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Analysis of the microbiota of refrigerated chopped parsley after treatments with a coating containing enterocin AS-48 or by high-hydrostatic pressure

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ABSTRACT

Parsley can be implicated in foodborne illness, yet chopped parsley is used as an ingredient or garnish for multiple dishes. The aim of the present study was to determine the effect of two different treatments on the bacterial diversity of parsley: (i) coating with a pectin-EDTA solution containing the circular bacteriocin enterocin AS-48, and (ii) treatment by high hydrostatic pressure (HHP) at 600 MPa for 8 min. Control and treated parsley were stored in trays at 5 °C for 10 days. Both treatments reduced viable counts by 3.7 log cycles and retarded growth of survivors during storage. The bacterial diversity of the chopped parsley was studied by high throughput sequencing (Illumina Miseq). Bacterial diversity of control samples mainly consists of *Proteobacteria* (96.87%) belonging to genera *Pseudomonas* (69.12%), *Rheinheimera* (8.56%) and *Pantoea* (6.91%) among others. During storage, the relative abundance of *Bacteroidetes* (mainly *Flavobacterium* and *Sphingobacterium*) increased to 26.66%. Application of the pectin-bacteriocin-EDTA coating reduced the relative abundance of *Proteobacteria* (63.75%) and increased that of *Firmicutes* (34.70%). However, the relative abundances of certain groups such as *Salmonella*, *Shigella* and *Acinetobacter* increased at early storage times. Late storage was characterized by an increase in the relative abundance of *Proteobacteria*, mainly *Pseudomonas*. Upon application of HHP treatment, the relative abundance of *Proteobacteria* was reduced (85.88%) while *Actinobacteria* increased (8.01%). During early storage of HHP-treated samples, the relative abundance of *Firmicutes* increased. Potentially-pathogenic bacteria (*Shigella*) only increased in relative abundance by the end of storage. Results of the present study indicate that the two treatments had different effects on the bacterial diversity of parsley. The HHP treatment provided a safer product, since no potentially-pathogenic bacteria were detected until the end of the storage period.

1. Introduction

Parsley (*Petroselinum crispum* Mill.) is a popular culinary vegetable native to the countries of the Mediterranean region and is widely used as a flavoring and aromatic food additive (Díaz-Maroto, Pérez-Coello, & Cabezudo, 2002; Simon & Quinn, 1988; Zhang, Chen, Wang, & Yao, 2006). Parsley is also used in folk medicine for the treatment of different diseases, and its bioactive constituents exhibit a wide range of pharmacological properties, including antioxidant, hepatoprotective, brain protective, anti-diabetic, spasmolytic, immunosuppressant, anti-platelet, gastroprotective, estrogenic, antibacterial and antifungal activity (Farzaei, Abbasabadi, Reza, Ardekani, & Rahimi, 2013). Consumption of foods containing parsley has been implicated in food-borne illness. A summer outbreak of severe gastroenteritis followed by haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura was reported in children after consumption of sandwiches containing parsley contaminated with verotoxigenic *Cytrobacter freundii* (Tschape et al., 1995). Parsley was also the implicated or suspected source of unrelated restaurant-associated enterotoxigenic *Escherichia coli* (ETEC) outbreaks in Minnesota (Naimi et al., 2003). In each of the outbreak-associated restaurants, parsley was chopped, held at room temperature, and used as an ingredient or garnish for multiple dishes. Parsley was also reported to be the vehicle for *Shigella* species (*Shigella sonney*, *Shigella boydi*) implicated in foodborne shigellosis (Centers for Disease Control and Prevention, CDC, 1999; Chan & Blaschek, 2005; Naimi et al., 2003). Manure and irrigation with contaminated water are main sources for enteric pathogens in fresh produce. Spray irrigation with water containing *Salmonella enterica* serotype Typhimurium (8.5 log CFU/ml) resulted in persistence of the bacteria in the phyllosphere and the rhizosphere for

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at least 4 weeks (Kisluk & Yaron, 2012). Furthermore, *E. coli* O157:H7 persisted for 154 to 217 days in soils amended with contaminated composts and was detected on lettuce and parsley for up to 77 and 177 days, respectively, after seedlings were planted (Islam, Doyle, Phatak, Millner, & Jiang, 2004). Another study on the microbiological quality of fresh produce revealed that total coliforms increased during the packing process in the case of cilantro and parsley (Johnston et al., 2005).

Fresh chopped parsley may have a profitable market in different food sectors (such as hotels, restaurants and catering services) as a ready-to use ingredient for food preparation. However, cutting and manipulation may shorten the shelf life of parsley considerably and at the same time release nutrients that may enhance bacterial growth. Among the different methods tested in food preservation, non-thermal methods such as bacteriocins (Galvez, Lucas, Pérez-Pulido, & Grande-Burgos, 2014), activated coatings (Cagri, Ustunol, & Ryser, 2004; Dhall, 2013), or high hydrostatic pressure (HHP) treatments (Balasubramaniam, Martínez-Monteagudo, & Rockendra, 2015; Yaldagard, Mortazavi, & Tabatabaie, 2008) are gaining interest in the preservation of fresh produce, since they may be considered natural preservation methods or have only a low or no impact on the food properties and small, bioactive molecules present in the food (Balasubramaniam et al., 2015). Enterocin AS-48 is a broad-spectrum circular bacteriocin with a generally-recognised as safe (GRAS) status (Cebrián et al., 2012; Grande-Burgos, Pérez-Pulido, López-Aguayo, Gálvez, & Lucas, 2014). The aim of the present study was to determine the effect of two preservation methods on bacterial diversity of chopped parsley stored under refrigeration. One method was based on the application of enterocin AS-48 incorporated in a pectin coating containing EDTA, and the other was based on application of a HHP treatment. High-throughput DNA sequencing technology (HTS) is becoming increasingly popular since it allows in-depth study of the microbial diversity in food (Ercolini, 2013; Kergourlay, Taminiau, Daube, & Champomier Vergès, 2015). Since there are no previous studies on the microbiota of chopped parsley or how it can be affected by application of food preservation practices, HTS was applied to study the bacterial diversity of chopped parsley before and after application of the preservation treatments.

2. Methods

2.1. Sample preparation, treatments, and microbiological analysis

Parsley (*Petroselinum crispum*, 1 kg) was bought at a local supermarket and cut into 2–3 cm long pieces with sterile scissors on a sterile beaker under aseptic conditions. The cut parsley was thoroughly mixed with a sterile spatula and divided into three batches (each one in duplicate). Batch A served as untreated control. Batch B was coated by immersion for 10 min in a pectin solution containing 1.0% pectin from apples (Sigma-Aldrich, Madrid, Spain), 100 mM EDTA (Sigma-Aldrich), 0.5% acetic acid (Sigma-Aldrich) adjusted to pH 5.0 and 40 μg/ml enterocin AS-48 prepared as described in a previous study (López Aguayo, Grande Burgos, Pérez Pulido, Gálvez, & Lucas, 2016). The pectin solution was prepared as described elsewhere (Jiang, Neetoo, & Chen, 2011; López Aguayo et al., 2016). After the coating treatment, the samples were allowed to drain for 1 min on sterile filter paper in a biosafety cabinet (Telstar, Madrid, Spain). Then the filter paper was replaced and the samples were allowed to dry for one hour in the cabinet. Batch C was placed inside polyethylene polyamide plastic bags containing an acidified saline calcium chloride solution (1% calcium chloride – PanReac, Barcelona, Spain; 0.5% acetic acid and 0.85% NaCl – Sigma-Aldrich). The bags were sealed and treated by high hydrostatic pressure for 8 min at 600 MPa and room temperature by using a Stansted Fluid Power LTD HHP equipment (SFP, Essex, UK) as described elsewhere (Toledo del Árbol et al., 2016a). After application of the HHP treatment, the bags were opened and the samples were drained and allowed to dry on sterile filter paper in a biosafety cabinet as described above. Samples from the three duplicate batches were deposited in 11 × 8 × 5 cm (length, width, height; Bandesur, Jaen, Spain) plastic trays (5 g per tray), and the trays were stored at 5 °C for 10 days. At days 0, 3, 5 and 10, three trays were removed from each batch replicate (six trays in total per batch) for microbiological analysis. For each tray, the content of the tray was transferred to a sterile stomacher bag containing 20 ml of sterile buffered peptone water and pummeled for 3 min in a Stomacher 80 (Seward, Worthing, UK). One aliquot (1 ml) of the homogenate was transferred to a sterile test tube, serially diluted on sterile saline solution and plated in triplicate on trypticase soya agar (TSA, Scharlab, Barcelona, Spain). After incubation at 30 °C for 24 h, the average viable cell counts were calculated for each batch (expressed as log colony forming units - CFU - per gram of sample).

2.2. DNA extraction

Aliquots (5 ml) from the remaining homogenates obtained for the three samples corresponding to each batch replicate as described above were mixed in a sterile 50 ml test tube and centrifuged at 600 × *g* for 5 min in order to remove solids from the vegetable tissue. An aliquot (1.5 ml) of the resulting supernatant was then transferred to an Eppendorf test tube and centrifuged at 13,500 × *g* for 5 min to recover microbial cells. The pellet was resuspended in 0.5 ml sterile saline solution each. Then, Propidium Monoazide (PMA™, Biotium, UK) was added to block subsequent PCR amplification of the genetic material from dead cells (Nocker, Cheung, & Camper, 2006; Nocker, Sossa-Fernandez, Burr, & Camper, 2007) as described by Elizaquivel, Sánchez, and Aznar (2012). DNA from PMA-treated cells was extracted by using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich), following instructions provided by the manufacturer. The resulting DNA from the two batch replicates and same sampling point was pooled into a single sample. DNA concentration and quality were measured with a NanoDrop spectrophotometer (Thermo Scientific, United Kingdom).

2.3. DNA sequencing and analysis

The sequence of the V3–V4 region of 16S rRNA gene was used as the taxonomic basis to estimate bacterial populations present in the samples (Caporaso et al., 2011) using Illumina technology. Library preparation and sequencing was done at the facilities of Fundación Parque Científico de Madrid (Madrid, Spain). The quality of the DNA was determined by agarose gel electrophoresis. Accurate concentration of DNA in the samples was determined using a fluorimetric method with Quant-IT PicoGreen reagent (Thermo Fischer, Madrid, Spain) in a Quantifluor ST fluorometer (Promega, Alcobendas, Madrid). The oligonucleotide primers used for the first PCR reaction were 16SV3-V4-CS1 ACACTGACGACATGGTTCTACACCTACGGGNGGCWGCAG (forward) and 16SV3-V4-CS2 5′ TACGGTAGCAGAGACTTGGTCTGACTACHVGGGTATCTAATCC (reverse), where the underlined regions are the CS1 and CS2 Fluidigm adapter nucleotide sequences, while the non-underline sequences are locus-specific sequences targeting conserved regions within the V3 and V4 domains of prokaryotic 16S rRNA genes (Klindworth et al., 2013). Each PCR reaction contained DNA template (~10–12 ng), 5 μl forward primer (1 μM), 5 μl reverse primer (1 μM), 12.5 μl Q5® High-Fidelity 2 × Master Mix (New England Biolabs, Ipswich, MA, USA), and PCR grade water to a final volume of 25 μl. PCR amplification was carried out as follows: 98 °C × 30 s, 20 cycles of 98 °C × 10 s, 50 °C × 20 s, 72 °C × 20 s, then 72 °C × 2 min and held at 4 °C. PCR products were visualized using agarose gel electrophoresis. Successful PCR products were cleaned using AMPure XP magnetic bead based purification (Beckman Coulter, Brea, CA, USA). Afterwards, a second PCR was applied under the same conditions as above (except that only 8 cycles were completed) to add the individual barcode to

each of the samples, as well as to incorporate Illumina-specific sequences in the amplicon libraries. Individual libraries were analyzed using a Bioanalyzer 2100 (Agilent, Madrid) to estimate the concentration of the specific PCR products and a pool of samples was made in equimolar amounts. The pool was further cleaned with AMPure XP magnetic beads, and the exact concentration of the library was measured by real time PCR using Illumina specific primers (Kapa Biosystems, Wilmington, MA, USA). Paired-end sequencing of the library was performed on an Illumina MiSeq sequencer (San Diego, CA, USA) using the MiSeq Reagent Kit (v3) with the longest read length set to 2 × 300 base pairs (bp). After demultiplexing, paired end reads were joined together with the fastq-join program (https://expressionanalysis.github.io/ea-utils/). Only reads that had quality value (QV) scores of ≥ 20 for > 99% of the sequence were extracted for further analysis. All sequences with ambiguous base calls were discarded. After filtering, sequence reads were assigned to operational taxonomic units (OTUs) based on sequence similarity for each read to 16S rRNA genes from the NCBI nt database by using BLASTN function (Era7 Bioinformatics, Granada, Spain). Each read was assigned to the taxon corresponding to the Best Blast Hit over a threshold of similarity (e < 1E-15). The sequencing output files were deposited in the Sequence Read Archive (SRA) service of the European Bioinformatics Institute (EBI) database under Accession Number PRJEB20528.

2.4. Statistical analysis

Data on viable cell counts for the different treatments and storage times were analyzed with two-way ANOVA (Microsoft Excel). The Shannon Wiever (H′) and Simpson (D) biodiversity indexes were calculated with Excel programme. Data on OTUs with relative abundances ≥ 1.5% obtained for the different treatments and storage times were analyzed by principal component analysis (PCA) with Pearson correlation coefficient (r; $P < 0.05$) (XLSTAT 2014 evaluation version (2014.1.03, Addinsoft, France). Correlations were defined as very weak (0.00–0.19), weak (0.20–0.39), moderate (0.4–0.59), strong (0.60–0.79) or very strong (0.80–0.99).

3. Results

3.1. Effect of treatments on microbial load

Viable cell counts in the control samples increased non-significantly ($P > 0.05$) from 6.3 to 6.8 log CFU/g at day 3, but then increased significantly ($P < 0.05$) during the remaining storage period, reaching 9.4 log CFU/g at day 10 (Table 1). Application of the pectin-bacteriocin-EDTA solution reduced viable cell counts significantly ($P < 0.05$) by 3.7 log cycles compared to the untreated controls. During storage of the coated samples, viable counts remained low for the first days, and only increased significantly ($P < 0.05$) by day 10, reaching 4 log CFU/g. Nevertheless, viable counts in the coated samples were always significantly lower ($P < 0.05$) at all storage times compared to untreated controls. The samples treated by HHP also showed a significant ($P < 0.05$) reduction of 3.7 log cycles at time 0. Furthermore, viable counts did not increase significantly ($P > 0.05$) during the first 7 days of storage, although they reached a significantly ($P < 0.05$) higher concentration of 4.9 log CFU/g at day 10. Viable counts obtained for the HHP treatment were significantly lower ($P < 0.05$) compared to untreated controls at all storage points, but they were only significantly different ($P < 0.05$) than the samples coated with the pectin-bacteriocin-EDTA solution at day 10 of storage.

Table 2
Number of sequences (reads) and observed diversity for 16S rRNA amplicons analyzed in this study.

Sample	N° of reads	Shannon-Wiener index (H′)	Simpson index (D)
C0	85,124	3.28	0.91
C3	121,350	3.46	0.92
C5	121,959	3.87	0.96
C10	185,489	3.94	0.96
H0	58,520	3.95	0.95
H3	16,478	1.92	0.49
H5	99,269	3.17	0.91
H10	85,054	3.51	0.99
PB0	47,453	2.77	0.85
PB3	59,028	1.94	0.68
PB5	90,251	3.58	0.93
PB10	104,288	3.68	0.95

3.2. Changes in bacterial diversity after treatments and during storage

The number of filtered reads ranged from 24,965 to 199,585 (Table 2). Most OTUs recovered from control samples at time 0 (96.87%) belonged to *Proteobacteria* (Fig. 1A). These were followed in relative abundance by *Bacteroidetes* (1.89%), *Firmicutes* (0.77%) and *Actinobacteria* (0.45%). At genus level (Fig. 1B), *Proteobacteria* were represented mainly by OTUs belonging to genus *Pseudomonas* (69.12%), followed by *Rheinheimera* (8.56%), *Pantoea* (6.91%) and sequences belonging to an unidentified Gamma proteobacterium (5.96%). During storage of controls samples, the relative abundance of *Proteobacteria* decreased while *Bacteroidetes* increased (reaching 26.66% at day 10). At genus level, there was a marked decrease in the relative abundance of *Pseudomonas* (down to 30.41% at day 10) and an increase in the relative abundances of other groups such as *Rheinheimera*, *Flavobacterium* and *Sphingobacterium*.

The changes in bacterial diversity observed after HHP treatment involved a reduction in the relative abundance of *Proteobacteria* (85.88%) and an increase of *Actinobacteria* (8.01%) (Fig. 1A). There was also a remarkable change at genus level (Fig. 1B). The relative abundance of *Pseudomonas* was reduced to 21.05% after HHP treatment, while several other genera (*Enterobacter*, *Sphingomonas*, and others) increased. The microbiota of the treated samples also changed during storage. *Proteobacteria* decreased further at day 3, while several groups of *Firmicutes* (*Paenibacillus*, *Enterococcus*, Uncultured low G + C

Table 1
Viable counts (total aerobic mesophiles) of control parsley samples and samples treated by high hydrostatic pressure (HHP) or coated with pectin plus bacteriocin (PB). Samples were stored at 5 °C.

	Viable counts (Log_{10} CFU/g ± SD) at different storage times (days)			
Treatment	0	3	7	10
Control	6.3 ± 0.05	6.8 ± 0.09	7.8 ± 0.09[a]	9.4 ± 0.06[a]
HHP	2.6 ± 0.29[b]	2.1 ± 0.10[b]	3.2 ± 0.04[b]	4.9 ± 0.09[a–c]
PB	2.6 ± 0.07[b]	2.3 ± 0.42[b]	3.4 ± 0.24[b]	4.0 ± 0.52[a,b]

SD, standard deviation.
[a] Counts were significantly higher ($P < 0.05$) compared to their respective time 0 sample value.
[b] Counts were significantly lower ($P < 0.05$) compared to all sample values of the untreated controls.
[c] HHP counts were significantly higher ($P < 0.05$) compared to counts from PB treatment at day 10.

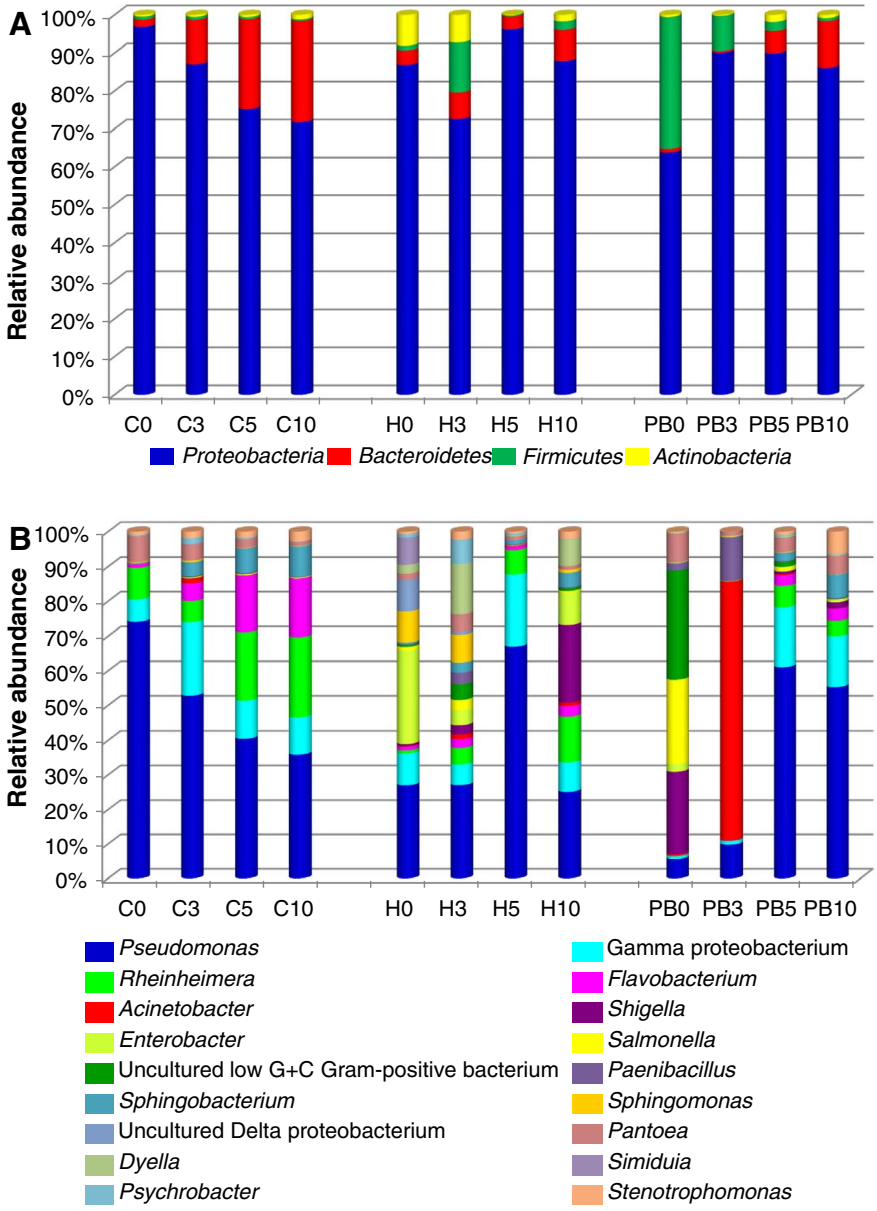

Fig. 1. Relative abundance of OTUs based on paired-end 16S rRNA gene sequencing analysis of DNA from parsley. Controls (C), samples treated with a pectin-bacteriocin-EDTA (PB) coating containing enterocin AS-48 and samples treated by high-hydrostatic pressure (H) were stored at 5 °C. Sampling was performed at days 0, 3, 5 and 10. OTUs were sorted by *Phylum* (A) or *Genus* (B).

Gram-positive bacterium, and others) increased. However, the relative abundance of *Proteobacteria* increased again during late storage. This involved mainly OTUs for *Pseudomonas*, Gamma proteobacterium and *Rheinheimera* at day 7. At day 10, however, there was a decrease in the relative abundance of *Pseudomonas* and an increase of the Enterobacteria *Shigella* (18.87%), *Enterobacter* (7.90%) and *Serratia* (2.80%).

Application of the pectin-bacteriocin-EDTA coating induced great changes in bacterial diversity of the samples at early storage. At time 0, the relative abundance of *Proteobacteria* was reduced to 63.75% while *Firmicutes* increased to 34.70% (Fig. 1A). OTUs for genus *Pseudomonas* were reduced to 5.01%, while OTUs from other *Proteobacteria* (mainly *Salmonella* and *Shigella* and to a less extent *Pantoea*) and *Firmicutes* (Uncultured low G + C Gram-positive bacterium and *Enterococcus*) increased in their relative abundances (Fig. 1B). This was a transient change, since OTUs for the Enterobacteria decreased below 1% by day 3, a storage point where *Acinetobacter* became the main OTU (70.60%) among *Proteobacteria* followed by *Paenibacillus* (11.68%) among *Firmicutes*. During late storage, there was a remarkable increase in the relative abundances of OTUs belonging to *Pseudomonas* and Gamma proteobacterium (and to a less extent also for other genera such as *Stenotrophomonas*, *Erwinia*, *Duganella* and *Janthinobacterium*) and also a higher relative abundance of *Bacteroidetes* represented mainly by *Chryseobacterium*, *Sphingobacterium* and *Flavobacterium*.

Calculation of biodiversity indexes indicated at decrease in both Shannon-Wiever and Simson's indexes after application of the pectin-bacteriocin-EDTA coating or the HHP treatment followed by an increase in both index values during prolonged storage in all cases (Table 2). For the control samples, principal component analysis (PCA) of the data at genus level revealed highest positive correlations between samples from early storage (C0 and C3) and between samples from late storage (C5, C10) as expected from the observed changes in microbial composition (Table 3, Fig. 2A). Samples from HHP treatment taken at time 0 (H0) showed the lowest positive correlations with HHP-treated samples H3 and H5, and did not show any positive correlation ($P > 0.05$) with H10 samples (Table 3, Fig. 2B). These results can be related with the changes in the microbial composition occurring in the

Table 3
Correlations between controls and samples treated by HHP or coated with pectin bacteriocin (PB) at different storage times.

Variables	C0	C3	C5	C10	H0	H3	H5	H10	PB0	PB3	PB5	PB10
C0	**1**											
C3	**0.9504**	**1**										
C5	**0.8725**	**0.8906**	**1**									
C10	**0.8058**	**0.8316**	**0.9906**	**1**								
H0	**0.6130**	**0.5966**	0.4521	0.3884	**1**							
H3	**0.8518**	**0.8051**	**0.6909**	**0.6207**	**0.5466**	**1**						
H5	**0.9711**	**0.9917**	**0.8773**	**0.8148**	**0.6282**	**0.8343**	**1**					
H10	**0.6592**	**0.6440**	**0.6509**	**0.6328**	0.4655	**0.5977**	**0.6695**	**1**				
PB0	− 0.0243	− 0.0898	− 0.1615	− 0.1904	− 0.1786	− 0.0490	− 0.0554	0.1543	**1**			
PB3	0.0360	0.0260	− 0.0409	− 0.0605	− 0.1021	− 0.0698	0.0283	− 0.0966	− 0.1464	**1**		
PB5	**0.9794**	**0.9916**	**0.8845**	**0.8201**	**0.6185**	**0.8367**	**0.9977**	**0.6662**	− 0.0313	0.0211	**1**	
PB10	**0.9727**	**0.9845**	**0.8786**	**0.8167**	**0.5992**	**0.8187**	**0.9852**	**0.6619**	− 0.0628	0.0042	**0.9904**	**1**

Significant correlations at $P < 0.05$ are highlighted in bold.

HHP-treated samples during storage. H0 samples also had no positive correlation with control samples from late storage (C5, C10), but they still had significant positive correlations ($P < 0.05$) with control samples from early storage (C0, C3). The samples treated with the pectin-bacteriocin-EDTA coating from storage days 0 and 3 (PB0, PB3) showed no positive correlation with any of the other samples (Table 3, Fig. 2C,D), in agreement with the great changes induced by this treatment on the bacterial diversity of the samples. By contrast, the coated samples from late storage (PB5, PB10) had very strong positive correlation ($P < 0.05$) between them and also had positive correlations that were strong or very strong with control samples and with the samples treated by HHP (Table 3, Fig. 2C,D). These results would suggest a late recovery of the initial microbiota from the treatment with pectin-bacteriocin-EDTA.

4. Discussion

According to results from the present study, chopped parsley had a high microbial load that was able to proliferate during refrigerated storage, reaching high cell concentrations and leading to food spoilage. A previous study reported microbial loads comprised between 3 and 5 log CFU/g for parsley from retail stores in Warsaw (Poland) (Garbowska, Berthold-Pluta, & Stasiak-Różańska, 2015). Only one batch of parsley was used for the present study. Keeping in mind that differences in microbial load and microbial composition are expected to occur between different batches, for the present study it was important to focus on a single batch in order to compare the effects of the different treatments on bacterial diversity and on the changes occurring during storage. The bacterial microbiota of the studied

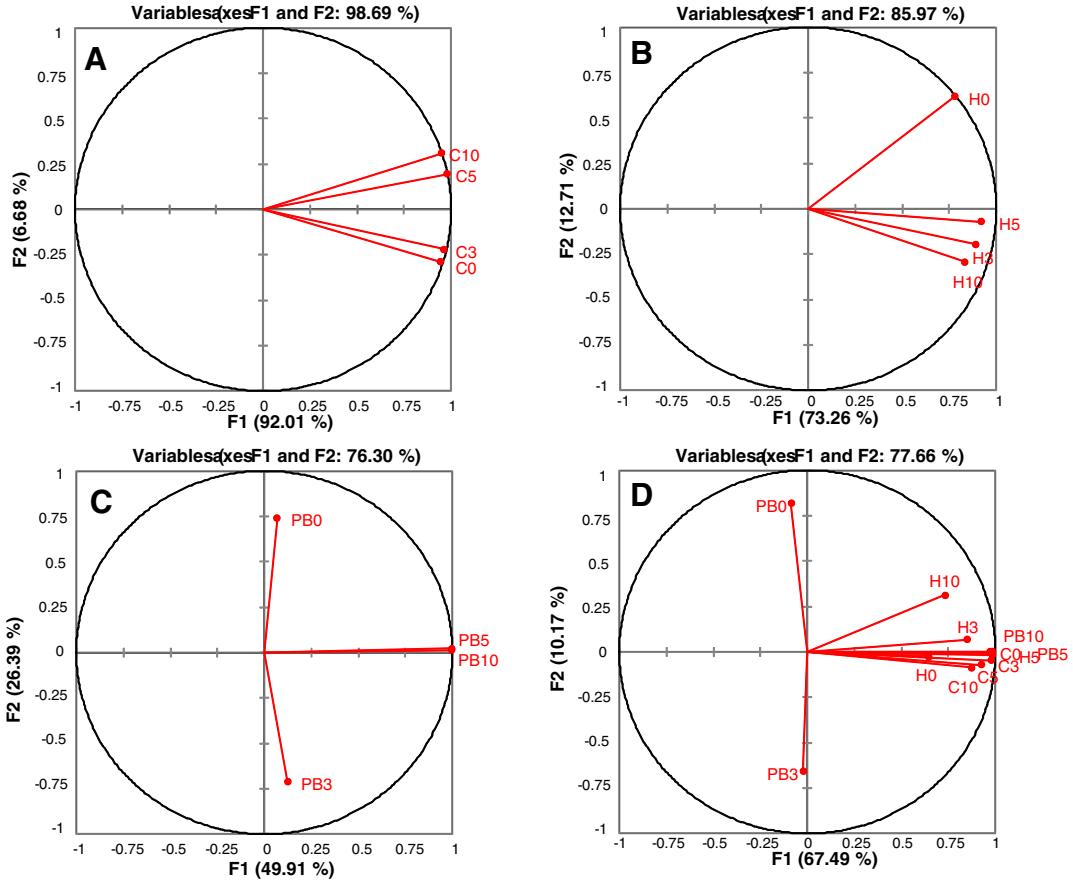

Fig. 2. Principal component analysis of sample variables (storage time and treatments) of parsley. A, untreated controls (C). B, samples treated with high hydrostatic pressure (H). C, samples treated with a pectin-bacteriocin-EDTA (PB) coating containing enterocin AS-48. D, all samples. The numbers indicate storage time (days).

parsley was composed mainly of *Proteobacteria* of genus *Pseudomonas*. This bacterial group has also been reported to be highly represented in the microbiota of other vegetable foods (Lee et al., 2013; Pérez Pulido, Toledo, Grande, Gálvez, & Lucas, 2015; Toledo del Árbol et al., 2016b). During refrigerated storage, the main change in the bacterial diversity detected in control samples involved a decrease in the relative abundance of *Pseudomonas* and an increase in the relative abundance of other plant-associated bacteria of the phylum *Bacteroidetes*, mainly *Flavobacterium* and *Sphingobacterium*. The changes in relative abundance of bacterial groups and the succession of bacterial communities during storage of parsley could be explained in terms of ammensalism and nutrient availability. An early predominance of *Pseudomonas* could result in rapid exhaustion of nutrients for this bacterial group and/or accumulation of toxic metabolic end products. This situation could lead to decrease of *Pseudomonas* populations and proliferation of other bacteria capable of using other alternative nutrients available and/or being more tolerant to toxic metabolic products.

Application of a pectin-bacteriocin-EDTA coating reduced viable counts and delayed bacterial growth till the end of storage. Enterocin AS-48 acts on the bacterial cytoplasmic membrane and is active mainly on Gram-positive bacteria (Grande-Burgos et al., 2014). Its inhibitory spectrum can be extended to Gram-negative bacteria in combination with outer-membrane disturbing agents such as EDTA (Ananou, Gálvez, Martínez-Bueno, Maqueda, & Valdivia, 2005). Furthermore, EDTA has been reported to inhibit bacterial growth by depriving microorganisms from essential growth factors such as Mg^{2+}, Ca^{2+}, and Fe^{2+} and thus inhibiting many enzymes that need these cations as cofactors (Banin, Brady, & Greenberg, 2006). Therefore, it would be expected that the combined treatment would reduce a microbial load composed mainly of Gram-negatives. However, the impact of the combined treatment on the different members of the bacterial community in the treated parsley could be influenced by many different factors including the degree of sensitivity to the bacteriocin-EDTA combination, the capacity of the different bacteria to grow inside the plant tissue (where diffusion of the bacteriocin would be expected to be very limited compared to the plant surface) or the inactivation of bacteriocin molecules by microbial and/or tissue proteases, among other factors. This could explain the succession of bacterial communities during storage of the treated samples. An early effect of the coating treatment was a marked reduction of the relative abundance of *Pseudomonas* while other bacterial groups (particularly *Salmonella*, *Shigella* and an uncultured low G + C Gram-positive bacterium) increased transiently. Although *Salmonella* and *Shigella* were rapidly displaced by other groups (mainly *Acinetobacter*) by day 3 and were not detected during further storage, there is a concern that they may cause foodborne illness. Enteric microorganisms have developed several inducible mechanisms for surviving transient acid stress (Chan & Blaschek, 2005; Foster, 2000; Foster & Moreno, 1999). Since the coating used in the present study was acidified to pH 5.0 with acetic acid, we could suggest a higher tolerance of enteric bacteria to other stress conditions in the tested food after induction of acid stress.

The drastic change in bacterial diversity observed in the coated samples at day 3 involved a remarkably high increase in the relative abundance of OTUs for *Acinetobacter*. Based on the capacity of this bacterium to use acetate as the sole carbon source, Baumann (1968) developed a minimal medium acidified with acetate for enrichment and isolation of *Acinetobacter* from soil and water. OTUs for *Acinetobacter* had a very low relative abundance of 1.23% in the untreated controls. It is tempting to suggest that the acetate used in the coating could have selected for *Acinetobacter* in the treated samples. In addition, transient inhibition of competitors (e.g., *Pseudomonas*) by the activated coating could have provided an advantage for proliferation of *Acinetobacter*. Previous studies have reported the isolation of *Acinetobacter* spp. from vegetables (Berlau, Aucken, Houang, & Pitt, 1999; Houang et al., 2001; Jackson, Randolph, Osborn, & Tyler, 2013; Rafei et al., 2015). Furthermore, pyrosequencing studies revealed the presence of *Acinetobacter* spp. as endophyte in tomato leaves (Romero, Marina, & Pieckenstain, 2014). While the majority of *Acinetobacter* species are nonpathogenic, environmental organisms, those species adapted to clinical environments are now causing serious health problems (Wong et al., 2017). Further studies based on challenge tests needed to be carried out in order to determine the influence of treatments based on activated coatings on survival and proliferation of *Acinetobacter* on parsley and other vegetable foods.

During late storage (days 5 and 10), the bacterial composition of samples treated with the pectin-bacteriocin-EDTA coating resembled that of untreated controls at early storage times (day 0 and day 3). These results would suggest recovery of the initial microbiota after a transient period of inhibition by the activated coating. Dilution of the coating with the vegetable tissue fluid, consumption of acetate and possible inactivation of the bacteriocin by proteases could account for loss of the inhibitory effects of the coating during late storage.

Application of HHP treatment on parsley achieved a similar reduction in viable cell counts as the activated coating, and also delayed microbial growth during refrigerated storage. The HHP treatment was applied under the same acidic conditions (0.5% acetic acid) as the activated coating. However, HHP treatment had a different effect on the bacterial populations in the parsley compared to the activated coating. Since pressure distribution in the food samples is homogeneous, the effect of HHP treatment would be independent of the endophytic or epiphytic location of the microbiota. Furthermore, not all bacterial groups are equally sensitive to pressure treatment or may not have the same capacity to repair HHP-induced sublethal cell damage in the environment of the tested food. While *Pseudomonas* had the greatest reduction in relative abundance after application of the HHP treatment, other bacterial groups typically associated with plants or soil (such as *Sphingomonas*, *Rahnella* or *Dyella*) seemed to prevail, suggesting that they were less sensitive to the HHP treatment applied on parsley. The population of *Pseudomonas* seemed to recover by day 5 as shown by the increase in its relative abundance, but then decreased again during late storage possibly due to nutrient limitation and/or competition by other bacterial groups. Compared to the activated coating, the main Enterobacteria detected in the HHP-treated samples was *Enterobacter*. The presumptive pathogenic *Salmonella* and *Shigella* were not detected or they had very low relative abundances in the HHP-treated samples, except for *Shigella* at day 10 of storage. Data from late storage suggest that Enterobacteria were one of the main bacterial groups increasing in relative abundance in the HHP-treated samples when the population of *Pseudomonas* decreased.

There are no previous studies on application of HHP treatments for preservation of parsley. From the microbiological point of view, the results from the present study suggest that HHP could be applied to reduce the microbial load of chopped parsley. There is a growing interest in using HHP treatments for decontamination of fresh produce, as exemplified by a study on application of this technology for decontamination of green onions from *Salmonella* and *Escherichia coli* O157:H7 (Neetoo, Nekoozadeh, Jiang, & Chen, 2011). Nevertheless, further work based on challenge tests with specific pathogenic strains is needed to assess the efficacy of using HHP for inactivation of foodborne pathogens in parsley.

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