Food Control 125 (2021) 107894

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Processing of leafy vegetables matters: Damage and microbial community structure from field to bag

E. Mulaosmanovic [a,*], T.U.T. Lindblom [b], S.T. Windstam [a,c], M. Bengtsson [d], A.K. Rosberg [a], L. Mogren [a], B.W. Alsanius [a]

[a] Swedish University of Agricultural Sciences, Department of Biosystems and Technology, Microbial Horticulture Unit, PO Box 103, SE-23053, Alnarp, Sweden
[b] Swedish University of Agricultural Sciences, Department of Crop Production Ecology, Plant Ecology Unit, PO Box 7043, SE-75007, Uppsala, Sweden
[c] State University of New York at Oswego, Department of Biological Sciences, 7060 NY-104, Oswego, NY, 13126, USA
[d] Swedish University of Agricultural Sciences, Department of Plant Protection Biology, Chemical Ecology Unit, PO Box 102, SE-23053, Alnarp, Sweden

ARTICLE INFO

Keywords:
Damage
Escherichia coli O157:H7
Microbiota
Post-harvest
Spinach
Swiss chard

ABSTRACT

Leafy vegetables undergo abiotic and biotic stresses, and a series of processing steps that cause mechanical injury. Breaching the epidermis alters phyllosphere structural and nutrient conditions, resulting in successional shifts in leaf microbiota and entry of human pathogens. This study examined damage during processing of baby leaves (Swiss chard, spinach) and concomitant microbial successional events. Machine-harvesting, washing, and packaging caused major phyllosphere perturbations, with increasing levels of leaf damage. Older leaves showed most damage, but plant species was influential. Diversity estimates of bacterial and fungal communities revealed shifts in microbiota post-harvest, particularly after the washing step. Relative abundance of *Pseudomonadaceae* and *Enterobacteriaceae* increased from field to bag. Bacterial species specific to different harvesting and processing steps replaced core microbiota species. While processing is unavoidable, procedures that mitigate leaf damage can enhance shelf-life and food safety.

1. Introduction

Baby leaf vegetables are usually eaten raw, with no sanitization step between field and bag. In Sweden, leafy greens are mainly field-grown, machine-harvested, and cooled to 4 °C directly post-harvest to maintain quality. During processing, the leaves are washed to remove soil and debris, dried, and bagged (Fig. 1). Each processing step causes perturbation and possible leaf damage (Kays, 1999; Snowdon, 1990), which can significantly alter the leaf micro-environment and associated microbes. Harvesting and post-harvest injuries also impair the physical quality and chemical characteristics (nutrient leakage) of packaged leafy vegetables (Ariffin, Gkatzionis, & Bakalis, 2017). Damage to the epidermis alters the 3-D landscape, increases surface area, and disrupts internal leaf structure, causing water loss (Aruscavage, Miller, Lewis Ivey, Lee, & LeJeune, 2008; Tukey & Morgan, 1963), localized cell death (Iakimova & Woltering, 2018), and passive leakage of nutrient-rich cellular fluids (Leveau & Lindow, 2001). A single damaged leaf can affect a whole bag, shortening shelf-life (Ariffin et al., 2017). Therefore, leaf tissue damage reduces sensory and nutritional product attributes.

Catabolism governs post-harvest processes in leaves, so modifying the leaf environment (e.g., low temperature, high humidity) can slow the deterioration rate (De Frias et al., 2018; Van den Berg, 1981). During leaf deterioration, organic and inorganic nutrients are released to the nutrient-scarce phyllosphere. Solutes leached from wounds provide carbon and nitrogen, prolonging survival of microbial pathogens (Aruscavage et al., 2008; Aruscavage, Phelan, Lee, & LeJeune, 2010; Brandl, 2008). Spoilage bacteria inhabiting plant surfaces and soil may proliferate in wounds and produce cell wall-degrading enzymes (Pérombelon, 2002), leading to cell necrosis and tissue maceration (Abbott & Boraston, 2008). While most pathogen contamination occurs in-field, harvesting and processing provide opportunities for cross-contamination (Wells & Butterfield, 1997). Machine-harvesting increases leaf exposure to soil or manure (Buchholz, Davidson, Marks, Todd, & Ryser, 2012; Fallon, Rios, & Fonseca, 2011). Under Swedish legislation only drinking water is used for washing, and use of chlorine for surface sanitation of fresh produce is not allowed. According to Swedish Food Agency, if chlorine is added to wash water it is considered a food additive, however chlorine is not on the list of permitted food

* Corresponding author.
E-mail address: Emina.Mulaosmanovic@slu.se (E. Mulaosmanovic).

https://doi.org/10.1016/j.foodcont.2021.107894
Received 26 November 2020; Received in revised form 10 January 2021; Accepted 13 January 2021
Available online 18 January 2021

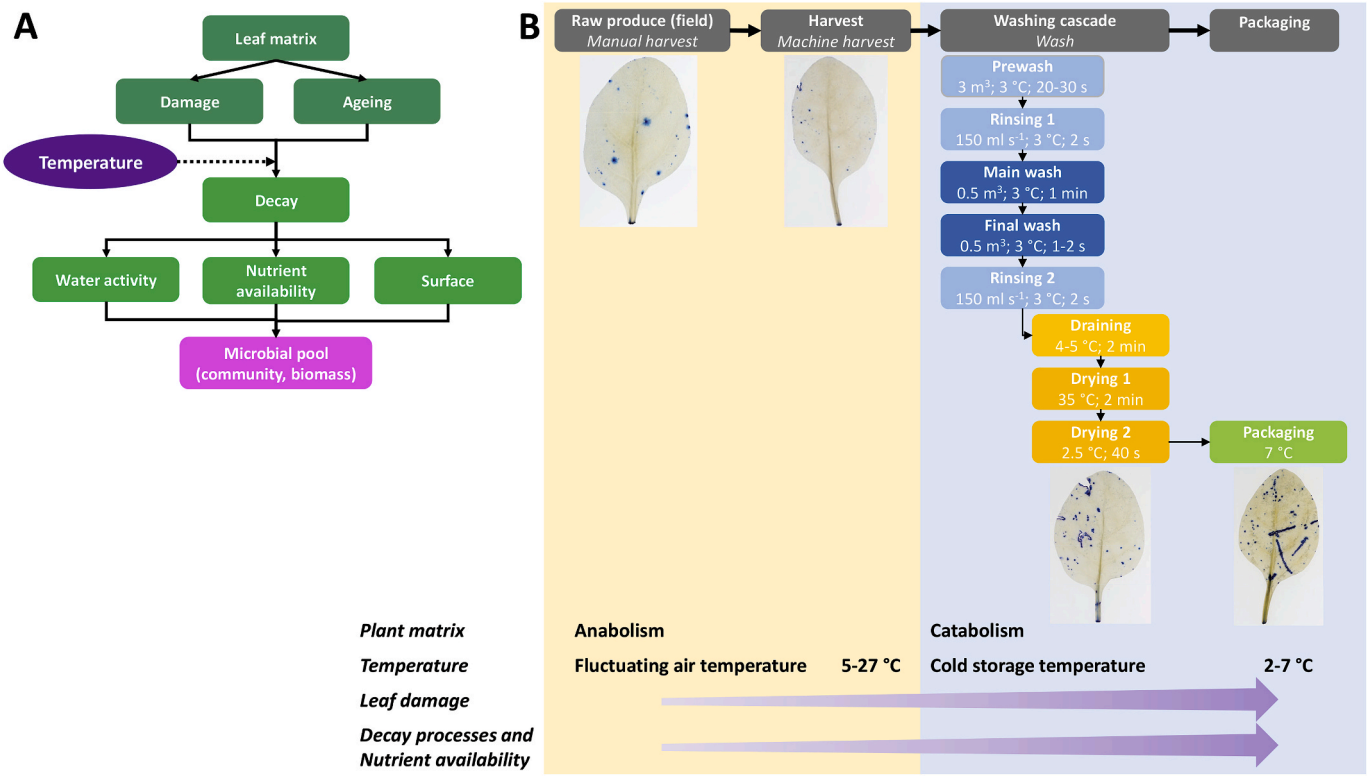

Fig. 1. (A) Steps in which damage to baby leaves is caused by abiotic and biotic factors and leaf ageing, which increases water activity, releases nutrients, and increases surface area, affecting microbial community composition and biomass. (B) Crucial steps from field to bag, environmental conditions, and duration of exposure during the steps considered in this study. (Illustration: B. Alsanius; Photos: E. Mulaosmanovic).

additives (LIVSFS 2007:15). Wash-water is replaced every 4.5 h during processing, and leafy vegetables of different species and origin can be washed with the same wash water. Washing lines are cleaned once, at the end of the day (Mauritzon, 2020). Although washing reduces overall microbial load (Gil, Selma, López-Gálvez, & Allende, 2009), standard washing post-harvest is insufficient for complete inactivation of human pathogens present on baby leaves (Rosberg, Darlison, Mogren, & Alsanius, 2020). Biotic lesions caused by plant pathogens, e.g., *Pseudomonas* spp., *Xanthomonas campestris* (Hartmann et al., 2017), *Bremia lactucae* (Simko, Zhou, & Brandl, 2015), and *Erwinia* spp. (Wells & Butterfield, 1997), are a possible internalization route for human pathogens such as *Escherichia coli* O157:H7 into plant tissue (Von Holy, Lindsay, & Beuchat, 2006).

The indigenous leaf microbiota comprises multiple phylloepiphytes (Lopez-Velasco, Carder, Welbaum, & Ponder, 2013), including bacteria (numerically dominant), fungi, and yeasts (Lindow & Brandl, 2003). Harvesting and post-harvest processing damage can drive successional shifts in microbial community structure by changing the leaf environment from hydrophobic to hydrophilic and enhancing adherence of bacteria. Injuries are preferred habitats (Brandl, 2008) and entry points to the leaf interior for microorganisms (Aruscavage et al., 2008; Aruscavage et al., 2010; Brandl, 2008). Plant-associated microbial communities have been studied with respect to pre-harvest factors and events (seasonal changes, site characteristics, host genotype) (Dees, Lysøe, Nordskog, & Brurberg, 2015; Ding & Melcher, 2016; Williams, Moyne, Harris, & Marco, 2013) and fertilization strategy (Ai et al., 2018; Darlison et al., 2019). However, post-harvest events, especially their role in leaf matrix modification, have received limited attention.

Damage morphometry and location at harvest and post-harvest, and effects on the microbiota, have not been described/quantified for leafy vegetables. Therefore, we quantified damage and characterized size, shape, and position of lesions inflicted on Swiss chard and spinach baby leaves during harvesting and post-harvest. We also assessed concomitant shifts in the phyllosphere microbiota. This paper highlights the impact of harvest and processing on leaf tissue integrity and the microbiota and therefore contributes to improved handling of leafy vegetables along the industrial processing chain, to maintain quality and food safety.

2. Materials and methods

2.1. Leaf sampling

Swiss chard (*Beta vulgaris* subsp. *cicla*) and spinach (*Spinacia oleracea* L.) were field-grown for four weeks on a conventional farm in southern Sweden (55°50′24.36″N, 13°5′48.552″E). To determine in-field (baseline) damage and microbiota, baby leaf samples were collected with scissors in field, from a 1 m^2 area for each replicate (n = 6). Samples from the same field were machine harvested, washed and packaged (commercial facility) on the same day. Machine harvest was performed using Ortomec harvesting machine, with a band-blade system that cuts crops at 1.27 cm (1/2 inch) above the bed top, and leaf samples in category "machine harvested" were collected at the end of the band. After washing in the processing plant, leaves were drained and dried by hot (35 °C, 2 min) and cold (2.5 °C, 40 s) air stream (Fig. 1), then sampled immediately for category "washed". Samples in category "packaged" were prepared in the same manner as for category "washed", then packaged in 200 g bags (OPP film), with high perforation and open atmosphere, and one bag was considered a replicate for microbiota assessment. At all sampling points, leaf samples were placed in plastic boxes to avoid additional damage during transport to the laboratory, and used for damage quantification and microbial community assessment. Samples in categories "baseline", "machine harvested" and "washed" were processed the same day, while "packaged" samples were processed the next day, after being stored for 24 h at 4 °C.

2.2. Damage detection, quantification, and classification

Damage was detected and quantified as described in Mulaosmanovic et al. (2020). To enhance the contrast between healthy and damaged tissue, chlorophyll was removed by soaking whole detached leaves in a solution of ethanol (Solveco, 95%) and acetic acid (Acros Organics, 99.6%) (3:1 (v/v) ratio). To visualize leaf tissue damage, whole leaves were stained with trypan blue (0.01% TB; Sigma Aldrich), which only stains cells with damaged membranes (Tran, Puhar, Ngo-Camus, & Ramarao, 2011). Stained leaves were photographed on a LED-light table (Canon EOS 5D Mark IV camera with Canon EF 50 mm 1:1.4 lens, manual exposure mode, shutter speed 1/125, aperture 6.3, ISO 160).

Leaf and lesion morphometric parameters were automatically quantified using LiMu image analysis program (Mulaosmanovic et al., 2020), with data exported as text files. Results comprised information from the barcode, and leaf and lesion morphometric and location parameters (leaf area, number of lesions per leaf, lesion area, distance of lesions from midrib and leaf edge, and eccentricity).

Leaf area and "leaf age": Based on area (pixels) and known area of objects included in leaf images (1 cm^2), measured areas were converted to cm^2 (1 $cm^2 \approx 55000$ px). For "age" classification, leaves were classified as first true leaves ($\geq$23 cm^2) or second true leaves (<23 cm^2).

Number of lesions, absolute and relative damage: Lesions were enumerated on leaf scale (total number of lesions per leaf). Absolute damage was expressed in pixels (px), as sum of individual lesion areas per leaf. Relative damage per leaf area was calculated as:

$$Damage = \left(\frac{lesion\ \ area}{leaf\ \ area}\right) \times 100\ \ [\%] \quad \text{(I)}$$

Size-based classification of lesions: All individual lesions (stained areas) were classified based on size into: microlesions (1 px; single cell lesions), mesolesions (2–200 px), or macrolesions (>200 px).

Shape-based classification of lesions: Shape was characterized based on the *eccentricity* value of individual lesions, calculated as:

$$Eccentricity, e = \frac{c}{a} \quad \text{(II)}$$

where c is distance from center to the focus and a is distance from the

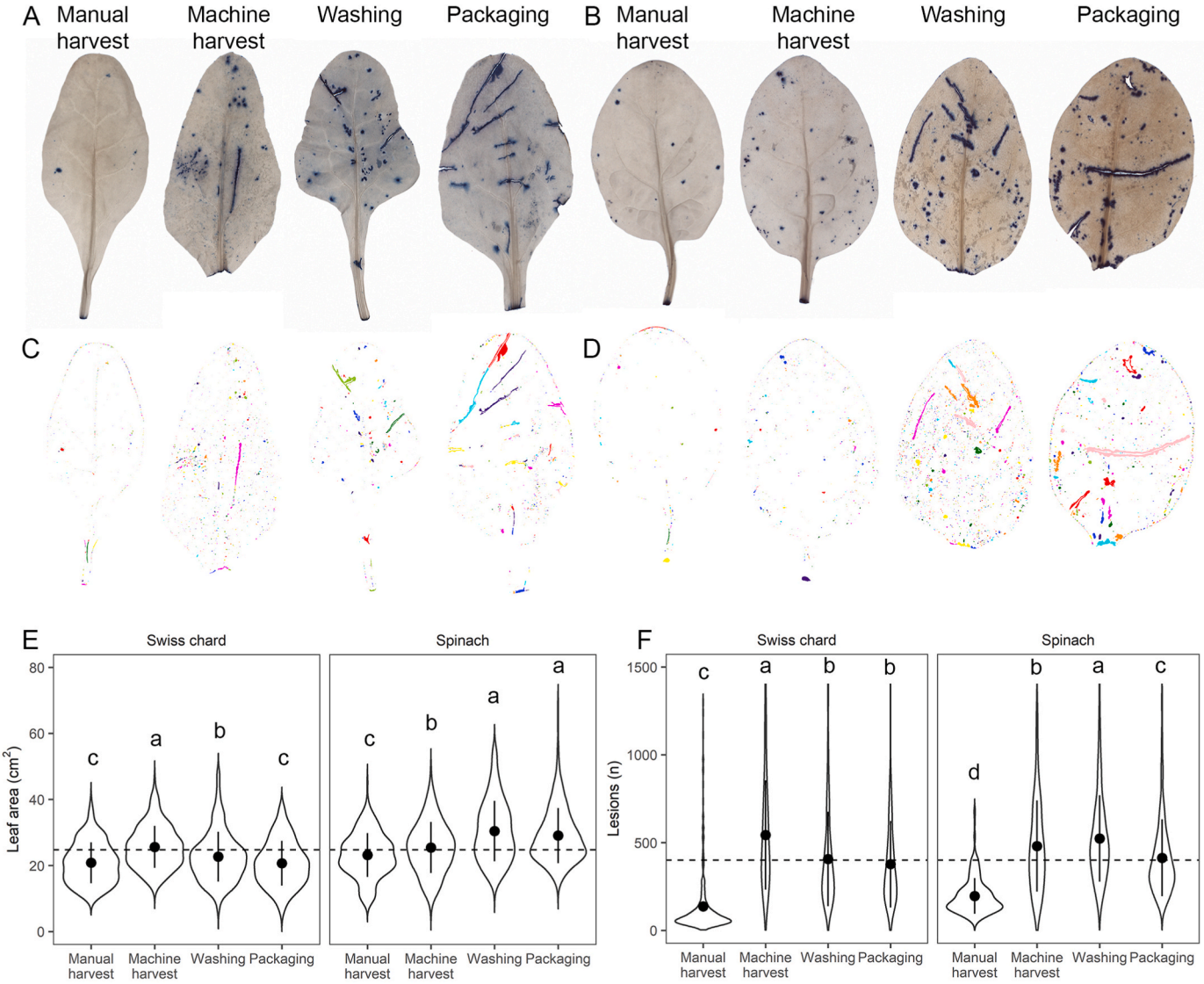

Fig. 2. (A, B) Detection and (C, D) quantification of leaf damage on Swiss chard (A, C) and spinach (B, D) leaves in-field ('baseline') and after machine-harvesting, washing, and packaging. (E) Leaf area and (F) number of lesions, quantified by trypan blue-staining of whole leaves and digital image analysis. Dashed horizontal line in violin plots (E, F) represents overall mean across steps for both species. Black dots within violins indicate within-step mean ± SD (n = 300). Different letters above violin plots indicate significant differences ($p \leq 0.05$) between processing steps (non-parametric Kruskal-Wallis test, followed by Dunn's post-hoc test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

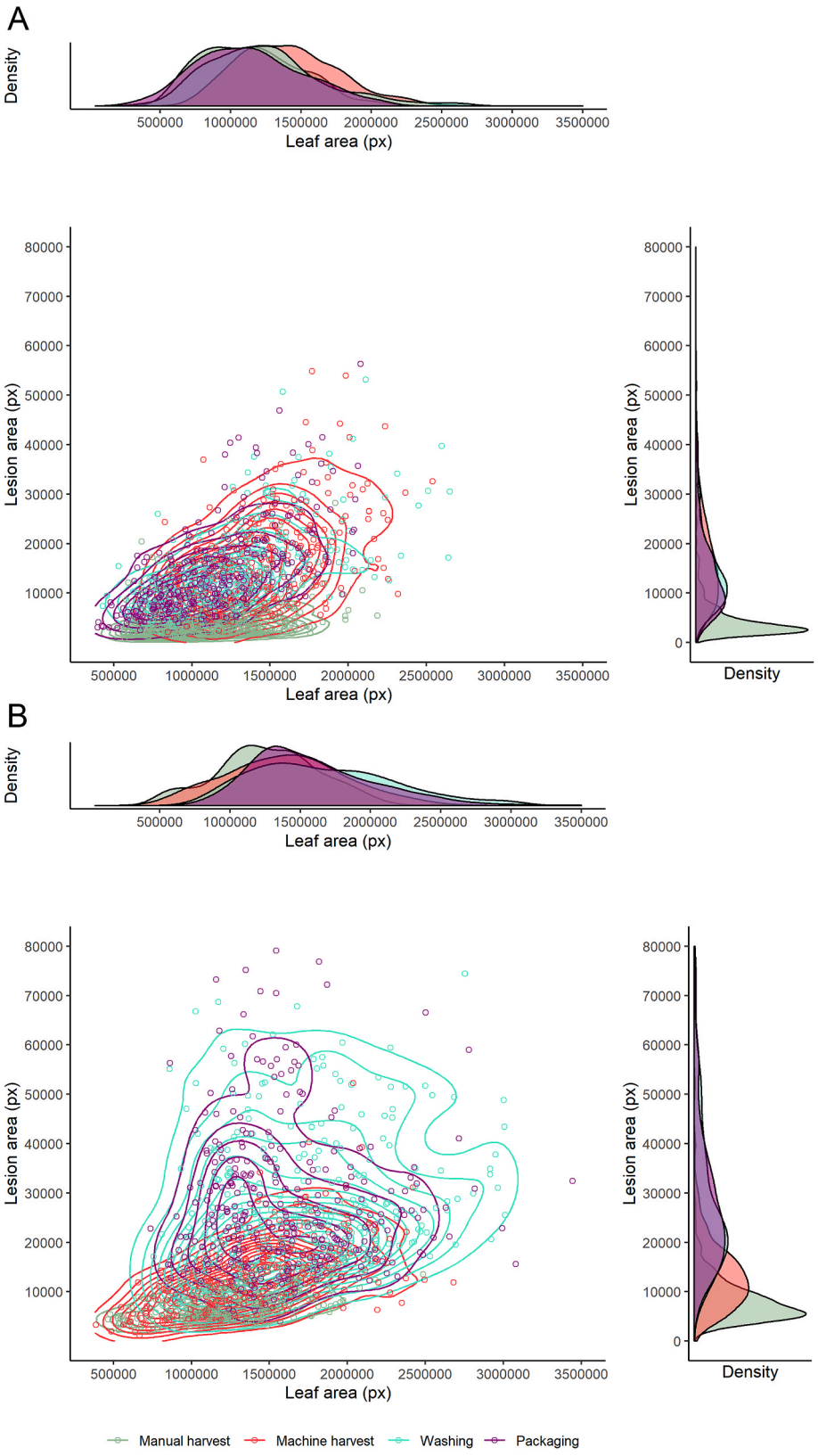

Fig. 3. Scatter marginal density plots showing leaf area distribution and associated lesion area on (A) Swiss chard and (B) spinach leaves (n = 300). Leaf and lesion areas are measured in pixels (px), and grouped by processing steps (in-field (baseline), machine-harvesting, washing, packaging).

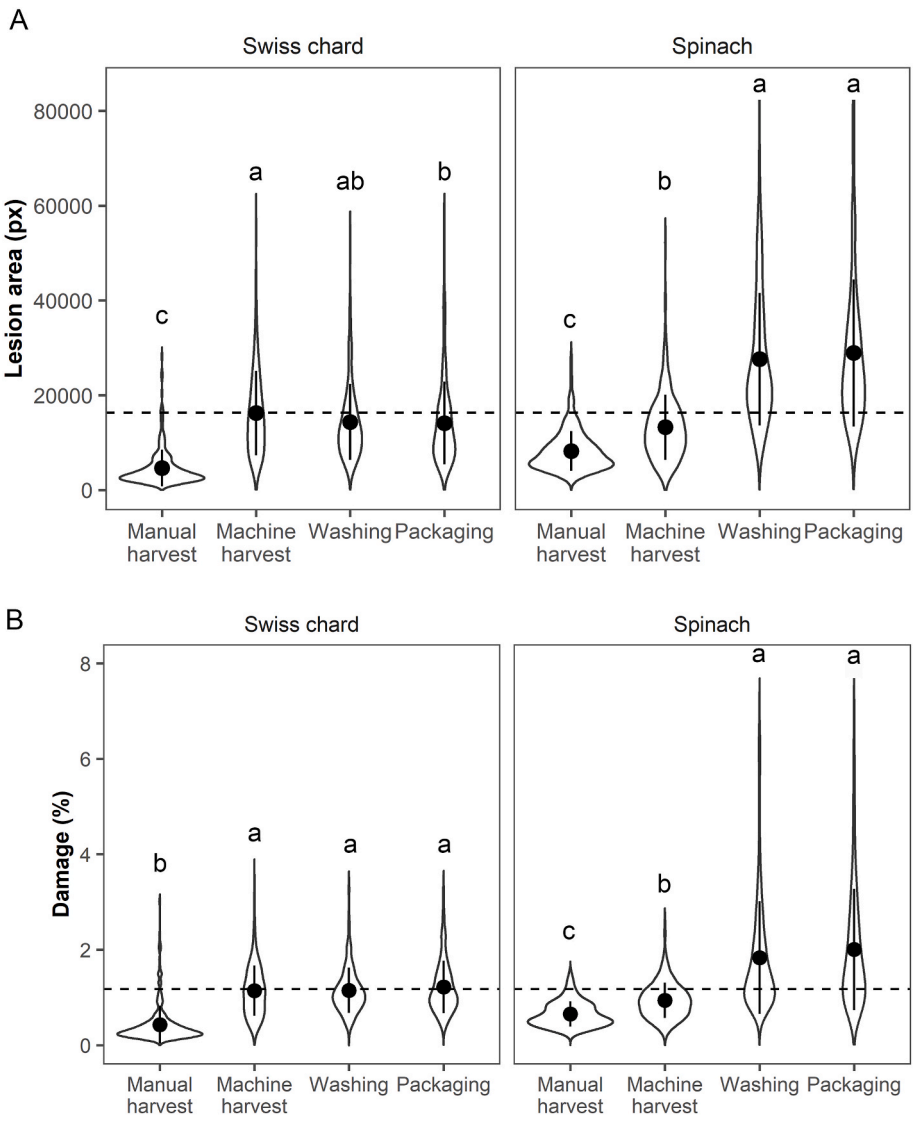

Fig. 4. (A) Absolute and (B) relative lesion area on Swiss chard and spinach leaves in the processing steps in-field (baseline), machine-harvesting, washing, and packaging. Absolute lesion area, measured in pixels (px), represents the sum of all lesions on leaf scale, relative lesion area is the percentage of leaf area covered with lesions. Dashed horizontal line in violin plots is overall mean across all steps for both species. Black dots within violins indicate within-step mean ± SD (n = 300) Different letters above violin plots indicate significant differences ($p \leq 0.05$) between processing steps (non-parametric Kruskal-Wallis test, followed by Dunn's post-hoc test).

center to the vertex.

Eccentricity value ranges from 0 (perfect circle) to 1. Lesions with eccentricity 0–0.9 were classified as round (dots) and lesions with eccentricity >0.9 as cuts.

Position-based classification of lesions: To depict lesion spatial patterns at leaf scale, the relative position of individual lesions was determined as distance from the midrib and the leaf edge:

$$Position = \frac{(distance\ to\ central\ line - distance\ to\ edge)}{(distance\ to\ central\ line + distance\ to\ edge)} \quad \text{(III)}$$

Position values ranged from −1 to 1 and lesions were classified as: edge lesions (0.5–1), leaf blade lesions (−0.5 to 0.5; area between midrib and edge on both sides of leaf), or midrib lesions (−1 to −0.5).

Combined size- and shape-based classification of lesions: Eccentricity and lesion area (px) values were combined into two size-based categories, small (<200 px; microlesions and mesolesions) and large (≥200 px; macrolesions). All lesions were then grouped into four categories: small round, large round, small cut, and large cut lesions.

Combined position- and shape-based classification of lesions: Position and shape parameter (eccentricity) were combined and all lesions were divided into six categories: leaf blade round, edge round, midrib round, leaf blade cut, edge cut, and midrib cut lesions.

2.3. Effect of processing on Swiss chard and spinach microbiota

The phyllosphere microbial community of (i) manually-harvested unwashed (baseline) and (ii) machine-harvested, commercially (iii) washed, and (iv) packaged (24h post-packaging) Swiss chard and spinach leaves was assessed as described previously (Darlison et al., 2019). Leaves (10 g; n = 6) were aseptically transferred to sterile plastic bags with filter (Grade Products Ltd., Coalville, UK) containing 50 mL 0.01M phosphate-buffered saline solution (PBS), and massaged for 2 min at standard speed in a Smasher® (Biomérieux Industry, Durham NC, USA). Bacterial and fungal cells were collected from the suspension by centrifugation of 20 mL at 5000×*g* for 10 min (Centrifuge 5804, Eppendorf AG, Hamburg, Germany), and the pellet obtained was re-suspended in 2 mL 0.01M PBS and centrifuged for 10 min at 13400×*g* (Minispin Centrifuge, Eppendorf AG, Hamburg, Germany). The pellet obtained was stored at −80 °C until DNA extraction with the Zymo-BIOMICS™ DNA Miniprep kit (Cat. No: D4300, Zymo Research, USA).

To evaluate phyllosphere bacterial and fungal communities, samples were analyzed with 300 bp paired-end read (llumina MiSeq V3) at LGC Genomics GmbH (Berlin, Germany). To assess bacterial communities, the 16S ribosomal gene was targeted using forward primer 799F (Chelius & Triplett, 2001) and reverse primer 1115R (Reysenbach & Pace, 1995). The ITS region was targeted using forward primer fITS7 (Ihrmark

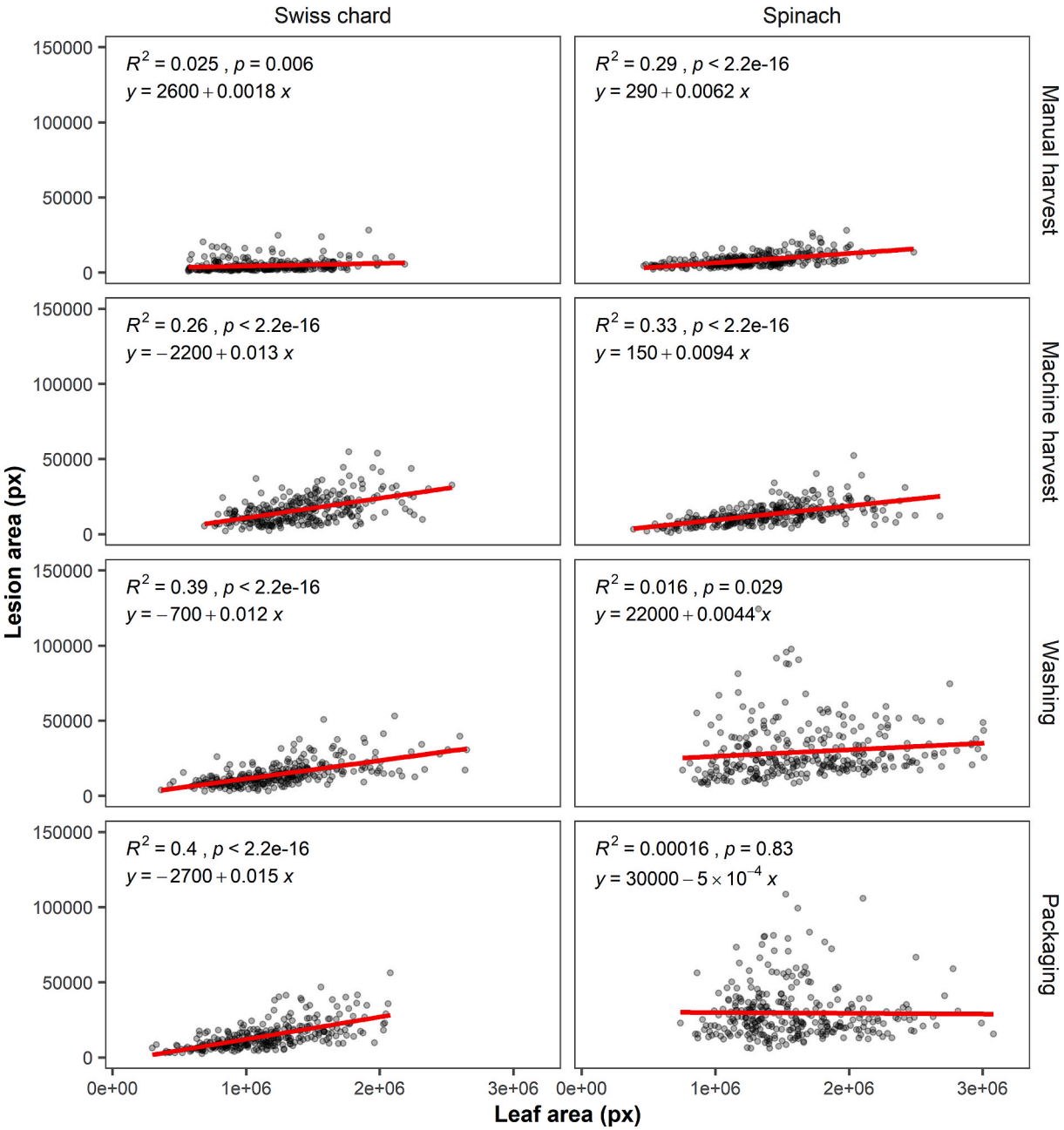

Fig. 5. Regression analysis. Regression line (red) for the model: $y_{Lesion\ \ area\ \ (px)} = \beta_0 + \beta_1 x_{Leaf\ \ area}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2012), while reverse primer ITS4R (White, Bruns, Lee, & Taylor, 1990) was used for fungal communities. The sequencing data were pre-processed and quality-assessed by the bioinformatics service at LGC Genomics GmbH, using Mothur software version 1.35.1 (Schloss et al., 2009). Demultiplexing of sequence reads was performed with Illumina bcl2fastq 2.17.1.14 software. A detailed description of data generation can be found in Supplement File S1.

2.4. Statistical analysis

All analyses except generation of Venn diagrams were performed in R Studio (version 3.6.1) (RStudioTeam). Differences in mean values of morphometric variables between the plant species were tested by nonparametric Kruskal-Wallis test. Pairwise multiple-comparison was carried out using Dunn's post-hoc test, with Holm correction to adjust significance for multiple comparisons. Package *ggplot2* was used for plotting and *ggpubr* for customization in ggplot2 plots. Coefficient of determination (R^2) was calculated using *stat_cor()* function. A linear regression model was created using the *lm()* function. Microbiome data analysis followed the R script modified after Shetty Sudarshan, Lahti Leo, Hermes Gerben, & Hauke Smidt (2020) (Version v3.0). Samples with <3000 reads were excluded from the BIOM file used to analyze microbial community composition, as were sequences identified as mitochondrial. The alpha-diversity of microbial communities between processing steps was estimated by the Shannon and Chao1 indices, using the function *estimate_richness()* from the phyloseq package (McMurdie & Holmes, 2013). Beta-diversity was calculated using weighted UniFrac in the *distance* function and the *ordinate* function in the phyloseq package, with data filtered based on prevalence and total count. To identify the taxa most influenced by species and treatment, Anova followed by

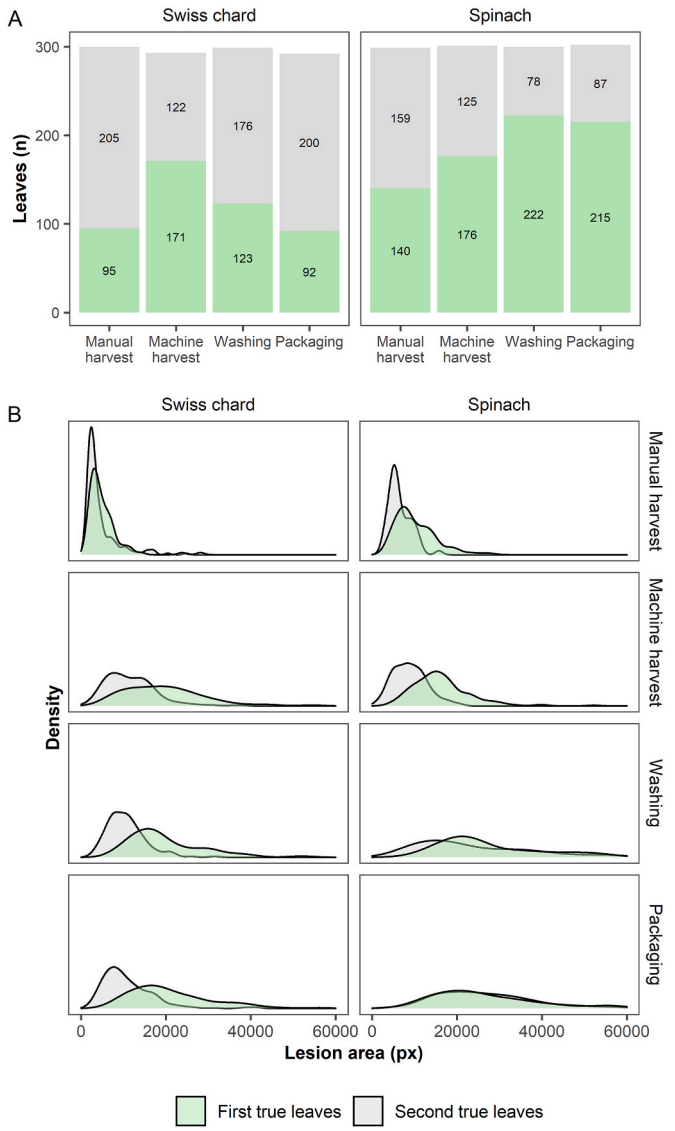

Fig. 6. (A) Size-based 'age' classification of leaves (n = 300) into first true leaves (≥23 cm^2) and second true leaves (<23 cm^2). (B) Lesion area in pixels (px) measured separately on first and second true leaves in different processing steps (field (baseline), machine-harvesting, washing, packaging).

Tukey test ($p < 0.05$) was performed. Membership-based Venn diagrams, showing treatment-unique and shared OTUs, were created using Metagenomics Core Microbiome Exploration Tool (MetaCoMET) (Wang, Xu, Gu, & Coleman-Derr, 2016), with OTUs relative abundance threshold 0.01%, sample absolute read-count threshold 100, and cut-off value 0.3.

3. Results and discussion

3.1. Baseline leaf damage and microbiota

For both Swiss chard (hereafter referred to as "chard") and spinach (except bagged spinach), baseline leaves had fewest lesions, with chard having fewer lesions than spinach (Fig. 2, Supplement Tables S1 and S2). Absolute and relative lesion area were also smallest on baseline leaves for both species, with similar relative lesion area for chard and spinach (0.4% and 0.7%, respectively), but greater absolute lesion area on spinach (Figs. 3 and 4). The latter may partly be due to leaf size, which was positively correlated with total lesion area for both species at baseline (Fig. 5). Baseline spinach leaves were larger than chard leaves (Fig. 2), so relative damage provided a better comparison than absolute damage. Another reason for skewness in damage estimates was the distribution of first and second true leaves, as first true leaves had greater lesion area (Fig. 6). At baseline, first true leaves represented 31.7% and 46.8% of chard and spinach leaves, respectively (Fig. 6). For both crops, most lesions (>95%) at baseline were mesolesions (Fig. 7B), and most were round (Fig. 8B). Most lesions (round or cuts), were located on leaf edges for both chard and spinach (Fig. 9B, Fig. S3). Chard had more midrib damage than spinach, for both cuts (1.8 *vs*. 0.7%) and round lesions (11.2 *vs*. 5.2%). As the midrib is the main vein, midrib damage could release more nutrients than leaf edge or leaf blade damage. While the baseline damage was relatively minor compared with that during processing, completely intact leaves were rare, which should be considered in studies on phyllosphere microbiology to avoid confounding.

Baseline spinach had lower bacterial species richness (Chao1) than subsequent processing steps, but higher species relative abundance (Fig. 12B) and evenness (Shannon index) than all samples except washed and packaged spinach (Fig. 10A–B). Baseline chard leaves had similar overall bacterial diversity to processed leaves (Fig. 10A–B). Tenzin, Ogunniyi, Ferro, Deo, and Trott (2020) found lower Chao1 and Shannon values for unwashed spinach samples immediately post-harvest than in different washing/disinfection treatments. Baseline bacterial Chao1 diversity values for spinach, but not chard, were lower than reported by Darlison et al. (2019) and Rosberg et al. (2020). Baseline bacterial Shannon diversity values were similar to those reported previously (Darlison et al., 2019; Rosberg et al., 2020). Baseline fungal diversity on both spinach and chard was similar to that in subsequent processing steps (Fig. 10C–D), although baseline chard had the second highest species richness (Fig. 10D). Fungal Chao1 diversity values for spinach leaves were similar to those described previously (Darlison et al., 2019), while fungal Shannon diversity values were slightly higher. For bacterial beta-diversity, baseline chard co-clustered with machine-harvested leaves, but not post-harvest steps, while baseline spinach clustered weakly with leaves from all subsequent steps (Fig. 11A–B). Rosberg et al. (2020) found that washing caused a bacterial diversity shift in spring-grown, but not autumn-grown, spinach and rocket. For fungal beta-diversity, baseline chard co-clustered with all other leaves, whereas baseline spinach co-clustered only with machine-harvested leaves (Fig. 11C–D). Baseline chard was dominated by the bacterial phyla Proteobacteria and Deinococcus-Thermus, particularly *Pseudomonadaceae*, *Moraxellaceae*, and *Deinococcaceae*. Baseline spinach was dominated by Proteobacteria, Firmicutes, and Actinobacteria (Fig. 12A–B), as reported previously for manually-harvested spinach leaves (Darlison et al., 2019; Leff & Fierer, 2013; Lopez-Velasco et al., 2013; Lopez-Velasco, Welbaum, Boyer, Mane, & Ponder, 2011; Rosberg et al., 2020; Tenzin et al., 2020). The three families occurring in highest relative abundance on baseline spinach were *Burkholderiaceae*, *Bacillaceae*, and *Enterobacteriaceae* (Fig. 12B). Ascomycetes was the dominant fungal phylum in baseline chard and spinach, and in subsequent processing steps (Fig. 12C). Basidiomycota was the second most prevalent phylum on chard, while Basidiomycota and Chytridiomycota were equally abundant on spinach (Fig. 12C), partly confirming previous findings (Darlison et al., 2019). The bacterial core microbiota of chard and spinach was similar on phylum level, and comprised Proteobacteria (62%–82%), Firmicutes (7%–13%), and Actinobacteria (3–7%). Deinococcus-Thermus comprised 23% of the chard core microbiota, but only 0.8% of the spinach core biota. The core biota of both was dominated by *Pseudomonadaceae* (29–41%), *Enterbacteriaceae* (8–18%), and *Burholderiaceae* (7–22%), with *Pseudomonas* the most abundant genus on both chard and spinach (29–41%), as previously reported for baby leaves (Rosberg et al., 2020). Baseline and machine-harvested chard shared the highest numbers of OTUs (bacteria 1.6%, fungi 5.3% OTUs). The largest group of treatment-specific OTUs was found on baseline leaves in all cases except for the bacterial community on spinach, where most specific

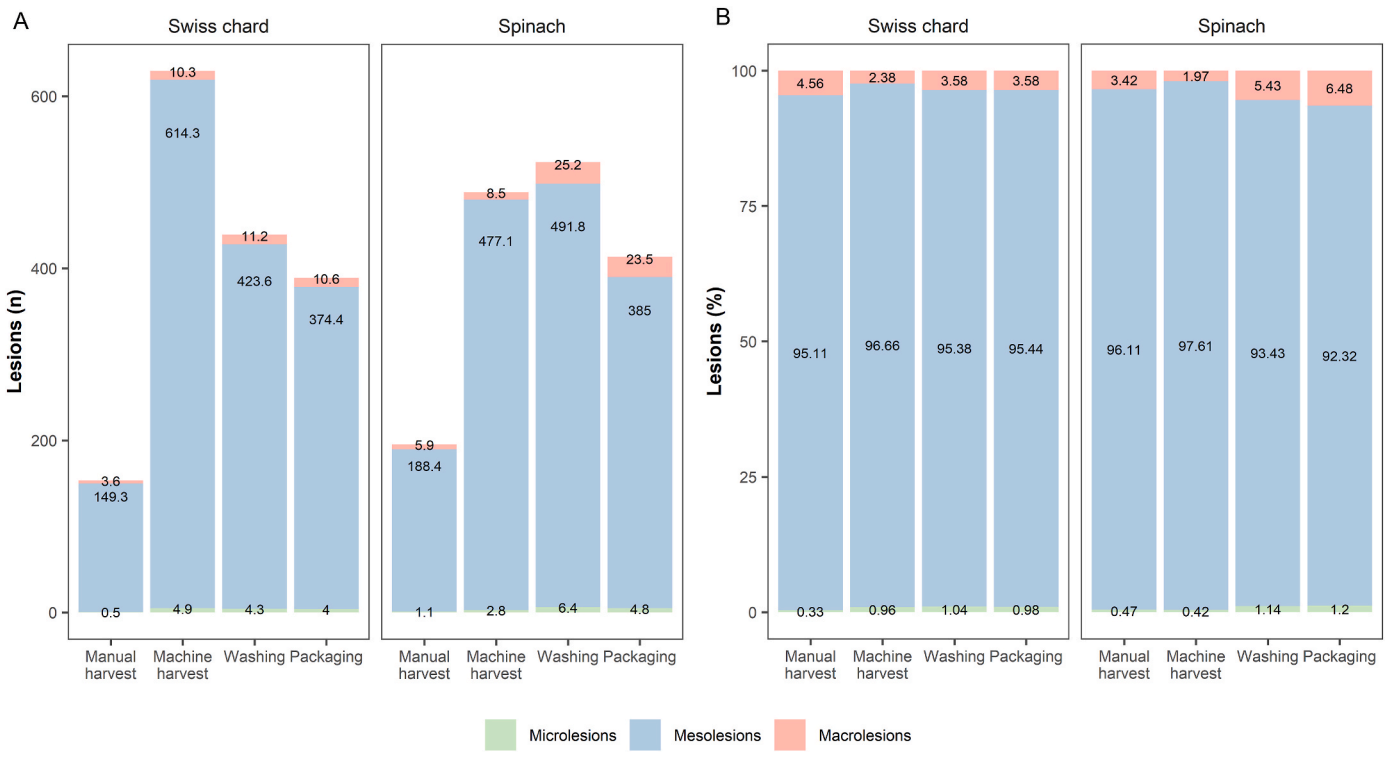

Fig. 7. (A) Absolute (and (B) relative distribution of each lesion size-based class on leaves, grouped by processing steps (in-field (baseline), machine-harvesting, washing, packaging). All individual lesions (stained areas) were classified into microlesions (1px), mesolesions (2–200 px), and macrolesions (>200 px).

OTUs were found on machine-harvested leaves. Baseline-specific bacterial OTUs predominantly comprised *Pseudomonas* (15–28%) on both plant species, *Acinetobacter* (44%) on chard, and unclassified *Enterobacteriaceae* (38%) on spinach. In both plant species, genera with <3% relative abundance comprised a high proportion of baseline-specific bacterial OTUs. Fungal microbiota were stable from field to bag, with less dominance of step-specific fungal OTUs (core: chard 22.8%, spinach 34%), than for the bacterial core biota. The fungal core microbiota of chard and spinach were similar on phylum level and dominated by Ascomycota (67–78%) and Basidiomycota (20–32%), but contained plant pathogens such as *Botrytis caroliniana*, *Fusarium oxysporum*, *Gibberella intricans*, and *Blumeria graminis*. These fungal species can cause lesion formation and plant tissue rot (Ezrari, Lahlali, Radouane, Tahiri, & Lazraq, 2020; Fernández-Ortuño, Li, Wang, & Schnabel, 2012).

3.2. Machine-harvested leaf damage and microbiota

Harvesting represents a major transition for the plant matrix from autotrophic to heterotrophic, i.e., from anabolic to catabolic processes, with influences of leaf matrix, temperature, leaf damage, decay, and microbial pools (Ariffin et al., 2017; Mogren et al., 2018; Rosberg et al., 2020).

Machine-harvesting significantly increased relative damage to both crops (Figs. 2 and 4A), partly due to the higher proportion of first true leaves (Fig. 6A). Mesolesions and small round lesions dominated the size and shape classes for both species, but the proportion of macrolesions on both was reduced compared with baseline (Figs. 7 and 8). Midrib lesions (round and cuts) (Fig. S3) increased for both chard and spinach (Fig. 9). Therefore, machine-harvesting damages leaves and the midrib area, resulting in nutrient leaching and driving shifts in the resident microbiota (Leveau & Lindow, 2001). This was observed especially for the bacterial community on spinach.

Compared with baseline, machine-harvesting did not significantly change alpha- or beta-diversity of bacteria or fungi on chard, but increased bacterial species richness (Chao1) and decreased species abundance and evenness on spinach (Figs. 10 and 11). On spinach, the bacterial community showed increasing representation of Proteobacteria, while other phyla decreased (Fig. 12). Machine-harvesting also seemed to introduce/favor *Enterobacteriaceae* on spinach leaves. Bacterial OTUs specific to machine-harvesting were dominated by *Acinetobacter* (43–74%) and *Pseudomonas* (10–14%) (Fig. 13).

3.3. Washing damage and microbiota

Washing to remove impurities was performed in a commercial processing facility using tapwater without sanitizing agents. Studies have found impaired wash-water quality in commercial facilities due to reuse in processing leaves from different batches (Grudén, Mogren, & Alsanius, 2016). Washing alters conditions on the leaf surface due to (i) cooling of plant biomass, (ii) centrifugation or exposure to combined air jets and short-time, high-temperature drying (Grudén et al., 2016), and (iii) aging of detached plant biomass (Medina, Tudela, Marín, Allende, & Gil, 2012).

Washing did not damage chard, but increased relative damage to spinach leaves (Fig. 4B). The significant relationship between leaf size and lesion area enabled comparison using relative damage (Fig. 5). Spinach leaves are convex, with the leaf tip folding towards the abaxial side. Forces applied during washing and drying (Fig. 1) may enhance breakage of the slightly folded spinach leaf tips, while smaller chard leaves can avoid such damage. This could partly explain the increase in cuts (Fig. 8, Fig. S2), and overall lesion area (Fig. 4) on washed spinach, but not chard leaves (Fig. 6, Fig. S1). The proportion of first true leaves in washed leaves decreased for chard (41%) and increased for spinach (74%) (Fig. 6). Mesolesions continued to dominate lesion size but macrolesions began to appear, comprising 3.6% and 5.4% of all lesions on chard and spinach, respectively (Fig. 7). Midrib cuts also increased (Fig. S3), to comprise 2.9% and 1.5% for chard and spinach, respectively, while for spinach, round midrib lesions increased to 8.8% (Fig. 9).

In terms of bacterial and fungal alpha-diversity, washing only

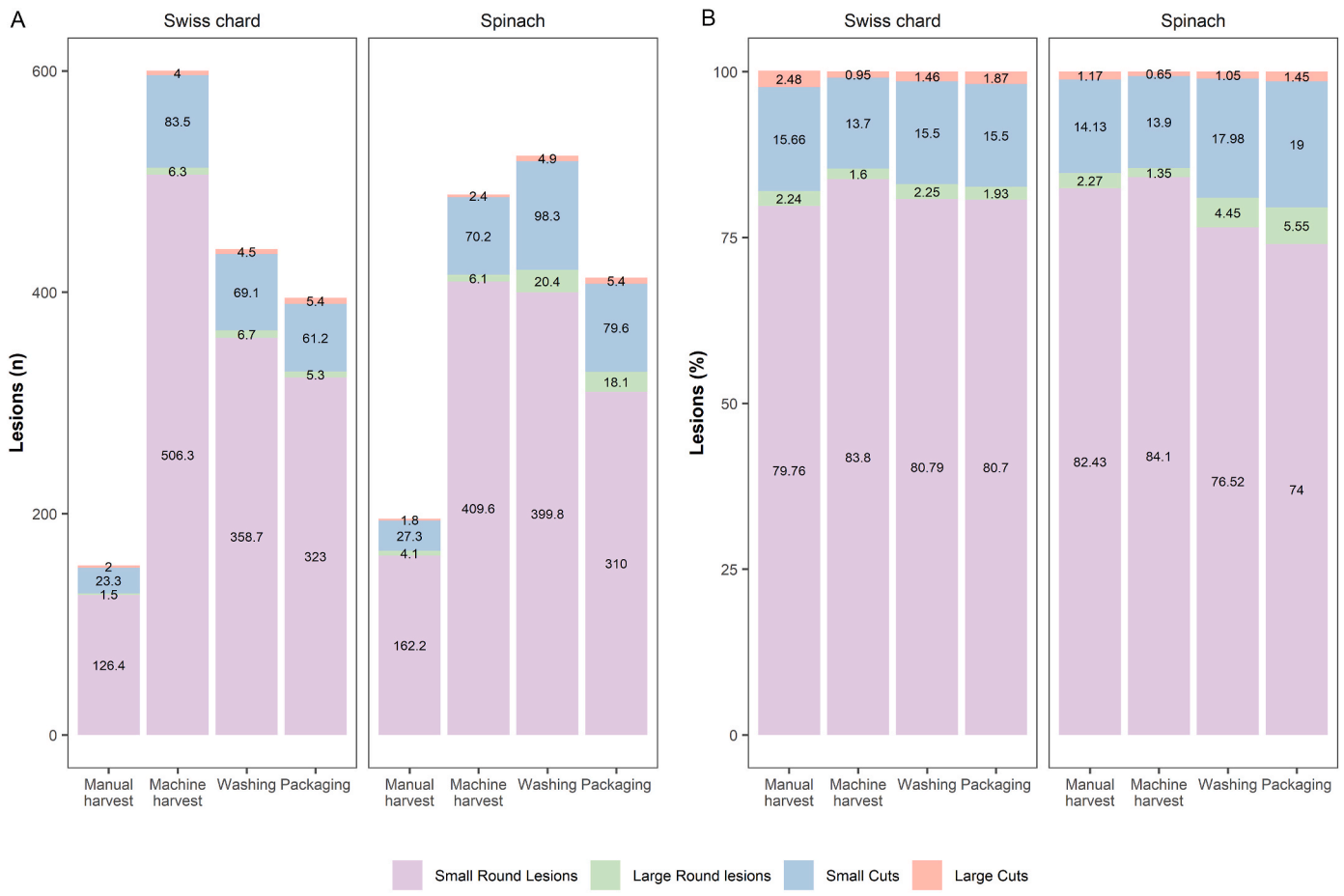

Fig. 8. (A) Absolute and (B) relative distribution of each lesion size and shape-based class on leaves, grouped by processing steps (in-field (baseline), machine-harvesting, washing, packaging). All individual lesions (stained areas) were classified into: small and large round lesions, and small and large cut-shaped lesions.

affected (decreased) fungal species richness on chard (Fig. 10). Tenzin et al. (2020) made similar findings when using tapwater for washing. However, Rosberg et al. (2020) found that commercial washing significantly reduced Chao1 and Shannon diversity on spring-grown, but not autumn-grown, spinach. Thus differences between studies could be a result of seasonal differences (Ding & Melcher, 2016). In terms of beta-diversity, bacterial communities on washed chard and fungal communities on washed spinach leaves co-clustered with communities in the leaf packaging step (Fig. 11). Spinach bacterial communities formed a single cluster, with some overlap with baseline and packaged leaves, while chard fungal communities co-clustered with communities from packaged leaves, with some overlap with baseline leaves (Fig. 11). Similarly, Rosberg et al. (2020) observed differences in beta-diversity from field sampling through processing (including washing). Washing caused significant shifts in relative abundance of Proteobacteria, which increased on both chard and spinach (Fig. 12), as observed in previous studies (Rosberg et al., 2020). However, Tenzin et al. (2020) observed increased relative abundance of Actinobacteria and Firmicutes, but decreased Proteobacteria, on tap-water washed leaves compared with unwashed. At family level, relative abundance of *Moraxellaceae* decreased and *Pseudomonadaceae* increased on both chard and spinach (Fig. 12). On chard, relative abundance of *Comamonadaceae* also decreased and *Burkholderiaceae* and *Enterobacteriaceae* increased. Although *Pseudomonas* was abundant on baseline leaves, its absolute abundance on washed samples increased 4.5-fold on chard and 5.4-fold on spinach compared with previous processing steps. Rosberg et al. (2020), also observed significant increases in relative abundances of *Enterobacteriaceae* and *Pseudomonas* spp. post-washing, together with no significant reduction in viable counts during washing without sanitizer or even substantially increased total aerobic counts and *Enterobacteriaceae* counts compared with non-washed leaves. Our results indicate that washing can allow cross-contamination by *Pseudomonadaceae* (spinach and chard) and *Enterobacteriaceae* (chard), as their proportions and absolute values increased substantially immediately post-washing. Slight (0.5%) relative damage on leaves can still support increases in *E. coli* O157:H7 populations, with pathogen numbers being saturated at 9.8% relative damage (Mulaosmanovic, Windstam, Vågsholm, & Alsanius, 2021). For fungal phyla, both chard and spinach showed an increase in Basidiomycota, with a decrease in Ascomycota on chard and Zygomycota on spinach. Bacterial OTUs specific to washing were *Pseudomonas* (80–83%) and *Acinetobacter* (2–13%) on both plant species (Fig. 13).

3.4. Packaging leaf damage and microbiota

Overall, packaged spinach leaves showed more damage than chard leaves (Figs. 3 and 4). Packaged leaves had around 3.7-fold more damage compared with baseline (Fig. 4A), but not compared with washed leaves (Fig. 4). Relative abundance of macrolesions increased significantly on packaged spinach, but not chard, with spinach having almost twice as many macrolesions as chard (Fig. 7B). Packaged spinach, but not chard, showed a substantial increase in relative abundance of cuts (Fig. S2). Relative abundance of large (chard and spinach) and small (spinach) cuts increased markedly post-packaging compared with washed samples (Fig. 8B).

Packaging did not affect alpha-diversity of bacterial and fungal communities (Fig. 10). For beta-diversity, packaged chard and spinach samples closely co-clustered with washed samples, and differed only

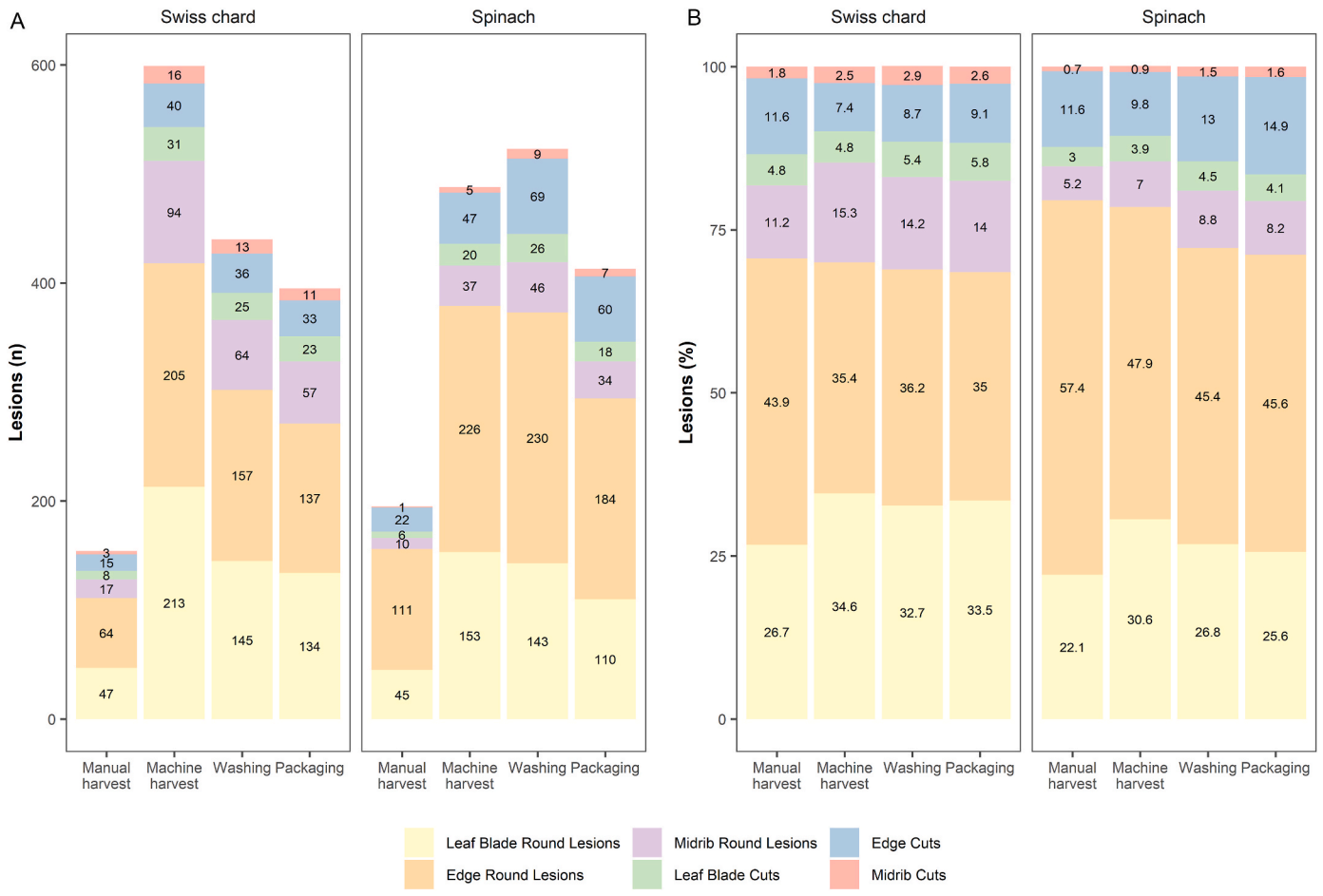

Fig. 9. (A) Absolute and (B) relative distribution of each lesion position- and shape-based class on leaves, grouped by processing steps (in-field (baseline), machine-harvesting, washing, packaging). All individual lesions were classified into: round-shaped and cut-shaped lesions on leaf blade, edge, and midrib, respectively.

from baseline samples (except for fungal communities on chard), and bacterial communities on machine-harvested spinach (Fig. 11). Phylum level diversity (relative abundance >2%) of bacteria and fungi decreased from field to bag. Packaged leaves were dominated by the bacterial phyla Proteobacteria and Firmicutes (chard and spinach) and Deinococcus-Thermus (chard). However, the proportions of Firmicutes, Deinococcus-Thermus, and Actinobacteria increased on packaged chard leaves, whereas the relative abundance of Actinobacteria decreased on spinach leaves (Fig. 12). Packaged chard leaves were dominated by *Pseudomonadaceae*, *Enterobacteriaceae*, and *Deinococcaceae*, and spinach leaves by *Pseudomonadaceae*, *Moraxellaceae*, and *Enterobacteriaceae* (Fig. 12A–B). On chard leaves, increased relative abundance of *Deinococcaceae* was observed post-packaging. *Pseudomonadaceae* and *Enterobacteriaceae* are reported to dominate bacterial populations in cold storage (Lund, 1992; Rosberg et al., 2020; Vankerschaver, Willocx, Smout, Hendrickx, & Tobback, 1996). Interestingly, relative abundance of *Enterobacteriaceae* and *Moraxellaceae* increased post-packaging on both crops, partly supporting recent findings by Rosberg et al. (2020). Increased relative abundance of *Pseudomonadaceae* and *Moraxellaceae* has been observed previously (Tenzin et al., 2020), and has been correlated with spoilage of baby leaves at cold-storage temperatures (Tatsika, Karamanoli, Karayanni, & Genitsaris, 2019). Changes in environmental (cold-chain) and nutritional conditions on the leaf surface caused by leaf damage might cause this shift in relative abundance of bacterial phyla after packaging. The most prominent representative of *Moraxellaceae* was *Acinetobacter*, a food-spoilage bacterial genus (Battey & Schaffner, 2001; Zhu et al., 2018) previously found on fresh produce (Rosberg et al., 2020). *Acinetobacter* (6–20%) and *Pseudomonas* (74–83%) accounted for high proportions of all packaging-specific OTUs, but their proportion in the core microbiota (always present) was substantially lower (*Acinetobacter* 0–1%; *Pseudomonas* 21–41%). Cold storage favors growth of *Acinetobacter* and *Pseudomonas* spp., including the pectinolytic pseudomonads (Nguyen-the & Carlin, 1994), due to their psychrotrophic lifestyle. They can outcompete mesophilic species for the nutrients released by wounding and accelerate spoilage of packaged produce (Andreani & Fasolato, 2017). Ascomycota and Basidiomycota were the dominant fungal phyla on packaged chard and spinach (Fig. 12C). Family-level relative abundance of fungi on packaged chard and spinach was similar to that on washed leaves (Fig. 12D). Unlike the bacterial core biota, the fungal core biota was not substantially impacted by packaging (Fig. 13).

3.5. Implications of processing steps

Chard and spinach baby leaves accumulated damage during processing (Figs. 1B, Fig. 2A–B). Leaf damage causes a temporary flush of carbon- and nitrogen-containing compounds in the nutrient-poor phyllosphere (Aruscavage et al., 2010; Mercier & Lindow, 2000; Tukey, 1970). Damage alone may be insufficient in explaining nutrient leakage, as not all lesions were equal (Figs. 2, 4, 7–9). The relationship between accumulated damage and nutrient concentration may not be linear. We suspect that midrib damage gives more prominent nutrient pulses than equivalent leaf damage, since midribs are the main conduit for photosynthates and minerals.

Conditions change from hydrophobic to hydrophilic in damaged areas, altering leaf physicochemistry and allowing microorganisms to

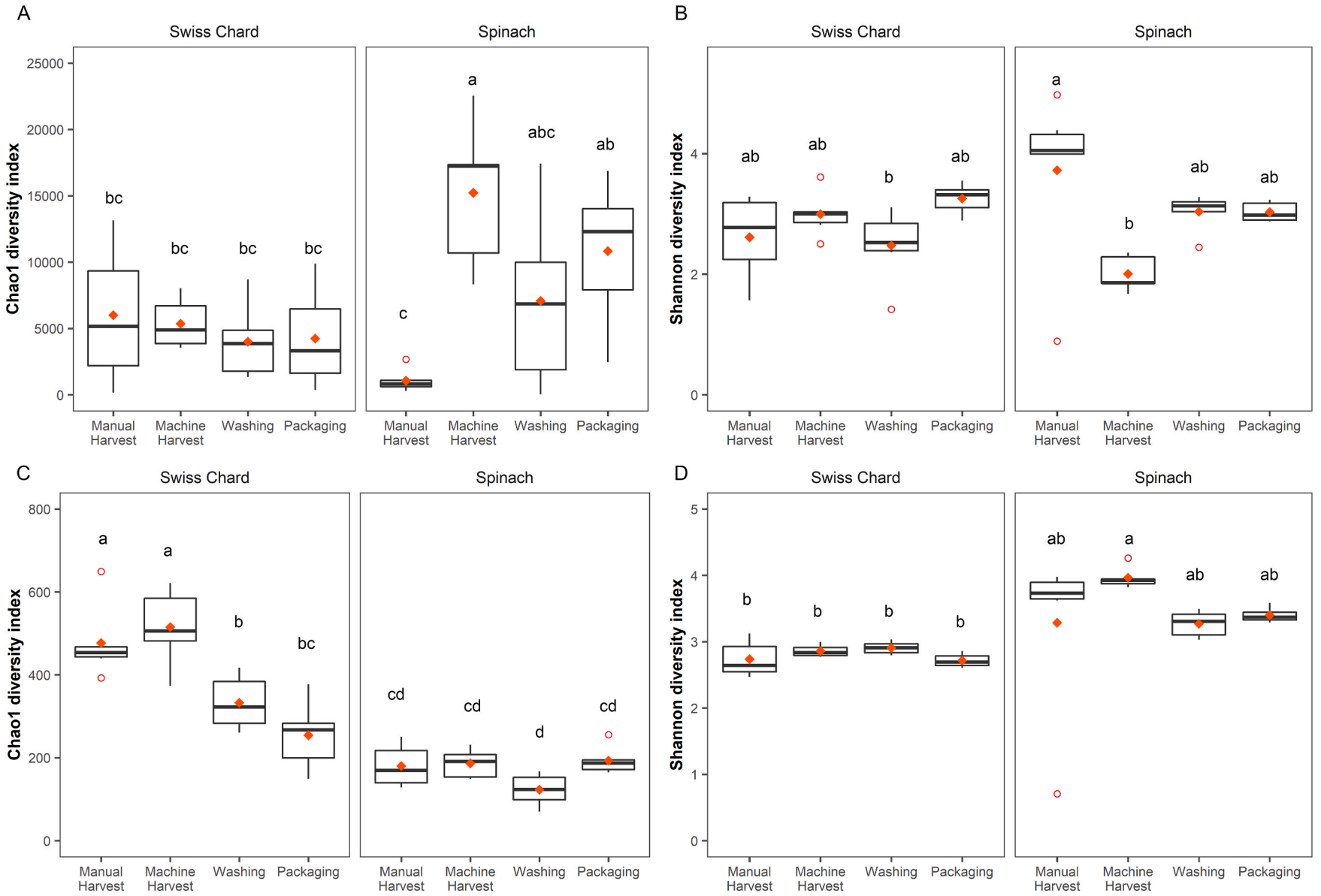

Fig. 10. Alpha-diversity of (A, B) bacterial, and (C, D) fungal communities. Chao1 (A, C) and Shannon (B, D) diversity indices, grouped by processing step (in-field (baseline), machine-harvesting, washing, packaging). Different letters above boxes indicate significant differences ($p \leq 0.05$) between processing steps and plant species.

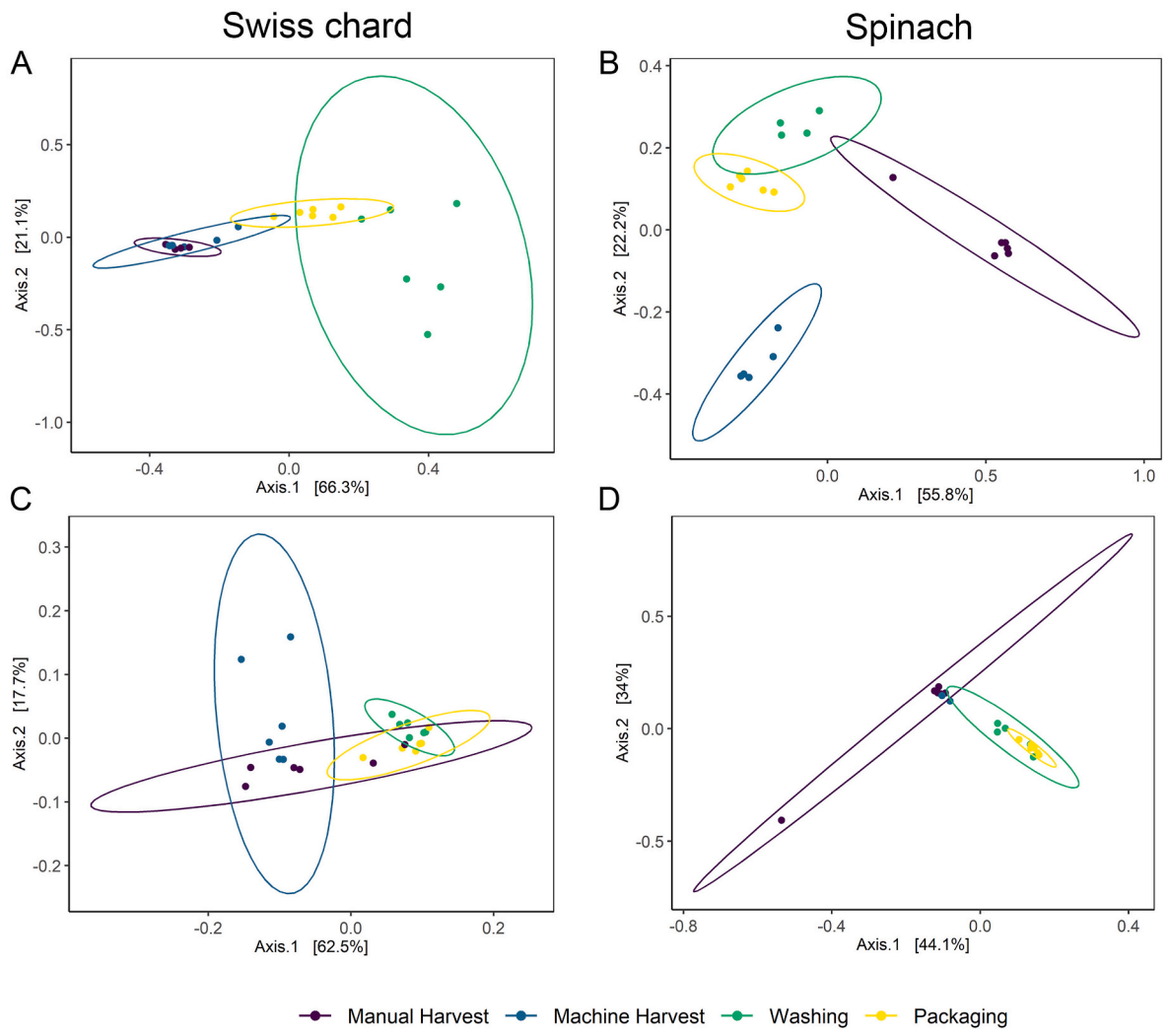

Fig. 11. Beta-diversity based on weighed UniFrac distances (PCoA plots) for 16S rRNA bacterial communities (A, B) and ITS2 fungal communities (C, D), of Swiss chard and spinach, grouped by processing steps (in-field (baseline), machine-harvesting, washing, packaging).

adhere. Damage also alters the 3-D landscape of the phyllosphere (Mulaosmanovic, Windstam, Vågsholm, & Alsanius, 2021), exposing surface areas previously unavailable for colonization by resident microbes and potential pathogens. A recent study found that artificially induced damage enabled ample *E. coli* O157:H7 colonization at damage sites (Mulaosmanovic, Windstam, Vågsholm, & Alsanius, 2021).

Spinach has extremely high respiration rate (Kader, 2002), thus mechanical damage stimulates additional respiration, and accelerates water loss (Escalona, Aguayo, Martínez-Hernández, & Artés, 2010; Kader, 2002). Although not investigated here, because of damage induced from field to bag, it is expected that respiration rate increases given the increased surface area, thus more sugar becomes available with wounding. In damaged plants defense mechanism activates, requiring more energy, thus increasing respiration (Fonseca, Oliveira, & Brecht, 2002). Oxidation of phenolic compounds (tissue browning) occurs as a result of damage, and this process requires increased oxygen consumption. This effect perseveres in the tissue adjacent to the damaged area, modifying the metabolism and quicker decay (Kader, 2002). Increased respiration and leached nutrients may support multiplication of spoilage microorganisms (Artés & Allende, 2005; Mogren et al., 2018). Harvesting and post-harvest processing has indirect consequences for microbes, through damage releasing more nutrients and increasing surface area, and direct consequences, e.g., during washing, which removes microbes or potentially introduces pathogen through cross-contamination (Gil et al., 2009; Grudén et al., 2016; Rosberg et al., 2020). Different processing steps have different direct/indirect effects, so measured damage does not directly reflect the microbiota.

The bacterial (2.3-fold) and fungal (2.9-fold) core microbiota on chard was larger than that on spinach, and each processing step caused shifts. The core microbiota was small (chard 1.2%; spinach 0.3% OTUs) (Fig. 13), and most OTUs were specific to processing steps. *Enterobacteriaceae* (unclassified 4–10%; *Pantoea* 4–8%) and *Burkholderiaceae* (*Burkholderia* 7–20%), families containing plant and human pathogens, comprised a significant fraction of the core biota. High abundance of *Enterobacteriaceae* on spinach, lettuce, and sprouts has been reported previously (Leff & Fierer, 2013). *Enterobacteriaceae* are recognized plant colonizers and plant microhabitats are known reservoirs, including for potentially human pathogenic bacteria (Brandl, 2006; Erlacher, Cardinale, Grosch, Grube, & Berg, 2014). Since baby leaves are consumed raw, these families in the core biota (always present), could pose health concerns. The greatest shift, at family- and OTU-level, was seen for washing (Figs. 11–13), which had both indirect and direct effects on the microbiota.

For resident microbes, processing causes perturbations that result in successional shifts, as shown for the chard and spinach microbiota (Figs. 11–13). Abundance of 38 bacterial families and one group of unclassified bacteria found on baseline chard and spinach leaves was not maintained, as three and six families increased in abundance for chard

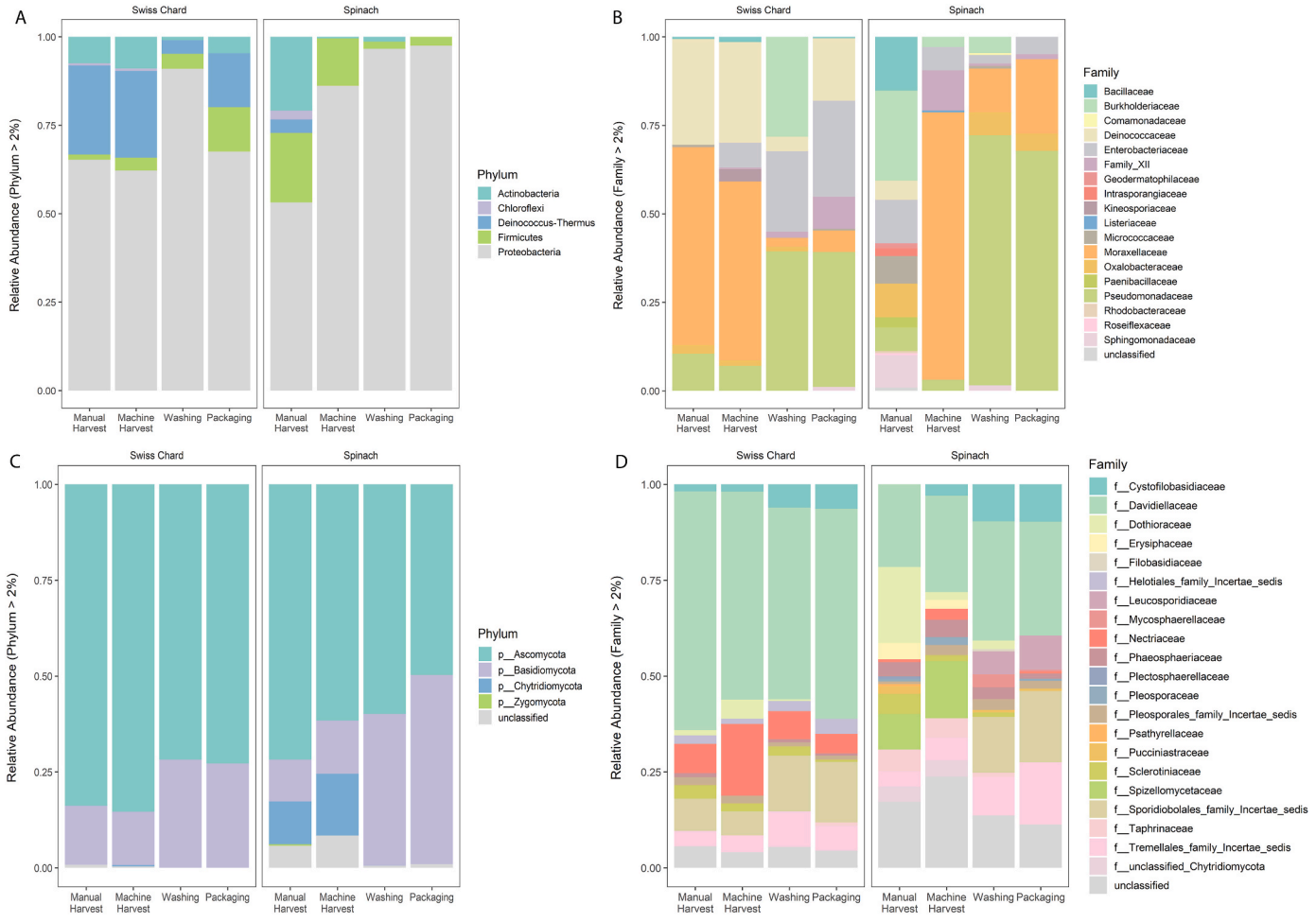

Fig. 12. Relative abundance (>2%) of bacterial and fungal phyla (A, C) and families (B, D) on Swiss chard and spinach leaves, grouped by processing steps (in-field (baseline), machine-harvesting, washing, packaging).

and spinach, respectively, while 26 and 27 families declined. Similarly, more than half of 25 fungal families and one group of unclassified fungi detected on baseline chard and spinach increased (3 and 7 families, respectively) or declined (14 and 7 families, respectively) in abundance during processing (Fig. S4).

Damaged leaf areas provide nutrients for microbial growth (Zagory, 1999). For *Pseudomonas fluorescens*, sugars are limiting for colonization, with microbial density being directly correlated with leaf sugar content (Mercier & Lindow, 2000). Microbiota shifts due to leaf damage and other direct effects during processing may affect the shelf-life and sensory quality of baby leaves, with one damaged leaf spoiling the whole bag (Ariffin et al., 2017). This may be because damage stimulates food spoilage microbes, or because cooling and washing remove other competitors, making niches available. For example, *Pseudomonadaceae* (mainly genus *Pseudomonas*) were present on both baseline and machine-harvested chard and spinach leaves, but their abundance was increased by washing and maintained by bagging (Fig. 12B and Fig. S4). *Pseudomonas* spp. are causal agents in food spoilage (Andreani & Fasolato, 2017; Federico et al., 2015). In beets (including chard), bacterial infections caused by *Pseudomonas syringae* pv. *aptata* can result in spot-like leaf lesions (Koike, Henderson, Bull, Goldman, & Lewellen, 2003). *Pseudomonadaceae* includes plant pathogens and soft-rot pathogens, which may be directly involved in decreasing shelf-life.

Damage appears to be unavoidable in the baby leaf processing. To enhance shelf-life and food safety, treatments that mitigate damage or impede deterioration are needed.

4. Conclusions

Lesion area increases from field to bag, at rates depending on plant species and leaf age. Most lesions are round and medium-sized, but severe cuts to leaf edge and midrib are inflicted during harvesting and post-harvest processing. Bacterial diversity and abundance decrease from harvesting to bag. Bacterial, but not fungal, species specific to different harvesting and processing steps substantially exceed core microbiota species. Abundance of *Pseudomonadaceae*, especially the genus *Pseudomonas* comprising diverse spoilage microorganisms and plant pathogens, increases during washing. *Enterobacteriaceae* shows a similar trend. *Moraxellaceae* abundance is reduced by washing, but it quickly recolonizes processed leaves due to its psychrotrophic lifestyle, cold-chain exclusion of mesophilic competitors, and nutrient release from damaged sites on leaves.

Funding

This study was part of the “Safe Salad”’ project [grant number: 2012-2107] and “Peculiar scent of contaminated leafy greens” project [grant number: 2017-00511] funded by the Swedish Research Council for Sustainable Development (FORMAS), Stockholm, Sweden (PI: Beatrix Alsanius).

CRediT authorship contribution statement

E. Mulaosmanovic: Conceptualization, Data curation, Formal

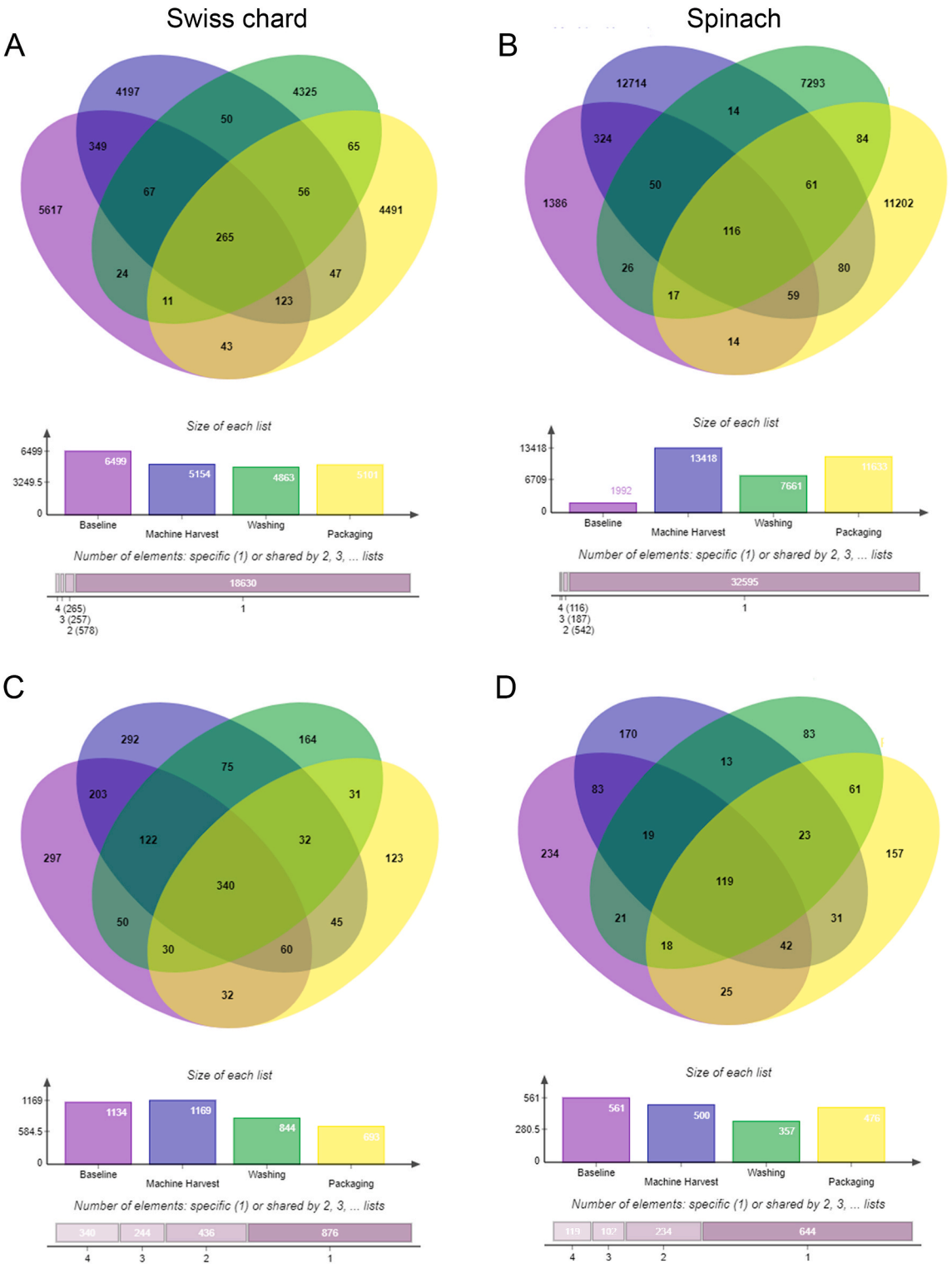

Fig. 13. Size of core microbiota of 16S rRNA bacterial communities (A, B) and ITS2 fungal communities (C, D), and OTUs unique to different processing steps (in-field (baseline), machine-harvesting, washing, packaging) on Swiss chard and spinach leaves.

analysis, Investigation, Methodology, Visualization, Writing - original draft. **T.U.T. Lindblom:** Data curation, (image analysis data). **S.T. Windstam:** Supervision, Writing - review & editing. **M. Bengtsson:** Supervision, Writing - review & editing. **A.K. Rosberg:** Data curation, Writing - review & editing. **L. Mogren:** Supervision, Writing - review & editing. **B.W. Alsanius:** Funding acquisition, Supervision, Writing - review & editing.

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.

Acknowledgements

The authors gratefully acknowledge Vidinge Grönt AB, Teckomatorp, Sweden for successful cooperation and for setting the plant material to our disposal. We would like to thank Jan-Eric Englund and Adam Flöhr for statistical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.107894.

References

Abbott, D. W., & Boraston, A. B. (2008). Structural biology of pectin degradation by Enterobacteriaceae. *Microbiology and Molecular Biology Reviews, 72*(2), 301–316.

Ai, C., Zhang, S., Zhang, X., Guo, D., Zhou, W., & Huang, S. (2018). Distinct responses of soil bacterial and fungal communities to changes in fertilization regime and crop rotation. *Geoderma, 319*, 156–166.

Andreani, N. A., & Fasolato, L. (2017). Pseudomonas and related genera. In *The microbiological quality of food* (pp. 25–59). Elsevier.

Ariffin, S. H., Gkatzionis, K., & Bakalis, S. (2017). Leaf injury and its effect towards shelf-life and quality of ready-to-eat (RTE) spinach. *Energy Procedia, 123*, 105–112.

Artés, F., & Allende, A. (2005). Processing lines and alternative preservation techniques to prolong the shelf-life of minimally fresh processed leafy vegetables. *European Journal of Horticultural Science, 70*(5), 231.

Aruscavage, D., Miller, S. A., Lewis Ivey, M. L., Lee, K., & LeJeune, J. T. (2008). Survival and dissemination of Escherichia coli O157: H7 on physically and biologically damaged lettuce plants. *Journal of Food Protection, 71*(12), 2384–2388.

Aruscavage, D., Phelan, P. L., Lee, K., & LeJeune, J. T. (2010). Impact of changes in sugar exudate created by biological damage to tomato plants on the persistence of Escherichia coli O157: H7. *Journal of Food Science, 75*(4), M187–M192.

Battey, A., & Schaffner, D. (2001). Modelling bacterial spoilage in cold-filled ready to drink beverages by Acinetobacter calcoaceticus and Gluconobacter oxydans. *Journal of Applied Microbiology, 91*(2), 237–247.

Brandl, M. T. (2006). Fitness of human enteric pathogens on plants and implications for food safety. *Annual Review of Phytopathology, 44*, 367–392.

Brandl, M. T. (2008). Plant lesions promote the rapid multiplication of Escherichia coli O157: H7 on postharvest lettuce. *Applied and Environmental Microbiology, 74*(17), 5285–5289.

Buchholz, A. L., Davidson, G. R., Marks, B. P., Todd, E. C., & Ryser, E. T. (2012). Quantitative transfer of Escherichia coli O157: H7 to equipment during small-scale production of fresh-cut leafy greens. *Journal of Food Protection, 75*(7), 1184–1197.

Chelius, M., & Triplett, E. (2001). The diversity of archaea and bacteria in association with the roots of Zea mays L. *Microbial Ecology, 41*(3), 252–263.

Darlison, J., Mogren, L., Rosberg, A. K., Grudén, M., Minet, A., Liné, C., … Uhlig, E. (2019). Leaf mineral content govern microbial community structure in the phyllosphere of spinach (Spinacia oleracea) and rocket (Diplotaxis tenuifolia). *The Science of the Total Environment, 675*, 501–512.

De Frias, J. A., Luo, Y., Zhou, B., Turner, E. R., Millner, P. D., & Nou, X. (2018). Minimizing pathogen growth and quality deterioration of packaged leafy greens by maintaining optimum temperature in refrigerated display cases with doors. *Food Control, 92*, 488–495.

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. *Applied and Environmental Microbiology, 81*(4), 1530–1539.

Ding, T., & Melcher, U. (2016). Influences of plant species, season and location on leaf endophytic bacterial communities of non-cultivated plants. *PloS One, 11*(3).

Erlacher, A., Cardinale, M., Grosch, R., Grube, M., & Berg, G. (2014). The impact of the pathogen Rhizoctonia solani and its beneficial counterpart Bacillus amyloliquefaciens on the indigenous lettuce microbiome. *Frontiers in Microbiology, 5*, 175.

Escalona, V. H., Aguayo, E., Martínez-Hernández, G. B., & Artés, F. (2010). UV-C doses to reduce pathogen and spoilage bacterial growth in vitro and in baby spinach. *Postharvest Biology and Technology, 56*(3), 223–231.

Ezrari, S., Lahlali, R., Radouane, N., Tahiri, A., & Lazraq, A. (2020). First report of Fusarium equiseti causing pre-and postharvest fruit rot on zucchini in Morocco. *Journal of Plant Pathology, 102*(1), 251-251.

Fallon, S., Rios, D., & Fonseca, J. (2011). Scrutinizing the sanitizing process involving aqueous chlorine from field to fresh-cut iceberg lettuce. In *Paper presented at the international conference on postharvest and quality management of horticultural products of interest for tropical regions 906*.

Federico, B., Pinto, L., Quintieri, L., Carito, A., Calabrese, N., & Caputo, L. (2015). Efficacy of lactoferricin B in controlling ready-to-eat vegetable spoilage caused by Pseudomonas spp. *International Journal of Food Microbiology, 215*, 179–186.

Fernández-Ortuño, D., Li, X., Wang, F., & Schnabel, G. (2012). First report of gray mold of strawberry caused by Botrytis caroliniana in North Carolina. *Plant Disease, 96*(6), 914-914.

Fonseca, S. C., Oliveira, F. A., & Brecht, J. K. (2002). Modelling respiration rate of fresh fruits and vegetables for modified atmosphere packages: A review. *Journal of Food Engineering, 52*(2), 99–119.

Gil, M. I., Selma, M. V., López-Gálvez, F., & Allende, A. (2009). Fresh-cut product sanitation and wash water disinfection: Problems and solutions. *International Journal of Food Microbiology, 134*(1–2), 37–45.

Grudén, M., Mogren, L., & Alsanius, B. (2016). Processing of green leaf produce: Microorganisms associated with process water and produce. In *Paper presented at the III international conference on fresh-cut produce: Maintaining quality and safety 1141*.

Hartmann, R., Fricke, A., Stützel, H., Mansourian, S., Dekker, T., Wohanka, W., et al. (2017). Internalization of Escherichia coli O157: H7 gfp+ in rocket and Swiss chard baby leaves as affected by abiotic and biotic damage. *Letters in Applied Microbiology, 65*(1), 35–41.

Iakimova, E. T., & Woltering, E. J. (2018). The wound response in fresh-cut lettuce involves programmed cell death events. *Protoplasma, 255*(4), 1225–1238. https://doi.org/10.1007/s00709-018-1228-y

Ihrmark, K., Bödeker, I., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., … Clemmensen, K. E. (2012). New primers to amplify the fungal ITS2 region–evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology, 82*(3), 666–677.

Kader, A. A. (2002). *Postharvest technology of horticultural crops* (3rd ed.). University of California Agriculture and Natural Resources: ANR Publication 3311 (Chapter 4).

Kays, S. J. (1999). Preharvest factors affecting appearance. *Postharvest Biology and Technology, 15*(3), 233–247.

Koike, S., Henderson, D., Bull, C., Goldman, P., & Lewellen, R. (2003). First report of bacterial leaf spot of Swiss chard caused by Pseudomonas syringae pv. aptata in California. *Plant Disease, 87*(11), 1397-1397.

Leff, J. W., & Fierer, N. (2013). Bacterial communities associated with the surfaces of fresh fruits and vegetables. *PloS One, 8*(3).

Leveau, J. H., & Lindow, S. E. (2001). Appetite of an epiphyte: Quantitative monitoring of bacterial sugar consumption in the phyllosphere. *Proceedings of the National Academy of Sciences, 98*(6), 3446–3453.

Lindow, S. E., & Brandl, M. T. (2003). Microbiology of the phyllosphere. *Applied and Environmental Microbiology, 69*(4), 1875–1883.

Lopez-Velasco, G., Carder, P. A., Welbaum, G. E., & Ponder, M. A. (2013). Diversity of the spinach (Spinacia oleracea) spermosphere and phyllosphere bacterial communities. *FEMS Microbiology Letters, 346*(2), 146–154.

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. *Journal of Applied Microbiology, 110*(5), 1203–1214.

Lund, B. M. (1992). Ecosystems in vegetable foods. *Journal of Applied Bacteriology, 73*, 115s–126s.

Mauritzon, J. (2020). *The possibilities of recycling wash water at Vidinge* (Master Thesis). Available online: https://lup.lub.lu.se/student-papers/search/publication/9004204. (Accessed 23 December 2020).

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS One, 8*(4).

Medina, M. S., Tudela, J. A., Marín, A., Allende, A., & Gil, M. I. (2012). Short postharvest storage under low relative humidity improves quality and shelf life of minimally processed baby spinach (Spinacia oleracea L.). *Postharvest Biology and Technology, 67*, 1–9.

Mercier, J., & Lindow, S. (2000). Role of leaf surface sugars in colonization of plants by bacterial epiphytes. *Applied and Environmental Microbiology, 66*(1), 369–374.

Mogren, L., Windstam, S., Boqvist, S., Vågsholm, I., Söderqvist, K., Rosberg, A. K., … Alsanius, B. (2018). The hurdle approach–A holistic concept for controlling food safety risks associated with pathogenic bacterial contamination of leafy green vegetables. A review. *Frontiers in Microbiology, 9*(1965). https://doi.org/10.3389/fmicb.2018.01965

Mulaosmanovic, E., Lindblom, T. U. T., Bengtsson, M., Windstam, S. T., Mogren, L., Marttila, S., … Alsanius, B. W. (2020). High-throughput method for detection and quantification of lesions on leaf scale based on trypan blue staining and digital image analysis. *Plant Methods, 16*(1), 62. https://doi.org/10.1186/s13007-020-00605-5

Mulaosmanovic, E., Windstam, S. T., Vågsholm, I., & Alsanius, B. W. (2021). Size Matters: Biological and Food Safety Relevance of Leaf Damage for Colonization of Escherichia coli O157:H7 gfp+. *Frontiers in Microbiology, 11*(3458). https://doi.org/10.3389/fmicb.2020.608086

Nguyen-the, C., & Carlin, F. (1994). The microbiology of minimally processed fresh fruits and vegetables. *Critical Reviews in Food Science and Nutrition, 34*(4), 371–401.

Pérombelon, M. (2002). Potato diseases caused by soft rot erwinias: An overview of pathogenesis. *Plant Pathology, 51*(1), 1–12.

Reysenbach, A., & Pace, N. (1995). *Reliable amplification of hyperthermophilic archaeal 16S rRNA genes by the polymerase chain reaction*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. Archaea: A Laboratory Manual.

Rosberg, A. K., Darlison, J., Mogren, L., & Alsanius, B. W. (2020). Commercial wash of leafy vegetables do not significantly decrease bacterial load but leads to shifts in bacterial species composition. *Food Microbiology*, 103667.

RStudioTeam. (2018). *Integrated development for R (version 3. 6. 1)*. Boston, MA: RStudio, Inc.. Available online: http://www.rstudio.com/.Rstudio.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., … Robinson, C. J. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology, 75*(23), 7537–7541.

Shetty Sudarshan, A., Leo, L., Hermes Gerben, D., & Hauke, S. (2020). *Microbial bioinformatics introductory course material 2018 (Version v3.0)*. Zenodo. https://doi.org/10.5281/zenodo.1436630

Simko, I., Zhou, Y., & Brandl, M. T. (2015). Downy mildew disease promotes the colonization of romaine lettuce by Escherichia coli O157: H7 and Salmonella enterica. *BMC Microbiology, 15*(1), 1–9.

Snowdon, A. L. (1990). *A colour atlas of post-harvest diseases and disorders of fruits and vegetables. Volume 1: General introduction and fruits*. Wolfe Scientific Ltd.

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.

Tenzin, S., Ogunniyi, A. D., Ferro, S., Deo, P., & Trott, D. J. (2020). Effects of an eco-friendly sanitizing wash on spinach leaf bacterial community structure and diversity. *Applied Sciences, 10*(8), 2986.

Tran, S. L., Puhar, A., Ngo-Camus, M., & Ramarao, N. (2011). Trypan blue dye enters viable cells incubated with the pore-forming toxin HlyII of Bacillus cereus. *PloS One, 6*(9), Article e22876.

Tukey, H., Jr. (1970). The leaching of substances from plants. *Annual Review of Plant Physiology, 21*(1), 305–324.

Tukey, H., & Morgan, J. (1963). Injury to foliage and its effect upon the leaching of nutrients from above-ground plant parts. *Physiologia Plantarum, 16*(3), 557–564.

Van den Berg, L. (1981). The role of humidity, temperature, and atmospheric composition in maintaining vegetable quality during storage. In *ACS symposium series* (pp. 95–107). https://doi.org/10.1021/bk-1981-0170.ch008

Vankerschaver, K., Willocx, F., Smout, C., Hendrickx, M., & Tobback, P. (1996). The influence of temperature and gas mixtures on the growth of the intrinsic micro-organisms on cut endive: Predictive versus actual growth. *Food Microbiology, 13*(6), 427–440.

Von Holy, A., Lindsay, D., & Beuchat, L. R. (2006). Vectors and conditions for preharvest contamination of fruits and vegetables with pathogens capable of causing enteric diseases. *British Food Journal, 108*(1), 38–53. https://doi.org/10.1108/00070700610637625

Wang, Y., Xu, L., Gu, Y. Q., & Coleman-Derr, D. (2016). MetaCoMET: A web platform for discovery and visualization of the core microbiome. *Bioinformatics, 32*(22), 3469–3470.

Wells, J., & Butterfield, J. (1997). Salmonella contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. *Plant Disease, 81*(8), 867–872.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications, 18*(1), 315–322.

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).

Zagory, D. (1999). Effects of post-processing handling and packaging on microbial populations. *Postharvest Biology and Technology, 15*(3), 313–321.

Zhu, S., Wu, H., Zhang, C., Jie, J., Liu, Z., Zeng, M., et al. (2018). Spoilage of refrigerated Litopenaeus vannamei: Eavesdropping on acinetobacter acyl-homoserine lactones promotes the spoilage potential of shewanella baltica. *Journal of Food Science & Technology, 55*(5), 1903–1912.