

The Microbiology of Microgreens Grown in Controlled Environment Chambers under ISS Conditions.

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Microgreens have been identified as a new type of pick-and-eat salad crop that can be utilized in space crop production systems. The majority of traditionally grown leafy green crops can be grown as microgreens, in addition to crops such as legumes, sunflower, buckwheat, most herbs, and corn, presenting hundreds of microgreen crop options. Notably, microgreens are nutrient dense, high in beneficial compounds like antioxidants, Vitamins C and K, and exhibit a variety of desirable flavors and textures. The short growth cycles (7-14 days), low water requirements and volume optimization potential make them a viable option for sustainable production of nutritious and flavorful crops in space. The crop production team at Kennedy Space Center is investigating the food safety aspects of microgreens grown under spaceflight relevant conditions for crew consumption. Microbiological analysis and screening for potential foodborne pathogens was performed on over 20 varieties of microgreens that have demonstrated positive horticultural attributes. Additionally, a comparison of microgreens grown hydroponically under ISS environmental conditions and similar varieties from local markets was completed to collect baseline data on the microbial load on microgreens. In an effort to improve microgreen quality, strategies to reduce the microbial load were tested, including bulk seed sanitization, harvest age, exposure to high blue light, and post-harvest chemical disinfection. The efficacy of a citric acid-based produce wash currently used for ISS grown produce and 1% H_2O_2 were investigated at different exposure times for reduction in bacterial and fungal counts on a variety of microgreens. Limited log reduction was achieved depending on exposure time. Our testing also demonstrated that seed sanitization impacted microbial load on microgreens and systems.

Nomenclature

APH = advanced plant habitat
CFU = Colony Forming Units
DI = De-ionized
HEA = Hektoin Enteric Agar
IMA = Inhibitory Mold Agar
ISS = International Space Station
LED = Light emitting diode

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Ppm = Parts per million
PPFD = Photosynthetic photon flux density

I. Introduction

THE food production team at NASAs Kennedy Space Center (KSC) is evaluating a myriad of candidate crop species that have the potential to deliver a highly nutritious and palatable component to the astronaut's diet in space crop systems[1-4]. Crop production on the International Space Station (ISS) has successfully been demonstrated in the Veggie and Advanced Plant Habitat (APH) plant growth hardware, resulting in harvests and crew consumption of seven cultivars of leafy greens and one chili pepper variety[5,6]. An evaluation of microgreens is being done at KSC for nutritional content, organoleptic acceptability, and microbiological quality, for eventual selection for space crop production. Microgreens have gained in popularity in recent years and are especially suited for indoor controlled environment agriculture[7,8]. They are easily grown and are harvested between 7-14 days. Terrestrially, they are used in fresh salads and other dishes to enhance flavor. The body of research on microgreens is relatively recent and limited, although with the popularity as a commercial crop, publications on microgreens research are increasing. Microgreens are highly nutritious in phytonutrients like antioxidants, Vitamins C and K, however nutritional attributes depend on growth conditions such as lighting and growing substrate as well as the particular plant variety [8]. Like sprouts, microgreens are harvested as young plants, and while they share some characteristics with sprouts in the ways they are cultivated, only the shoot of the microgreen is consumed, excluding any root material and like other leafy greens, they are usually consumed raw. Cultivation methods and consumer culinary practices are considerations for the evaluation of microbiological quality and safety.

In an effort to characterize the microbiology of cultivars of microgreens being considered for space crop production, we conducted a survey of bacterial and fungal load as well as food safety screening for *Escherichia coli*, coliforms, *Salmonella* sp and *Staphylococcus aureus* on 36 different microgreen varieties grown in controlled environment chambers with ISS environmental conditions. A comparative microbial analysis of similar market available microgreens was also conducted. Additionally, we investigated methods to reduce microbial risk associated with the quality and consumption of microgreens. This investigation included the development of a method to sanitize seeds in bulk resulting in no loss in seed viability and concomitantly, a reduction in seed microbial load. The U.S. Food and Drug Administration (USFDA) recommends that sprout growers prewash their seeds in a 20,000-ppm calcium hypochlorite ($Ca(OCl)_2$) solution to kill human pathogens that might attach to the seeds[9] , however a chlorine gas method is utilized in our testing to eliminate liquid submersion of seeds. The efficacy of post-harvest chemical sanitization methods including a produce wash currently used on ISS grown and consumed produce was tested on harvested microgreens.

The effect of seed density and harvest height on the total microbial load of the edible portion of microgreens is also reported here. These data will contribute to the understanding of microbial risk and mitigation for the cultivation and consumption of this novel crop for space agriculture.

II. Methods

A. Cultivation and Harvest

Figure 1. Microgreens in growth chamber.

As part of KSC's new crop production work, a prescreening and down selection process of 36 different microgreen species (Appendix A) were tested in a Percival reach-in environmental chamber (Figure 1). Microgreens used for other tests such as seed and produce sanitization were cultivated the same way. Microgreens were seeded onto a sterile hemp mat contained in a hydroponic tray cleaned with 3% H_2O_2. Seeds were weighed and sowed across the mat at consistent pre-determined densities. Hoagland's solution (1/2 strength) was pumped from a reservoir continuously through the mat and chamber environmental conditions were maintained at 23°C, 3000 ppm CO_2 and 50% relative humidity to simulate ISS conditions. LED lighting (OSRAM Phytofy)

was set in the following configuration: Blue (450 nm) = 23%; Green (521nm) = 27%; Red (660 nm) = 50%. to provide a total of 150 PPFD with a photoperiod of 16 hours on/8 off (Fig. 1).

After 10 days of growth, microgreens were harvested by cutting shoots above the root mat with a sharp knife blade (Figure 2). Since microbiological analysis was to be performed, the harvesting blade and gloves were sanitized with 70% ethanol to minimize contamination between crops. Three, approximately five-gram samples were collected for microbiological analysis, after the total yield was weighed. For the seed sanitization study, twelve cultivars were used to compare the difference between microgreens sown with sanitized and unsanitized seeds.

Figure 2. Harvest of microgreens for microbiological analysis. (A) Mat removal (B) 3 microgreen varieties before harvest (C) Harvest (D) Collecting microbiological samples.

B. Microbiological Analysis

A 1:10 weight-volume dilution was prepared in sterile blender bags for each sample with sterile buffered peptone water (BPW) and the bags were then blended in a Bag blender (Interscience) for 2 minutes. After, 100 µL aliquots were plated and spread onto trypticase soy agar (TSA) and inhibitory mold agar (IMA) plates at dilutions of -4, -5, and -6 for TSA and -1, -2, and -3 for IMA, the plates were then incubated at 30° C for 48 hrs for TSA and 120 hrs for IMA. After incubation, the plates were counted manually and counts were recorded in a excel spreadsheet that calculated and determined colony forming units per gram fresh weight (CFU gfw-1) of sample.

Screening was done for certain potentially pathogenic microbes using selective and differential media and selective *Salmonella* enrichment. One mL of the sample was placed onto an *E. coli/coliform* and Staph express Petrifilm (3M, Minneapolis, MN) and incubated for 24 hours. After incubation, the films were observed for the presence of a positive pathogen and counted following manufacturers protocols. For the *Salmonella* screening the procedure requires the use of different growth media to enrich and then selectively discriminate *Salmonella* species within a set of samples. Samples were initially placed in a 35˚ C incubator for 24 hours to pre-enrich all the species present in the samples. Then, the samples were placed into a Rappaport-Vassiliadis (RV) media broth and placed in a 35˚ C incubator for another 24 hours to allow for selective enrichment of *Salmonella* species. Following this incubation period, the samples were then streaked on Hektoen Enteric (HE) agar for isolation and placed back in the 35˚ C

incubator until colonies appeared, roughly 24-72 hours. Following incubation, colonies typical of *Salmonella* sp if present, were identified. Microbiological testing is based on FDA procedures for the microbiological analysis of foods[10].

C. Seed Sanitization

Seeds were sanitized based on a modification of a previously utilized sanitization method used for seeds in Veggie experiments[11]. Since microgreens require several grams of seeds, a larger volume chamber method was developed to hold up to five glass 100 x 15 mm petri dishes (Figure 3). The chamber is a 2.5 L anaerobic incubation chamber providing an adequate seal to prevent escape of the chlorine gas. Chlorine gas is generated in the jar when 5.3 mLs of hydrochloric acid (HCL) is added to 115 mL chlorine bleach. Operations were performed under a fume hood. Seeds were weighed and poured into the petri dishes in a mono-layer to ensure exposure to the gas for one hour. Germination and microbiological testing were done on each seed variety. Five seeds of each variety were placed onto sterile filter paper moistened with sterile DI water in petri dishes and observed daily for germination. For assessment of microbial growth, five seeds were placed each onto TSA and IMA, incubated at 30˚ C and observed for bacterial and fungal growth.

Figure 3. Seed sanitizing chamber

D. Market Comparison

Five varieties of microgreens, Radish, Kale, Cilantro, Broccoli, and Pea were purchased from a local market and kept refrigerated for 48 hours until analysis. Microbiological analysis was performed as described in this paper and each variety was sampled and analyzed in triplicate.

E. Post-harvest Sanitization Testing.

After harvest, microgreens were sanitized using either 1% H_2O_2 or 1% Pro-San (Microcide, Sterling Heights, MI) an organic acid based produce wash. Pro-San was tested as a foam as well as a wipe soaked in the disinfectant which is the current method used on ISS for produce cleaning. Sterile water and no treatment served as controls. Three cultivars were grown and used for this evaluation, Cressida Cress (*Lepidium sativum*), Tokyo Bekana (*Brassica rapa* var. chinensis), and 'Waltham 29' Broccoli (*Brassica oleracea* var. italica). After harvest, microgreens were placed into a ziplock bag and sanitizer or sterile water was added. Microgreens were gently shaken in the bag containing sanitizer for 1 minute, followed by 3 rinses with sterile DI water. For the wipe method, microgeens were placed on a sterile 6 x 6 poly wipe (Kimberly Clark) soaked with 1% Pro-San. A second wipe was placed on top and gently pressed onto the microgreens for 30 seconds. Three approximately five gram samples were collected from each treatment and processed as described in section B, microbiological analysis. Following this test, a second test was performed to investigate the effect of a longer exposure time of 3 minutes. Tokyo Bekana was exposed to Pro-San Foam, 1% H_2O_2 and sterile DI for 1 and 3 minutes. The microgreens were rinsed and processed as described previously.

F. Harvest Height and Seed Density

Harvest height was tested by growing two varieties of microgreens, Tokyo Bekana and Black Oil Sunflower (*Helianthus annuus*) and harvesting at 7, 10 and 14 days. The distance from the top remained consistent with each harvest (~40 mm), while, with time, the stem length, or distance from the root mat increased.

To examine the effect of microbial load on seed planting density, seeds were planted at full density, 2/3 and 1/3 density, grown for 10 days, harvested and processed for microbiological analysis. Planting densities for each variety are listed in Appendix A.

III. Results

A. Survey of 36 Cultivars

Figure 4 shows the microbial counts in terms of colony forming units (CFU) per gram of fresh weight of harvested microgreens. It should be noted that none of the seeds used in this study were sanitized prior to planting. However, the hemp mats, reservoir, and troughs that the microgreens were grown on were sterilized prior to each planting. Additionally, all the species in each test shared the same reservoir for the duration of each test which could have affected the microbial loads across the different species. Overall, microgreens tend to have higher microbial counts per gram fresh weight in comparison to other leafy greens grown in controlled environments at KSC. The grey and yellow lines on the graph illustrates the NASA aerobic plate count (APC) and Yeast/Molds standards for non-thermostabilized foods, respectively. It should be noted that there are no current NASA standards for freshly grown foods in these controlled habitats. Therefore, mitigation efforts need to be implemented in order to lower the overall microbial loads and prevent contamination with potential pathogens on freshly harvested microgreens.

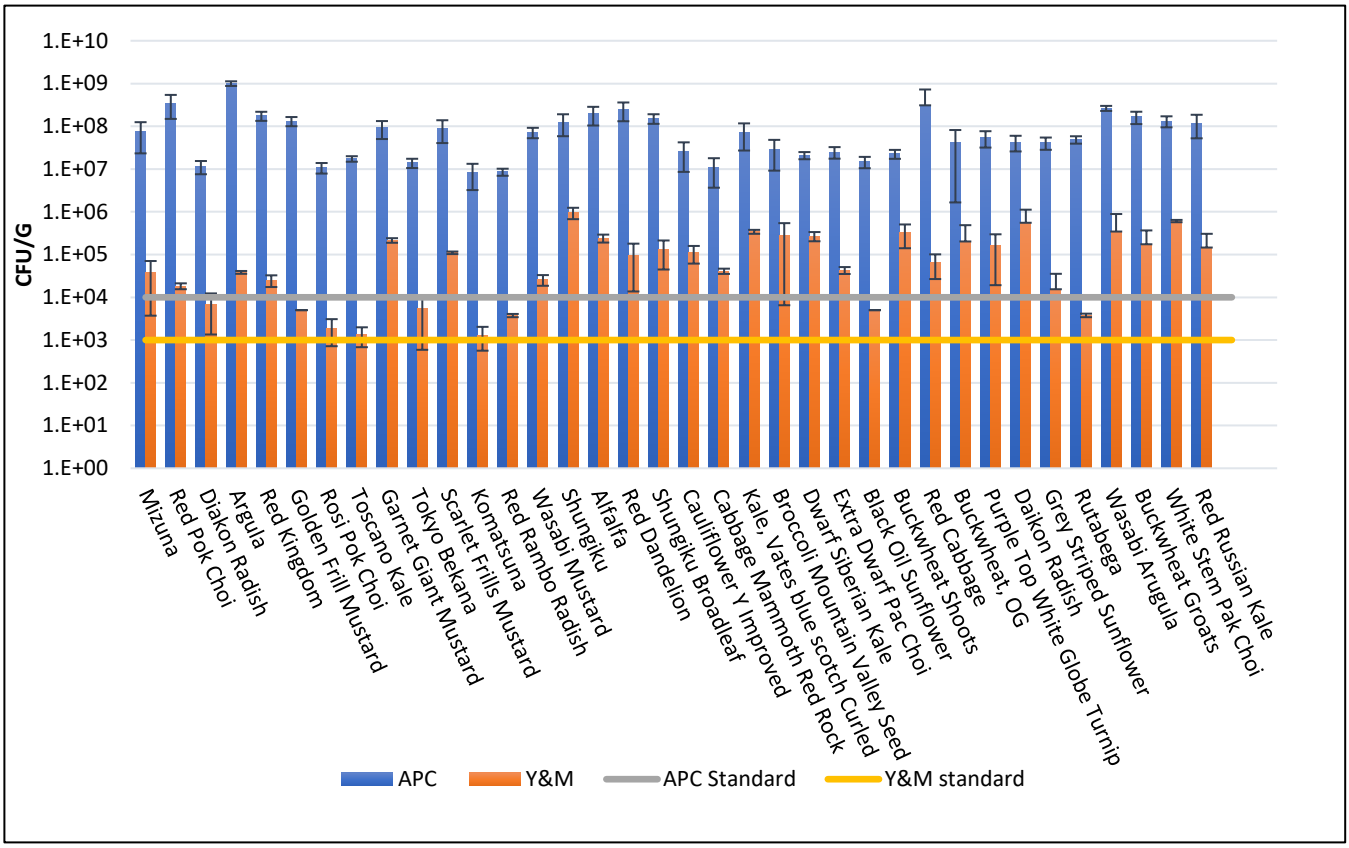

Figure 4. Aerobic plate counts on TSA and yeast and mold counts on IMA (CFU/g) from microgreen samples. N=3 and error bars represent standard deviation.

Screening for potential pathogens yielded negative results, neither *E. coli*, *S. aureus*, nor *Salmonella* were detected in any of the microgreen samples. The presence of coliforms was detected on multiple microgreen samples but were not identified as *E. coli*. Bacterial counts and the presence of bacteria in the family, *Enterobacteriaceae* (include non-fecal coliforms) that serve as an indicator of contamination of processed foods, irrigation water and food processing surfaces are frequently present in raw foods like fresh produce making those tests less relevant as risk indicators for fruits and vegetables. However, the presence of coliforms may represent a risk to the consumer and can be mitigated by implementing sanitization efforts pre and post-harvest.

B. Seed Sanitization

Figure 5 illustrates the effect of seed sanitization on the bacterial and fungal counts on ten-day old microgreens. Bacterial counts on TSA were not reduced in any of the plants grown from sanitized seeds. In the case of Tokyo Bekana, Cressida Cress, Mizuna (*Brassica rapa*), 'Vates Blue Scotch Curled' kale (*Brassica oleracea* var. acephala), and Alfalfa (*Medicago sativus*) the counts were actually higher when grown from sanitized seeds. Seed sanitizing did have an effect on lowering the fungal counts on eight of the twelve varieties tested. It was also noted that fungal growth on the hemp mat was observed to be less when the seeds were sanitized.

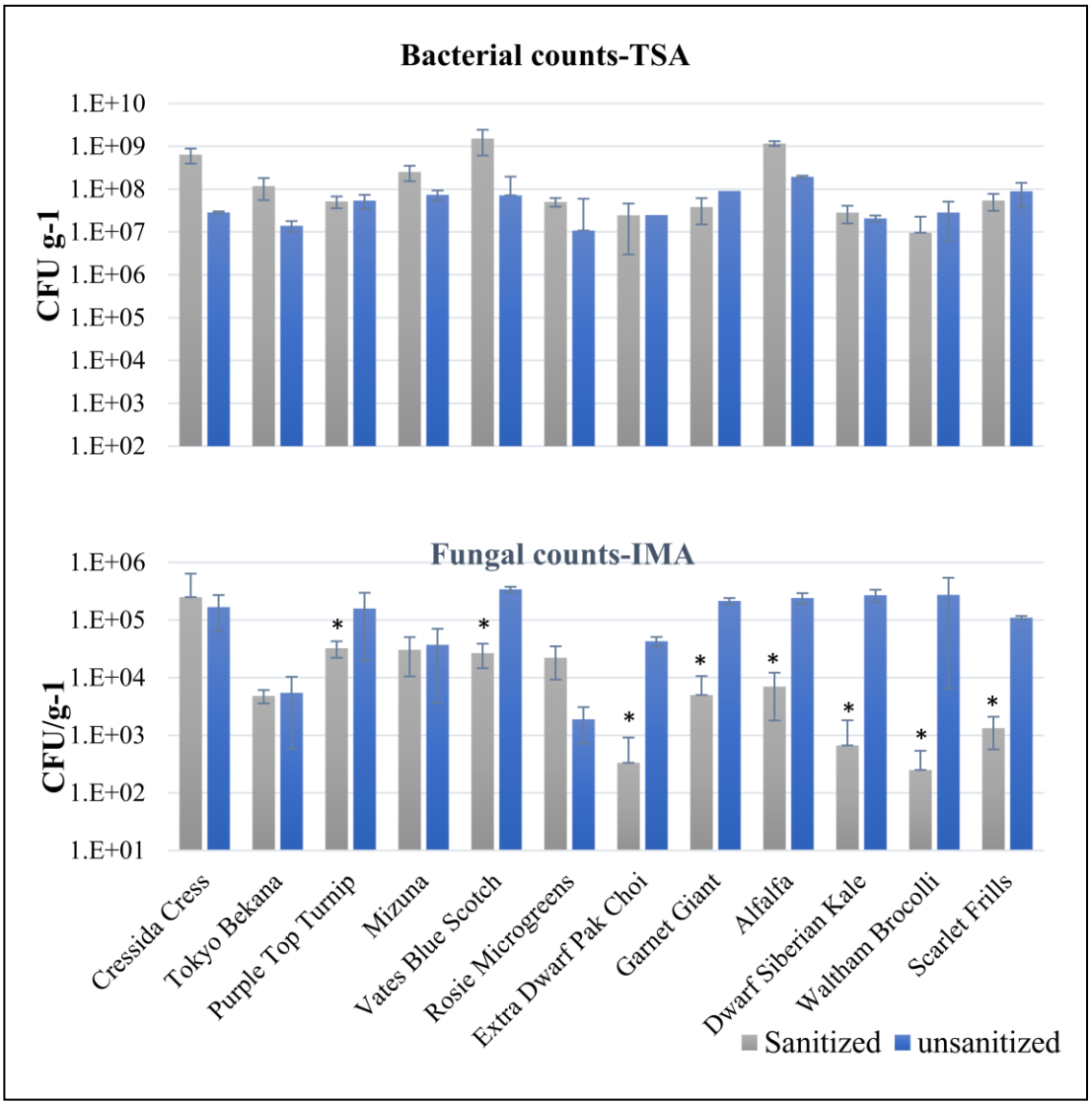

Figure 5. Bacterial and fungal counts (CFU/g) on microgreens grown with and without seed sanitizing. *Significantly lower fungal counts.

C. Market Comparison

Bacterial counts (APC) on the market procured microgreens were higher than those grown in the chamber except for cilantro, however fungal counts were higher in the chamber grown microgreens (Table 1) when comparing microgreens sewn from unsanitized seeds. The fungal counts from microgreens grown from sanitized seeds were lower than comparable varieties from the market, below 1 x 10^3 (data not shown).

Table 1. Average APC and fungal counts (CFU/g) on market and chamber grown microgreens (n=3). Chamber grown are those cultivated from unsanitized seeds. ND=no data.

	APC		Fungal	
	Market	Chamber	Market	Chamber
Radish	1.8 x 10^8	1.2 x 10^7	5.1 x 10^3	6.8 x 10^3
Cilantro	3.7 x 10^7	1.7 x 10^8	4.1 x 10^3	ND
Kale	6.8 x 10^7	2.1 x 10^5	1.6 x 10^3	2.7 x 10^5
Pea	1.0 x 10^8	2.9 x 10^4	8.8 x 10^2	8.2 x 10^3
Broccoli	4.1 x 10^8	2.1 x 10^5	9.8 x 10^2	2.8 x 10^5

D. Sanitization tests

The effectiveness of the sanitizing methods tested was minimal. ProSan foam was the most effective with a 1.13 log reduction on Cressida cress (Table 2).

Table 2. Efficacy of sanitizers shown as log reduction in bacterial counts on TSA. ND=no data.

	Log reduction (Bacterial CFU/g)					
	Water	1% H_2O_2 1 min	1% H2O2 3 min	ProSan Foam 1 min	ProSan Foam 3 min	ProSan wipe
Tokyo Bekana	0.59	1.06	0.25	0.89	0.94	0.58
Cressida Cress	0.39	0.69	ND	1.13	ND	0.49
Broccoli	0.67	0.93	ND	0.96	ND	0.91

E. Harvest Height and Seed Density

At harvest the height of the microgreens was measured in three different places on the mat. Tokyo Bekana did not vary significantly between 7 and 14 days. Sunflower had an average height of 40 mm at day 7 and 10 but by day 14 the average height was 94 mm. Sunflowers were harvested 40 mm from the top, leaving approximately 50 mm stem height at day 14 harvest. The rational for harvesting further away from the rooting mat, if possible, is to minimize the contribution of the rooting mat microbial load on the edible shoots. A significant difference between day 7 and day 14 harvests from both crops could be seen in the fungal counts and bacterial counts were lower on the sunflower microgreens (Figure 6).

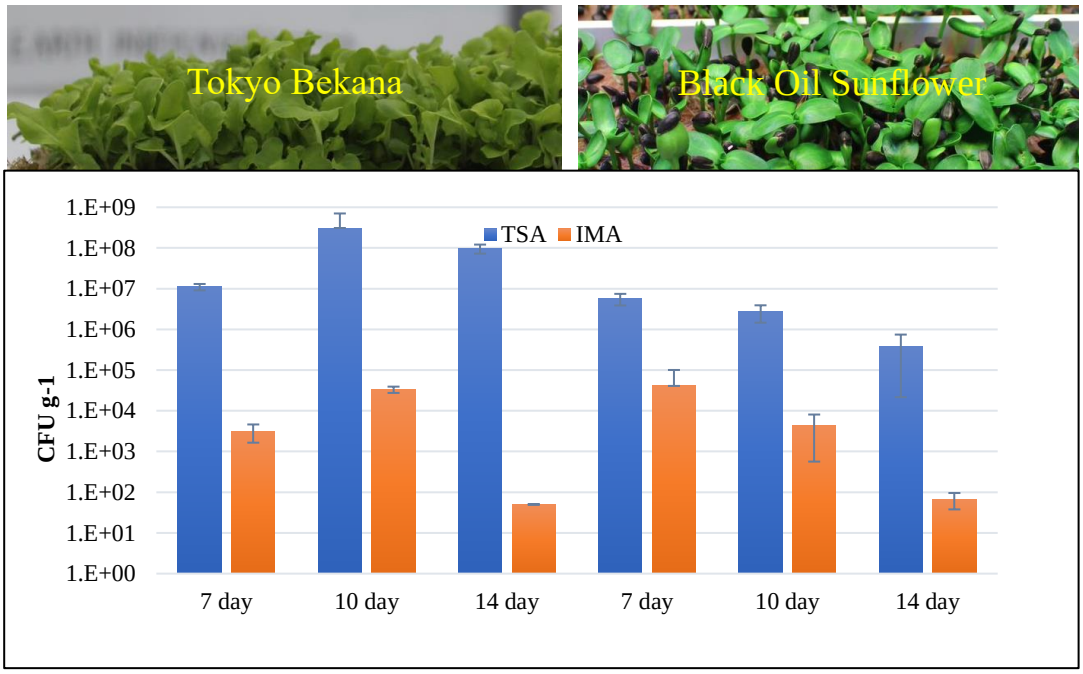

Figure 6. Bacterial (TSA) and fungal counts (IMA) (CFU/g) on microgreens harvested at the same height (40mm) from the cotyledons at 7, 10 and 14 days. N=3, bars represent standard deviation.

IV. Conclusion

Microgreens are being considered by NASA as a pick and eat salad crop for space system agriculture. Our work here describes the microbiological quality of microgreens and possible practices to minimize microbiological risks to the consumer and the quality of the crop. We show that seed sanitizing reduces the fungal load on the edible portion of the plant and that some horticultural practices, such as harvest height may impact microbial load. These data and additional future studies on post-harvest cleaning will contribute to defining critical control points in the cultivation of microgreens.

Appendix A

Crop	Variety	Scientific Name	Planting density g/4 x 4 in	Total g planted/tray (7, 4x4 sections)
Mizuna		*Brassica rapa*	0.9	5.6
Red Pac Choi	Red	*Brassica rapa chinensis*	1	5.6
Daikon Radish (white)	Daikon	*Raphanus sativus*	2	14
Arugula		*Eruca sativa*	1	7
Golden Frills Mustard	Golden Frills	*Brassica juncea*	0.8	5.6
Mizuna	Red Kingdom	*Brassica japonica*	1	5.6
Pok Choi	Rosi	*Brassica rapa chinensis*	1	7
Kale	Toscano	*Brassica oleracea*	1	7
Mustard	Garnett Giant	*Brassica juncea*	0.9	6.3
Chinese Cabbage	Tokyo Bekana	*Brassica rapa chinensis*	0.9	6.3
Mustard	Scarlett Frills	*Brassica juncea*	0.8	5.6
Komatsuna		*Brassica rapa perviridis*	1	7
Radish	Red Rambo	*Raphanus sativus*	2	14
Mustard	Wasabi	*Brassica juncea*	1	6.3
Shungiku		*Glebionis coronaria*	2	7
Alfalfa		*Medicago sativus*	1.5	10.5
Dandelion	Red	*Chichorium intybus*	1	3.5
Shungiku	Broadleaf	*Glebionis coronaria*	2	7
Cauliflower	Y Improved	*Brassica oleracea botrytis*	1	7
Cabbage	Mammoth Red Rock	*Brassica oleracea capitata*	1	7
Kale	Vates blue scotch curled	*Brassica oleracea acephala*	1	7
Broccoli	Mountain Valley Seed	*Brassica oleracea italica*	1	7
Kale	Dwarf Siberian	*Brassica oleracea acephala*	1	7
Extra Dwarf Pac Choi	Extra Dwarf	*Brassica rapa chinensis*	1	7
Sunflower	Black Oil	*Helianthus annuus*	10	7
Buckwheat	Shoots	*Fagopyrum esculentum*	1	7
Cabbage	Red	*Brassica oleracea capitata*	1	7
Buckwheat, OG		*Fagopyrum esculentum*	1	7
Turnip	Purple Top White Globe	*Brassica rapa*	1	7
Radish	Daikon, red	*Raphanus sativus*	2	7
Sunflower	Grey Striped	*Helianthus annuus*	7	49
Rutabega		*Brassica napobrassica*	2.3	16.1
Wasabi Arugula	Wasabi	*Diplotaxis erucoides*	0.8	7
Buckwheat	Groats	*Fagopyrum esculentum*	8	66.5
Pac Choi	White Stem. Extra Dwarf	*Brassica rapa chinensis*	1	7
Red Russian Kale	Red Russian	*Brassica napus*	1	7
Sunflower	Grey Striped	*Helianthus annuus*	7	49

Rutabega		*Brassica napobrassica*	2.3	16.1
Arugula	Wasabi	*Diplotaxis erucoides*	0.8	7
Buckwheat	Groats	*Fagopyrum esculentum*	8	66.5
Pac Choi	White Stem, Extra Dwarf	*Brassica rapa chinensis*	1	7
Kale	Red Russian	*Brassica napus*	1	7
Tatsoi		*Brassica rapa*	0.9	5.6
Cress	Cressida	*Lepidium sativum*	0.9	5.6
Kohlrabi	Purple	*Brassica oleracea*	0.9	5.6
Brussell Sprouts		*Brassica oleracea*	0.9	5.6
Collards	Vates	*Brassica oleracea*	0.9	5.6
Lettuce Mix, Mucelum		*n/a*	0.9	7
Cress	Upland	*Barbarea verna*	1	7
Lentils		*Lens culinaris*	7	49
Orach		*Atriplex hortensis*	2	14
Basil	Dark Opal	*Ocimum basilicum*	1.4	9.8
Shiso		*Perilla frutescens*	2	14
Cress	Persian	*Lepidium sativum*	1	7
Canteloupe		*Cucumis melo*	3	21
Quinoa		*Chenopodium quinoa*	?	14
Chia		*Salvia hispanica*	1	7
Pea	Dwarf Grey Sugar	*Pisum sativum*	17	119
Pea	Dun	*Pisum sativum*	17	119
Pea	Mammoth Melting Sugar	*Pisum sativum*	19	133
Swiss Chard	(yellow)	*Beta vulgaris*	3.5	24.5
Beet	(Bulls Blood)	*Beta vulgaris*	3.5	24.5
Fennel		*Foeniculum vulgare*	2	14
Mung Beans		*Vigna radiata*	11	77
Kohlrabi	(white)	*Brassica oleracea*	1	7
Pac Choi	White Stem	*Brassica rapa*	1	7

Acknowledgments

We would like to thank Larry Koss for assistance in building and maintenance of the hydroponic growing systems used in these studies. This work is funded by NASA HRP.

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