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Culture-Independent Analysis of Microbial Communities Associated with Hydroponically Grown Living Lettuce

Abby Matthews
University of Mississippi. Sally McDonnell Barksdale Honors College

CULTURE-INDEPENDENT ANALYSIS OF MICROBIAL COMMUNITIES ASSOCIATED WITH HYDROPONICALLY GROWN "LIVING LETTUCE"

By

Abby Christine Matthews

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Approved by

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Advisor: Dr. Colin Jackson

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Reader: Dr. Ryan Garrick

_____

Reader: Dr. Erik Hom

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ABSTRACT

Culture-Independent Analysis of Microbial Communities Associated with Hydroponically Grown "Living Lettuce"

The bacteria associated on and within a plant make up that plant's microbiome. Given recent interest in how microbes can affect human health, as well as developments in DNA sequencing that allow us to examine microbial communities without culture techniques, a number of recent studies have investigated the composition of the microbial communities on edible plants. Leafy green salad vegetables pose an added interest, since they are consumed without sterilization or cooking. This study investigated the bacterial composition of endophytic and epiphytic communities of hydroponically grown green leaf lettuce that is sold with the roots intact and referred to as "living lettuce." In addition to examining the composition of the bacterial communities associated with living lettuce, comparisons were made to conventionally grown and packaged lettuce, both at the time of purchase and following refrigerator storage to reflect the time of consumption. The structure of bacterial communities was determined using next generation sequencing of the 16S rRNA gene. Bacterial communities on living lettuce were significantly different from those observed on conventional lettuce. While differences between the dominant phyla and subphyla of communities of the two types of lettuce were observed on the day of purchase, community composition became more similar following two weeks of refrigeration. As expected, community diversity decreased across both types of lettuce

following refrigerator storage. Major taxa observed were consistent with results from previous studies of the bacterial communities of salad greens, and there were no significant populations of known human pathogens such as *Salmonella* spp. and *Escherichia coli*. This study demonstrates that the bacteria associated with hydroponically grown living lettuce, while different than that of traditionally grown and packaged lettuce, presents no apparent risk to the consumer in terms of its associated bacteria.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Introduction

The biosphere is dependent on the various activities of microbial organisms, and the majority of Earth's biodiversity is microbial. Despite this, human understanding of bacteria and other microorganisms is vastly limited, with their existence not even discovered until Leeuwenhoek observed them with a microscope in the 17th century (Pace 1997). Even centuries after the discovery of microorganisms, the study and classification of microbes was limited to those that could be grown via pure-culture. Over the last several decades our perspective on microbial diversity has changed drastically, beginning with Carl Woese's development of a phylogenetic tree that classified organisms as either Eukarya, Bacteria, or Archaea. Woese based his tree on the 16S ribosomal RNA (rRNA) gene sequence, a conserved phylogenetic marker that can be reliably amplified via Polymerase Chain Reaction using a range of primers (Pace 1997, Nelson 2013). Following Woese, Norman Pace further increased the understanding of microbial diversity by identifying microorganisms by obtaining their genetic material directly from the environment, without a need for pure culture, and using sequencing techniques to examine the 16S rRNA gene. Pace's work provided the means to identify microorganisms from natural environments without having to rely on culture-based techniques. The application of these approaches to thousands of different environments has shown that previous views of microbial diversity were greatly limited, with uncultivated microorganisms making up over 99% of the microorganisms existing in

nature (Hugenholtz 1998). As newer (next-generation) DNA sequencing methods have been developed over the last 10-15 years, mankind is beginning to realize that organisms also serve as a habitat for diverse microorganisms, and the concept of a "microbiome", the community of microorganisms in or on a specific organism or environment, has emerged (Nelson 2013).

Like other organisms, plants host a community of bacteria, which make up a plant's microbiome. Plant-associated microorganisms perform a variety of necessary functions, such as suppressing disease, stimulating growth, and promoting stress resistance (Berg et al. 2014). The microbial communities associated with agricultural plants have been gaining recognition in the biological community because of their potential to create more efficient and sustainable agricultural practices by increasing plant production and reducing chemical input and emission of greenhouse gasses (Hunter et al. 2013). Furthermore, plant-associated bacteria have gained attention in recent years because of potential relationships to human health in terms of the spread of food-borne disease and the contribution of edible plant diversity to our own gut microbiomes (Berg et al. 2014). The specific location on the plant is an important factor to consider when examining the impacts of their microbiome, with two distinct biomes existing above the surface of the soil: the phyllosphere (the leaf surface) and the endosphere (the inside of the plant; Turner et al. 2013).

The term phyllosphere, coined in 1950 and derived from the Greek word 'fyllo' meaning leaf, refers to the above-ground surface of a plant (Lugtenberg 2015). The microbes that colonize the phyllosphere are known as epiphytes, and can occupy the

phyllosphere up to 10^7 cells per cm^2. The phyllosphere has the potential to be a highly diverse and dynamic environment, and epiphytes are affected by changes in moisture, temperature, and ultraviolet radiation (Turner et al. 2013). Microorganisms colonize the surfaces of leaves in a variety of processes. While those already present in buds will be the first to colonize the developing leaf, the majority of the microorganisms inhabiting the developed phyllosphere will arrive from sources such as precipitation, dust, wind, and contact with insects and other wildlife. Colonization is also affected by leaf characteristics such as the topography and waxiness of the leaf's surface. (Lugtenberg 2015).

In addition to epiphytes on the surface of the leaf, plants are also colonized by endophytes which reside in the endosphere, the area within plant tissue. Like epiphytes, endophytes can be diverse, and may occupy vascular tissue, the intercellular apoplast, and within dead cells (Turner et al. 2013). Endophytes generally do not cause disease, and some endophytes contribute to plant fitness and development (Hardoim et. at 2013). Colonization of the endosphere can occur through a number of plant surfaces such as leaves, stems, and flowers; however, the majority of colonization occurs through the rhizosphere, the area of the plant below the soil. Bacteria enter the endosphere through openings in the roots, and can then be translocated to different areas of the plant. The endosphere is a less-dynamic environment than the phyllosphere, providing microbes with nutrients and protection against environmental stresses (Lindow and Brandl 2003). As well as naturally occurring endophytes, human pathogens can exist as endophytes, even if only temporarily. Some pathogens in the endosphere of edible plants such as spinach and lettuce have been linked to outbreaks of food-borne illnesses such as

Salmonella spp. and *Escherichia coli* (Waitt et al. 2013, Jackson et al. 2015). Such salad vegetables can be a major source of illness, and produce is attributed with causing 46% of all foodborne illness outbreaks in the United States, an annual economic burden of $39 billion (Waitt et al. 2013).

Given the increasing concerns involving plant-associated bacteria and their potential effects on human health, this experiment focused on examining the microbiome of green leaf lettuce. Lettuce is the second-largest vegetable crop produced in the United States in terms of production by weight, and is a common produce in many Western diets (Barbosa 2015). In particular, this study focuses on "living lettuce," which is hydroponically grown in greenhouses and packaged with its roots intact in plastic clamshell containers. This lettuce is increasing popular with a shelf life of 18 days, compared to the 7 day shelf life of traditionally packaged green leaf lettuce (Waitt et al. 2013). An additional factor in its rising popularity is the benefit of growing crops hydroponically. Hydroponic farming presents substantially higher crop yields and conservation of water, compared to conventional field-grown methods (Barbosa 2015). Lettuce grows faster in a hydroponic environment, where nutrients can be supplied more precisely and water is not a limiting factor (Waitt et al. 2013). While living lettuce presents a favorable alternative to conventionally-grown lettuce, Good Agricultural Practices (guidelines set by the Food and Agricultural Organization of the United States that intend to minimize the risk of microbial food safety hazards) have not been developed for hydroponically grown leafy greens. While hydroponic production may limit risk factors associated with contamination from wildlife, insects, and environmental run-off, lettuce may be exposed to microbial contamination via human contact or through

water (Waitt et al. 2013). Additionally, these vegetables are often consumed raw and unwashed, presenting a potential risk to the consumer.

The aim of this study was to examine and compare the bacterial communities of hydroponically grown living lettuce and traditionally grown and packaged green leaf lettuce. Since humans consume both the epiphytic and endophytic communities, these communities were examined in unison, rather than separately. This experiment aimed to determine the community composition of the associated bacteria at the point of consumer consumption, so samples were taken on two different occasions following purchase. Culture-independent next generation 16S rRNA gene sequencing was used to analyze samples and make comparisons of bacterial populations.

Methods

Sample Acquisition and Processing

Six heads of green leaf lettuce (*Lactuca sativa*) were purchased from a grocery store in Oxford, Mississippi on September 23, 2016. Three of the samples were hydroponically grown living lettuce, packaged with intact roots in sealed, plastic clamshell containers. The remaining three samples were traditionally grown lettuce with roots removed, which were unpackaged at the grocery store but stored in sealed plastic bags upon purchase. Lettuce were chosen based on similarities in size and appearance. On the day of purchase, samples were collected by cutting 0.5 g portions of leaves that had been removed from each head of lettuce. Outer leaves were discarded, so the leaves sampled were representative of those a consumer would eat. Three leaves from each of the six lettuces were sampled, for a total of 18 samples. Samples were then frozen (-20 °C) until DNA could be extracted. The remaining lettuce was stored at 4 °C in a typical refrigerator for two weeks. At that time, on October 7, 2016, the same sampling process (three samples taken from each of the six plants) was followed again. Thus, a total of 36 samples were collected, 18 from the living lettuce (representing three samples from each of three plants taken on day of purchase and after two weeks of storage) and 18 from the conventional lettuce (also three samples from each of three plants taken on day of purchase and after two weeks of storage).

DNA Extraction and Sequencing

Frozen samples were thawed and DNA was extracted using a Mo Bio Laboratories PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc. Carlsbad, CA), a kit used to isolate microbial DNA from a variety of environmental samples. Detailed instructions on DNA extraction were provided by Mo Bio Laboratories Inc. Presence of recovered DNA was confirmed using agarose gel electrophoresis. The V4 region of the 16S rRNA gene was amplified and sequenced using a dual-index barcoded Illumina next-generation sequencing approach (Kozich et al. 2013, Jackson et al. 2015). Final sequencing was carried out at the University of Mississippi Medical Center (UMMC) Molecular and Genomics Core Facility on an Illumina MiSeq platform.

Sequence Analysis

Sequence data in the form of FASTQ files obtained from the sequencing process was analyzed using mothur, a software program developed by Patrick Schloss at the University of Michigan (Schloss et al. 2011). Following procedures recommended by Schloss, a series of system commands were used to remove ambiguous sequences and align sequences against the SILVA 16S rRNA database (Table 1). Chimeras (sequences that erroneously combined during PCR) were removed using mothur-incorporated UCHIME software. The remaining aligned sequences were classified using the Greengenes sequence database, and contaminant sequences (Eukarya, Archaea, chloroplast, mitochondria, and unknown sequences) were removed from the dataset. Bacterial sequences were then classified into operational taxonomic units (OTUs), a term

used to describe closely related individuals. OTUs were determined based on sequence similarity, where with >97% similarity constitutes the same OTU (Schloss et al. 2011). Bacterial communities from each sample were examined based on presence and relative abundance of OTUs. Samples were compared in terms of Alpha and Beta diversity to determine whether there was a difference between the bacterial communities associated with the living lettuce and conventional lettuce.

Table 1: Summary of commands used within mothur software package used to analyze 16S rRNA sequence data obtained from lettuce samples.

Command	**Function**
make.contigs	Processes FASTQ files
screen.seqs	Filters sequences that fail to meet defined criteria
unique.seqs	Removes identical sequences
count.seqs	Counts the number of unique sequences in each sample
align.seqs	Aligns sequences to SILVA database
filter.seqs	Removes erroneous, non-informative sequences
pre.cluster	Combines sequences that are nearly-identical
chimera.uchime	Identifies potentially chimeric sequences
remove.seqs	Removes chimeric sequences
classify.seqs	Classifies sequences using Greengenes database
remove.lineage	Removes unwanted lineages (eukarya, archea, chloroplast, mitochondria)
cluster.split	Groups sequences into OTUs
make.shared	Determines how many times each OTU is detected in samples
count.groups	Determines number of sequences in each sample
classify.otu	Identifies OTUs
dist.shared	Creates matrix based on presence and abundance of OTUs
nmds	Gives coordinates for comparisons on a plot via non-metric multidimensional scaling

Results

Successful extraction of DNA from lettuce samples was confirmed using agarose gel electrophoresis (Figure 1). After aligning and extracting erroneous sequences using mothur software, a sample from living lettuce on the day of purchase (3C), living lettuce two weeks after purchase (9A), and conventional lettuce two weeks after purchase (10C) were found to have an insufficient amount of valid sequences for analysis, and were removed from the dataset. The total number of bacterial 16S rRNA gene sequences recovered across the 33 remaining samples was 1,030,383. The number of sequences recovered per sample was not evenly distributed (Table 2, Figure 2), ranging from 70,193 recovered from a conventional lettuce sample two weeks after purchase (11B) to 4,420 from a living lettuce sample two weeks after purchase (7B) (Table 2). Operational taxonomic units (OTUs) were classified based on 97% similarity, yielding a total of 8,802 distinct OTUs across all samples. As with sequences, the number of OTUs found in each sample varied (Figure 3), ranging from 1,677 OTUs found in a living lettuce sample the day of purchase (3A) and 52 OTUs found in a conventional lettuce the day of purchase (10C). Since samples with more sequences typically have more OTUs, a standardized number of OTUs was found for each sample, based on the number of OTUs per 4,420 sequences (the lowest number of sequences recovered from an individual sample) (Table 2, Figure 3). Through this method, an average of 52 OTUs were detected in each sample. This ranged from 89 OTUs found in a living lettuce sample collected the

day of purchase (1C), to 31 OTUs found in a conventional lettuce sample two weeks after purchase (11B). Across all samples, the five most abundant OTUs were identified (in order from most abundant to least abundant) as *Pseudomonas* sp. Unclassified Enterobacteriaceae, *Epulopiscium* sp. Unclassified Gemmataceae, and *Methylotenera* sp.

The portions of major phyla and subphyla present in each sample were examined, and the mean percentage was determined for each of the four sample types: living lettuce the day of purchase, conventional lettuce the day of purchase, living lettuce two weeks after purchase, and conventional lettuce two weeks after purchase (Table 3, Figure 4). In all samples, the most abundant subphylum was the Gammaproteobacteria, which accounted for an overall mean of 74.2% of sequences. Following Gammaproteobacteria, other major phyla and subphyla included Alphaproteobacteria, Bacteroidetes, Firmicutes, Betaproteobacteria, Planctomycetes, Acidobacteria, Verrucomicrobia, Cyanobacteria, and Chloroflexi (Table 3, Figure 4). While Gammaproteobacteria was the most abundant subphyla in each sample set, the portions of other major phyla varied from sample to sample. For example, Firmicutes made up 4.07% of the sequences obtained from leaves of living lettuce on the day of purchase, but only 0.79% of sequences from living lettuce samples taken two weeks later. The proportion of Alphaproteobacteria increased after two weeks on samples taken from both living lettuce (+7.16%) and conventional lettuce (+3.57%), as well as the portion of Bacteroidetes (+8.98% and +2.37%, respectively).

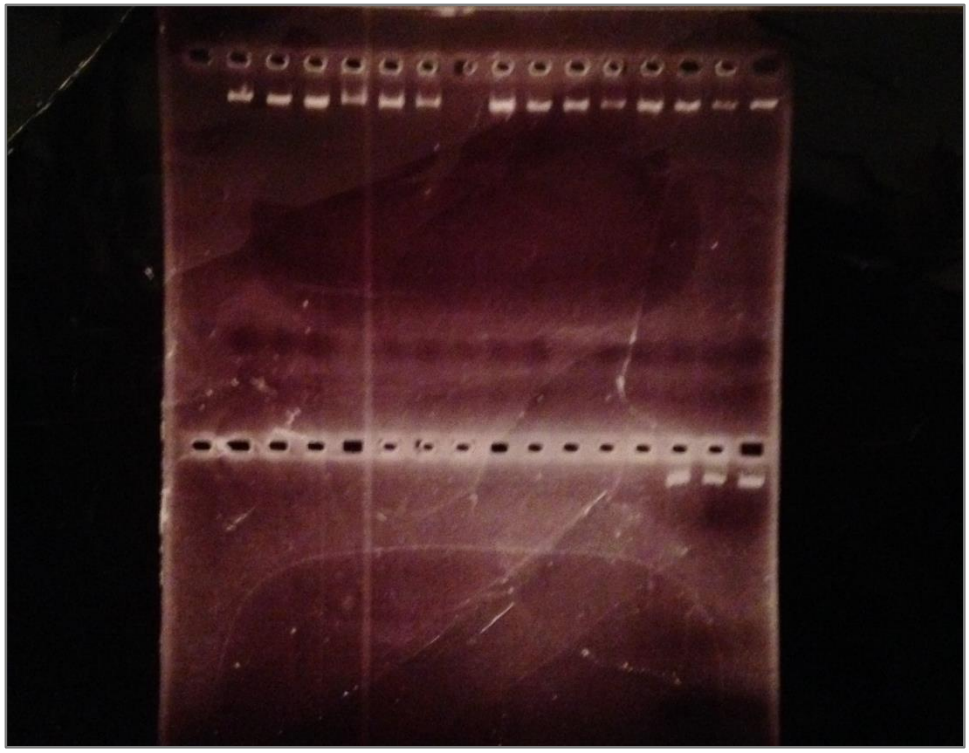
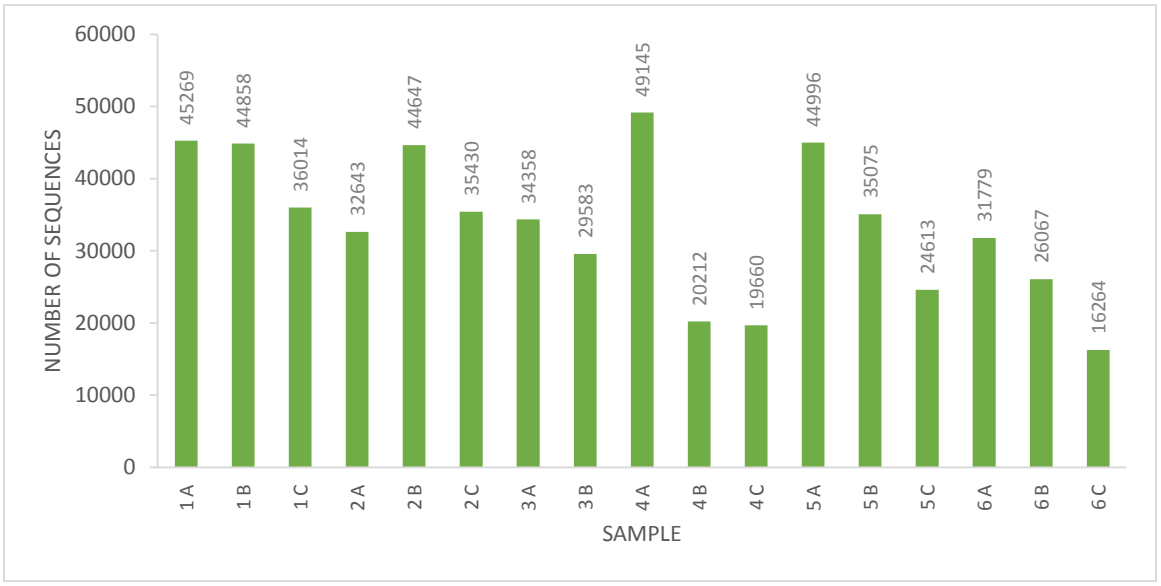


Figure 1. DNA extracted from green leaf lettuce was confirmed via agarose gel electrophoresis. Two gels were used to accommodate all 36 samples (18 samples per gel). Samples were placed in wells in order (1A, 2A, 3A, 2A, 2B…). The presence of DNA was indicated by the bands below the wells.

Table 2. Number of bacterial 16S rRNA gene sequences and OTUs (standardized to 4,420 sequences) recovered from lettuce samples on day of purchase and two weeks after purchase. Rows labeled 1A–3B represent samples from hydroponically grown living lettuce, while 4A—6C represent samples obtained from conventionally grown and packaged lettuce the day of purchase. Rows labeled 7A—9C represent samples from hydroponically grown living lettuce, while 10A—12C represent samples obtained from conventionally grown and packaged lettuce two weeks after purchase.

Day of Purchase			Two Weeks after Purchase		
Sample	**Sequences Recovered**	**OTUs**	**Sample**	**Sequences Recovered**	**OTUs**
1A	45269	68	**7A**	9655	41
1B	44858	48	**7B**	4420	48
1C	36014	89	**7C**	13422	48
2A	32643	50	**8A**	9070	42
2B	44647	50	**8B**	62284	45
2C	35430	49	**8C**	60323	42
3A	34358	56	**9B**	17225	57
3B	29583	49	**9C**	12409	51
4A	49145	53	**10A**	27158	54
4B	20212	62	**10B**	6428	55
4C	19660	69	**11A**	52274	38
5A	44996	65	**11B**	70193	31
5B	35075	59	**11C**	49074	44
5C	24613	47	**12A**	39998	43
6A	31779	49	**12B**	52597	44
6B	26067	53	**12C**	25514	47
6C	16264	60			

A)



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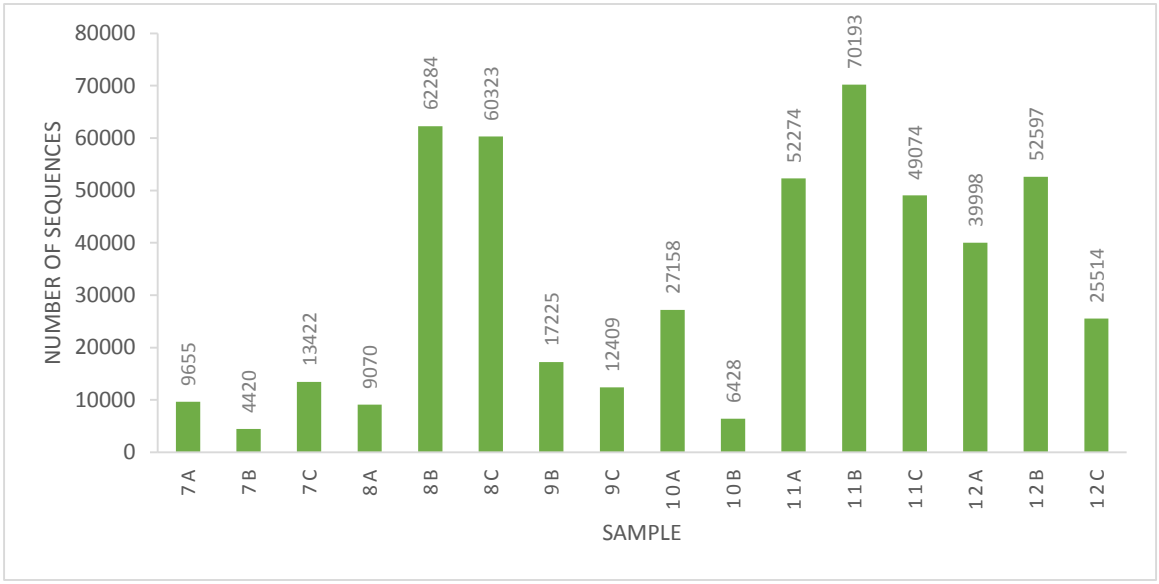
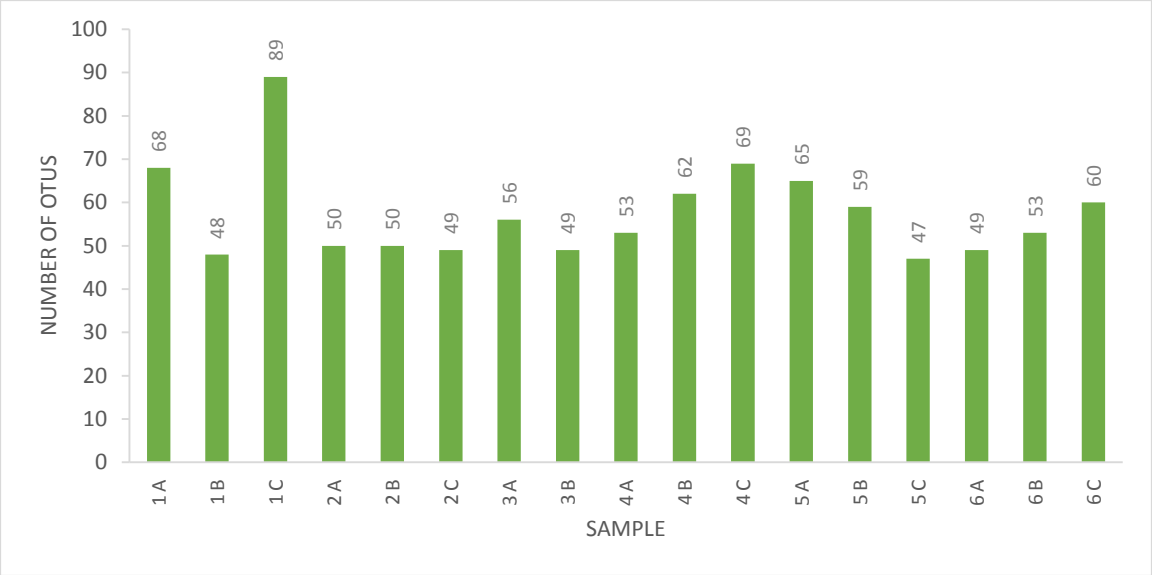


Figure 2. Number of bacterial 16S rRNA gene sequences recovered from lettuce samples on day of purchase (A) and two weeks after purchase (B). Bars labeled 1A—3C and 7A—7C represent samples from hydroponically grown living lettuce, while 4A—6C and 10A—12C represent samples obtained from conventionally grown and packaged lettuce.

A)



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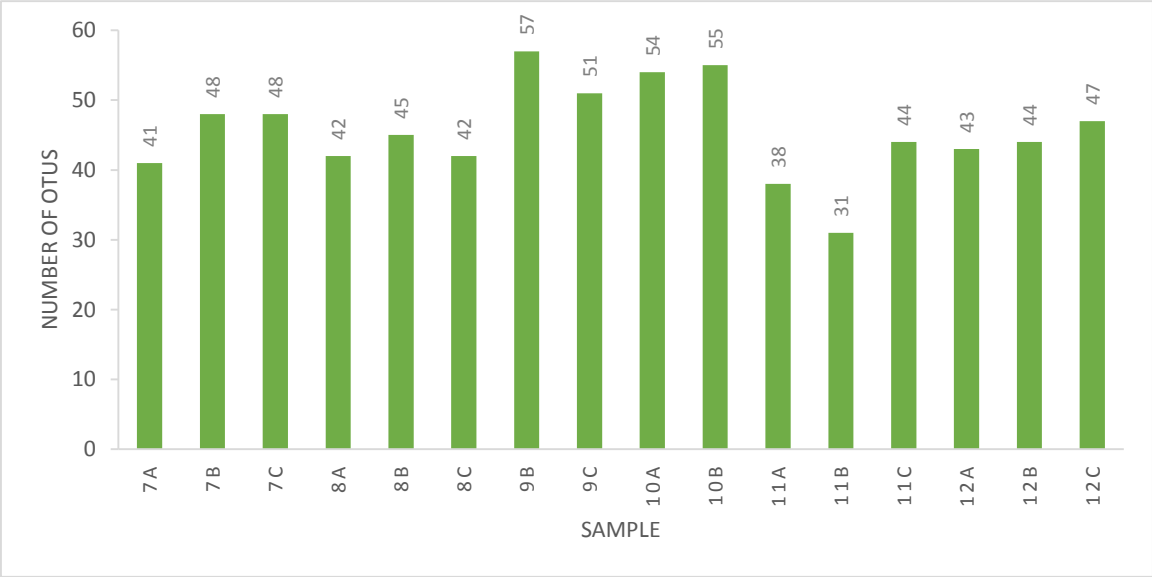

Figure 3. Number of operational taxonomic units (OTUs) found in lettuce samples on day of purchase (A) and two weeks after purchase (B) after standardization to 4,420 sequences. Bars labeled 1A—3C and 7A—9C represent samples from hydroponically grown living lettuce, while 4A—6C and 10A—12C represent samples obtained from conventionally grown and packaged lettuce.

Table 3. Proportion of major bacterial phyla and subphyla associated living and conventional green leaf lettuce. Columns labeled Living 1 and Conventional 1 account for the mean percentages of major phyla of samples taken the day of purchase, while columns labeled Living 2 and Conventional 2 account for the mean percentages of major phyla of samples taken after two weeks of refrigeration.

Phylum or Subphylum	**Living 1**	**Conventional 1**	**Living 2**	**Conventional 2**
Gammaproteobacteria	64.28%	83.25%	66.21%	77.11%
Alphaaproteobacteria	5.72%	6.44%	13.60%	10.01%
Bacteroidetes	3.63%	5.37%	13.06%	7.74%
Firmicutes	7.32%	0.42%	0.27%	0.16%
Betaproteobacteria	1.96%	1.47%	4.08%	3.78%
Planctomycetes	4.76%	0.40%	0.29%	0.09%
Acidobacteria	2.26%	0.50%	0.37%	0.10%
Verrucomicrobia	1.22%	0.37%	0.16%	0.09%
Cyanobacteria	1.07%	0.06%	0.03%	0.06%
Chloroflexi	0.23%	0.08%	0.10%	0.01%
Other	7.55%	1.64%	1.84%	0.85%

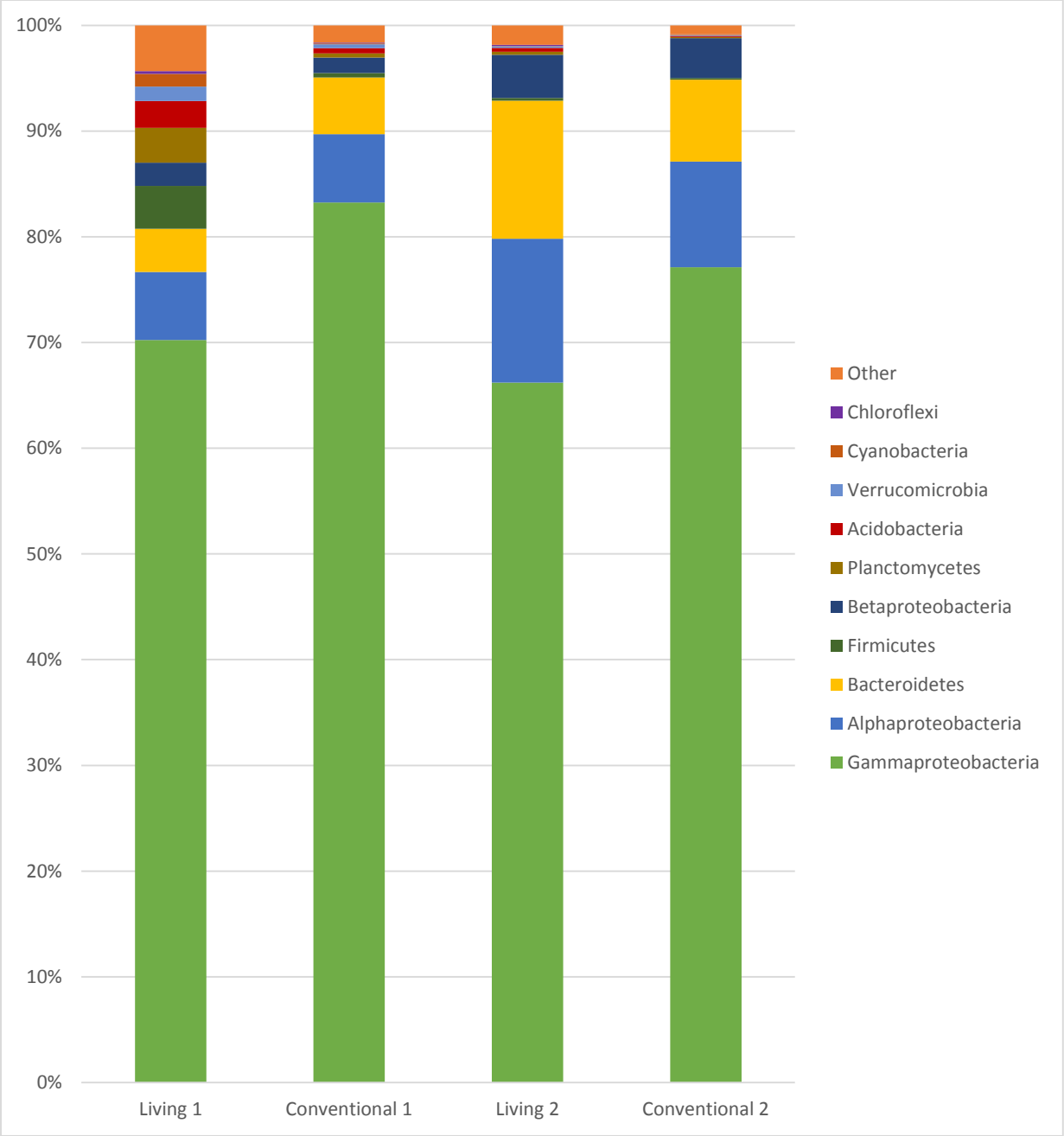

Figure 4. Proportion of major bacterial phyla and subphyla associated with living and conventional green leaf lettuce. Columns labeled Living 1 and Conventional 1 account for samples taken the day of purchase, while columns labeled Living 2 and Conventional 2 account for samples taken two weeks later.

Alpha diversity of each sample was determined through the Inverse Simpson Index, which is a quantitative measure that accounts for the number of OTUs present, as well as their relative abundance in each sample (Table 4). A higher score in the Inverse Simpson Index indicates a more diverse community. The samples with the highest scores came from two living lettuce on the day of purchase (1C with a score of 183.10 and 1A with a score of 48.82) and one conventional lettuce on the day of purchase (6C with a score of 52.51). The samples with the lowest Inverse Simpson Scores came from two conventional lettuce following 2 week of refrigeration (8C with a score of 8.37 and 7A with a score of 6.55) and one living lettuce collected on the day of purchase (1B with a score of 6.17). Eight of the 10 samples with the lowest Inverse Simpson scores were collected two weeks after refrigeration, while nine out of 10 samples with the highest scores were collected the day of purchase.

Variation in community structure between samples was examined through the Bray-Curtis dissimilarity index, which compares samples based on the presence or absence of OTUs, as well as the relative abundance of those OTUs. The comparison between samples was visualized through both nonmetric multidimensional scaling (NMDS) and a dendrogram (Figure 5, Figure 6). NMDS provides each sample with a coordinate based on its similarities with other samples. Samples with more similar communities will be located more closely together on the plot, while samples with more dissimilar communities will be further apart. Based on the relative location of samples on the NMDS ordination, it appeared that samples obtained from living lettuce two weeks after purchase (7A—9C), were more similar to samples taken from living lettuce the day of purchase (1A—6C) than samples taken from conventional lettuce on either day. The

difference between populations on living and conventional lettuce were found to be significantly different according to AMOVA ($F=2.564$, $p=0.001$) and ANOSIM ($R=0.281$, $p<0.001$).

Table 4. Alpha diversity of bacterial communities obtained from lettuce samples through Inverse Simpson Index. Rows labeled 1A—3C represent samples from hydroponically grown living lettuce, while 4A—6C represent samples obtained from conventionally grown and packaged lettuce the day of purchase. Rows labeled 7A—9C represent samples from hydroponically grown living lettuce, while 10A—12C represent samples obtained from conventionally grown and packaged lettuce two weeks after purchase.

Sample	**Inverse Simpson**	**Sample**	**Inverse Simpson**
1A	48.82	**7A**	6.55
1B	6.17	**7B**	11.94
1C	183.10	**7C**	10.11
2A	16.16	**8A**	9.37
2B	14.39	**8B**	8.71
2C	24.73	**8C**	8.37
3A	26.63	**9B**	11.77
3B	10.90	**9C**	16.95
4A	13.69	**10A**	17.67
4B	44.71	**10B**	17.97
4C	37.28	**11A**	9.20
5A	29.54	**11B**	8.95
5B	23.63	**11C**	10.48
5C	16.20	**12A**	11.02
6A	22.16	**12B**	11.40
6B	24.67	**12C**	11.67
6C	52.51		

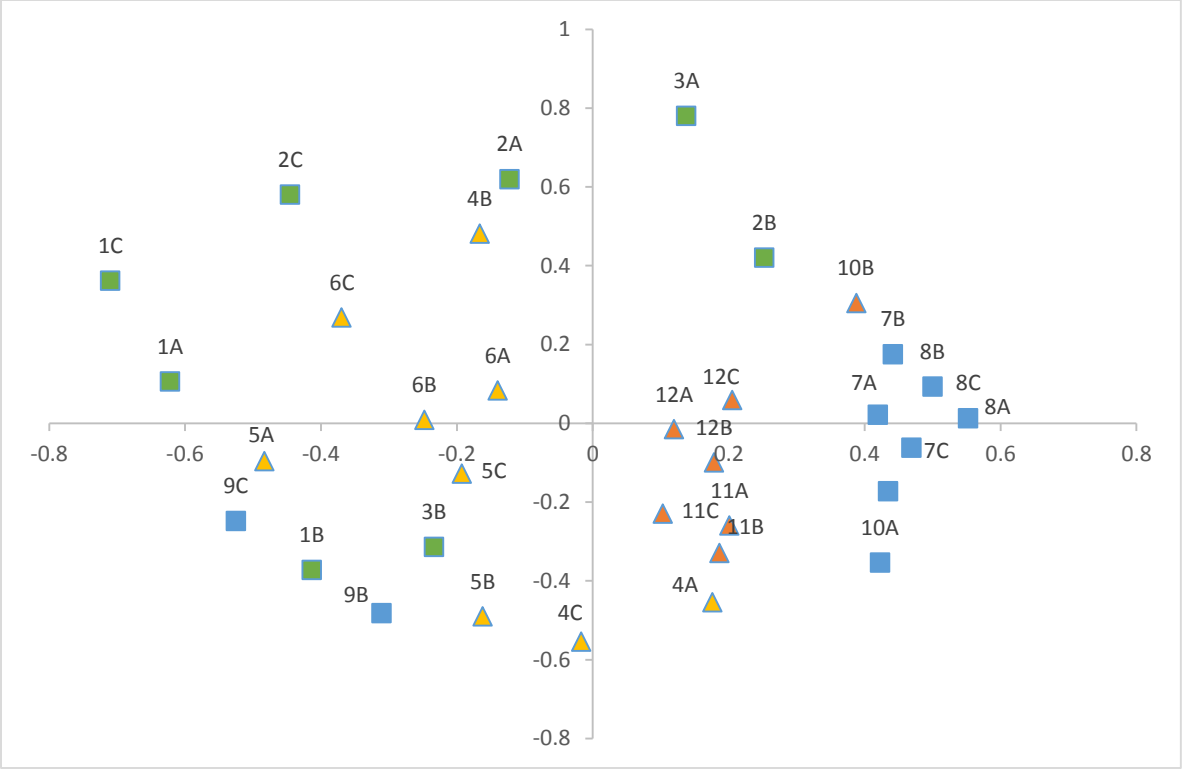

Figure 5. NMDS plot representing similarities between bacterial communities associated with samples obtained from living lettuce the day of purchase (green squares), conventional lettuce the day of purchase (yellow triangles), living lettuce two weeks after purchase (blue squares), and conventional lettuce two weeks after purchase (orange triangles). Points located more closely on the plot represent communities with more similar compositions based on the relative abundance of OTUs.

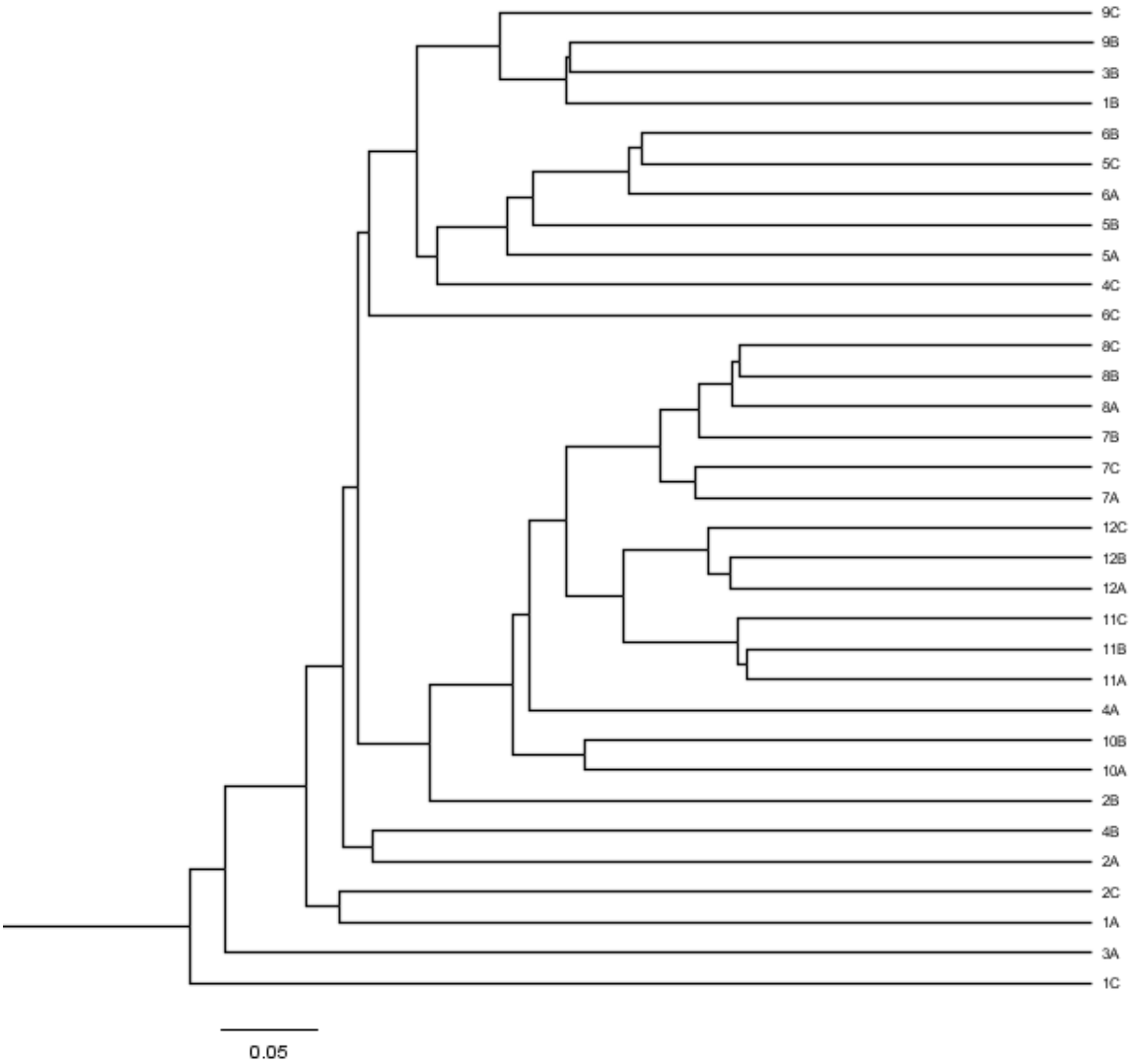

Figure 6. Dendrogram representing community similarity based on Bray-Curtis Dissimilarity Index. Samples labeled 1A—3C and 7A—7C represent samples from hydroponically grown living lettuce, while 4A—6C and 10A—12C represent samples obtained from conventionally grown and packaged lettuce.

Discussion

This study investigated the bacterial communities associated with green leaf lettuce. Specifically, it compared the communities of hydroponically grown lettuce that is packaged and sold "living" with roots intact to those associated with conventionally grown and packaged lettuce. Samples were taken from leaves of both types of lettuce on the day of purchase, as well as after two weeks of refrigeration, with the intention to determine samples representative of bacterial communities present upon purchase as well as following refrigerator storage that might more accurately reflect the time of consumption. Analyses of sequence data obtained from samples were made based on major phyla and subphyla observed in samples, the number of sequences and OTUs obtained from each sample, and measures of alpha and beta diversity.

Through 16S rRNA gene sequencing, bacteria from eight different phyla were found, and sequences belonging to the phylum Proteobacteria were further identified by their subphyla. The dominant phylum was the Proteobacteria, with the subphyla Gammaproteobacteria making up 74.2% of all recovered sequences. Previous studies have similarly found Gammaproteobacteria to be the most abundant group on green leaf lettuce (Jackson et al. 2013). However, the portion of Gammaproteobacteria sequences found in samples taken from living lettuce on the day of purchase was 13% lower than the portion observed in conventional lettuce taken the same day. Other major phyla and subphyla found in conventional lettuce samples consisted of Alphaproteobacteria and

Bacteroidetes. While living lettuce samples taken the day of purchase showed similar proportions of Alphaproteobacteria, Firmicutes made up the next largest phyla, rather than Bacteroidetes. While Bacteroidetes was the second most dominant phylum in previous studies of lettuce, Firmicutes was found to be the second most dominant phylum in studies done on spinach leaves (Jackson et al. 2013, Lopez-Velasco et al. 2011). The portions of major phyla and subphyla in samples taken from living lettuce after two weeks of refrigeration more closely resembled those of samples taken from conventional lettuce taken at the same time. The three most abundant phyla found in both living and conventional lettuce samples at this time were Gammaproteobacteria, Alphaproteobacteria, and Bacteroidetes.

The two most abundant OTUs found throughout all samples were identified as *Pseudomonas* sp. and unclassified members of the Enterobacteriaceae family, which is consistent with previous studies done on salad greens (Jackson et al. 2013, Lopez-Velasco et al. 2011). Members of the Enterobacteriaceae family are often present in phyllosphere communities because of their ability to survive on environments that experience fluctuations in nutrient availability, exposure to UV radiation, and temperature (Hunter et al. 2010). Bacteria belonging to the genus *Pseudomonas* are less commonly the dominant members of the phyllosphere, but they have been shown to inhabit the endosphere in many leafy green vegetables (Jackson et al. 2013).

Differences between communities from living lettuce were determined to be significantly different than those on conventionally grown and packaged lettuce. This could be the result of a number of factors, given the dynamic nature of plant-microbe

interactions. Host determinants such as metabolism and immune function can affect community assembly and growth (Lebeis 2014). Additionally, living lettuce are packaged and sold with intact root systems, which are home to bacterial populations known as the rhizosphere (Turner et al. 2013). Bacteria inhabiting the rhizosphere have the potential to translocate to the endosphere and phyllosphere through plant vasculature, which may have an effect on these communities (Melotto et al. 2014, Turner et al. 2013).

Despite being significantly different, communities found in samples taken from both types of lettuce were found to be more diverse than those taken two weeks after refrigeration at 4°C. Previous studies conducted on leafy greens have found that phyllosphere diversity decreases after prolonged cold storage (Lopez-Velasco et al. 2011, Jackson et al. 2015). The decrease in diversity during storage is possibly due to the temperature and atmospheric conditions during refrigeration. Bacteria capable of surviving in cold environments are known as psychotrophs, and have been linked to the spoilage of refrigerated foods such as leafy greens. A common psychotroph often deemed responsible for the spoilage of foods is the Gammaproteobacteria *Pseudomonas* sp. (Kraft 1992), which was a major OTU identified in samples from this study. The growth of *Pseudomonas* from prolonged storage in a cold environment is a possible explanation for the decrease in diversity across samples, and the greater similarity between living and conventional lettuce bacterial communities after two weeks of refrigeration.

While green leaf lettuce has previously been associated with the outbreak of foodborne illnesses due to pathogens such as *E. coli* and *Salmonella* (Waitt et al. 2014),

data from this study did not show significant presence of these bacteria on hydroponically grown living lettuce. Other studies performed to determine the risk of these pathogens on hydroponically grown lettuce returned similar results, concluding that the hydroponic farming of lettuce provides a microbiologically safe means of farming, given the observation of reasonable sanitary practices (Riser et al.1984). In addition to sanitary farming practices, storage at a proper temperature has been found to be significant in reducing the risk of human pathogens. Consumers storing lettuce at temperatures greater than or equal to 12 °C face an increased risk of *Salmonella* and *E. coli*, which have been found to grow on the phyllosphere and rhizospheres of improperly refrigerated living lettuce (Waitt et al. 2014).

The bacterial communities associated with any living organism are affected by variations in the environment, with communities of plants being affected by factors such as temperature, nutrient availability, and exposure to UV radiation. With a growing interest on the human microbiome and its relationship to human health, the microbiome associated with leafy green vegetables such as green leaf lettuce is of interest to because of their presence on and within the leaves at the time of consumption. In addition, bacterial communities associated with lettuce have been linked to outbreaks of foodborne illnesses, which pose a threat to human health. This study demonstrated that the bacterial communities associated with hydroponically grown living lettuce pose no apparent threat to human health, as they show no significant difference to the microbiomes of conventionally grown and packaged green leaf lettuce at the time of consumption. When proper sanitary measures and storage recommendations are observed, there is no significant risk of foodborne illness caused by pathogens. Given the information

obtained in this and similar studies, the benefits of hydroponic farming, and the prolonged shelf life of lettuce sold with intact roots, living lettuce is a produce alternative that many consumers may find favorable to lettuce traditionally grown and packaged.

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