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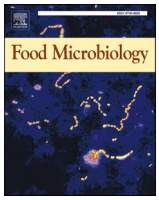

Commercial wash of leafy vegetables do not significantly decrease bacterial load but leads to shifts in bacterial species composition

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ABSTRACT

Production of leafy vegetables for the "Ready-to-eat"-market has vastly increased the last 20 years, and consumption of these minimally processed vegetables has led to outbreaks of food-borne diseases. Contamination of leafy vegetables can occur throughout the production chain, and therefore washing of the produce has become a standard in commercial processing. This study explores the bacterial communities of spinach (*Spinacia oleracea)* and rocket (*Diplotaxis tenuifolia)* in a commercial setting in order to identify potential contamination events, and to investigate effects on bacterial load by commercial processing. Samples were taken in field, after washing of the produce and at the end of shelf-life. This study found that the bacterial community composition and diversity changed significantly from the first harvest to the end of shelf-life, where the core microbiome from the first to the last sampling constituted <2% of all OTUs. While washing of the produce had no reducing effect on bacterial load compared to unwashed, washing led to a change in species composition. As the leaves entered the cold chain after harvest, a rise was seen in the relative abundance of spoilage bacteria. *E. coli* was detected after the washing indicating issues of cross-contamination in the wash water.

1. Introduction

The consumption of leafy green vegetables, such as baby leaves of spinach, rocket, and other ready-to-eat, minimally processed vegetables, has increased over the last two decades. Worldwide production of spinach increased from 9.5 million tons in year 2000 to almost 27 million in 2018 (FAO, 2019). In Sweden alone, one of the large retail chains reported that the sales of ready-to-eat, prepacked salad mixes increased from 600,000 bags in 2005 to nearly 40 million bags in 2016 (Söderqvist, 2017). Consumption of minimally processed leafy greens has been known to cause outbreaks of food-borne illnesses through contamination with human pathogens, such as *Escherichia coli* O157:H7, *Listeria* and *Salmonella*. Mogren et al. (2018) listed outbreaks related to leafy vegetables in Europe and North America between the years 2000 and 2016 and found 29 outbreaks from which 15 lead to several hundreds of affected persons, and 5 outbreaks that lead to deaths as a direct cause of eating contaminated leafy vegetables. Contamination can occur throughout the production chain; in field, at harvest, washing, and packaging of the produce. Use of low quality irrigation water regarding abundance of human enteric pathogens, untreated manure, unhygienic farm equipment and wash water, are examples of potential sources of human pathogens (Mogren et al., 2018). In the production of ready-to-eat leafy vegetables there are few tools available that are able to eradicate potential pathogens on the produce before consumption (Gil et al., 2015). Washing of produce, with or without addition of sanitizing agents, is the main intervention step for removal of microorganisms, however, wash water has also been identified as a source for cross-contamination of human enteric pathogens (Allende et al., 2008; Holvoet et al., 2012). Gruden et al. (2016) showed a high increase of heterotrophic bacteria, slow-growing bacteria, and intestinal enterococci in the process water of a commercial facility when produce were exposed to the wash water. With process water being reused for several hours in commercial washing and packaging establishments this shows a clear potential for cross contamination between different batches of leafy greens processed during the same day. While washing is a good practice for removal of soil and other foreign materials, Uhlig et al. (2017) showed an approximate reduction of 1 $\log_{10}$ colony forming units (CFU) g^{-1} for total aerobic bacteria and 0.5 $\log_{10}$ CFU g^{-1} of *Enterobacteriaceae* in a simulated household washing procedure. The reduction was dependent on the force of water flow suggesting that a physical force is needed for removal of bacteria. In the same trial leaves were inoculated with *E.coli* and the reduction rate was measured after

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immersing the leaves in potable water 5 times; the results showed no significant reduction. Even with the use of different sanitizing agents, the efficiency in reduction of enteric pathogens differ depending on which sanitizer is being used (Banach et al., 2015). Pezzuto et al. (2016) showed that several sanitizing agents were efficient for reduction of *Listeria monocytogenes*, but sodium hypochlorite was the only sanitizer efficient against *Salmonella enterica*. The highest efficiency reported concerning reduction of total bacterial load is <90% with potable water (Uhlig et al., 2017), and <99% when a sanitizing agent has been used (Pezzuto et al., 2016). However, the 99% reduction was only possible when the initial load of bacteria was high (7 log CFU/g). At a lower initial load of bacteria (3 log CFU/g), the efficiency of the washing procedure was at best 1 log, but for many washing procedures the efficiency was negligible (Pezzuto et al., 2016).

Survival of human pathogens on leaf surfaces is depending on several factors; e.g. tolerance to UV radiation, competition with the natural microbiota of the phyllosphere, as well as nutrient and water availability (Brandl, 2006). Leaves with mechanical or biotic damages tend to be more prone to attachment and multiplication of human enteric pathogens due to an increased flow of nutrients available for microbial growth compared to intact leaves (Brandl, 2008). Microorganisms on the leaf surface are to a large part protected from changes in abiotic conditions through biofilm formation. It has been estimated that 10–40% of the bacterial population is growing in protective, multispecies, biofilms (Morris et al., 1998). It is of importance to follow the entire production chain in order to identify if, when, and where contamination events take place. It is also of importance to investigate if a contamination happening in the primary production is still present after washing and packaging, and if such an event has a general effect on the phyllosphere microbial community structure. Previous studies have investigated the fate of *E.coli*, and other human pathogens, on leafy greens by adding pathogens and investigating survival over time (Alam et al., 2014; Islam et al., 2004; Williams and Marco, 2014). In the case of *E.coli*, the survival time was dependent on the amount of inoculum where a higher inoculum lead to longer survival. However, even with lower inoculum levels the pathogen was still detectable after 72 h (Alam et al., 2014). This

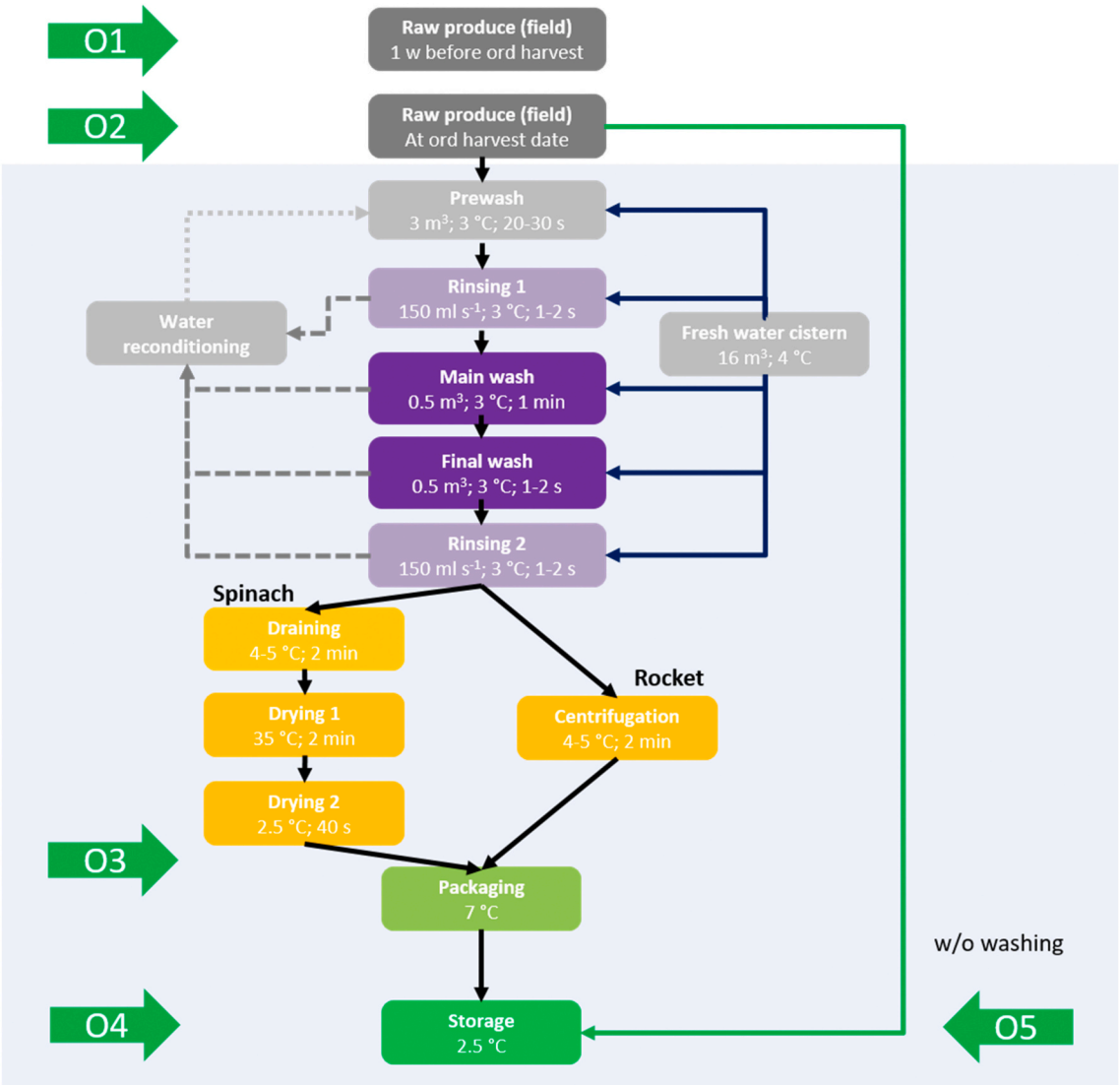

Fig. 1. Flow chart illustrating the process of washing, drying and packaging of spinach and rocket leaves at the commercial facility. Large, green arrows indicate sampling positions. Blue colored background indicate cool temperatures. Prewash, main wash and final wash are water baths, while rinsing steps are showers with fresh water. The prewash is the first water bath that produce enter where most of the particles are washed off. This water is a mixture of fresh water and reconditioned water from the following washing steps where only fresh water is used. Water reconditioning consists of a sedimentation tank where solids are removed. Illustration modified from Grudén et al., (2016). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

means that a contamination with a pathogen during primary production, especially at the later stage, could easily be transferred in to the processing stage where also cross-contamination with other produce could occur.

The objectives of this study were to investigate changes that occur in the bacterial communities of the phyllosphere of spinach and rocket along the production chain, to identify potential contamination events, and to investigate effects on bacterial load by commercial processing. The hypotheses were: i) the phyllosphere microbial community composition changes with production event, ii) season is a driver of microbial community composition of the phyllosphere, and iii) natural contamination with *E. coli* can occur at any point in the production chain, iv) commercial wash of leafy greens reduces bacterial load compared to unwashed. Spinach (*Spinacia oleracea)* and rocket (*Diplotaxis tenuifolia)* were chosen as model crops due to their popularity among Swedish consumers.

2. Materials & methods

2.1. Sample collection

Samples of conventionally grown spinach (*Spinacia oleracea*) and rocket (*Diplotaxis tenuifolia*) were collected in spring and autumn of 2016 from a commercial farm in the south of Sweden. Both seasons consisted of five sampling occasions: 1) approximately one week before harvest, 2) at harvest, 3) after washing of the produce, 4) at the end of shelf life of the washed and stored product, and 5) at the end of shelf-life of unwashed leaves from the same batch, which was the control for comparison to washed and stored leaves. The occasions are referred to as O1, O2, O3, O4, and O5 hereafter (Fig. 1). Due to crop rotation practices the samples taken in the autumn were not grown in the same field as those in spring, however they were in close vicinity to each other. For each sampling occasion six replicate samples were collected. Sampling occasions O1 and O2 were performed in field and each replicate sample consisted of all edible plant parts, cut manually using scissors, from a 1 m^2 area. All sampling equipment was sterilized between replicates and aseptic techniques were used. After the second sampling event, the rocket and spinach leaves were commercially harvested with machines, and transported to the nearby washing and packing facility where an unbroken cold chain of 4 °C was kept. Samples of unwashed leaves (O5) were collected when the leaves had been mounted onto the conveyor belt prior to the washing station, and stored in plastic bags in the pre-washing room at 4 °C until the end of shelf-life. After the commercial washing (potable water only) spinach leaves were dried with hot air flow of 35 °C, while rocket leaves were dried through centrifugation. After drying, leaves were collected and analyzed (O3). Washed and dried leaves were commercially packed and bags were randomly chosen for analysis; these bags were left until the end of shelf-life at the facility at 4 °C. Shelf-life was estimated by the staff at the washing and packing facility based on the quality of incoming produce. For detailed information of sampling dates, see Table 1. Special care was taken to ensure that samples from O3–O5 were from the same production lot as O1 and O2. For each occasion, 6 replicate samples were taken giving a total of 120 (60 spinach; 60 rocket) samples for processing.

Table 1
Dates of sampling occasions of spinach (*Spinacia oleracea*) and rocket (*Diplotaxis tenuifolia*) in 2016. O1) 1 week prior to harvest, O2) at harvest, O3) after washing, O4) washed and stored, and O5) unwashed and stored.

	Spring		Autumn	
	Spinach	Rocket	Spinach	Rocket
O1	May 27	May 30	Aug. 31	Sept. 6
O2	June 1	June 3	Sept. 6	Sept. 9
O3	June 8	June 8	Sept. 8	Sept. 13
O4	June 15	June 15	Sept. 19	Sept. 23
O5	June 15	June 15	Sept. 19	Sept. 23

2.2. Culture dependent analysis

From each replicate, 10 g of leaves were suspended in 40 mL Tris buffer (0.01 M Tris-HCL, Merck KGaA, Darmstadt, Germany; pH 5.63) in sterile, disposable, plastic bags (A6 PLAST, 400 mL, Olstykke, Denmark) and mechanically processed in a Smasher™ (Smasher™ Lab Blender, AES-Chemunex, Bruz, France) at normal mode for 30 s. Serial dilutions of the resulting liquid were made using 0.85% NaCl, and 100 μL aliquots were used for viable counts to quantify total aerobic counts, *Enterobacteriaceae*, *E. coli*, total coliforms, and *Enterococci* (Table 2).

2.3. Culture-independent analysis

For analysis of phyllosphere bacterial community composition, 10 g of leaves from each replicate sample were placed in a sterile plastic bag containing a filter (Separator 400, 180 mm * 300 mm * 70 μm; Grade Products Ltd., Coalville, UK), and 40 mL phosphate-buffered saline solution (PBS, 0.01 M, pH 7.4, Sigma-Aldrich) was added. Leaves were mechanically processed using a Smasher™ (Smasher™ Lab Blender, AES-Chemunex, Bruz, France) at normal mode for 2 min. From the resulting suspension, 20 mL were transferred to sterile Falcon tubes and centrifuged at 5000×*g* for 15 min (Centrifuge 5804, Eppendorf AG, Hamburg, Germany). The supernatant was discarded and the pellet transferred to 1.5 mL cryo tubes and stored at −80 °C. DNA extraction of the pellets was carried out with the KingFisher Cell and Tissue DNA Kit (Thermo Scientific, Vantaa, Finland) using a DNA extraction robot (KingFisher Duo, Thermo Scientific, Vantaa, Finland), according to the manufacturer's instructions.

2.4. Illumina sequencing and data processing

Bacterial community composition was analyzed with Illumina MiSeq, 300 bp paired-end read targeting the V3 region, by LGC Genomics GmbH (Berlin, Germany). Primers used for the 16S rRNA gene were 785F and 1064R.

Data from the sequencing was analyzed by the bioinformatics service of LGC Genomics GmbH (Berlin, Germany), who handled the data pre-processing and generated OTU count tables in BIOM format. Briefly, quality control and filtering of sequences was performed using the MOTHUR pipeline (version 1.35.1). Primer and barcode sequences, as well as chimeras, were removed and reads with a final length of <100 bases were discarded. Sequences were then aligned against the 16S Mothur-Silva SEED r119 reference alignment and sequences from other domains of life were removed. Clustering of operational taxonomic units (OTUs) was carried out at the 97% identity level using the *cluster.split* method, and *de novo* phylogenetic trees were generated with the FastTree method.

The original Illumina MiSeq sequence data have been submitted to the NCBI Sequence Read Archive (SRA) under BioProject ID number PRJNA660350.

Table 2
Overview of agar media used for enumeration of different microbial groups.

Target organism	Agar	Incubation conditions	Manufacturer
Total aerobic bacteria	Tryptic soy agar (TSA), conc. 0.1x	25 °C; 72 h	Difco
Enterobacteriaceae	Violet red bile agar (VRBD)	37 °C; 24 h	Merck
E. coli and coliforms	Brilliance *E.coli*/ Selective Coliform agar	37 °C; 20 h	Oxoid Ltd.
Enterococci	Bile Aesculin agar	37 °C; 18 h	Lab M Ltd.

2.5. Statistical analyses

The number of microorganisms, determined by viable count, in the different sampling occasions were analyzed with analysis of variance (ANOVA), followed by Tukey's test after logarithmic transformation (x' = log(CFU + 1)). Fold increase was calculated on geometric means of sampling occasions O4 and O5 as compared to sampling occasion O2. The BIOM file was used for analysis of bacterial community composition. All statistical analyzes were performed in R Studio (www.rstudio.com). Sequences identified as 'mitochondria' were removed and samples with <3000 reads were excluded, resulting in a total of 111 samples for further analyzes. Shannon and Chao1 indices were used to estimate alpha diversity with the function *estimate_richness* of the phyloseq package (McMurdie and Holmes, 2013), in order to determine changes in bacterial community diversity and richness at the different sampling occasions. For beta diversity calculations, weighted UniFrac using the *distance* and *ordinate* functions in the phyloseq package was used, based on data filtered according to prevalence and total count. The phyloseq package was also used to create plots and community composition figures with *ggplot2* (Wickham, 2009). Venn diagrams, based on membership, were produced using the Metagenomics Core Microbiome Exploration Tool (MetaCoMet), comprising the OTUs above 0.01% of total reads (Wang et al., 2016).

3. Results

3.1. Population sizes of culturable bacteria on leaves

Numbers of colony forming units, from different bacterial groups, changed between the sampling occasions; in all cases an increase was seen in colony forming units per gram leaf (CFU g^{-1}) from sampling occasion O1 to O5 (Table 3). Results showed that washing of the produce rather increased than decreased the bacterial load; although a significant increase only occurred for total aerobic counts at the production of spinach in spring, and for *Enterobacteriaceae* in rocket produced in the autumn. Fold increase represents how many times the number of colony forming units increased from the commercial harvest occasion (O2) in field, to the end of shelf life of both washed and unwashed leaves. Washing of the leaves had little to no effect on the increase in bacterial load during storage.

In 30% of the sampling occasions *E. coli* was detected on the leaves; however, it was not detected in all replicate samples at those occasions. For rocket, *E. coli* was present in sampling occasions O1 and O2 in both spring and autumn, and in spinach it occurred after washing (O3) in spring and after washing and storage (O4) in autumn. The number of CFU g^{-1} fresh produce ranged from $\log_{10}$ 1.3–4 in rocket, and from $\log_{10}$ 0.8–4 in spinach.

3.2. Bacterial diversity

For characterization of the changes in bacterial community composition of spinach and rocket leaves along the production chain, Illumina MiSeq sequencing was done. A total of 1,880,997 raw sequence reads was generated with 17,029 unique operational taxonomic units (OTU). Samples with less than 3000 reads were excluded, leaving 111, out of 120, samples for further statistical analyses. Bacterial sequence reads ranged from 3142 to 38,271 per sample library.

The alpha diversity was assessed with Shannon and Chao1 indices, grouped by plant species, harvest occasion, and season. The Shannon diversity index showed a clear decrease in diversity from the first sampling occasion (O1) to the last (O5), with the same pattern repeated independent of plant species and growing season. Richness of species also significantly decreased in all cases, except for spinach grown in spring where the richness was highest at harvest (O2) (Fig. 2).

At a relative abundance of >2% the phyllosphere of spinach and rocket was mainly occupied by members of Proteobacteria, Firmicutes, Bacteriodetes, and Actinobacteria (Fig. 3). In rocket, Firmicutes was more prominent in the unwashed and stored leaves (O5), while Bacteriodetes were more commonly occurring in the washed and stored produce (O4). Spinach in spring showed a similar pattern, while the autumn harvest of spinach did not. Within the Bacteriodetes phylum, *Flavobacteriaceae* was more commonly occurring in the washed and stored, than in the unwashed, produce. Most of the *Flavobacteriaceae* were unclassified genera, although a large part belonged to *Flavobacterium*. A closer look at the Proteobacteria phylum showed that both *Pseudomonas* and unclassified *Enterobacteriaceae* increased to a high degree over the plant production cycle (Table 4). *Enterobacteriaceae* was most common in the spring production, while *Pseudomonas* was equally common in spring and autumn. *Pseudomonas* increased noticeably after washing (O3) while *Enterobacteriaceae* was occurring to a high degree in

Table 3
Mean, standard deviation, and fold increase of colony forming units in the phyllosphere of spinach and rocket at five different sampling occasions: O1) 1 week prior to harvest, O2) at harvest, O3) after washing, O4) washed and stored, and O5) unwashed and stored. Fold increase of geometric means from O4 and O5 compared to O2 (W=washed produce, U=unwashed produce). Means that do not share a letter are significantly different.

			$\log_{10}$ mean cfu/g fw leaf ±standard deviation							
Crop	Season	Occasion	Total aerobic counts	Fold increase	*Enterobacteriaceae**	Fold increase	*Enterococcus*	Fold increase	Coliform bacteria	Fold increase
Spinach	Spring	O1	4.9 ± 0.2 *a*		2.5 ± 1.6 *a*		3.0 ± 0.2 *a*		1.8 ± 1.5 a	
		O2	4.2 ± 0.3 *b*		2.8 ± 0.4 *a*		2.4 ± 0.2 *a*		2.6 ± 0.5 a	
		O3	5.6 ± 0.2 *c*		4.5 ± 0.2 *b*		4.0 ± 0.2 *a*		4.5 ± 0.4 a	
		O4	7.0 ± 0.2 *d*	W: 526	5.4 ± 0.1 *b*	W: 414	4.7 ± 0.1 *b*	W: 168	5.8 ± 0.1 b	W:1645
		O5	6.6 ± 0.2 *d*	U: 240	5.2 ± 0.3 *b*	U: 221	4.4 ± 1.0 *b*	U: 92	5.8 ± 0.2 b	U:1800
	Autumn	O1	5.4 ± 0.1 *a*		4.8 ± 0.2 *ab*		4.4 ± 0.1 *a*		3.5 ± 1.0 a	
		O2	5.9 ± 0.2 *ab*		4.4 ± 0.2 *b*		3.5 ± 0.4 *b*		4.0 ± 0.4 a	
		O3	5.9 ± 0.3 *ab*		4.4 ± 0.3 *b*		3.1 ± 0.2 *b*		4.2 ± 0.3 a	
		O4	6.4 ± 1.3 *ab*	W: 3	5.4 ± 0.4 *c*	W: 11	4.1 ± 0.3 *a*	W: 4	5.3 ± 0.3 b	W: 18
		O5	7.3 ± 0.4 *b*	U: 22	5.3 ± 0.4 *ac*	U: 9	4.5 ± 0.4 *a*	U: 9	5.4 ± 0.3 b	U: 21
Rocket	Spring	O1	5.1 ± 1.2 *a*		2.9 ± 0.6 *a*		3.0 ± 0.5 *a*		1.8 ± 1.1 a	
		O2	4.2 ± 0.4 *a*		3.1 ± 0.6 *ab*		1.6 ± 0.5 *b*		2.9 ± 1.5 a	
		O3	4.2 ± 2.4 *a*		3.7 ± 0.2 *b*		2.3 ± 0.9 *ab*		3.0 ± 0.3 a	
		O4	6.3 ± 1.4 *a*	W: 115	5.0 ± 0.1 *c*	W: 75	4.0 ± 0.4 *c*	W: 270	5.1 ± 0.2 b	W: 165
		O5	6.5 ± 0.2 *a*	U: 203	5.0 ± 0.2 *c*	U: 77	4.3 ± 0.2 *c*	U: 493	5.1 ± 0.1 b	U: 177
	Autumn	O1	5.9 ± 0.4 *a*		3.8 ± 0.5 *a*		3.1 ± 0.3 *a*		3.2 ± 0.4 ab	
		O2	6.1 ± 0.6 *a*		3.5 ± 0.8 *a*		3.6 ± 0.8 *ab*		2.7 ± 1.3 b	
		O3	6.3 ± 0.1 *ab*		4.7 ± 0.2 *b*		4.0 ± 0.2 *bc*		4.4 ± 0.3 bc	
		O4	7.7 ± 0.3 *c*	W: 41	5.0 ± 0.3 *b*	W: 32	4.6 ± 0.3 *c*	W: 11	4.9 ± 0.3 c	W: 181
		O5	7.0 ± 0.1 *bc*	U: 9	4.7 ± 0.2 *b*	U: 15	4.1 ± 0.2 *bc*	U: 3	4.3 ± 0.3 bc	U: 45

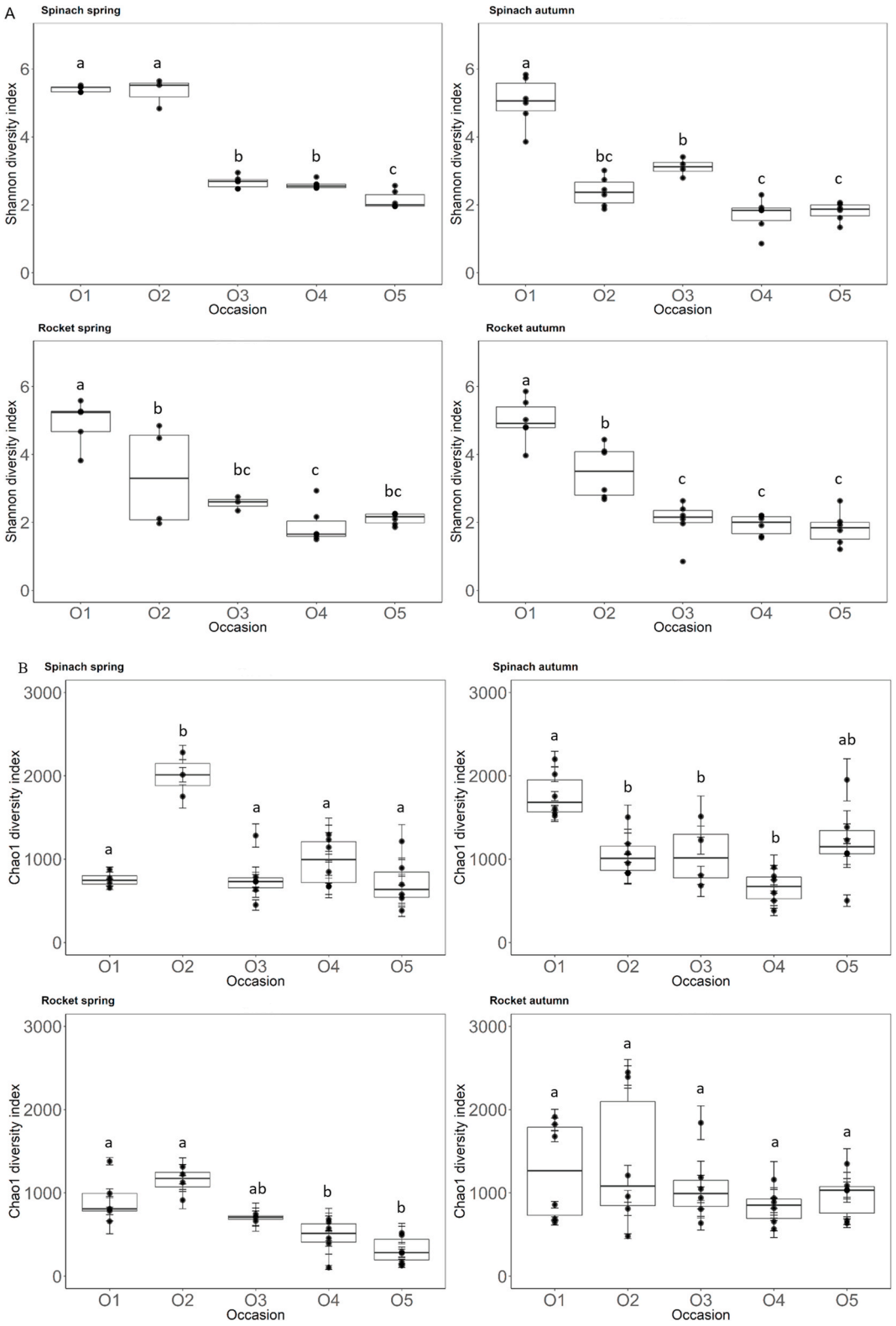

Fig. 2. Shannon (A) and Chao1 (B) indices based on relative abundance of bacterial communities in the phyllosphere of spinach and rocket at five different sampling occasions: O1) 1 week prior to harvest, O2) at harvest, O3) after washing, O4) washed and stored, and O5) unwashed and stored. Boxes that do not share a letter indicate significant differences.

the stored product. The *Enterobacteriaceae* mostly consisted of unclassified genera. Among the classified genera *Buttiauxella*, *Erwinia*, *Buchnera*, *Pantoea*, and *Escherichia-Shigella* were found. The *Escherichia-Shigella* occurred one week prior to harvest of rocket in spring (O1) to a level of 0.06% of the total Proteobacteria, and at harvest of rocket in the autumn (O2) with 0.2% of total Proteobacteria. Within the Firmicutes order, *Family_XII* was highly abundant, especially in the autumn harvests, and consisted only of the *Exiguobacterium* genus. Other bacterial families of high abundance within Firmicutes were *Bacillaceae* and *Paenibacillaceae*. Genera within Proteobacteria that occurred in

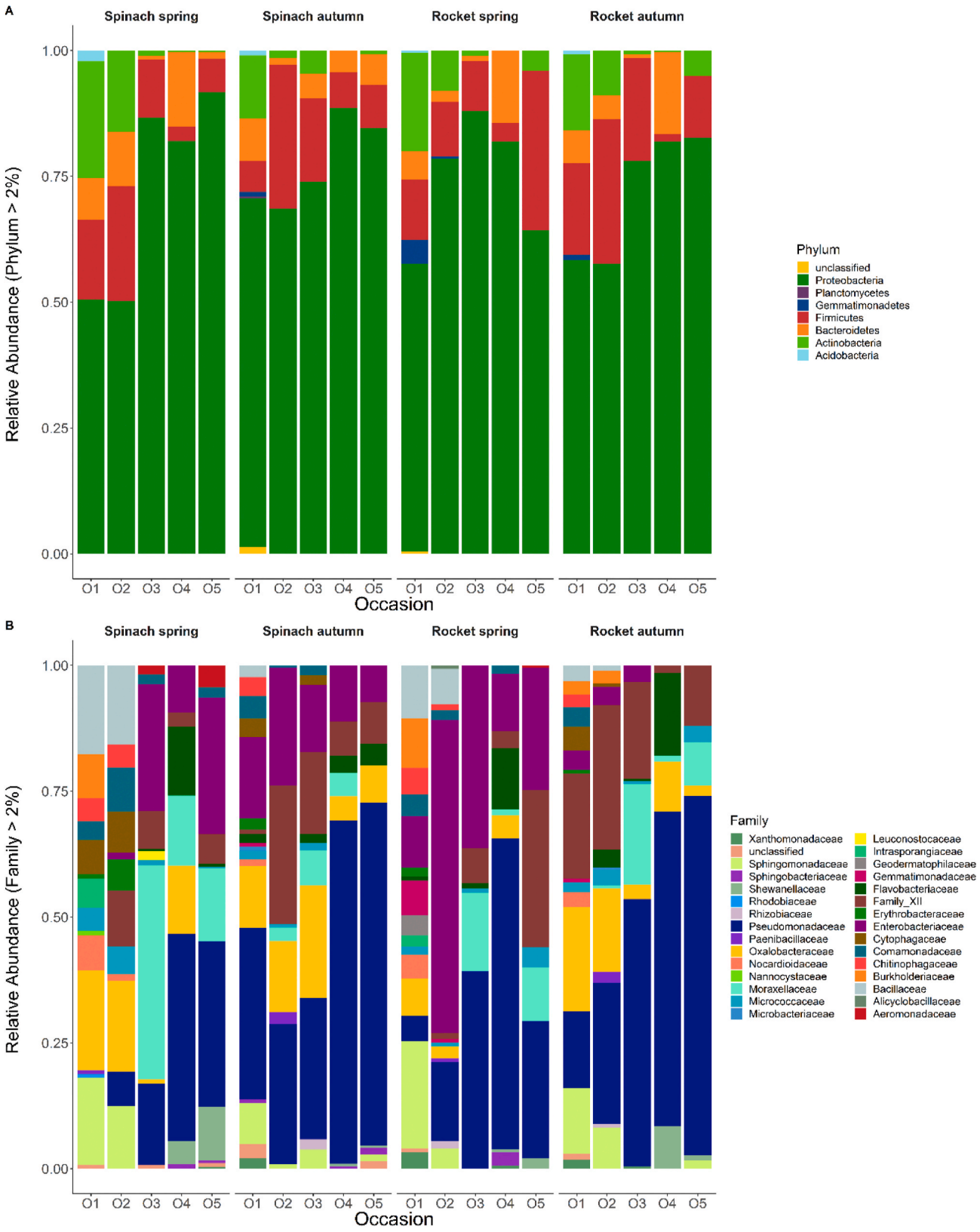

Fig. 3. Relative abundance of bacterial phyla (A) and families (B) in the phyllosphere of spinach and rocket at five different sampling occasions: O1) 1 week prior to harvest, O2) at harvest, O3) after washing, O4) washed and stored, and O5) unwashed and stored.

Table 4

A Relative abundance of bacterial genera of rocket phyllosphere within the Proteobacteria phylum at five different sampling occasions: O1) 1 week prior to harvest. O2) at harvest. O3) after washing. O4) washed and stored, and O5) unwashed and stored. Genera at an abundance >2% are presented. **B** Relative abundance of bacterial genera of spinach phyllosphere within the Proteobacteria phylum at five different sampling occasions: O1) 1 week prior to harvest. O2) at harvest. O3) after washing. O4) washed and stored, and O5) unwashed and stored. Genera at an abundance >2% are presented.

A	***D. tenuifolia* spring**					***D. tenuifolia* autumn**				
	Sampling occasion					**Sampling occasion**				
Proteobacteria genera	**O1**	**O2**	**O3**	**O4**	**O5**	**O1**	**O2**	**O3**	**O4**	**O5**
Alphaproteobacteria										
Methylobacterium								2.87 ± 0.35		2.68 ± 0.72
Microvirga						2.13 ± 0.26	2.51 ± 0.74			
Rhizobium				2.03 ± 0.89	2.05 ± 1.12		2.14 ± 0.51	2.09 ± 0.80	2.10 ± 0.68	
Pseudolabrys	2.08 ± 0.85									
Acetobacteraceae; unclassified	6.48 ± 1.71	3.99 ± 1.72								
Skermanella										2.20 ± 1.05
Candidatus_Alysiosphaera						3.07 ± 0.73				
Sphingomonas	6.89 ± 3.03	4.66 ± 0.57	5.67 ± 1.38	2.95 ± 1.77	3.52 ± 3.57	4.90 ± 0.57	6.72 ± 1.60	7.16 ± 1.23	5.14 ± 1.42	5.75 ± 0.90
Betaproteobacteria										
Comamonadaceae; unclassified	4.60 ± 1.42	6.67 ± 1.87	8.98 ± 1.34	4.87 ± 5.29	2.25 ± 1.65	5.32 ± 0.81	4.33 ± 0.54	3.11 ± 1.07	2.16 ± 0.69	3.25 ± 1.04
Duganella									4.18 ± 2.57	
Massilia	2.76 ± 0.83	2.84 ± 0.57	2.25 ± 1.12	4.95 ± 1.14	2.32 ± 1.83	3.49 ± 0.80	4.38 ± 1.14	5.85 ± 1.17	6.05 ± 1.42	5.12 ± 0.67
Oxalobacteraceae; unclassified	3.58 ± 0.96	2.96 ± 1.58		7.82 ± 4.80		5.14 ± 0.78	6.58 ± 1.75	4.73 ± 1.30	9.22 ± 5.27	4.61 ± 2.14
Deltaproteobacteria										
Myxococcales; 0319-6G20; unclassified						2.15 ± 0.57				
Haliangium						2.80 ± 0.72				
Gammaproteobacteria										
Aeromonas					2.03 ± 1.12					
Shewanella				2.16 ± 0.91	2.39 ± 0.67				2.88 ± 1.84	
Erwinia					3.64 ± 1.88					
Pantoea					2.39 ± 0.67					
Enterobacteriaceae; unclassified		5.02 ± 4.62	7.86 ± 3.33	11.75 ± 7.52	25.17 ± 3.37		2.36 ± 1.01	6.75 ± 1.38	4.17 ± 1.24	2.84 ± 1.24
Aquicella	2.91 ± 0.78									
Acinetobacter			5.31 ± 1.06	3.16 ± 0.98	8.63 ± 3.20			7.71 ± 3.67		
Psychrobacter					2.74 ± 0.95					
Pseudomonas	2.51 ± 1.09	3.17 ± 1.04	13.29 ± 3.56	31.70 ± 8.01	26.49 ± 3.43	2.04 ± 0.80	6.57 ± 3.53	22.50 ± 6.22	37.62 ± 6.86	23.33 ± 7.15
Pseudomonadaceae; unclassified									2.34 ± 0.99	
Stenotrophomonas					2.39 ± 0.67					
Xanthomonadaceae; unclassified	2.63 ± 0.89	2.18 ± 1.23		2.08 ± 2.28		2.78 ± 0.68	2.39 ± 0.65	2.02 ± 0.83		

B	***S. oleracea* spring**					***S. oleracea* autumn**				
	Sampling occasion					**Sampling occasion**				
Proteobacteria genera	**O1**	**O2**	**O3**	**O4**	**O5**	**O1**	**O2**	**O3**	**O4**	**O5**
Alphaproteobacteria										
Brevundimonas			2.25 ± 0.41							
Devosia	2.46 ± 0.54									
Methylobacterium			2.07 ± 0.35					2.07 ± 0.74	2.22 ± 0.57	

(*continued on next page*)

Table 4 (*continued*)

B	*S. oleracea* spring					*S. oleracea* autumn				
	Sampling occasion					Sampling occasion				
Proteobacteria genera	**O1**	**O2**	**O3**	**O4**	**O5**	**O1**	**O2**	**O3**	**O4**	**O5**
Microvirga	2.80 ± 0.64	2.83 ± 1.34				2.04 ± 0.34				
Rhizobium								3.21 ± 0.82	2.42 ± 0.68	
Acetobacteraceae; unclassified		2.83 ± 2.58								
Candidatus_Alysiosphaera						2.50 ± 0.87				
Altererythrobacter								2.28 ± 0.44		
Sphingomonas	5.02 ± 0.81	5.38 ± 2.24	4.19 ± 0.72			3.94 ± 0.63	5.73 ± 0.86	7.66 ± 0.90	5.53 ± 1.75	6.44 ± 2.41
Betaproteobacteria										
Comamonadaceae; unclassified	5.29 ± 0.85	8.80 ± 3.79	7.01 ± 1.27	4.69 ± 1.69	3.72 ± 2.82	4.07 ± 0.47	5.29 ± 2.27	5.32 ± 1.86	2.59 ± 0.99	2.35 ± 1.24
Duganella				2.30 ± 1.21						
Massilia	3.87 ± 0.93	5.20 ± 2.98	4.26 ± 0.84	5.36 ± 0.39	3.65 ± 2.68	3.21 ± 0.43	5.07 ± 1.10	6.22 ± 0.77	5.95 ± 1.37	5.14 ± 2.30
Oxalobacteraceae; unclassified	4.55 ± 0.84	4.53 ± 1.49	3.58 ± 0.63	15.22 ± 4.44		5.24 ± 2.40	8.30 ± 3.18	8.23 ± 2.89	6.03 ± 2.93	6.11 ± 1.83
Deltaproteobacteria										
Myxococcales; 0319-6G20; unclassified	2.11 ± 0.83					2.64 ± 1.00				
Haliangium						2.64 ± 0.86				
Gammaproteobacteria										
Shewanella				2.88 ± 0.71	3.07 ± 1.25					
Enterobacteriaceae; unclassified			10.36 ± 3.50	11.94 ± 3.52	29.16 ± 8.36		6.54 ± 4.21	3.99 ± 0.90	9.22 ± 3.63	11.21 ± 3.69
Acinetobacter			7.33 ± 0.81	5.32 ± 2.15	5.67 ± 3.89			3.00 ± 1.30	4.04 ± 2.00	
Pseudomonas		2.84 ± 1.31	11.14 ± 1.59	23.54 ± 4.20	26.22 ± 4.52	3.34 ± 1.56	8.95 ± 1.10	10.14 ± 1.78	30.13 ± 4.15	27.72 ± 6.08
Xanthomonadaceae; unclassified	3.26 ± 0.90					3.02 ± 0.79	2.39 ± 0.97			

relatively large abundances as the leaves entered the processing facility, i.e. low temperatures, were *Aeromonas*, *Acetobacteriaceae*, *Acinetobacter*, *Altererythrobacter*, *Brevundimonas*, *Duganella*, *Erwinia*, *Methylobacterium*, *Pantoea*, *Rhizobium*, *Shewanella*, *Skermanella*, and *Stenotrophmonas*.

Beta diversity metrics showed a clear distinction between the sampling occasions in field (O1 and O2) and sampling occasions occurring after the spinach and rocket had entered the cold chain (O3, O4 and O5). Except for spinach produced in autumn, where O2–O5 were overlapping to a large degree, and washed produce (O3) had a higher similarity to unwashed and stored (O5), than to washed and stored (O4) produce (Fig. 4).

3.3. Core microbiome

The core microbiome of rocket and spinach leaves, based on presence and absence of bacteria present in an abundance of >0.01%, was established to investigate how many of the bacterial OTUs that were present throughout the production cycle, and how many were specific to certain sampling occasions (Fig. 5). For both rocket and spinach, the number of bacterial OTUs present in all sampling occasions were higher during the autumn harvest than the spring harvest. In the autumn, the core microbiome constituted 1.2% for rocket and 1.8% for spinach, while in spring the corresponding figures were 0.7% and 0.8% respectively. In all cases, the number of specific OTUs were higher one week prior to harvest (O1) than in the other occasions. The bacterial core microbiome of rocket and spinach were very similar to each other in both seasons on phylum and genus level: Proteobacteria (from 76% to 85%), Firmicutes (from 9% to 18%), Bacteriodetes (from 4% to 6%), and Actinobacteria (from 1% to 3%). *Pseudomonas* was by far the most commonly occurring genus in both plant species and seasons with a relative abundance of 35%–53%. The two first sampling occasions (O1 and O2), for each plant species and season, shared the largest number of OTUs when all sampling occasions were compared. The highest similarity was between O1 and O2 in the spring production of spinach where 9% of the OTUs were shared. As a comparison, only 0.07% of OTUs were shared between the sampling one week prior to harvest (O1) and the unwashed and stored produce (O5).

4. Discussion

On a macroscale the phyllosphere microbiome is subject to constant changes in both abiotic and biotic conditions from the environment in which the plant is growing. On a microscale the leaf itself is a highly diverse landscape offering an abundance of microclimates which favors the coexistence of a multitude of microorganisms (Leveau, 2006). Several studies have shown that the phyllosphere microbiome is dynamic and subject to change as external conditions are fluctuating, leading to variations in both composition and abundance (Alsanius et al., 2017; Copeland et al., 2015; Darlison et al., 2019a, 2019b; Dees et al., 2015; Ding and Melcher, 2016; Grady et al., 2019; Gu et al., 2018; Jackson et al., 2013; Lopez-Velasco et al., 2011; Luo et al., 2019; Park et al., 2015; Rastogi et al., 2012; Sylla et al., 2013; Williams and Marco, 2014). The novelty of our study is the investigation of the changes that occur in the phyllosphere microbiome of leafy green vegetables and natural contamination events of *E. coli* in the entire production chain, from field to end of storage, in a commercial setting.

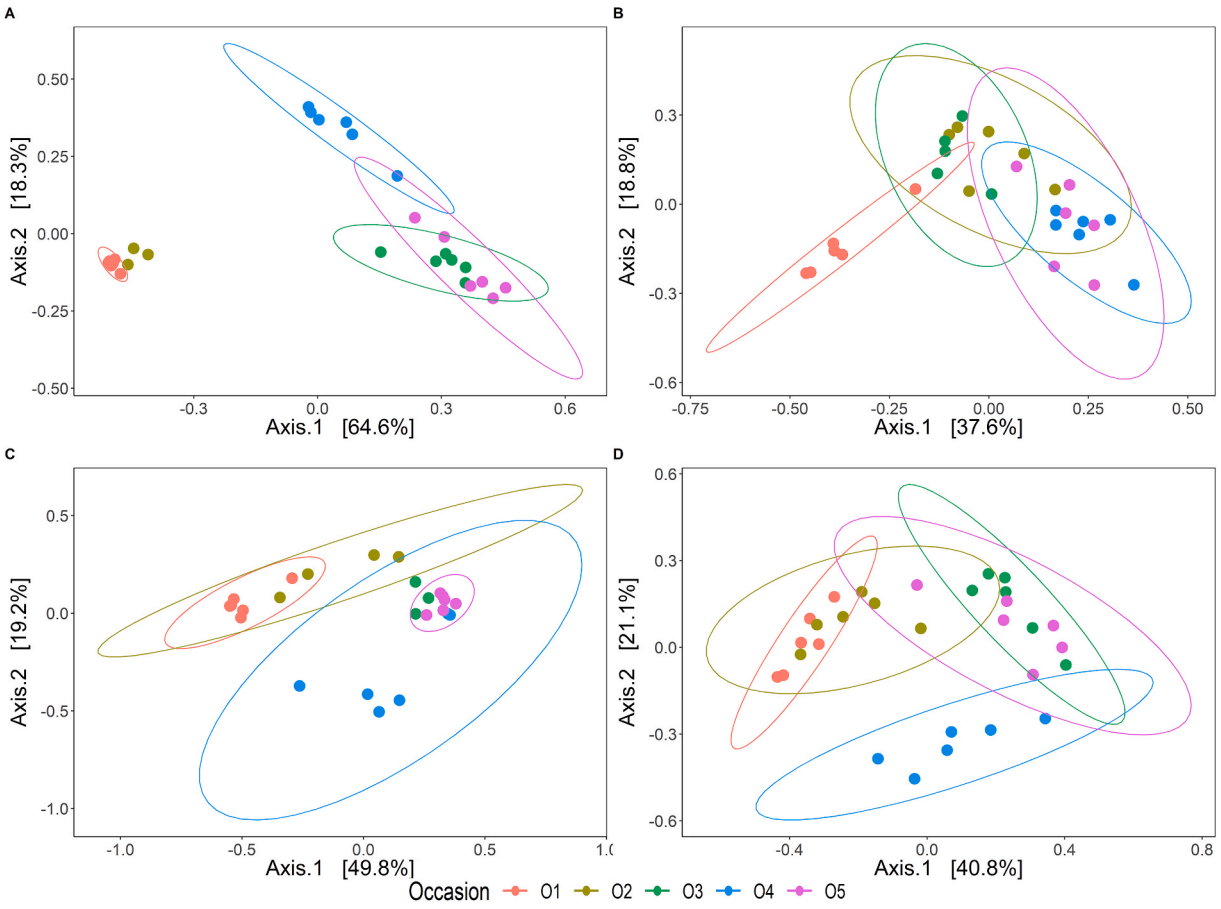

Fig. 4. PCoA plots on beta diversity based on weighted UniFrac distances for 16S rRNA bacterial communities grouped by species and season (spinach spring (A) and autumn (B), rocket spring (C) and autumn (D)) at five different sampling occasions: O1) 1 week prior to harvest, O2) at harvest, O3) after washing, O4) washed and stored, and O5) unwashed and stored.

Washing, with and without sanitizer, is a well-established step in all commercial processing of leafy vegetables. Its main objective is to remove debris, soil particles, animal droppings and pesticide residues from the final product (Gil et al., 2011). The new market of "Ready--to-Eat" products implies that the packaged baby leaf and pre-cut leafy vegetable do not need additional preparation steps before consumption and are of high hygienic quality. Our results indicate that viable counts of selected microbial groups from commercially washed and non-washed leafy produce at the end of shelf life was higher than after harvest at the field site. In several cases, the occurrence of viable counts after wash exceeded microbial numbers in non-washed produce. Furthermore, no significant reduction in viable counts were found between produce at harvest (O1, O2) and after washing (O3). In fact, viable counts of *Enterobacteriaceae* and total aerobic counts increased significantly after the washing step at two occasions. This increase could be a result of contamination from the harvesting machines, and also an indication of the time spent in storage before washing. However, the fact that there was no difference in bacterial load between washed and unwashed leaves at the last sampling occasions remains, i.e., washing had no positive effect on the end result. Other studies investigating microbial load of leafy vegetables have focused on the use of sanitizers in wash water (Baert et al., 2009; Banach et al., 2017; Pezzuto et al., 2016), or on decontamination of the wash water itself (Banach et al., 2015; Turantas et al., 2018; Van Haute et al., 2015). Our finding questions the use of resources for the washing step in a sustainable production chain. It is, however, of importance to mention that according to Swedish legislation, the present washing facility does not use any sanitizer in the wash water.

The EU regulation (EC) No. 2073/2005 restricts acceptable abundance of the index organism *E. coli* to 100–1000 CFU/g with counts <100 CFU/g and >1000 CFU/g as satisfactory and non-satisfactory hygienic quality, respectively. It excludes the presence of *Salmonella* spp. (not detected in 25 g). In the present study, *E. coli* occurred randomly at different steps, and at times, far above of satisfactory quality. Its detection could not be predicted from the previous or later sampling events. Reuse of wash water (Gruden et al., 2016) as well as cross contamination during processing are often named as possible contamination steps (Allende et al., 2008; Holvoet et al., 2012), which supports our finding of high *E. coli* levels after wash. Söderqvist et al. (2019) stated that the probability of infection upon consuming leafy vegetables is 90% lower during spring than autumn. However, in their data prevalence of *Enterobacteriaceae*, which was used as a basis for risk analysis, was considerably lower in spring than autumn samples which biased the outcome. In contrast, the presence of high numbers of *E. coli* could not be attributed to one of the crops nor seasonal effects in the present study.

Based on the metagenomic analysis of leaf inhabiting bacteria using 16S rRNA, harvest and cooling occur to be the main game changers with respect to microbial diversity. This can be explained both with respect to changes in environmental and nutritional conditions. Harvest is the final step of crops' autotrophic lifestyle (Mogren et al., 2018), whereas the postharvest period is a catabolic stage, marked by breakdown of the plant biomass. Along with harvest, but also during washing and packaging, the leaf biomass is injured (Mulaosmanovic, Lindblom, et al., submitted), which may accelerate the breakdown of non-damaged leaves (Ariffin et al., 2017). Leaf surface alterations allow passive release of organic compounds, leading to a nutritionally richer environment, but also a modified landscape for colonization

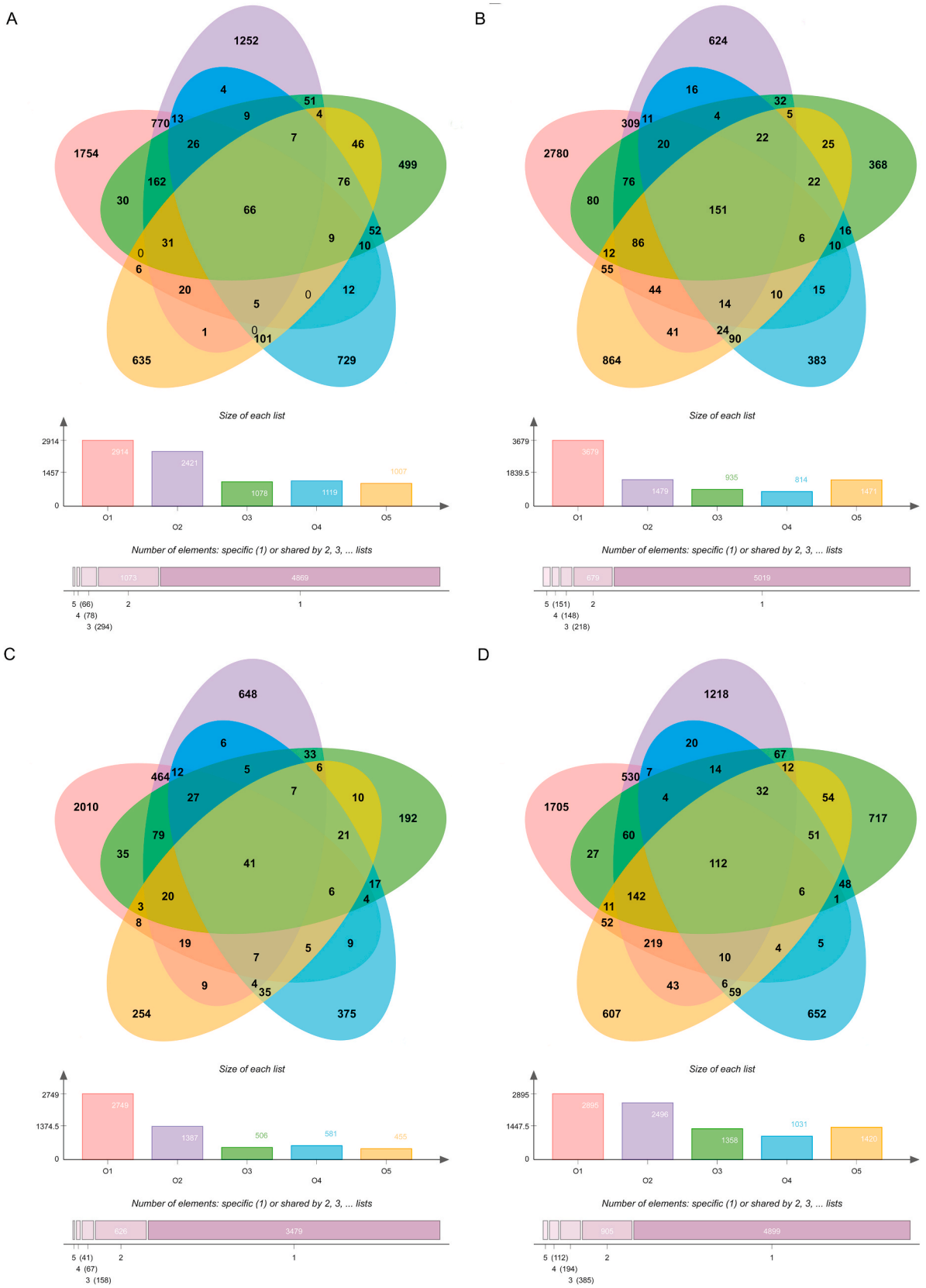

Fig. 5. Venn diagrams of 16S rRNA bacterial communities grouped by species and season (spinach spring (A) and autumn (B), rocket spring (C) and autumn (D)) showing the core microbiome at five different sampling occasions: O1) 1 week prior to harvest, O2) at harvest, O3) after washing, O4) washed and stored, and O5) unwashed and stored.

(Mulaosmanovic, Windstam, et al., submitted). Our results show that the bacterial core biome attributes only to a very small extent to the bacterial metagenome of leafy vegetables at different steps during harvest and postharvest, despite of seasonal variations. Furthermore, alpha diversity is strongly affected by the entrance to the cold chain, displaying lower Shannon index especially for spinach after harvest. However, the change in community composition and diversity appears to be declining over time as well, i.e., leaves have a higher bacterial diversity one week before than at ordinary harvest. The differences may be explained by leaf age. Exudates from young lettuce leaves were shown to be richer in organic C- and N-compounds than middle and older leaves (Brandl and Amundson, 2008). The proportion of larger and physiologically older leaves is higher at ordinary harvest than one week earlier, and may thus induce the transition in diversity. Also, the crop landscape differs, as leaf coverage and interactions within the crop stand changes considerably during the last seven days before harvest.

The largest change in population dynamics occurred as the leaves were harvested and entered the cold chain of the processing factory. Similar to the results reported by Lopez-Velasco et al. (2011), where leaves of spinach were stored in 4 °C for 15 days, the dominance of Proteobacteria and particularly of *Enterobacteriaceae* species and *Pseudomonas* spp. in our study were very distinct. *Pseudomonas* spp. have repeatedly been connected to spoilage of "Ready-to-eat" leafy vegetables (Federico et al., 2015; Lee et al., 2013; Liao et al., 1988), and was by far the most prominent bacteria present in the stored produce.

Cross contamination and leaf damage may explain the differences observed in beta diversity and relative abundance between cold stored materials. Although the bacterial metagenome of cold stored material overlapped with respect to beta diversity, the relative abundance of *Flavobacteriaceae* (mainly *Flavobacterium* and unclassified genera of *Flavobacteriaceae*) was significantly higher in washed than unwashed leaves at expiring date. In contrast, high abundance of taxa within Bacteriodetes (*Exigobacterium* and genera within *Bacillaceae*) were found in the bacterial biome of unwashed leaves. In the processing plant, the main wash water is kept in the basin for approximately 6 h due to regulations on water usage. This wash water could potentially harbor *Flavobacterium* spp. which thrive on soil particles and other residues from the leaves (Kolton et al., 2016). Gu et al. (2018) found that *Flavobacterium* spp. grew rapidly in storage, especially at temperatures above 4 °C. Also, Flavobacteria are well-known for their wide range of extracellular enzyme formation which enables them to digest easily degradable polymers to more recalcitrant biopolymers; specifically metabolism of plant cell wall-associated carbohydrates such as xylose, arabinose and pectin (Kolton et al., 2016). Another bacterium of interest, which occurred in both plant species in both washed and unwashed stored product, is *Acinetobacter*. This bacterium has commonly been associated with food spoilage of cold stored food (Battey and Schaffner, 2001). This, in combination with the increase in damage during processing (Mulaosmanovic, Lindblom, et al., submitted), may be indicators for the proceeding biological turn-over of the leaf biomass, concomitantly causing higher nutrient availability, and change in the abundance of fast growing, opportunistic microorganisms.

The present study was conducted in a large, commercial processing plant, following the Swedish processing regulations. In conclusion, leaf wash without sanitizer combined with cold storage does not lead to the anticipated low bacterial load. Addition of sanitizers might be assumed as an optimal solution for a safer product. However, even with very high sanitizer concentrations the desired effect has not always been achieved (Banach et al., 2015; Pezzuto et al., 2016). From the perspective of wise resource use, it is therefore questionable if washing has an added value for a sustainable food system and measures along the food chain need to be thoroughly reconsidered, *e.g.*, washing closer in time to consumption and reductions in estimated shelf-life. As *E. coli* was detected also after the washing, this step might inhabit more disadvantages than benefits. The main conclusion of this study is that cold storage and washing without sanitizer changes the bacterial diversity and community structure compared to unwashed, favoring the relative abundance of spoilage bacteria of leafy vegetables such as spinach and rocket.

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