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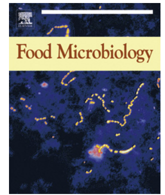
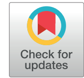


Shifts in spinach microbial communities after chlorine washing and storage at compliant and abusive temperatures



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ABSTRACT

Fresh produce, like spinach, harbors diverse bacterial populations, including spoilage and potentially pathogenic bacteria. This study examined the effects of produce washing in chlorinated water and subsequent storage on the microbiota of spinach. Baby spinach leaves from a commercial fresh-cut produce processor were assessed before and after washing in chlorinated water, and then after one week's storage at 4, 10, and 15 °C. Microbial communities on spinach were analyzed by non-selective plating, qPCR, and 16S rDNA amplicon sequencing. Bacterial populations on spinach, averaging 6.12 ± 0.61 log CFU/g, were reduced by 1.33 ± 0.57 log after washing. However, populations increased by 1.77–3.24 log after storage, with larger increases occurring at higher temperature ($15 > 10 > 4$ °C). The predominant phylum identified on unwashed spinach leaves was Proteobacteria; dominant genera were *Pseudomonas* and *Sphingomonas*. Bacterial communities shifted significantly after chlorine washing and storage. Several Proteobacteria species, such as *Stenotrophomonas* sp. and *Erwinia* sp., were relatively tolerant of chlorine treatment, while species of *Flavobacterium* and *Pedobacter* (phylum Bacteroidetes) grew rapidly during storage, especially at abusive temperatures. *Cupriavidus* sp. and *Ralstonia* sp. showed significant increases after washing. After storage, microbial communities on spinach appeared to shift back toward the pre-washing distributions.

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1. Introduction

Fresh vegetables and fruits harbor large and diverse bacterial communities, with populations ranging from 2 to 8 log CFU/g, which may potentially include pathogenic and spoilage bacteria (Leff and Fierer, 2013; Nicholl et al., 2004; Ottesen et al., 2013; Yang et al., 2001). These complex bacterial communities may strongly influence the likelihood that exogenous bacteria, such as foodborne pathogens, can survive and proliferate on produce (Lopez-Velasco et al., 2012; Shi et al., 2009).

Although washing is a critical process during the preparation of fresh-cut produce for the ready-to-eat market, this process is also prone to permitting pathogen cross-contamination that could increase risks to food safety (FDA, 2008). Several studies have investigated the efficacy of sanitizer types and concentrations for preventing pathogen cross-contamination during washing processes (Luo et al., 2012; Suslow, 2001; Weng et al., 2016). Chlorine (in the form of HOCl) is widely used by the produce industry as a sanitizer; it provides an economical and efficient microbial reduction that has minimal adverse impacts on food quality (Luo et al., 2012). The concentrations of chorine on fresh produce processing lines are usually monitored periodically and replenished as needed (Cornell, 1996; Suslow, 2001). However, the low occurrence of foodborne pathogen contamination makes it impractical to directly evaluate how well sanitization works against these targeted bacteria in production settings (Denis et al., 2016). Instead, the total bacterial counts, such as SPC (standard plate count) and APC (aerobic plate count), have been commonly used for evaluation purposes (FSANZ, 2001; Maturin and Peeler, 2001). Yet, the ability of

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the diverse bacterial species to survive on produce varies during the sanitization process (Jackson et al., 2013). There are also different levels of risks that could be associated with produce safety after the washing process. For example, fresh-cut produce needs storage temperatures controlled at $\leq$ 41 °F ($\leq$5 °C) (FDA, 2008, 2012). Temperature abuse can result in the rapid growth of spoilage bacteria and, if present, foodborne pathogens (Luo et al., 2010).

The consumption of spinach has been increasing rapidly in the US since 2009 (PBHF, 2015). Several studies have attempted to profile the microbial communities on fresh produce including spinach (Leff and Fierer, 2013; Lopez-Velasco et al., 2010). These studies showed that the diversity and composition of bacterial communities on produce were associated with food commodity and farming practice, and that different plant types and parts were likely to harbor distinctive microbial communities (Leff and Fierer, 2013; Ottesen et al., 2013). The knowledge on the microbial community composition and dynamics on fresh produce impacted by processing and storage would be of critical importance for better understanding the interactions of the microbial community with potentially contaminating pathogens and for developing effective intervention strategies. Recent advances in next generation sequencing technology and bioinformatics analyses provide more powerful tools to identify microbial communities in greater depth, which can lead to an enhanced understanding of the dynamics of diverse bacterial taxa of the microbial ecology in a deeper level (Callahan et al., 2017; Goodwin et al., 2016; Kumar et al., 2015; Oulas et al., 2015).

This study analyzed the effect of produce washing in chlorinated water on the population and composition of bacterial communities on spinach collected from commercial processing lines. The impact of compliant versus abused storage temperatures on the shift in bacterial communities on spinach were also evaluated. The DNA-modifying dye propidium monoazide (PMA) was applied during sample preparation to ensure the focus on viable microbial communities on spinach samples (Chiao et al., 2014; Nocker et al., 2010). Quantitative-PCR (qPCR) was performed and estimated 16S rDNA gene copy values were analyzed along with relative abundance data to estimate the dynamics of diverse bacterial communities during the processing and storage (Chiao et al., 2014; Liang et al., 2015).

2. Materials and methods

2.1. Spinach and wash water sample collection and processing

Fresh baby spinach samples were obtained in December 2016 and January 2017 from a commercial processor in the Mid-Atlantic region of US. The spinach for the first and second samplings (batches) was organically grown in Salinas, CA, and Yuma, AZ, respectively, and shipped to the establishment for processing. Chlorinated water was used for spinach sanitation in a traditional double-flumed washing system at 4 °C; with targeted dwelling time of 20 s in each flume. Wash water pH and free chlorine were maintained using an automated dosing system at customary levels consistent with most industry practices. On each sampling day, 4 portions of unwashed spinach (100 g each) and 4 temporally matching finished packages of washed spinach (500 g) were collected at the beginning of the production shift (Early trial), when the organic load in the wash water was expected to be low; and at the end of the work shift of the spinach line (Late trial), when the organic load in the wash water was expected to be higher. These wash water samples (1 L) were collected from the primary and secondary washing flumes at time points matching spinach samples. Spinach and water samples were stored on ice and delivered to laboratory for processing within 5 h.

The unwashed spinach samples were immediately processed in the laboratory for bacterial enumeration and DNA extraction. The washed spinach samples were split into 4 sets: one set was processed at the same time as the unwashed spinach samples, and the other three sets were tested after storage at the temperatures of 4, 10, and 15 °C for 7 days. For each sampling event, each type of samples was processed in quadruplet. In total, 80 spinach samples including 16 unwashed, 16 washed, and 48 stored samples (16 at each storage temperature) were tested in this study. From each spinach sample, 20 g portions were transferred to sterile Whirl-Pak filter bags (Nasco, Fort Atkinson, WI) with 50 ml phosphate-buffered saline (PBS, pH 7.2, Thermo Fisher Scientific) in quadruplicates. These spinach leaves were fully immersed in buffer, and sonicated at 40 kHz for 2×1 min with 30 s pulses in a sonicator waterbath (Branson, Richmond, VA), followed by gentle hand massaging. An aliquot was taken for bacterial plate counts, and the remaining filtrate was concentrated for DNA purification.

Real-time pH and free chlorine readings of the flume water were recorded at the time of sampling. Total dissolved solids (TDS, mg/L), turbidity (nephelometric turbidity units (NTU)), and chemical oxygen demand (COD, mg/L) were determined using established procedures (Weng et al., 2016).

2.2. Bacterial enumeration

For plate count (the culture method), 100 µl of the filtrate of each sample and its 10-fold serial dilution was spiral plated on Tryptic Soy Agar plates with polysorbate and lecithin (TSA-PL, Sigma-Aldrich, St. Louis, MO), and Vegitone Plate Count Agar plates (PCA-V, Sigma-Aldrich) in duplicates. TSA-PL is recommended for the isolation of microorganisms from the environment for surface sanitization evaluation (Salfinger and Tortorello, 2015). PCA-V with plant peptone has been reported to provide higher recovery rates and better growth performance (Sigma-Aldrich, 2005). Plates were incubated at 30 °C for 2 days and colonies were enumerated using a Flash & Go automatic colony counter (IUL, Barcelona, Spain). Total bacterial population was also estimated by qPCR, as described in the section below.

2.3. PMA treatment and DNA extraction

After sonication, the filtrate (45 ml) was centrifuged for 2 min at 1000 g to remove coarse debris and soil particles, and then for an additional 15 min at 4500 g to precipitate bacterial cells. The precipitated cells were further concentrated by micro-centrifugation (14,000 g for 10 min). Finally, pelleted cells were re-suspended in 500 µl PBS with 50 µM DNA-modifying dye propidium monoazide (PMA, Biotium, Fremont, CA). The cell suspension was incubated in the dark for 10 min at room temperature, followed by exposure to light from a 650-W halogen lamp (Sachtler R651HS; Camera Dynamics, Inc., Valley Cottage, NY) for 10 min. The cell suspension was kept on ice as described in a previous study (Chiao et al., 2014). Subsequently, cells were washed with PBS twice and collected by centrifugation at 14,000 g for 10 min.

The cells were re-suspended in 150 µl TE buffer (Thermo Fisher Scientific) containing 10 mg/ml lysozyme (Epicentre, Madison, WI) and 5 mg/ml proteinase K (Eicentre), and then incubated at 37 °C for 10 min. Bacterial DNA of each sample was extracted using the DNeasy Powersoil kit (Qiagen, Gaithersburg, MD) following the supplier's instructions and stored at −80 °C.

2.4. Quantitative real-time PCR (qPCR)

qPCR targeting a highly conserved 180 bp portion of 16S rDNA gene (Clifford et al., 2012) was performed on an Mx3005P QPCR

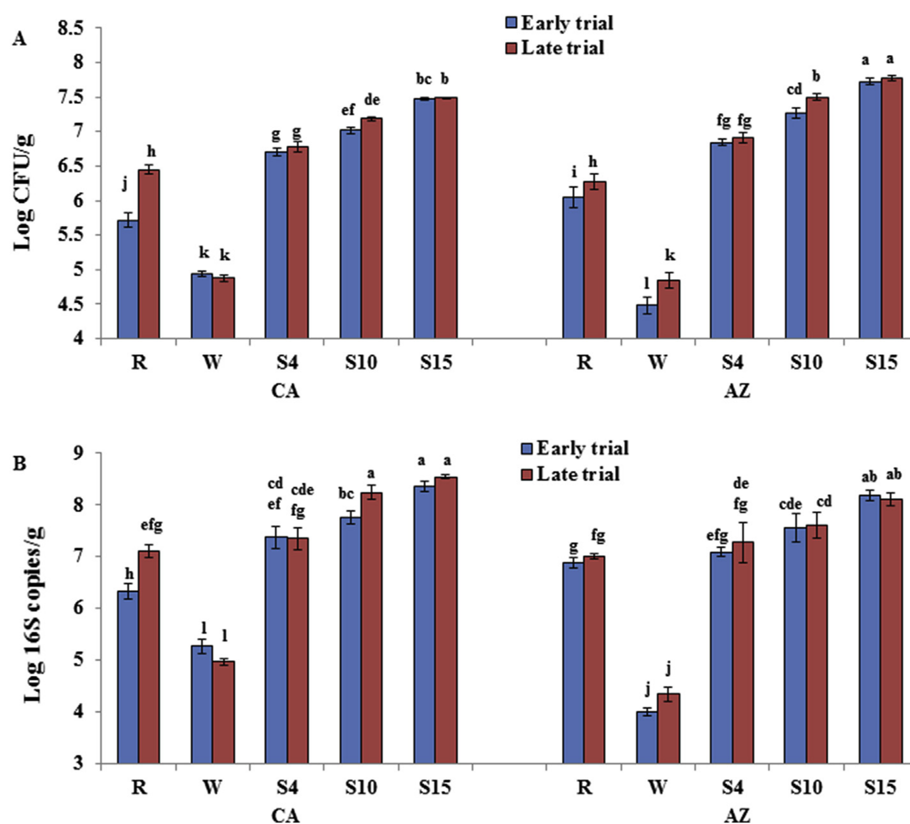

Fig. 1. Total mesophilic aerobic plate count (A) and **16S rDNA quantification of live bacteria (B) on spinach subjected to chlorine washing** and **storage at three different temperatures.**

R: Unwashed spinach; W. Washed Spinach; S: Storage at indicated temperature for one week. CA and AZ indicate regions where spinach was grown. Blue and red bars indicate Early and Late production shifts when spinach was sampled. Sixteen replicates were tested for each type of sample.

Bars indicate standard error (SE), and different letters above the bars indicate statistical difference at $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

system (Agilent technology Inc. Savage, MD) to estimate the total 16S rDNA copy numbers. Each 25 μL reaction mix contained 12.5 μL of 2 × QuantiTect GotoTaq qPCR Master Mix (Promega, Madison, WI), 1 μL of forward and reverse primers to a final concentration of 0.5 μM, 8.5 μL water, and 2 μL DNA templates with dilution if necessary. Standard curves were generated in every qPCR run using serial dilutions of chromosomal DNA extracted from *E. coli* of known concentration using the same method (NENUN, 2010). Cycle threshold numbers and baseline were determined automatically using the Noiseband algorithm. All PCR were performed in triplicate. The inferred absolute abundances of identified taxa in the spinach DNA samples were calculated by multiplying the total 16S rDNA copy numbers of each sample and the relative abundance (RA) of each taxon in the sample (Liang et al., 2015). RA was calculated based on the 16S amplicon sequencing data as described below.

2.5. 16S rDNA amplicon sequencing and sequence analysis

Sample DNAs were processed for 16S amplicon sequencing following the Earth Microbiome Project protocol (Caporaso et al., 2011, 2012) using MiSeq (Illumina). The barcode primer sets, 515F - 806R, were used to amplify the 16S rDNA fragment (Apprill et al., 2015; Parada et al., 2016).

MiSeq sequence data were sorted based on unique barcodes and quality-controlled using the Quantitative Insights Into Microbial Ecology (Qiime2, version 2017.6, https://docs.qiime2.org/2017.6/) with plugins demux (https://github.com/qiime2/q2-demux), dada2 (Callahan et al., 2016) and feature-table (McDonald et al., 2012). Alpha and beta diversity analyses were performed using plugins alignment (Katoh and Standley, 2013), phylogeny (Price et al., 2010), diversity (https://github.com/qiime2/q2-diversity), and emperor (Vazquez-Baeza et al., 2013). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/), which had been trimmed to include the v4 region of 16S rDNA, bound by the 515F/806R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. Taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier), taxa (https://github.com/qiime2/q2-taxa) and composition (Mandal et al., 2015).

2.6. Statistical analysis

With plate count enumeration and qPCR estimation, bacterial CFU values and 16S rDNA copy numbers were log transformed, and analysis of variance (ANOVA) was performed to analyze the differences in both bacterial populations by plate count and 16S rDNA copy numbers by qPCR, across different types of spinach samples. Pearson's correlation coefficients were calculated to evaluate the correlations between the dynamics of bacterial population and 16S rDNA copy numbers of different types of samples. The levels of top five bacterial species on CA or AZ spinach in different types of samples during the washing and storage processes were also analyzed using ANOVA. The

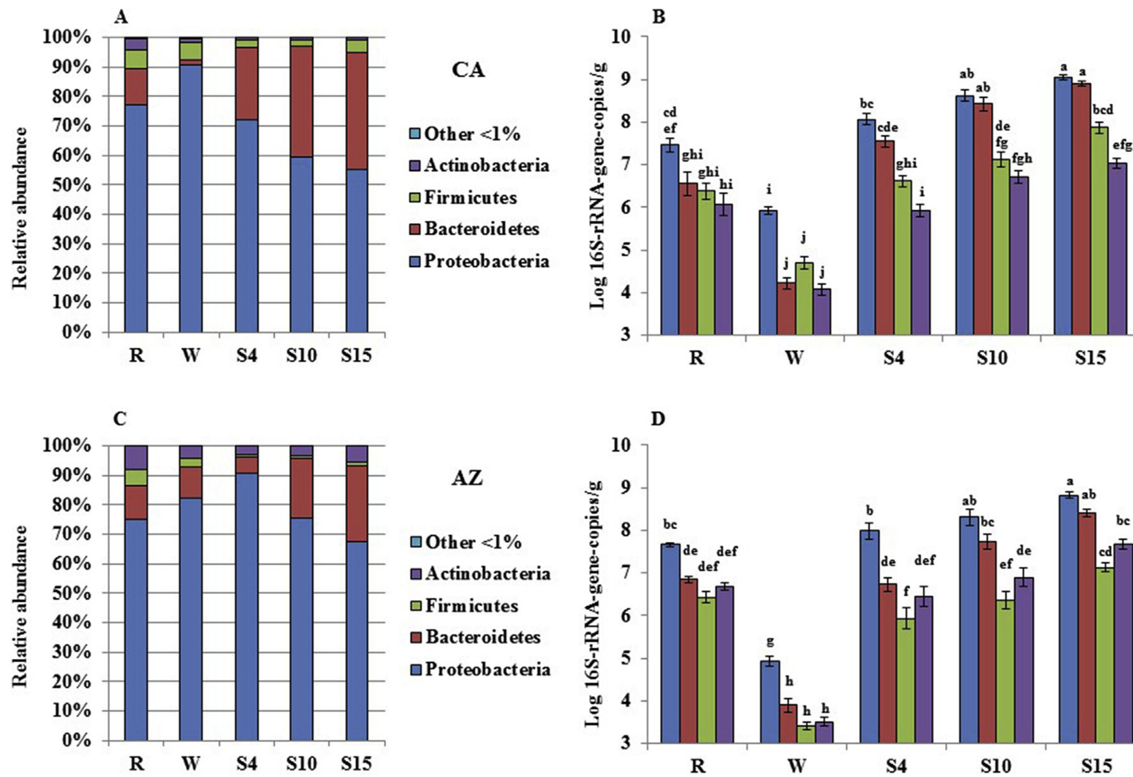

Fig. 2. Shift of major bacteria phyla (>1%) on spinach samples of California (A, B) and Arizona (C, D) in each type of samples. A and C are relative abundance values of major taxa; B and D are corresponding bacterial levels calculated based on qPCR estimation of 16S rDNA copy numbers in each type of samples (8 samples/type).

R: Unwashed spinach; W. Washed Spinach; S: Storage at indicated temperature for one week. Bars represent standard errors, and different letters above the bars indicate statistical difference at $p < 0.05$. Only phyla comprising >1% (average relative abundance) of the bacteria identified in at least one type of samples were included.

differences of alpha diversity indexes, including observed OTUs, Shannon index, evenness, and Faith's Phylogenetic Diversity (faith-pd), and beta diversity analyses using Jaccard, Bray-Curtis, Unweighted, and Weighted UniFrac methods, among different types of samples were analyzed using the Kruskal–Wallis H test and the Permutational multivariate analysis of variance (PERMANOVA) analysis, respectively, in Qiime 2. Statistical analysis was performed using SAS (SAS release 9.3, SAS Institute Inc., Cary, North Carolina). Except when stated otherwise, P values of <0.05 were considered statistically significant.

3. Results

3.1. Dynamics of bacterial populations on spinach impacted by chlorine washing and storage temperatures

The processing establishment from which spinach and wash water samples were obtained used a customized automatic pH and free chlorine dosing system to control the quality of wash water. Real-time online readings indicated that free chlorine and pH levels were stably maintained at the set ranges throughout the period of sampling. As expected, the other water quality parameters, including turbidity, TDS, and COD, in primary flume wash water were higher than in the secondary flume wash water. However, the differences in water quality between the Early and Late trials, although statistically significant, were less than expected (Table S1).

Two non-selective media were used to determine total mesophilic aerobic bacterial populations on spinach subjected to washing and storage. There was no significant difference between the plate count results on TSA-PL and PCA-V plates (Data not shown), and therefore the data presented here were based on calculations using average cell counts on both media. (Fig. 1).

Bacterial populations on spinach grown in CA decreased by 0.8 and 1.6 log in Early and Late trials, respectively. Similarly, for spinach grown in AZ, bacterial populations were reduced 1.6 log in the Early trial, and 1.4 log in the Late trial, after washing (Fig. 1A). By the end of production shift, the wash water turbidity, TDS, and COD values in both flumes were higher than at the Early stage of the shift (Table S1); however, in terms of reducing the bacterial populations on spinach, the effectiveness of washing was not significantly different between the two trials ($P = .072$).

After one week of storage, mesophilic aerobic bacteria on spinach proliferated in a temperature dependent manner (Fig. 1A). The population on washed CA spinach increased by 1.83 ± 0.06, 2.19 ± 0.04, and 2.57 ± 0.01 logs after one week storage at 4, 10, and 15 °C, respectively. The proliferation of bacteria on AZ spinach was significantly higher, increasing by 2.25 ± 0.06, 2.75 ± 0.07, and 3.12 ± 0.04 logs at 4, 10, and 15 °C, respectively.

3.2. Correlation of qPCR estimates of 16S rDNA copies with plate counts

qPCR was conducted for all samples to estimate 16S rDNA copies (Fig. 1B). On average, 16S rDNA copy number decreased by 1.60 logs and 2.76 logs after chlorine washing for spinach from CA and AZ, respectively. After one week of storage, 16S rDNA copies on CA spinach samples increased by 2.24, 2.88, and 3.33 logs at 4, 10, and 15 °C, and the copies on AZ spinach samples increased by 3.01, 3.41, and 3.97 logs, respectively. These qPCR estimates of bacterial 16S rDNA copy numbers were significantly correlated with population changes calculated by the plate count method ($P < .01$).

Table 1
Change in relative abundance (RA, %) of major bacterial species (RA>1%) on spinach after chlorine wash and after storage at different temperature.[a]

Species	Chlorine Wash		Storage			
	Initial (R)	Differential (W-R)	Initial (W)	Differential (S-W)		
				S4	S10	S15
Spinach from CA						
Pseudomonas sp. 1	27.92	12.53	40.45	-8.79	-7.44	-15.29
Pseudomonas sp. 2	14.16	-13.13	1.03	15.28	2.86	1.77
Acinetobacter sp.	13.40	-12.57	0.83	-0.53	-0.35	-0.42
Flavobacterium succinicans	4.25	-3.86	0.39	10.31	12.29	10.75
Shewanella sp.	3.51	-2.95	0.56	4.12	0.52	4.51
Psychrobacter sp.	3.49	-3.30	0.19	-0.14	-0.18	-0.18
Erwinia sp.	2.94	21.94	24.88	-23.30	-24.07	-24.33
Sphingomonas sp.	2.52	-0.24	2.28	-0.72	-0.60	-1.35
Flavobacterium sp. 1	2.33	-2.15	0.18	1.57	2.88	3.41
f-*Micrococcaceae* sp. 1	2.21	-2.01	0.20	0.18	0.26	0.24
Carnobacterium viridans	2.18	-2.05	0.13	-0.07	-0.13	-0.12
Pseudomonas viridiflava	1.91	4.32	6.23	-4.60	-4.91	-4.59
Chryseobacterium sp.	1.85	-1.25	0.60	5.15	5.34	5.47
Janthinobacterium lividum	1.79	0.72	2.51	2.90	3.02	2.79
Planomicrobium sp.	1.59	-1.53	0.06	-0.06	-0.06	-0.06
Sphingobacterium faecium	1.40	-1.28	0.12	2.07	5.93	8.27
Paenibacillus sp.	1.38	3.88	5.26	-2.72	-3.70	-2.71
Pedobacter sp. 1	1.35	-0.72	0.63	2.28	5.64	5.90
Janthinobacterium sp.	1.25	2.66	3.91	-1.83	-2.19	-2.88
f-*Enterobacteriaceae* sp.	1.16	2.50	3.66	-0.87	-1.64	-1.81
Agrobacterium sp.	0.65	0.73	1.38	0.25	3.71	3.79
Flavobacterium sp. 2	0.42	-0.39	0.03	0.43	1.87	1.29
f-*Comamonadaceae* sp.	0.19	-0.15	0.04	0.06	0.40	1.07
Stenotrophomonas sp.	0.14	1.14	1.28	-0.59	-0.41	0.34
f-*Paenibacillaceae* sp.	0.12	-0.01	0.11	0.01	0.17	1.23
Pedobacter sp. 2	0.02	0.00	0.02	0.42	1.36	1.74
Other species<1%	5.89	-2.87	3.02	-0.77	-0.58	1.19
Spinach from AZ						
Sphingomonas sp.	30.23	-25.46	4.77	-4.25	-4.15	-4.39
Pseudomonas sp. 1	12.40	18.22	30.62	-5.80	1.19	1.44
Pseudomonas viridiflava	7.11	-3.93	3.18	-2.50	-2.22	-2.31
Acinetobacter sp.	6.40	-6.28	0.12	0.41	1.74	0.77
f-*Microbacteriaceae* sp. 1	5.84	-4.95	0.89	-0.75	-0.63	-0.71
Sphingobacterium faecium	5.06	-4.91	0.15	1.02	5.34	8.59
Agrobacterium sp.	4.33	-3.95	0.38	-0.28	-0.05	0.54
Pedobacter sp. 1	4.24	5.63	9.87	-7.42	-5.42	-4.56
Exiguobacterium sp.	4.22	-3.43	0.79	-0.67	-0.60	-0.64
Janthinobacterium sp.	2.72	1.52	4.24	-3.44	-2.33	-2.27
f-*Oxalobacteraceae* sp.	2.59	2.24	4.83	-4.75	-4.76	-4.78
Chryseobacterium sp.	1.82	-1.49	0.33	0.28	2.57	2.44
f-*Micrococcaceae* sp. 1	1.67	-0.21	1.46	0.50	0.78	1.42
f-*Moraxellaceae* sp.	1.37	-1.37	0.00	0.00	0.00	0.00
Psychrobacter sp.	1.35	2.40	3.75	-0.72	-1.78	-2.22
f-*Enterobacteriaceae* sp.	1.35	-1.14	0.21	-0.11	-0.15	-0.12
Erwinia sp.	0.91	3.36	4.27	-3.61	-3.17	-3.62
Shewanella sp.	0.76	-0.20	0.56	8.37	13.41	14.78
Janthinobacterium lividum	0.66	0.21	0.87	-0.30	0.68	0.45
Pseudomonas sp. 2	0.35	15.02	15.37	34.20	1.45	-7.12
Flavobacterium succinicans	0.15	-0.12	0.03	0.49	2.40	3.18
Stenotrophomonas sp.	0.12	1.32	1.44	-1.33	-1.25	-1.32
f-*Microbacteriaceae* sp. 2	0.12	1.38	1.50	-1.37	0.10	0.35
Paenibacillus sp.	0.07	0.99	1.06	-0.74	-0.66	-0.20
Flavobacterium sp. 1	0.06	-0.04	0.02	0.08	1.17	2.45
Pedobacter sp. 2	0.01	0.13	0.14	0.24	1.76	1.56
Cupriavidus sp.	<0.01	2.91	2.91	-2.91	-2.91	-2.91
Ralstonia sp.	<0.01	2.18	2.18	-2.18	-2.18	-2.18
Flavobacterium sp. 2	<0.01	0.02	0.02	0.14	1.88	0.93
f-*Micrococcaceae* sp. 2	<0.01	0.18	0.18	0.19	-0.01	1.58
Other species<1%	4.11	-0.20	3.91	-2.85	-2.28	-1.19

[a] Included are bacterial species with RA>1% in any sample of the processed samples. The top 5 species that showed the greatest increase or greatest decrease in RA after treatment (Chlorine wash or storage at indicated temperature) are marked by red and blue fonts.

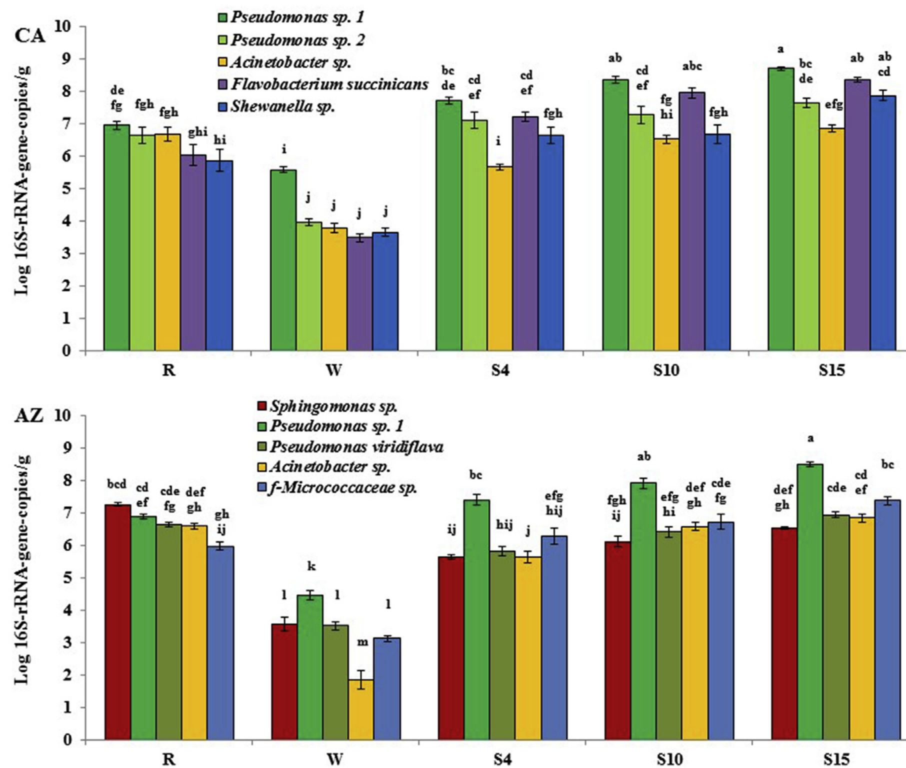

Fig. 3. Population changes of the top five bacteria species of unwashed spinach samples from California (CA) and Arizona (AZ) in each type of samples (8 samples/type). R: Unwashed spinach; W. Washed Spinach; S: Storage at indicated temperature for one week. Eight replicates were tested for each type of sample. Bars represent standard errors, and different letters above the bars indicate statistical difference at $p < 0.05$.

3.3. Spinach microbial community composition as determined by 16S rDNA amplicon sequencing

The v4 region of 16S rDNA was targeted for sequencing analyses, and the sequencing data have been submitted to NCBI (Accession No.: PRJNA417268). After 16S rDNA amplicon sequencing of the 80 spinach samples, a total of 15,892,604 raw paired reads (each pair is composed of reads generated by the forward and reverse primers) were generated, with a range of 45,869 to 410,478 reads per sample. Paired-end merge and quality control analyses using Qiime2 resulted in 11,636,527 reads in total, with a range of 36,382 to 281,388 reads per sample. Queries to the Greengene database using these sequences identified a total of 673 OTUs in the 80 spinach samples, including 2 archaea, 644 bacteria, 11 eukaryotes (chloroplast and mitochondria), and 16 unassigned OTUs. All eukaryote reads, which counted for 0.033% of the total 11,636,527 reads, and unassigned reads (0.039%) were purged prior to data analysis of bacterial communities on spinach (Dataset S1). The bacteria OTUs were classified into 223 species within 197 genera and 14 phyla (Dataset S2). An additional 6890 bacterial reads (0.059% of the total reads) could only be assigned to the domain Bacteria.

Four of these 14 phyla had relative abundance of >1%. Proteobacteria was the phylum with the highest abundance in all types of spinach samples, accounting for 73.7% of the total reads, which was in agreement with previous reports by Lopez-Velasco et al. (2011). and Leff et al. (Leff and Fierer, 2013). The phyla with the next highest RA on spinach was Bacteroidetes (20.0%), followed by Firmicutes (3.3%) and Actinobacteria (2.9%) (Fig. 2). At genus and species level, all the most abundant species (top five) belonged to the phylum Proteobacteria, except for one species of family Microbacteriaceae (phylum Actinobacteria). There were differences in the composition of major bacteria species (>1% RA) identified on unwashed spinach produced in CA and AZ (Table 1, Fig. 3). The ubiquitous *Pseudomonas* spp., highly fitted for competing with other bacteria in food and food production environments (Hibbing et al., 2010; Langsrud et al., 2016), were highly abundant in all spinach samples. Yet, two undetermined species (RA: 27.92% and 14.16%) of genus *Pseudomonas* were the top species on unwashed CA spinach, while the top two species on unwashed AZ spinach were *Sphingomonas* sp. (30.23%) and *Pseudomonas* sp.1. Other species among the top five in RA for both CA or AZ unwashed samples include *Acinetobacter* sp., *Flavorbacterum succinicans*, *Shewanella* sp., and *Micrococcaceae* sp.

Based on alpha and beta diversity analyses, there was no significant difference between Early and Late trial samples for all tested alpha diversity indexes and matrix distances (data not shown). This was consistent with plate count results which indicated great similarities in bacterial populations between the Early and Late trials samples. Therefore, the 16S rDNA sequencing analysis data of the Early and Late trials were combined for presentation.

3.4. Bacterial community shift impacted by chlorine wash

Proteobacteria was the dominant phylum identified on unwashed spinach from both CA (RA: $77.14 \pm 3.43\%$) and AZ ($75.05 \pm 1.86\%$). After the washing and packaging process, Proteobacteria RA increased to $90.52 \pm 3.43\%$ and $82.30 \pm 3.20\%$ on spinach grown in CA and AZ, respectively (Fig. 2A and C). Considering the population of microbial communities had been reduced by chlorine washing, these increases in RA corresponded to reductions of 1.60 log and 2.77 log in Proteobacteria specific 16S rDNA on CA and AZ spinach, respectively (Fig. 2B and D). The RA of the other major bacteria phyla decreased by varying extents which

Table 2
Changes in relative abundance (%) and 16S rDNA copies (log10) in bacterial species LEAST and MOST affected by chlorine wash.

Species	Change in RA			Change in 16 S rDNA copies (log10)		
	RA (%)		Ratio (R/W)	16S rDNA		Differential (W-R)
	R	W		R	W	
CA Spinach						
Cupriavidus sp.[a]	<0.01	0.20	<28	1.62 ± 0.62	3.22 ± 0.07	1.60
Ralstonia sp.[a]	<0.01	0.15	<15	1.72 ± 0.65	3.09 ± 0.06	1.37
Stenotrophomonas sp.[a]	0.14	1.23	9	4.59 ± 0.11	3.92 ± 0.20	−0.67
Erwinia sp.[a]	2.94	24.88	8	5.91 ± 0.08	5.36 ± 0.08	−0.55
f-*Microbacteriaceae* sp. 1[a]	0.19	0.84	4	4.70 ± 0.06	3.68 ± 0.17	−1.02
Paenibacillus sp.[a]	1.38	5.26	4	5.53 ± 0.09	4.65 ± 0.14	−0.88
Pseudomonas viridiflava	1.91	6.23	3	5.73 ± 0.11	4.67 ± 0.17	−1.06
f-*Enterobacteriaceae* sp.	1.16	3.66	3	5.49 ± 0.11	4.52 ± 0.09	−0.97
Janthinobacterium sp.	1.25	3.91	3	5.55 ± 0.12	4.53 ± 0.12	−1.02
Agrobacterium sp.	0.65	1.38	2	5.18 ± 0.14	4.11 ± 0.09	−1.07
All species	**100**	**100**	**1**	**7.57 ± 0.17**	**5.97 ± 0.09**	**−1.60**
Acinetobacter lwoffii	0.31	0.01	1/31	5.00 ± 0.17	0.33 ± 0.33	−4.66
Planomicrobium sp.[a]	1.59	0.06	1/28	5.72 ± 0.21	2.69 ± 0.14	−3.03
f-*Micrococcaceae* sp.	0.22	0.01	1/24	4.51 ± 0.42	0.90 ± 0.44	−3.61
Arthrobacter sp.	0.65	0.03	1/20	4.99 ± 0.40	1.58 ± 0.48	−3.41
Psychrobacter sp.	3.49	0.19	1/18	5.82 ± 0.36	3.13 ± 0.16	−2.70
Acinetobacter sp.[a]	13.40	0.83	1/16	6.65 ± 0.21	3.78 ± 0.14	−2.87
Carnobacterium viridans	2.18	0.13	1/16	5.78 ± 0.28	3.05 ± 0.12	−2.73
f-*Bacillaceae* sp.	0.16	0.01	1/16	4.59 ± 0.32	1.29 ± 0.39	−3.30
Pseudomonas sp. 2	14.16	1.03	1/14	6.63 ± 0.24	3.95 ± 0.10	−2.69
Flavobacterium sp. 2	0.42	0.03	1/13	4.81 ± 0.41	1.84 ± 0.42	−2.97
Flavobacterium sp. 1	2.33	0.18	1/13	5.57 ± 0.41	2.94 ± 0.23	−2.63
Hymenobacter sp.	0.24	0.02	1/12	4.55 ± 0.18	1.88 ± 0.32	−2.67
Wautersiella sp.	0.15	0.01	1/12	4.42 ± 0.40	1.35 ± 0.42	−3.07
Sphingobacterium faecium[a]	1.40	0.12	1/11	5.59 ± 0.29	2.98 ± 0.12	−2.61
f-*Comamonadaceae* sp.	0.19	0.04	1/5	4.76 ± 0.28	1.90 ± 0.43	−2.86
AZ Spinach						
Cupriavidus sp.[a]	<0.01	2.91	<291	0.42 ± 0.42	3.41 ± 0.08	2.99
Ralstonia sp.[a]	<0.01	2.18	<218	0.41 ± 0.41	3.27 ± 0.09	2.86
f-*Comamonadaceae* sp.	<0.01	0.59	<59	0.45 ± 0.45	2.69 ± 0.59	2.24
Pseudomonas sp. 2	0.35	15.37	44	4.52 ± 0.67	4.06 ± 0.22	−0.46
Paenibacillus sp.[a]	0.07	1.06	15	4.54 ± 0.13	2.95 ± 0.11	−1.59
Stenotrophomonas sp.[a]	0.12	1.50	13	4.86 ± 0.08	2.99 ± 0.19	−1.87
f-*Microbacteriaceae* sp. 1[a]	0.12	1.44	12	4.83 ± 0.10	2.79 ± 0.17	−2.04
Pedobacter sp. 2	0.01	0.14	14	2.95 ± 0.65	1.54 ± 0.29	−1.41
f-*Paenibacillaceae* sp.	0.01	0.10	10	2.03 ± 0.77	1.39 ± 0.33	−0.64
Erwinia sp.[a]	0.91	4.27	5	5.72 ± 0.06	3.63 ± 0.10	−2.09
All species	**100**	**100**	**1**	**7.79 ± 0.06**	**5.02 ± 0.10**	**−2.77**
f-*Moraxellaceae* sp.	1.37	0	0	5.80 ± 0.17	0	−5.80
Paracoccus sp.	0.64	0	0	5.59 ± 0.07	0	−5.59
Acinetobacter sp. [a]	6.40	0.12	1/55	6.58 ± 0.08	1.85 ± 0.28	−4.74
Mycoplana sp.	0.38	0.01	1/53	5.37 ± 0.08	0.56 ± 0.29	−4.80
Sphingobacterium faecium[a]	5.06	0.15	1/33	6.49 ± 0.05	1.99 ± 0.20	−4.50
Methylobacterium sp.	0.12	0.00	1/28	4.84 ± 0.08	0.39 ± 0.27	−4.45
Agrobacterium sp.	4.33	0.38	1/11	6.41 ± 0.06	2.29 ± 0.36	−4.12
Planomicrobium sp.[a]	0.59	0.10	1/6	5.49 ± 0.12	1.53 ± 0.34	−3.96

Included species with 16S rDNA amplicons reduced by < 0.5 log less than the overall reduction (Least impacted, including those with amplicon increase, above "All Species" labeled in bold) and by < 1 log more than the overall reduction (Most impacted, below "All Species") after chlorine washing. Only species with RA > 0.1% in either before or wash sample are included.
R: Unwashed (raw); W: Washed.
Letter f-in front denotes undetermined species that has been assigned to the family level has been assigned to the family level.
[a] Indicate species present on both CA and AZ spinach.

corresponded to significant reductions in 16S rDNA after the washing process.

The community shifts at genus-species level were more remarkable (Table 1, Fig. 2). Among the top five species on unwashed CA spinach, the RA of *Pseudomonas* sp. 1 increased significantly after the washing process, while RA of the other four decreased.

Table 2 lists bacterial species most versus least affected by chlorine washing, based on the change in 16S rDNA copies. The 16S rDNA copy number reduction for 10 species on CA spinach and 10 on AZ spinach was at least 0.5 log lower than the respective overall reduction (1.60 and 2.77 logs) counting all the taxa. Interestingly, 16S copies for *Cupriavidus* sp. and *Ralstonia* sp. on both CA and AZ spinach, and f-*Comamonadaceae* sp. on AZ spinach, significantly increased after chlorine washing and packaging, from nearly undetectable to up to 2.9% RA; this possibly indicates exogenous contamination during or post washing. In addition, *Stenotrophomonas* sp., *Erwinia* sp., f-*Microbacteriaceae* sp. 1, and *Paenibacillus* sp. on both CA and AZ spinach, and *Pseudomonas* sp. 2 on AZ spinach had very little reduction of 16S copies, suggesting potentially greater resistance to chlorine washing. In contrast, 15 species on CA and 8 species on AZ spinach showed 16S rDNA reduction at least 1 log higher than the overall reduction (Table 2). Several species had reduction more than 2

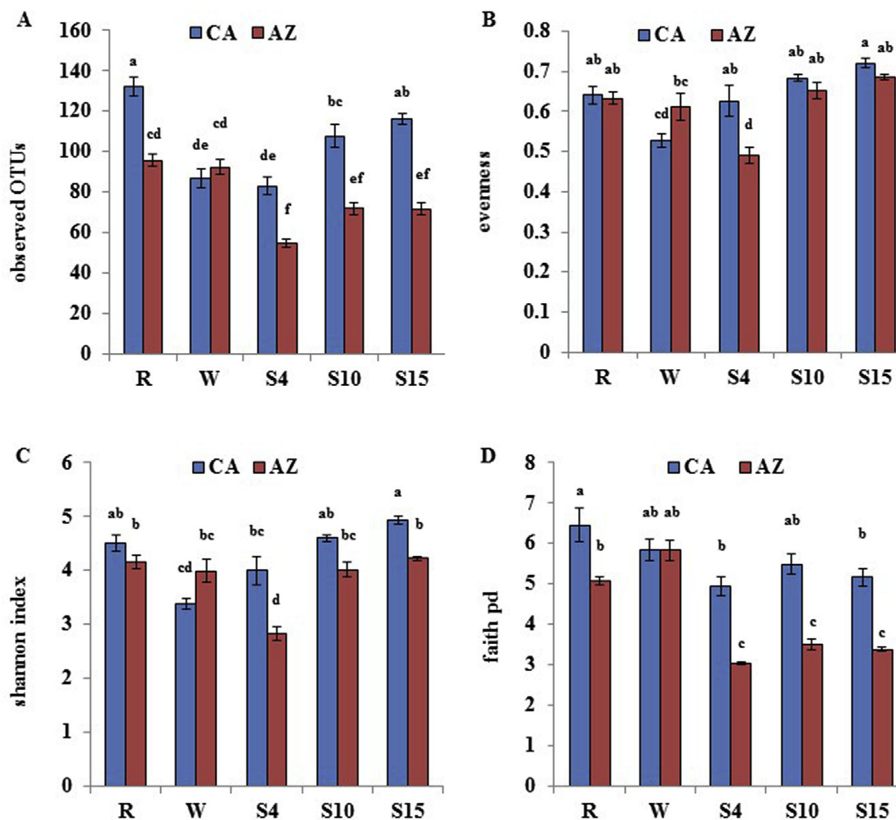

Fig. 4. Alpha diversity indexes of bacterial community on spinach samples of California (CA) and Arizona (AZ). (A). Observed OTUs; (B). Evenness; (C). Shannon index; and (D). Faith-pd.

R: Unwashed spinach; W. Washed Spinach; S: Storage at indicated temperature for one week. Eight replicates were tested for each type of sample. Bars represent standard errors, and different letters above the bars indicate statistical difference at $p < 0.05$.

logs than overall, including *Acinetobacter lwoffii*, and f- *Micrococcaceae* sp. on CA spinach, and f- *Moraxellaceae* sp., *Paracoccus* sp., and *Mycoplana* sp. on AZ spinach. Species in the relevant genera or families seemed to have diverse natural habitats, including soil, water, and plants (Lakshmi et al., 2013; Lee and Lee, 2013; Loy and Brodersen, 2014; Rodriguez-Alonso et al., 2009; Sun et al., 2016).

The observed OTUs, evenness, and Shannon index of bacterial communities on CA spinach were all reduced after washing process, while the differences of these alpha diversity indexes between unwashed and washed AZ spinach were not significant (Fig. 4). Before washing, the observed OTUs (Fig. 4A) and faith-pd (Fig. 4D) of bacterial communities on unwashed CA spinach was higher than that from AC spinach. PCoA analysis, based on the composition of the bacterial OTUs, showed that the eight replicates of unwashed or washed samples from both batches (CA and AZ) clustered closely (Fig. S1A), indicating the shift of bacterial communities on spinach after washing process and the reproducibility of the sequencing profiles.

3.5. Shifts in bacterial communities on spinach impacted by storage temperatures

Although the alpha diversity indexes, including observed OTUs, Shannon index, evenness, and faith-pd, of washed samples (W in Fig. 4) were not significantly different between the two batches (CA and AZ), after one week of storage, the values of these four indexes were lower for AZ spinach samples than for CA spinach across all three temperature conditions (S4, S10, and S15 in Fig. 4). Based on the composition of bacterial communities, the bacterial communities on stored spinach samples (Day 7) were well separated from those on the initial washed sample (Day 0) for both batches (Fig. S1B). Beta diversity metric distances between samples stored at 10 and 15 °C (S10 and S15) were closer than that to samples stored at 4 °C (S4).

RA of phylum Proteobacteria decreased and that of Bacteroidetes increased significantly on both CA and AZ spinach samples after one week storage at all temperatures, except for AZ spinach that had been stored at 4 °C (Fig. 2A and C). The changes in RA were significantly affected by storage temperature (15 > 10 > 4 °C). The absolute 16S rDNA copy numbers of all the four major phyla increased during storage (Fig. 2B and D). Proteobacteria remained the most abundant phylum in all samples despite decreases in its RA.

At genus-species level, the community shift on washed spinach was significant after storage at all temperatures examined. *Pseudomonas* spp. remained the predominant species after storage at all temperatures (Fig. 3). On CA spinach, all five species that increased the most in RA after washing also showed the greatest loss in RA during storage; nearly all those that showed the greatest gains in RA after storage were those that had experienced significant losses of RA after washing (Table 1). Similar, although less evident, trends were also observed with AZ spinach, except for *Sphingomonas* sp., which continued to decline to RA <1% after storage at all temperatures (Table 1).

Table 3 lists bacterial species that exhibited the highest (at least 1 log higher than overall) and lowest (at least 1 log less than overall) proliferation after storage, based on changes in number of

Table 3
Changes in 16S rDNA amplicons (log10 16S rDNA/g) of bacterial species with highest and lowest proliferation after storage at 4, 10, and 15 °C.

Species	Initial	Increase (S-W)		
	(W)	S4	S10	S15
CA (log 16S rRNA gene)				
Flavobacterium succinicans [a]	3.48 ± 0.13	3.71	4.46	4.86
Sphingobacterium faecium [a]	2.98 ± 0.12	3.49	4.63	5.23
Flavobacterium sp. 1[a]	2.94 ± 0.23	3.43	4.36	4.86
Pedobacter sp. 2[a]	2.31 ± 0.12	3.33	4.49	5.13
Flavobacterium sp. 2	1.84 ± 0.42	4.00	5.27	5.57
Wautersiella sp.	1.35 ± 0.42	3.65	4.49	5.43
All Species	**5.97 ± 0.09**	**2.24**	**2.88**	**3.33**
Erwinia sp.	5.36 ± 0.08	0.97	1.38	1.66
Cupriavidus sp. [a]	3.22 ± 0.07	−1.84	−2.17	−2.66
Ralstonia sp. [a]	3.09 ± 0.06	−2.15	−2.56	−3.09
AZ (log 16S rRNA gene)				
Shewanella sp.	2.48 ± 0.23	4.27	4.89	5.64
Flavobacterium succinicans[a]	0.97 ± 0.29	4.70	5.68	6.37
Sphingobacterium faecium[a]	1.99 ± 0.20	4.00	5.11	5.91
Flavobacterium sp. 1[a]	0.92 ± 0.29	4.00	5.55	6.17
Pedobacter sp. 2[a]	1.54 ± 0.29	4.04	5.09	5.64
f-*Bacillaceae* sp.	0.77 ± 0.23	4.67	4.65	5.12
All Species	**5.02 ± 0.10**	**3.01**	**3.41**	**3.97**
f-*Oxalobacteraceae* sp.	3.55 ± 0.15	0.82	−0.35	0.78
Cupriavidus sp.[a]	3.41 ± 0.08	−2.02	−1.49	−2.85
Ralstonia sp. [a]	3.27 ± 0.09	−2.85	−1.78	−2.69
f-*Comamonadaceae* sp.	2.69 ± 0.09	−2.69	−2.69	−2.69
Carnobacterium viridans	2.45 ± 0.22	1.73	0.40	−1.83
Acinetobacter lwoffii	2.06 ± 0.33	−2.06	−2.06	−1.30
f-*Bradyrhizobiaceae* sp.	1.94 ± 0.08	−1.94	−1.94	−1.94
Luteibacter rhizovicinus	1.75 ± 0.29	0.59	1.43	0.00
Planomicrobium sp.	1.53 ± 0.34	−1.53	−0.96	−1.53
Pseudomonas fragi	1.50 ± 0.46	−0.30	0.43	−0.81

Included bacteria species with 16S rDNA increase at least 1 log higher (above "All Species" labeled in bold) or lower (below "All Species") than the average bacterial increase at any of the three storage temperatures.
W: Washed spinach without storage, S: storage at indicated temperature for 1 week. Letter f-in front denotes undetermined species that has been assigned to the family level.
[a] Indicate species present on both CA and AZ spinach.

16S rDNA copies. *Flavobacterium succinicans, Sphingobacterium faecium, Flavobacterium* sp. 1, and *Pedobacter* sp. 2 were among those with the highest log increases on both CA and AZ spinach. In contrast, *Cupriavidus* sp., *Ralstonia* sp., and f-*Comamonadaceae* sp., which had significant increases in 16S rDNA copies after washing and packaging, all showed negative proliferation after storage, dropping to undetectable to <2 log 16S rDNA copies/g.

The 16S rDNA levels for most of bacterial species increased more significantly at 15 °C and 10 °C than at 4 °C, on both CA and AZ spinach. However, a few species exhibited greater amplicon increases after storage at 4 °C rather than either at 10 or 15 °C (Table 4). One of these, *Carnobacterium viridians*, a lactic acid producing bacterium (LAB), was identified from both CA and AZ samples and appears to prefer growing at low temperatures. *Cupriavidus* sp. and *Ralstonia* sp. showed no proliferation and declined more slowly at 4 °C than either at 10 or 15 °C (except at 10 °C on AZ spinach).

4. Discussion

In this study, plate counting, qPCR, and 16S rDNA amplicon sequencing were used to examine the effects of chlorine washing and storage at compliant (4 °C) or abused temperatures (10 and 15 °C) on the population and diversity dynamics of bacterial communities on spinach leaves. Among the over 600 OTUs identified on spinach samples using 16S rDNA sequencing, approximately 15%, 67%, and 99% were assigned to species, genus, and family levels, respectively, which indicates a high efficiency of bacterial identification. Fewer than 0.04% of the total paired-end reads remained unassigned, and 0.06% could only be classified to the Bacteria domain (Dataset S1), which may denote novel sequences that have not been documented in the Greengenes database. However, it cannot be ruled out that they resulted from sequencing errors.

Microbial community analyses that are based only on sequence RA data may result in misleading interpretations (Ban et al., 2015; Lovell et al., 2015). Therefore, instead of relying solely on the proportion data, qPCR estimation of 16S rDNA copies was incorporated to compare the absolute abundances of specific microbial taxa at each identification level (phyla to species) across different samples. The estimated bacterial 16S rDNA level by qPCR followed the same trend as plate count data (Fig. 1). The calculated copy numbers were about 1–1.5 log higher than the plate counts, which is consistent with the multiple copies of 16S rDNA and the presence of bacterial cells unable to grow on the agar plates, such as certain psychrotrophs and so-called viable but non culturable (VBNC) bacterial cells (Ramamurthy et al., 2014).

Across all spinach samples, Proteobacteria was the most prevalent phylum, and at the genus level *Pseudomonas* and/or *Sphingomonas* were the most dominant. The dominant presence of *Pseudomonas* is consistent with its ubiquitous presence on produce and in the food environments; this genus is highly capable of competing with other bacteria in food and food production environments (Hibbing et al., 2010; Langsrud et al., 2016). *Acinetobacter* sp., one of the top five species obtained from unwashed spinach from both CA and AZ, is known for causing spoilage of cold stored foods (Battey and Schaffner, 2001).

Washing leafy greens with chlorine water in a conventional double flume system typically reduces microbial load by 1–2 logs (Luo et al., 2012; Nou and Luo, 2010). This study, while registering comparable reduction in mesophilic aerobic populations, showed that this reduction was not uniform across bacterial communities. Bacteria such as *Erwinia* sp., *Stenotrophomonas* sp., f-*Microbacteriaceae* sp., and *Paenibacillus* sp. seemed to exhibit higher resistance to chlorine washing. It is possible that some species among these are phytopathogens (Barth et al., 2009; Young et al., 1996) and might be adapted to adhere more tightly to the spinach surface. Some species in these genera have already been shown to have resistance to heavy metals and disinfectants (Khan et al., 2016; Ryan et al., 2009).

Interestingly, *Cupriavidus* sp. and *Ralstonia* sp. (and f-*Comamonadaceae* sp. in case of AZ spinach) showed significant increases after the chlorine wash process, which could indicate exogenous contamination during or after washing. *Ralstonia* spp. are soil-, water-, and plant-associated, and certain species can cause plant diseases (Allen et al., 2005a, b). *Ralstonia insidiosa* has been readily isolated in produce processing plants (Liu et al., 2013). *Cupriavidus* is Gram-negative, motile, rod-shaped organisms with oxidative metabolism. Heavy metal resistance has been described for both *Cupriavidus* and *Ralstonia* (Goris et al., 2001; Monsieurs et al., 2011). *Cupriavidus* and *Ralstonia* both have great potentials of biofilm formation (Fairbrother et al., 2013; Lerch et al., 2017; Liu et al., 2016). The increase of these two bacteria on washed samples may indicate the presence of such biofilms in water supply or food contact surfaces and/or might be associated with the tolerance to disinfectants.

After storage, bacterial populations on washed spinach significantly increased, whether at compliant (4 °C) or abused (10 and 15 °C) temperatures; the increase rate was significantly affected by storage temperature ($P < .05$). Although Proteobacteria had higher total population, several fast-grown Bacteroidetes species contributed most significantly to the bacterial community shift during the storage. Higher storage temperatures resulted in higher

Table 4
Bacterial species showing greater proliferation on spinach stored at 4 °C than at either 10 or 15 °C.

Species	16S rDNA level (log copies/g)			
	W	S4	S10	S15
CA Spinach				
Overall	**5.97 ± 0.09**	**8.21 ± 0.14**	**8.85 ± 0.13**	**9.30 ± 0.06**
f-*Microbacteriaceae* sp.	3.30 ± 0.11	5.00 ± 0.12	4.81 ± 0.69	4.86 ± 0.70
Cupriavidus sp.*	3.22 ± 0.07	1.39 ± 0.68	1.05 ± 0.69	0.57 ± 0.57
Psychrobacter sp.	3.13 ± 0.16	4.78 ± 0.21	2.57 ± 0.98	3.34 ± 0.98
Ralstonia sp.	3.09 ± 0.06	0.95 ± 0.62	0.53 ± 0.53	0
*Carnobacterium viridians**	3.05 ± 0.12	4.30 ± 0.65	0	1.53 ± 1.00
AZ Spinach				
Overall	**5.02 ± 0.10**	**8.02 ± 0.19**	**8.43 ± 0.18**	**8.99 ± 0.08**
Pseudomonas sp. 2	4.06 ± 0.22	7.71 ± 0.20	7.55 ± 0.14	7.82 ± 0.11
f-*Oxalobacteraceae* sp.	3.55 ± 0.15	4.36 ± 0.66	3.19 ± 0.94	4.32 ± 0.95
Cupriavidus sp.*	3.41 ± 0.08	1.39 ± 0.68	1.92 ± 0.73	0.56 ± 0.56
*Carnobacterium viridians**	2.45 ± 0.22	4.18 ± 0.62	2.85 ± 0.84	0.61 ± 0.61
Arthrobacter sp.	1.16 ± 0.44	3.82 ± 0.86	2.63 ± 1.00	6.05 ± 0.15
f-*Bacillaceae* sp.	0.77 ± 0.23	5.44 ± 0.29	5.42 ± 0.24	5.89 ± 0.16

Only species with RA > 0.1% in the washed or stored at any temperature are included. Red font indicates the storage temperature of maximal proliferation.
*indicate species present on both CA and AZ spinach.
W: Washed (no storage); S: Stored at indicated temperature.
Letter f-in front denotes undetermined species that has been assigned to the family level.

RA increase of Bacteroidetes (Fig. 3A and C). This observation differs from a previous study (Lopez-Velasco et al., 2011), which showed a major RA increase of phylum Proteobacteria after 15-day storage at 4 and 10 °C. Three of the 4 fast growing Bacteroidetes bacteria on both CA and AZ spinach are species of *Flavobacterium*, which can be found in a variety of environments (Dong et al., 2013; Good et al., 2015). In addition, the *Wautersiella* sp. and *Chryseobacterium* sp., which proliferated rapidly on stored CA and AZ spinach samples, respectively, both also belong to phylum Bacteroidetes (Table 3). *Sphingomonas faecium* and *Shewanella* sp. (Phylum Proteobacteria) were also among the species which grew rapidly during storage periods (Tables 1 and 3).

This study examined the microbiome shift on spinach affected by post-harvest processing and storage. Many pre-harvest environmental factors, such as growth locality, seasonality, and cultivation practices, which can strongly influence the initial microbiome on fresh produce (Leff and Fierer, 2013), were not the main consideration in this study. Using spinach from diverse growing regions (CA vs AZ), it was observed that many of the species with the greatest gains in RA after chlorine washing, especially those suspected to be exogenous contaminants, showed big drops in RA during storage; yet several of those species losing RA after the chlorine wash process gained in RA during the same storage period. Thus, the microbial community on spinach apparently shifted back, becoming more similar to the community prior to chlorine washing. The exception to this phenomenon was *Sphingomonas* sp., which had RA>30% before chlorine wash, but dropped to RA<1% after chlorine wash and storage (Table 1). Setting that exception aside, these observations suggests that the post chlorine shock storage generally favors the growth of spinach indigenous microbiota and disadvantage exogenous contaminants. Most foodborne pathogens do not belong to the native microbiota on fresh produce, although several studies have showed relative long-term survival of enteric pathogens, including *E. coli* O157 and *Salmonella*, on fresh produce (Allen et al., 2005a, b; Chua et al., 2008; Markland et al., 2013). However, in most of these studies large populations of pathogens were used for inoculation, which might overwhelm the native microbial community. It is reasonable to assume that in most naturally occurring contamination events, foodborne pathogens enter the community as minor members; it would be interesting to further examine the community responses to such contaminations.

5. Conclusion

In this study, the shifts in microbial communities on spinach after being washed with chlorine at commercial operations and after storage at compliant and abusive temperatures were examined. The most affected bacterial taxa and bacteria contributing most significantly to the community shifts were identified. The results provided contextual information about the microbial ecology of the diverse bacterial communities on spinach during fresh-cut produce processing and the subsequent storage, which can help guide further studies on the interactions of microbes on produce, the prevention of contamination with foodborne pathogens, and spoilage. Understanding the dynamics and population heterogeneity of microbial community in a complex food system can assist the development of safer, more robust, and efficient food production processes.

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Appendix A. Supplementary data

Figure S1. PCoA score plot based on weighted UniFrac metrics show overall structure changes of bacteria communities on spinach samples after washing (**A**) and storage processes (**B**).

Table S1. Water parameters in flume 1 (prime) and **flume 2 (secondary) during the washing process in sampled organic baby spinach line of the commercial factory.** Samples used in batches a and b were grown and harvested in California (CA) and Arizona (AZ), respectively.

Dataset S1. Sequencing read numbers of identified archaea and bacteria OTUs.

Dataset S2. Bacterial population (log 16S gene copy numbers) of

each sample at genera level.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.01.002.

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