blue-rich treatment over 58 days for five plants in each treatment for most of the growth period, where daily average water use per pillow was 34 and 39 mL in the red- and blue-rich treatments, respectively. VEG-04A ground water use was even higher than flight, while levels were similar to flight in VEG-04B. For VEG-04B, approximately half of the water was used prior to harvest 1, and the remainder split between harvests 2 and 3.

## Data collection

### Organoleptic methods

A 9-point hedonic scale (where 1 = dislike extremely – 9 = like extremely) was used to assess acceptability overall and sensory attributes for Appearance, Color, Aroma, Flavor, and Texture. A 5-point, 'just-about-right' scale (3 = just-about-right, <3 = too little, >3 = too much) was used to assess Crispness, Tenderness, and Bitterness. Ground samples were harvested at 28, 42, and 56 DAI and shipped chilled overnight to the Johnson Space Center (JSC), stored overnight at 4°C, disinfected with 200 ppm chlorine solution, rinsed with water, and dried at room temperature prior to panel quantitative affective testing. The JSC Sensory Evaluation Center was used for ground organoleptic acceptability tests, and produce was presented to an untrained panel who were asked to evaluate the produce. Panelists were isolated during testing and provided with palate cleansers before and between samples (Catauro and Perchonok 2012). N ≥ 25 subjects evaluated the produce per harvest with a total of $n$ = 79 evaluations.

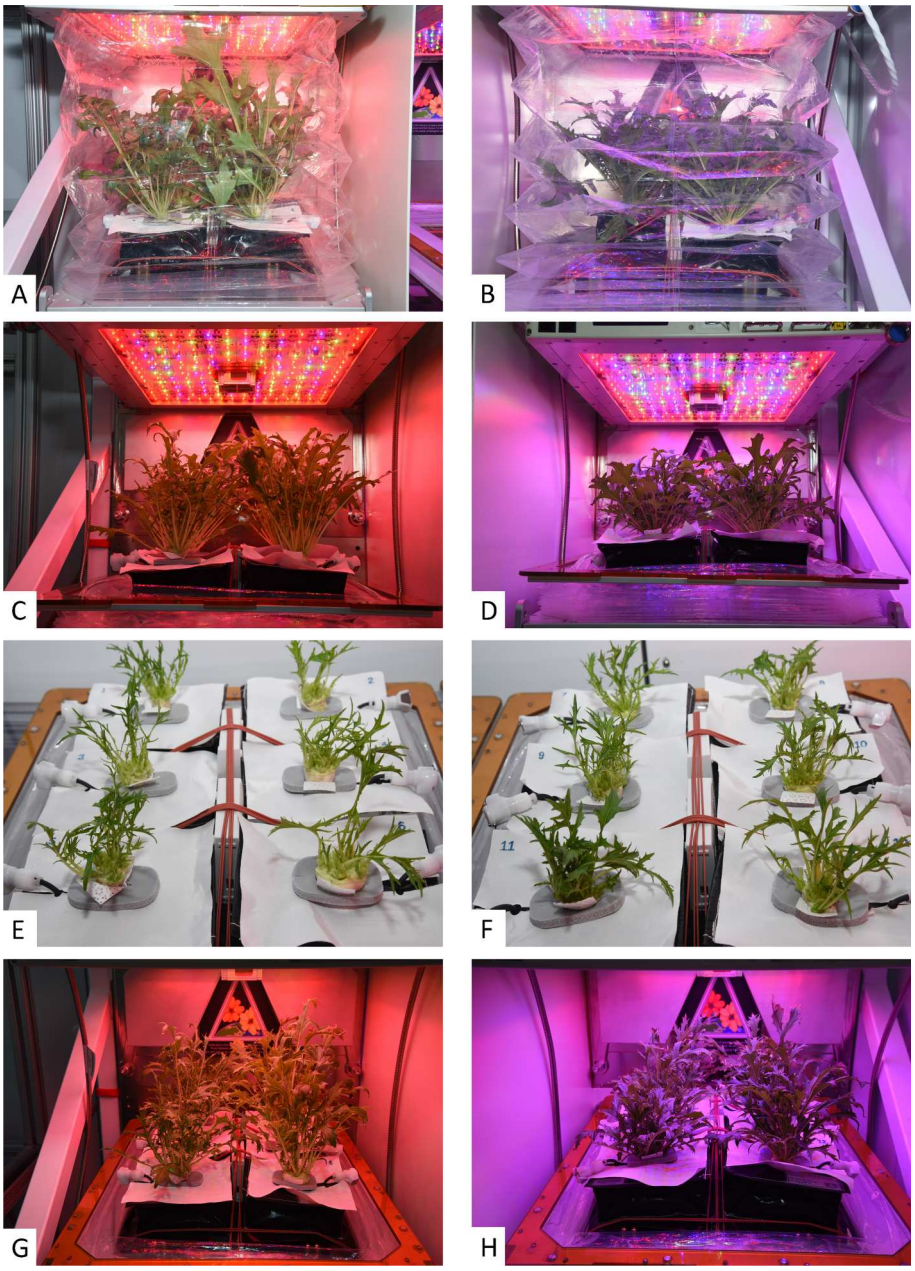


**Figure 1.** Ground control photos of VEG-04A red-rich **(A)** and blue-rich **(B)** light treatments at the single, final harvest (35 DAI); VEG-04B red-rich light treatment before **(C)** and after **(E)** and blue-rich light treatment before **(D)** and after **(F)** the second cut-and-come again harvest (43 DAI); and VEG-04B red- **(G)** and blue-rich **(H)** light treatments at final harvest (58 DAI).

Flight samples were evaluated at 35 DAI for VEG-04A and at 29, 43, and 58 DAI for VEG-04B, under similar procedures as the pre-flight ground testing. After harvest, leaves for consumption were sanitized with ProSan® wipes and placed in a sanitized plant container, with a requirement that produce must be consumed within two hours after sanitization or be re-sanitized. The crew were instructed to rinse their mouths with water from a drink bag to cleanse their palate before sampling one or two leaves larger than 7.5 cm from the sanitized plant container for one of the light treatments, record their evaluation in the data collection tool, and then repeat with both palate cleansing and evaluation with leaves from the second light treatment. First and second light treatments for evaluation varied by crew member, and upon completion the remainder of the sanitized produce could be consumed with meals at crew discretion. $N = 2–3$ subjects per harvest with a total of $n = 14$ evaluations. All human subject data were approved by the JSC Institutional Review Board (Flight Assessment via PRO 2457; Ground Assessment via STUDY00000281-SFSL Sensory). All astronauts received informed consent briefings and consented to provide these data. Informed written consent was obtained prior to subject participation for ground testers.

### *Return sample analysis*

Sample processing and elemental, oxygen radical adsorption capacity (ORAC), total phenolic content, and microbiological analyses were conducted using the same procedures as in previous Veggie flight studies (Khodadad et al. 2020). Additional microbiological analysis methodology specific to VEG-04A and VEG-04B are described below.

Pillow wick and substrate samples were prepared similarly as the leaf and pillow root samples. Swab samples were placed in sterile phosphate-buffered saline (PBS) with 0.3% Tween 80 and vortexed at high speed for 30 s. Water samples were diluted and plated similar to sample extracts.

The VEG-04B isolate, identified as *S. aureus*, was confirmed using whole genome sequencing (WGS) on the Illumina MiSeq sequencer. DNA was isolated from a pure culture using the Qiagen Microbial Cell DNA Isolation Kit (Qiagen, Inc., Carlsbad, CA), tagged with a unique barcode using the Illumina DNA Library Prep Kit (Illumina, Inc., San Diego, CA), and then sequenced. Analysis of the genomic sequences were completed using Average Nucleotide Identity (ANI) to identify the microbe to its nearest relative(s).

These methods have been adapted from the FDA Bacteriological Analytical Manual (https://www.fda.gov/food/science-research-food/laboratory-methods-food) and were used previously on produce grown in Veggie on the ISS (Khodadad et al. 2020; Hummerick et al. 2021).

### Statistical analysis

Measures were analyzed using regression models defined by the interaction of categorical fixed effects Experiment (VEG-04A or VEG-04B), Location (flight or ground), and Treatment (red-rich or blue-rich). Robust standard errors addressed non-homogenous variance across conditions. An F-test on the model determined significance of at least one combination being different before conducting pairwise comparisons. Expected marginal means were used for estimation and comparisons, the latter of which included between Experiments, between Treatments, and between Locations, holding other conditions constant. Analyses were conducted in SAS v9.4 with the GLIMMIX procedure using the LSMEANS statement for estimation and comparisons. For plant-specific measures (e.g. biomass), random effects were incorporated for plant position in the Veggie unit. Similarly, survey response analyses incorporated subject-specific random intercepts. Likert-sale survey responses were visualized using the Likert package in R.

Microbiological counts (log transformed) between treatments and consecutive harvests in Veg-04B were compared with a one-way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA).

## Results and discussion

### Organoleptic acceptability

Figure 2 shows the flight and ground organoleptic acceptability data. The assessment in flight showed that all mizuna samples were generally well-liked (Figure 2(A)). There was a minor indication that mizuna grown under red-rich light was liked slightly more than under blue-rich light, but this preference was not significant ($P > 0.05$). The evaluation of ground samples showed that, on average, all samples were acceptable, and only minor differences were observed between scores for mizuna grown with red-rich versus blue-rich light (Figure 2(B)). Ground scores were significantly lower compared to spaceflight scores.

In assessments of 'just-about-right' parameters in flight, most evaluations scored 'just-about-right' (Figure 2(C)). The scores indicated that mizuna grown under red-rich light was less bitter but also less crisp than mizuna grown under blue-rich light. Ground 'just-about-right' parameters showed that most subjects scored the samples as 'just-about-right'; however, mizuna grown under both light treatments was considered bitter (Figure 2(D)). There was no difference observed between harvests for either the flight or ground samples.

Comparing ground to flight samples indicated that scores were generally lower for ground samples, and that ground samples were considered more bitter. Potential explanations for the differences observed between ground and flight include the limited subject number in flight, which might have skewed the data. Also, lack of fresh foods in flight may have enhanced the positive perception of fresh mizuna. The evaluation of plain mizuna, without condiments, enabled comparison to flight samples, but this is not a traditional consumption method. Another possibility is that mizuna really is better when grown in spaceflight. The use of ProSan® wipes in flight versus the standard chlorine rinse on the ground may have impacted flavor as well, and future testing should be done to assess any flavor differences that sanitization methods may confer. Additional testing of other leafy crops and fresh produce in spaceflight and in ground-based analogs where fresh food is restricted may help us better understand these results. Overall, both light treatments produce highly acceptable mizuna in flight, with some slightly more negative values in overall acceptability and flavor in the blue-rich treatment canceled out by slightly insufficient or excess crispness, tenderness, and bitterness in the red-rich treatment. Regardless of the cause(s) of the perceived flavor, increased acceptability in flight supports the inclusion of plants in long-duration exploration missions.

### Crop survival & stress

Horticultural and operational lessons learned from VEG-04A were implemented for VEG-04B, which increased the number of mizuna plants that grew for the entire duration of the latter study. VEG-04A had an excessive water environment for the seedlings early in the study, which contributed to one-third of the mizuna plants in flight (1 of 6 in the red-rich treatment; 3 of 6 in the blue-rich treatment) and one-half of the mizuna plants in the ground control (3 of 6 in the red-rich treatment; 3 of 6 in the blue-rich treatment) dying and being replaced with pillow blanks. Pillow blanks were placed directly on the root mat, and the black surfaces may have contributed to heat absorption in the root mat and remaining pillows, exacerbating plant stress. VEG-04A was ultimately conducted for a week longer (to 35 DAI) than originally anticipated due to the plant stress and loss earlier in the study.

After VEG-04A, testing was conducted at KSC to more closely examine the challenges experienced and to refine plans for VEG-04B watering amounts, schedule, and anomaly operations. Whereas the white pillow shades were removed in the VEG-04A experiment after the canopy closed, these were left in place for the entire VEG-04B experiment to maintain nearly equivalent temperatures inside the pillows between the two light treatments when plants were repetitively harvested. The watering schedule was improved to better control the moisture and salt content of the germination wicks, especially early in the study when the seedlings were most vulnerable. Less water was added to the root mat (Supplemental Table 1), and the root mat was watered at 16 DAI instead of at initiation, when the plants shifted to exponential growth and quickly produced vegetation. Other changes in water amount and frequency were implemented, and these contributed to better and more uniform plant establishment.

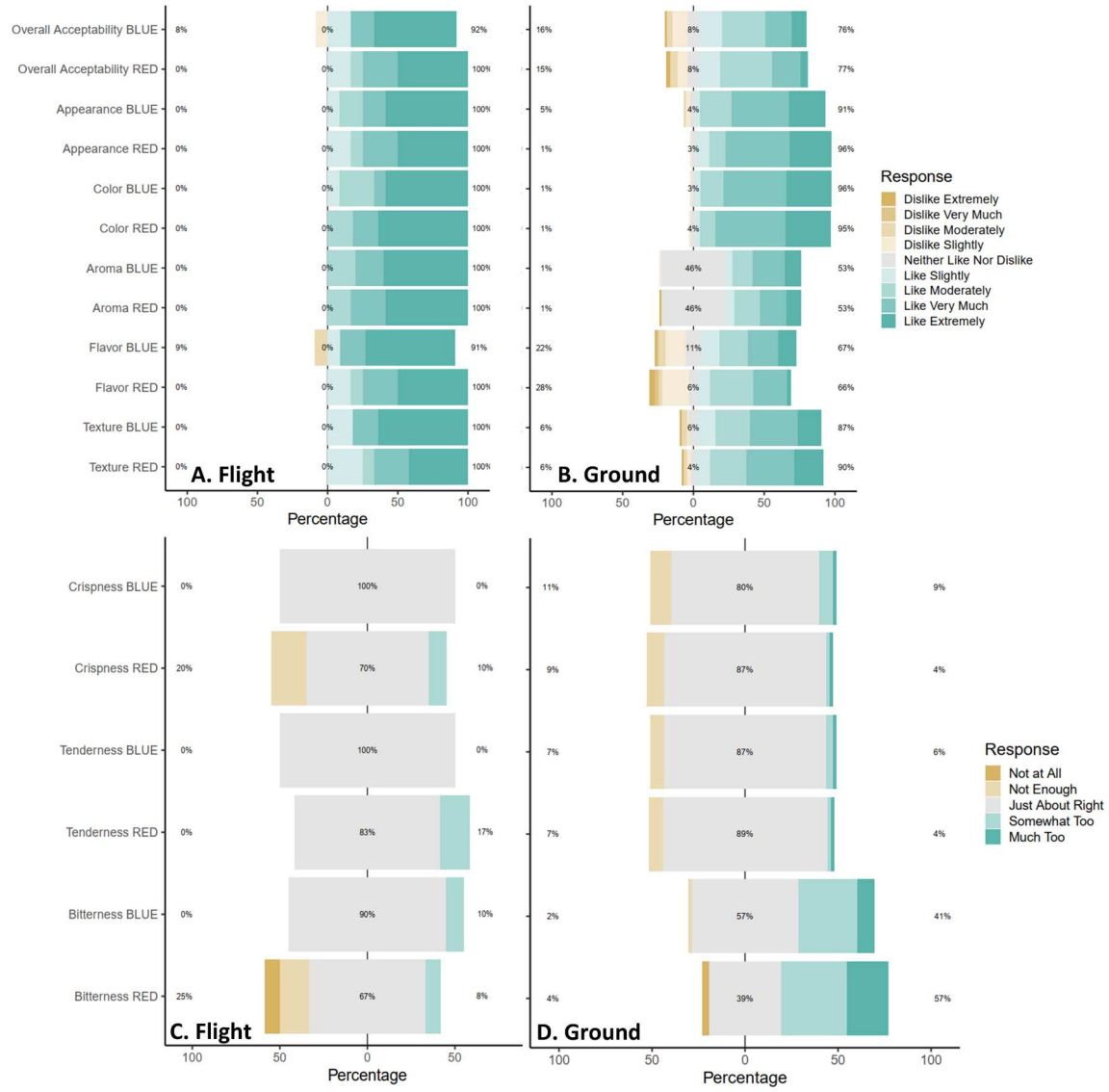


**Figure 2.** Sensory evaluation and organoleptic acceptability data for **(A)** flight hedonic parameters, **(B)** ground hedonic parameters, **(C)** flight 'just-about-right' parameters, and **(D)** ground 'just-about-right' parameters.

Adding more watering activities highlighted a trade-off of a manually operated plant growth system like Veggie. Crew time for plant care is a limited resource (Poulet et al. 2021), and it becomes necessary to balance crew time and any psychological benefits of caring for plants versus crop success, the latter of which optimizes fresh produce production and could create a positive psychological experience that outweighs the additional activities.

Additionally, delaying the thinning activity from 7 DAI in VEG-04A to 13 DAI in VEG-04B increased the likelihood that the remaining seedling in each pillow would survive. When a plant failed, the removed pillow was replaced with a white pillow shade, instead of a black pillow blank, to reduce thermal radiation absorption. These modifications improved crop success: five of the six VEG-04B plants in the flight red-rich treatment (one plant died after harvest 1), five of the six plants in the flight blue-rich treatment (one plant died prior to harvest 1), and all ground control plants survived.

## *Biomass production*

Surviving plants and adjusted harvest dates affected the consistency of total fresh edible biomass across treatments and flight/ground location in VEG-04A. Mizuna produced 293 and 97 g in the red-rich ($n = 5$) and blue-rich ($n = 3$) flight treatments, respectively, and 141 and 121 g in the red-rich ($n = 3$) and blue-rich ($n = 3$) ground treatments, respectively.

For VEG-04B, the red-rich treatment had lower total fresh edible biomass ($n = 6$ until harvest 1, then $n = 5$; 186.80 g) than the blue-rich treatment ($n = 5$; 222.85 g). Like VEG-04A, the ground control had higher production in the red-rich treatment ($n = 6$; 293.62 g) than in the blue-rich treatment ($n = 6$; 223.54 g).

More advanced statistical analyses were not conducted for total biomass due to low sample size and statistical power, highlighting the importance of conducting future studies in spaceflight and with ground analogs to increase our ability to interpret data more confidently from such research.

When we adjusted fresh biomass production to per surviving plant, we found no location or lighting treatment effect, nor an effect between VEG-04A and VEG-04B in flight ($P > 0.05$; Figure 3). Biomass per plant was greater only on the ground for VEG-04B than VEG-04A for both the red-rich ($P \leq 0.01$) and blue-rich ($P \leq 0.05$) treatments.

Although total fresh biomass per plant was fairly consistent across treatments, biomass production rate decreased across harvests when plants were grown for cut-and-come-again

harvests in VEG-04B (Figure 3). Mizuna in flight was lower in the final harvest ($P \leq 0.001$) for both lighting treatments, and the biomass production rate decreased after the first harvest on the ground in the blue-rich treatment ($P \leq 0.001$) and after the first ($P \leq 0.001$) and second ($P \leq 0.01$) harvests in the red-rich treatment. Finally, mizuna biomass production was lower in flight than on the ground for both the first ($P \leq 0.01$) and final ($P \leq 0.01$) harvests in the red-rich treatment. In three of the four treatments (both VEG-04A single harvests and the VEG-04B red-rich treatment), flight-grown plants were slightly smaller than ground-grown, possibly due to unique stresses of the spaceflight environment such as fluid dynamics in microgravity leading to root zone stress and non-uniform airflow leading to reduced gas exchange (Porterfield 2002; Liao et al. 2004; Poulet et al. 2020).

Related research on cut-and-come-again harvests with mizuna in Veggie analog hardware have reported decreasing biomass production across harvests (Morsi et al. 2023). Biomass results with brassica mustard greens similar to mizuna, like 'Amara' mustard (*Brassica carinata*) and kale (*Brassica napus* L. subsp. Pabularia cv. 'Red Russian') have also shown no clear benefits in yield with cut-and-come-again harvesting in Veggie, and another brassica, pak choi (*Brassica rapa* subsp. chinensis cv. 'Extra Dwarf'), produced half as much biomass with a longer, cut-and-come-again harvest protocol (Bunchek et al. 2021). However, if cut-and-come-again is selected for prolonged growth and to reduce crew time and the launch mass of supplies, a blue-rich light treatment would be recommended to support better overall growth. For a single harvest approach, a red-rich treatment seems to support better growth in flight and on the ground. Generally, flight-grown plants yielded less than their ground-grown counterparts.

## Nutrient analysis

Mizuna had higher concentrations of secondary metabolites like phenolic compounds and mineral nutrients like iron in VEG-04A than VEG-04B (Tables 3a and 3b), which may indicate that these accumulate at a higher rate during the plants' exponential growth phase and decline thereafter. The specific content of phenolic compounds was higher in VEG-04A for the red-rich ($P \leq 0.001$) and blue-rich (flight $P \leq 0.01$; ground $P \leq 0.05$) treatments. Iron was higher in VEG-04A (flight $P \leq 0.001$; ground $P \leq 0.01$) but was not influenced by lighting treatment. Khodadad et al. (2020) also reported higher specific contents of multiple nutrients, including iron and phenolics, when red romaine lettuce (*Lactuca sativa* cv 'Outredgeous') was grown on the ISS and ground for one month with a single harvest, versus with a cut-and-come-again harvest approach for two months. However, as iron is already high in the crew pre-packaged diet, iron accumulation in mizuna may not be as high of a priority as other nutrients.

Conversely, magnesium and manganese were higher in VEG-04B ($P \leq 0.01$), which showed that other nutrients accumulate over time. Mizuna grown under blue-rich light also had more manganese ($P \leq 0.01$), except for in VEG-04A flight ($P > 0.05$) which could be a result of plant stress during that study. Magnesium was slightly higher in the blue-rich treatment across experiments and locations, although this effect was not significant ($P > 0.05$). Nonetheless, blue-rich light has been reported to have a significantly greater positive influence on magnesium accumulation than red-rich light in other leafy greens like 'Outredgeous' lettuce (Mickens et al. 2018).

Calcium accumulation rates were similar to manganese, with the greatest concentrations when mizuna was grown under blue-rich light in VEG-04B ($P \leq 0.01$). However, as changing the level of calcium is not desired due to possible negative side effects of consuming higher amounts (Smith et al. 2021), growing mizuna under a single-harvest protocol like VEG-04A would be recommended to limit calcium accumulation in the leaves.

Additionally, some elements were higher in spaceflight than on the ground, including sulfur in both studies (VEG-

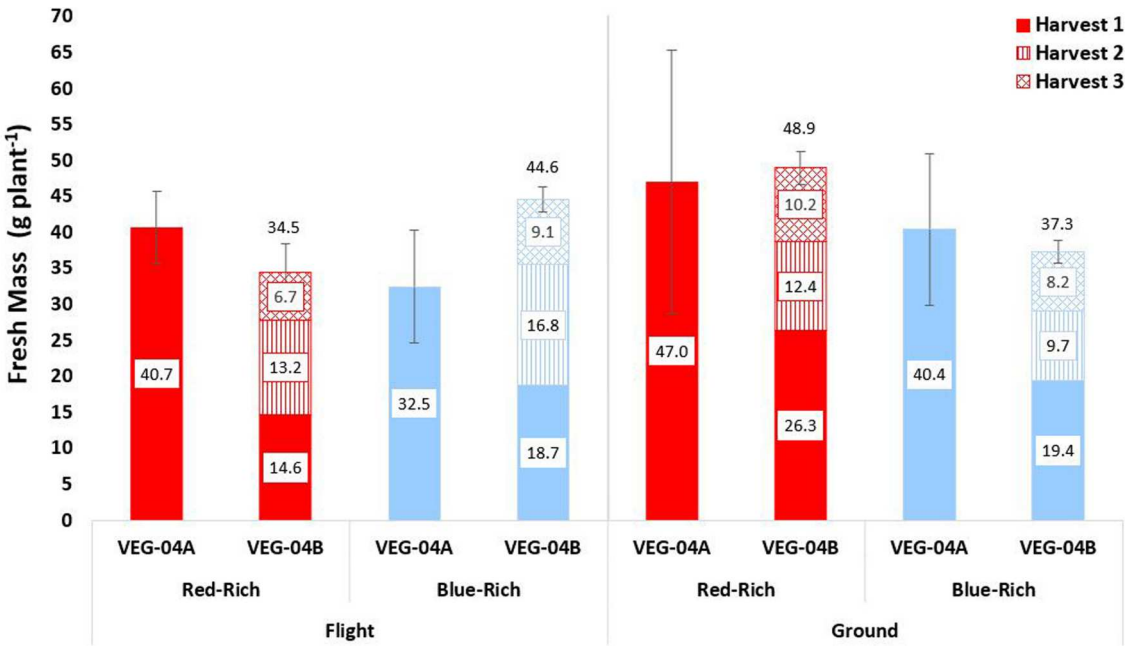


**Figure 3.** Average fresh edible biomass per plant in VEG-04A and VEG-04B, with standard error bars displayed. Greater standard error bars displayed values for VEG-04A are attributed to greater plant stress and death compared to VEG-04B. Numbers displayed above bars for VEG-04B are the cumulative biomass per plant values across all 3 harvests.

04A $P \leq 0.05$; VEG-04B $P \leq 0.01$), and for magnesium ($P \leq 0.05$), potassium ($P \leq 0.01$), sodium ($P \leq 0.01$), and iron ($P \leq 0.001$) in VEG-04B, where flight versus ground effects were likely more pronounced in the longer experiment. While high concentrations of sulfur-containing compounds like glucosinolates and methylcystiensulfoxide in brassicas like mizuna can cause the leaves to taste spicier and/or more bitter (Neugart et al. 2018), glucosinolates help plants recover from wounding and cell damage, and offer human health benefits like cancer prevention, anti-inflammation, and endocrine disruptors for diabetes prevention (Wiesner et al. 2013; Hanschen et al. 2017; Mickens et al. 2019).

Light treatment did not affect many mineral nutrients, including iron, phosphorus, potassium, sodium, or sulfur. No differences were found ($P > 0.05$) for copper or zinc at any test level. In general, the blue-rich light treatment increased accumulation of the nutrients desired to supplement the crew diet more than the red-rich. The harvest protocol or growth duration of a crop may need to be selected based on the desired nutrients to supplement, and nutrient trade-offs may occur: some nutrients increase with longer growth cycles, while others decrease. One potentially beneficial outcome is that nutrient accumulation in mizuna grown in flight was found to be higher than on the ground for many nutrients. We recommend repeating VEG-04A to better differentiate true nutrient effects versus confounding plant stress effects that were experienced in this study.

### Microbial counts on leaf tissue

Both bacterial and fungal counts (colony-forming unit, CFU $g^{-1}$) from the VEG-04A ground control samples were significantly lower than flight samples for the red-rich treatment, but not the blue-rich treatment (Figure 4). Count data from ground control leaf samples included a comparison of leaves pre- and post-cleaning. While the mean did not indicate a statistical difference between the two cleaning stages ($P > 0.05$), the samples that were not cleaned had at least one sample that was higher than any of the cleaned leaves (Figure 4). Flight samples returned to KSC for analysis had not been cleaned with ProSan® wipes. Statistical analysis indicated a difference ($P \leq 0.05$) between light treatments in flight plants in VEG-04A (Figure 4), and when considering individual samples in both flight and ground control samples, two of the three samples from the blue-rich treatment fell below the lowest value for bacterial (aerobic plate count, APC) and yeast and mold counts in the red-rich treatment (Figure 4).

Bacterial and fungal counts (CFU $g^{-1}$) on leaves harvested during VEG-04B increased with each subsequent harvest, starting at $\leq 100$ CFU $g^{-1}$ in the first harvest for both ground and flight (Figure 5). Counts in VEG-04A averaged $1.7 \times 10^6$ CFU $g^{-1}$ in the red-rich treatment and $5.2 \times 10^4$ CFU $g^{-1}$ in the blue-rich treatment. The bacterial count from the second harvest flight samples was not higher than the first in both lighting treatments; however, fungal counts did increase from averages of 70–$4.3 \times 10^3$ CFU $g^{-1}$ in the red-rich treatment and $1.9 \times 10^2$ to $2.0 \times 10^3$ CFU $g^{-1}$ in the blue-rich treatment. Fungal counts from the ground control samples remained low with most at, or below, detection limit, with the exception of one sample from the last harvest in the blue-rich treatment. The third, final harvest yielded the highest counts overall, notably in the flight samples. Mean bacterial and fungal counts were $5.2 \times 10^4$ and $1.2 \times 10^7$ CFU $g^{-1}$, respectively, for the red-rich treatment and $2.1 \times 10^4$ and $2.5 \times 10^5$ CFU $g^{-1}$, respectively, for the blue-rich treatment. These counts fell within the range of the VEG-04A sample counts with only one harvest.

Mizuna has been grown previously in Veggie in technology demonstrations VEG-03D and VEG-03E, as well as in the Russian Lada plant growth hardware on the ISS (Hummerick et al. 2011). Microbiological analyses were done on ground and flight samples from both systems, where microbial counts ranged from $1.4 \times 10^3$ to $2.6 \times 10^7$ CFU $g^{-1}$ bacteria and $2.4 \times 10^4$ to $1.3 \times 10^5$ CFU $g^{-1}$ fungi (Hummerick et al. 2011, 2021). In Lada, cut-and-come-again harvesting was tested with mizuna, and as in VEG-04B, bacterial and fungal counts increased with each subsequent harvest (Hummerick et al. 2011). Significant increases in CFU $g^{-1}$ with consecutive harvests may be a result of increased human handling, as well as leaf exudates released due to cutting increasing available nutrients on leaf surfaces. While a repeated harvest may impact harvest yield, it may also contribute to an increased microbial load, which may be a factor in determining the best harvest method.

### Microbial counts on pillow components

Pillow components included the wick material, substrate, and roots. VEG-04A bacterial counts (CFU $g^{-1}$) were comparable between flight and ground control materials and were highest for roots, followed by wick, and then substrate. The CFU $g^{-1}$ in substrate was lower in the flight red-rich treatment than those in the blue-rich treatment. Average fungal counts were higher in the flight samples and highest

**Table 3a.** Elemental nutritional analysis with ICP-OES of mizuna mustard leaf samples, with Least Square Means (µg $g^{-1}$ dry mass) and standard errors displayed for VEG-04A flight red ($n = 5$) and blue ($n = 3$), ground red ($n = 3$) and blue ($n = 3$); VEG-04B flight red ($n = 5$) and blue ($n = 5$), and ground red ($n = 6$) and blue ($n = 6$).[a,b]

| | | | Al | B | Cu | Fe | Mn | Na | Zn |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | µg $g^{-1}$ dry mass | | | |
| VEG-04A | Flight | Red | 23 (2.7) | 93 (12) | 8.1 (0.7) | 67 (6.2) | 306 (30) | 828 (100) | 38 (5.0) |
| | | Blue | 23 (3.2) | 124 (16) | 11 (1.5) | 85 (6.6) | 248 (4.2) | 613 (128) | 40 (5.5) |
| | Ground | Red | 22 (4.3) | 74 (1.8) | 12 (1.9) | 64 (7.1) | 166 (13) | 630 (98) | 34 (4.7) |
| | | Blue | 24 (2.5) | 85 (10) | 10 (1.2) | 62 (4.6) | 213 (11) | 479 (57) | 26 (5.0) |
| VEG-04B | Flight | Red | 11 (1.3) | 72 (8.7) | 8.9 (0.4) | 56 (5.1) | 150 (15) | 785 (144) | 29 (1.3) |
| | | Blue | 21 (3.3) | 88 (9.3) | 10 (1.0) | 61 (5.4) | 218 (20) | 836 (85) | 34 (3.2) |
| | Ground | Red | 52 (6.7) | 55 (5.5) | 10 (0.8) | 34 (1.9) | 212 (12) | 262 (66) | 30 (1.2) |
| | | Blue | 55 (7.3) | 66 (4.8) | 12 (1.0) | 40 (3.1) | 255 (17) | 274 (59) | 31 (1.1) |
| *ANOVA* | | | | | | *P-Value* | | | |
| E × L × T | | | *** | ** | ns | *** | *** | *** | ns |

[a]Abbreviations: Al, aluminum; ANOVA, analysis of variance; B, boron; Cu, copper; E, experiment (VEG-04A, VEG-04B); Fe, iron; L, location (Flight, Ground); Mn, manganese; Na, sodium; ns, nonsignificant; T, treatment (red-rich, 'Red;' blue-rich, 'Blue'); Zn, zinc.
[b]Significance of model terms shown as: ns, $P > 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

**Table 3b.** Elemental nutritional analysis with ICP-OES of mizuna mustard leaf samples, with Least Square Means (mg $g^{-1}$ dry mass) and standard errors displayed for VEG-04A flight red ($n = 5$) and blue ($n = 3$), ground red ($n = 3$) and blue ($n = 3$); VEG-04B flight red ($n = 5$) and blue ($n = 5$), and ground red ($n = 6$) and blue ($n = 6$), except for total phenolic content and average ORAC, which had smaller sample sizes if insufficient plant material remained after analysis of other nutrients.[a,b,c,d].

| | | | Ca | K | Mg | *P* | S | Phenolic | ORAC |
|---|---|---|---|---|---|---|---|---|---|
| | | | mg $g^{-1}$ dry mass | | | | | | µM TE $g^{-1}$ dry mass |
| VEG-04A | Flight | Red | 6.3 (0.6) | 39 (2.7) | 8.4 (0.8) | 5.1 (0.6) | 6.9 (0.8) | 18 (1.2) | 133 (14) |
| | | Blue | 5.6 (0.2) | 34 (6.4) | 7.5 (0.3) | 5.2 (1.3) | 6.9 (1.4) | 27 (4.4) | § |
| | Ground | Red | 4.3 (0.3) | 29 (4.4) | 5.4 (0.1) | 4.1 (1.1) | 4.5 (0.8) | 18 (0.4) | 125 (11) |
| | | Blue | 5.2 (0.3) | 27 (1.2) | 6.4 (0.4) | 3.6 (0.5) | 4.5 (0.5) | 24 (5.5) | 137 (5.9) |
| VEG-04B | Flight | Red | 7.3 (1.3) | 32 (3.0) | 8.2 (1.3) | 3.1 (0.2) | 6.2 (0.8) | 9.4 (0.5) | 99 (21) |
| | | Blue | 8.9 (0.7) | 37 (4.1) | 11 (0.9) | 3.9 (0.5) | 8.0 (0.9) | 11 (0.3) | 133 (9.3) |
| | Ground | Red | 5.9 (0.3) | 21 (1.3) | 7.3 (0.4) | 2.6 (0.2) | 3.6 (0.2) | 11 (0.7) | 97 (7.9) |
| | | Blue | 6.8 (0.4) | 21 (0.8) | 8.5 (0.6) | 2.9 (0.2) | 3.9 (0.2) | 11 (0.5) | 135 (12) |
| *ANOVA* | | | | | | *P-Value* | | | |
| E × L × T | | | *** | *** | *** | ** | *** | *** | § |

[a]Abbreviations: ANOVA, analysis of variance; Ca, calcium; E, experiment (VEG-04A, VEG-04B); K, potassium; L, location (Flight, Ground); Mg, magnesium; ORAC, oxygen radical antioxidant capacity; *P*, phosphorus; S, sulfur; T, treatment (red-rich, 'Red;' blue-rich, 'Blue'); TE, Trolox® equivalent.
[b]Significance of model terms shown as: **, $P \leq 0.01$; ***, $P \leq 0.001$.
[c]Different sample sizes for total phenolic content: VEG-04A flight red ($n = 4$) and blue ($n = 2$), VEG-04B flight blue ($n = 3$); for average ORAC: VEG-04A flight red ($n = 4$) and blue ($n = 2$, of which 2 are excluded outliers), VEG-04A ground blue ($n = 3$, of which 1 is an excluded outlier), VEG-04B flight red ($n = 4$) and blue ($n = 3$).
[d]§ = Data omitted due to suspected outlier effects.

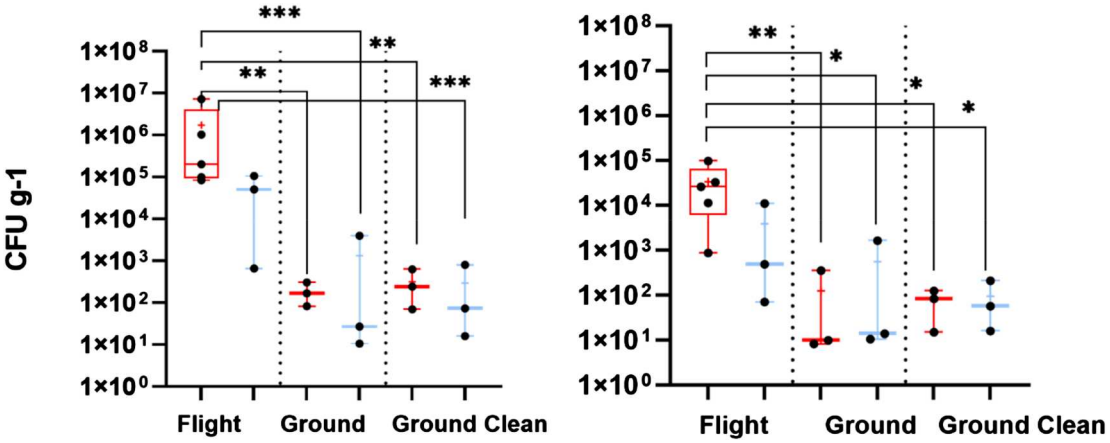


**Figure 4.** Box and whisker plots of VEG 04A **(A)** bacterial and **(B)** fungal counts on mizuna leaves from flight and ground experiments. Plants from one harvest were analyzed in both flight and ground controls before and after cleaning. Red boxes indicate the red-rich light treatment; blue boxes are the blue-rich treatment. Whiskers represent the min and max values, symbols are individual sample values, + represents the mean, and the horizontal line is the median.

in the flight substrate samples. This trend was not evident in the ground controls (Figure 6(A and B)).

The wick material from VEG-04B pillows had the highest bacterial counts in both flight and ground control pillow components and the highest fungal counts in the flight samples. Wick bacterial and fungal counts in VEG-04B were higher ($P < 0.05$) than counts from VEG-04A wicks. Counts from the red-rich treatment wick were lower than the blue-rich treatment in the flight samples, but were the same for the ground control wick samples. The counts in both flight substrate samples were higher for the blue-rich treatment (Figure 6(C and D)). Factors that may influence

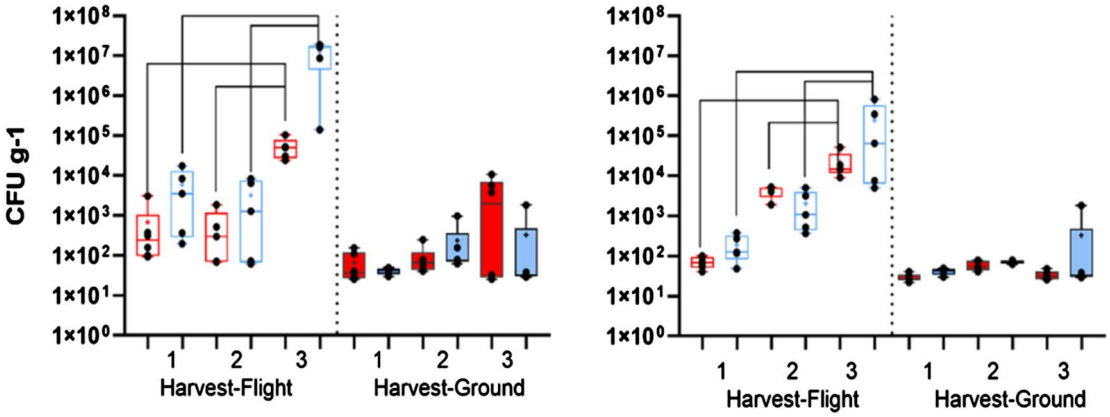


**Figure 5.** Box and whisker plots of VEG 04B **(A)** bacterial and **(B)** fungal counts on mizuna leaves from flight and ground experiments. Plants from three harvests were analyzed in both flight and ground controls. Red boxes indicate the red-rich light treatment; blue boxes are the blue-rich treatment. Whiskers represent the min and max values, symbols are individual sample values, + represents the mean, and the horizontal line is the median. Brackets indicate differences ($P < 0.0001$; except fungal counts from harvest 1 vs 2 in the blue-rich treatment, $P = 0.0037$) between the harvest means only, in each light treatment.

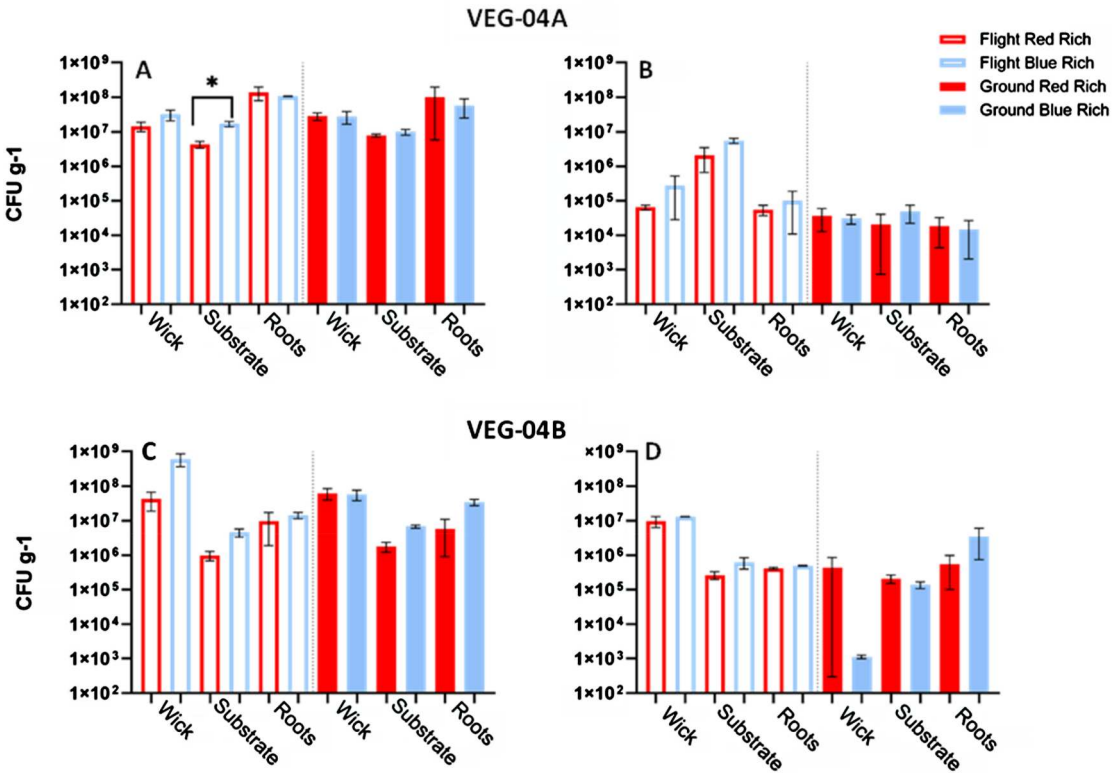


**Figure 6.** Bacterial **(A, C)** and fungal **(B, D)** counts on pillow components from VEG-04A and VEG-04B from flight and ground experiments. Red bars indicate the red-rich light treatment; blue bars are the blue-rich treatment. Data are presented in log scale. Bars indicate means with standard error of the mean. Brackets indicate $P < 0.05$ between pillow component light treatment pairs.

microbial density in the pillow components may be temperature and moisture differences between the blue-rich and red-rich treatments. Although implementing pillow shades reduced the thermal difference, relative humidity was still higher in the blue-rich treatment in both VEG-04A and VEG-04B in spaceflight (Table 2). Relative humidity was also higher in VEG-04B than VEG-04A for both light treatments, likely due to the higher number of surviving plants and subsequent growth and transpiration.

Humidity influences microbial growth and community dynamics on built environment surfaces, including the ISS, and the plant phyllosphere (Aung et al. 2018; Sielaff et al. 2019; Schuerger 2021; Schuerger et al. 2021a). A study by Dannemiller et al. (2017) investigating the effect of humidity on microbial growth in carpet and household dust found that equilibrium relative humidity (ERH) above 80% had a profound effect on growth rates of fungi and microbial community structure, and that airborne microbes could originate from fungi grown at ERH levels of $\geq 85\%$. Relative humidity levels in the Veggie flight units were consistently above 80%, with the highest average values in the blue-rich treatment in VEG-04B at 89.8% during the day cycle and 91.9% during the night cycle (Table 2).

### Microbial counts on surfaces

Bacterial counts on Veggie surfaces ranged from below detection limits to $1.1 \times 10^6$ CFU per swab in the VEG-04A flight unit for the red-rich treatment followed by the blue-rich treatment in the VEG-04B flight unit (Table 4). The highest average bacterial count, $1.52 \times 10^5$ CFU per swab, was found in the red-rich treatment for VEG-04A flight. All ground controls had lower average counts than flight units. Ground controls had lower temperature and relative humidity (Table 2) and were housed inside a growth chamber with limited access. Lab coats, hair and beard caps, and gloves were worn during all ground activities, whereas astronauts were only required to wear gloves during flight activities. These factors likely contributed to the lower microbial counts on ground control surfaces. The same flight experiments with the highest bacterial counts, VEG-04A flight red-rich and VEG-04B flight blue-rich, also had the highest average fungal counts (Table 5). No consistent trend was observed based on sample location within the Veggie units. A possible explanation for the high bacterial and fungal counts found on swab locations 2, 3, and 5 in VEG-04A flight may be localized contamination due to plant debris on the pillow and bungee surfaces or leaf contact with the bellows surface combined with the presence of moisture, allowing microbial proliferation.

### Pathogen screening on leaf tissue

All mizuna samples tested negative for *E. coli* and *Salmonella spp*. VEG-04B flight samples from plant #5 (red-rich treatment, front left pillow) yielded and confirmed *S. aureus* at 88 CFU $g^{-1}$ at harvest 2, and *S. aureus* was detected but not quantified from plant #5 at the final harvest 3. The identification of this isolate of *S. aureus* was confirmed by 16S rDNA and WGS. The sequencing results indicated there was a 99.86% identity match to *S. aureus* strain DSM20231 and a 99.85% match to *S. aureus subsp. aureus* NCTC 8325, the latter being one of the more common strains of *S. aureus*. While we screen for this organism as an indication of transfer of human associated bacteria, it is a commensal organism on human skin and quite ubiquitous in the environment (Kadariya et al. 2014). For foodborne illness to occur, the bacterium must grow and produce enough toxin to cause 'food poisoning'. The enterotoxins are produced in the exponential phase

**Table 4.** Bacterial counts (CFU per swab) on Veggie surfaces.

| | VEG-04A | | | | VEG-04B | | | |
|---|---|---|---|---|---|---|---|---|
| | Red-rich | | Blue-rich | | Red-rich | | Blue-rich | |
| SURFACE | Flight | Ground | Flight | Ground | Flight | Ground | Flight | Ground |
| 1 | 2450 | 25 | <25 | <25 | <20 | 400 | 5,660 | 380 |
| 2 | $1.1 \times 10^6$ | <25 | <25 | <25 | 3340 | 20 | 1,220 | 60 |
| 3 | <25 | 150 | <25 | <25 | 900 | <20 | 8,100 | <20 |
| 4 | <25 | 150 | 50 | 25 | 40 | <20 | 700 | <20 |
| 5 | $1.13 \times 10^5$ | <25 | 125 | <25 | <20 | 1,700 | <20 | <20 |
| 6 | 425 | <25 | 25 | <25 | <20 | 560 | 480 | 3,980 |
| 7 | 300 | <25 | 125 | <25 | 240 | 20 | 60 | 2,320 |
| 8 | <25 | 50 | <25 | <25 | <20 | 980 | 4,260 | 4,780 |
| AVERAGE | 152,031 | 44 | 53 | 25 | 511 | 465 | 2,563 | 1,448 |
| ST. DEV. | 360,185 | 41 | 42 | 0 | 1158 | 570 | 2,861 | 1,853 |

Note: Surface sample locations 1–8 are described in Supplemental Figures 1–3. Mean and standard deviation calculations include detection limits.

of growth, and typically enough toxin will be produced when the cell density reaches $10^5$–$10^8$ CFU $mL^{-1}$ (Seo and Bohach 2007; Montville and Matthews et al. 2008). Few outbreaks of *S. aureus* foodborne illness have been linked to leafy greens, presumably because leaf surfaces are not an optimal growth environment for these bacteria. However, under more favorable conditions and high inoculum density, *S. aureus* has the potential to infect plants in nature (Prithiviraj et al. 2005).

## Bacterial isolates

Five bacterial isolates were identified from VEG-04A flight leaf samples and eight from ground control samples (Supplemental Table 2). All five species found on the flight samples were also isolated from the ground controls. Sixteen bacterial species were identified from the VEG-04B flight leaf samples, and nine out of the sixteen species were also isolated on the VEG-04B ground control leaves. A total of eleven species were isolated from the ground control samples. Two species, *Staphylococcus warneri* and *S. saprophyticus*, were identified on both VEG-04A and VEG-04B flight and ground samples. Common species among flight and ground samples may indicate a common source such as seed or Veggie components prepared on the ground, or the ubiquity of these bacteria in the environment may suggest the possibility of the presence of the isolates independent of source in both ground and flight samples. With the exception of the *S. aureus* recovered in VEG-04B, none of the bacteria are common causes of foodborne illness.

In the pillow components (Supplemental Table 3), eight bacteria were identified from VEG-04A flight samples, while seven were isolated and identified from the ground controls, with only one species, *Ralstonia pickettii,* identified from both flight and ground samples of both VEG-04A and VEG-04B. This bacterium was one of two identified in water samples taken from the flight and ground experiments. Nine bacteria were identified from the VEG-04B pillow samples, while ten were isolated from the ground controls; four of these species were shared across flight and ground control: *Azospirillum lipoferin*, *Microbacterium spp.*, *Paenibacillus pabuli/taichungensis*, and *Sphingomas parapaucimobilis. Clavibacter michageneisis subsp. tessellarius* was isolated from VEG-04B flight pillows and from the flight and ground leaf tissue. This bacterium is a host-specific plant pathogen that causes leaf spot in wheat (Li et al. 2018). Isolation of plant pathogens in plant growth units on the ISS should be continued in the future, and efforts to minimize sources of contamination and decontamination after use is an important aspect of an integrated pest/pathogen management plan (Schuerger 2021).

Several bacteria isolated from the pillow components are beneficial to plants. For example, *Azospirillum lipoferum* is an endophyte that has been reported to enhance crop yields, increase nitrogen content in plants, mitigate drought stress, and produce plant growth-promoting hormones (Steenhoudt and Vanderleyden 2000; Abdel Latef et al. 2020). Several genera isolated, including *Bacillus, Brevundimonas*, *Burkholderia, Enterobacter, Paenibacillus, Paraburkholderia, Pseudomonas,* and *Sphingomonas*, are known to exhibit plant growth-promoting traits such as antifungal activity, phosphate solubilization, and siderophore and plant hormone production (Compant et al. 2005; Glick 2012; Handy et al. 2021; Madhaiyan et al. 2021; Heo et al. 2022; Katsenios et al. 2022; Gómez-Godínez et al. 2023). Conversely, some of the same bacteria can cause disease in humans, especially if the host immune system is compromised. For example, cases of human infection caused by *Burkholderia spp.* have been documented, primarily in Cystic Fibrosis patients and nosocomial infections (Zlosnik et al. 2020).

**Table 5.** Fungal counts (CFU per swab) on Veggie surfaces.

| | VEG-04A | | | | VEG-04B | | | |
|---|---|---|---|---|---|---|---|---|
| | Red-rich | | Blue-rich | | Red-rich | | Blue-rich | |
| SURFACE | Flight | Ground | Flight | Ground | Flight | Ground | Flight | Ground |
| 1 | 1,850 | <25 | <25 | <25 | 100 | <20 | 5,440 | <20 |
| 2 | 1,925 | <25 | <25 | <25 | 1,060 | <20 | 6,120 | <20 |
| 3 | 57,750 | 1,075 | <25 | <25 | 1,340 | <20 | 4,440 | <20 |
| 4 | 100 | <25 | 75 | <25 | 340 | <20 | 1,700 | <20 |
| 5 | 50 | <25 | 50 | <25 | 720 | 20 | 1,400 | <20 |
| 6 | 200 | <25 | 175 | <25 | 1,380 | <20 | 1,720 | <20 |
| 7 | 25 | <25 | 525 | <25 | 1,220 | <20 | 3,260 | <20 |
| 8 | 25 | 25 | <25 | <25 | 20 | <20 | 40 | <20 |
| AVERAGE | 7,739 | 147 | 109 | 13 | 773 | 11 | 3,015 | 10 |
| ST. DEV. | 18,918 | 351 | 166 | 0 | 522 | 3 | 2,015 | 0 |

Note: Surface sample locations 1–8 are described in Supplemental Figures 1–3.

Eleven isolates were identified from the VEG-04A surface samples – seven from flight and four from ground. None of the isolates identified were found on the surfaces of the VEG-04B flight and ground units or the VEG-04A leaf and pillow samples (Supplementary Table 4). Thirteen different bacteria were isolated and identified from the VEG-04B flight unit surface samples and ten from the ground units. Two bacteria found on the blue-rich flight unit, *Microbacterium marytipicum* and *Sphingomonas parapaucimobilis*, were also present in the leaf tissues and pillow components. *M. marytipicum* was isolated from both the red- and blue-rich Veggie units as well as associated leaf and pillow materials. The bacteria isolated from both flight and ground control Veggie surfaces were *Bacillus spp*., *Enterobacter cloacae subsp. dissolvens, Enterococcus casseliflavis, Leclercia adecarboxylata, Lysinibacillus fusiformis, Paraburkholderia caryophylli, Pseudomonas oryzihabitans, Sphingomonas parapaucimobilis,* and *Sprosarcina koreensis.* None of these were unique to the ground control. Five bacteria were found only on the flight units: *Brevibacterium iodinum, Brevibacterium otitidis, Corynebacterium pilosum, Staphylococcus warneri*, and *Stentrophomonas maltophila.*

Overall, nineteen different bacterial species were identified from the VEG-04A samples and forty from the VEG-04B samples, with only five common to VEG-04A and VEG-04B. Using culture-based methods to grow and isolate bacteria and fungi provides limited representation of the entire microbial community that may be present in the various samples associated with the plants grown in Veggie. In a characterization of microbial communities associated with ISS surfaces by Sielaff et al. (2019), cultivable and qPCR amplicon sequenced microbial populations were surveyed. Forty-six percent of the bacteria and 40% of the fungi detected by DNA sequencing could also be cultured. This percentage is high compared to other environments like clean rooms, where only 1–10% can be cultured. Cultivating and identifying microbial isolates enables additional characterization of individual bacteria and fungi that may pose a risk to crew health and food safety. Many of the same genera identified in VEG-04 samples have been identified previously on the ISS (Castro et al. 2004; Venkateswaran et al. 2014; Yamaguchi et al. 2014; Lang et al. 2017; Sielaff et al. 2019) as well as on Veggie plants and surfaces on the ISS (Khodadad et al. 2020; Hummerick et al. 2021).

### Fungal isolates

A total of six genera of fungi were isolated and identified from flight and ground samples from VEG-04A. From the flight surface samples, *Aspergillus unguis, Fusarium oxysporum, Penicillium spp., Penicillium citrinum*, and *Rhodotorula mucilaginosa* were identified, while only *Penicillium spp.* was isolated from the ground-control samples. *Aspergillus spp., Alternaria spp., Fusarium spp., Purpureocillium lilacinum*, and *R. mucilaginosa* were identified from the VEG-04A leaf samples, while all but *Alternaria spp.* and *Fusarium spp.* were isolated from the ground controls. *F. oxysporum* and *R. mucilaginosa* were identified in every flight VEG-04A pillow substrate and root sample. VEG-04B sample isolates included those isolated from VEG-04A, except no *Alternaria spp.* or *P. lilacinum* were recovered. *Aspergillus, Penicillium,* and *Purpureocillium* are common genera in a wide range of habitats including plant rhizospheres, soil, air, and water. *F. oxysporum* has been recovered in Veggie plant and associated hardware samples since the VEG-01C experiment with zinnia (*Zinnia hybrida* cv. 'Profusion') in 2015 (Khodadad et al. 2020; Hummerick et al. 2021; Schuerger et al. 2021a). Compounded by excess humidity and water accumulation on plant tissues in the Veggie flight unit, fungal growth subsequently identified as *F. oxysporum* damaged zinnia leaves and stems and was also shown to be an opportunistic pathogen in ground studies (Schuerger et al. 2021a). *Alternaria alternata* is a common endophytic fungal species isolated from plants that can also be pathogenic, causing leaf spot in brassica crops like mizuna (DeMers 2022). However, no indication of symptomatic disease was present on the leaves from VEG-04A where *A. alternata* was recovered.

### Microbiological safety

Results of the present study show that microorganisms of concern may grow on mizuna tissues, especially in an environment shared with humans, even if safe-handling procedures are practiced. Future work is needed in this area to identify better methods to clean hardware and to ensure safety of crops consumed in flight. This challenge is considerable given restrictions of mass, water, types of cleaning and sanitizing solutions, and testing capabilities. Solutions that fit within these limitations are required for crops to become an integral part of a food system to prevent the disastrous effects that foodborne illness could have on a space mission.

## Conclusions

This study found that the organoleptic acceptability of mizuna was not as influenced by lighting treatment as by location, with flight-grown produce perceived as more acceptable. A cut-and-come-again harvest approach for mizuna has neither apparent benefits on yield, nor major negative impacts, although crop regrowth after each harvest decreased over time. Mizuna yield was generally lower in flight, likely due to negative responses to the spaceflight environment. However, this has not been consistently observed for all crops grown in space. Especially now that crop biomass can be measured in flight with the MMD, it is recommended that biomass be included as a standard parameter of data collection in future studies to better observe this effect across crops. The red-rich treatment yielded higher for single harvests, while the blue-rich treatment was better for cut-and-come-again in flight, but not on the ground. The blue-rich treatment positively affected accumulation of nutrients more than the red-rich treatment, and nutrient accumulation in flight was higher than on the ground for many nutrients. Due to this variability, the desired harvest program/growth duration should be decided based on the desired nutrients, acknowledging that nutrient trade-offs may occur. The effect of lighting treatment on microbial load was inconclusive, since blue-rich light resulted in lower bacterial and fungal counts on leaves than red-rich in the VEG-04A experiment but not in VEG-04B. The cut-and-come-again harvest approach resulted in a significant increase of both bacteria and fungi in flight and a slight effect in the ground controls.

In general, low sample sizes and spaceflight-imposed stresses can make it difficult to assess differential light

responses. Longer-duration, repetitive harvesting may change nutrients differentially and can increase microbial loads, but this approach may be more sustainable in certain mission scenarios where resources or crew time may be limited. Flight-grown mizuna was generally more acceptable to tasters and had higher nutrient levels, but yields tended to be lower and microbial loads were higher. Similar testing with different crops is recommended to discern crop-specific effects from general plant responses to the spaceflight environment, harvest approach, light treatment, or a combination of factors. Ground studies can form a basis of decision-making for future space crops and crop-growth approaches, but flight testing is still needed to confirm selections until enough flight studies are performed to generalize flight impacts for different crops.

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## Author contributions

All authors contributed to the design of the work with JB, MH, LS, and MY completing research and data analysis. All authors contributed to the development of the manuscript and final version to be published.

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No potential conflict of interest was reported by the author(s).

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