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Microbiome convergence following sanitizer treatment and identification of sanitizer resistant species from spinach and lettuce rinse water

Ganyu Gu[a,b], Andrea Ottesen[c], Samantha Bolten[a], Yaguang Luo[a], Steven Rideout[b], Xiangwu Nou[a,*]

[a] Environmental Microbiology and Food Safety Laboratory, USDA ARS, Beltsville, MD, United States of America
[b] Eastern Shore Agricultural Research and Extension Center, Virginia Tech, Painter, VA 23420, United States of America
[c] Center for Food Safety and Applied Nutrition, US FDA, College Park, MD 20740, United States of America

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ABSTRACT

Fresh produce, as a known or suspected source of multiple foodborne outbreaks, harbors large populations of diverse microorganisms, which are partially released into wash water during processing. However, the dynamics of bacterial communities in wash water during produce processing is poorly understood. In this study, we investigated the effect of chlorine (FC) and peracetic acid (PAA) on the microbiome dynamics in spinach and romaine lettuce rinse water. Treatments with increasing concentrations of sanitizers resulted in convergence of distinct microbiomes. The resultant sanitizer resistant microbiome showed dominant presence by *Bacillus* sp., *Arthrobacter psychrolactophilus*, *Cupriavidus* sp., and *Ralstonia* sp. Most of the FC and PAA resistant bacteria isolated from spinach and lettuce rinse water after sanitation were gram positive spore forming species including *Bacillus*, *Paenibacillus*, and *Brevibacillus* spp., while several PAA resistant *Pseudomonas* spp. were also isolated from lettuce rinse water. Inoculation of foodborne pathogens altered the microbiome shift in spinach rinse water under PAA treatment, but not in lettuce rinse water or FC treated samples. These inoculated foodborne pathogens were not isolated among the sanitizer resistant strains.

1. Introduction

Diverse microbial communities, including members of potentially pathogenic and spoilage bacteria, compose the native microbiota on fresh produce (Gu et al., 2018; Leff and Fierer, 2013; Nicholl et al., 2004; Ottesen et al., 2013). Fresh produce microbiomes are determined by the physiochemical characteristics of the commodities, as well as the environmental characteristics and agricultural managements during growth and processing (Gu et al., 2018; Leff and Fierer, 2013; Ottesen et al., 2019). Fresh-cut leafy greens are required to be appropriately washed during processing for the ready-to-eat market (FDA, 2008). Sanitizers including chlorine and peracetic acid (PAA) are commonly applied during fresh produce washing and processing to reduce the potential presence of foodborne pathogens and spoilage bacteria, and to prevent post-harvest cross contamination (Luo et al., 2018; Van de Velde et al., 2014). Both chlorine and PAA are oxidants, which can cause oxidation of thiol groups of bacterial proteins, resulting in metabolic inhibition (Denyer and Stewart, 1998; Maris, 1995).

In a typical washing process, a major proportion of native microbes on leafy green surface are dislodged and released into flume water. While effective sanitation inactivates most of the bacteria in water, this pool of complex microbial reservoir could raise a potential risk of cross-contamination in less than optimally managed processes (Luo et al., 2012; Luo et al., 2011). The dynamics of microbiome shifts in wash water treated at different levels of sanitizers have not been well studied. A better understanding of this process could benefit the improvement of mitigation strategies for foodborne pathogens and spoilage bacteria during produce processing. In addition, while inoculations with foodborne pathogens are essential for evaluating the effectiveness of variously washing processes, the impact of introducing large inoculums on the dynamics of exist microbial communities during washing is poorly understood.

In addition to traditional culture dependent methods, the development and improvement of high-throughput (HT) sequencing technology and bioinformatics analysis provide advanced tools to identify complex microbial communities faster, cheaper, and in greater depth (Goodwin et al., 2016; Soon et al., 2013). In this study, plate counting, PMA-qPCR, and 16S rRNA gene HT sequencing were performed to investigate the microbiome shift in spinach and lettuce rinse water after treatment at different concentrations of free chlorine (FC) and PAA. The impact of

* Corresponding author.
E-mail address: xiangwu.nou@ars.usda.gov (X. Nou).

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inoculation of foodborne pathogens, including Shiga-toxin producing *Escherichia coli*, *Salmonella enterica*, and *Listeria monocytogenes*, in rinse water on the change of microbial composition after sanitation was evaluated. Isolates resistant to FC and PAA in produce rinse water were also identified by 16S rRNA gene sequencing.

2. Materials and methods

2.1. Preparation of produce rinse water

Fresh baby spinach was obtained from a commercial fresh produce processor prior to washing. Untrimmed loose-head romaine lettuce was purchased from a whole-sale company in Maryland. After trimming, the remaining darker green leaves, which were likely to carry higher microbial load than the inside less exposed leaves, were selected to prepare rinse water. Spinach and lettuce leaves (640 g) were washed in 1400 mL sterile water in a large Whirl-Pak bags with 0.3 mm mesh filter (Nasco, Fort Atkinson, WI, USA) by hand massaging for 20 times, followed by sonicating 2 min at 40 kHz in a sonicator waterbath (Branson, Richmond, VA), and then hand massaging for additional 20 times. The filtered rinsates for spinach (SRW) and Romaine lettuce (LRW) were collected for sanitizer treatment.

2.2. Foodborne pathogen inoculum

Nine strains of foodborne pathogens including Shiga-toxin producing *E. coli*, *S. enterica*, and *L. monocytogenes* were used for inoculation in this study. The *E. coli* strains consisted one O104:H4 (isolated from sprouts) and two O157:H7 (isolated from spinach or beef) and were all ampicillin resistant. The *S. enteria* strains include one Newport isolated from mango, one Poona from cantaloupe, and the type strain Typhimurium ATCC 14028. The *L. monocytogenes* strains included two 1/2a and one 1/2b serotypes which were isolated from 2011 cantaloupe outbreaks. Stored bacterial strains were incubated in Tryptic Soy Broth (TSB; BD, Franklin Lakes, NJ) at 37 °C overnight. The nine bacterial strains were re-suspended in phosphate buffered saline (PBS, pH 7.2) to approximately 8 log CFU/mL, and mixed as a single cocktail inoculum.

2.3. Sanitizer treatment

Chlorine stock solutions (5,000, 500 and 50 mg/L of FC) were prepared using sodium hypochlorite (Clorox, Oakland, CA) with hydrochloric acid as acidulant adjusting to pH 6.5. PAA stock solutions (5,000, 500 and 50 mg/L) were prepared by diluting Tsunami (Ecolab, St. Paul, MN) without pH adjustment. The filtered rinsates (SRW and LRW), with or without inoculation of foodborne pathogens to approximately 6 log CFU/mL using the 9-strain cocktail as prepared above, were dispensed into tubes in 10 mL aliquots. Pre-determined volumes of appropriate sanitizer stock solution were added into the aliquots of rinsates to achieve 0.5 to 100 mg/L initial sanitizer concentrations (FC: 0.5, 5, 10, 30, 50 mg/L; PAA: 0.5, 5, 10, 30, 50, 100 mg/L). Sterile water (0 mg/L) was used as control. The treatment was terminated after 30 s exposure by adding in 100 μL 10% sodium thiosulfate for sanitizer neutralization. Four replicates were performed in the experiment.

2.4. PMA treatment and DNA extraction

A portion (1 mL) of each sample (except samples treated with 50 mg/L FC or 100 mg/L PAA) was centrifuged at 14,000*g* for 10 min. The collected bacterial pellet was resuspended in PBS and treated with DNA-modifying dye propidium monoazide (PMAxx, Biotum, Fremont, CA) for selective detection of viable bacteria as described previously (Gu et al., 2018). PMAxx treated cells were re-suspended in 150 μL Tris-EDTA buffer (Thermo Fisher Scientific, Waltham, MA) containing 10 mg/mL lysozyme (Epicentre, Madison, WI) and 5 mg/mL proteinase K (Epicentre), 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 provider's instructions and stored at −80 °C.

2.5. Bacterial enumeration

Total mesophilic aerobic bacteria (MAB) counts for each sample were determined by plate counting on Tryptic Soy Agar (TSA, BD). TSA plates were incubated at 30 °C for 2 days and colonies were enumerated using a Flash & Go automatic colony counter (IUL, Barcelona, Spain). The detection limit of plate counting was 1 log CFU/mL.

Quantitative real-time PCR (qPCR) was performed to estimate the total 16S rRNA gene copy numbers of bacteria in each sample as describe in the previous study (Gu et al., 2018). qPCR targeting a highly conserved 180 bp portion of 16S rRNA gene (Clifford et al., 2012) was performed on an Mx3005P QPCR system (Agilent technology Inc. Savage, MD). 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). All qPCR tests were performed in triplicate. The detection limit of qPCR was 1.7 log 16S copies/mL.

2.6. 16S rRNA gene HT sequencing and sequence analysis

DNAs of 80 spinach and lettuce rinse water samples (10 sanitation treatments × 2 produce type × 4 replicates) and a parallel set of 80 samples inoculated with foodborne pathogens were processed for 16S HT sequencing following the Earth Microbiome Project protocol (Caporaso et al., 2012; Caporaso et al., 2011) using MiSeq (Illumina). The barcode primer sets, 515F - 806R, were used to amplify the 16S rRNA gene 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 2018.4, https://docs.qiime2.org/2018.4/) 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% 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). The inferred absolute abundances of identified taxa in each sample were calculated by multiplying the total 16S rRNA gene copy numbers and the relative abundance (RA) of each taxon in the sample (Liang et al., 2015). The genome numbers of inoculated *E. coli*, *S. enterica*, and *L. monocytogenes* strains were estimated by dividing the 16S copies of identified species by its 16S gene counts in a single genome (Stoddard et al., 2015; Vetrovsky and Baldrian, 2013). The detection limit of foodborne pathogens by 16S HT sequencing in this study was 1 log genome copies/mL.

2.7. Isolation of chlorine and PAA resistant bacteria from produce rinse water

Bacterial colonies on TSA plates from spinach and lettuce rinse water samples treated with 30 mg/L or higher concentrations of FC or PAA were streaked on new TSA plates for purification and further identification. The resistance of isolated bacteria to FC or PAA was

confirmed by applying 50 mg/mL of the corresponding sanitizer in 5 log CFU/mL bacterial suspension for 30 s, and recovering from TSA plates after 2-day incubation at 30 °C. Purified isolates from each sample with distinct morphology on TSA were selected and identified by sequencing the nearly full length of 16S rRNA gene (Frank et al., 2008). In brief, colonies were incubated in TSB at 30 °C for 48 h. Bacterial suspensions were heated at 97 °C for 10 min, and centrifuged at 10,000*g* for 1 min. Supernatant was used as template for PCR amplification using primers 27F (5′-AGAGTTTGATYMTGGCTCAG, where Y is C or T and M is A or G) and 1492R (5′-TACCTTGTTACGACTT) and GoTaq Green Master Mix (Promega, Madison, WI). PCR products were purified and sequenced by a commercial service provider (Eurofins Genomics, Louisville, KY). The Lasergene software package (DNASTAR, Inc., Madison, WI) and NCBI BLAST were used for sequence analysis to identify resistant strains.

2.8. Statistical analysis

With plate count enumeration, PMA-qPCR estimation, and community analysis, bacterial CFU values, 16S rRNA gene copy numbers, and genome numbers of inoculated foodborne pathogens, were log transformed, and analysis of variance (ANOVA) was performed to analyze the differences in bacterial populations across different types of produce rinse water samples under different sanitizer treatments. Pearson's correlation coefficients were calculated to evaluate the correlations between the two detection methods among different types of samples. Correlation analysis was also conducted to compare the levels of bacterial species between produce rinse water and inoculated samples. The differences of alpha diversity indexes, including evenness and Shannon index, and beta diversity analyses using Jaccard method 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). Tukey's Honestly Significant Difference was used to compare means. Except when stated otherwise, P values of < 0.05 were considered statistically significant.

3. Results

3.1. Reduction of total bacterial populations after sanitation

Plate counting and PMA-qPCR were conducted for all spinach and lettuce rinse water samples treated with a series of concentrations of FC or PAA to measure bacterial populations (Fig. 1). Plate counts on TSA was used to measure total mesophilic aerobic bacteria (MAB), while PMA-qPCR was used to estimate total viable bacteria (TVB) based on the copy numbers of bacterial 16S rRNA genes. In both cases, the reduction in bacterial counts was correlated to sanitizer concentrations. MAB counts in spinach rinse water (SRW) were reduced by 4.1 and 3.5 logs after treatment with 50 mg/mL FC (Fig. 1A) and 100 mg/mL PAA (Fig. 1C), respectively. For lettuce rinse water (LRW), treatment with 50 mg/mL FC and with 100 mg/mL PAA resulted MAB reduction of 1.7 (Fig. 1A) and 1.3 (Fig. 1C) logs, respectively. The MAB counts measured by plating on TSA and the TVB counts estimated by PMA-qPCR was significantly correlated ($P < 0.001$). Inoculation of foodborne pathogens did not obviously affect the total bacterial populations in most rinse water samples, except TVB levels in PAA treated SRW samples. The correlation of total bacterial counts in inoculated and non-inoculated rinse water samples were significant ($P < 0.01$).

3.2. Spinach and lettuce rinsate microbiomes

The V4 region of 16S rRNA gene was targeted for sequencing analyses, and the sequencing data have been submitted to NCBI (Accession No.: PRJNA498218). After 16S rRNA gene HT sequencing of the 160 samples, a total of 11,848,323 raw paired reads (each pair is composed of reads generated by the forward and reverse primers) were generated, with a range of 14,360 to 152,537 reads per sample. Paired-end merge and quality control analyses using Qiime2 resulted in 9,760,827 reads in total, with a range of 12,789 to 127,440 reads per sample. Queries to the Greengene database using these sequences identified a total of 3018 OTUs in the 160 samples, including 18 archaea, 2915 bacteria, 72 eukaryotes (chloroplast and mitochondria), and 13 unassigned OTUs. All eukaryote reads, which counted for 1.08% of the total 9,760,827 reads, and unassigned reads (0.16%) were purged prior to data analysis of produce rinse water microbiomes. The 2933 bacteria and archaea OTUs were classified into 28 phyla, 558 genera, and 682 species. Among the nearly 3000 OTUs identified from rinse water samples of spinach and lettuce, at least 51%, 86%, and 97% were assigned to species, genus, and family levels, respectively. About 0.16% of the total paired-end reads remained unassigned, and 2.23% could only be classified to the Bacteria domain, which may denote sequences of bacterial species that were not documented in the last updated version of Greengenes database.

The HT sequencing for all samples identified 26 and 22 phyla in spinach and lettuce rinse water, respectively. Four phyla of them had average RA of > 1% (Fig. 2). Proteobacteria was the phylum with the highest abundance in all types of rinse water samples, accounting for 78.15% and 82.16% of the total reads from spinach and lettuce, respectively. The phyla with the next highest average RA in rinse water was Firmicutes (spinach, 13.90%; lettuce, 10.59%), followed by Actinobacteria (spinach, 5.48%; lettuce, 1.94%) and Bacteroidetes (spinach, 1.84%; lettuce, 0.17%).

At the genus and species levels, spinach and lettuce rinse water showed distinct microbiomes based on the presence of taxa with RA > 2% (Fig. 3). Although *Pseudomonas* spp. showed dominant presence in both spinach (*P. veronii* and 2 other species, 60.98% combined) and lettuce (*P. veronii*, *P. viridiflava*, and other species, 68.63% combined) rinse water, *Janthinobacterium* sp. (10.04%) and an unidentified species of family *Enterobacteriaceae* (5.61%) in spinach rinse water, and an unidentified species of family *Xanthomonadaceae* (15.46%) and *Erwinia* sp. (4.17%) in lettuce rinse water were among the top 5 most abundant taxa prior to sanitizer treatment.

3.3. Sanitizer treatment and microbiome convergence

Bacterial populations in spinach and lettuce rinse water were significantly reduced by treatment with increasing concentrations of sanitizers, regardless of the inoculation by foodborne pathogens (Fig. 1). Viable populations of the inoculated foodborne pathogens were estimated based on PMA-qPCR quantification and 16S rRNA gene HT sequencing (Fig. S1). The decrease of estimated genome numbers followed the same trends as that of the total viable bacterial populations when treated with increased doses of sanitizers.

This change was reflected by a gradual shift of the microbiomes. For both spinach and lettuce rinse water, treatment with higher concentrations of sanitizers significantly reduced the RA of Proteobacteria, while increasing Firmicutes, Actinobacteria, and other less represented phyla (Fig. 2). At genus/species level, the top 5 most abundant species were all reduced to minor presence when FC was applied as sanitizer to either spinach or lettuce rinse water. On the other hand, when PAA was applied, some of the most abundant genus/species retained high RA such as *P. viridiflava* and an unknown species of family *Xanthomonadaceae* (Fig. 3).

Randomly selected reads (12,670) from each of the 160 rinse water samples were subjected to alpha and beta diversity analyses. The evenness and Shannon index of bacterial communities in water samples showed similar trends to changes of total bacteria after sanitation, except the lettuce rinse water samples treated with 30 and 50 mg/L PAA, which might be associated with the dominance of resistant bacteria from tested romaine lettuce (Fig. S2). Interestingly, application of

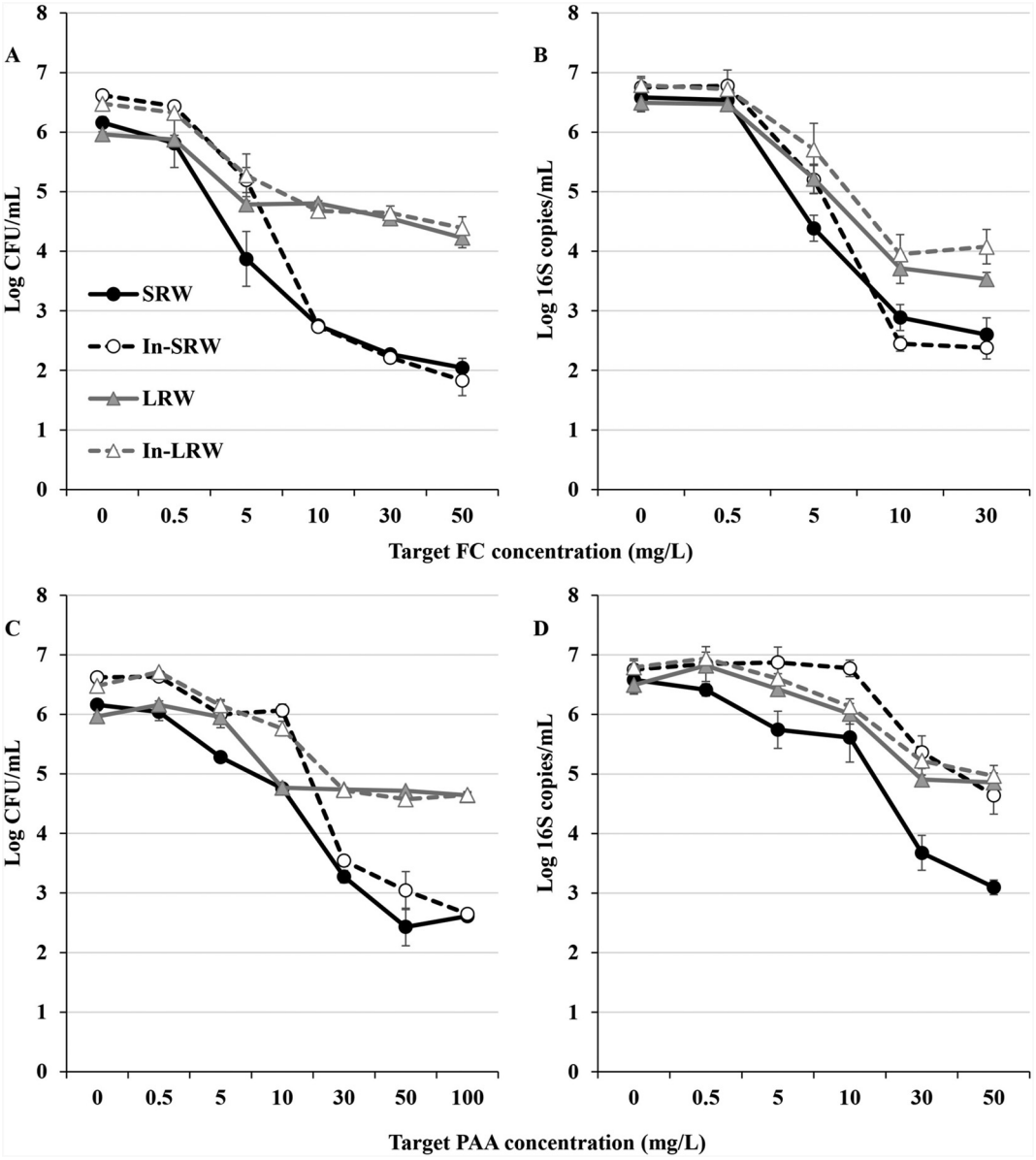

Fig. 1. Total viable bacterial counts detected by non-selective plating and by PMA-qPCR.
Plate count measurement of total bacterial populations in produce rinse water treated with free chlorine (A) and peracetic acid (C); PMA-qPCR quantification of bacterial 16S rRNA gene copy numbers in produce rinse water treated with free chlorine (B) and peracetic acid (D). SRW, Spinach rinse water; LRW, Lettuce rinse water; In-, inoculated with foodborne pathogens. FC, free chlorine; PAA, peracetic acid. Bars represent standard errors.

increased concentrations of sanitizers, especially FC, resulted in increased microbiome similarity in rinse water samples, irrespective of spinach or lettuce rinse water being treated (Fig. 4). This convergence of distinct microbiomes would reflect an immergence of a new microbiome that is increasingly resistant to sanitizers.

The sanitizer resistant microbiomes were mainly composed of bacterial species that showed low RA prior to sanitizer treatment. This was also true when produce rinse water was inoculated with foodborne pathogens at about the same level of total native bacteria (~6 log CFU/mL). For example, microbiomes between non-inoculated produce rinse water and inoculated samples after 30 mg/L FC or 50 mg/L PAA treatments were significantly correlated ($P < 0.0001$). Table 1 lists the top 5 species (bold font) with the highest RA in spinach and lettuce rinse water after treatment with 30 mg/L FC and with 50 mg/L PAA, which often includes *Arthrobacter psychrolactophilus*, *Bacillus* sp., *Cupriavidus* sp., and *Ralstonia* sp. An exception is the lettuce rinse water microbiome following 50 mg/mL PAA treatment, where the top 5 most abundant species included these abundantly presented in untreated lettuce rinse water, including *Pseudomonas viridiflava* and an unknown species of family *Xanthomonadaceae*. Inoculation with foodborne pathogens did not obviously change the prevalent bacterial species after sanitation, except for that in spinach rinse water treated with 50 mg/L PAA.

3.4. Isolation and identification of sanitizer resistant microbiota

In an attempt to identify and characterize sanitizer resistant microbiota in spinach and lettuce rinse water, the treated rinsates were plated on non-selective medium TSA. Representative and morphologically diverse colonies were selected and re-streaked for purification. After confirming the enhanced sanitizer resistance by exposing low concentration (5 log CFU/mL) of cells to 50 mg/L FC or PAA, these selected strains were identified to species by sequencing the full length 16S rRNA gene (Table 2). The culturable sanitizer resistant microbiota

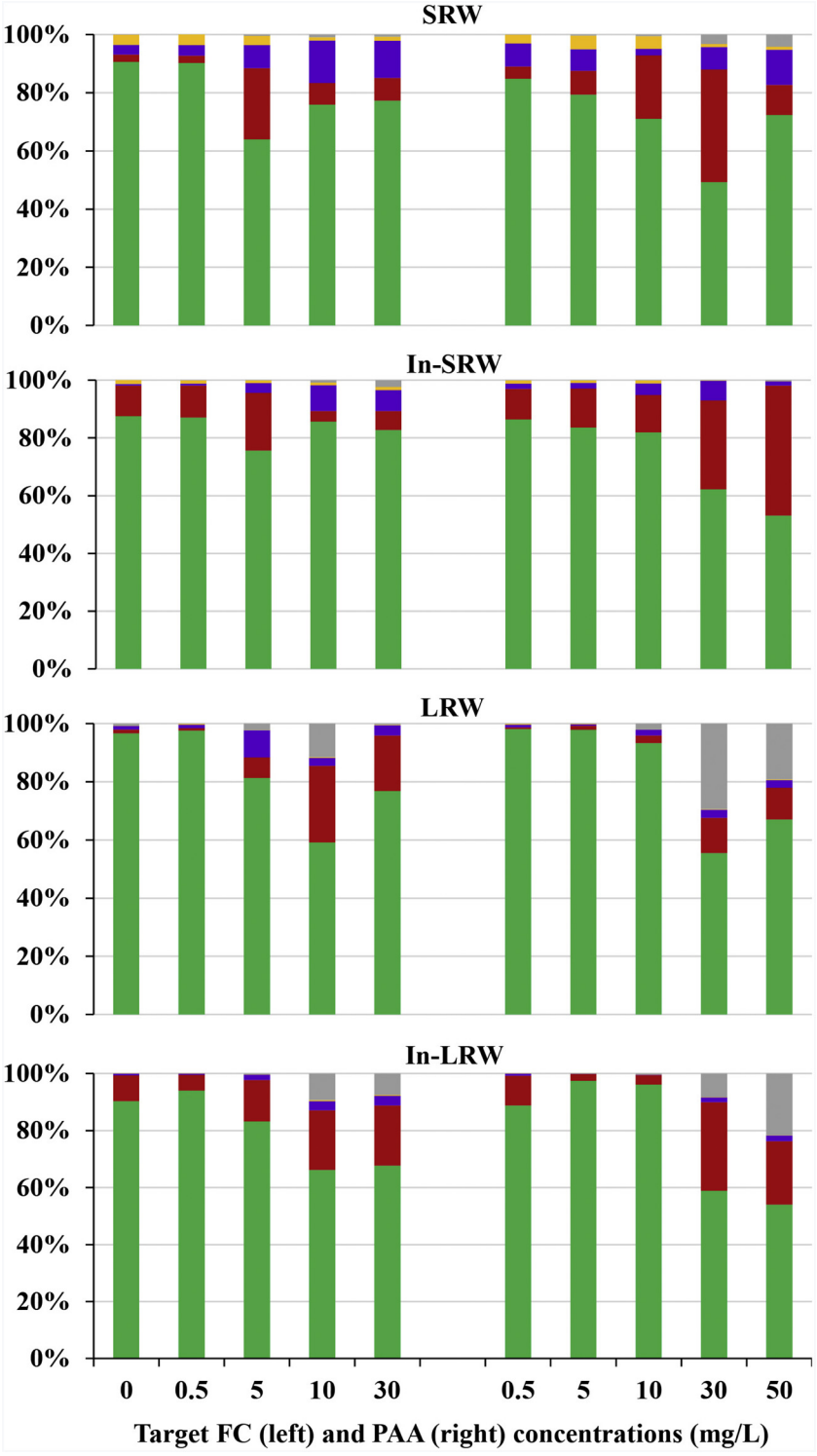

Fig. 2. Relative abundance (RA) of the top four bacteria phyla in spinach and lettuce rinse water after 30 s sanitation at different concentrations of FC and PAA.
Proteobacteria; Firmicutes; Actinobacteria; Bacteroidetes; other phyla.

were most represented by species of *Bacillus* and other Gram positive spore forming species, except that, in the case of lettuce rinse water treated with PAA, *Pseudomonas* species were well represented. The frequent isolation of *Bacillus* spp. was consistent with the HT sequencing results (Table 1) that *Bacillus* sp. was among the most prevalent in the sanitizer treated rinse water samples. Following the same trend, *Pseudomonas* spp. were well represented among the sanitizer resistant isolates from PAA treated lettuce rinse water, and the corresponding 16S rRNA gene copy number was among the top 5 most abundant ones. On the other hand, some other bacterial species, such as *Cupriavidus* sp. and *Ralstonia* sp., were missing among the sanitizer resistant isolates, despite the abundant presence detected by HT sequencing in both FC and PAA treated produce rinse water samples.

4. Discussion

In this study, washing freshly harvested baby spinach leaves and romaine lettuce outer leaves in sterile water generated bacterial suspensions containing about 6 log CFU/mL of mesophilic aerobic bacteria, as determined by plating counts on TSA. This estimate was

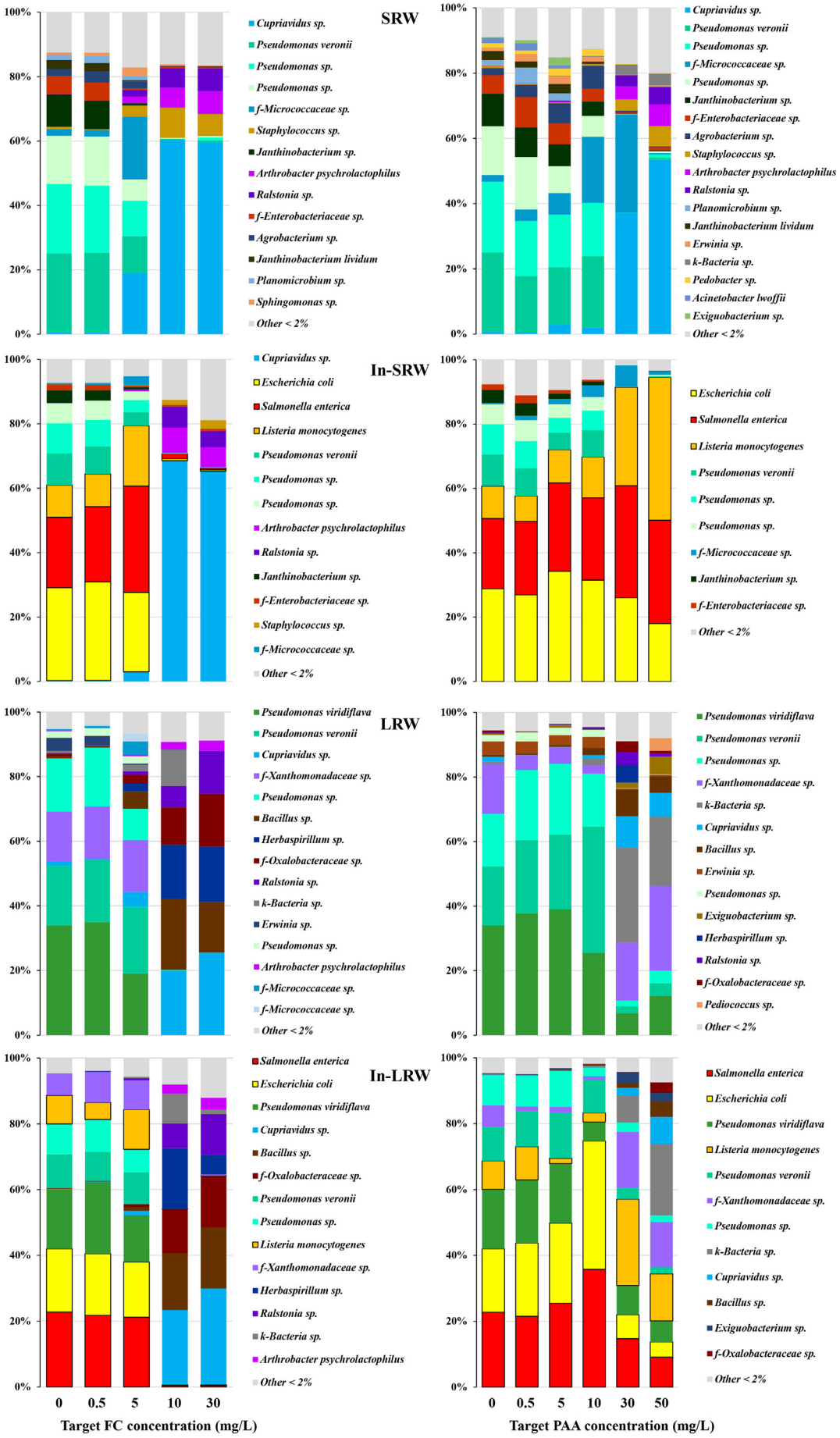

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Fig. 3. RA of the most abundant bacterial species (RA > 2%*) in spinach and lettuce rinse water after 30 s sanitation at different concentrations of FC and PAA. *, only taxa comprising > 1% (average RA) of the bacteria identified in at least one type of samples were included.

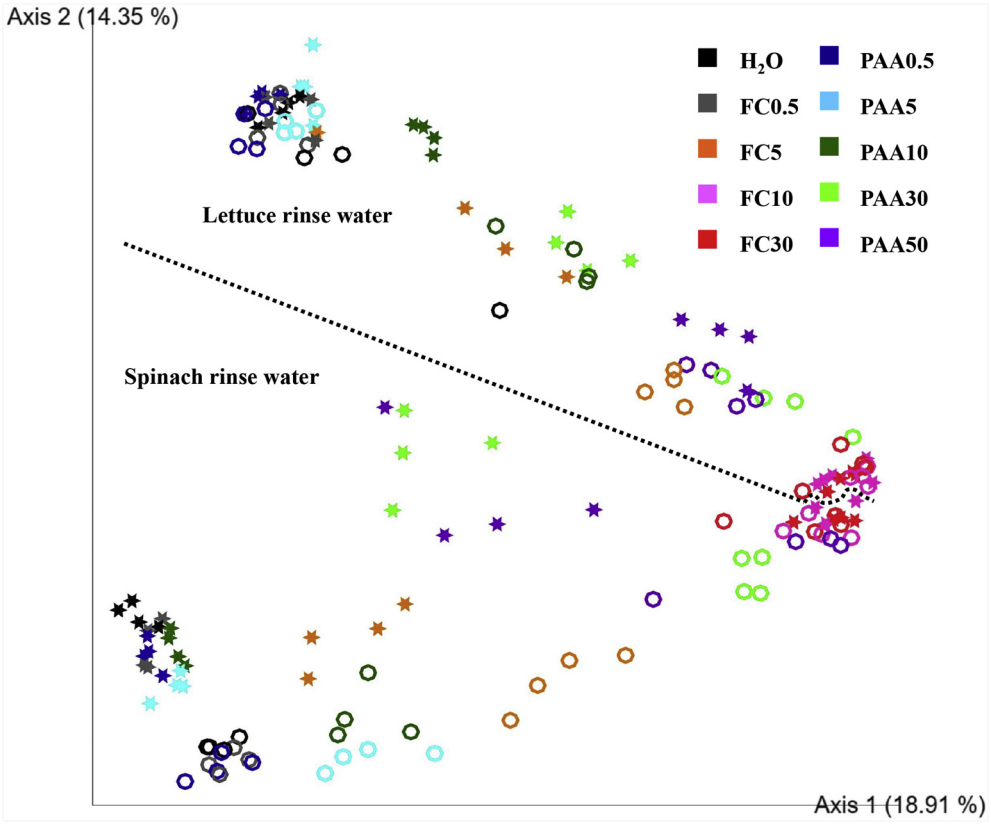

Fig. 4. Microbiome relatedness in produce rinse water after sanitation of FC and PAA at different concentrations.
Principle coordinate analysis (PCA) was performed to compare bacterial communities in rinse water samples (denoted by ring, SRW and LRW) and inoculated samples with foodborne pathogens (represented by star, In-SRW and In-LRW). Different colors of rings and stars denote the 10 sanitation treatments: H_2O, control; numbers (0.5–50) following FC and PAA denote applied concentrations of sanitizers (mg/L).

Table 1
List of the most abundant bacterial species[a] after sanitizer treatment.

Bacterium species	Rinse water			Inoculated rinse water[b]		
	H_2O[c]	FC 30	PAA 50	H_2O[c]	FC 30	PAA 50
In spinach rinse						
Arthrobacter psychrolactophilus	0.89 ± 0.89	**1.46 ± 0.11**	**1.93 ± 0.05**	0	**1.17 ± 0.09**	0.23 ± 0.23
Bacillus sp.	1.38 ± 0.80	**0.53 ± 0.15**	0.90 ± 0.07	0	0.18 ± 0.09	0
Cupriavidus sp.	4.38 ± 0.02	**2.37 ± 0.10**	**2.82 ± 0.05**	4.10 ± 0.15	**2.20 ± 0.08**	**1.76 ± 0.09**
f-Enterobacteriaceae	5.33 ± 0.11	0.38 ± 0.23	1.11 ± 0.09	5.01 ± 0.10	**0.40 ± 0.10**	0
f-Micrococcaceae	4.89 ± 0.17	0	1.12 ± 0.05	4.37 ± 0.09	0	**1.73 ± 0.18**
Planomicrobium sp.	4.78 ± 0.14	0.01 ± 0.01	0.27 ± 0.27	3.90 ± 0.19	0.02 ± 0.02	**1.40 ± 0.18**
Pseudomonas sp.	5.76 ± 0.10	0.42 ± 0.23	0.71 ± 0.37	5.72 ± 0.10	0.18 ± 0.06	**1.19 ± 0.22**
Pseudomonas veronii	5.97 ± 0.11	0.32 ± 0.32	0.50 ± 0.38	5.74 ± 0.10	0	**1.21 ± 0.15**
Ralstonia sp.	3.38 ± 0.01	**1.46 ± 0.09**	**1.82 ± 0.11**	3.30 ± 0.12	**0.90 ± 0.27**	0.82 ± 0.09
Staphylococcus sp.	4.18 ± 0.18	**1.43 ± 0.22**	**1.88 ± 0.09**	3.19 ± 0.18	**0.83 ± 0.19**	0.72 ± 0.09
Vibrio sp.	0.71 ± 0.71	0.50 ± 0.14	**1.12 ± 0.02**	0.57 ± 0.57	0.35 ± 0.04	0.25 ± 0.11
In lettuce rinse						
Arthrobacter psychrolactophilus	1.22 ± 1.22	**2.00 ± 0.09**	2.75 ± 0.05	0	**2.63 ± 0.16**	2.99 ± 0.13
Bacillus sp.	4.90 ± 0.15	**2.72 ± 0.05**	**3.55 ± 0.09**	5.01 ± 0.04	**3.34 ± 0.15**	**3.62 ± 0.13**
Cupriavidus sp.	4.99 ± 0.27	**2.93 ± 0.07**	**3.73 ± 0.06**	4.98 ± 0.09	**3.54 ± 0.14**	**3.88 ± 0.12**
Exiguobacterium sp.	4.58 ± 0.20	0.14 ± 0.14	**3.60 ± 0.09**	4.85 ± 0.14	0.30 ± 0.30	**3.28 ± 0.07**
f-Oxalobacteraceae	3.67 ± 1.23	**2.59 ± 0.30**	2.74 ± 0.07	4.58 ± 0.11	**3.24 ± 0.11**	3.04 ± 0.34
f-Xanthomonadaceae	6.26 ± 0.09	0.49 ± 0.08	**4.27 ± 0.06**	6.56 ± 0.12	1.22 ± 0.29	**4.05 ± 0.04**
Pseudomonas viridiflava	6.63 ± 0.13	0.28 ± 0.10	**3.92 ± 0.14**	7.00 ± 0.06	0.83 ± 0.08	**3.73 ± 0.05**
Ralstonia sp.	4.28 ± 0.27	**2.46 ± 0.31**	2.82 ± 0.06	3.26 ± 1.09	**3.15 ± 0.10**	3.05 ± 0.14

[a] 16S rRNA gene copy numbers [log (copies + 1)/mL] of the top 5 bacterial species (bold) identified by high-throughput sequencing in rinse water samples treated with 30 mg/L free chlorine (FC) or 50 mg/L peracetic acid (PAA). Letters sp. indicate species identified to genus level, and letter f in front denotes undetermined species that has been assigned to the family level.
[b] Inoculated with a cocktail of 9 strains of foodborne pathogens;
[c] Control samples treated with sterile water.

Table 2
Bacterial species isolated from produce rinse water treated with sanitizers[a].

Bacterium species	Spinach rinse		Lettuce rinse	
	FC	PAA	FC	PAA
Bacillus amyloliquefaciens			x	x
Bacillus aryabhattai			x	
Bacillus australimaris	x	x		
Bacillus cereus			x	
Bacillus clausii		x		
Bacillus endophyticus		x		
Bacillus filamentosus		x		
Bacillus foraminis		x		
Bacillus galliciensis		x		
Bacillus glycinifermentans	x			
Bacillus licheniformis	x			
Bacillus litoralis	x			
Bacillus marisflavi			x	
Bacillus massiliensis	x			
Bacillus megaterium		x	x	
Bacillus nealsonii		x		
Bacillus niabensis	x	x		
Bacillus paralicheniformis	x			
Bacillus pocheonensis	x			
Bacillus pumilus	x	x		
Bacillus safensis	x	x		
Bacillus sp.	x	x	x	x
Bacillus subtilis	x	x		
Bacillus toyonensis			x	
Bacillus velezensis		x	x	x
Brevibacillus nitrificans	x			
Brevibacterium frigoritolerans	x			
Paenibacillus lautus	x	x		
Planococcaceae sp.	x			
Pseudomonas moraviensis				x
Pseudomonas rhizosphaerae				x
Pseudomonas rhodesiae				x
Pseudomonas sp.				x
Pseudomonas viridiflava				x
Sporosarcina aquimarina		x		
Sporosarcina newyorkensis		x		
Staphylococcus hominis			x	
Terribacillus halophilus		x		
Terribacillus saccharophilus			x	x

* Treatment for 30 s. at 30 mg/L FC, 50 mg/L PAA, or higher doses of sanitizers.

consistent with the total viable bacterial concentration determined using quantitative real-time PCR targeting a conserved region in the 16S rRNA gene, following PMA treatment (Fig. 1). These bacterial suspensions also represented rich and diverse microbiomes, with over 600 bacterial species being identified by HT sequencing based microbial community analyses (Fig. 3). The changes in response to sanitizer treatment in the suspensions could provide valuable insights into the microbiome dynamics in commercial fresh produce wash water using chlorine or PAA as sanitizers.

In commercial operations for leafy green processing, chlorine and PAA are among the most commonly used sanitizers, with FC at 10–15 mg/L or PAA at 80 mg/L as control point (Simons, 2000; Tudela et al., 2019). In this study, while both treatments with chlorine at FC < 10 mg/L and PAA < 30 mg/L resulted significant reduction in MAB (determined by non-selective plating) and TVB (determined by PMA-qPCR), residual bacterial survival was observed even at the highest sanitizer concentration applied (Fig. 1). The persistence of bacterial populations in wash water could also raise concerns for potential survival and cross-contamination of foodborne pathogens and spoilage bacteria during fresh produce processing (Holvoet et al., 2012; Luo et al., 2011). Therefore, understanding the dynamics of complex microbial communities in wash water and identifying resistant bacteria to applied sanitizers may improve the sanitation strategies and hence mitigate the risk of cross-contamination during food processing.

HT sequencing based community analyses revealed chlorine and PAA resistant microbiomes from treated spinach and romaine lettuce rinse water, which are mostly composed of bacteria species that showed low RA in the rinse water prior to sanitizer treatment. *Bacillus* spp., *A. psychrolactophilus*, *Cupriavidus* sp., and *Ralstonia* sp. were among those with the highest RA after sanitizer treatment (Table 1). This microbiome shift reflected the selective sanitation on residential microbiota in wash water. In some cases, *Pseudomonas* spp. were also among the most abundant following PAA treatment, which indicated different sanitation effect for FC and PAA on various bacterial species. The foodborne pathogens, inoculated as a 9-strain cocktail at a density comparable to that of the total native microbiota, were not observed as component of chlorine-resistant microbiome, but were observed after PAA treatment.

The putative sanitizer resistant microbiomes were partially validated by non-selective plating to isolate sanitizer resistant bacterial strains following the treatment. The culturable bacteria isolated from sanitizer treated rinse water were mostly identified as *Bacillus* spp. or closely related genera that produced endospores, although *Pseudomonas* spp. were also recovered from lettuce rinse water treated with PAA. Various *Bacillus* spp. as Gram-positive and spore forming bacteria isolated from drinking and waste water samples were shown chlorine resistant (Ding et al., 2019; Paes et al., 2012; Roi et al., 2015). *Pseudomonas* is common inhabitant on produce and in water (Gu et al., 2019; Gu et al., 2018; Vaz-Moreira et al., 2012), and many species have been reported to possess resistance to multiple antibiotics (Papapetropoulou et al., 1994; Rosenberg and Duquino, 1989; Vaz-Moreira et al., 2012). However, non-selective plating on TSA failed to recover *A. psychrolactophilus*, *Cupriavidus* and *Ralstonia* spp. from sanitizer treated rinse water, albeit the high abundance of the corresponding 16S rRNA gene copies in those samples. Similarly, none of the inoculated foodborne pathogens were recovered after sanitizer treatment, albeit the detection of corresponding 16S rRNA genes after PAA treatment. The failure in recovering these bacterial species, which sometimes showed high abundance by PMA-qPCR, by non-selective plating could suggest the induction of the viable but non-culturable (VBNC) state (Oliver, 2010; Purevdorj-Gage et al., 2018). Future studies to determine the possible presence of sanitizer (especially PAA) induced VBNC populations of foodborne pathogens during washing and processing would benefit the risk assessment and process management for food safety.

Microbial communities in the spinach and lettuce rinse water constituted two distinct microbiomes, manifested as distant clusters on a principle coordinate analysis (PCoA) plot (Fig. 4). With the application of increasing concentrations of sanitizers, especially chlorine, the distance between the clusters shortened, suggesting increased similarity between the microbiome composition. This convergence of distinct microbiomes suggested a core sanitizer resistant microbiome composed of limited bacterial taxa with increased tolerance to sanitizer treatment. Thus, constituents of such sanitizer resistant microbiome would more likely survive in commercial flume water for fresh produce processing, and potentially assimilate the microbiota on the end products. Foodborne pathogens, especially those considered of significant concern for fresh produce industry, including Shiga-toxin producing *E. coli*, *S. enterica*, and *L. monocytogenes*, were shown unlikely to be components of the sanitizer resistant microbiota. However, it should be noted that the changes in microbiome were observed with only two types of fresh produce and the potential impact of wash water organic load was not considered in this study. More robust examination of wash water for more diverse commodities, especially that for commercial fresh produce processing, could provide insights into the nature of sanitizer resistant microbiome, and its implication in potential interactions with foodborne pathogens.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108458.

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