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Review

Emerging Perspectives on the Natural Microbiome of Fresh Produce Vegetables

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Abstract: Plants harbor a diverse microbiome existing as bacterial populations on the leaf surface (the phyllosphere) and within plant tissues (endophytes). The composition of this microbiome has been largely unexplored in fresh produce vegetables, where studies have tended to focus on pathogen detection and survival. However, the application of next-generation 16S rRNA gene sequencing approaches is beginning to reveal the diversity of this produce-associated bacterial community. In this article we review what is known about the composition of the microbiome of fresh produce vegetables, placing it in the context of general phyllosphere research. We also demonstrate how next-generation sequencing can be used to assess the bacterial assemblages present on fresh produce, using fresh herbs as an example. That data shows how the use of such culture-independent approaches can detect groups of taxa (anaerobes, psychrophiles) that may be missed by traditional culture-based techniques. Other issues discussed include questions as to whether to determine the microbiome during plant growth or at point of purchase or consumption, and the potential role of the natural bacterial community in mitigating pathogen survival.

Keywords: microbiome; bacteria; salad produce; herbs; phyllosphere; endophytes; 16S rRNA

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1. Introduction

The last few decades have seen rapid growth in the fields of microbial ecology and environmental microbiology, such that natural microbial communities are now recognized as being much more diverse than previously thought. While much of the early research in this field focused on using molecular techniques to characterize the diversity of microbial assemblages in soils and waters; from the late 1990s onwards there has been an increased awareness of the natural microbiota associated with plants. Just as there has been a research focus on determining the make-up of the complex microbiome of animals (and especially mammals) and its role in health and disease, the importance of the plant microbiome is beginning to be examined, whether in the context of its role in plant growth and disease resistance, or in the context of human consumption of plant material (e.g., [1]).

The natural bacterial populations associated with plants are present on both aboveground (stem, leaves, flowers, fruits, *etc.*) and belowground (roots, tubers, *etc.*) structures. That roots have an associated microbial community (the rhizosphere) has long been recognized, and aboveground portions of plants (primarily leaves) also have such a community (the phyllosphere). While agricultural scientists are likely aware of the potential for microorganisms to be found naturally on the plant surface, the idea that plants also contain many microbial populations living inside of them (endophytes) is less well known. However, virtually every study that has tested for the presence of endophytes inside of plant samples has found them, so it is likely that every plant species is colonized by at least one endophytic bacterial species. While most endophytes are likely to be non-pathogenic to humans, a number of pathogenic bacteria can become internalized as at least temporary endophytes within leaves. These and other endophytes present an interesting problem from the viewpoint of consumer safety as no amount of washing or vegetable preparation will remove them, and while this is not a potential issue with the consumption of cooked vegetables, it may be with the consumption of raw vegetables in salads. Here we review the state of our current knowledge of the microbial assemblages associated with produce vegetables and present perspectives on how these assemblages relate to consumer health. We also demonstrate how next-generation sequencing technologies can provide valuable insights into the composition of these assemblages, paying particular attention to an understudied vegetable type, fresh herbs.

2. General Characteristics of Phyllosphere Bacterial Communities

Leaves of plants present an area that is available for potential microbial colonization and growth, whether they are on the leaf surface (the phylloplane) or within the leaf itself as endophytes. As such, microbial populations in or on leaves can be abundant, and the phyllosphere community can be taxonomically diverse [2]. The leaf surface itself can be a harsh environment for microbial growth, with microorganisms being potentially exposed to extreme fluctuations in moisture availability, ultraviolet variation, temperature, and nutrient availability [2,3]. However, phyllosphere adapted bacteria may be capable of modifying their local environment, increasing the leakage of nutrients from the host plant and producing extracellular polysaccharides to resist desiccation and help attachment [4]. Studies of the phyllosphere using traditional culture-based approaches have been a part of agricultural and environmental microbiology since at least the 1950s, although for obvious reasons many studies have tended to focus

on the detection of known plant pathogens (e.g., species of *Xanthomonas*, *Erwinia* and *Pseudomonas*) or human pathogens from a food safety standpoint. However, the development and application of molecular (largely 16S ribosomal RNA) approaches in microbial ecology, has led to a more thorough understanding of the diversity and complexity of leaf-associated bacterial communities.

Building upon Carl Woese's work demonstrating that ribosomal RNA (rRNA) gene sequences could be used to determine bacterial phylogeny [5], microbial ecology underwent a dramatic shift in the 1980s and 1990s in that it became feasible to directly obtain bacterial DNA from environmental samples, polymerase chain reaction (PCR)-amplify bacterial 16S rRNA genes, and then to use those genes to examine bacterial diversity either through DNA sequencing or through techniques such as denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP) analysis. Such analyses revealed that microbial communities were much more complex than previously believed and that perhaps as few as 1% of bacteria had been identified using traditional culture techniques [6,7]. Initially, these molecular microbial ecology studies focused on marine systems (likely because of easier analysis), with a greater emphasis on soils from the mid-1990s onwards [8]. However, despite an increased interest in the microbial diversity of soils from an agricultural standpoint, even including the plant rhizosphere [9–13]; initially, only a few studies applied this concept to examine the natural microbial diversity on the aboveground structures of plants.

Yang *et al.* [14] used DGGE to examine the natural bacterial communities in the phyllosphere of seven agriculturally important plant species: cotton (*Gossypium hirsutum*), corn (*Zea mays*), sugar beet (*Beta vulgaris*), green bean (*Phaseolus vulgaris*), Valencia and navel oranges (both *Citrus sinensis*), and the grapefruit-pomelo hybrid OroBlanco (*C. grandis* × *C. paradisi*). DGGE banding patterns indicated that leaves sampled from different individuals of the same plant species tended to show similar bacterial community profiles, but the profiles for the different species were generally unique. While the phyllosphere communities on the two orange varieties examined (Valencia, navel) were similar, they were very different from those on the other citrus variety (OroBlanco) [14]. Sequence analysis of 17 of the dominant DGGE bands obtained from the Valencia orange samples showed that only four (identified as *Enterobacter agglomerans*, *Bacillus pumilus*, *Acinetobacter* sp., and a member of the Cytophagales) represented established phyllosphere taxa. The other sequences included representatives of the Alpha-, Gamma-, and Deltaproteobacteria that had not previously been reported to inhabit the phyllosphere. This was in contrast to the results of the same analysis conducted on bacteria growing in mixed culture in BIOLOG plates, which were predominantly members of *Pseudomonas*, *Erwinia*, *Acinetobacter*, and *Enterobacter* [14]; all genera that are recognized phyllosphere inhabitants based on traditional culture techniques. Thus, using culture-independent 16S rRNA based methods revealed a phyllosphere community that was very different, and presumably more diverse, than the one that would have been determined through culture-dependent methodologies.

The last 10–15 years has seen a rapid increase in the application of rRNA-based methods to describe leaf-associated assemblages. DGGE and other approaches have been used to characterize the bacterial community in the phyllosphere of food crops, such as corn (*Zea mays*) [15], pepper (*Capsicum annuum*) [16], cucumber (*Cucumis sativus*) [17], and spinach (*Spinacia oleracea*), celery (*Apium graveolens*), broccoli (*Brassica oleracea var. italica*), and cauliflower (*Brassica oleracea var. botrytis*) [18]. Similarly, the diversity and structure of the bacterial community in the phyllosphere of a number of tree species has been described [19–22]. More than basic descriptions of community structure,

16S rRNA techniques have also been used to examine how the phyllosphere community is influenced by environmental factors, such as ultraviolet light [15] and rainfall [23], as well as pesticide [24,25] or biological control agent [16,26] application. At an ecological level, both spatial [20] and temporal [21] patterns in the composition of the phyllosphere have been examined. Thus, while our understanding of the phyllosphere initially lagged behind our knowledge of soil or rhizosphere microbiology, these, and other, culture-independent studies are leading us to insights into both the composition of leaf-associated microbial communities, and how these communities interact with the host plant [27,28].

3. Bacterial Communities Associated with Leaves of Salad Produce Vegetables

Fresh produce vegetables such as lettuce, spinach, *etc.* present an example of leaves that may be consumed by humans without the use of preparation methods that would remove or kill the associated bacterial community. As such, studies of phyllosphere or endophytic bacterial assemblages on these plants are interesting from both a basic scientific and applied aspect. From an applied viewpoint, the presence of potential human pathogens is of interest given that these are not likely to be removed prior to consumption. Most studies of bacteria in produce have used culture-dependent methods to determine the culturable pathogenic populations present, or have examined the recovery of bacteria following intentional exposure to pathogens such as *Escherichia coli* O157:H7, *Salmonella*, or *Listeria* [29–33]. Direct visualization of bacterial cells in the phyllosphere through microscopy has also been used to examine the microbial colonization of leafy vegetables [29,32–34]. Together, these studies indicate that pathogenic bacteria are capable of colonizing leaf surfaces and internal tissues (*i.e.*, they can be part of both phyllosphere and endophytic assemblages), and thus are not necessarily removed by washing. Both *Salmonella* and *E. coli* have been found to be capable of becoming endophytic and colonize the interior tissues of plants [30,33,35–38], although the ability to colonize and persist within the leaf varies between specific strains of bacteria. The ability of these human pathogens to survive and persist on the leaf surface is also less than that of the naturally occurring bacterial populations that reside there. For example, *Salmonella enterica* serovar Thompson has been shown to be capable of colonizing the phyllosphere of the herb cilantro (*Coriandrum sativum*), but was not as resilient to environmental conditions on the leaf surface as native bacterial epiphytes [32]. Similarly, plant-associated *E. coli* strains that were specifically isolated from cabbage roots were more proficient at colonizing the surface of alfalfa sprouts than an O157:H7 clinical isolate [39]. These examples highlight the importance of considering the naturally occurring microbiome on leafy produce, as it may be more persistent than, and even interact with, potential pathogens.

Most of the earlier studies on the presence and persistence of potential human pathogens on salad produce relied on the use of selective microbiological culture media and biochemical tests for the identification of bacterial isolates [29,32]. However, as with general microbial diversity studies, this has been supplemented by culture-independent molecular approaches using 16S rRNA gene amplification and subsequent community profiling by techniques, such as DGGE, T-RFLP, DNA sequencing, and/or microarrays [34,40]. More recently, metagenomic or next-generation sequencing approaches have been used [1,41–43].

An interesting question regarding the microorganisms present on consumable produce is when to sample, or even broader, at what time point do we say that the leaf-associated bacteria that are present

are the representative bacterial community? The naturally occurring microbiome that is associated with the plant (*i.e.*, the true phyllosphere and endophyte community) should probably be defined as the microbial populations that are present on or in vegetables growing in the field. However, from the standpoint of the consumer, the microbial populations present at the point of sale, or even the time of consumption, may be more relevant, and both phyllosphere and endophytic microorganisms may differ at these different time points. DGGE of 16S rRNA genes showed reduced bacterial diversity in the phyllosphere of lettuce (*Lacutuca sativa var. capitata*) samples collected from grocery stores compared to samples taken directly from a farm field site, suggesting a simplification of the phyllosphere community during processing of salad vegetables [44]. Furthermore, commercially pre-bagged, refrigerated versions of the same lettuce samples showed evidence of the presence of additional bacterial populations (notably *Pseudomonas libaniensis*) [44] indicating the potential for either contamination or the selective growth of specific bacterial populations during processing and storage. A similar observation has been made for spinach (*Spinacia oleracea*), for which numbers of culturable Pseudomonadaceae and Enterobacteriaceae were found to increase after 12 days of cold storage [29]. The latter study is probably a more reliable assessment of changes that occur post-harvest and following storage, as changes in DGGE profiles or in the composition of 16S rRNA gene clone libraries represent a proportional change in community composition not a change in the absolute number of organisms. Thus, for example, while the presence of *Pseudomonas libaniensis* in clone libraries generated from lettuce samples following refrigerated storage [44] could indicate the growth of that species, it could also indicate a reduction in overall bacterial numbers following refrigeration, especially of more dominant taxa, so that *P. libaniensis* could only then be detected. Regardless, it seems reasonable to assume that the microbiota on vegetables are likely to change following harvest, and while the microbiome present in fresh produce vegetables at the time of purchase and consumption may not entirely be indicative of that present in the growing plant, it does represent the microbial populations to which consumers are potentially exposed, and may be more relevant to study.

Two recent studies took that viewpoint and used 16S rRNA techniques to survey the bacterial populations present in the phyllosphere [41] or in both the phyllosphere and internalized within the leaves [1] of vegetables obtained directly from grocery stores/supermarkets. Both report 16S rRNA gene sequences identified as representing members of the Gammaproteobacteria to be the most abundant sequence types detected, with the Enterobacteriaceae being particularly prevalent. Sequences identified as Enterobacteriaceae were proportionally much more abundant on fresh salad vegetables such as lettuce and spinach, than they were on apples, peaches or grapes [41]. Clustering 16S rRNA gene sequences together using a 97% similarity criterion to define operational taxonomic units (OTUs; a surrogate for species), suggested the presence of around 40–70 different OTUs or species on the lettuce or spinach leaf surface [1,41]. Thus, while not as diverse as soils or natural waters, the leaf-associated community of consumable fresh produce is quite diverse, and consumers are likely being exposed to 50 or more species of bacteria during salad consumption. While many of these populations are likely to be plant symbionts or pathogens, some of the bacterial genera and species detected in those studies include strains that can be human pathogens [1].

Taken together, these studies suggest that there are some families and genera of bacteria that are commonly found in the phyllosphere of leafy salad vegetables. Members of the Enterobacteriaceae have been consistently detected, and are often among the most abundant members of the phyllosphere community [34,40–43]. Members of the genus *Pseudomonas* have been found to be among the dominant

bacterial populations in spinach [42] and lettuce [40,43], as well as in bagged salad mixes containing lettuce, red cabbage, and carrots [34]. However, species of *Pseudomonas* are not always dominant members of the phyllosphere, as shown in the survey of Leff and Fierer [41] which found that the entire family Pseudomonadaceae accounted for less than 5% of the bacterial 16S rRNA gene sequences obtained from spinach or lettuce, compared to Enterobacteriaceae which accounted for 38% (lettuce) or 58% (spinach) of the sequences obtained. This same variability is seen in the less abundant bacterial populations detected in phyllosphere studies, where certain bacterial genera can account for >1% of the community in some studies but are not detected at all in others. Thus, despite the generally widespread occurrence of the Enterobacteriaceae and, to a lesser extent, the genus *Pseudomonas*, there does not appear to be a consistent core set of taxa that are found in the phyllosphere of all produce vegetables.

While there may be no consistent core taxa, environmental conditions during produce growth and storage likely impact the leaf-associated bacterial community. Although they are almost always abundant, Enterobacteriaceae have been reported as being more prevalent in the phyllosphere of conventionally grown spinach and lettuce compared to those under organic cultivation [41]. At least that was the case for produce sampled at the point of purchase: the opposite trend was reported when lettuce was sampled after being freshly harvested, when organically grown plants showed higher numbers of Enterobacteriaceae than conventionally grown ones [45]. However, these two studies may not be entirely comparable as they differed in the style of analysis used (culture-dependent *vs.* molecular) as well as in the time of sampling. A comparison of the plant-associated bacterial communities on six varieties of organically and conventionally grown salad produce sampled at point of purchase revealed no consistent differences in community composition between the two growth approaches [1]. Regardless of the influence of cultivation style, other environmental variables during growth such as soil moisture, organic content, or nutrient (fertilizer) availability could also affect microbial community composition.

As previously mentioned, storage undoubtedly has an influence on the leaf-associated microbial community. When fresh-cut spinach was stored at 10 °C, numbers of both Enterobacteriaceae and Pseudomonadaceae increased at least 1000-fold over 12 days [29]. Storage under refrigeration also lowered the diversity and richness of the phyllosphere community on spinach [42], and the temperature of storage influenced how much the community changes over the storage period. After 15 days of storage, spinach samples that were held at 15 °C harbored bacterial communities that were more similar to the original microbiome than samples that were stored at 10 °C [42]. Changes in the bacterial community associated with bagged lettuce mixes have also been reported following storage at 10 °C, with an increase in the relative abundance of Enterobacteriaceae and a decrease in the relative abundance of *Pseudomonas* [34]. Interestingly, when the bagged lettuce mixes were stored at refrigerator temperature (4 °C), the decrease in *Pseudomonas* was less pronounced and this genus was still the dominant taxonomic group, as it was prior to storage [34]. Thus, refrigerated storage might help retain the natural microbiome, while extended storage at cool, but not cold, temperatures might be more likely to promote shifts in the phyllosphere community, and potentially favor pathogenic strains.

4. Bacterial Communities Associated with Consumable Herbs: A Demonstration of Next-Generation Sequencing Approaches

Studies of microbial diversity, even those using culture-independent molecular techniques, are limited by the ability of the technique used to adequately sample the microbiome in question. With thousands to millions of bacteria cells present on a leaf surface, even 16S rRNA gene based methods such as DGGE or cloning and Sanger sequencing of amplified 16S rRNA gene fragments only reveal a miniscule subset of the bacteria that are present. The last few years, however, have seen the emergence of next-generation sequencing technologies that allow a far more in-depth profiling of bacterial assemblages to occur. Such approaches can readily yield thousands or tens of thousands of partial 16S rRNA gene sequences from a single sample of plant material (often from just 0.1–0.5 g of material), providing much more coverage of the community present, and increasing the ability to detect less common taxa. Such approaches were used by the previously mentioned studies of fresh produce at the point of consumer purchase [1,41] suggesting that those studies may well be better assessments of the bacterial component present on fresh vegetables than those before. That said, even those studies only assessed around 2000 [1] or 200 (this lower amount because more vegetables were sampled) [41] sequences, largely because they were based around the Roche 454 next-generation sequencing platform. Illumina next-generation sequencing has replaced 454 as the procedure of choice, and emerging protocols on that platform now facilitate more in-depth sampling of microbiome structure [46,47]. This approach is beginning to be used to characterize various plant-associated bacterial communities, with studies being reported for the phyllosphere of neo-tropical rainforest trees [48] and wetland plants [49]. However, other than a recent study examining how pesticide application can change the phyllosphere of tomatoes [50], there have been few, if any, studies that have used Illumina next-generation sequencing to characterize the bacterial communities on fresh fruit or vegetables. Here, we demonstrate the use of this platform to describe the natural bacterial assemblages present on a generally ignored subset of fresh vegetables, fresh herbs, and suggest some guidelines for using next-generation sequencing approaches in this context.

4.1. Sample Collection, Processing, and 16S rRNA Gene Illumina Sequencing

Samples of six fresh herbs were purchased from an Oxford, Mississippi, USA, grocery store in September 2014. Herbs were basil (*Ocimum basilicum*), chives (*Allium schoenoprasum*), dill (*Anethum graveolens*), mint (*Mentha spicata*), rosemary (*Rosmarinus officinalis*), and thyme (*Thymus vulgaris*). All were packaged in polymer film-sealed plastic containers, labeled as products of the USA. For each herb type, three representative leaves/fronds were sampled aseptically and 0.1 g weighed and used for DNA extraction. DNA was extracted from the leaf sample using a PowerPlant DNA Isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA). Thus, 16S rRNA gene sequences obtained reflect those of bacterial populations in the phyllosphere and potential endophytes. An alternative approach would have been to remove bacterial cells from the leaf surface prior to extracting DNA from them, and this has been done for some studies [41–44]. While that approach has the advantage of minimizing the co-amplification of chloroplast and mitochondrial sequences (which typically can amplify with bacterial specific primers), it prevents the sampling of endophyte populations, which may be as relevant as phyllosphere populations in regards to food safety. Furthermore, for this specific study, removing

bacteria from the leaf surface would have been difficult given the small size of the particular leaf samples used. Given the large number of sequences per sample that can be generated using next-generation sequencing, we recommend extracting DNA from the intact plant material rather than leaf washes, as the ability to detect the entirety of the bacterial community associated with the plant (*i.e.*, both phyllosphere and endophyte populations) likely outweighs the potential loss of some data to chloroplast or mitochondrial sequence reads.

A dual-index barcoding approach was used for Illumina next-generation sequencing whereby each sample was amplified with bacterial specific 16S rRNA gene primers, each tagged with a specific 8-nucleotide barcode [47]. These primers amplify a 250 nucleotide long region of the V4 variable region of the 16S rRNA gene. The specific amplification approach used involves a single round of PCR, thereby limiting the risk of amplification artifacts [47]. Following amplification, amplicons from all samples were pooled, and the assembled library spiked with 5% PhiX to increase nucleotide base diversity prior to sequencing (a necessary step for bacterial community analysis by this method). The final library was sequenced on an Illumina MiSeq instrument, via two index sequencing reads, at the University of Mississippi Medical Center (UMMC) Molecular and Genomics Core Facility. Illumina MiSeq-based sequencing was chosen as it reflects a suitable balance of a large number of sequence reads per run (and therefore per unit cost), the ability to obtain high quality data, and the ability to obtain reads in excess of 200 nucleotides long, thereby facilitating more accurate sequence classification. While the Illumina HiSeq or NextSeq systems can provide higher sample throughput, the MiSeq system is currently more commonly used for 16S rRNA gene studies, and at a cost at or below U.S. $100,000 is more suitable for individual investigators or testing laboratories [46,47].

4.2. Data Processing and Bioinformatics Pipeline

Raw sequence data (fastq files) were downloaded and accessed via the 16S rRNA bioinformatics software package mothur [51,52]. Sequences were processed along a bioinformatics pipeline following the general guidelines of Kozich *et al.* [47], which are optimized for Illumina MiSeq-generated 16S rRNA gene data. Briefly, contigs were assembled using forward and reverse reads and screened to only include those with a maximum length of 275 bp and no base ambiguities (*i.e.*, sequence contigs had to have identical base calls for both the forward and reverse read). Sequences were aligned against the SILVA 16S rRNA database [53] and misaligned sequences deleted. Sequences were clustered together by roughly 1% sequence similarity to account for potential amplification and sequencing errors, and chimeras removed using UCHIME [54]. Valid sequences were classified using the Greengenes [55] 16S rRNA classification scheme, and erroneous (archaeal, eukaryotic) sequences removed. Remaining bacterial sequences were grouped into operational taxonomic units (OTUs) based on >97% sequence similarity. As stated, these procedures follow published recommendations for this type of data [47], which are also routinely updated online [56]. These procedures were developed after a series of studies that assessed issues such as the impacts of clustering level, chimera removal procedure, alignment and classification reference, and OTU similarity criterion on bacterial community analysis [56] and we have found them suitable for sequences amplified from a range of samples including plant material, soils, and waters.

4.3. Taxonomic Composition of Bacterial Communities on Fresh Herbs

The microbiome of each of the herbs examined was generally dominated by members of the Firmicutes, Bacteroidetes, and Proteobacteria (Table 1). Of the latter, Alpha- and Gamma- sub-phyla were the most prevalent. However, the proportional abundance of each of these phyla in the sequence dataset varied between herb species with members of the Firmicutes being more prevalent in/on basil, rosemary, and thyme, and the Proteobacteria (especially Gammaproteobacteria) being more prevalent in/on dill and, to a lesser extent, chives (Table 1). A substantial proportion of the sequences recovered from mint were also classified as Proteobacteria, although these were Alphaproteobacteria and their proportional abundance was highly variable between samples (Table 1). Other major bacterial groups that were detected in relatively common proportions (accounting for >1% of the sequence dataset) were the Bacteroidetes (accounting for an average of 6.1%–17.9% of sequences, depending upon the herb species) and Actinobacteria (an average of 0.3%–6.5% of sequences).

Table 1. Major phyla and subphyla of bacteria associated with prepackaged fresh herbs sampled from a grocery store in Mississippi, USA, as determined from high throughput 16S rRNA gene sequencing. Numbers represent mean (+/− SE) percentage of total sequence reads obtained from 3 samples per herb that were identified as that phylum.

Phylum	Basil	Chives	Dill	Mint	Rosemary	Thyme
Acidobacteria	0.02 (0.01)	0.07 (0.07)	0.10 (0.04)	0.04 (0.03)	0.01 (0.00)	0.01 (0.01)
Actinobacteria	0.26 (0.04)	1.00 (0.13)	6.52 (1.46)	0.74 (0.10)	0.36 (0.05)	0.67 (0.07)
Bacteroidetes	6.1 (1.26)	13.7 (7.14)	6.1 (2.73)	17.9 (3.34)	12.2 (1.28)	13.1 (1.21)
Firmicutes	84.2 (1.87)	51.6 (24.19)	13.4 (5.33)	48.8 (23.94)	82.2 (1.02)	81.5 (0.74)
Lentisphaerae	0.02 (0.01)	0.08 (0.04)	-	0.13 (0.07)	0.07 (0.01)	0.10 (0.02)
Proteobacteria	9.2 (3.08)	28.9 (26.74)	73.6 (4.32)	31.99 (27.29)	5.05 (1.05)	4.40 (0.54)
Alpha-	1.41 (0.48)	0.22 (0.17)	2.08 (0.52)	25.56 (22.82)	2.36 (0.58)	0.15 (0.05)
Beta-	0.28 (0.04)	0.78 (0.29)	1.12 (0.36)	2.65 (1.60)	0.46 (0.09)	0.51 (0.04)
Gamma-	7.29 (2.63)	27.70 (26.94)	70.33 (4.10)	3.40 (3.01)	1.89 (0.51)	3.40 (0.58)
Delta-	0.17 (0.02)	0.23 (0.12)	0.02 (0.00)	0.37 (0.14)	0.33 (0.04)	0.34 (0.07)

The total number of valid bacterial sequences obtained from each individual sample averaged 18,140, but ranged from 5129 to 47,548. Compared to prior molecular surveys of bacterial assemblages on fresh vegetables, this represents a substantial increase in sequencing depth, but raises the question as to how much sequence data is needed to accurately assess the phyllosphere and endophyte communities associated with such samples. From analyses of Roche 454 next-generation sequencing datasets, 1000 valid sequences have been found to capture 90% of the patterns in community structure between samples (beta-diversity), while 5000 sequences were necessary to accurately predict total diversity within a sample (alpha-diversity) [57]. However, those numbers were derived from analyses of sediment samples, and bacterial communities in sediments are generally more diverse than those associated with plants. Thus, while we recommend a minimum of 5000 sequences to be analyzed from a sample in order to accurately assess the bacterial community present, in practice 2000–3000 sequences is likely to be enough to obtain a reasonable determination of community composition for plant samples. Rarefaction curves of the number of distinct OTUs observed as a function of sampling effort suggested that similar

sequencing depth (2000–5000 sequences per sample) was sufficient to describe the bacterial assemblages associated with fresh salad produce [1]. However, the more reads per sample the greater the ability to detect rare taxa, and if the goal is to detect specific bacterial populations (e.g., pathogens) that may account for a minor proportion of the total bacterial community, then greater sequencing depth may be needed.

The dominant specific bacterial populations (as determined from proportional abundance of each OTU) associated with the fresh herb samples included various members of the Firmicutes, Bacteroidetes, and Proteobacteria (Table 2). An unclassified member of the Bacillaceae represented by far the most numerous sequence type followed by sequences identified as *Streptococcus* sp. and *Prevotella copri*. Other major OTUs included *Pseudomonas* sp., *Bacillus cereus*, and *Bacteroides* sp. (Table 2). While some sequences could clearly be identified to the species level, others could only be identified to genus, or even less resolved (e.g., the dominant unclassified Bacillaceae, as well as unclassified members of the Enterobacteriaceae and Planococcaceae). This highlights a potential limitation of next-generation sequencing in that sequence read lengths are currently limited to a few hundred bases (in this case 250 bp). However, even with that limitation some patterns are apparent. For example, a number of well-represented OTUs were identified as taxa that are obligate anaerobic bacteria (e.g., *Prevotella copri*, *Bacteroides* sp., *Phascolarctobacterium* sp., *Catenibacterium* sp.). These taxa represent bacteria that are typically found in the human large intestine, and were initially described after isolation from fecal samples [58–60]. Thus, their presence in prepackaged fresh herbs suggests that those herbs show evidence of past fecal contamination. Regular microbiological culture methods would not necessarily have detected these organisms because of their anaerobic culture requirements, demonstrating that while culture-independent methods, such as next-generation sequencing may have limitations, they can be a useful tool in detecting the presence of intestinal microbial populations.

Table 2. Identities of the most prevalent bacterial populations (OTUs) associated with prepackaged fresh herbs (basil, chives, dill, mint, rosemary, thyme) sampled from a grocery store in Mississippi, USA, as determined from high throughput 16S rRNA gene sequencing. Number of reads represents the total number of sequence reads identified as that taxon, out of a total of 392,272 reads.

OTU	Phylum (Class)	Number of Reads
Unclassified Bacillaceae	Firmicutes (Bacilli)	204,483
Streptococcus sp.	Firmicutes (Bacilli)	22,984
Prevotella copri	Bacteroidetes (Bacteroidia)	22,597
Pseudomonas sp.	Proteobacteria (Gamma)	15,955
Bacillus cereus	Firmicutes (Bacilli)	12,823
Bacteroides sp.	Bacteroidetes (Bacteroidia)	10,045
Phascolarctobacterium	Firmicutes (Clostridia)	6124
Bacteroides sp.	Bacteroidetes (Bacteroidia)	5231
Psychrobacter sp.	Proteobacteria (Gamma)	4293
Unclassified Enterobacteriaceae	Proteobacteria (Gamma)	4240
Faecalibacterium prausnitzii	Firmicutes (Clostridia)	4232
Unclassified Planococcaceae	Firmicutes (Bacilli)	3485

Table 2. *Cont.*

Methylobacterium adhaesivum	Proteobacteria (Alpha)	2821
Catenibacterium sp.	Firmicutes (Erysipelotrichi)	2738
Pseudomonas veronii	Proteobacteria (Gamma)	2702
Sphingomonas sp.	Proteobacteria (Alpha)	2572
Agrobacterium sp.	Proteobacteria (Alpha)	2546
Sphingomonas sp.	Proteobacteria (Alpha)	2445
Sphingomonas sp.	Proteobacteria (Alpha)	2327
Unclassified Rikenellaceae	Bacteroidetes (Bacteroidia)	1950
Arthrobacter psychrolactophilus	Actinobacteria (Actinobacteria)	1943
Portiera aleyrodidarum	Proteobacteria (Gamma)	1868
Exiguobacterium sp.	Firmicutes (Bacilli)	1733
Sutterella sp.	Proteobacteria (Beta)	1633
Alistipes putredinis	Bacteroidetes (Bacteroidia)	1600

Three taxa (*Psychrobacter* sp., *Arthrobacter psychrolactophilus*, *Exiguobacterium* sp.) detected as important OTUs were bacterial species that are psychrophilic or psychrotolerant (*i.e.*, cold tolerant). The presence of sequences affiliated with these groups likely arises from the refrigerated storage of the produce sampled, as they are unlikely to be abundant in the natural herb phyllosphere. This confirms the accepted idea that the bacterial community associated with produce likely does change during processing and storage, with an increased prevalence of psychrotolerant groups following refrigeration. Indeed, given that the herbs sampled were packaged in plastic containers sealed with polymer file, the high prevalence of OTUs that would be classified as anaerobic likely also reflects microaerophilic storage conditions, and while those OTUs may have originated from fecal contamination, their relative abundance appears to have increased during storage. This provides some support for the argument to sample at point of sale, rather than at point of growth, if the goal is to determine the bacterial community present on produce from a consumer standpoint [1,41]. Further supporting the idea that changes in the herb phyllosphere and endophytic communities must have occurred during processing and storage is the finding that relatively few of the dominant OTUs (essentially just species of *Methylobacterium*, *Agrobacterium*, and *Sphingomonas*) would be regarded as primarily plant-associated bacteria.

4.4. Overall Summary and Conclusions of the Example Study

Overall, the results of the herb study demonstrate that next-generation sequencing technologies such as Illumina 16S rRNA gene sequencing can serve a useful function in the analysis of bacterial communities associated with fresh produce such as herbs. These approaches are rapidly becoming more affordable and easier to perform, and in terms of labor intensity tend to be more efficient than culture-based approaches. In terms of time expenditure, processing and DNA extraction from all herb samples was accomplished with 1–2 days of purchase, and could realistically be completed within a few hours by a dedicated technician. Illumina 16S rRNA gene library preparation takes another few days, with the actual sequencing run taking 24–48 h. Thus, with a dedicated laboratory, it would be possible to generate complete community data in <2 weeks. In this particular study, the bacterial assemblages associated with herbs were found to be dominated by members of the Firmicutes and Gammaproteobacteria, although there was some variation between the particular herbs sampled.

Plant-associated taxa were relatively scarce, supporting previous findings [34,42] that phyllosphere and endophyte communities can change during storage, whether from refrigeration or being packaged in modified atmospheres. These changes appeared to result in a higher presence than expected of 16S rRNA sequences identified as being from psychrophilic organisms, and a substantial number of sequences classified as coming from anaerobic taxa. The latter finding is particularly alarming as many of the genera identified (e.g., *Bacteroides*, *Catenibacterium*, *Phascolarctobacterium*, *Prevotella*) are associated with the human large intestine so are typically only detected in fecal samples. Thus, the herbs likely were subject to fecal contamination at some point, and these bacterial populations increased during the storage period. Given the obligate anaerobic nature of these taxa, they would not have been detected by routine microbiological culture, demonstrating that the use of culture-independent approaches can provide useful insights into bacteria present on fresh produce.

5. Conclusions

Assessing the natural microbiome of consumable plants is important, as natural microbial assemblages may reduce the likelihood of pathogen colonization or survival. For example, reduced levels of *Salmonella enterica* colonization have been observed in lettuce that has a more diverse endophyte community [35]. The mechanism for this pathogen reduction with increased endophyte diversity is not conclusively known, but may well be an increased likelihood of antagonists to *S. enterica* being present with increased overall diversity. Additional support for this concept comes from a study examining the viability of *E. coli* O157:H7 on romaine lettuce, where the phyllosphere bacterial diversity in plants that had culturable *E. coli* O157:H7 cells differed from that on plants where the *E. coli* was no longer viable [61]. That native plant-associated microorganisms can act as competitors to potential human pathogens such as *Salmonella* species and *E. coli* O157:H7 has been shown in lettuce and alfalfa sprouts [62,63], suggesting that even in the absence of specific antagonistic interactions, natural phyllosphere and endophytic communities may limit the presence and abundance of pathogenic bacteria by simply outcompeting them in the living plant. Determining the structure of these communities might therefore provide insights into produce-borne outbreaks of disease, and even lead to the development of tools to assess the likelihood of these outbreaks occurring [1]. Intentional addition of competitive native microbiota has even been proposed as a potential method to reduce enteropathogen contamination of fresh produce [64].

Whether it is most important to determine the microbiome of fresh produce vegetables in the field or at point of sale/consumption is debatable. From the viewpoint of the potential for the natural microbial community to mitigate the growth and persistence of pathogenic bacteria, then the composition of the phyllosphere community in the field may be most important. However, a number studies have shown that the composition and diversity of this community changes during processing, handling, and storage [29,34,42,44], so it could be questioned as to whether the field community is as important if exposure to pathogens occurs post-harvesting. From the viewpoint of the consumer, the bacterial populations that are in or on the produce at point of sale, or more specifically the point of consumption, are the most relevant, as these are the bacteria to which they are being exposed. Interestingly, there have been few, if any, studies that have determined the changes that may occur in produce-associated microbial communities in the time between purchase and consumption, even though that time could well span a number of days. More

in-depth tracking of the changes that can occur over the entire period from growth through harvesting, processing, storage, purchase, and final consumption is certainly merited.

The methodologies used to assess the composition of the bacterial community on produce need to be current. The use of culture-dependent methods is certainly justified when the goal is to determine the presence or viable abundance of specific bacterial populations such as pathogens or indicator species, whose culture requirements are known, and for which specific selective and differential growth media exist. However, culture-independent molecular methods allow the entire bacterial community to be examined, facilitating a more thorough examination of the microbiome present. Emerging technologies such as next-generation sequencing can be used to detect populations that may be missed by standard culture approaches (such as the anaerobic taxa detected on the herb samples described above), and as they become more affordable are likely to compete with traditional culture approaches in routine assessment and monitoring of produce. That said, when we have used culture-dependent approaches and culture-independent next-generation sequencing to analyze the same produce samples, we found that while sequencing revealed the presence of more bacterial taxa, the dominant taxa in our sequence libraries were the same ones detected by the culturing approach [1]. Thus the two approaches are best viewed as complementary and, ideally, both would be used in analyses of fresh produce vegetables.

Just as microbial ecologists have come to understand the complexity and diversity of naturally occurring bacterial assemblages in soils and waters, we are now beginning to grasp the diversity of the phyllosphere and endophytic communities in agricultural crops. Determining this diversity is especially important for crops such as fresh salad produce as they are not extensively processed or cooked prior to consumption. Understanding the complexity of these communities could lead to the development of new pathogen control mechanisms [64] and a greater understanding of pathogen survival and persistence. Regardless of potential future developments, it's becoming clear that leafy salad vegetables harbor a diverse set of phyllosphere and endophytic populations, which consumers must continually be being exposed to. The impact of this native microbiome on consumer health, whether positive or negative, has been largely unexplored.

Raw bacterial sequence data (fastq files) from the herb samples analyzed as part of this manuscript have been deposited in the NCBI Sequence Reads Archive (SRA) under Project accession number SRP052782. Individual herb samples have SRA Sample accession numbers SRS826210, SRS826225, SRS826710, SRS826711, SRS826712, SRS826713, SRS826714, SRS826715, SRS826716, SRS826717, SRS826718, SRS826719, SRS826720, SRS826721, SRS826722, SRS826723, SRS826724, SRS826726, SRS826727, SRS826728, SRS826729, SRS826730, SRS826731, and SRS826732.

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Author Contributions

Colin R. Jackson reviewed the literature and wrote the initial draft of the manuscript with assistance from Heather L. Tyler; Bram W. G. Stone performed the next-generation sequencing experiments in Section 4; Colin R. Jackson performed the bioinformatics analyses on the sequencing data and generated Tables 1 and 2. All three authors reviewed and edited the final submission.

Conflicts of Interest

The authors declare no conflict of interest.

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