

**Microbiological sampling methods and sanitation of edible
plants grown on ISS**

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Microbiological sampling methods and sanitation of edible plants grown on ISS

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Pathogenic microbes on the surfaces of salad crops and growth chambers pose a threat to the health of crew on International Space Station. For astronauts to safely consume space-grown vegetables produced in NASA's new vegetable production unit, VEGGIE, three technical challenges must be overcome: real-time sampling, microbiological analysis, and sanitation. *Raphanus sativus* cultivar Cherry Bomb II and *Latuca sativa* cultivar Outredgeous, two salad crops to be grown in VEGGIE, were inoculated with *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), a bacterium known to cause food-borne illness. Tape- and swab-based sampling techniques were optimized for use in microgravity and assessed for effectiveness in recovery of bacteria from crop surfaces. Rapid pathogen detection and molecular analyses were performed via quantitative real-time polymerase chain reaction using LightCycler® 480 and RAZOR® EX, a scaled-down instrument that is undergoing evaluation and testing for future flight hardware. These methods were compared with conventional, culture-based methods for the recovery of *S. Typhimurium* colonies. A sterile wipe saturated with a citric acid-based, food-grade sanitizer was applied to two different surface materials used in VEGGIE flight hardware that had been contaminated with the bacterium *Pseudomonas aeruginosa*, another known human pathogen. To sanitize surfaces, wipes were saturated with either the sanitizer or sterile deionized water and applied to each surface. Colony forming units of *P. aeruginosa* grown on tryptic soy agar plates were enumerated from surface samples after sanitization treatments. Depending on the VEGGIE hardware material, 2- to 4.5-log₁₀ reductions in colony-forming units were observed after sanitization. The difference in recovery of *S. Typhimurium* between tape- and swab-based sampling techniques was insignificant. RAZOR® EX rapidly detected *S. Typhimurium* present in both raw culture and extracted DNA samples.

I. Introduction

Pathogenic microbes on the surface of salad crops and growth chambers pose a threat to the health of crew on International Space Station (ISS). Lada Vegetable Production Unit (VPU) was plant growth hardware developed by the Russian Federal Space Agency (FKA) that produced salad crops on ISS. Microbiological samples recovered

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from Lada hardware exhibit microbial densities higher levels than are allowed for safe consumption after successive harvests¹. As a result of practicing inadequate post-harvest sanitization techniques, American astronauts have been prevented from consuming produce grown in Lada. Microbiological detection techniques on space station are currently limited to a total microbial load count without organismal identification. These data are insufficient for determining whether pathogens are present.

Improved sampling and detection methods, as well as a means to mediate the threat of pathogenic infection, are needed to improve crew safety, especially in light of the possible deployment of VEGGIE (Orbitec, Inc.), the new VPU commissioned by NASA, on ISS in December 2013 or early 2014. The goals of this comparative study are threefold: to determine representative microbiological sampling methods, and to optimize rapid detection of pathogens with quantitative real-time polymerase chain reaction (qRT-PCR), and to design a simple yet effective method of sanitizing the surfaces of crops and production units. These methods were optimized to require as little time and effort as possible by astronauts for use in microgravity.

Methods of rapid pathogen detection were explored using a scaled-down PCR instrument called RAZOR® EX (Biofire Diagnostics, Inc.) was compared with the detection capabilities of a full-scale instrument, LightCycler® 480 (Roche, Inc.). RAZOR® EX is currently undergoing evaluation for its potential as flight hardware.

Raphanus sativus cv. Cherry Bomb II (radish) and *Latuca sativa* cv. Outredgeous (lettuce) were the two model organisms studied for surface contamination to test microbiological sampling techniques. These techniques included a hands-free, “pop-up,” tape-based method¹, a swab stick method, and a bag maceration method for comparison. Samples were taken at the top, middle, and bottom surfaces of each organism. These surface locations are described in more detail in section II-B.

The commercially available, food grade sanitizer PRO-SAN® (Microcide, Inc.) has been shown to be a viable candidate for repeated sanitation³. To affirm the effectiveness of this sanitizer for crops and growth chambers, initial testing included a comparison of log₁₀ reduction of colony-forming units (CFU)/cm² per sanitizer on the two plastics used for the majority of surfaces in VEGGIE. These sanitizing agents were applied with a dry wipe saturated in each solution.

II. Materials and Methods

All methods were performed with aseptic technique while wearing sterile gloves.

A. Sanitization of VEGGIE Materials

An isolated colony of *P. aeruginosa* was incubated in 25 mL of tryptic soy broth (TSB) in a sterile Erlenmeyer flask on a shaker for at 35°C for 18 h. The TSB was transferred to 50 mL centrifuge tube and was centrifuged at 5,000 rpm for 10 min. The supernatant was decanted, and pellet was resuspended in sterile diH₂O. Absorbance was measured at 540 nm using a ThermoSpectronic™ GENESYS™ 20 spectrophotometer, and an extract from the resuspended pellet was diluted as necessary to achieve density ~10⁸ CFU/mL.

Sanitization methods were tested on the surfaces of Teflon® perfluoroalkoxy (PFA) fluoroplastic film (DuPont, Inc.) from the flexible bellows enclosure and of DuPont™ Teflon®-coated Kevlar® (TCK) fabric from the root mat with integrated fluid reservoir. A second sheet of TCK was also cut into eighteen 5-cm² squares (coupons). To ensure cleanliness of surfaces before inoculation, 70% EtOH was sprayed generously and let stand for 10 s. Then the surfaces were each wiped with a clean paper towel and let dry in a biological safety cabinet (BSC). Surfaces were then exposed to the UV lamp in the BSC for 30 min. Eighteen 5-cm² squares, divided by strips of tape on both solid sheets, and nine coupons were each inoculated with 100 µL of ~10⁹ CFU/mL *P. aeruginosa* culture suspended in TSB. Inocula were spread evenly on each square using yellow, plastic spreaders. Surfaces were allowed to dry for at least 1 h till fully dry before applying treatment.

The sanitizer tested was 1% PRO-SAN®, a citric acid-based, food grade sanitizer. PRO-SAN® was compared with sterile diH₂O and a control for effectiveness in reduction of microbial counts on the materials listed above. The control was not wiped. Three replicates each of the three treatments were performed on each surface. Six 5-cm² polypropylene wipes (Kimberly Clark, Inc.) per material and forceps were autoclaved in pouches. In the BSC, individual wipes were submerged in either sterile sanitizer or sterile diH₂O with sterile forceps. After saturating each wipe, it was held with forceps and allowed to drip. One wipe was used to sanitize each inoculated square for 30 s using forceps. After wiping, each wipe was placed in a labeled, sterile 50-mL centrifuge tube. Controls (3 squares per surface and 3 coupons) were not subjected to the wiping procedure.

Buffered peptone water (PBW) was diluted using 4.5 mL sterile diH₂O dilution blanks, and 100 µL was plated onto tryptic soy agar (TSA). For the sanitizer treatment, 1:10, 1:100, and 1:1E3 dilutions were used; for the water



Figure 1. Growing Outredgeous and Cherry Bomb II. Lettuce and radish cultivars are being grown in a growth chamber under controlled environmental conditions.

surface: at the leaf base and the outer edge of the leaf at both middle and top. All markings were made on the flattest spots possible to ensure ease of sampling with tape- and swab- based methods.

Tape-based sampling was performed by removing a pre-cut strip of tape from the “pop-up” dispenser, holding the sides while pressing the center of the strip onto the inoculant location, and placing the strip into a glass petri dish containing 3 mL TSB⁴ as shown in Figure 2. Swab-based sampling entailed pressing and rubbing a sterile stick swab in a back-and-forth motion across the sampling site for 10 s. The bag method included placing an entire radish into a sterile, plastic bag containing a relative amount of TSB per weight and macerating with a bag mixer for 2 min.

Each square was inoculated with 10 μ L *S. Typhimurium* in the BSC. Radish and lettuce were set to dry completely for at least 1 hr. After crops dried, all 3 sampling methods were performed on 3 of each crop. Samples were added to 15-mL culture tubes with 5 mL of TSB. Buffer extracts from each sampling method were serially diluted and 100 μ L of the appropriate dilution was plated onto TSA and Brilliant Green Agar (BGA). The plates were incubated at 37°C for 48 h and enumerated after 48 h. The remaining buffer was incubated for 6 h at 37°C for enrichment of the *S. Typhimurium* culture. A 2-mL extract of each enriched culture was transferred to a 2-mL centrifuge tube and stored at -20°C for molecular analysis.

C. Enumeration of CFU

CFU were counted using a manual colony counter at 1.75x magnification. A dot of permanent marker was made as each CFU was counted. CFU were generally only counted on plates with 30 to 300 CFU present; however, this was not always possible. For plates with lower than 30 CFU present, the total number was counted and recorded. For plates with greater than 300 CFU present, a three-step process was followed to ensure a representative count was recorded: 1) three 1-cm² squares were marked where CFU density looked representative of whole plate; 2) each square was enumerated; 3) average CFU was calculated; 4) average was multiplied by plate surface area (56.5 cm²).

treatment, 1:100, 1:1E3, and 1:1E4 dilutions were used; and for the unsanitized controls, 1:1E4, 1:1E5, and 1:1E6 dilutions were used. Plates were incubated at 37°C for 48 h, and CFU were counted after 48 h.

B. Microbiological Sampling of Salad Crops

Radish and lettuce were grown in soil in an environmental control chamber (Figure 1) at 50% relative humidity with a 16 h photoperiod (23°C/18°C) with 40% metal halide, 40% high pressure sodium, and 20% fluorescent light sources.

Leaves were removed from 9 radishes with sterile scissors, and 9 leaves of lettuce were removed from the base by hand. Three 1-cm² squares were marked on each radish and lettuce leaf. On radishes, squares were marked at the top near the leaf base, the middle of the body, and the bottom near the root base. On lettuce leaves, squares were marked on the upper



Figure 2. Tape-based Sampling of Cherry Bomb II. 1-cm² squares were sampled with adhesive tape via the “pop-up” method in a biological safety cabinet.

D. Isolation of Genomic DNA

A 200- μ L sample from each sampling method replicate was pipetted into a new 2-mL centrifuge tube. Serial dilutions were made by pipetting 1.8 mL of sterile diH₂O into each tube and vortexing the tube for 5 s. A spectrophotometer was blanked with 1 mL of sterile diH₂O, after which absorbance at 540 nm was measured with 1 mL of each diluted sample. Absorbance between 0.1 and 1 Abs units was desired.

One colony of *S. Typhimurium* was pipetted into 25 mL TSB and was incubated in a 35°C shaker for 125 rpm for 18 h. Two hundred μ L of culture was pipetted into a 2 mL centrifuge tube. DNA was extracted, measured for absorbance, and serially diluted to create known standards against which unknown samples could be compared using the LightCycler® 480. DNA was serially diluted 1:5; 1:10; 1:100; 1:1,000; 1:5,000; and 1:10,000 with sterile diH₂O. Dilutions were vortexed for 5 s between each successive dilution. 1:10 dilutions were made from stocks of each sampling method. Genomic DNA was isolated from diluted culture using the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.). The Experienced User Protocol provided in the MO BIO Kit has been summarized as follows:

1. Add 1.8 ml of microbial culture to a 2-mL centrifuge tube and centrifuge at 10,000 x g for 30 seconds at room temperature. Decant the supernatant and spin the tubes at 10,000 x g for 30 seconds at room temperature and completely remove the media supernatant with a pipette tip.
2. Resuspend the cell pellet in 300 μ L of MicroBead Solution and gently vortex to mix. Transfer resuspended cells to MicroBead Tube.
3. Add 50 μ L of Solution MD1 to the MicroBead Tube.
4. Secure MicroBead Tubes horizontally using the bead beater. Vortex at maximum speed for 1 min.
5. Make sure the 2-mL MicroBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at 10,000 x g for 30 seconds at room temperature.
6. Transfer the supernatant to a clean 2-mL centrifuge tube.
7. NOTE: Expect 300 to 350 μ L of supernatant.
8. Add 100 μ L of Solution MD2 to the supernatant. Vortex for 5 seconds. Then incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 min at 10,000 x g.
10. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2-mL centrifuge tube. Expect approximately 450 μ L in volume.
11. Add 900 μ L of Solution MD3 to the supernatant and vortex for 5 seconds.
12. Load about 700 μ L into the spin filter and centrifuge at 10,000 x g for 30 seconds at room temperature. Discard the flow through, add the remaining supernatant to the spin filter, and centrifuge at 10,000 x g for 30 seconds at room temperature. Discard all flow through liquid.
13. Add 300 μ L of Solution MD4 and centrifuge at room temperature for 30 seconds at 10,000 x g.
14. Discard the flow through.
15. Centrifuge at room temperature for 1 min at 10,000 x g.
16. Being careful not to splash liquid on the spin filter basket, place spin filter in a new 2-mL centrifuge tube.
17. Add 50 μ L of Solution MD5 to the center of the white filter membrane.
18. Centrifuge at room temperature for 30 seconds at 10,000 x g.
19. Discard spin filter.

Extracted DNA samples were serially diluted with sterile diH₂O in new centrifuge tubes and vortexed for 5 s. Concentration and quality of 1 μ L DNA was measured for each dilution using a NanoDrop 1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.) at 260 nm. Desired 260 nm/280 nm values were 1.7 to 1.9. DNA was frozen at -20°C for storage.

E. qRT-PCR

The LightCycler® 480 was used to quantitatively compare *S. Typhimurium* DNA against *S. Typhimurium* genomic DNA standards. The RAZOR® EX and LightCycler® 480 were used to assess effectiveness in rapid detection of pathogens. The RAZOR® EX was only used for positive versus negative detection but detected both raw culture and extracted DNA, whereas LightCycler® was used for quantitative results with extracted DNA only. The samples that were run on the LightCycler® 480 included standards, two negative controls, and two replicates of each of three following samples: enriched bagged lettuce, three enriched swabbed lettuce, three pre-enriched swabbed radish, and three enriched swabbed radish. The samples run on RAZOR® EX included two replicates of enriched cultures, pre- and post-enriched swabbed radish, standards, and a positive and negative control.

PCR preparations were done in a laminar flow hood. SYBR Green I Master (Roche) was used for LightCycler® 480. A 20- μ L reaction of 15 μ L master mix and 5 μ L template DNA was added to each well. 15 μ L master mix was composed of 4.5 μ L sterile PCR grade H₂O (Roche), 0.25 μ L forward primer (Invitrogen), 0.25 μ L reverse primer (Invitrogen), and 10 μ L SYBR Green I Master. Then 5 μ L template DNA was added to each well, and the plate was sealed. Two experimental replicates of three biological replicates of lettuce and radish were compared with two

experimental replicates of six standards. Two negative controls, with sterile PCR grade H₂O instead of template, were added as well.

The enzyme activation program was executed for one cycle at 95°C for 10 min. For quantification, the amplification program was executed for 40 cycles at 95°C for 15 s; annealing at 55°C for 10 s; and extension at 72°C for 15 s on single acquisition mode. The melting curve program was executed for one cycle at 95°C for 30 s; 65°C for 30 s; and 95°C continuously. The cooling program was executed for one cycle at 40°C for 30 s.

The RAZOR® EX (Figure 3) was used to detect *S. Typhimurium* in diluted sample (1:10) from culture grown in TSB on a shaker (~18 h at 35°C), diluted post-enriched sample (1:10) from middle of swabbed radish #2 (RSM2); genomic DNA from diluted pre- and post-enriched sample (1:10) from RSM2; and genomic DNA from diluted *S. Typhimurium* standard (1:5). A negative control (PCR H₂O) and positive control (*S. Typhimurium* genomic DNA standard) were compared against samples.

The final concentrations of reagents used for RAZOR® RT-PCR were as follows: 1x Taq360 Buffer, 2.5 mM MgCl₂, 200 µM ea dNTPs, 900 nM forward primer, 900 nM reverse primer, 100 nM TaqMan Probe, 0.025 U/µL AmpliTaq GoldEnzyme, variable to 150 µL PCR H₂O, variable template DNA. For this experiment, 1 µL template was used for cDNA samples. For raw culture samples, 100.00 µL culture was used. The program was run with a denaturing cycle at 94°C for 4 min followed by annealing at 60°C for 1 min followed by 54 cycles of denaturing at 94°C for 15 s and annealing at 60°C for 60 s.

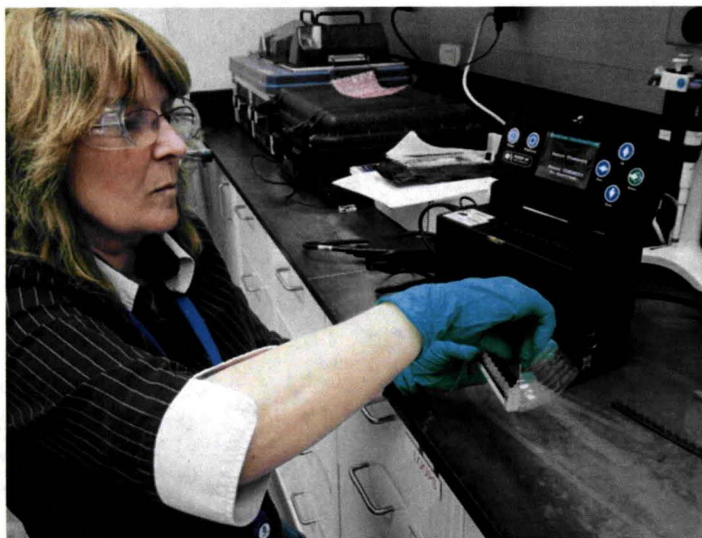


Figure 3. Loading *S. Typhimurium* into RAZOR® EX.

RAZOR® EX rapidly detected *S. Typhimurium* in both raw

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III. Results and Discussion

A. Sanitization

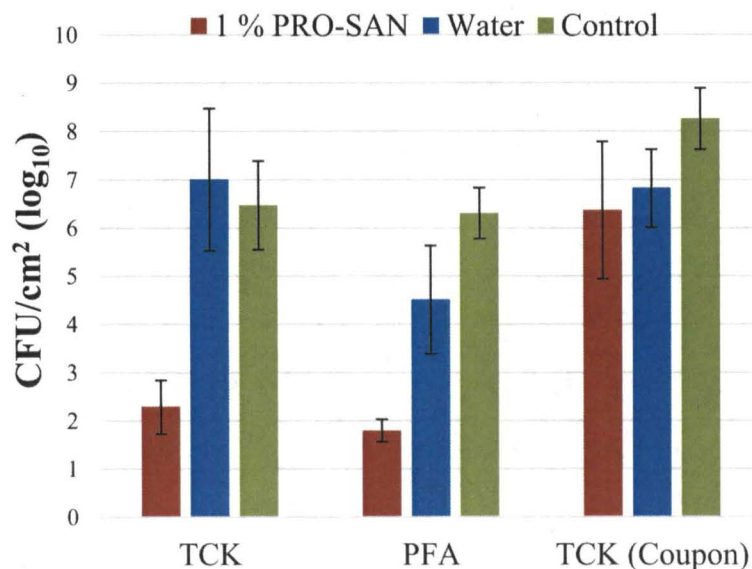


Figure 4. Average microbial density per material.

Microbial density was lowest on sheets of TCK and PFA after sanitizing with 1 % PRO-SAN versus other treatments. Error bars are located two standard deviations away from average.

Data indicate that 1 % PRO-SAN® is significantly more effective at sanitizing sheets of the materials used as most of the surfaces within the VEGGIE system as displayed in Figure 4; however, the 1 % PRO-SAN® did not show a significant effectiveness at sterilizing coupons of TCK. Sterile diH₂O had an insignificant effect on the microbial density present on TCK; however, water was slightly significantly more effective than the control treatment on PFA. It is speculated that the TCK coupons were not effectively sanitized with either sanitizer because they had free range of motion during treatment. As such, it was a difficult challenge to wipe each coupon while avoiding contact with other surfaces.

As shown in Figure 5, the reduction in microbial density on sheets of TCK and PFA was observed to be upward of

4 log₁₀ CFU/cm² after 1 % PRO-SAN was applied. Sterile, deionized water actually decreased the sterility of TCK sheets more than the control. Water reduced CFU/cm² by almost 2 orders of magnitude on PFA and by over 1 order of magnitude on TCK coupons.

B. Microbiological Sampling

Displayed in Figure 6, differences in microbial density from plate counts were shown to be insignificant between each sampling method. Enrichment of culture did, however, show an increase in microbial density across all samples taken by swab- and bag-based methods. Samples were not taken from pre-enriched culture via the tape-based method because all bacteria adhered to the adhesive on the tape. As such, a comparison of pre- and post-enriched samples gathered with tape could not be performed.

C. Rapid Detection of Pathogens with qRT-PCR

LightCycler® 480 successfully detected *S. Typhimurium* in every sample tested from both radish and lettuce. Enriched radish DNA was approximately 10 times more concentrated than pre-enriched radish DNA. Bagged lettuce DNA was approximately 10 times more concentrated than swabbed lettuce DNA. Data are not shown.

Results suggest that the presence of *S. Typhimurium* was detected in enriched culture samples as well as both pre- and post-enriched extracted DNA samples by RAZOR® EX even without prior extraction of DNA. Pre-enriched culture samples were not tested.

IV. Conclusion

Sanitization with 1 % PRO-SAN® wipes was most effective at sterilizing *P. aeruginosa* on TCK and PFA sheets. The methods for wiping TCK coupons will be improved for future experiments. Since both tape- and swab-based sampling methods were equally effective, the tape-based method would probably be most effective in microgravity because of the ability for an astronaut to secure the tape dispenser with an elastic strap around the wrist while sampling. Only enriched culture samples were tested for pathogen detection with RAZOR® EX, and experiments must be conducted to explore whether *S. Typhimurium* can be detected from samples of raw, pre-enriched culture. Other pathogenic microbes, including *P. aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* will be tested in the future. If pathogens in raw sample can be detected with

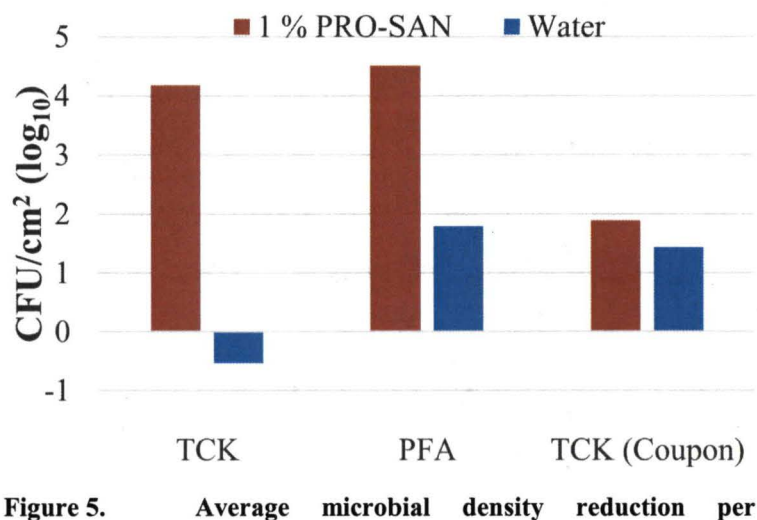


Figure 5.
material.

Sanitizing with wipes saturated with 1 % PRO-SAN reduced microbial

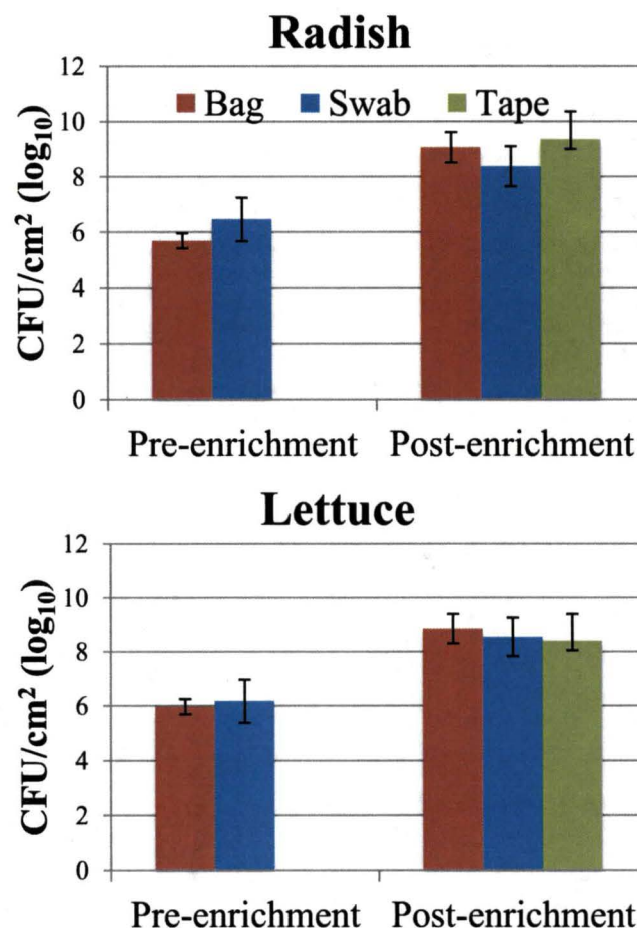


Figure 6. Average microbial density on plates of pre- and post-enriched samples from radish and lettuce. Enriched samples from both radish and lettuce exhibited higher microbial densities than pre-enriched samples in both bag and swab treatments. There is no significant difference in the recovery of microbes between sampling methods. Error bars are located one standard deviation

RAZOR® EX, then the time requirement by astronauts for rapid pathogen detection with RAZOR® EX would decrease; however, reagent preparation time will also need to be minimized with future research. In order to confirm the viability of RAZOR® EX at testing a variety of other pathogens, there is a need to optimize PCR reagent concentrations for other pathogenic microbes. Low limits of pathogen density in samples must be researched to know how much sample is required by RAZOR® EX. The remaining samples from lettuce and radish will be tested on both the LightCycler® 480 and RAZOR® EX as this project continues.

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