11-574 - Hummerick

The effect of plant cultivar, growth media, harvest method and post harvest treatment on the microbiology of edible crops.

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Systems for the growth of crops in closed environments are being developed and tested for potential use in space applications to provide a source of fresh food. Plant growth conditions, growth media composition and harvest methods can have an effect on the microbial population of the plant, and therefore should be considered along with the optimization of plant growth and harvest yields to ensure a safe and palatable food crop. This work examines the effect of plant cultivar, growth media, and harvest method on plant microbial populations. Twelve varieties of leafy greens and herbs were grown on a mixture of Fafard #2 and Arcillite in the pillow root containment system currently being considered for the VEGGIE plant growth unit developed by Orbitec. In addition, Sierra and Outredgeous lettuce varieties were grown in three different mixtures (Fafard #2, Arcillite, and Perlite/Vermiculite). The plants were analyzed for microbial density. Two harvest methods, "cut and come again" (CACA) and terminal harvest were also compared. In one set of experiments red leaf lettuce and mizuna were grown in pots in a Biomass Production System for education. Plants were harvested every two weeks by either method. Another set of experiments was performed using the rooting pillows to grow 5 varieties of leafy greens and cut harvesting at different intervals. Radishes were harvested and replanted at two-week intervals. Results indicate up to a 3 $\log_{10}$ difference in microbial counts between some varieties of plants. Rooting medium resulted in an approximately 2 $\log_{10}$ lower count in the lettuce grown in arscillite then those grown in the other mixtures. Harvest method and frequency had less impact on microbial counts only showing a significant increase in one variety of plant.

Post harvest methods to decrease the bacterial counts on edible crops were investigated in these and other experiments. The effectiveness of PRO-SAN and UV-C radiation is compared.

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I. Introduction

Human presence in space beyond low earth orbit, whether it is a base on the moon or Mars, will likely include earth like components such as plant growth systems. Resupply of critical life support elements, food, oxygen, and water would be limited, therefore plants could provide atmospheric and wastewater regeneration to a closed ecological system as well as provide a source of fresh and nutritious food. Understanding the microbial ecology of crops grown in controlled environment plant growth systems is important in assuring the quality and safety of the crop as a food source. Plant growth conditions, nutrition and plant species are some of the factors that can influence the microbial population inhabiting plant surfaces (1). The microbial community naturally present in the phyllosphere of a plant may impact the ability of other microorganisms, perhaps human pathogens, to colonize the leaf surface. The microbial load on crops intended for consumption by astronauts must fall within an acceptable range according to NASA microbiological standards set for food. Currently the limit for aerobic bacteria on a non-thermostabilized food item is $\leq 10^4$ colony forming units (CFU) (2). Studies done to determine the microbial numbers in field and retail samples of a variety of leafy green produce items report total aerobic bacteria levels in the range of 10^4 to 10^7 CFU/gram (3,4). Research continues on the development of systems for the growth of salad crops in closed environments for space mission scenarios (1, 2). One such system, the VEGGIE plant production unit developed by Orbitec as a low supply "salad machine" is currently being tested and has been approved and safety tested for deployment on the International Space Station (Stutte etal). The VEGGIE unit used in these experiments delivers water and nutrients to the plants by a capillary matting system: a reservoir connected to an absorbent root mat combined with a containment system that holds the seed and rooting material in "pillow" like packets. The pillows have been tested and successfully grown varieties of lettuce.(Stutte et al) One advantage of using plant containment like the pillow in a system intended for use in microgravity is that the seed, and rooting media can be pre-packed and disposed of as a unit or reused individually. Additionally, a vegetable production unit (VPU) currently housed on the Sveda module of the International Space Station (ISS) has been studied with respect to plant growth conditions, microbial risk and psychological benefits of producing edible crops in space. Previous studies done with Mizuna(*Brassica juncea* var. *japonica)* grown in the Lada VPU showed an increase in bacterial and fungal counts with subsequent harvests from the same root module while the effectiveness of a food sanitizer decreased on the crops with higher microbial counts[3].

The work presented here examines multiple factors that have been shown to affect the microbial density present on edible plants grown in other vegetable production unit configurations including, growth medium, harvest method, and plant cultivar. Harvest methods examined in this study were the "cut and come again" (CACA) approach versus terminal harvest of the plants. The CACA type harvest not only allows prolonged use of the same growing media through subsequent harvests decreasing the need for resupply of rooting, growth media and seeds as well as labor involved in set-up and planting. Another advantage is that leafy greens can be harvested as desired eliminating storage requirements for uneaten material. Two separate sets of experiments were done to compare harvest methods. Firstly, two salad crops, Mizuna (*Brassica juncea* var. *japonica)* and Red Leaf lettuce (*Lactuca sativa* var. '*Outredgeous*') which have been used in studies testing a variety of plant growth systems specifically designed for closed environments (2) were grown in Biomass Production System for education (BPsE). These are similar in configuration to the VEGGIE plant growth systems. We examined the microbial densities, both bacterial and fungal, after sequential harvests and reuse of the same growth medium contained in pots. The second set of experiments compared the microbiology of 5 different species of plants grown in the pillow root containment system containing the same growth medium but harvested by the 2 methods described for the first experiment. The plant cultivars used in this experiment were selected based on studies done at KSC demonstrating successful growth in the pillow root containment systems( ). Included in these studies was a comparison of the microbial counts on the phyllosphere of 12 cultivars of herbs, leafy greens and one legume (pea) as well as a comparison of two cultivars of lettuce, Outredgeous and Sierra grown in 3 types of rooting media, Fafard #2, Perlite, and Arcillite. Finally, we tested 2 methods of post harvest sanitization and the effect of harvest method on the efficacy of the sanitizer PRO-SAN® to reduce microbial counts.

This work emphasizes factors that have an effect on the microbiological counts and should be considered in the initial planning of plant growth systems to meet the standards required by NASA food safety

II. Materials and Methods

A. Cultivar and Growth medium evaluation:

1. Plant growth:

Plants were grown using the rooting pillows designed to contact the root mat water delivery reservoir. Pillows were hand constructed using 15 cm x 10 cm static shielding bags (4" x 6" reclosable static shielding bags, Uline, Pleasant Prairie, WI) with a 7.5 cm x 11.5 cm piece of Nitex nylon mesh heat sealed to the surface that comes into contact with the mat to allow capillary wicking. Pillows were filled with 100mL of media and fertilizer and sealed with the "ziplock" closure. Slits (two/pillow) were cut on the top surface and Nitex wicking was threaded through to accommodate seeds and facilitate moisture retention through germination.

Plants used for microbial analysis were from one set of all the cultivars grown in Fafard #2 and one set each of Outredgeous and Sierra grown in 3 types of rooting media, Fafard #2, Perlite, and Arcillite. The 2 plants grown in one pillow were pooled samples. Shoot tissue was cut from the plants and weighed using aseptic technique and placed into sterile blender bags (BagLight, Interscience Labs, Weymouth, MA). Sterile DI water was added to each sample in a 1:10 weight/volume ratio. Bags containing sample and diluent were placed in a bag mixer (Interscience Labs, Weymouth, MA) and blended for 2 minutes to remove microbes from plant surfaces. The sample extracts were serially diluted in sterile de-ionized water and plated in duplicate onto Difco R2A agar (BD, Franklin lakes, NJ). Plates were incubated at 28°C for 48 hours before enumeration of colonies to determine colony-forming units (CFU) per gram of tissue.

B. 2 species harvest method:

2. Plant Growth Conditions.

Mizuna and Red Leaf lettuce were planted in pots containing arscellite, clay based soil, and 7.5 grams/ liter of Nutricote® fertilizer. Crops were grown in a CEC (ECG, Chagrin Falls, OH) which controlled temperature, relative humidity, and carbon dioxide levels. Within the CEC, pots were contained within a BPSe (Fig. 1) which controlled water using a gravity flow system keeping water levels constant as well as light with an adjustable light panel. Parameters for the CEC were checked daily and set as follows: temperature 23.0^0 Celsius, relative humidity 55%, and CO2 1200ppm. Light was checked and adjusted twice per week with a LI-COR model LI-189 photometer so that PPFD averaged 200μmol/m2/s on the plant surface.

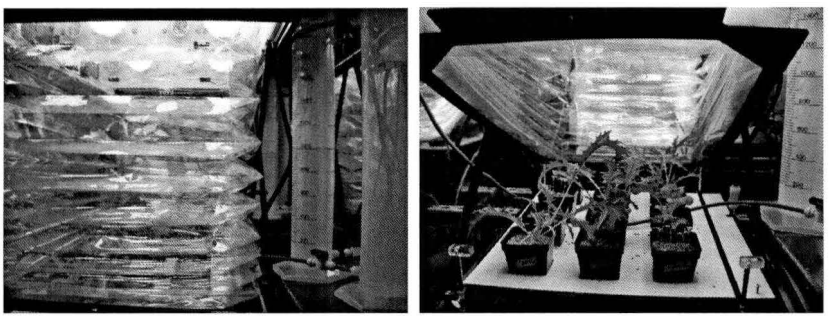

Figure 1. BPSe configuration inside of a CEC

3. Harvesting.

On the day of the harvest, the tray containing the pots was removed from the BPSe. On the first week of harvest all pots were thinned to three plants per pot. All plant material was sampled including roots to get a baseline of all culturable microorganisms that might grow on the plants. For the "cut and come again" method leaves were cut with a sterile scalpel within one inch from the base of the stem, leaving the smallest leaves for the next harvest. This method was uniform for all weeks after the first. On days 14, 28, and 42 the plants intended for replanting were fully removed from the pot and the root tissue was detached with a sterile scalpel. New seeds were the planted into the pots. Day 21 and 35 samples for this treatment were treated similarly as week one, except the root tissue was removed. On days 14, 28, and 42 harvested plants were weighed and half the plant tissue was sanitized for 3 minutes with PRO-SAN®, a citric acid based commercial sanitizer intended for use on fresh fruits and vegetables as well as food preparation surfaces. All samples were weighed and placed into sterile sampling bags; 10-50mLs of water was

then added depending on plant mass. Samples were then macerated in a Bagmixer® 400 lab blender (Interscience, Rockland, MA) to remove microorganisms from the plant.

C. 5 Species harvest method and cultivar evaluation in the pillow root matting

4. Plant Growth Conditions

Five species of plants were used in these studies: 'Outredegous' lettuce (Lactuca sativa cv. 'Outredgeous'), Mizuna (Brassica rapa cv.. nipposinica), 'Sierra' lettuce (Lactuca sativa cv. 'Sierra'),, 'Tokyo Bekana' Chinese cabbage (Brassica rapa var. chinensis 'Tokyo Bekana'), and 'Cherry Bomb II' radish (Raphanus sativus L. cv. Cherry Bomb II). These cultivars were selected based on the experiments done using 13 cultivars described in this paper. Seeds were planted in pillows, 2/slit and thinned at day 7 of growth to one plant per slit. Twelve pillows were plnated per species leaving four for for each harvest frequency.

The pillows were designed for the VEGGIE unit, but were grown in controlled environment chambers (CEC). The plants were grown in CEC to mimic the condition in the VEGGIE unit. The temperature was set for 28°C during the day and 24°C at night. The relative humidity was 70% during the day and 75% during the night. The photoperiod was 16 hours of light and 8 hours of darkness. The light was kept at approximately 150 µmol m-2s-1 with CO2 kept at 1200 ppm continuous. These environmental conditions were maintained using computerized control and checked daily.

Figure 2. Pillow arrangement for tubs. Pillows were randomly assigned to position using a random number generator.

5. Microbiological analysis.

After maceration in the Bag-mixer, sample diluents was precessed and analysed by the following methods:

Total plate counts:Sample solutions were serially diluted in sterile DI water and plated onto Plate Count Agar (PCA) (Difco, Becton Dickenson and Co. Sparks, MD) for aerobic plate counts (APC). Inhibitory Mold Agar (IMA) (Difco, Becton Dickenson and Co. Sparks, MD) was used for yeast and mold counts (YM). PCA plates were incubated at 25^0 C for up to 48 hours. IMA plates were incubated at room temperature for 5 days. Duplicates of each plate within the 25-250 colonies range, or lowest possible dilution (direct sample) were counted and averaged. Averages were used to calculate colony forming units per gram of fresh plant weight (CFU/g fwt).

Acridine orange direct counts: To calculate total direct counts (TDC), a portion of each sample was fixed with formalin to a final concentration of 2% and sonicated using a Sonic Dismembrator 550 (Fisher Scientific, Waltham, MA). Samples were stained with acridine orange, a DNA stain, and filtering through a 25mm black polycarbonate 0.2µm filter (Millipore Corp., Bedford, MA).2 Filters were mounted on a microscope slide with a drop of immersion oil and covered with a cover slip. Cells were enumerated from images acquired digitally (Olympus DP71 camera) through a Zeiss Axioscope 2 microscope (Carl Zeiss, Thornwood, NY) with a halide arc lamp (Xcite 120, Exfo life Sciences. Mississauga, Ontario). Since this staining method stains both living and dead cells, data collected will be used to determine if the total amount of bacteria on the plant varies with each harvest method. These data will also be used to conclude if PRO-SAN® is killing the plant microorganisms, or merely being washed off.

Oxygen biosensor method for community level physiological profiling: The BD Oxygen Biosensor System (BD-OBS) (BD Biosciences, Bedford ,MA) 96 well plates were used to measure the consumption of oxygen by microbes inoculated into the wells containing different carbon substrates. The bottom of the wells contains a silicone gel and an oxygen sensitive fluorophore. As the oxygen concentration decreases in the wells (as with microbial respiration), the fluorescent signal increases, enabling a measurement of substrate utilization by the overlaying microbial inoculum3. Forty microliters of 8 sterile substrate solutions (300 ppm, sucrose, fructose, mannose, acetate, coumerate, asparagine and lysine) were loaded into rows of wells of a plate. 200 ul of sample diluent was added to

each well containing substrate yielding a final substrate concentration of 50 ppm. Plates were covered with an adhesive film (Titer top, Diversified Biotech, Boston, MA) and the fluorescent signal read every 15 minutes for 48 hours on a Synergy plate reader (Biotek, Winooski, VT.) with filters for 485nm excitation and 590 nm emission wavelengths. For the analysis of the response to substrates, a normalized relative fluorescent unit (NRFU) was derived by dividing the measured fluorescent value measured at any time point by the value reached at 1 hour. The minimum response time (hours) to each substrate (when the NRFU reached a value of 1.1), was determined to assess microbial community physiological differences between plant cultivar and harvest method.

6. Data Analysis

Microbial enumeration data were transformed to log10 values. The effect of the harvest method and sequence on bacterial and fungal density, and total bacterial count, were analyzed using a 2 way ANOVA with the difference between treatments at time intervals being determined using the Bonferroni multiple comparison test (Graphpad Prism 4.00 for Windows, Graphpad Software, San Diego, Ca)

III. Results and Discussion

A. Cut vs. Replant Harvest microbial density.

Bacterial counts as indicated by the APC showed a decrease in culturable aerobic bacteria with each subsequent harvest of both plant types using the "cut and come again" method of harvest. After 28 days of Mizuna growth and 35 days of red leaf lettuce growth, the fungal counts increased steadily until the day 42 final harvest. Conversely the counts from the replanted crops did not show a significant decrease or increase over time. No difference was seen between 7 day old plants or 14 day old plants after consecutive replants in the same growth medium. (Fig. 2). The total cell count derived from direct observation of acridine orange stained cells followed the same trend as the APC, i.e. a decrease in counts after day 28 and 35 on Mizuna and Red Leaf lettuce respectively. These results suggest that microbial

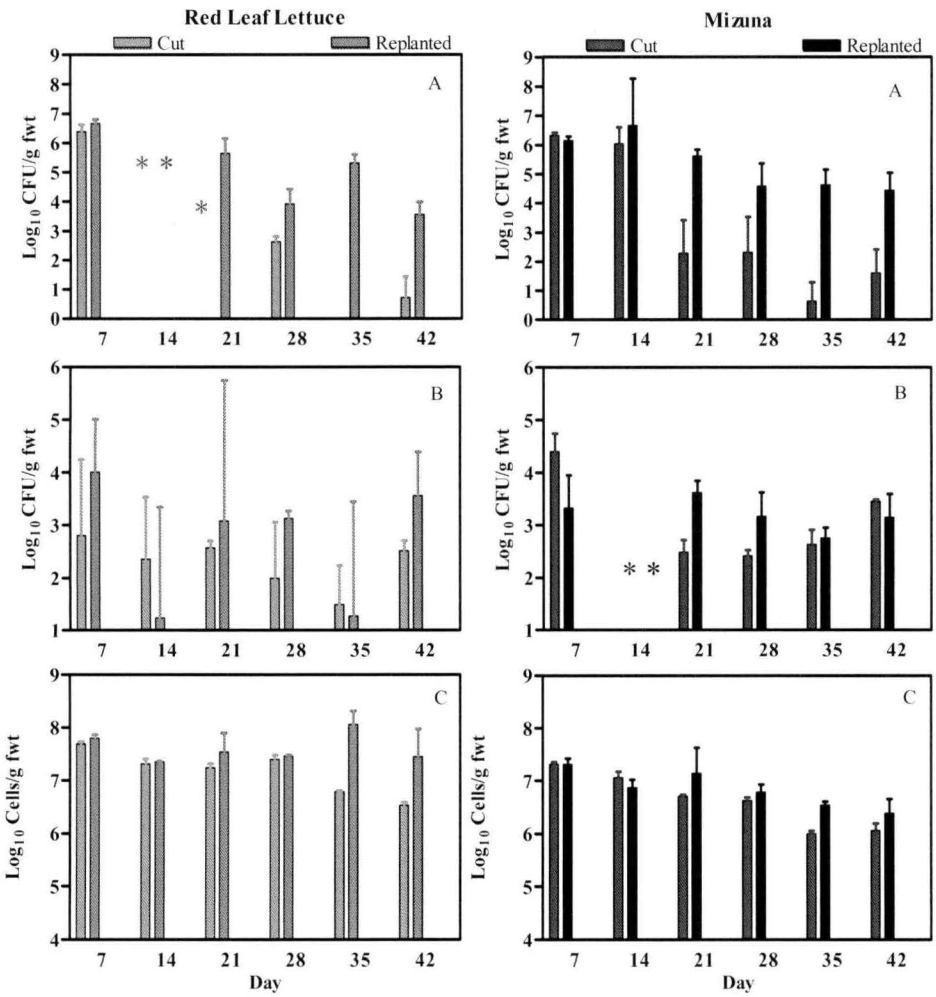

Figure 2. Microbial counts (A=APC, B= YM, C=TDC) on the phyllosphere (ex. whole seedling was used on day 7) of Red Leaf lettuce and Mizuna with replanting or cut harvest. *Below detection limit (Red Leaf lettuce APC Day 14=4.6, Day 21=3.4 CFU and YM Mizuna Day 14= 2.1 CFU).

density on the phyllosphere of both crops is dependent on the age of the plant (shown by a decrease over time) and not the harvest method.

Washing harvested leaves for three minutes in 2% PRO-SAN® followed by a thorough rinse was effective in reducing bacterial and fungal counts on mizuna by up to 4 $\log_{10}$ (Fig. 3 A). A 5 log reduction in APC was achieved using this method on the 14 day old mizuna harvested by the cut method. With increased age of the plant the effectiveness of the sanitizer decreased as seen on the 42 day harvests. Log reductions between 1 and 2 were seen on these mizuna plants with both harvest methods. This could indicate a shift in the microbial community to bacterial types that are more resistant to the sanitizing agent. Fungal counts were reduced by 2-3 $\log_{10}$ irrespective of harvest method. The TDCs were not affected by the PRO-SAN® treatment (Fig. 3 C) indicating that the action was biocidal on the culturable bacteria and fungi and not simply a physical removal of the cells via rinsing of the leaves. If this were the case a difference in total cell count would have been seen between the sanitized and unsanitized leaves.

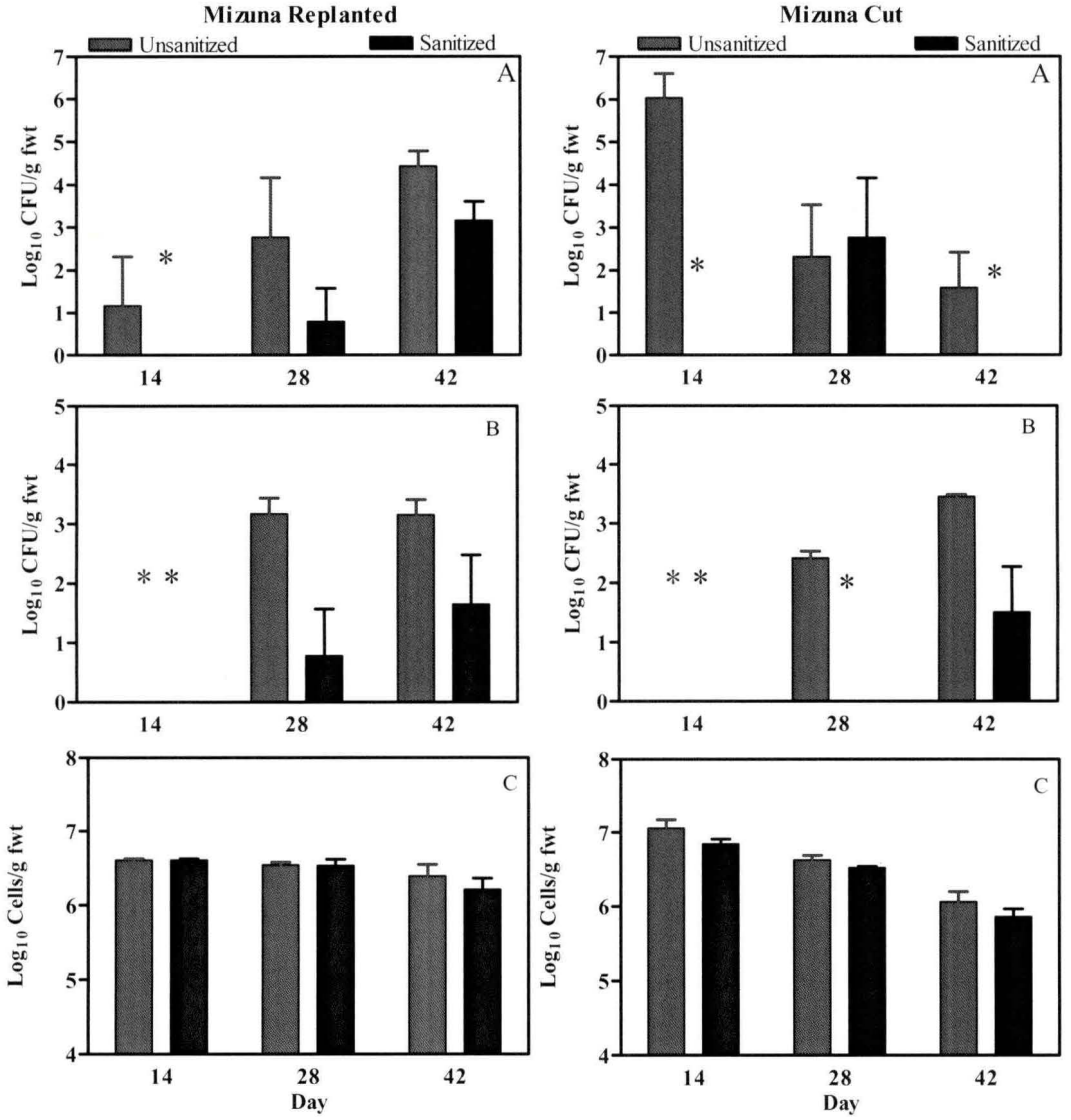

Figure 3. Microbial counts (A=APC, B= YM, C=TDC) on the phyllosphere of Mizuna with replanting or cut harvest before and after sanitizing with PRO-SAN®. *Below detection limit as indicated by asterisk.

The red leaf lettuce showed a similar trend in the replanted plants in that the log reduction in bacterial counts at Day 42 (the 3rd harvest) was less then at day 28 and there was no significant difference between the sanitized and

unsanitized plant APCs after the 3rd harvest.(Fig. 4) Some of the bacterial and fungal counts were below detection limits so log reductions in numbers could not be determined for these samples.

NASA requirements state that a non-thermostabilized food product must not exceed an aerobic plate count of $2x10^4$ CFU/g for any single sample or $1x10^4$ for any two samples from a lot and that yeasts and molds should not exceed 1000 CFU/g for any single sample. In our studies, sanitization with PRO-SAN® achieved these standards

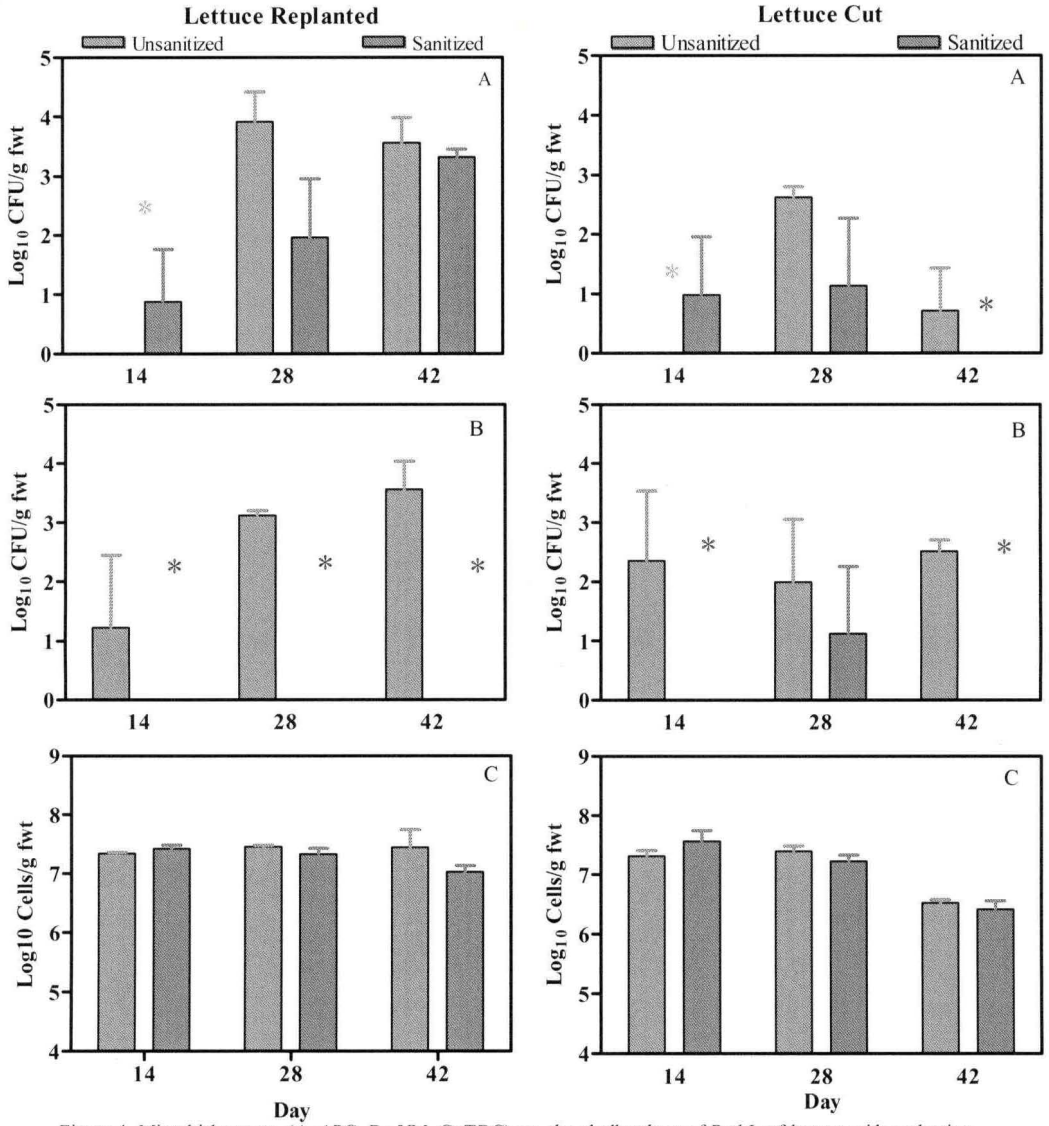

Figure 4. Microbial counts (A=APC, B= YM, C=TDC) on the phyllosphere of Red Leaf lettuce with replanting or cut harvest before and after sanitizing with PRO-SAN®. *Below detection limit as indicated by asterisk.

CLPP.

To determine possible temporal shifts in the microbial communities residing on the phyllosphere of the two crops over the sequential harvest cycles and grow outs, community level physiological profiles were performed on the washes from harvested plants. Generally, significant differences ($P<.01$) in substrate utilization as indicated by minimum response time could be seen between plant types and harvest methods at different time points (Data not shown). No significant differences were determined for individual plant types over time based on these analysis. Variation in microbial communities with different plant species is known and has been attributed to different factors including the topography of the leaves and chemical content of the plant (6) which could explain the difference seen between the two species examined.

B. Species harvest method and cultivar evaluation in the pillow root matting

Significant differences in aerobic plate counts (APC), yeast and mold (Y+M) counts, and AODC were found between the cultivars. ($P<.0001$ Figure 3). The two lettuces, 'Outredgeous' and 'Sierra', had less average bacterial colonization and Y+M counts than the two Mizuna and Chinese Cabbage, both in the Brassica family. Mizuna had the highest bacterial and fungal counts at all three harvest methods. However, this may be caused by the impact of its more open outward leaf growth on microbial populations than the close growth of lettuce leaves (Hunter et. al., 2010). It should also be noted that the pillows were grown in tubs instead of the enclosed VEGGIE unit. The tubs were exposed to the open environment, making the plant more susceptible to microbes.

No significant differences between weekly, bi-weekly, and monthly harvests for APC were observed except in one instance between Chinese Cabbage harvested weekly and monthly (TBM and TBW) at $P<0.05$. The data does not support the original hypothesis that multiple harvests would cause increased microbial loads due to the plant tissue being exposed to the environment with each cutting. Monthly harvest pillows were wounded and exposed to the environment momentarily before being placed in a stomacher bag, while weekly harvest pillows were handled and wounded more frequently. However, the bacterial load of the weekly pillows was still lower than the monthly for three of the four species.

There were no significant variations between harvest methods for Y+M counts. The monthly and weekly fungal counts for 'Outredgeous' lettuce (OL) were below detection limit As a result, the data cannot be used to support that harvest methods affected counts. However, for the other three species, fungal counts were higher for the weekly harvest than the monthly, which was opposite the observation seen in the microbial counts. Since fungal growth can have antibiotic properties, the increased fungal counts in weekly harvests may have influenced the microbial counts.

Cell counts were also found to differ significantly between harvest methods and cultivars. However, the images used for the counts were affected by large amounts of plant matter. Despite the efforts to sonicate and filter the matter, the images still contained significance amounts of matter.

These data suggest that in the plant growth systems tested here i.e. BPse and the pillow rooting mat system harvest method did not result in a significant increase in microbial load as was seen in the Lada VPU. Specific cultivars had a higher microbial density then others. When rooting medium was compared, plants grown in arcellite had signifantly lower bacterial counts then those grown in Farfard or Peat medium. These results suggest that through the effective selection of rooting medium and cultivar based on the understanding of the microbial ecology of the system, microbiological standards could be achieved.

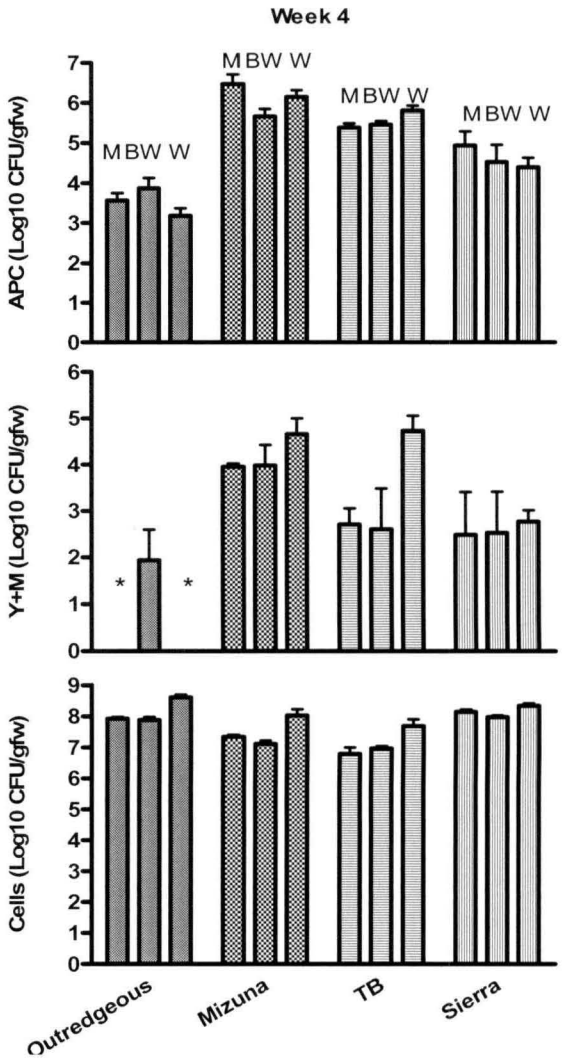

Figure 3. Aerobic plate counts, yeast +mold counts, and cell counts of plant species and harvest methods. Bars represent mean + 1 standard deviation. * represents counts that were below detection limit for that harvest method.