

# Bacterial Communities Associated with Surfaces of Leafy Greens: Shift in Composition and Decrease in Richness over Time

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The phyllosphere is colonized by a wide variety of bacteria and fungi; it harbors epiphytes, as well as plant-pathogenic bacteria and even human pathogens. However, little is known about how the bacterial community composition on leafy greens develops over time. The bacterial community of the leafy-green phyllosphere obtained from two plantings of rocket salad (*Diplotaxis tenuifolia*) and three plantings of lettuce (*Lactuca sativa*) at two farms in Norway were profiled by an Illumina MiSeq-based approach. We found that the bacterial richness of the *L. sativa* samples was significantly greater shortly (3 weeks) after planting than at harvest (5 to 7 weeks after planting) for plantings 1 and 3 at both farms. For the second planting, the bacterial diversity remained consistent at the two sites. This suggests that the effect on bacterial colonization of leaves, at least in part must, be seasonally driven rather than driven solely by leaf maturity. The distribution of phyllosphere communities varied between *D. tenuifolia* and *L. sativa* at harvest. The variability between these species at the same location suggests that the leaf-dwelling bacteria are not only passive inhabitants but interact with the host, which shapes niches favoring the growth of particular taxa. This work contributes to our understanding of host plant-specific microbial community structures and shows how these communities change throughout plant development.

The phyllosphere is a habitat on the surface of plant leaves colonized by a variety of bacteria, yeasts, and fungi (1). It harbors epiphytes, as well as plant-pathogenic bacteria and even human pathogens. Microbial populations on leaf surfaces are highly influenced by rapid fluctuations in UV radiation, temperature, and humidity and are restricted by limited access to nutrients (1, 2). Resident bacteria on leaves can have neutral, negative, or positive influences on their host plants by serving as pathogens, preventing leaf colonization by pathogens, or acting as growth promoters (3). Traditionally, phyllosphere bacteria have been characterized by using culture-based approaches and much of the work on produce-associated bacteria has focused on a small number of pathogenic species. Culture-based methods will not include bacteria that are not able to grow on standard artificial media or are slow growing. This limits our understanding of the phyllosphere microbial community's ecology, genetics, and physiology (4). Bacterial communities associated with leafy greens have already been described by several culture-independent studies. "First-generation" molecular techniques have been used to describe variation in community structure in the context of leaf surface properties and microbial interactions (5), seasonal variation in the community structure (6), and monitoring of bacterial communities in the food chain (7). The introduction of culture-independent methods, in particular, microbial profiling using high-throughput sequencing to study microorganisms, has revealed more complexity and diversity of the phyllosphere microbiota and has dramatically changed the landscape of microbial ecology (5, 7–10). The 454 pyrosequencing platform has been used in different studies of bacterial communities associated with leafy greens (11–15), but Illumina MiSeq has lately been the dominant platform for microbial profiling.

Previous studies have shown that the impact of leaf age and seasonal variations on the phyllosphere communities is not clear (14–18). It can be hypothesized that phyllosphere communities will change with time because of a decline in the nutrient supply when the leaf matures, selection of specific microbiota by different

leafy greens, or weather effects. The objective of our study was to determine the succession patterns in microbial communities on the surface of leafy greens throughout the growing season (April to September) to see how the communities change over time and with leaf maturity. Because of the increased concern about vegetables as vehicles for transmission of human pathogens, we also wanted to investigate how frequently potential human pathogens were present in conventionally grown leafy greens at different maturity stages.

## MATERIALS AND METHODS

**Sample collection.** Conventionally grown lettuce (*Lactuca sativa*) and rocket salad (*Diplotaxis tenuifolia*) were collected from two farms in southeastern Norway during the growing season of 2013 (April to September), for microbial profiling of the phyllosphere bacteria. Farm Vestfold is located near the sea in Vestfold County, a region known for early-season vegetable production. Farm Buskerud is located at the head of the Drammensfjord, 55 km north of Farm Vestfold. Both farms use overhead irrigation; the water used at Farm Vestfold comes from the public drinking water supply and that used at Farm Buskerud comes from a nearby river. The soil type at both farms is sandy clay loam. *L. sativa* seedlings were locally produced in greenhouses and planted either on bare soil (Farm Vestfold) or on plastic-covered beds (Farm Buskerud). *L. sativa*

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**TABLE 1** Samples of leafy greens collected at two farms in southeastern Norway throughout the growing season of 2013 that were subjected to microbial profiling of the phyllosphere-inhabiting bacteria

Farm and leafy green (variety)	Planting	Day/mo of sampling (no of samples sequenced)		Plant age (wk)
		3 wk <sup>a</sup>	Harvest	
<b>Vestfold</b>				
<i>L. sativa</i> (Little Gem)	1	15/5 (3)	12/6 (4)	9
<i>L. sativa</i> (Little Gem)	2	03/7 (4)	17/7 (4)	7
<i>L. sativa</i> (Little Gem)	3	20/8 (4)	13/9 (4)	8.5
<b>Buskerud<sup>b</sup></b>				
<i>L. sativa</i> (Iceberg)	1	23/5 (3)	17/6 (4)	9.5
<i>L. sativa</i> (Frillice)	2	29/7 (4)	19/8 (4)	8
<i>L. sativa</i> (Frillice)	3	19/8 (3)	17/9 (4)	10
<i>D. tenuifolia</i> (Rocket)	1		08/7 (3)	5
<i>D. tenuifolia</i> (Rocket)	2		29/7 (3)	5

<sup>a</sup> The plants that were sampled 3 weeks after planting in the field were 5 weeks old upon sampling.

<sup>b</sup> For the purpose of this work, communities developing on the different lettuce varieties at Farm Buskerud were assumed to be similar.

heads were harvested by hand. *D. tenuifolia* was directly sown on raised beds, and the leaves were harvested by cropping machines. *L. sativa* samples were collected twice per planting (3 weeks after planting in the field and at harvest) and from three subsequent plantings. Four samples were collected per field, and each sample consisted of three *L. sativa* heads. Two leaves were picked from each *L. sativa* head (six leaves in total), one from the outer leaf circle and one from the inner leaf circle. Samples were also taken from *L. sativa* plants the day they were planted in the field (day zero). However, we were not able to amplify bacterial DNA from these samples; hence, they were not included in the sequencing experiment. *D. tenuifolia* was collected just after harvest with cropping machines from three subsequent plantings. Three samples were taken from two plastic bags freshly packed (not washed) for retail at the farm. The type of leafy green and replicates are described in detail in Table 1 (for some of the *L. sativa* samples, sequencing results were obtained from only three out of four subsamples because of sequencing errors). The leaves were briefly rinsed in running distilled water to remove traces of soil and pooled in an Erlenmeyer flask. A 50-ml salt-Tween solution (0.15 M NaCl, 0.1% Tween 20) (19) was added to each sample in the flask, and it was placed in a rotary shaker (100 rpm) at room temperature (RT) for 15 min. Aliquots of 100  $\mu$ l of the solution were plated on nutrient glucose agar (NGA; 23 g nutrient agar [Difco], 5 g yeast extract, 10 g glucose, 1,000 ml distilled water) and incubated at RT. Bacteria in the solution were collected on 0.2- $\mu$ m filters by vacuum filtration. The filters were stored at  $-20^{\circ}\text{C}$  prior to molecular analysis.

**DNA isolation from filters and pure bacterial cultures.** The filters were ground in liquid nitrogen with a pestle and mortar, and added directly to the lysis buffer of the DNeasy Plant minikit (Qiagen, GmbH, Hilden, Germany). From each NGA plate, up to 10 phenotypically different single bacterial colonies were repeatedly streaked onto new NGA plates to obtain pure cultures. The isolates were grown on NGA agar plates at RT and stored in glycerol stocks at  $-80^{\circ}\text{C}$ . One loop with bacterial growth from the pure culture was suspended in 1 ml sterile MilliQ water and incubated at  $100^{\circ}\text{C}$  on a heating block. The lysate was used directly as the template for PCR amplification.

**Analysis of the 16S rRNA gene by Sanger sequencing.** A 500-bp fragment of the 16S rRNA gene (variable regions V6 to V8) was amplified for sequence analysis by PCR with the primer pair F985PTO/R1378 (F985PTO, 5'-AACGCGAAGAACCCTTACSC-3'; R1378, 5'-CGGTGTG TACAAGGCCCGGAACG-3') (20). The PCR mixture (25  $\mu$ l) con-

tained 0.4 mM each primer, 0.2 mM deoxynucleoside triphosphates, 1.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 0.25  $\mu$ l bovine serum albumin, and 2.5  $\mu$ l template. The PCR cycle consisted of denaturation at  $95^{\circ}\text{C}$  for 3 min; 35 cycles of  $95^{\circ}\text{C}$  for 1 min,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 30 s; and a final extension at  $72^{\circ}\text{C}$  for 5 min. PCR products were analyzed on a 1% agarose gel in TBE buffer by electrophoresis. Sequencing was performed at GATC Biotech, and the sequences were assembled and analyzed with CLC Main Workbench 6.

**Microbial profiling with Illumina MiSeq.** Fifty-one *L. sativa* and *D. tenuifolia* samples (Table 1) were selected for sequencing with the Illumina MiSeq platform. The NEXTflex 16S V4 Amplicon-Seq kit (Bioo Scientific, Austin, TX), which amplifies the fourth hypervariable (V4) domain of the microbial 16S rRNA, was used to label all of the samples prior to sequencing according to the manufacturer's instructions. The samples were analyzed by agarose gel electrophoresis (1% agarose in TBE buffer), and the DNA fragments of the expected size were excised from the gel under UV light. The DNA amplicons were purified with the QIAquick gel extraction kit (Qiagen, GmbH, Hilden, Germany). Sequencing was performed at the Norwegian Sequencing Center, Oslo, Norway, on the Illumina MiSeq platform by the 300-bp paired-end protocol.

**Sequence processing, analysis, and community comparisons.** Sequences were preprocessed and analyzed with Mothur v1.33 (21) by following the Mothur standard operating procedure (22, 23). In short, the sequences were trimmed and processed and aligned by using the Bacterial SILVA SEED database as the template ([http://www.mothur.org/wiki/Silva\\_reference\\_files](http://www.mothur.org/wiki/Silva_reference_files)). Sequences that originated from chloroplasts and mitochondria were removed from the data set. Potentially chimeric sequences were detected and removed with chimera uchime (24), and the remaining sequences were called "filtered sequences." The aligned filtered sequences were classified with Trainset9\_032012.pds from the Ribosomal Database Project (25) and clustered into operational taxonomic units (OTUs) defined by 97% similarity.

The sequence libraries were further characterized to determine the degrees of diversity and similarity of the microbial communities present on the different samples. The Shannon (H0) and Simpson (1/D) indices were used to establish relative diversity levels, and Chao I was used to provide estimates of species richness that might be expected if more exhaustive sampling were done (see Table S1 in the supplemental material). Pairwise comparison of a subset (based on 9,549 random sequences from each sample) with weighted UniFrac (26) was performed to test if the bacterial communities in the fields were significantly different. Metastats, which is based on a nonparametric *t* test, Fisher's exact test, and the false-discovery rate (27), was used to find OTUs that were statistically significantly different when groups were compared ( $P < 0.05$ ). The phylogenetic  $\beta$ -diversity tree was made with tools from Interactive Tree Of Life (28). In an effort to investigate if different *L. sativa* varieties hosted different bacterial families, we analyzed the abundances of selected bacterial families in the three *L. sativa* varieties Little Gem, Iceberg, and Frillice at 3 weeks after planting and at harvest, also including *D. tenuifolia* at harvest.

**Nucleotide sequence accession number.** The sequences obtained in this study are available in the European Nucleotide Archive database under project identification number PRJEB6233.

## RESULTS

Altogether, 51 samples of conventional field-grown leafy greens (*D. tenuifolia* and *L. sativa*) were collected from two farms in southeastern Norway during three subsequent plantings in the growing season of 2013 (Table 1).

**Identification of culturable bacteria by Sanger sequencing.** Aliquots of the rinsing solution from *L. sativa* samples were plated onto NGA agar, and a total of 162 bacterial isolates were obtained. Sequencing of part of the 16S rRNA gene showed that the isolates represented 26 genera and four bacterial phyla, *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*. *Pseudomonas* (30%)

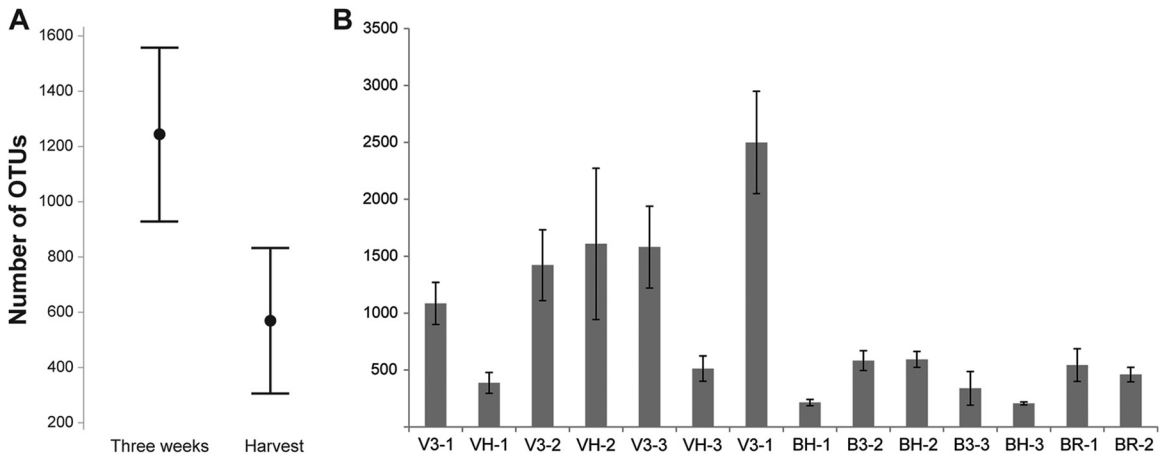


FIG 1 (A) Mean numbers of OTUs obtained from *L. sativa* samples collected 3 weeks after planting and at harvest at Farm Buskerud and Farm Vestfold. Also shown are the 95% confidence intervals. (B) OTU abundances on planting samples of *L. sativa* and *D. tenuifolia* (BR) from Farm Buskerud (B) and Farm Vestfold (V) at 3 weeks (3) and at harvest (H). Standard-error bars are shown. The number after the hyphen in each designation on the x axis is the planting number.

was the most abundant genus, followed by *Arthrobacter* (12%), *Pantoea* (10%), and *Acinetobacter* (8%).

**Comparison of phyllosphere communities with Illumina MiSeq.** Out of 51 samples of leafy greens (Table 1), a total of

4,363,870 filtered bacterial 16S rRNA sequences were obtained by MiSeq sequencing (see Table S1 in the supplemental material). There was a significantly higher number of OTUs from samples collected at 3 weeks after planting (Fig. 1A) than from samples

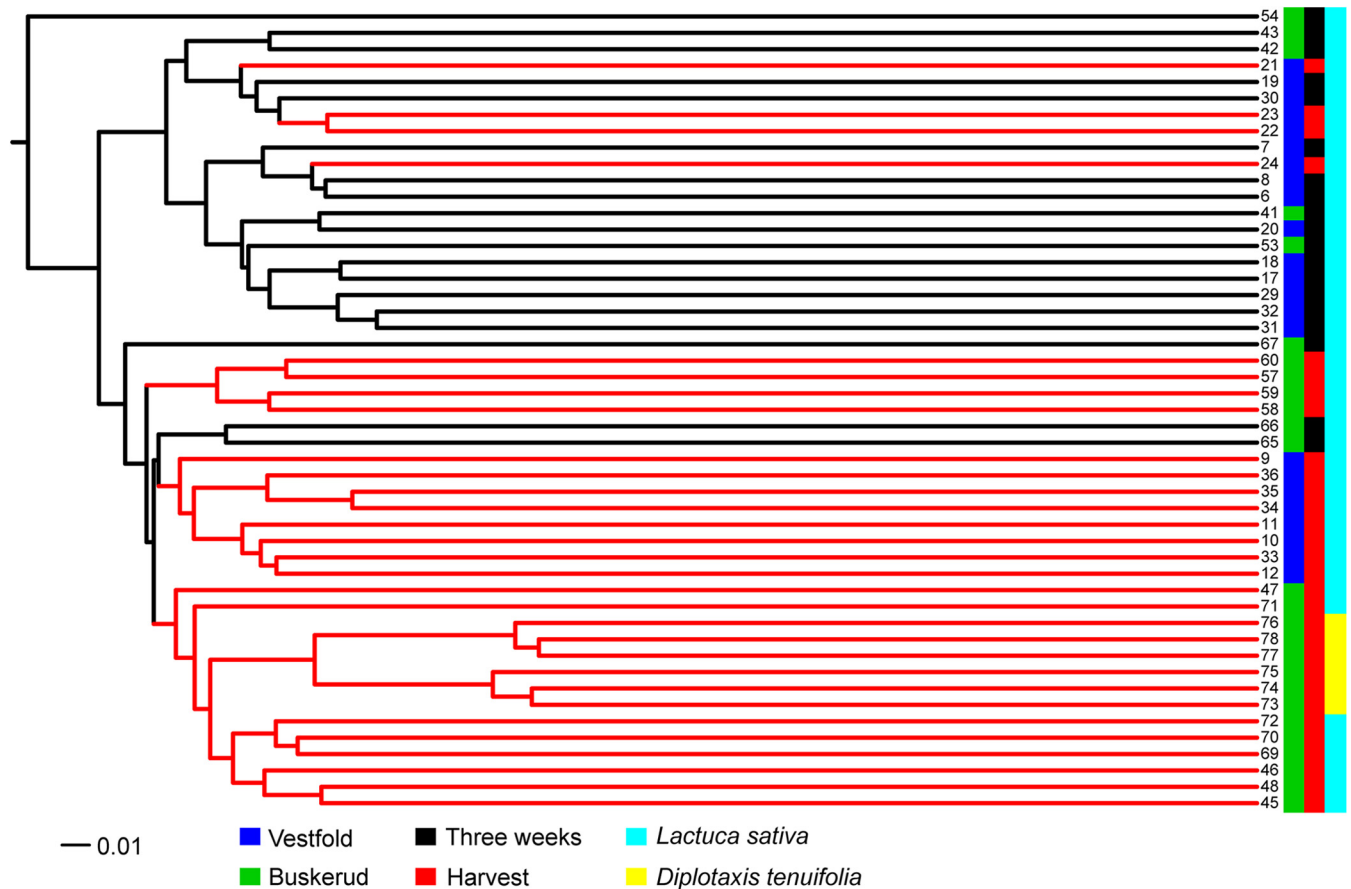


FIG 2  $\beta$ -Diversity tree of all of the samples made with jclass calculator (Jaccard index) on the basis of a subset of 9,549 random sequences from each sample. Samples: 6 to 8, V3-1; 9 to 12, VH-1; 17 to 20, V3-2; 29 to 32, V3-3; 33 to 36, VH-3; 41 to 43, B3-1; 45 to 48, BH-1; 53 to 56, B3-2; 57 to 60, BH-2; 65 to 67, B3-3; 69 to 72, BH-3; 73 to 75, BR-1; 76 to 78, BR-2. Code: Farm Buskerud, B; Farm Vestfold, V; 3 weeks after planting, 3; harvest, H.

TABLE 2 Comparison of bacterial communities on leafy greens at different harvest time points at Farm Buskerud and Farm Vestfold

Bacterial phylum and genus	Avg % representation ± SE						
	<i>L. sativa</i> (Vestfold) at:		<i>L. sativa</i> (Buskerud) at:		<i>L. sativa</i> (Vestfold and Buskerud) at:		<i>D. tenuifolia</i> at harvest
	3 wk	Harvest	3 wk	Harvest	3 wk	Harvest	
<i>Actinobacteria</i>	13 ± 3.3	6 ± 3.1	9 ± 3.5	4 ± 1.5	11 ± 2.3	4 ± 1.8	7 ± 1.5
<i>Arthrobacter</i>	5 ± 1.3	3.5 ± 2.6	3.2 ± 1.0	<1 ± 0.2	4.5 ± 0.9	2 ± 1.4	2.5 ± 0.7
<i>Nocardioiodes</i>	2 ± 0.6	<1 ± 0.2	1 ± 0.7	<1 ± 0.2	1.5 ± 0.4	<1 ± 0.1	1 ± 0.8
<i>Rhodococcus</i>	<1 ± 0.1	<1 ± 0.03	<1 ± 0.2	<1 ± 0.3	<1 ± 0.1	<1 ± 0.1	1 ± 1.2
<i>Bacteroidetes</i>	6 ± 0.22	6 ± 0.1	2 ± 0.4	6.3 ± 1.2	4 ± 0.8	6 ± 0.9	24 ± 4.1
<i>Chryseobacterium</i>	<1 ± 0.4	2 ± 0.8	0.4 ± 0.1	2 ± 0.8	<1 ± 0.2	2 ± 0.5	9 ± 1.3
<i>Flavobacterium</i>	1.2 ± 0.4	1.6 ± 0.6	0.5 ± 0.2	<1 ± 0.2	<1 ± 0.3	1 ± 0.4	11 ± 1.2
<i>Hymenobacter</i>	1.5 ± 0.6	<1 ± 0.2	0.7 ± 0.2	2 ± 1.5	1 ± 0.3	1.5 ± 0.7	<1 ± 0.0
<i>Firmicutes</i>	3 ± 1.6	1.5 ± 0.7	6 ± 0.6	1 ± 0.4	5 ± 1.0	1 ± 0.5	7 ± 6.3
<i>Exiguobacterium</i>	1.5 ± 0.9	<1 ± 0.04	4 ± 1.3	1 ± 0.9	2.5 ± 0.9	<1 ± 0.4	6 ± 5.8
<i>Proteobacteria</i>	73 ± 6.2	86 ± 4.6	76 ± 10.5	87 ± 12	76 ± 3.8	87 ± 2.9	60 ± 9.0
<i>Acinetobacter</i>	<1 ± 0.6	<1 ± 0.02	1 ± 1.1	3 ± 1.0	1 ± 0.6	1.5 ± 0.8	1 ± 1.1
<i>Alkanindiges</i>	4 ± 1.8	12 ± 5.2	1 ± 0.4	3 ± 0.5	2.5 ± 1.0	7 ± 3.1	<1 ± 0.0
<i>Brevundimonas</i>	<1 ± 0.3	1.5 ± 0.9	<1 ± 0.2	<1 ± 0.1	<1 ± 0.2	<1 ± 0.5	2.5 ± 0.6
<i>Duganella</i>	8 ± 3.5	9 ± 3.9	5 ± 1	29 ± 0.8	7 ± 1.8	19 ± 4.8	5.5 ± 1.6
<i>Massilia</i>	12 ± 4	4 ± 0.8	13 ± 5.4	8 ± 3.2	12 ± 3.0	6 ± 1.6	8 ± 3.5
<i>Methylophilus</i>	<1 ± 0.3	2 ± 0.5	<1 ± 0.1	<1 ± 0.7	<1 ± 0.2	1.5 ± 0.5	1 ± 0.7
<i>Pantoea</i>	5 ± 3.6	14 ± 7.2	15 ± 10	5 ± 2.4	10 ± 5.6	10 ± 4.0	5 ± 4.6
<i>Pseudomonas</i>	26 ± 10	22 ± 7.0	22 ± 10	24 ± 5.1	24 ± 6.6	23 ± 3.9	25 ± 11.5
<i>Rhizobium</i>	<1 ± 0.1	4 ± 1.9	<1 ± 0.2	2 ± 0.9	<1 ± 0.1	3 ± 1.1	1 ± 0.2
<i>Sphingomonas</i>	5 ± 0.4	5 ± 0.7	4 ± 1.3	8 ± 3.9	4.5 ± 0.6	6 ± 2.0	4.5 ± 3.0
<i>Xanthomonas</i>	<1 ± 0.03	2 ± 0.6	3 ± 1.3	<1 ± 0.03	1.5 ± 0.9	1 ± 0.5	2.5 ± 1.9
Other <sup>a</sup>	5 ± 0.14	1 ± 0.15	3 ± 0.11	1 ± 0.02	4 ± 0.1	2 ± 0.04	2 ± 0.16
Unclassified/other <sup>b</sup>	25 ± 3.4	14 ± 3.6	22 ± 8.0	8 ± 0.7	23 ± 1.0	11 ± 3.0	13 ± 0.8

<sup>a</sup> *Acidobacteria*, *Armatimonadetes*, *Chlamydiae*, *Chlorobi*, *Chloroflexi*, *Deinococcus*, *Thermus*, *Fusobacteria*, *Gemmatimonadetes*, *Nitrospora*, *Planctomycetes*, *Spirochaetes*, *Tenericutes*, *Verrucomicrobia*, “*Candidatus* BRC1,” “*Candidatus* OD1,” “*Candidatus* OP11,” “*Candidatus* TM7,” “*Candidatus* WS3,” and unclassified taxa.

<sup>b</sup> About 640 taxa are included in the “other” category.

collected at harvest, according to a basic one-way analysis of variance with Tukey’s range test ( $P = 0.002$ ). These results were not consistent in all of the samples, and the second planting (V3-2 and VH-2, B3-2 and BH-2) was separated from the others at both locations with similar OTU abundances at 3 weeks and at harvest (Fig. 1B). A higher number of genera was also found at 3 weeks after planting (540 genera) than at harvest (472 genera).

The weighted UniFrac method showed that the bacterial communities identified in the samples collected at harvest and 3 weeks after planting were significantly different ( $P < 0.001$ ). The comparison of *L. sativa* sampled 3 weeks after planting and at harvest was illustrated by  $\beta$ -diversity phylogeny with jclass calculator (Jaccard index) on the basis of the weighted UniFrac distances (Fig. 2). The  $\beta$ -diversity tree shows that samples collected 3 weeks after planting mainly clustered separately from the samples collected at harvest.

**Identification of bacterial community compositions by Illumina MiSeq.** The distribution of sequences from the *L. sativa* samples collected 3 weeks after planting could be assigned to the phyla *Proteobacteria* (76%), *Actinobacteria* (11%), *Firmicutes* (5%), and *Bacteroidetes* (4%) (Table 2; Fig. 3 and 4A). The dominant phyla associated with *L. sativa* leaves at harvest could be

assigned to *Proteobacteria* (87%), *Bacteroidetes* (6%), and *Actinobacteria* (4%) (Table 2; Fig. 3 and 4A). Members of the phylum *Proteobacteria* were also the most abundant bacteria (60%) found in the *D. tenuifolia* samples collected at harvest (Table 2; Fig. 3B and 4A).

The most common genus overall on the *L. sativa* and *D. tenuifolia* samples was *Pseudomonas* (Table 2), which was present on all of the *L. sativa* samples and all but one of the *D. tenuifolia* samples. *Pantoea* and *Sphingomonas* were the only genera identified on all of the samples. The predominant families across all of the samples were *Pseudomonadaceae* (23%), *Oxalobacteraceae* (22%), and *Enterobacteriaceae* (10%) (Fig. 4B). The genera *Salmonella* and *Escherichia*, which include potentially harmful human pathogens, were not found in our study. The lactic acid bacterial genera *Aerococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Weissella* were present on the samples but at very low abundances.

The distribution of bacterial families among different *L. sativa* varieties was investigated (Fig. 5). Members of the family *Enterobacteriaceae* were more abundant on Frillice (12%) than on the other varieties at 3 weeks after planting. At harvest, members of the family *Oxalobacteraceae* were more abundant on Iceberg (43%) and Frillice (34%) than on Little Gem (14%). There were

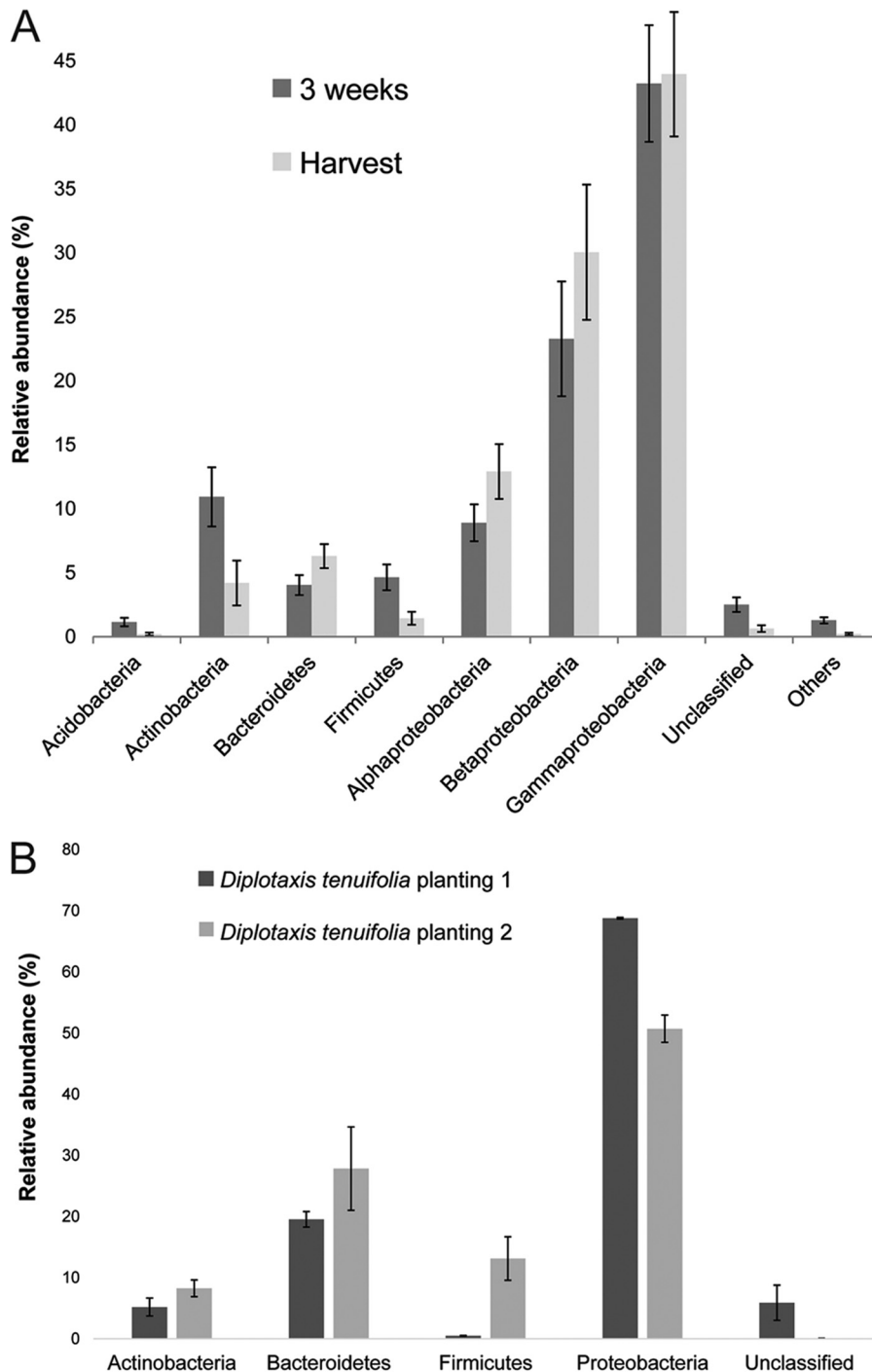


FIG 3 Relative abundances (percent) of phyla in *L. sativa* samples after 3 weeks and at harvest (A) and in *D. tenuifolia* plantings 1 and 2 (B). Standard error bars are shown.

more *Flavobacteriaceae* bacteria (21%) on *D. tenuifolia* and more *Sphingomonadaceae* bacteria (11%) on Frillice than on the other varieties at harvest.

**Phyllosphere bacterial communities varied according to season.** To investigate more closely the main bacterial distribution at 3 weeks after planting and at harvest and the possible seasonal effect observed in both plantings 2 and 3, we used the Metastats method (27) to statistically evaluate the differential abundance of

the OTUs in selected groups (see Table S2 in the supplemental material). This analysis showed that *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, and *Verrucomicrobia* were the significantly different phyla 3 weeks after planting, while members of the phylum *Proteobacteria* (genera *Duganella*, *Pedobacter*, and *Rhizobium*) were most abundant at harvest. Samples collected at harvest at the second planting, from both Buskerud and Vestfold, showed similar microbial profiles regarding phyla and genera.

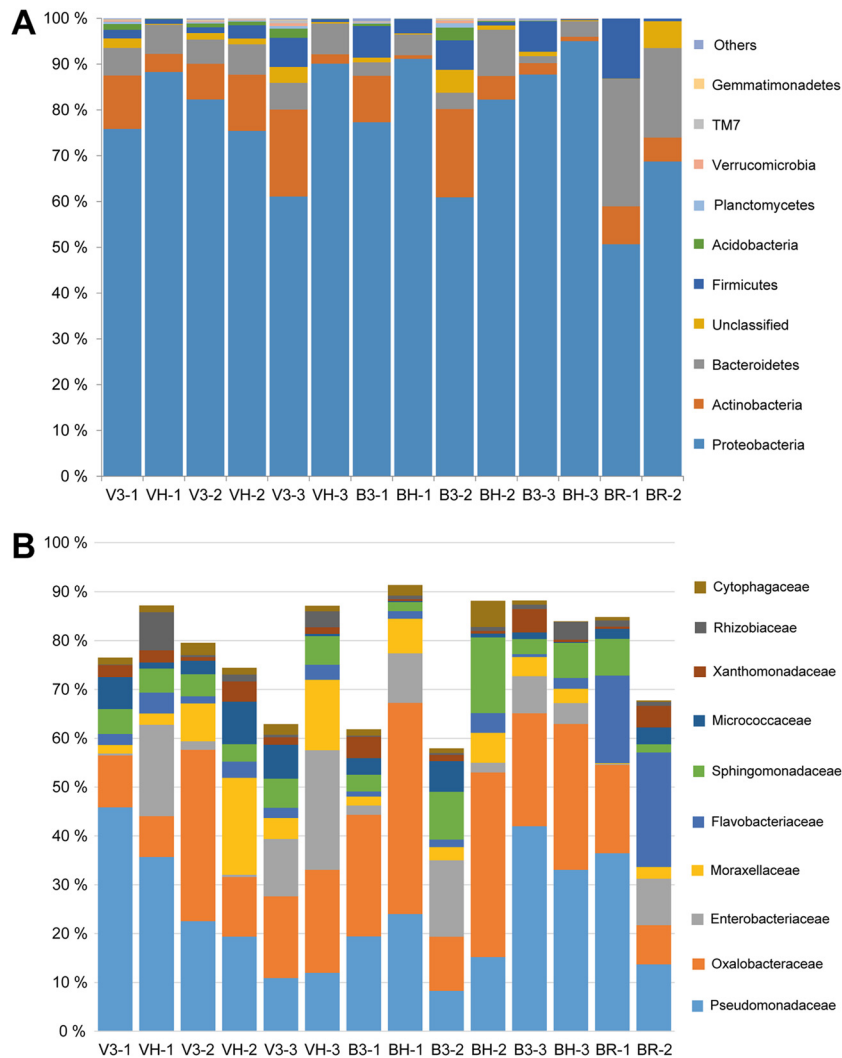


FIG 4 Relative abundances (percent) of bacterial phyla (A) and the most abundant families (B) on *L. sativa* and *D. tenuifolia* samples.

## DISCUSSION

This comprehensive study used Illumina MiSeq to study bacterial communities associated with leafy greens across plant development and time. Illumina-based 16S rRNA gene sequencing has higher accuracy and greater throughput than previously used pyrosequencing (29), thus allowing, in principle, deeper insight into the microbial communities of the leafy-green phyllosphere.

The present study identified *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* as the most prevalent phyla in the phyllosphere of leafy greens, regardless of culture or culture-independent analysis. This is consistent with previous studies of bacterial communities on leafy greens (11, 13–15, 30). Our results showed that *Proteobacteria* and *Bacteroidetes* were the most abundant phyla at harvest. This is consistent with the findings of Jackson et al. (11). However, *Firmicutes* replaced *Bacteroidetes* as one of the dominant phyla in other surveys of the leafy-green phyllosphere (13–15).

Comparison of the bacterial diversity on *L. sativa* leaves during the three plantings revealed some significant trends. We found that the bacterial richness of *L. sativa* samples was significantly

greater shortly after planting (3 weeks) than at harvest (5 to 7 weeks after planting) for plantings 1 and 3 at both sites. This means that the bacterial communities in the phyllosphere of our leafy-green samples changed with the maturation of the plants, and it may indicate that plants exert a selective force on the colonizing bacteria. A few studies have already investigated the impact of leaf age and seasonal effects on phyllosphere communities (14–18). Our findings are consistent with previous studies. Thompson et al. (16) reported that the greatest number of bacterial species could be isolated from young plants. A study of the phyllosphere of spinach showed that the largest numbers of bacteria were present on newly emerged leaves (18). The richness and abundance of the spinach phyllosphere bacterial community decrease as the plant leaf matures (18). On the other hand, a study of leaf age and the seasonality of the tree species *Quercus ilex* in the Mediterranean forest revealed an increase in the richness of epiphytic bacteria with increasing time of colonization (17). With respect to planting 2 in our study, the bacterial richness remained consistent at both sites, and they also had similar microbial profiles that separated them from plantings 1 and 3 (Fig. 1B; see Table S2 in the

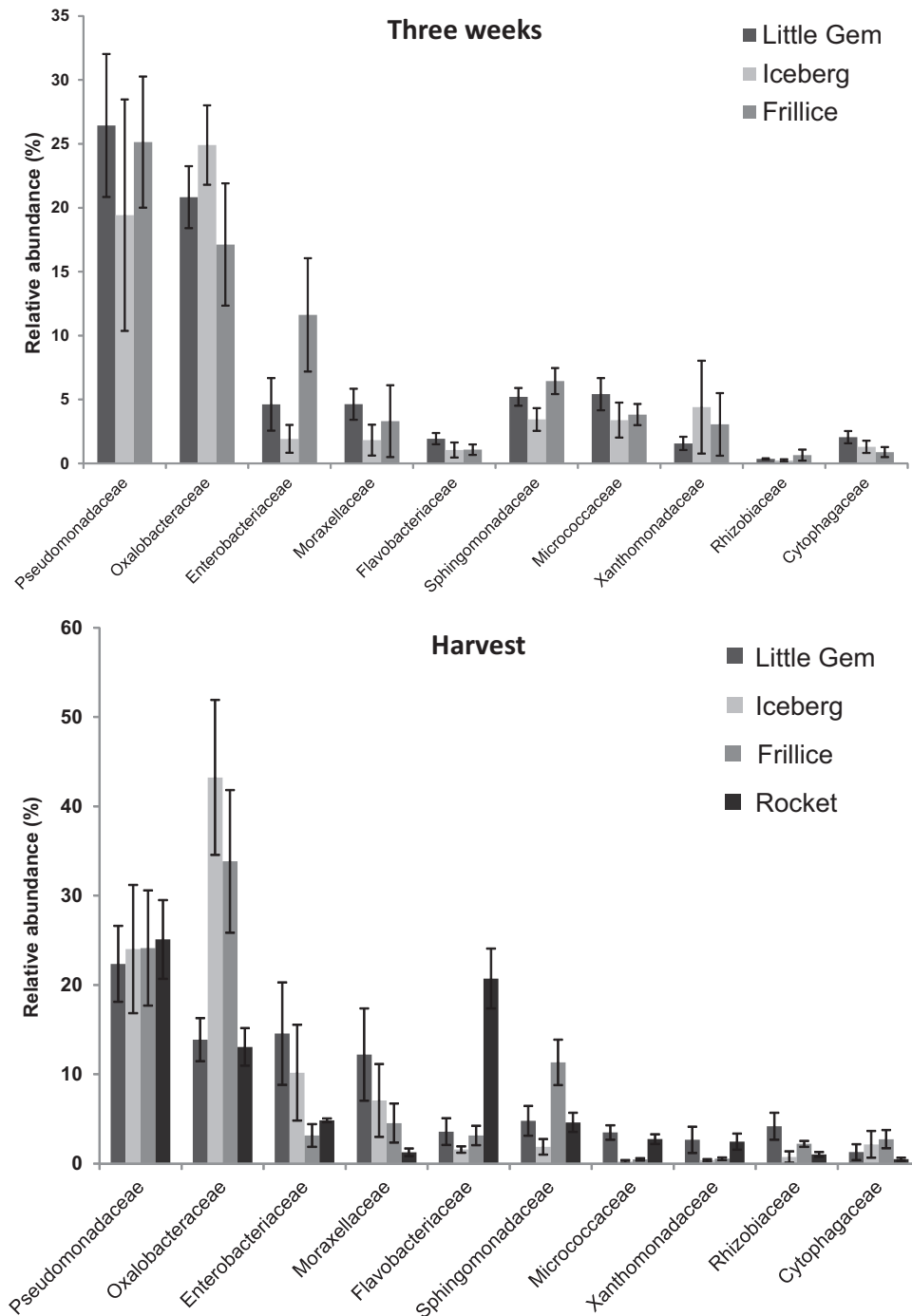


FIG 5 Relative abundances of selected bacterial families on *D. tenuifolia* and three varieties of *L. sativa* (Frillice, Iceberg, and Little Gem).

supplemental material). Figure 6 shows that planting 2 had a higher temperature at harvest at both locations than plantings 1 and 3, which may partly explain the separation of planting 2 from plantings 1 and 3. The  $\beta$ -diversity tree (Fig. 2) showed that not all of the samples collected 3 weeks after planting and at harvest clustered. Two samples, VH-2 collected at harvest in Vestfold and B3-3 collected 3 weeks after planting in Buskerud, were in the “3 weeks after planting” and “harvest” clades, respectively (Fig. 2), suggesting that the bacterial community composition changes

with season and not solely with leaf maturation. This confirms the findings of Williams et al. (15), where, for the first time, a seasonal effect was reported. Other studies have also observed seasonal variations in terms of the bacterial community composition of the phyllosphere of different plant species (8, 14, 31), but clearly, further in-depth studies on seasonal effects are needed.

*Actinobacteria* were more prevalent in the samples collected at harvest at Farm Vestfold (6%) than in those collected at harvest at Farm Buskerud (4%) (Table 2). This is likely due to the fact that

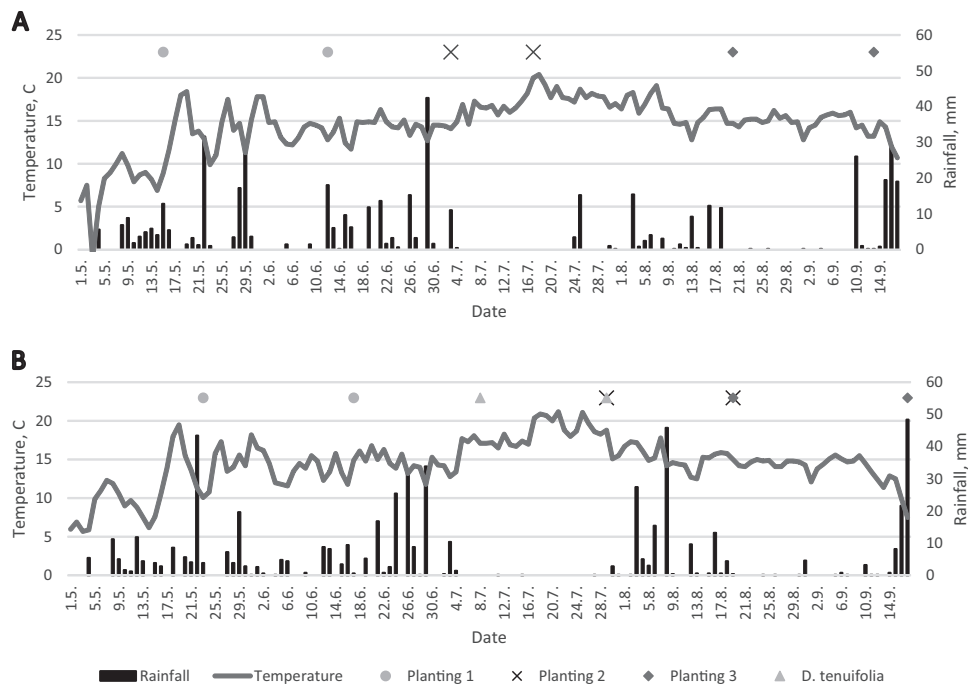


FIG 6 Daily mean temperatures and accumulated rainfall amounts at Farm Vestfold (A) and Farm Buskerud (B) from 1 May through 17 September 2013. Sampling dates are indicated for *L. sativa* plantings 1, 2, and 3 (3 weeks after planting and at harvest) and *D. tenuifolia* (two samplings at harvest).

the *L. sativa* plants at Farm Vestfold were exposed to open soil while the soil at Farm Buskerud was covered with plastic film. Consequently, the *L. sativa* plants at Farm Vestfold could easily be colonized by soil bacteria since the plastic barrier was lacking and *Actinobacteria* are abundant in soil (32). Our study may suggest that the growing environment (soil) also affects the composition of bacterial communities associated with leafy greens. *D. tenuifolia* and *L. sativa* at Farm Buskerud were irrigated with water from the same source (a river) and grown on the same type of soil. However, river water cannot be considered a homogeneous inoculum source for different crops at a single farm. Recently, airborne bacteria have been shown to be important in forming initial phyllosphere communities (33–35). Further studies of air, soil, and water samples should be done to identify possible inoculum sources. It should be mentioned that all of our *L. sativa* samples were sown in a greenhouse and grown for 2 weeks before planting in the field, but previous studies have shown that *L. sativa* plants grown indoors have lower total bacterial quantities than those grown in the field and that field microbiota can successfully colonize plants grown indoors (35). This suggests that the greenhouse period did not greatly influence the lettuce microbiota studied.

A notable variability in the distribution of phyllosphere communities in the *D. tenuifolia* samples compared to those in the *L. sativa* samples was observed at harvest, despite the fact that they were grown on the same type of soil at Farm Buskerud (sandy clay loam). This supports other studies showing a pronounced interspecies variability in phyllosphere communities (3, 36). The large variability of phyllosphere communities between *D. tenuifolia* and *L. sativa* at the same location and the observation that there was a shift in bacterial diversity during the planting suggest that the leaf-dwelling bacteria are not only passive and random inhabitants but are indeed influenced by the leafy-green species they colonize.

*Pantoea* and *Sphingomonas* were the only genera identified in all of our samples. This is in contrast to other surveys of leafy greens, where *Pseudomonas*, *Bacillus*, *Massilia*, and *Arthrobacter* were among the genera present in all of the samples (11, 14). Bulgarelli et al. (30) included *Pseudomonas*, *Pantoea*, *Massilia*, and *Sphingomonas* as part of the core phyllosphere communities. *Pseudomonas* bacteria were also found in several other studies to dominate the phyllosphere (5–7, 13, 14). *Sphingomonas* bacteria were identified in all our samples, in contrast to previous investigations of the leafy-green phyllosphere (14). However, *Sphingomonas* bacteria have been reported to be prevalent in other studies of the leaf microbiota on other plants (31, 37) and are known to contribute to plant health (30). A comprehensive knowledge of the drivers of bacterial community structure in the phyllosphere is of the utmost importance in developing new strategies for plant protection.

The genera identified in this study also include important plant pathogens like *Pantoea*, *Pseudomonas*, *Rhodococcus*, and *Xanthomonas*, as well as plant symbiotic *Rhizobium*. The genus *Xanthomonas* was present in 44 of 51 samples in various abundances (0.1 to 14%). In contrast, *Xanthomonas* was present in only 33 of 88 samples in a survey in California (14). *Pantoea*, which is a member of the *Enterobacteriaceae* family, is also found to be one of the major genera in other studies of the leafy-green phyllosphere (5, 11, 14). The genus *Pantoea* includes several species that are generally associated with plants as either epiphytes or pathogens, and some species can cause disease in humans (38). One sample (no. 54, from B3-2, Fig. 2) was separated from the clades, and we found that most of the sequences (97%) belonged to genus *Pantoea*. This suggests a plant pathogen infection; however, no symptoms were visible on the *L. sativa* plants. Other genera in the *Enterobacteriaceae* family, such as *Cronobacter*, *Proteus*, *Providencia*, and *Yersinia*, were also prevalent across all of the samples. Other



genera in the *Enterobacteriaceae* family that include potentially harmful human pathogens, such as *Salmonella* and *Escherichia*, were not found in our study. Our study did not confirm the findings of Williams et al. (15), which identified OTUs from the *Enterobacteriaceae* family that were not classified in any known genera and therefore suggested the presence of novel bacterial species unique to the phyllosphere.

This study provides novel ecological insight into the development of bacterial communities on leafy greens. A shift in bacterial community composition and richness could be observed over time throughout the growing season. We suggest that host-microbe interactions play a role over time in shaping niches favoring the growth of particular taxa. A notable degree of variability in the distribution of phyllosphere communities could be observed among leafy-green species.

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