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The live bacterial load and microbiota composition of prepacked "ready-to-eat" leafy greens during household conditions, with special reference to *E. coli*

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ABSTRACT

Ready-to-eat (RTE) leafy greens are popular products that unfortunately have been associated with numerous foodborne illness outbreaks. Since the influence of consumer practices is essential for their quality and safety, the objective of this study was to analyze the microbiota of RTE products throughout shelf life during simulated household conditions. Products from different companies were analyzed in terms of plate counts, and resealed and unopened packages were compared. High bacterial loads were found, up to a total plate count of 9.6 $\log_{10}$ CFU/g, and *Enterobacteriaceae* plate counts up to 6.0 CFU/g on the expiration date. The effect of consumer practice varied, thus no conclusions regarding resealed or unopened bags could be drawn. The tested products contained opportunistic pathogens, such as *Enterobacter homaechei*, *Hafnia paralvei* and *Pantoea agglomerans.* Amplicon sequencing revealed that the relative abundance of major taxonomic groups changed during shelf life; *Pseudomonadaceae* and *Xanthomonadaceae* decreased, while *Flavobacteriaceae* and *Marinomonadaceae* inceased. Inoculation with *E. coli* CCUG 29300^T showed that the relative abundance of *Escherichia-Shigella* was lower on rocket than on other tested leafy greens. Inoculation with *E. coli* strain 921 indicate growth at the beginning of shelf-life time, while *E. coli* 731 increases at the end, seemingly able to adapt to cold storage conditions. The high levels of live microorganisms, the detection of opportunistic pathogens, and the ability of *E. coli* strains to grow at refrigeration temperature raise concerns and indicate that the shelf life may be shortened to achieve a safer product. Due to variations between products, further studies are needed to define how long the shelf-life of these products should be, to ensure a safe product even at the end of the shelf-life period.

1. Introduction

Every year, over 9 million people suffer from foodborne illness in the US alone, and during 1998–2008 leafy-green vegetable products were the largest single vector for such illnesses (22 %) (Painter et al., 2013). In Europe, EFSA has ranked leafy greens at the top of the list of non-animal food vectors of food-borne illnesses (EFSA, 2013). Leafy green products are often eaten raw, and contamination of pathogenic bacteria can occur throughout the whole production chain. Ready-to-eat leafy green products are subjected to processing before packaging, including cutting, which enhances bacterial growth by creating surfaces on which bacteria can attach, grow and internalize (Allende et al., 2006; Gleeson and O'Beirne, 2005; Solomon and Matthews, 2005). Once the leaves are contaminated, the pathogen can spread across batches and cannot be eliminated unless heated (Allende et al., 2003; Doyle and Erickson, 2008; Jensen et al., 2015; Uhlig et al., 2017).

Ready-to-eat leafy green vegetables are often packed with modified atmosphere (MAP) that contains a mixture of 0.5–3 % oxygen, 5–15 % CO_2 and the rest N_2 (Oliveira et al., 2015) in order to suppress oxidation and prolong shelf-life, an economically beneficial strategy. . However, microorganisms, pathogens included, may still multiply in this environment. As a consequence, MAP removes the sensory signs of spoilage and creates aa concealed risk for the consumer (Farber, 1991; Luo et al., 2010).

The culturable microbiota after processing usually range from 3.0 to 6.0 $\log_{10}$ CFU/g, and frequently occurring are families of *Pseudomonadaceae* and *Enterobacteriaceae* (Ragaert et al., 2007), as are genera of *Arthrobacter*, *Acinetobacter*, *Chryseobacterium*, *Flavobacterium* and

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Pantoea (Dees et al., 2015; Jackson et al., 2013). De Giusti et al. (2014) saw differences in total mesophilic and *E. coli* counts of RTE leafy green products depending on post-harvest practices. At the first day of the shelf life time, 54.8 % of the products had counts exceeding $\log_{10}$ 7.0 CFU/g, the reference standard for total mesophilic count for ready-to-eat salads proposed by PHLS Advisory Committee for Food and Dairy Products in the UK F. Arienzo et al. (2020) discovered high aerobic counts (above 10^6 CFU/g) after the second day after opening packages of romaine lettuce, rocket and lamb's lettuce, and surprisingly high prevalence of *Salmonella*.

Data from 16S amplicon sequencing show that the microbial composition of leafy greens is dominated by the phylum Proteobacteria (up to 90 %), followed by Firmicutes, Actinobacteria and Bacteriodetes. The consistently dominant genera seem to be *Pseudomonas* (Jackson et al., 2013; Lopez-Velasco et al., 2011; Tatsika et al., 2019; Williams et al., 2013). During cold storage, a decrease in community richness has been observed, and species of well-known psychotrophs seem to be favored, such as certain members of the *Enterobacteriaceae* family, and the genera *Pseudomonas*, *Methylobacterium* and *Pantoea* (Lopez-Velasco et al., 2011; Tatsika et al., 2019).

The microbial composition may also interact with invading pathogens. Söderqvist et al. (2017) saw that after inoculation and storage with *Y. enterocolitica* and L. *monocytogenes*, relative abundances of Enterobacteriales and Bacillales increased. Williams et al. (2013) saw that lettuce plants that contained *E. coli* O157:H7 after irrigation had lower aerobic plate counts and a distinctly different bacterial diversity.

Household conditions such as storage temperature and consumer practices are crucial components for the safety of ready-to-eat vegetable products. However, it remains unclear how consumer practices, such as consuming only a part of the content and then resealing the package and storing it in the fridge for a few days, may affect pathogen multiplication and the validity of the stated shelf-life period. The atmosphere in the package will change after opening, and it is not known what effects this may have on the microbiota of the product. Moreover, pathogen growth on these produces have often been studied with type strains (Gleeson and O'Beirne, 2005; Luo et al., 2010; Söderqvist et al., 2017; Williams et al., 2013), but it remains unclear how potentially pathogenic wild strains originally found on leafy greens behave during household conditions.

This study combines viable cell count and colony identification with next generation sequencing in order to evaluate the microbial hygienic status in retailed ready-to-eat leafy green products, with and without modified atmosphere packaging (MAP). The aim was to study bacterial load and community composition during simulated household conditions. The evaluation was based on consumer practices such as opening and closing of ready-to-eat lettuce packages, and cold storage throughout the shelf life time. Additionally, microbiota changes after inoculation of three different *E. coli* strains (type strain and two wild strains) are followed during the same conditions. This study provided information on the microbial development and quality at the point of consumption, and how it depends on consumer practices, which is essential to the safety of these products.

2. Materials and methods

The study follows three independent trials, each involving different product batches:

i. Culture-dependent analysis of the native microbiota of leafy green products from different companies, stored under household conditions
ii. Culture-dependent analysis of the microbiota of rocket, inoculated with three different *E. coli* strains
iii. Culture-independent analysis of the microbiota of leafy green products, both uninoculated and inoculated with two different *E. coli* strains.

2.1. Sampling and E. coli inoculation for culture-dependent analysis

Packages (á 65–70 g) of rocket (*Eruca vesicaria*, conventionally grown and organic), spinach (*Spinacia oleracea*) and a mixed leafy green product containing friseé (*Cichorium endivia* var. *crispum*), red lettuce (*Lactuca sativa* var. *crispa*) and red mangold (*Beta vulgaris*), were chosen from four different Sweden based processing and packaging companies (Table 1). The varieties chosen for analysis represent the dominating leafy greens in commercially available products. Rocket from company A and from company C were both grown in Sweden, and the other product batches were imported from Italy. Mixed leafy greens from company D was the only product packed in modified atmosphere (MAP). The products were purchased at local supermarkets in south Sweden and brought directly to the laboratory. For each product batch, 18 packages were bought simultaneously on the day of delivery (first day of the shelf life time) and stored at 4 °C. Eight products with varying shelf-life times (as stated on the package) were analyzed according to the sampling scheme in Fig. 1, designed to simulate household practices such as opening the bag, taking out a portion and closing it again before putting it back into the fridge. Packages were fully opened before sampling to ensure sufficient atmosphere exchange. Six packages were used on each sampling occasion for each product, and one sample was taken per package.

For *E. coli* inoculation, packages of a rocket batch from company D (randomly selected) were injected separately with *E. coli* CCUG 29300^T, and two wild strains isolated from romaine lettuce, *E. coli* 731 and *E. coli* 921 respectively. On the day of delivery, 300 µl of *E. coli* was injected to each bag (to generate10^6 CFU/g leafy greens as described by Gleeson and O'Beirne (2005)) and shaken carefully to mix as previously performed by Gleeson and O'Beirne (2005). The samples were stored and sampled as described in Fig. 1. Samples from the first six packages were taken out prior to inoculation to check for inherent *E. coli* presence.

2.2. Sampling and E. coli inoculation for culture-independent analysis

For next generation sequencing (NGS), 18 packages (á 65 g) from company C, of rocket, spinach and a mixed leafy green product containing green Batavia (*Lactuca sativa* var. *capitata crispum*), Tatsoi (*Brassica rapa* var. *narinosa*) red lettuce and rocket respectively, were sampled according to the scheme in Fig. 2. DNA of samples from six packages of each product type were immediately extracted and stored at −18 °C. Three packages of each type were inoculated with *E. coli* CCUG 29300^T and three packages with *E. coli* 731 wild strain as described in Section 2.1, and stored at 4 °C until the expiration date (day 7). The remaining 6 packages per type were stored unopened at 4 °C until the expiration date.

Table 1
Four different leafy green product types from four different companies were included in this study. Products were analyzed with viable cell count and next generation sequencing (NGS), and inoculated with three different strains of *E. coli*.

Identification method Inoculation strain	Viable cell count and Sanger Sequencing	Next Generation Sequencing
Uninoculated	Mix[c, d], rocket[a,c,d], organic rocket[c], spinach[b,d]	Mix[c], rocket[c], spinach[c]
E. coli CCUG 29300^T	Rocket[d]	Mix[c], rocket[c], spinach[c]
E. coli 731	Rocket[d]	Mix[c], rocket[c], spinach[c]
E. coli 921	Rocket[d]	

[a] Product packaged by company A.
[b] Product packaged by company B.
[c] Product packaged by company C.
[d] Product packaged by company D.

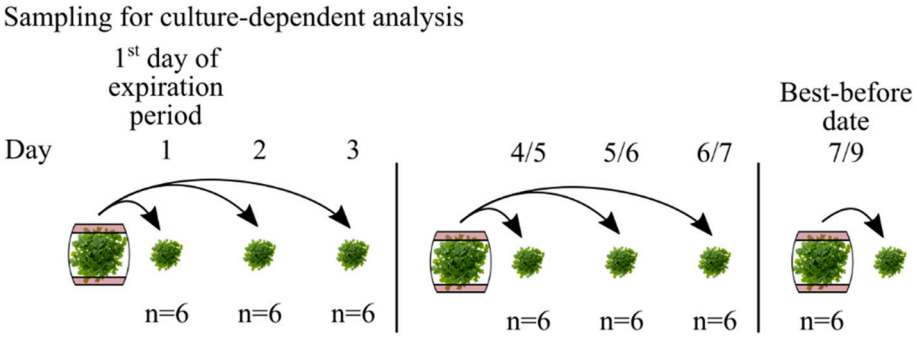

Fig. 1. Sampling scheme for culture-dependent analysis. On the first day of the shelf life time, 18 packages of ready-to-eat leafy greens were purchased. Six packages were opened on the first day, and the packages were then resealed, stored at 4 °C and sampled again the next two days. On day 4 or 5, depending on stated shelf life time, 6 unopened packages were sampled and resampled the next two days (day 5 or 6 and on day 6 or 7). On the expiration date (day 7 or 9), the last six packages were sampled. Sampling days differ according to the shelf-life time of the product.

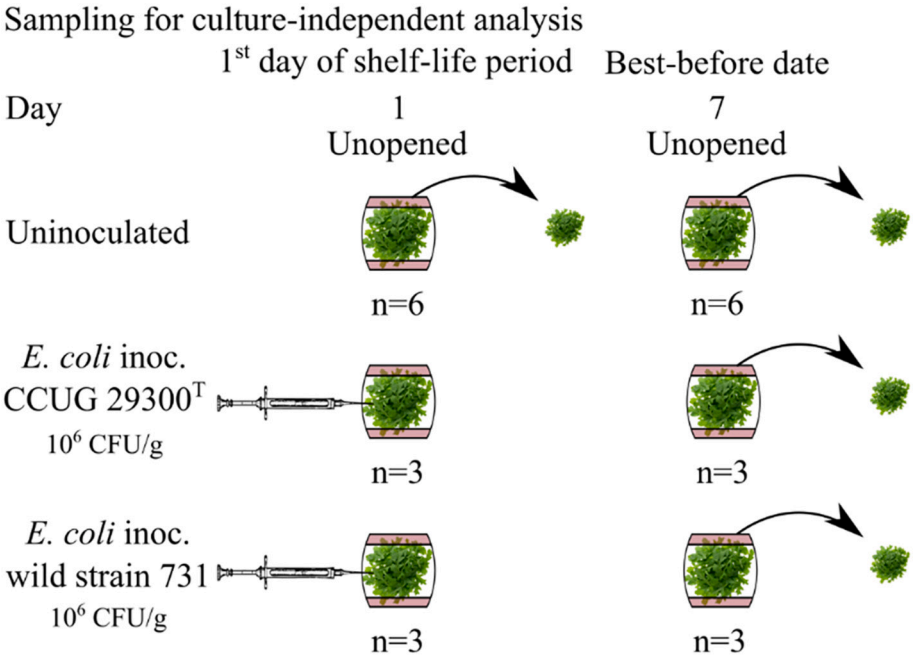

Fig. 2. Sampling scheme of leafy green mix, rocket and spinach for culture-independent analysis. On the first day of the shelf-life, uninoculated samples were taken, and *E. coli* inoculation was performed by injection. On the expiration date (day 7), samples from uninoculated and *E. coli* inoculated packages were analyzed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Viable cell count

Viable cell count was performed on 10 g samples as described by Uhlig et al. (2017). Each sample was mixed with 90 ml physiological saline and peptone in a Laboratory Blender Stomacher 400 (Seward Medical, London, UK). For *Enterobacteriaceae* plate count, diluted samples were applied to Violet Red Bile Dextrose agar (VRBD) (Merk Millipore, Darmstadt, Germany) and incubated at 37 °C for 24 h. For total aerobic plate count, samples were applied to Tryptic Soy Agar (TSA) (Fluka, Missouri, USA) and incubated at 30 °C for 72 h. *E. coli* inoculated samples were additionally spread on Brilliance *E. coli* Coliform Selective Agar (ECBA) (Oxoid, Basingstoke, UK) and incubated at 37 °C for 24 h. After enumeration, two randomly selected colonies per sample from TSA and VRBD plates were isolated and stored in Hogness' freezing medium (Ahrné et al., 1989) at −80 °C until further analysis by Sanger sequencing.

2.4. Sanger sequencing

DNA extraction, PCR, gel electrophoresis, Sanger sequencing of the 16S rRNA gene and data treatment were performed according to (Uhlig et al., 2017). Briefly, isolates were suspended in 0.5 ml physiological saline followed by bead beating on an Eppendorf Mixer (model 5432, Eppendorf, Hamburg, Germany) and after centrifugation, the supernatant was used for Polymerase Chain Reaction (PCR). Primers ENV1 (5'-AGAGTTTGATIITGGCTCAG-3′) and ENV2 (5'-CGG ITA CCT TGT TAC GAC TT-3′) (Eurofins Genomics, Ebersberg, Germany, (Pettersson et al., 2003) were used with TopTaq DNA Polymerase (Qiagen, Netherlands) according to the manufacturers' inststructions. PCR products were confirmed by gel electrophoresis and subsequently sent for sequencing at Eurofins Genomics (Ebersberg, Germany). The sequences were trimmed to between 590 and 788 bp depending on sequence quality and compared to type strains at the Ribosomal Database project (RDP) by Seqmatch (Cole et al., 2013).

2.5. Next generation sequencing

DNA extraction was performed according to Tatsika et al. (2019), with some modifications. In total, 72 samples of 2 g of leaves were placed in test tubes with 20 ml PBS (Oxoid Ltd., Blastingstoke, UK) and thereafter sonicated for 10 min. The leaves were removed and the remaining liquid centrifuged 20 min at 11600 xg. The supernatant was discarded, and the remaining pellet was stored at −18 °C until further processing by DNA extraction. DNA extraction was performed using the

Nucleiospin® Soil Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. DNA concentration was measured using Qubit ™ 1× ds DNA HS Assay Kit (Life Technologies Corporation, Eugene, OR, USA). The PCR primers B969F (ACGCGHNRAACCTTACC) and BA1406R (ACGGGCRGTGWGTRCAA) (Eurofins Genomics, Ebersberg, Germany) were used with PCR reagents Kapa HiFi Hotstart Ready Mix (Kapa Biosystems Pty (Ltd), Salt River, Cape Town, South Africa) to amplify 470 bp of the V6-V8 hyper variable region of the 16S rRNA gene (Tatsika et al., 2019). The following PCR program was used: 3 min at 95 °C, 25 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a hold period of 5 min at 72 °C. The PCR products were purified using AMPure XP beads (Beckman Coulter Genomics, Brea, CA, USA). An index PCR was run with forward adaptors 5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′ and reverse adaptors 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′ and the Nexter-aXT Index Kit v2 Set A (Illumina, San Diego, CA, USA) at 95 °C for 3 min followed by 8 cycles of 95 °C for 30 s, and 72 °C for 5 min. The products were again measured with Qubit (Life Technologies). The length of the amplified fragments was measured on random samples with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbrunn, Germany) and Agilent DNA kit (Agilent Technologies, Vilnius, Lithuania), according to manufacturer's instructions after both PCR runs. The indexed samples were diluted to 4 nM with resuspension buffer (Illumina) and sequenced on Illumina Miseq with Miseq reagent kit v3 (600-cycle) according to manufacturer's instructions. PhiX (Illumina) was used as internal control. The final loading volume was 600 µl.

The data was demultiplexed by Illumina CASAVA 1.8 and filtered using DADA2 in Qiime (Callahan et al., 2016). The sequences were trimmed at 25 bp on the left end, and at 275 bp on the right end.

2.6. Calculations and statistical analysis

The colony count data was analyzed using SigmaPlot version 13.0 (SPSS Inc., Chicago, USA) The differences between all groups were evaluated by Kruskal-Wallis one-way ANOVA on ranks. The differences between two experimental groups were assessed by Mann-Whitney rank sum test and results of $p \leq 0.05$ were considered statistically significant. Data is presented in boxplots, where the ends of the whisker are set at 1.5*IQR (interquartile range) above the third quartile (Q3), and 1.5*IQR below the first quartile (Q1). Outliers are defined as values outside this range. Colony count data was also analyzed by principal component analysis (PCA) using Scikit-learn v. 0.24.1 (Pedregosa et al., 2011).

The taxonomic classification of the NGS data was made in Qiime 2 (Bolyen et al., 2019) using a Naïve-Bayes classifier against the SILVA 132-database (Quast et al., 2013). Further calculations of the NGS data were then performed in R version 3.6.3 (R Team, 2006), Vegan package version 2.5 (Oksanen et al., 2019). Abundance levels were compared group wise by Kruskal-Wallis one-way analysis of variance on ranks, and two groups were compared with Mann-Whitney *U* test. α-diversity was calculated using Shannon index, and the differences between groups were evaluated by Kruskal-Wallis one-way ANOVA on ranks. The β-diversity was calculated with weighed UniFrac, analyzed with principal component analysis (PCoA) and evaluated statistically by the Adonis function in R version 3.6.3, using multivariate analysis of variance with distance matrices (Anderson, 2001). The differential relative abundance between groups was analyzed on genus level by DESeq2 (Anders and Huber, 2010) with a *p*-value cut-off at 0.001.

3. Results

3.1. Viable cell count and Sanger sequencing of the microbiota of leafy green products stored under household conditions

The total aerobic plate count from all products on day one varies from 5.8 to 7.7 $\log_{10}$ CFU/g (Table 2). The total aerobic plate count of mixed leafy greens from company C, rocket (all companies), organic rocket, and spinach from company B increased significantly ($p \leq 0.05$) throughout the whole shelf life time. Rocket from company A contained a total aerobic plate count of 9.6 $\log_{10}$ CFU/g at the expiration date. Total count of mixed leafy greens and spinach from company D, and rocket from company C stayed almost constant during the whole shelf-life time. Aerobic plate counts of organic rocket were higher than conventionally grown rocket from the same company.

The *Enterobacteriaceae* plate count varied from 2.0 to 5.0 $\log_{10}$ CFU/g on day one of the shelf-life time (Table 3). The counts of unopened packages of rocket (all companies) and spinach from company B increased significantly ($p \leq 0.05$) throughout the whole shelf lifetime. *Enterobacteriaceae* plate counts of organic rocket were higher than conventionally grown rocket from the same company.

A PCA analysis was applied to all viable cell count data to visualize the variation in viable cell count between the different products (Fig. 3). The data matrix was composed of the total aerobic plate counts and *Enterobacteriaceae* plate counts for all products, and the first two principal components (PCs) were calculated. PC1 represented 57.85 % of the variation, and PC2 14.54 %. The PCA analysis shows strong positive correlations between all sampling points during the shelf-life time. The bacterial load of rocket samples from company C was different from samples of rocket from other companies. The two spinach groups were similar to each other. Large differences between products exist, and no correlations between product type (spinach, rocket or mix) or manufacturer were found.

The identities of the rRNA gene (16S) Sanger sequenced bacterial isolates from rocket, mixed leafy greens and spinach belong to the phyla of Actinobacteria, Proteobacteria, Firmicutes and Bacteriodetes. A

Table 2
Total aerobic count throughout the whole shelf-life time of ready-to-eat leafy green mix (Mix), rocket, organic rocket and spinach from different companies, indicated by subscripted letters A-D. Sampling days differ according to the shelf-life time of the product.

Total aerobic plate count[1]							
Day	1	2	3	4/5	5/6	6/7	7/9
Mix^{C}	7.7 (7.6–7.9)	8.4 (8.3–8.5)^{aa}	8.6 (8.5–8.8)^{aa}	8.4 (8.2–8.5)^{aa}	8.7 (8.7–8.9^{bb}	9.0 (8.9–9.0)^{bb}	8.9 (8.6–9.0)$^{aa, bb}$
Mix^{D}	7.2 (6.8–7.4)	6.9 (6.8–7.1)	7.1 (6.9–7.1)	7.3 (6.7–7.5)	7.4 (7.3–7.5)	7.7 (7.4–8.0)^{b}	7.5 (7.3–8.0)
Organic rocket^{C}	6.0 (5.6–6.4)	6.3 (5.9–6.7)	6.9 (6.2–7.5)	6.8 (6.6–6.9)^{aa}	7.2 (7.0–7.3)^{bb}	7.2 (7.0–7.4)^{bb}	7.3 (7.1–7.5)$^{aa, bb}$
Rocket^{A}	7.8 (7.8–7.9)	8.4 (8.3–8.4)^{aa}	8.4 (8.3–8.4)^{aa}	8.7 (8.6–8.8)^{aa}	9.2 (9.0–9.3)^{bb}	8.7 (8.4–8.8)	9.6 (9.5–9.8)$^{aa, bb}$
Rocket^{C}	5.8 (5.4–6.2)	6.5 (6.0–6.8)	6.4 (6.2–6.8)^{a}	6.1 (5.7–6.5)	5.9 (5.7–6.5)	6.5 (6.4–7.2)^{b}	6.4 (6.2–7.2)^{a}
Rocket^{D}	7.1 (6.9–7.2)	7.9 (7.7–7.9)^{aa}	7.7 (7.7–7.9)^{a}	8.0 (7.9–8.1)^{aa}	8.3 (8.2–8.5)^{bb}	8.7 (8.7–8.7)^{bb}	8.7 (8.6–8.7)$^{aa, bb}$
Spinach^{B}	7.1 (7.0–7.1)	7.3 (7.1–7.5)^{a}	7.6 (7.4–7.7)^{aa}	7.1 (6.8–7.2)	7.5 (7.0–7.7)	7.5 (7.3–7.6)^{b}	7.3 (7.3–7.6)$^{aa, bb}$
Spinach^{D}	6.3 (6.2–6.6)	6.6 (6.2–7.9)	6.5 (6.4–6.8)	6.6 (6.5–6.7)	6.6 (6.5–6.6)	6.6 (6.4–6.7)	6.7 (6.5–6.9)

[1] Counts expressed as median log10 CFU/g vegetable of six replicates with interquartile range (25–75 %).
[a] $p \leq 0.05$ compared to day 1.
[aa] $p \leq 0.01$ compared to day 1.
[b] $p \leq 0.05$ compared to day 4/5.
[bb] $p \leq 0.01$ compared to day 4/5.

Table 3
Enterobacteriaceae plate count throughout the whole shelf-life time of ready-to-eat leafy green mix (Mix), rocket, organic rocket and spinach from different companies, indicated by subscripted letters A-D. Sampling days differ according to the shelf-life time of the product.

Enterobacteriaceae plate count[1]							
Day	1	2	3	4/5	5/6	6/7	7/9
Mix^{C}	5.0 (5.0–5.1)	5.3 (5.2–5.4)^{aa}	5.9 (5.8–6.1)^{aa}	5.3 (5.2–5.5)^{aa}	5.7 (5.5–5.8)^{b}	5.9 (5.7–6.0)^{bb}	5.4 (5.0–5.6)
Mix^{D}	4.0 (3.1–4.6)	3.3 (2.9–3.7)	3.7 (3.0–4.1)	3.9 (3.2–4.1)	4.1 (4.0–4.1)	4.3 (3.6–4.9)	4.0 (3.3–4.8)
Organic rocket^{C}	4.5 (4.2–4.8)	4.7 (4.4–4.9)	5.1 (4.9–5.5)^{aa}	4.8 (4.6–5.1)^{a}	5.0 (4.9–5.1)	4.9 (4.8–5.1)	4.8 (4.7–5.0)^{aa}
Rocket^{A}	3.4 (3.3–3.7)	4.7 (4.2–5.2)^{aa}	>5.0	5.6 (5.4–5.7)^{aa}	5.9 (5.7–6.3)	5.3 (5.1–5.6)	6.0 (5.7–6.3)$^{aa,\ b}$
Rocket^{C}	2.2 (2.0–3.2)	3.1 (2.8–3.4)	4.0 (3.6–4.3)^{aa}	3.6 (3.3–3.8)^{a}	3.7 (3.4–4.0)	3.0 (2.8–3.2)^{b}	4.2 (3.9–4.7)$^{aa,\ b}$
Rocket^{D}	<3.0	3.4 (3.2–3.5)	3.8 (3.3–4.9)	3.0 (2.7–3.4)	3.7 (3.3–4.1)	4.7 (4.3–4.9)^{b}	4.0 (3.8–4.4)^{bb}
Spinach^{B}	2.0 (2.0–2.1)	2.2 (2.0–3.0)	2.0 (2.0–2.1)	2.2 (2.0–3.1)	2.5 (2.0–4.0)	3.8 (3.2–4.1)^{bb}	4.0 (3.5–4.4)$^{aa,\ bb}$
Spinach^{D}	2.5(2.0–3.0)	2.5 (2.0–3.0)	3.3 (2.7–3.8)	2.0 (2.0–2.1)	3.0 (2.9–3.3)^{bb}	2.0 (2.0–2.3)	3.5 (2.0–3.6)

[1] Counts expressed as median $\log_{10}$ CFU/g vegetable of six replicates with interquartile range (25–75 %).
[a] $p \leq 0.05$ compared to day 1.
[aa] $p \leq 0.01$ compared to day 1.
[b] $p \leq 0.05$ compared to day 4/5.
[bb] $p \leq 0.01$ compared to day 4/5.

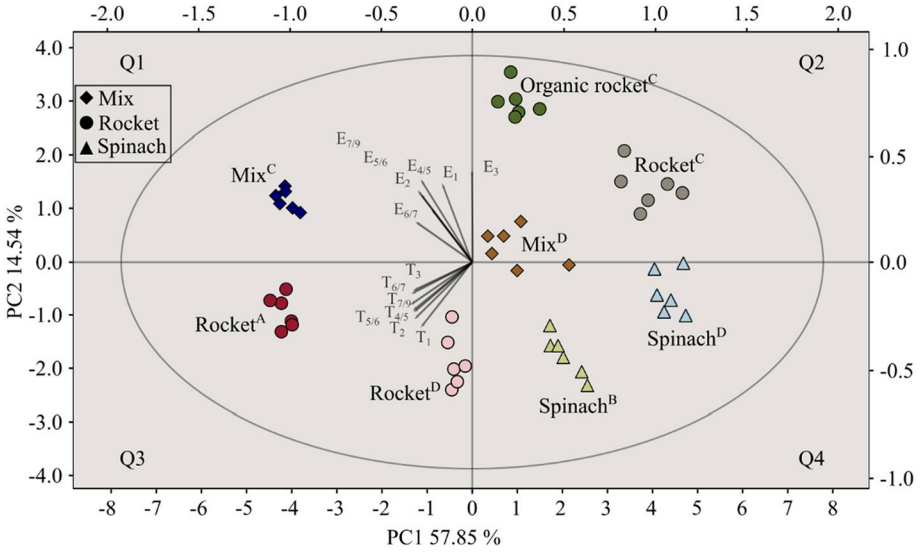

Fig. 3. PCA biplot of viable cell counts (total aerobic plate count and *Enterobacteriaceae* plate count) of ready-to-eat leafy green vegetables from different companies, indicated by subscripted letter A-D. ◆ = mixed leafy greens, ● = rocket, and ▲ = spinach. Sampling occasions are presented as vectors with T = Total aerobic plate count, E = *Enterobacteriaceae* plate count and sampling days superscripted. Ellipse: Hotelling T2 confidence interval 0.95. Vectors represent sampling point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Plate counts of *E. coli* inoculated rocket from company D throughout the whole shelf-life time. *E. coli* 731 and *E. coli* 921 are *coli* wild strains originating from leafy greens.

Day	1	2	3	5	7	8	9
Total aerobic plate count[1]							
E. coli CCUG 29300^{T}	6.8 (6.7–6.9)	7.7 (7.4–8.0)^{aa}	7.9 (7.7–8.2)^{aa}	8.9 (8.5–9.0)^{aa}	8.9 (8.6–8.9)	9.3 (9.1–9.5)^{b}	9.1 (8.9–9.2)^{aa}
E. coli 731	6.8 (6.5–6.8)	6.7 (6.6–7.1)	6.9 (6.9–7.4)	7.7 (7.4–7.9)^{aa}	8.4 (8.3–8.5)	7.8 (7.5–7.9)^{b}	8.1 (7.9–8.2)$^{aa,\ b}$
E. coli 921	7.8 (7.8–8.1)	8.0 (7.9–8.1)	8.6 (7.9–9.3)^{aa}	8.2 (8.1–8.5)^{aa}	8.1 (7.9–8.2)^{b}	8.6 (8.5–8.8)	8.8 (8.6–9.0)
Enterobacteriaceae plate count[1]							
E. coli CCUG 29300^{T}	5.2 (5.1–5.6)	4.8 (4.6–5.0)^{aa}	4.0 (3.9–4.6)^{aa}	4.7 (4.5–4.8)^{aa}	5.0 (4.5–5.0)	4.4 (3.9–4.4)^{bb}	4.2 (3.7–4.5)^{aa}
E. coli 731	4.4 (4.3–4.5)	3.7 (3.5–3.7)	3.3 (3.2–3.4)^{aa}	2.8 (2.6–2.9)^{a}	2.7 (2.7–2.8)	2.7 (2.5–2.9)	2.7 (2.5–2.9) ^{aa}
E. coli 921	5.1 (4.7–5.6)	5.0 (4.8–5.1)^{aa}	4.6 (4.2–4.9)	4.1 (3.3–4.5)^{a}	4.4 (3.9–5.3)	5.0 (4.5–5.2)	4.4 (3.7–4.9)
E. coli count[1]							
E. coli CCUG 29300^{T}	4.4 (4.2–4.8)	3.9 (3.7–4.1)^{a}	3.9 (3.3–4.3)	3.9 (3.7–4.1)^{a}	3.9 (3.7–4.0)	3.5 (3.3–4.2)b	3.8 (3.4–4.5)
E. coli 731	4.4 (4.3–4.5)	3.7 (3.6–3.8)	3.4 (3.3–3.5)	2.9 (2.8–3.1)	2.8 (2.6–2.9)	2.9 (2.8–3.0)	3.1* (3.0–3.2)
E. coli 921	4.7 (4.5–5.0)	5.0 (4.7–5.4)^{aa}	5.2 (4.8–5.3)^{aa}	4.3 (3.3–5.0)^{aa}	4.5 (4.1–4.8)	4.1 (3.6–4.5)	4.1(3.5–4.6)^{aa}

[1] Counts expressed as median $\log_{10}$ CFU/g rocket salad of six replicates with interquartile range (25–75 %).
[a] Indicates $p \leq 0.05$ compared to day 1.
[aa] Indicates $p \leq 0.01$ compared to day 1.
[b] Indicates $p \leq 0.05$ compared to day 5.
[bb] Indicates $p \leq 0.01$ compared to day 5.
[*] Indicates $p \leq 0.05$ compared to day 7.

dominating part of the culturable bacteria belonged to the genus of *Pseudomonas*. *Aeromonas* and *Staphylococcus* species were also frequently found. Notable is also the presence of *Bacillus*, *Enterobacter*, *Hafnia* and *Pantoea* species.

3.2. Viable cell count of the microbiota of rocket inoculated with E. coli, stored under household conditions

The viable cell count of *E. coli* prior to the inoculation of rocket packages was under the detection limit (2 $\log_{10}$ CFU/g) (Table 4). The initial total aerobic plate counts vary from 6.8 to 7.8 $\log_{10}$ CFU/g and increase over time for all three *E. coli* strains. *Enterobacteriaceae* plate count decreased over the whole shelf-life period for all *E. coli* inoculated samples. Inoculation with wild *E. coli* strain 921 decreased the *Enterobacteriaceae* concentration in the beginning. The *E. coli* count was unchanged for samples inoculated with *E. coli* CCUG 29300^T, while an initial decrease was observed for wild *E. coli* strain 731 at the beginning, followed by an increase at the end. For wild *E. coli* strain 921, an initial increase was followed by a decrease for the rest of the shelf life time.

The identities of the rRNA gene (16S) Sanger sequenced bacterial isolates from *E. coli* inoculated rocket belong to the phyla of Actinobacteria, Proteobacteria, Firmicutes and Bacteriodetes. A dominating part of the culturable bacteria picked from the viable cell count plates belonged to the genus of *Pseudomonas*. Notable is the detection of *Pantoea,* and the dominating presence of *Enterobacter* in samples inoculated with *E. coli* 921. Opportunistic human pathogens were identified; *Enterobacter homaechei* in one leafy green mix sample, *Hafnia paralvei* in one spinach and one rocket sample, and *Pantoea agglomerans* in one

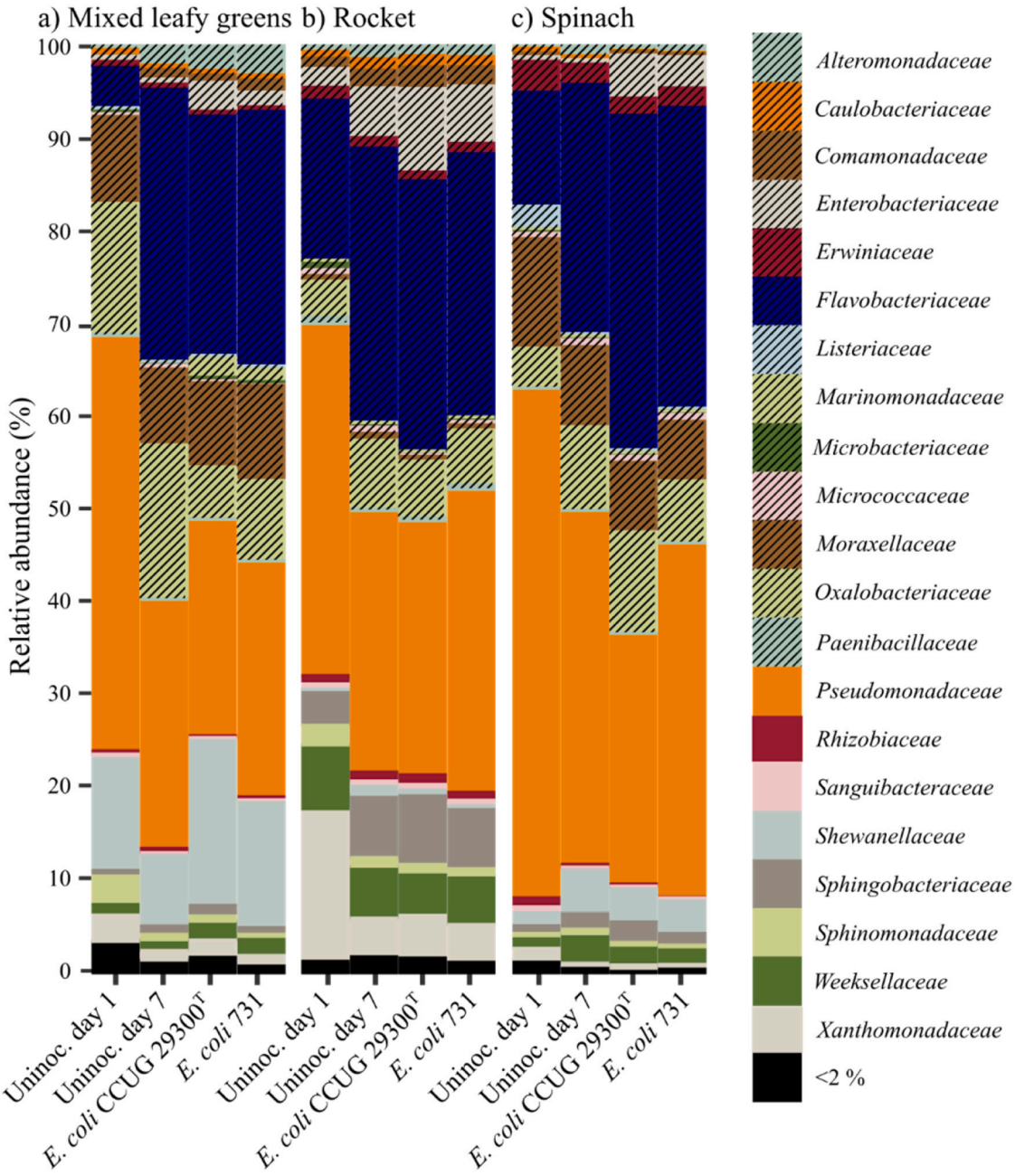

Fig. 4. Relative bacterial abundance at family level of ready-to-eat products from company C, uninoculated and inoculated with two different strains of *E. coli*. a) mixed leafy greens, b) rocket, c) spinach. *E. coli* CCUG 29300^T: sample inoculated with *E. coli* CCUG 29300^T, *E. coli* 731: sample inoculated with wild strain *E. coli* 731. *E. coli* inoculated samples were analyzed on day 7 (the expiration date).

rocket sample.

3.3. Next generation sequencing of the microbiota of leafy green products during shelf-life, with and without E. coli inoculation

The most prevalent phylum of leafy greens for rocket, spinach and mix samples was Proteobacteria, with a relative abundance of 94.0 % for mixed leafy greens, 82.3 % for spinach and 71.2 % for rocket on day 1. At the expiration date (after 7 days), the prevalence of Proteobacteria had decreased by 20–30 % in all samples. The second most prevalent phylum was Bacteriotetes, an increase was seen over time, from 6.4 % for mixed leafy greens on day 1, to 30.7 % on day 2 ($p < 0.01$), and for spinch; 13.2 on day 1 and 31.8 % on day 7 ($p < 0.01$). Firmicutes and Actinobacteria were also detected, constituting <2.5 % and 1 % of the total microbiota composition respectively.

Over all product types, the top two families with the highest relative abundances were *Pseudomonadaceae* (33.7 %) and *Flavobacteriaceae* (25.3 %) (Fig. 4). *Pseudomonadaceae* decreased over time for uninoculated rocket and spinach packages, whereas *Flavobacteriaceae* increased over time. Variations in the bacterial composition between the different product types were found. In mixed leafy greens (Fig. 4a), higher relative abundances of *Shewanellaceae* (13.0 %) and *Oxalobacteriaceae* (11.4 %) were detected. In the rocket (Fig. 4b), higher relative abundances of *Xanthomonadaceae* (7.4 %), *Sphingobacteriaceae* (6.3 %) and *Enterobacteriaceae* (5.7 %) were recorded. In spinach (Fig. 4c), relatively high levels of *Listeriaceae* (2.4 %, genus *Brochothrix*) and *Erwiniaceae* (2.2 %) were found. Higher relative abundance of *Moraxellaceae* was found in the mixed leafy greens and in the spinach (9.4 % and 8.8 % respectively). On genus level, higher relative *Escherichia-Shigella* abundance was found on *E. coli* inoculated spinach (5.0 %) and mixed leafy greens (3.5 %) compared to rocket (0.6 %, $p < 0.05$).

The α-diversity, based on amplicon sequence variants (ASVs) and calculated by Shannon diversity index did not vary between the different products. The values ranged from 4.9 and 5.5 on the first day and remained on the same levels on the expiration date. *E. coli* inoculation did not alter the Shannon index.

The β-diversity, based on ASVs and calculated by weighted unique fraction metric (UniFrac), considering the amount and phylogenetic distance between species, changed from start to end for all product types ($p = 0.003$), as seen in Fig. 5 as a shift to the left.

The differences in relative abundance on genus level of uninoculated leafy green products (Fig. 6a) indicate that for uninoculated mixed leafy greens and spinach, many amplicon sequence variants (ASVs) are at higher levels at the start than at the end of the shelf-life time. For rocket, the relative ASV abundances did not change over time. *E. coli* inoculation (Fig. 6b-c) rendered microbiota changes predominantly in mixed leafy greens. For spinach and rocket, the only change was higher relative *Escherichia-Shigella* abundance after inoculation.

4. Discussion

4.1. Viable cell count and Sanger sequencing of the microbiota of leafy green products stored under household conditions

Even though the visual appearance of the products included in the study did not differ, total aerobic plate counts of ready-to-eat leafy green vegetables vary greatly, already at the first day of the shelf-life time (5.8 to 7.7 $\log_{10}$ CFU/g (Table 2). Even wider ranges of bacterial load were found by Jackson et al. (2013), from 4.0 $\log_{10}$ CFU/g for green leaf lettuce to 8.7 $\log_{10}$ CFU/g for spinach. In the present study, the PCA biplot (Fig. 3) additionally shows that each replicate (each bag), within the same product is more similar to its own product batch than others, even if they are of the same vegetable type. This batch "individuality" is also reflected in the PCA loadings, suggesting that a high starting value correlates with high values for the rest of the shelf lifetime. A high total aerobic plate count additionally correlates with a high *Enterobacteriaceae* plate count. These results underline the importance of product hygiene practices during the production stage. Previous studies suggest that an initial contamination is difficult to remove (Castro-Rosas et al., 2012; Doyle and Erickson, 2008). The present results show that the same principle applies to the whole cultivable microbiota on leafy green vegetables.

Jackson et al. (2013) saw no correlations between higher or lower viable cell counts for organic produce compared to conventionally grown. Results in this study support those results; organic rocket in the present study had higher aerobic plate counts than conventionally grown from the same company, but higher values were found for conventionally grown rocket from other companies.

Rocket grown in Sweden from two different companies (A and C) contained both the highest and the lowest bacterial counts of all products tested. Their dissimilarity, also shown by the PCA analysis, visualizes the absence of correlation between bacterial load and country of origin, in this case Sweden and Italy. Moreover, the analyzed products also had different shelf-life times; company A, B and C had 9 days of shelf-life, while company C had 7 days. Rocket from company C had lower total aerobic plate counts than from company A and D, potentially favoring the shorter shelf-life time. However, mix from company C, together with rocket from company A contained the highest levels of both aerobic and *Enterobacteriaceae* plate count of all products tested. This suggests that the bacterial load for each product batch is independent of company and forecasted shelf-life. Further supporting this theory are the high variations within each vegetable type, in particular for

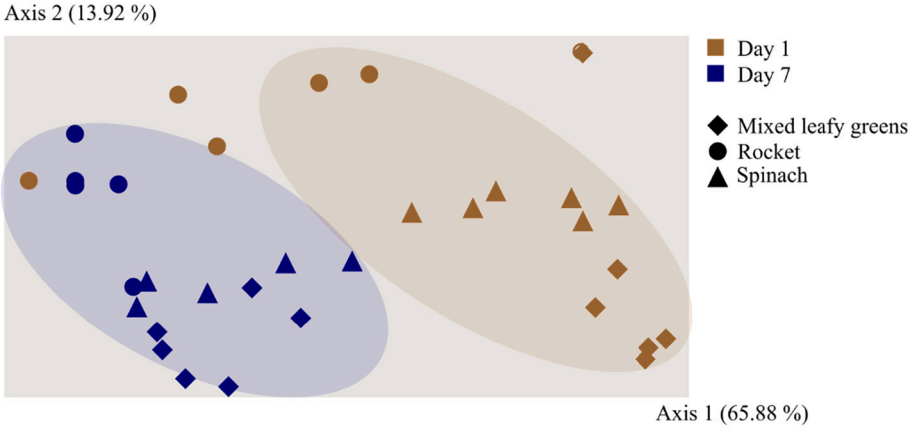

Fig. 5. Principal coordinate analysis (PCoA) of beta diversity based on the variation of amplicon sequence variants (ASVs) for uninoculated, stored ready-to-eat products, calculated by weighted unique fraction metric (unifrac) (Vázquez-Baeza et al., 2017).

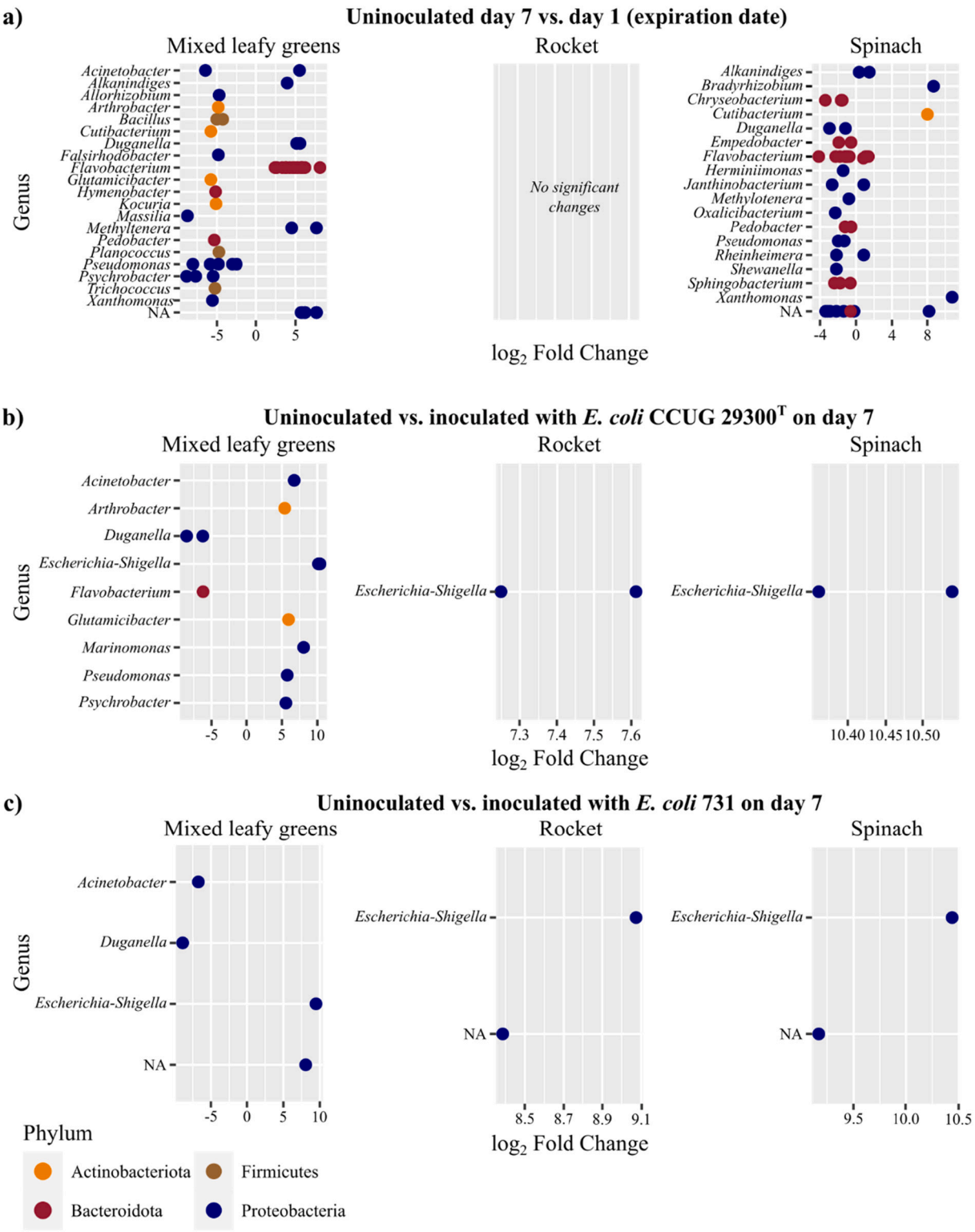

Fig. 6. Statistically significant differences ($p < 0.001$) in relative abundance of amplicon sequence variants (ASVs) in RTE products, expressed as $\log_2$ Fold change. Please note that the y-axes display different intervals. a) A positive $\log_2$ fold change values indicate higher relative ASV abundance on day 7, and negative values indicate higher relative ASV abundance on day 1. b) and c) A positive $\log_2$ fold change values indicate higher relative ASV abundance in *E. coli* inoculated samples, and negative values indicate higher relative ASV abundance in uninoculated samples. Multiple symbols within the same genus represent different ASVs assigned to the same genus. NA stands for unannotated ASVs.

rocket and mixed leafy greens (Table 2-3).

This is the first study designed to simulate household conditions and measure its effect on the microbiota of leafy green vegetables. By opening the bag, taking out some vegetables and putting it back in the fridge again, this pattern was simulated both at the beginning of the shelf-life time and at the end. Opening a bag changes its current atmospheric conditions, and higher plate counts were found in the opened packages for some products, while unchanged or lower concentrations

were found in others (Table 2-3). No specific product seemed to be more sensitive to the atmospheric change than any other. Interestingly, mix from company D contained modified atmosphere (MAP), but bacterial counts remained unchanged, regardless of unopened or opened bag. Research on the effect of modified atmosphere on the microbiota of leafy greens is not consistent (Ragaert et al., 2007). Barriga et al. (1991) showed that MAP mixtures of 3 % O_2 and 10 % CO_2 had no effect on total count and coliforms among others on leafy greens, which is in line with the results in this study also after simulating consumer practices. However, MAP at these concentrations reduces visual signs of deterioration, while not inhibiting pathogens such as *E. coli* O157:H7, leaving no signs for the consumer that the product may be harmful to eat (Luo et al., 2010).

The identities of the cultured isolates reveal that the dominant culturable genera in all leafy green vegetable products were *Pseudomonas*, a well-known plant endophyte (Jackson et al., 2013; Rosenblueth and Martínez-Romero, 2006; Söderqvist et al., 2017). Other typical plant commensals, such as *Arthrobacter*, *Aeromonas* and *Chryseobacterium* were commonly isolated, and also found in other studies (Dees et al., 2015; Jackson et al., 2013; Tatsika et al., 2019). The presence of skin bacteria such as *Staphylococcus epidermis* and *Staphylococcus warneri* indicate that contamination from humans in these products is not uncommon. Interestingly, opportunistic human pathogens, such as *Enterobacter homaechei*, *Hafnia paralvei*, and *Pantoea agglomerans*, were identified in leafy green mix, spinach and rocket samples. Despite the fact that opportunistic pathogens are frequently found on leafy green vegetables at the time of consumption (El-Sayed et al., 2013; Jackson et al., 2013; Szabo et al., 2000; Uhlig et al., 2017), leafy green products are continuously sold and marketed with a long shelf-life.

4.2. Next generation sequencing of the microbiota of leafy green products during shelf-life

To characterize the overall microbiota, including non-culturable organisms, of ready-to-eat leafy green vegetable products during the shelf-life time, next generation sequencing (NGS) was performed. The DNA extraction protocol for NGS analysis by (Tatsika et al., 2019) uses a small sample size of 2 g, which limits the representation of the whole bacterial population and increases the variability between samples by allowing only parts of the plant tissue to be analyzed. Taking this limitation into account, the microbiota composition for all leafy greens was overwhelmingly dominated by Proteobacteria (71.2–94.0 %), accompanied by Bacteriodetes, Firmicutes and Actinobacteria, as previously seen (Jackson et al., 2013; Lopez-Velasco et al., 2011; Rastogi et al., 2012). The microbiota composition of each product type (mix, rocket, spinach) differed, as previously observed (Dees et al., 2015; Söderqvist et al., 2017; Tatsika et al., 2019). In all product types, a shift in the microbiota composition occurred over time, mainly by an increased relative abundance of Bacteriodetes, mostly represented by a large increase in *Flavobacteriaceae* (Fig. 4). Simultaneously, a relative decrease in Proteobacteria was observed, mainly represented by *Pseudomonadaceae*. Previous studies indicate that certain members of the family *Pseudomonadaceae* may survive or even grow in refrigerated temperatures on leafy greens (Lopez-Velasco et al., 2011; Söderqvist et al., 2017). The decrease in relative abundance of *Pseudomonadaceae* seen in this study could be concluded as an effect of the explosive growth of *Flavobacteriaceae*. Rosberg et al. (2021) saw that the relative abundance of *Flavobacteriaceae* was significantly higher in washed than unwashed produce, thus the high levels seen in this study may have been a result of the washing process before packaging.

Microbiota changes over time in leafy green vegetables were shown by Lopez-Velasco et al. (2011), who noted a decrease in detected phyla from 11 to 4 after only one day of storage at 4 °C. Moreover, Söderqvist et al. (2017) noted an increase in Enterobacteriales after storage for7 days at 8 °C . In the present study, statistical differences in α-diversity (taking into account evenness and richness) were not found over time, but in β-diversity, a shift in microbiota composition could be observed. β-diversity was calculated with weighted unique fraction metric (UniFrac), considering the amount and phylogenetic distance between the amplicon sequence variants (ASVs) present in the sample. The differential relative abundance (Fig. 6) visualizes the change over time on genus level; many genera were present at day 1 but not at day 7. Rosberg et al. (2021) found a clear decrease in diversity for spinach and rocket during industrial production, the step preceding the process studied here, meaning that the changes in the microbial communities starts already at harvest and continues during cold storage.

By including large amounts of data from both culture-dependent and culture-independent techniques, not only does this study analyze both the viable, culturable fraction of the microbiota of leafy greens, but also the unculturable species present. Additionally, this study gives a unique opportunity to compare how well traditional culturing covers the scope of the microbiota detected by 16 S rRNA amplicon sequencing. It is said that culturing on agar media may only be able to cover 2 % of the total microbiota in environmental samples (Wilson et al., 1997). A part of the difference between the two techniques is that culturing only detects live bacteria, while amplicon sequencing detects all DNA present in the sample. This study used TSA and VRBD to target the total culturable mictobiota, and specifically *Enterobacteriaceae*, and culturing showed to match 18 of 48 (37.5 %) of the genera detected by amplicon sequencing. Culturing identified genera that were not detected by amplicon sequencing, and vice versa. In this comparison, it is important to consider that different batches of leafy greens were analyzed with the two different techniques, thus allowing for identification of unique taxa in both cases. Culturing still covered 7 out of the 10 most common genera from amplicon sequencing. These results are in line of the comparison of culturing and 454 pyrosequencing made by Jackson et al. (2013) and indicate that the microbial community of leafy greens are relatively easy to cultivate, compared to other environmental communities.

4.3. Viable cell count and next generation sequencing of the microbiota of rocket inoculated with E. coli, stored under household conditions

Rocket inoculated with *E. coli* CCUG 29300^T and wild strain *E. coli* 921 showed higher plate counts overall than after inoculation with *E. coli* 731 (Table 4). *Enterobacteriaceae* plate count decreases over time, but *E. coli* count did not follow the same pattern, suggesting that other *Enterobacteriaceae* genera are responsible for the decrease observed. Inoculation with *E. coli* 921 resulted in an increase in *E. coli* count at the beginning, while with *E. coli* 731, *E. coli* count instead increased at the end. It can be seen that both wild strains are able to grow in cold conditions, in contrary to the type strain, and also at different time points during the shelf-life time. The increase at the end with *E. coli* 731 may indicate a possible adaption to cold conditions. In previous studies on leafy greens, cold storage has on the contrary seen to prevent the growth of *E. coli* (Gleeson and O'Beirne, 2005; Luo et al., 2010; Söderqvist et al., 2017; Tomás-Callejas et al., 2011). The wild strains in this study were originally isolated from Romaine lettuce, and likely to be found on leafy vegetables.

The growth behavior of wild *E. coli* strains in their native environment has not previously been studied. As previously mentioned, other studies have focused on the growth of type strains on leafy greens during cold storage (Gleeson and O'Beirne, 2005; Luo et al., 2010; Söderqvist et al., 2017; Williams et al., 2013), which may not represent the real outcome. The growth of autochthonous strains may differ due to possible adaptation to temperature differences in the native environment, in contrast to type strains originating from other environments. Although their pathogenicity is not known, their growth patterns are worrying for the food safety of RTE leafy green products.

For next generation sequencing analysis, uninoculated samples were compared with samples inoculated with the *E. coli* CCUG 29300^T and with *E. coli* 731. The latter was chosen instead of *E. coli* 921 due to its

capacity to grow at the end of shelf-life and adapt to cold conditions. In uninoculated samples, the genus *Escherichia* could not be detected at the expiration date. Rocket had a smaller relative abundance of *Escherichia* than spinach and mix 7 days after inoculation with the type strain (0.6 % compared to 5.0 and 3.4 % respectively). Interestingly, the microbiota composition of rocket (Fig. 4) seemed to be very stable upon *E. coli* inoculation, in contrary to the microbiota of mix and spinach. The rocket microflora therefore seems to be more constant and resistant to bacterial invasion than both mix and spinach, a favorable trait in a hygiene perspective. A similar finding of an inherent inhibitory capacity of rocket was reported by Darlison et al. (2019), that observed significantly lower counts of the added *E. coli* O157:H7 on rocket, than on spinach and Swiss chard. However, it should be noted that the *Enterobacteriaceae* levels of uninoculated rocket samples are in the higher range among the products analyzed.

5. Conclusion

In this study, large amounts of data from both culture-dependent and culture-independent techniques were combined to give a unique insight into the microbiota of leafy greens and the development during cold storage at the time of consumption. From a consumer's point of view, the high concentrations of live bacteria on ready to eat leafy green products are concerning. The *Enterobacteriaceae* plate counts of several products exceeded 5 $\log_{10}$ CFU/g, which are very high levels for a ready-to-eat product. Identification of colonies picked from countable plates reveals that opportunistic pathogens, such as *Enterobacter homaechei*, *Hafnia paralvei*, and *Pantoea agglomerans* are present in these products at the time of consumption. The microbiota composition, as determined by next generation sequencing, is subject to a striking change over time, while the effect of added *E. coli* varies due to vegetable type. However, as seen by the plate counts, wild *E. coli* strains are able to grow even in refrigeration. The culturable bacterial content, as well as the effect of opened or closed packages seem to depend on the individual product batch rather than the vegetable type or the company. In most products, the bacterial growth occurs during the first three days, and thereafter remains on a relatively constant level. Should the bacterial load be kept on a reasonable number in these products, the shelf-life time may need to be shorter than the present recommendations. But due to the variations seen between products, further studies are needed to define how long the shelf-life of these products should be, to ensure a safe product even at the end of the shelf-life period.

CRediT authorship contribution statement

Conceptualization, methodology, supervision and validation Å.H. and G.M.; formal analysis, E.U.; investigation, E.U., N.N., Y.N., J.P., E. O., J.P, B.P.; data curation, E.U., A.K.; writing—original draft preparation, E.U., writing - review and editing, E.U., Å.H., G.M.; project administration, Å.H. All authors have read and agreed to the published version of the manuscript.

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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References

Ahrné, S., Molin, G., Ståhl, S., 1989. Plasmids in Lactobacillus strains isolated from meat and meat products. Syst. Appl. Microbiol. 11 (3), 320–325. https://doi.org/10.1016/S0723-2020(89)80031-1.

Allende, A., Aguayob, E., Artés, F., 2003. Microbial and sensory quality of commercial fresh processed red lettuce throughout the production chain and shelf life. Int. J. Food Microbiol. 91, 109–117.

Allende, A., McEvoy, J.L., Luo, Y., Artes, F., Wang, C.Y., 2006. Effectiveness of two-sided UV-C treatments in inhibiting natural microflora and extending the shelf-life of minimally processed ‘red oak leaf’ lettuce. Food Microbiol. 23 (3), 241–249. https://doi.org/10.1016/j.fm.2005.04.009.

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol. 11 (10), R106. https://doi.org/10.1186/gb-2010-11-10-r106.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26 (1), 32–46. https://doi.org/10.1111/j.1442-9993.2001.01070. pp. x.

Arienzo, A., Murgia, L., Fraudentali, I., Gallo, V., Angelini, R., Antonini, G., 2020. Microbiological quality of ready-to-eat leafy green salads during shelf-life and home-refrigeration. Foods 9 (10). https://doi.org/10.3390/foods9101421.

Barriga, M.I., Trachy, G., Willemot, C., Simard, R.E., 1991. Microbial changes in shredded iceberg lettuce stored under controlled atmospheres. J. Food Sci. 56 (6), 1586–1588. https://doi.org/10.1111/j.1365-2621.1991.tb08646.x.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37 (8), 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from illumina amplicon data. Nat. Methods 13 (7), 581–583. https://doi.org/10.1038/nmeth.3869.

Castro-Rosas, J., Cerna-Cortés, J.F., Méndez-Reyes, E., Lopez-Hernandez, D., Gómez-Aldapa, C.A., Estrada-Garcia, T., 2012. Presence of faecal coliforms, Escherichia coli and diarrheagenic E. coli pathotypes in ready-to-eat salads, from an area where crops are irrigated with untreated sewage water. Int. J. Food Microbiol. 156 (2), 176–180.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Cuske, C.R., Tiedje, J.M., 2013. Ribosomal database project: data and tools for high throughput rRNA analysis 42 (D1), D633–D642.

Darlison, J., Mieli, M., Bengtsson, T., Hartmann, R., Mogren, L., Vågsholm, I., Alsanius, B., 2019. Plant species affects establishment of E. coli O157:H7 gfp+ on leafy vegetables. J. Appl. Microbiol. 127 https://doi.org/10.1111/jam.14299.

De Giusti, M., Solimini, A.G., Cottarelli, A., De Vito, C., Aurigemma, C., Tufi, D., Marinelli, L., 2014. Temporal pattern of microbial indicators of ready-to-eat rocket salads during shelf life. Ann. Ist. Super. Sanita 50 (1), 90–95. https://doi.org/10.4415/ann_14_01_13.

Dees, M.W., Lysøe, E., Nordskog, B., Brurberg, M.B., 2015. Bacterial communities associated with surfaces of leafy greens: shift in composition and decrease in richness over time. Appl. Environ. Microbiol. 81 (4), 1530. https://doi.org/10.1128/AEM.03470-14.

Doyle, M.P., Erickson, M.C., 2008. Summer meeting 2007 the problems with fresh produce: an overview. J. Appl. Microbiol. 105 (2), 317–330.

Efsa, 2013. Scientific opinion on the risk posed by pathogens in food of non-animal origin. Part 1 (outbreak data analysis and risk ranking of food/pathogen combinations). EFSA J. 11 (1) https://doi.org/10.2903/j.efsa.2013.3025.

El-Sayed, S., Al-Holy, M., Osaili, T., Alshammari, E., Ashankyty, I., 2013. Microbiological quality of leafy green vegetables sold in the local market of Saudi Arabia. Ital. J. Food Sci. 25, 446–452.

Farber, J.M., 1991. Microbiological aspects of modified-atmosphere packaging technology - a review. J. Food Prot. 54 (1), 58–70. https://doi.org/10.4315/0362-028X-54.1.58.

Gleeson, E., O’Beirne, D., 2005. Effects of process severity on survival and growth of Escherichia coli and Listeria innocua on minimally processed vegetables. Food Control 16 (8), 677–685. https://doi.org/10.1016/j.foodcont.2004.06.004.

Jackson, C.R., Randolph, K.C., Osborn, S.L., Tyler, H.L., 2013. Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. BMC Microbiol. 13 (1), 274.

Jensen, D.A., Friedrich, L.M., Harris, L.J., Danyluk, M.D., Schaffner, D.W., 2015. Cross contamination of Escherichia coli O157:H7 between lettuce and wash water during home-scale washing. Food Microbiol. 46, 428–433.

Lopez-Velasco, G., Welbaum, G., Boyer, R., Mane, S., Ponder, M., 2011. Changes in spinach phylloepiphytic bacteria communities following minimal processing and refrigerated storage described using pyrosequencing of 16S rRNA amplicons. J. Appl. Microbiol. 110, 1203–1214. https://doi.org/10.1111/j.1365-2672.2011.04969.x.

Luo, Y., He, Q., McEvoy, J., 2010. Effect of storage temperature and duration on the behavior of Escherichia coli O157:H7 on packaged fresh-cut salad containing Romaine and Iceberg lettuce. J. Food Sci. 75, M390–M397. https://doi.org/10.1111/j.1750-3841.2010.01722.x.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Wagner, H., 2019. Vegan: Community Ecology Package. R Package Version 2.5-6.

Oliveira, M., Abadias, M., Usall, J., Torres, R., Teixidó, N., Viñas, I., 2015. Application of modified atmosphere packaging as a safety approach to fresh-cut fruits and vegetables – a review. Trends Food Sci. Technol. 46 (1), 13–26. https://doi.org/10.1016/j.tifs.2015.07.017.

Painter, J.A., Hoekstra, R.M., Ayers, T., Tauxe, R.V., Braden, C.R., Angulo, F.J., Griffin, P. M., 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. Emerg. Infect. Dis. 19 (3), 407–415.

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., Dubourg, V., 2011. Scikit-learn: machine learning in python. J. Mach. Learn. Res. 12, 2825–2830.

Pettersson, B., Ahrné, S., Wang, M., Jeppsson, B., Uhlén, M., Molin, G., 2003. The mucosa-associated bacteria from the sigmoid colon of nine healthy 60 years old individuals, identified by bacterial 16S rDNA.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41 (D1), D590–D596. https://doi.org/10.1093/nar/gks1219.

R Team, 2006. A language and environment for statistical computing. Computing 1. https://doi.org/10.1890/0012-9658(2002)083[3097:CFHIWS]2.0.CO;2.

Ragaert, P., Devlieghere, F., Debevere, J., 2007. Role of microbiological and physiological spoilage mechanisms during storage of minimally processed vegetables. Postharvest Biol. Technol. 44 (3), 185–194. https://doi.org/10.1016/j.postharvbio.2007.01.001.

Rastogi, G., Sbodio, A., Tech, J.J., Suslow, T.V., Coaker, G.L., Leveau, J.H.J., 2012. Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. ISME J. 6 (10), 1812–1822. https://doi.org/10.1038/ismej.2012.32.

Rosberg, A.K., Darlison, J., Mogren, L., Alsanius, B.W., 2021. Commercial wash of leafy vegetables do not significantly decrease bacterial load but leads to shifts in bacterial species composition. Food Microbiol. 94, 103667 https://doi.org/10.1016/j.fm.2020.103667.

Rosenblueth, M., Martínez-Romero, E., 2006. Bacterial endophytes and their interactions with hosts. Mol. Plant-Microbe Interact. 19 (8), 827–837. https://doi.org/10.1094/MPMI-19-0827.

Söderqvist, K., Osman, O., Wolff, C., Bertilsson, S., Vågsholm, I., Boqvist, S., 2017. Emerging microbiota during cold storage and temperature abuse of ready-to-eat salad. Infect. Ecol. Epidemiol. 7, 1328963. https://doi.org/10.1080/20008686.2017.1328963.

Solomon, E.B., Matthews, K.R., 2005. Use of fluorescent microspheres a a tool to investigate bacterial interactions with growing plants. J. Food Prot. 67, 2342-2253.

Szabo, E.A., Scurrah, K.J., Burrows, J.M., 2000. Survey for psychrotrophic bacterial pathogens in minimally processed lettuce. Lett. Appl. Microbiol. 30 (6), 456–460. https://doi.org/10.1046/j.1472-765x.2000.00747.x.

Tatsika, S., Karamanoli, K., Karayanni, H., Genitsaris, S., 2019. Metagenomic characterization of bacterial communities on ready-to-eat vegetables and effects of household washing on their diversity and composition. Pathogens 8 (1), 37. https://doi.org/10.3390/pathogens8010037.

Tomás-Callejas, A., López-Velasco, G., Camacho, A.B., Artés, F., Artés-Hernández, F., Suslow, T.V., 2011. Survival and distribution of Escherichia coli on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions. Int. J. Food Microbiol. 151 (2), 216–222. https://doi.org/10.1016/j.ijfoodmicro.2011.08.027.

Uhlig, E., Olsson, C., He, J., Stark, T., Sadowska, T., Molin, G., Håkansson, Å., 2017. Effects of household washing on bacterial load ans removal of Escherichia coli from lettuce and "ready-to-eat" salads. Food Sci. Nutr. 5 (6), 1215–1220. https://doi.org/10.1002/fsn3.514.

Vázquez-Baeza, Y., Gonzalez, A., Smarr, L., McDonald, D., Morton, J.T., Navas-Molina, J. A., Knight, R., 2017. Bringing the dynamic microbiome to life with animations. Cell Host Microbe 21 (1), 7–10. https://doi.org/10.1016/j.chom.2016.12.009.

Williams, T.R., Moyne, A.L., Harris, L.J., Marco, M.L., 2013. Season, irrigation, leaf age, and Escherichia coli inoculation influence the bacterial diversity in the lettuce phyllosphere. PLoS One 8 (7), e68642. https://doi.org/10.1371/journal.pone.0068642.

Wilson, M.J., Weightman, A.J., Wade, W.G., 1997. Applications of molecular ecology in the characterization of uncultured microorganisms associated with human disease. Rev. Med. Microbiol. 8 (2).