CENTER for PRODUCE SAFETY

CPS 2022 RFP
FINAL PROJECT REPORT

Project Title

Evidence for the industrial application of bacteriophages to control *Listeria monocytogenes* in leafy greens

Project Period

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Objectives

1. *To verify in an industrial setting the effectiveness of bacteriophages to control Listeria monocytogenes (Lm) growth while preserving the quality of leafy greens affected by seasonality.*
2. *To confirm the beneficial impact of bacteriophages controlling Lm growth at abusive temperatures and the interaction with the natural microbiota.*
3. *To estimate the cost-benefits of the industrial application of bacteriophages as a post-process treatment to control Lm.*

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FINAL REPORT

Abstract

Ensuring microbial safety in fresh-cut products, against *Listeria monocytogenes*, is a significant industry challenge. Effective control strategies are essential to prevent contamination and protect public health. Bacteriophages have emerged as a promising biopreservative strategy for controlling *L. monocytogenes* in fresh produce, demonstrated at laboratory scale. However, their practical application and efficacy in industrial settings remain uncertain. The project aims at the industrial validation of the application of bacteriophages as a prevention tool to control *L. monocytogenes* growth in leafy greens applied in a large-scale industrial operation when the quality of raw materials fluctuates by seasonality and abusive storage conditions. Three objectives were identified. The first objective was to verify, in an industrial setting, the effectiveness of bacteriophages in controlling *L. monocytogenes* growth while preserving the quality of leafy greens as affected by seasonality. The second objective was to confirm the beneficial impact of bacteriophages controlling *L. monocytogenes* growth at abusive temperatures and to assess their interaction with the natural microbiota. The third objective was to estimate the cost-benefits for the industrial application of bacteriophages as a post-process treatment. Conducted over one year, the project involved 20 trials on a commercial baby spinach processing line. A commercial bacteriophage solution, PhageGuard Listex™ (Listex™), at a concentration of 10^9 pfu/mL, was applied post-washing and drying, just before packaging on the vibration conveyor belt aiding leaf separation. Control and treated samples were stored at commercial temperature conditions (3 days at 4°C + 7 days at 7°C) as well as abusive temperatures (10 days at 10°C). The results of this project indicate that *L. monocytogenes* was not detected in any of the 600 samples over the 20 trials performed. The enumeration of *Listeria* spp. showed low levels in baby spinach, with only a slight reduction observed in the treated leaves compared to untreated ones. Statistical analysis indicated no significant differences in *Listeria* levels due to the Listex™ treatment. However, when considering the prevalence of *Listeria* spp. (i.e., the proportion of samples where *Listeria* spp. were detectable in >5 CFU/plate), a significant reduction was observed after storage in the treated samples, demonstrating the potential efficacy of Listex™. Quality characteristics of treated spinach related to the physiological metabolism, such as the headspace gas composition, overall sensory quality, and color, were not affected compared to the control leaves. The analysis of the bacterial community revealed that there were no significant changes in the diversity and structure of the microbiota in the control and Listex™-treated baby spinach. However, significant changes were observed in the microbial composition after storage, with shifts in the abundance of specific taxa. Regarding costs associated with Listex™, implementation costs include initial investment in application equipment, ongoing product costs, labor for application, training for proper use, and routine monitoring and testing. It is estimated that these costs will increase the cost of packaged leafy greens by 0.05-0.54 cents per bag. The findings indicate that the use of Listex™ on an industrial scale has the potential to reduce the prevalence of *Listeria* spp. in baby spinach while maintaining product quality.

Background

Listeria monocytogenes are bacteria that can be found ubiquitously in different environments such as soil and water, and especially in food-manufacturing environments (Ferreira et al., 2014). Historically, outbreaks have been closely linked to foods of animal origin, such as meats and dairy products. However, recent incidents have highlighted the risk of *L. monocytogenes* contamination

in fruits and vegetables, increasing a significant concern within the ready-to-eat fruit and vegetable industry (Tabit, 2018). This shift emphasizes the critical need for comprehensive environmental monitoring programs (EMPs) and stringent safety protocols to mitigate the risk of *L. monocytogenes* in fresh produce. In food processing environments, *L. monocytogenes* can adhere to and proliferate on surfaces and sites hard to reach, allowing the bacterium to establish on equipment used in processing, including conveyor belts, pipes, floors, and drainage systems. This leads to cross-contamination between contaminated contact surfaces and fresh produce. Controlling this pathogen becomes exceptionally challenging. Environmental monitoring programs and sanitation plans play pivotal roles in detecting the presence of *L. monocytogenes* in the processing environment and their control (Gil et al., 2023). Due to the bacterial persistence in postharvest environments and the challenges in achieving effective microbial control, intervention strategies including biocontrol agents, such as the application of bacteriophages viruses that infect and lyse bacterial cells, present a promising avenue against common foodborne pathogens such as *L. monocytogenes* (Hyla et al., 2022). The exploration of these post-process treatments to enhance safety in fresh produce production has garnered attention. While the efficacy of bacteriophages has been well-documented in meat and dairy products, research into their use in fresh whole and ready-to-eat plant-based products remains sparse. Bacteriophages offer a natural and targeted approach to combat bacterial pathogens, including *L. monocytogenes*, without affecting the product quality or safety over traditional chemical preservatives or antibiotics. However, regulatory concerns and the lack of large-scale industrial application studies have hampered their widespread adoption as biocontrol agents in the food industry. Advancing research at the industrial level is imperative and such efforts need to provide valuable data on the optimization and effectiveness of phage-based treatments, fostering confidence in their use as biopreservation methods.

In this project, applied research was conducted to develop a validated strategy for *L. monocytogenes* control in the food industry, particularly for the application of bacteriophages in fresh and ready-to-eat produce.

Research Methods

Processing plant

The industrial application was performed in a processing line at the Torre Pacheco plant (Florette Iberica, Murcia, Spain). There were three main challenges: 1. the method of application that included the device and the process operation steps; 2. the application of the proper concentration of phages by a fine, mist-like spray with no phage inactivation; and 3. the adequate coverage of the product surface by adding the lowest amount of water to the product to prevent any deterioration process from the water excess on the product and high cost.

PhageGuard Listex™

Following the supplier recommendations for the PhageGuard Listex™ application (Micreos, The Netherlands), the target concentration of the post-process treatment was 10^6 plaque forming units or PFU/g as the units of phages capable of lysing host cells and forming a plaque. For that, a working solution of bacteriophages (10^9 PFU/mL) was prepared by diluting 30 mL of the concentrated phages in 2 L of water in sterile conditions.

For the application of PhageGuard Listex™, a spraying system CO® device sprayed the working solution of bacteriophages at a flow rate of 3.33 mL/s established in our previous CPS-2020 project (Gomez-Galindo et al., 2023). We confirmed that there were no significant differences in

the concentration of phage in the working solution and the solution coming from the nozzle. The prototype was placed above the vibration conveyor belt after the pre-sorting inspection point before ascending the product to the packaging operation (**Figure 1**). Control samples were those not subjected to this post-harvest treatment. A total of 30 bags of 125 g each of treated and untreated baby spinach were processed per trial and transported to the lab (25 miles) for further analysis. Bags were stored for 10 days under commercial conditions (3 d at 4°C + 7 d at 7°C) and compared with those stored at abusive storage conditions (10°C).

Microbiological analyses

The levels of phages in the working solution (before and after applying the spray) and on the treated baby spinach were enumerated following the double-layer agar plaque assay protocol using the host strain *Listeria innocua* 2627. For the product, 10 g of treated baby spinach was mixed in 100 mL of 2% buffered peptone water (BPW, wt/vol) (Scharlab). The homogenate was filtered (Polyethersulfone syringe filters of 0.45 µM, Thermo Fisher Scientific, San Jose, CA, USA) to remove bacteria. Ten-fold serial dilutions were performed in SM buffer (5.8 g NaCl, 2.0 g MgSO4 7H2O, 50 mL1 M Tris-HCl pH 7.4, 2% gelatin in 1 L dH_2O) (Manohar et al., 2018). A double-layer agar assay protocol was used with 100 µL of the filtered homogenate placed on Luria Bertani (LB) agar plate plus an LB soft agar and *Listeria innocua* 2627 as the host strain. The plates were incubated at 30°C for 48 h. Bacteriophage levels were expressed as plaque-forming units per mL or gram (pfu/mL or g).

For the detection of *L. monocytogenes* in baby spinach, the standard method ISO-11290-1 with slight modifications was followed. First, a pre-enrichment was carried out with 50 g of baby spinach mixed with 225 mL of Half Fraser Broth incubated for 48 h at 30°C. Then, 1 mL was transferred to 9 mL of Fraser Broth and incubated at 37°C for 24 h. Potentially positive colonies for *L. monocytogenes* were isolated and grown in BHI (Oxoid, Thermo Fisher Scientific, San Jose, CA, USA) agar plates. The isolates were tested by conventional polymerase chain reaction (PCR) using a PCR System (Applied Biosystems® thermal cycler, Thermo Fisher Scientific) with specific primers to confirm the presence of *iap* and the virulence *hly* genes.

For the enumeration of *Listeria* spp., the aim was to lower the detection limit by increasing the sample size using filtration as a concentration step. A sample of 25 g was gently mixed with 225 mL of BPW using a smasher with a LoD <1 CFU/g. Membrane filtration of 25 mL was performed and filters were placed on OCLA plates. Enumeration of blue-green colonies was done after 36 h at 37°C on plates with more than 5 colonies.

Sensory quality

Changes in O_2 and CO_2 concentrations in the headspace of the bags were monitored each sampling day using a gas chromatograph (Shimadzu GC-14) equipped with a thermal conductivity detector (TCD). The gas was drawn from the bags using a septum attached to the bags and a calibrated syringe.

The sensory quality of baby spinach was examined to assess the impact of post-process Listex™ treatment after processing (d0) and after storage (d10) at commercial and abusive temperatures. The organoleptic attributes included the overall visual quality, off-odors, crispiness loss, decay, and yellowing. A five-member expert panel evaluated the samples that were coded (3 digits) and presented individually for independent evaluation. The overall visual aspect was scored on a continuous 0-10 scale (0= extremely unpleasant; 10=extremely pleasant) while off-odors, crispiness loss, decay and yellowing were scored on a continuous scale with a range of 0-10 (0= absence; 10= completely damaged).

Image acquisition, processing and color extraction

The image acquisition was done in an illuminator chamber with two LED lights placed on the left and right sides of the box ceiling. A camera, Canon EOS (70D) (Canon Europe, Amstelveen, The Netherlands), with the settings adjusted (Canon Lens: EF-S18-55mm f/3,5-5.6 IS,) was used to obtain the spinach images. Photography conditions were: 1/50 s II shutter speed, *ƒ*/5,6 ISO: 100 apertures and SpyderCheckr™RGB spectrum (v 1.3; Datacolor) as a reference color chart for calibration (**Figure 2**).

For image processing, the pliman (plant image analysis) package was used as the appropriate one for leaf analysis (Olivoto, 2022). For determining the color changes by the treatment and storage, the images were segmented for background removal and color extraction (**Figure 3A**). Different indexes (e.g., R, G, B, and R/R+G+B) were examined (**Figure 3B**) and the selection was confirmed by the density graphs (**Figure 3C**). For baby spinach, the G index was selected as the best one with two maximums, one for the background and another for the spinach (**Figure 3C**). Once the segmentation was done and the background removed (**Figure 3D**), the mean value of all the pixels was calculated for R, G and B. The RGB images were then converted into the CIE L* a* b* color scale (**Figure 3E**). The color characterization and changes in baby spinach were measured by using a*, hue and Chroma that indicated the chromatic color from green (-a*) to red (+a*), the hue and the Chroma as color saturation.

Microbial community analysis

Recovery of microbiota from baby spinach and DNA extraction

Fifty grams (g) of baby spinach were collected into Stomacher bags to which 200 mL of buffered peptone water (BPW) with 0.1% Tween 80® was added. The bags were carefully sonicated for 7 min, trying not to disrupt the tissue to minimize the chloroplast DNA interferences from the tissue. All volume of this suspension was centrifuged at 4000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in 1 mL of PBS, transferred to a 1.5 mL tube and centrifuged at 9000g for 10 min. The remaining supernatant was discarded and the pellet was stored at -20°C until further DNA extraction. Total DNA was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN®) according to the manufacturer's instructions, with slight modifications. Solution CD1 (800 µL) was added to the pellet carefully to homogenize and then transferred to a PowerBead Pro Tube. The bead tube was horizontally placed and vortexed at maximum speed for 10 min. After that, tubes were centrifuged at 15000g for 1 min and the supernatant was transferred to 2 mL tube. Solution CD2 (200 µL) was added to the tubes and vortexed for 5 s and then centrifuged at 15000g for 1 min at room temperature. Avoiding the pellet, the supernatants were transferred to 2 mL tubes, and Solution CD3 (600 µL) was added. After a 5 sec vortex, 650 µL of the lysates were added onto an MB Spin Column and centrifuged at 15000g for 1 min. The flow-through was discarded and the step was repeated to ensure that all the lysate was passed through the MB Spin Column. Five hundred µL of Solution EA were added to the MB Spin Column placed into 2 mL tubes. A new centrifuge step at 15000g for 1 min was performed and the flow-through was discarded, with the MB Spin Column being placed back into the same 2 mL collection tubes. Solution C5 (500 µL) was added and a new centrifugation was done. After discarding the flow-through, the tubes were again centrifuged at 16000g for 2 min to dry the ethanol that remained in the tubes. The Spin Column was placed into a 1.5 mL elution tube and 50 µL of RNAse free water was added to the center of the membrane filter. The tubes were one last time centrifuged at 15000g for 1 min. Then, the Spin Column was discarded and the DNA was stored at -20°C for further use.

Sequencing 16 rRNA

The V3-V4 hypervariable region of 16S rRNA from bacterial samples was sequenced using an Illumina V3 2x300bp kit on an Illumina MiSeq system (Illumina, San Diego). The library was prepared following Illumina protocols using barcoded primer sets, 515F and 799 R, which did not amplify chloroplasts present in the samples, as previously described (Artimova et al., 2022). Miseq sequence data quality was evaluated with FastQC (Andrews, 2010) and summarized with MultiQC (Ewels et al., 2016). Cutadapt (Marcel et al., 2011) trimmed primers and all untrimmed sequences were discarded. Sequences that did not contain primer sequences were considered artifacts. A mean of 93.2% of the sequences per sample passed the filtering. Adapter and primer-free sequences were processed sample-wise (independent) with DADA2 (Callahan et al., 2016) to eliminate PhiX contamination, trim reads (before median quality drops below 25 and at least 75% of reads were retained; forward reads at 266 bp and reverse reads at 252 bp, reads shorter than this were discarded), discard reads with > 2 expected errors, correct errors, merge read pairs, and remove polymerase chain reaction (PCR) chimeras. Taxonomic classification was performed by DADA2 and the database 'Silva 138.1 prokaryotic SSU' (Quast et al., 2013). Amplicon sequence variants ASV sequences, abundance and DADA2 taxonomic assignments were loaded into QIIME2 (Bolyen et al., 2019). Within QIIME2, the final microbial community data was visualized in a barplot, evaluated for sufficient sequencing depth with alpha rarefaction curves, and investigated for alpha (within-sample) and beta (between-sample) diversity.

Statistical analysis

All statistical analyses were performed in the R-studio program (3.3.2) and IBM SPSS Statistics 29 (SPSS, Chicago, IL). For each sample, species richness (observed species), diversity (Shannon, Simpson and Fisher's alpha, indices), and evenness (Chao1 and abundance-based coverage estimator, ACE) were calculated. Differences in observed species, alpha diversity, evenness measures and relative abundances of bacteria genera between control and Listex™ samples were obtained by applying the Kruskal-Wallis rank sum test between groups. Tukey's honest significant difference (HSD) test was applied for multiple comparisons among storage days and storage temperatures ($P \leq 0.05$). Bray-Curtis dissimilarity was selected as the beta (between-sample) diversity measure in this study. *P*-value was obtained by Permutational Multivariate Analysis of Variance (PERMANOVA). Principal Component Analyses (PCA) of Beta diversity using the Bray-Curtis distance metric were generated by QIIME2 visualized by EMPeror (Bolyen et al., 2019). The differential abundance analysis was performed using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC2) (Lin and Peddada, 2020). The challenge of differential abundance analysis is the compositional nature of microbiome data due to sampling and sequencing depth (the number of reads assigned to an ASV must be interpreted relative to the total number of reads obtained for that sample). Microbial absolute abundances were compared between study groups (baby spinach storage 10 days vs. 0 day).

Research Results / Outcomes and Accomplishments

Listex™ application and survival during storage

In all trials, bacteriophage levels were estimated to confirm the proper application of the treatment and the achievement of the target concentration. The results obtained indicate that the levels in the PhageGuard Listex™ working solution before and after spraying using *L. innocua* as the host bacteria met the supplier's recommendation (10^9 PFU/mL). The concentration of the bacteriophage solution remained unaffected by the nozzle pressure used during treatment application (**Figure 4**). The initial analysis of phage concentrations in the treated product revealed

levels below the minimum effective concentration needed for adequate inactivation of *L. monocytogenes* (10^6 PFU/g), possibly because phage analyses occurred 17 h after application, rather than immediately. Typically, the trials were performed in the evening at the end of the workday, making immediate analysis of phage levels impossible. To demonstrate that the desired phage concentration was achieved under industrial conditions, and to understand the reasons for the low concentration due to the delay between application and analysis, a parallel experiment was conducted. Phage levels were analyzed both immediately after application and again 17 h later. The findings confirmed that the bacteriophages were applied correctly at an industrial scale, especially in the vibratory conveyor used for leaf separation, ensuring the desired phage concentration on the surface of baby spinach (**Table 1**). Across all trials, bacteriophage levels decreased by about 1 log over the product shelf life, regardless of the storage temperature (**Figure 5**). The results obtained showed that this 'phage biocontrol' can be directly applied successfully by a fine, mist-like spray through multiple nozzles with no phage inactivation on the vibratory conveyor belt after the sorting defect selection before the packaging. However, this application only treats one side of the leaves and full coverage is not guaranteed, although the leaves fall into the bag and all the treated sides are in contact each other in the bag. As an alternative application, a timed spray onto the product after being released from a multi-weigher before falling into the bag can be possible. The multiple nozzles can be installed on a circular container and aimed fully automated at the product for 3D coverage. In this way the product surface can be the adequate coverage via spray adding the lowest amount of water to the product to prevent any deterioration process from the water excess on the product. The stability of the phage working solution was at least 48 h at 4°C.

Listex™ on controlling/reducing *Listeria* spp/*L. monocytogenes*

When examining the enumeration of *Listeria* spp. in baby spinach during 20 trials, low levels of *Listeria* spp. were observed with a slight reduction in treated leaves, although no statistical differences were found (**Figure 6**). However, to analyze the efficacy of bacteriophages against *Listeria* spp. more comprehensively, the prevalence of *Listeria* spp. in the samples was calculated. The results indicated that the bacteriophage treatment Listex™ significantly reduced the prevalence of *Listeria* spp. in baby spinach after storage (**Table 2**).

In a survey of UK RTE salad, McLauchlin et al. (2022) reported that 11/604 samples (1.82%) were positive for *L. monocytogenes,* with one sample exceeding >20 cfu/g and the remaining 10 samples <20 cfu/g. Given that our project also involved commercially washed leaves, a similar level of contamination with *L. monocytogenes* was anticipated. Unfortunately for the project, to corroborate effectiveness of Listex™ controlling natural contaminated samples, no *L. monocytogenes* contamination was detected in baby spinach among the 600 samples, including those after processing (d0) and after storage (d10). Our results only confirmed the presence of *Listeria* spp. after enrichment. As our project aims to demonstrate the reduction of *L. monocytogenes* by Listex™ using the commercially practical spray equipment and rates, we would have needed to confirm the treatment effectiveness by detecting more than 32 positive samples in untreated spinach. This implies that demonstrating the efficacy of Listex™ in controlling/reducing or eliminating *L. monocytogenes* contamination (assuming a 1% prevalence) would have required analyzing several thousand samples for conclusive proof of Listex™ efficacy. In line with the recommendation by the Chilled Food Association in the UK that helped us with this reflection, the approach requiring thousands of samples was clearly impractical in the current research project.

Listex™ on sensory quality and color of baby spinach

Quality attributes of treated spinach, such as headspace gas composition, overall sensory quality, and color, were evaluated and compared with untreated leaves. The oxygen (O_2) and carbon dioxide (CO_2) concentrations in the packaging headspace showed no significant differences between treated and control leaves, with the effect of storage temperature being the only notable factor (**Figure 7**). As expected, O_2 levels decreased from their initial values, particularly under conditions of abusive temperature, while CO_2 levels increased. A notable data dispersion was observed across the 20 trials, indicating that while some samples showed height metabolic activity significantly altering the packaging atmosphere, others exhibited minimal respiration, leading to only minor atmospheric changes with no distinction between treated and untreated leaves.

Listex™-treated baby spinach maintained similar overall visual quality to untreated leaves, with no significant differences noted after storage, either under refrigeration or abusive temperatures (**Figure 8**). The loss of crispness was especially pronounced after 10 days at 10°C, showing high variability across the samples analyzed (n=600) throughout the 20 trials, without any differences between treated and untreated products (**Figure 9**). Decay and off-odors increased at abusive temperatures, while refrigeration markedly reduced the development of decay and off-odors, again with no differences between treated and untreated leaves (**Figures 10 and 11**).

The effect of bacteriophages on color changes was assessed, not just subjectively by a trained panel but also through image analysis, providing an objective color measurement. Yellowing was scored similarly for baby leaves at day 0 and after 10 days of storage, showing no differences between Listex-treated and control leaves (**Figure 12**). Within the various parameters of the CIELAB (Lab*) color space measured, the hue angle was chosen as a parameter that reflected the type of color (red, yellow, green, and blue) without considering color saturation or lightness, with 90° denoting yellow and 180° green. According to **Figure 13**, no initial color changes were observed between treated and control leaves, nor after storage at refrigeration or abusive temperatures.

Listex™ on bacterial community composition of baby spinach

The sequencing of the 180 baby spinach samples produced a total of 19,669,022 reads, with a range of 29,956 to 160,626 per sample (average 109,272 reads per sample). After preprocessing, including checking sequence quality, removing primers, filtering quality, and removing PCR chimeras, a total of 5,296 amplicon sequence variants (ASVs) were obtained across all samples. Some sequences (181) were removed because the taxonomic string contained some mitochondria, and chloroplast (5115 ASVs passed). The sufficient depth of sequencing was shown by the rarefaction curves constructed from the 29956 counts obtained (**Figure 14**). The taxonomic classification performed by DADA2 resulted in the classification of 99.36% at the Phylum level, 98.62% at the Class level, 96.71% at the Order level, 92.58% at the Family level, 78.06% at the Genus level, and 28.7% at the Species level. The 5115 bacterial ASVs obtained represent 28 phyla, 326 families and 624 genera. The relative abundance (RA) of the top ten phyla, families and genera observed for the samples control and treated collected after treatment (day 0) and after day 10 storage at commercial and abusive temperatures are shown in **Figures 15, 16 and 17.**

In baby spinach samples control and Listex™ treated samples at day 0, *Proteobacteria* was the phylum with the highest relative abundance (RA), accounting for 60.2% and 64.6 % (average for control and Listex™ samples (n=30), respectively) of the total reads. The phylum with the next highest RA on spinach was *Firmicutes* with 31.8% and 25.6 %, for control and Listex™ samples, respectively, followed by *Actinobacteria*, (5.3% and 5.9% in control and Listex™ samples), and *Bacteroidota (*2.3% and 3.5%, respectively) (**Figure 15**). At family level, the most dominant taxa

were *Pseudomonaceae* (19.8% and 22.3% in control and Listex™ samples) *Moraxellaceae* (12.3% and 14.7%), *Exiguobacteraceae* (10.0% and 8.8%), Erwiaceae (9.1% and 5.2%) and *Oxalobacteraceae* (5.8% and 8.5%, respectively) (**Figure 16**). At the genus level, the most abundant genus in the baby spinach control and treated samples at day 0 were *Pseudomonas* (19.8 and 22.3 %), *Bacillus* (10.7 and 8.2), *Exiguobacterium* (10.0 and 8.8%), *Pantoea* (8.9 and 5.1%) and *Acenitobacter* (7.0 and 7.3 %) (**Figure 17**). All these families and genera mainly belonged to the most detected phyla *Proteobacteria* and *Firmicutes*. *Proteobacteria*, *Firmicutes* and *Actinobacterium* have been reported in previous studies as the most important phyla detected in the microbiota of baby spinach (Lopez-Velasco et al., 2011; Leff et al., 2013; Tenze et al., 2020). The more abundance genera detected in all the samples at day 0, *Pseudomonas*, *Bacillus* and *Pantoea*, have been previously suggested as part of the bacterial 'core' phyllosphere microbiota on leafy greens (Rastogi et al., 2011; Truchado et al., 2018).

After 10 days of storage at commercial or abusive temperatures, the proportions of phyla, families and genera detected in the baby spinach samples on day 0 shifted. At the phylum level, there was an increase in the percentage of *Proteobacteria* and *Bacteroidota*, and a decrease in the percentage of *Firmicutes and Actinobacteriota*, regardless of the storage conditions (**Figure 15**). These results are similar to those obtained by other authors who observed an increase in RA of *Proteobacteria* and *Bacteroidota* when baby spinach was stored at 4 and 10°C for 15 days and at 4, 10 and 15°C for 7 days, respectively (López-Velasco et al., 2011; Gu et al., 2018). A similar trend was observed at the family level, where there was a relative increase in the *Pseudomonaceae* and *Oxalobacteraceae*. However, the abundance of *Moraxellaceae*, *Exiguobacteraceae*, and *Erwiniaceae* decreased in the baby spinach samples stored at both commercial and abusive temperatures. Among the top ten genera detected in baby spinach analyzed in this study, an increase in the relative abundance of *Pseudomonas* and *Flavobacterium* was observed, while *Exiguobacterium* decreased during the shelf life of baby spinach stored at commercial or abusive temperatures (**Figure 17**). **Table 3** summarizes the main genera (those with an average abundance >1% in samples) observed in untreated baby spinach as control and Listex™ treated samples at day 0 and after 10 days of storage at different temperatures. In **Table 3**, it can be observed that storage conditions significantly reduced the RA of different genera such as *Bacillus*, *Exiguobacterium,* and increased the RA of *Flavobacterium*, *Glutamicibacter, Pseudomonas* and *Serratia*. Although the overall composition of the microbiota was very similar between the control and Listex™ samples as described above, we also assessed whether there were significant differences in relative abundance between storage time of 10 days and 0 days at the genus level using the ANCOMBC2 test (Lin and Peddada, 2020). The result showed significant differences in the relative abundance of several genera detected in both treatments. *Serratia, Shewanella, Empedobac*ter and several others had a log-fold change (LFC) greater than 0, indicating that they were significantly more abundant in the treated than in the control samples (**Figure 18**). Conversely, significantly higher abundances of the genera *Bacillus, Bradythizobium, Tabrizcola* and several others were detected in the control samples compared to the treated samples. The results suggested that in overall composition, the microbiota between the control and Listex™ was very similar but a slight shift in baby spinach microbiota between initial and 10-day storage at commercial or abusive was observed.

Listex™ on the diversity and structure of the bacterial community of baby spinach

To evaluate how Listex™ and storage temperatures impacted the microbial community of baby spinach, measures of alpha and beta diversity were calculated. Microbial alpha diversity measures microbial diversity within a sample. The alpha diversity index calculated were species richness (observed species), diversity (Shannon, Simpson and Fisher's alpha, indices), and evenness (Chao1 and ACE). **Figure 19** shows the alpha diversity index measures for all samples

(n=180). No significant differences between the untreated and Listex™ treated samples were detected in alpha diversity index measures, indicating that species richness, diversity and evenness were similar between samples (**Figure 19A**). The results suggested that Listex™ did not change within-sample bacterial alpha diversity. However, significant differences were observed among baby spinach at day 0 and after storage for 10 days at commercial and abusive temperatures (**Figure 19B**). A decrease in species evenness (Chao1 and ACE) measures of the bacterial community structure was detected between 0 and 10 days of storage, independently of storage temperature. For all baby spinach during storage for 10 days at 10°C (Control and Listex™ treated) the Shannon and Simpson diversity measures were significantly higher than those found for the samples stored at commercial temperature and baby spinach control (**Figure 19B**). Therefore, the alpha diversity of the bacterial community of baby spinach appears to be significantly influenced by storage time and temperature, independent of Listex™ treatment.

In terms of Beta diversity analysis, a comparable trend was observed. Bacterial beta diversity shows the difference in taxonomic abundance profiles across different groups of samples. The Principal Coordinates Analysis (PcoA), based on the Bray-Curtis distance matrix, showed a clear distinction between the initial microbiota of baby spinach and the samples stored for 10 days at commercial or abusive temperatures. Control and treated samples before storage were grouped via PcoA 1, which contributed 19.35% of the variation (**Figure 19 A**). On the contrary, the variance between treated and untreated samples was mainly separated by PcoA 2 (12.50%). When PcoA based on the Bray-Curtis distance matrix was performed with the control group, significant differences were observed between storage time and temperature storage conditions (**Figure 19 B**). A similar result was observed for the Listex™ group samples (**Figure 19 C**). The permutational multivariate analysis of variance (PERMANOVA) performed on the samples corresponding to each group indicated that significant differences were also observed between commercial and abusive temperatures (**Table 4**). These results showed that the application of Listex™ as a post-processing treatment did not influence the diversity and bacterial community structure of baby spinach. However, storage time and storage temperatures impacted the diversity and bacterial community structure of baby spinach.

Listex™ costs and benefits

The application of Listex™ as a post-process treatment designed to prevent/reduce/eliminate *L. monocytogenes* on leafy greens presents several potential costs and benefits:

<u>*Costs*</u>:

Initial investment: Implementing Listex™ in processing facilities may require an initial investment in purchasing the product itself, as well as any necessary adjustment in the processing line for the application. The purchase of the product is not only linked to the initial investment but also to the routine use of the treatment. Considering the price of the product and the amount of product that should be applied to the product, it is assume that the cost linked to the application of the product to leafy greens increase the price of the bags by 0.05-0.54 cents depending on the amount of product treated (**Table 5**).

Application equipment: Depending on the scale of operations, specialized equipment would be needed to apply Listex™ effectively. This could include spraying systems or fogging equipment for treating food surfaces.

Application costs: The process of applying Listex™ may require labor and other resources. This could involve hiring specialized personnel.

Training and education: Proper training and education of the personnel on the proper use and application of Listex™ is essential to ensure its effectiveness and compliance with regulatory standards. Costs may be incurred for employee training programs and educational materials.

Monitoring and testing: Regular monitoring and testing of food products and processing environments may be necessary to verify the efficacy of Listex™ treatment. This could involve laboratory analysis and on-site testing, which may incur additional costs as well as the regular analysis of phages to confirm that the concentration of phages is been applied and check that the spray nozzles are working.

Potential product cost: Depending on the duration of the effect, cost associated with the labeling may occur if the treatment is considered an additive and not a processing aid.

Overall, while there are costs associated with implementing Listex™ in processing facilities of fresh-cut products, there are also potential benefits in terms of enhanced food safety, extended shelf life, brand protection, and regulatory compliance that can outweigh these costs and contribute to the overall success of its use.

Benefits:

Enhanced food safety: The primary benefit of using Listex™ in processing facilities is the reduction or control of *L. monocytogenes* contamination in fresh-cut products. This helps to enhance food safety and reduce the risk of foodborne illness outbreaks associated with *Listeria* contamination. Bacteriophage preparation is highly specific to their bacterial hosts (*L. monocytogenes* and *Listeria* spp.), allowing for precise targeting of pathogens without harming beneficial microbiota. The use of Listex™ for the food safety cost will be the same, as the strict hygiene measures should still be conducted. Sanitation cannot be reduced due to the use of Listex™.

Extended shelf life: By controlling *L. monocytogenes*, Listex™ can reduce food waste and minimize losses for both processors and retailers. If the end of shelf life is determined by exceeding acceptable *Listeria* levels, the initial reduction of *Listeria* by Listex™ could contribute to shelf-life extension.

Reduced risk of recalls: Controlling *L. monocytogenes* contamination with Listex™ can help reduce the risk of product recalls due to food safety concerns. Recalls are costly and can damage brand reputation, so prevention is crucial for minimizing these risks.

Compliance with regulations: Using Listex™ can help processing facilities comply with regulatory requirements for controlling *L. monocytogenes* in food products. This can help avoid fines, penalties, and legal liabilities associated with non-compliance.

Protecting damage to brand reputation: Implementing Listex™ demonstrates a commitment to food safety and quality, which can help protect the brand reputation of processors. Consumers are increasingly concerned about food safety, and having measures in place to control *Listeria* can enhance consumer confidence in the brand.

Operational efficiency: Implementing Listex™ as part of a comprehensive food safety program can lead to more efficient operations in processing facilities. By reducing the risk of contamination and the need for corrective actions, Listex™ can streamline production processes and reduce downtime.

Market access: Some retailers and distributors may require suppliers to demonstrate adherence to strict food safety standards, including measures to control *L. monocytogenes*. Using Listex™ can help processing facilities meet these requirements and maintain access to key markets.

Market access and consumer preference: As consumer awareness of environmental and health issues grows, there may be market advantages associated with using bacteriophage treatments. Products labeled as certified organic may appeal to a broader consumer base.

Minimal environmental impact: Unlike chemical pesticides and antibiotics, bacteriophages are natural entities that do not persist in the environment or accumulate in food products. Their use

can therefore minimize environmental pollution and ecological risks associated with conventional pest control methods.

Overall, the use of Listex™ as a biocontrol agent offers a promising approach to addressing bacterial infections and enhancing food safety while potentially reducing costs and minimizing negative environmental impacts. However, further research, development, and regulatory approval are needed to fully realize the cost benefits and practical applications of bacteriophage-based treatments.

Summary of Findings and Recommendations

Summary of Findings

1. Listex™ application: This 'phage biocontrol' can be directly applied successfully by a fine, mist-like spray through multiple nozzles with no phage inactivation on the vibratory conveyor belt after the sorting defect selection before the packaging.

2. Control of *L. monocytogenes* by Listex™: The control strategy of using Listex™ as a bacteriophage cocktail to reduce/eliminate *L. monocytogenes* on baby spinach proposed in this project was not proven as naturally occurring *L. monocytogenes* was not present in any of the 600 samples analyzed in twenty independent experimental trials over 8 month-period.

3. Effectiveness of Listex™ controlling *Listeria* spp.: Listex™ has shown significant efficacy in reducing the prevalence of *Listeria* spp. in treated baby spinach.

4. Impact on sensory quality: Treatment with Listex™ does not significantly alter the sensory qualities such as color, overall visual appearance, or texture of baby spinach. The headspace gas composition, sensory quality, and color remained similar between treated and untreated samples.

5. Listex™ interaction with the natural microbiota: Listex™ does not affect the structure and diversity of the microbiota in baby spinach, but rather the conditions under which the spinach is stored.

6. Costs associated with Listex™: Implementation costs include initial investment in application equipment, ongoing product costs, labor for application, training for proper use, and routine monitoring and testing. The estimated increase in cost is 0.05-0.48 cents per package of leafy greens (175 g).

Recommendations

1. Optimize application processes: Develop and refine the application process to ensure consistent and effective dosing of Listex™. Consider automation to reduce labor costs and improve precision.
2. Regular monitoring and testing: Implement strict monitoring protocols to regularly assess the efficacy and proper application. This includes checking phage concentrations and ensuring that spray nozzles are functioning correctly.
3. Training programs: Invest in comprehensive training programs for personnel to handle and apply Listex™ correctly, ensuring the treatment's effectiveness and compliance with food safety regulations.
4. Further research and development: Continue research to explore the full potential of Listex™ in different types of fresh produce.

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APPENDICES

Publications and Presentations

Publications

Gil, M.I., Allende, A., Tudela, J.A., Truchado, P. Analyzing the Efficacy of Commercial Bacteriophages for Enhancing Food Safety in Industrial Baby Spinach Production. Food Control, *to be submitted*.

Gil, M.I., Illan, G., Allende, A., Truchado, P. Investigating Variations in the Microbiota of Commercial Baby Spinach Across Seasons and Post-Processing Treatment with Commercial Bacteriophages. Food Microbiology, *to be submitted*.

Presentations

"Evidence for the industrial application of bacteriophages to control Listeria monocytogenes in leafy greens". M. I. Gil; P. Truchado. Chilled Food Association (CFA) Technical Group meeting. Online. 27th February 2024.

"Are commercial bacteriophages an effective tool for food safety control of baby spinach in an industrial setting?". *P. Truchado; A. Allende; M. I Gil.* In: IAFP European Symposium, Geneva (Switzerland), 30 April 30 to May 2, 2024. Poster presentation.

"*Impacto de los bacteriófagos comerciales en la seguridad alimentaria de las espinacas baby: un enfoque industrial*". *P. Truchado; A. Allende; J. A. Tudela; M.I Gil*. In: XIV Congreso Nacional y XII Ibérico de Maduración y Postcosecha, Madrid, June 12 to June 14, 2024. Oral presentation.

Budget Summary

This project was awarded $98,050 in research funds. The project budget was mainly used for materials and supplies as well as sequencing costs required for the analysis of bacterial communities (~$42,086), and salaries and benefits (~$33,526). The funding received was adequate for the execution of the project. However, due to an extension of the project duration, the contract of the technician had to be extended, resulting in a slight budget overrun in the allocation of salaries and related indirect costs. This budget overrun has been compensated by under-expenditure in other budget categories, allowing us to stay within the overall project budget.

Tables 1–5 and Figures 1–20 (see below)

Tables and figures supporting the achievements of the project:

Table 1. Levels of bacteriophages immediately after application and 17 hours later.

Replicates	Just after application (Log pfu/g)	17 h after application (Log pfu/g)
A	6.35	4.90
B	6.01	5.23
C	6.27	4.90
D	6.06	4.89
E	6.08	5.01

Table 2. Prevalence of *Listeria* spp. in untreated (CT) and treated baby spinach (Listex™) after application (day 0) and storage (day 10) at commercial and abusive temperatures. Prevalence was calculated considering the number of positive samples (≥ 5 cfu/plate)/total samples analyzed.

	Application – Day 0	Storage – Day 10	
		3d at 4ºC + 7d at 7ºC	10d at 10ºC
CT	6/100	15/100	14/100
Listex™	5/100	6/100	8/100

Table 3. Relative abundance (RA, %) of major bacterial genus (RA>1%) in control and Listex™ treated baby spinach samples after application (day 0) and after storage (day 10) at commercial and abusive temperatures.

	Control			Listex™		
	day 0	**3d 4ºC+7d 10ºC**	**10d 10ºC**	**day 0**	**3d 4ºC+7d 10ºC**	**10d 10ºC**
Acinetobacter	7.0±8.2	1.4±1.5	2.7±2.7	7.3±8.3	1.4±0.8	2.5±1.7
Bacillus	**10.7±0.1a**	**0.3±0.5b**	**0.1±0.2b**	**8.2±5.3a**	**0.28±0.3b**	**0.10±0.1b**
Buttiauxella	0.3±0.38	0.9±0.7	2.8±2.6	0.5±0.7	0.9±0.7	2.2±2.1
Chryseobacterium	0.7±0.6	2.8±4.3	2.9±2.1	1.1±1.1	3.1±4.0	3.6±3.3
Comamonas	0.3±0.5	0.1±0.2	0.1±1.0	0.6±0.6	0.2±0.2	1.4±1.2
Duganella	0.6±0.5	2.1±1.5	1.8±1.3	0.88±0.5	2.08±1.6	1.5±0.9
Exiguobacterium	**10.0±10.9**	**3.8±3.55**	**3.5±2.61**	**8.8±9.9**	**3.3±4.5**	**2.6±1.9**
Flavobacterium	**1.0±0.6**	**6.2±2.5**	**6.9±3.3**	**1.7±1.0**	**7.3±3.0**	**6.8±1.6**
Glutamicibacter	**0.8±0.5b**	**2.3±1.9a**	**1.4±0.8a**	**0.6±0.4b**	**0.6±0.2b**	**1.3±0.9a**
Janthinobacterium	4.5±9.7	4.3±0.8	5.5±2.3	6.8±9.7	5.2±2.9	5.7±2.3
Klebsiella	1.0±0.7	1.9±0.4	4.2±3.0	0.9±0.6	3.8±4.5	4.1±1.8
Methylobacterium	1.6±2.4	0.0±0.0	0.0±0.0	0.7±0.6	0.0±0.0	0.0±0.0
Pantoea	8.9±8.5	6.7±3.2	7.1±3.2	5.2±3.3	6.9±5.8	5.6±2.9
Paracoccus	0.3±0.3a	0.0±0.0b	0.0±0.0b	0.7±0.88a	0.0±0.0b	0.0±0.0b
Pectobacterium	0.1±0.1	0.1±0.2	1.1±1.3	0.3±0.6	0.1±0.3	1.4±1.8
Pseudomonas	**19.9±15.1b**	**44.7±9.0a**	**36.4±5.4a**	**22.3±14.2b**	**42.6±8.7a**	**38.9±4.4a**
Psychrobacter	5.3±1.43	2.1±4.0	0.9±1.17	7.4±16.59	1.8±2.89	1.1±1.48
Ralstonia	1.5±2.4	0.0±0.0	0.0±0.00	0.4±0.4	0.0±0.0	0.0±0.0
Serratia	**0.2±0.1b**	**4.7±3.7a**	**2.5±2.1a**	**0.3±0.2b**	**2.3±1.4a**	**2.4±1.6a**
Shewanella	0.7±0.9	5.4±6.7	4.9±5.1	2.5±3.7	8.5±11.2	6.8±7.7
Sphingobacterium	0.2±0.1	1.4±1.6	2.0±1.5	0.4±0.3	1.7±1.4	1.6±1.0
Sphingomonas	2.5±3.8	0.1±0.1	0.0±0.0	0.8±0.7	0.1±0.1	0.0±0.0
Stenotrophomonas	0.4±0.3	0.7±0.7	1.7±1.02	0.4±0.4	0.8±0.7	1.1±0.6

Values are the median and standard deviation (n=30). Green bold letters indicate an increase in the genus while red bold letters indicate a decrease in the genus after storage. Different letters indicate significant difference between treated and untreated baby spinach (P<0.05)

Table 4. *P*-values and *pseudo-F* values resulting from bacteria metagenome comparisons determined using PERMANOVA based on Bray-Curtis dissimilarity matrices. Baby spinach microbiome comparisons included Listex™ treated baby spinach and untreated (Control) and two storage conditions at commercial and abusive temperatures.

	p-value	*pseudo-F*
Treatment (Control versus Listex™)		
Baby spinach day 0	0.383	1.021
Baby spinach 3 days 4ºC+7 days 10ºC	0.768	0.688
Baby spinach 10 days at 10°C	0.690	0.739
Temperature (Commercial versus Abusive)		
Baby spinach control	0.006	2.579
Baby spinach Listex™	0.064	1.783
Storage (day 0 versus 10 days)		
Baby spinach control 3 days 4ºC+7 days 10ºC	0.001	12.284
Baby spinach control 10 days at 10°C	0.001	12.267
Baby spinach Listex™ 3 days 4ºC+7 days 10ºC	0.001	9.867
Baby spinach Listex™ 10 days at 10°C	0.001	11.167

Table 5. Estimated cost of Listex™ application per bag, considering a 175 g bag of baby leaves.

Target concentration (pfu/g)	**1-liter PGL**	**Log reduction**	**kg treated**	**List price***	**Cost-in-use/kg in cents**	**Cost per bag (175 g) in cents**
1x 10^6	2x 10^{14}	~ or <1 log	200,000	$ 620	0.31	0.05
2 x 10^6	2x 10^{14}	1 log	100,000	$ 620	0.62	0.11
1x 10^7	2x 10^{14}	2 log	20,000	$ 620	3.1	0.54

*List price is indicative and dependent on volumes

Figure 1. Application device of bacteriophages on the vibration conveyor belt.

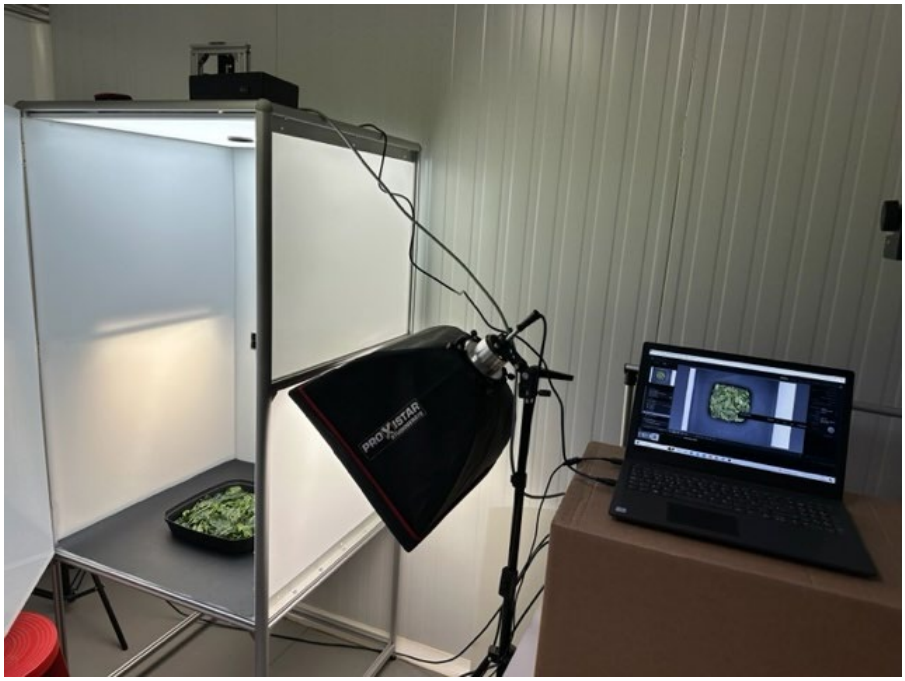


Figure 2. Spinach image acquisition in an illuminator chamber with two LED lights and a Canon camera.

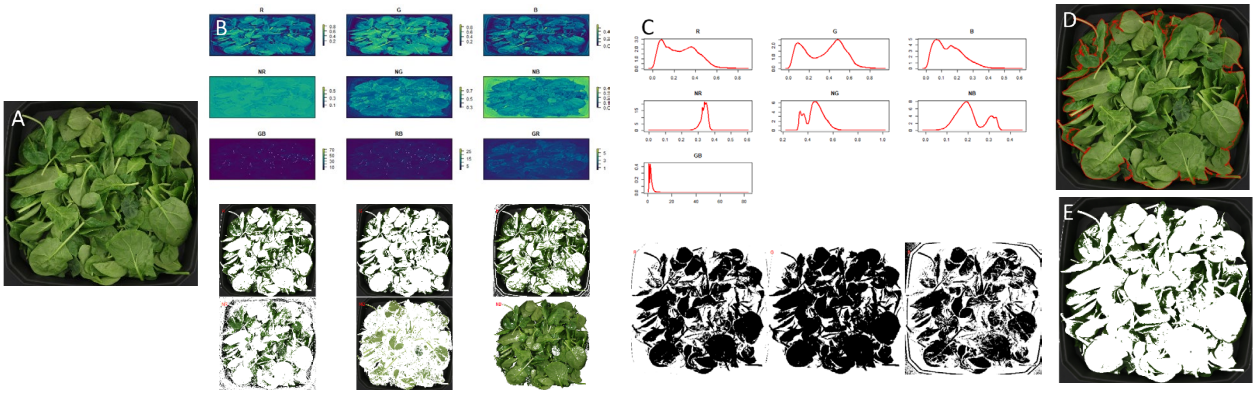


Figure 3. Plant image analysis to determine the color changes (L*, a*, b*, hue and Chroma) over the storage period in treated and untreated baby spinach.

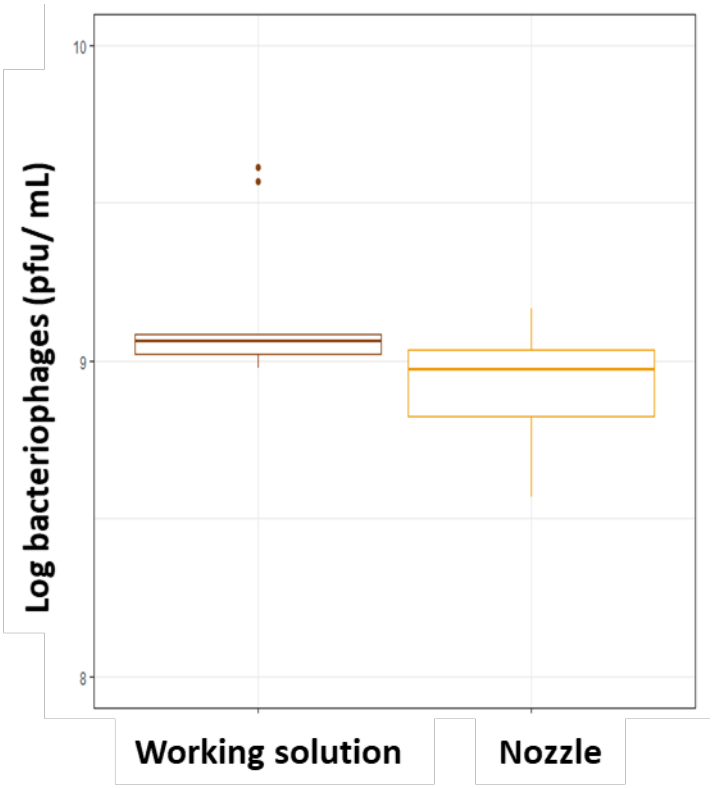


Figure 4. Levels of bacteriophages (Log pfu/mL) in the Listex™ working solution after preparation and after application by a nozzle spray.

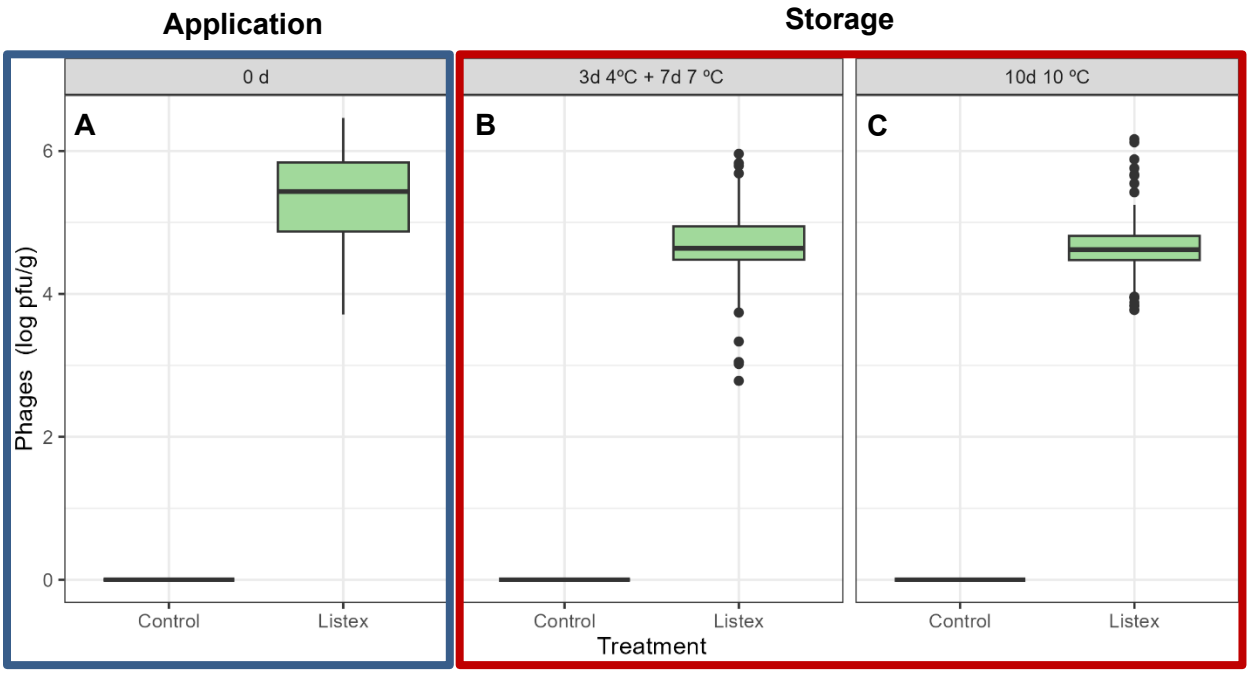


Figure 5. Levels of bacteriophages (Log PFU/g) in Listex™ treated and untreated (Control) baby spinach immediately after application (0 d) (A), after storage under commercial conditions (3d 4ºC + 7d 7ºC) (B), and under abusive storage conditions (10d 10ºC) (C). Box plots represent data from 20 trials (n=600), where the lower part of the box shows the 25th percentile and the upper part the 75th percentile. The dots represent outliers.

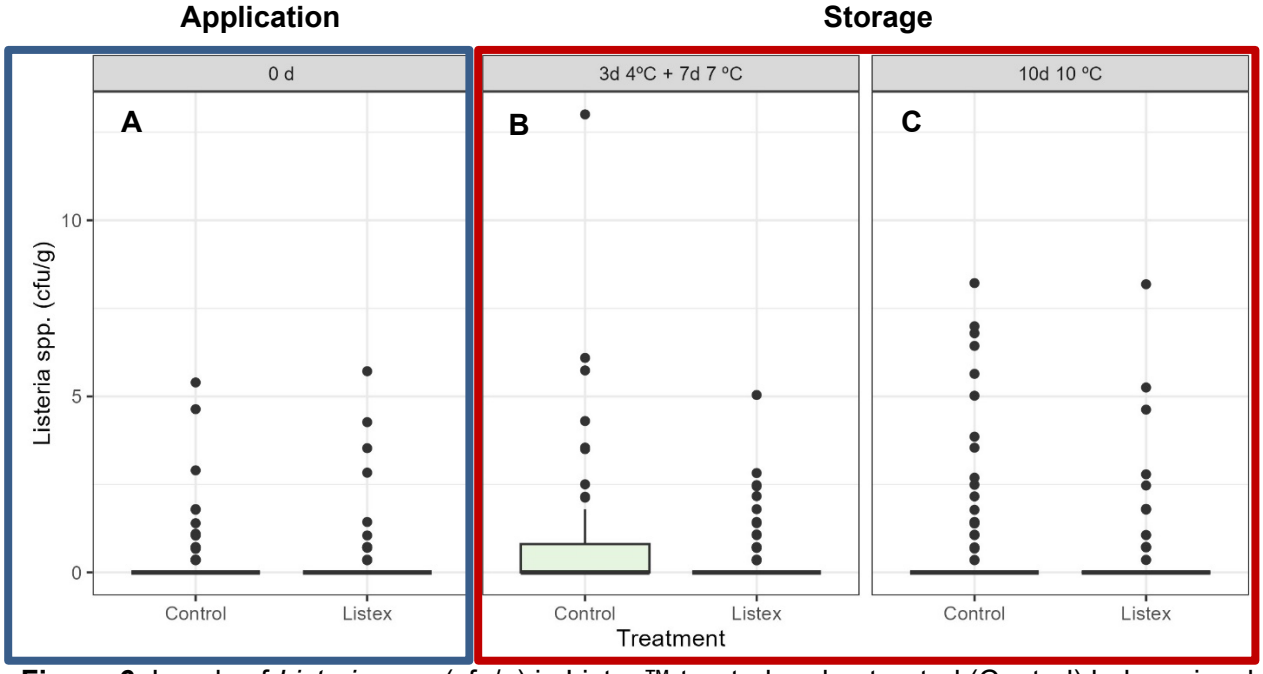


Figure 6. Levels of *Listeria* spp. (cfu/g) in Listex™ treated and untreated (Control) baby spinach immediately after application (0 d) (A), after storage under commercial conditions (3d 4ºC + 7d 7ºC) (B), and under abusive storage conditions (10d 10ºC) (C). Box plots represent data from 20 trials (n=600), where the lower part of the box shows the 25th percentile and the upper part the 75th percentile. The dots represent outliers.

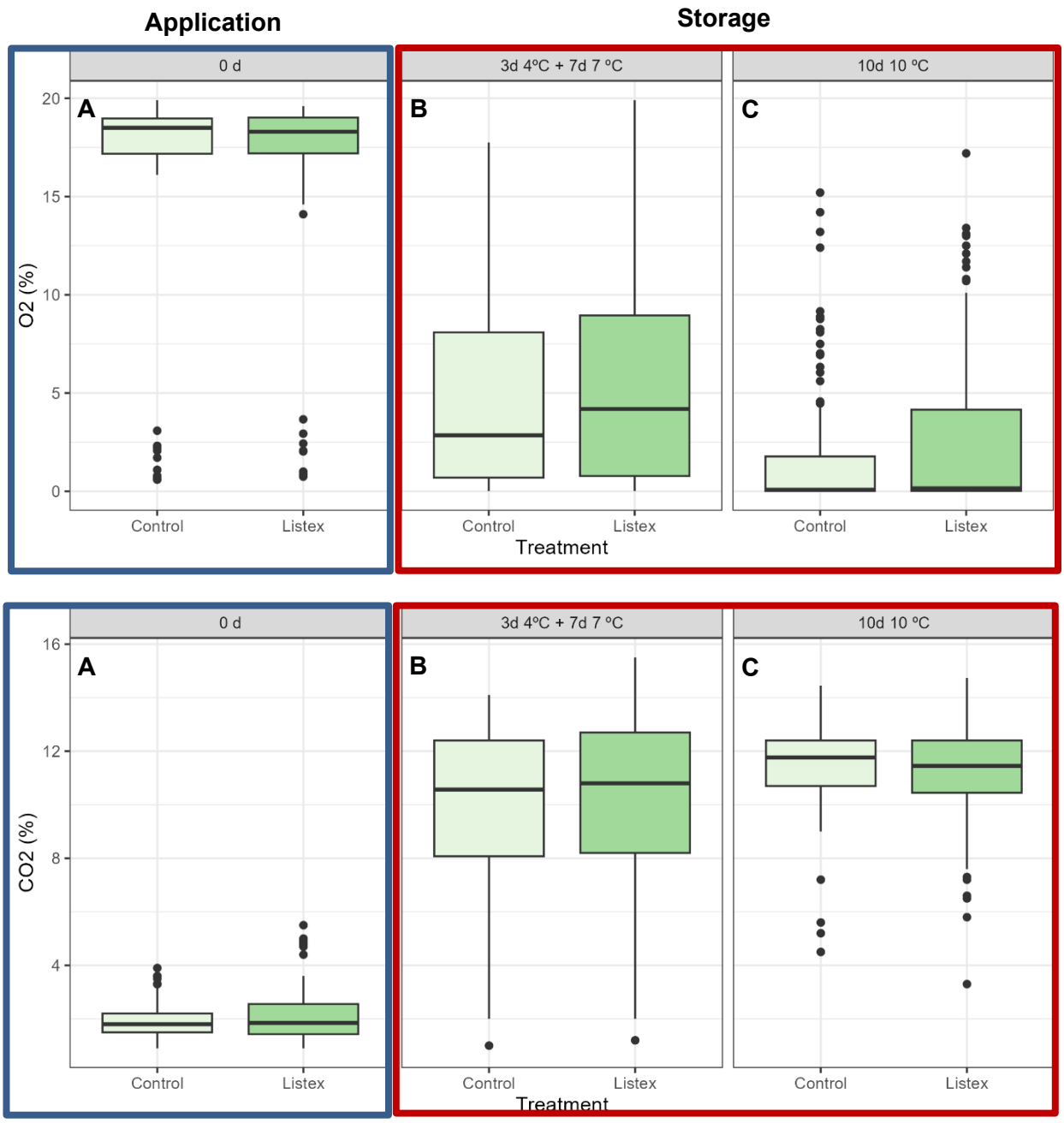


Figure 7. Changes in O_2 and CO_2 concentrations in Listex™ treated and untreated (Control) baby spinach immediately after application (0 day) (A), after storage under commercial conditions (3d 4ºC + 7d 7ºC) (B), and under abusive storage conditions (10d 10ºC) (C). Box plots represent data from 20 trials (n=600), where the lower part of the box shows the 25th percentile and the upper part the 75th percentile. The dots represent outliers.

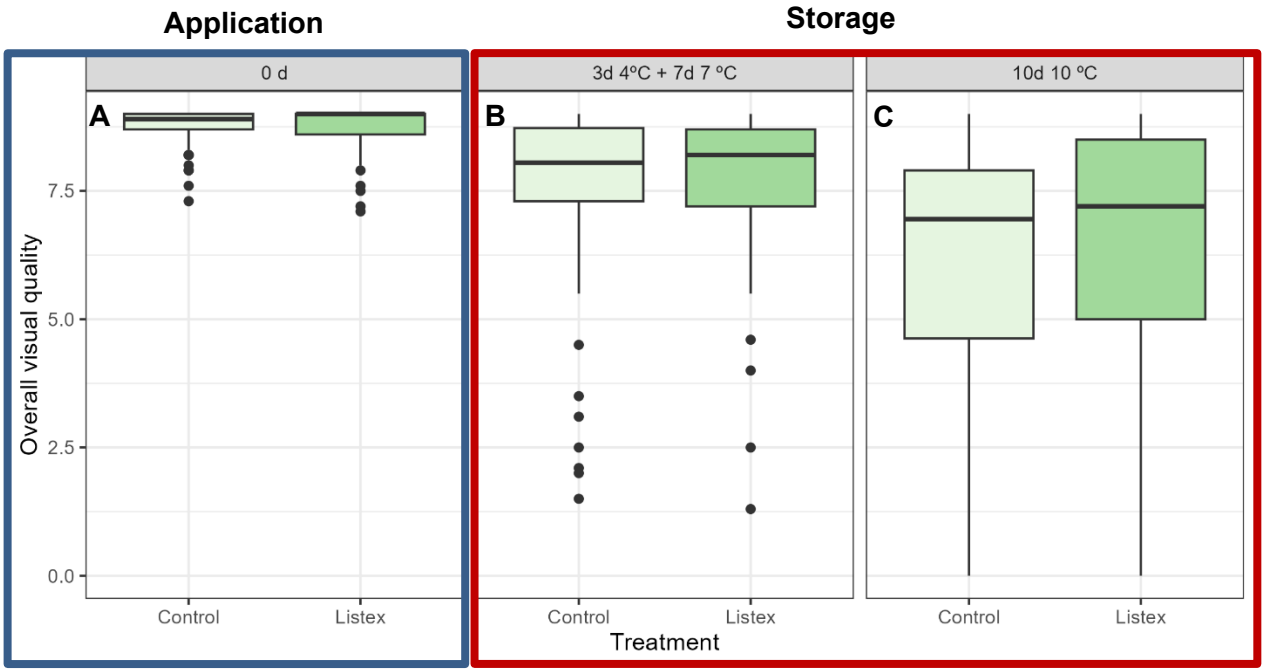


Figure 8. Changes in overall visual quality in Listex™ treated and untreated (Control) baby spinach immediately after application (0 d) (A), after storage under commercial conditions (3d 4ºC + 7d 7ºC) (B), and under abusive storage conditions (10d 10ºC) (C). Box plots represent data from 20 trials (n=600), where the lower part of the box shows the 25th percentile and the upper part the 75th percentile. The dots represent outliers.

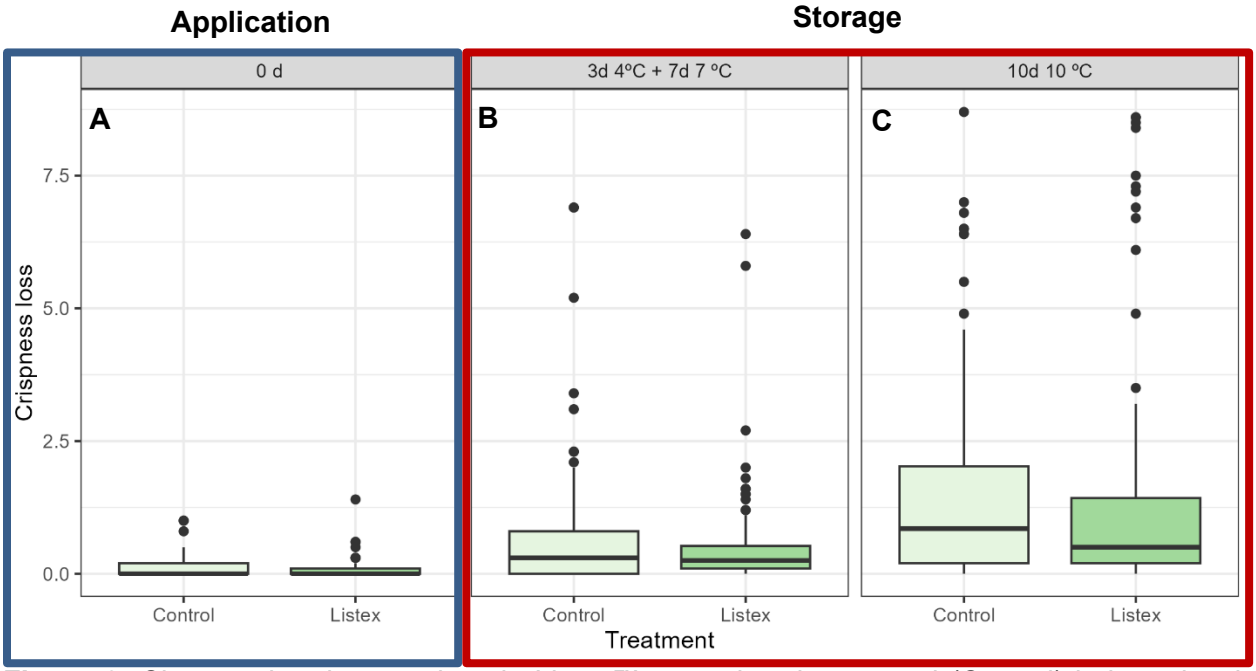


Figure 9. Changes in crispness loss in Listex™ treated and untreated (Control) baby spinach immediately after application (0 d) (A), after storage under commercial conditions (3 d 4ºC + 7 d 7ºC) (B), and under abusive storage conditions (10 d 10ºC) (C). Box plots represent data from 20 trials (n=600), where the lower part of the box shows the 25th percentile and the upper part the 75th percentile. The dots represent outliers.

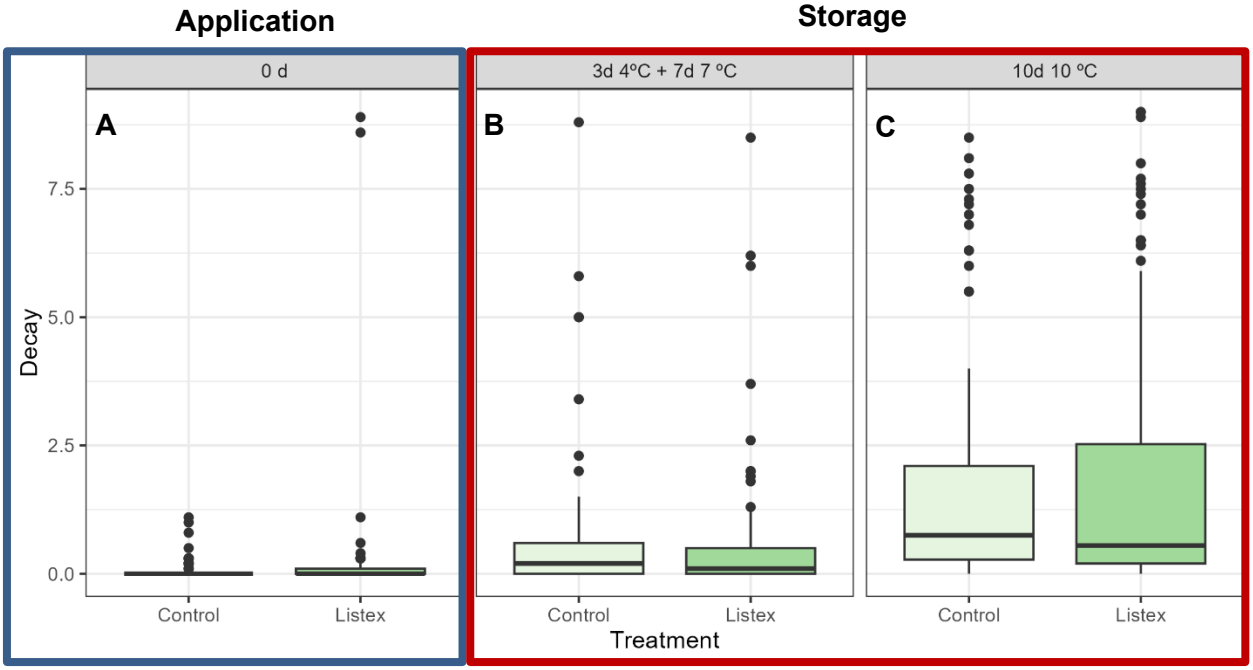


Figure 10. Changes in decay in Listex™ treated and untreated (Control) baby spinach immediately after application (0 d) (A), after storage under commercial conditions (3d 4ºC + 7d 7ºC) (B), and under abusive storage conditions (10d 10ºC) (C). Box plots represent data from 20 trials (n=600), where the lower part of the box shows the 25th percentile and the upper part the 75th percentile. The dots represent outliers.

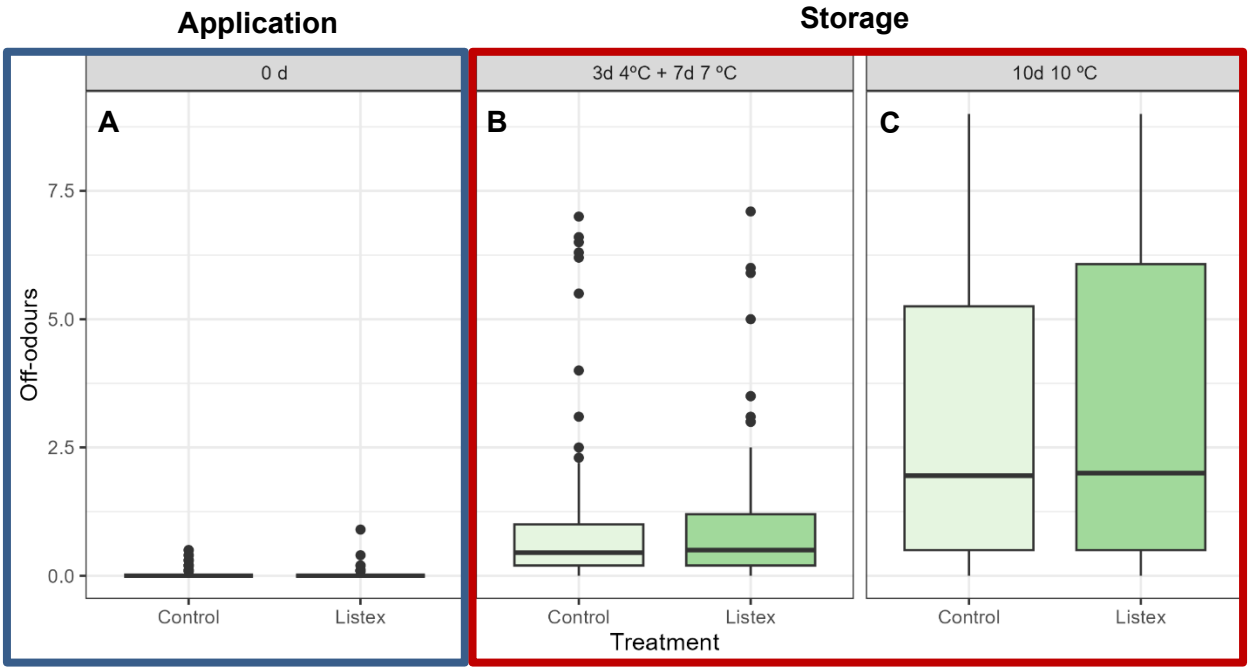


Figure 11. Changes in off-odors in Listex™ treated and untreated (Control) baby spinach immediately after application (0 d) (A), after storage under commercial conditions (3d 4ºC + 7d 7ºC) (B), and under abusive storage conditions (10d 10ºC) (C). Box plots represent data from 20 trials (n=600), where the lower part of the box shows the 25th percentile and the upper part the 75th percentile. The dots represent outliers.

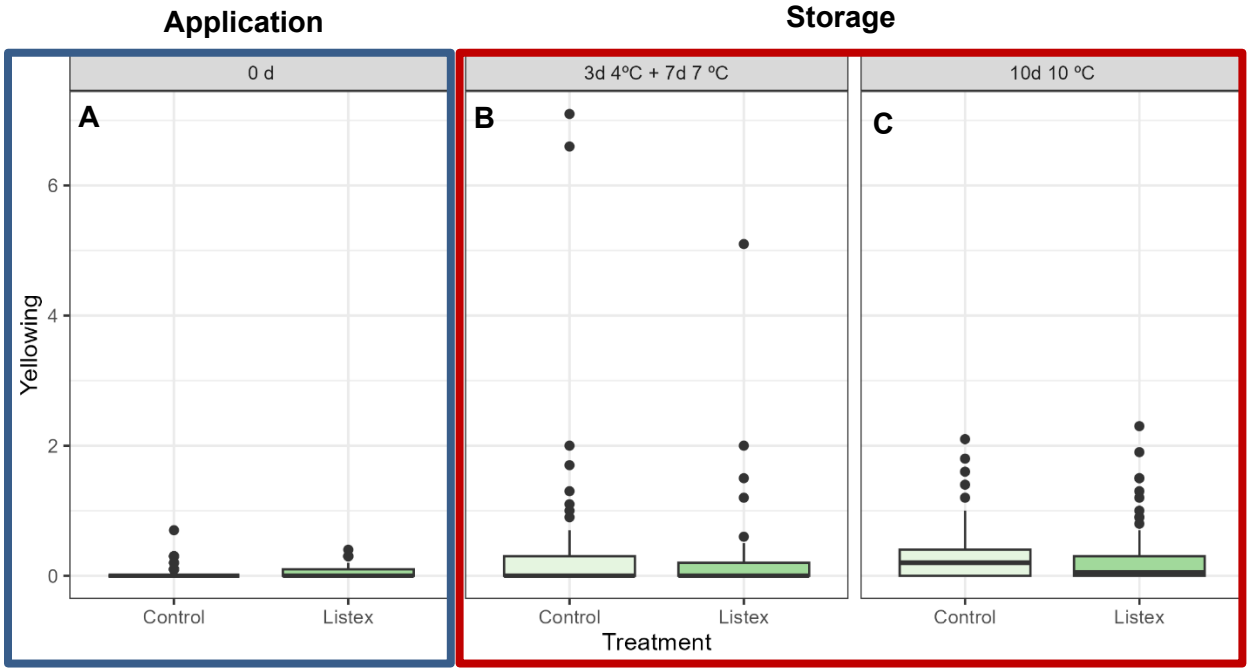


Figure 12. Changes in yellowing in Listex™ treated and untreated (Control) baby spinach immediately after application (0 d) (A), after storage under commercial conditions (3d 4ºC +7d 7ºC) (B), and under abusive storage conditions (10d 10ºC) (C). Box plots represent data from 20 trials (n=600), where the lower part of the box shows the 25th percentile and the upper part the 75th percentile. The dots represent outliers.

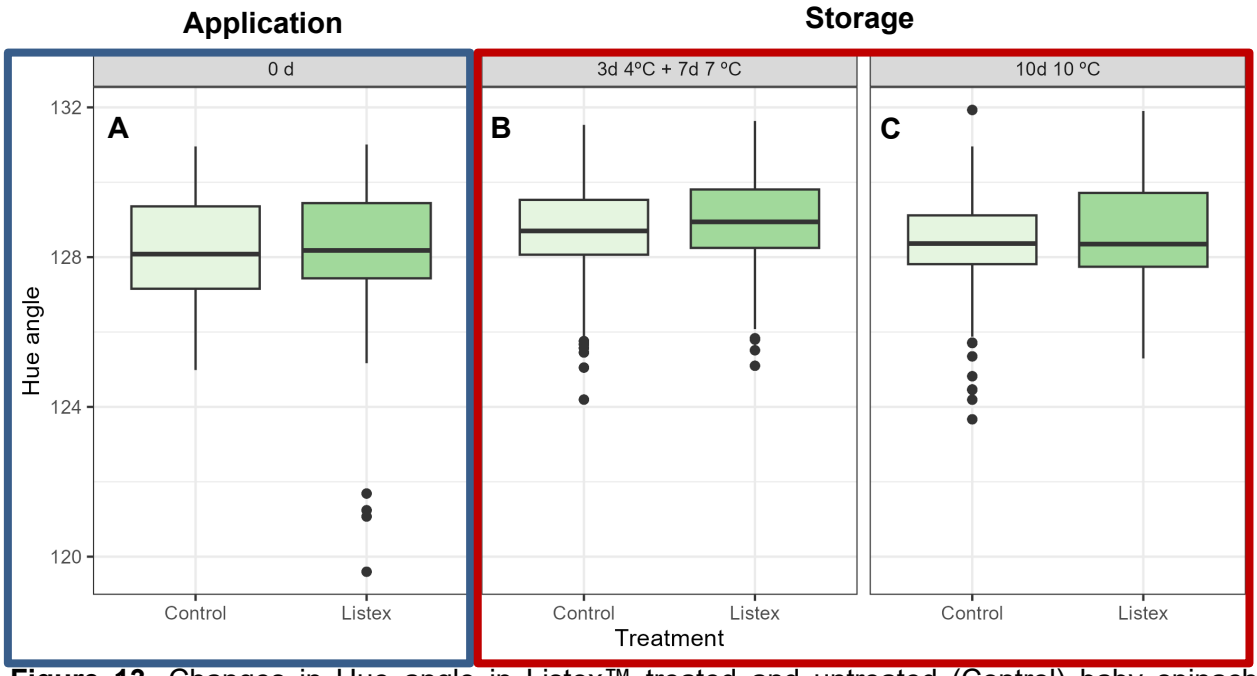


Figure 13. Changes in Hue angle in Listex™ treated and untreated (Control) baby spinach immediately after application (0 day) (A), after storage under commercial conditions (3d 4ºC + 7d 7ºC) (B), and under abusive storage conditions (10d 10ºC) (C). Box plots represent data from 20 trials (n=600), where the lower part of the box shows the 25th percentile and the upper part the 75th percentile. The dots represent outliers.

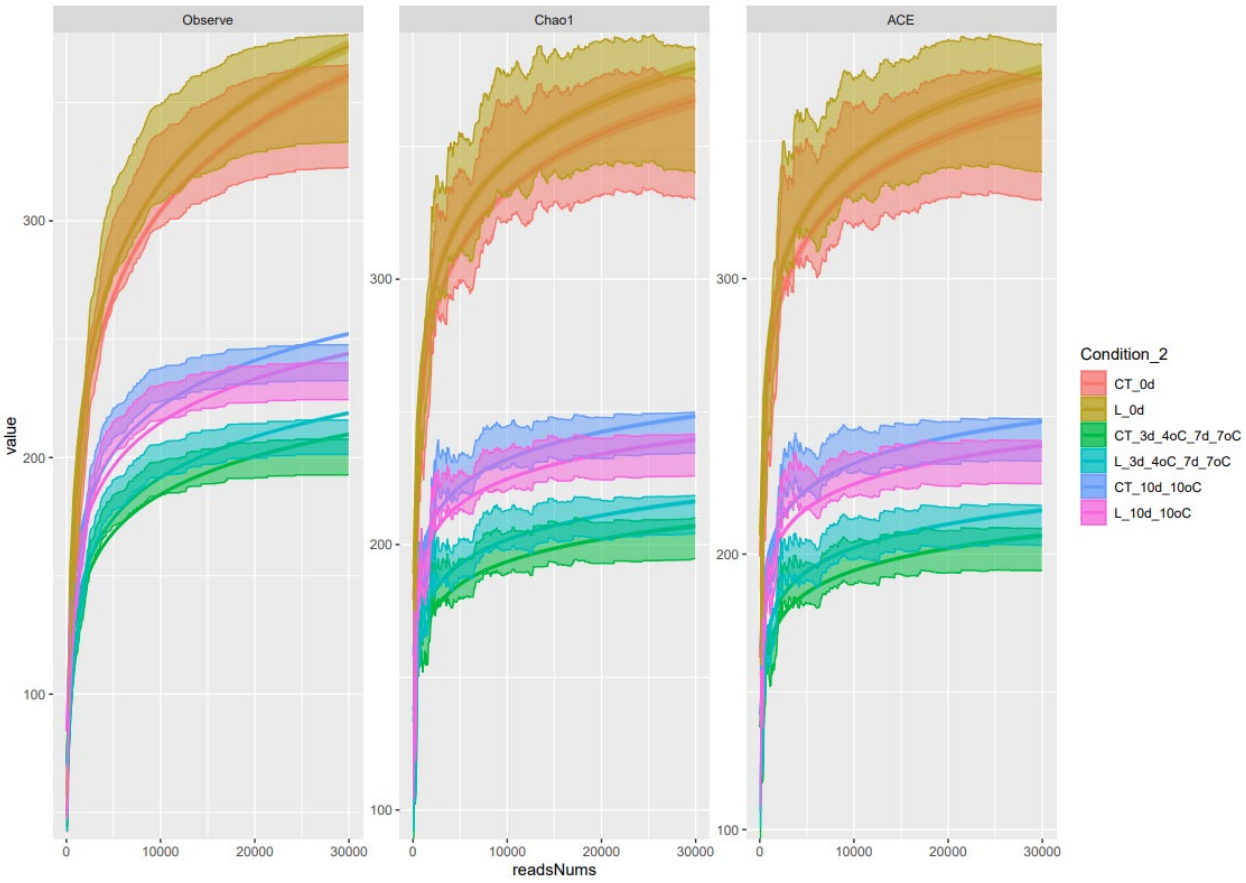


Figure 14. Observed species, Chao1, and abundance-based coverage estimator (ACE) phylogenetic distance whole tree rarefaction curves of Listex™ treated and untreated (Control) baby spinach immediately after application (0 day) (A), after storage under commercial conditions (3d 4ºC followed by 7d 7ºC) (B), and under abusive storage conditions (10d 10ºC).

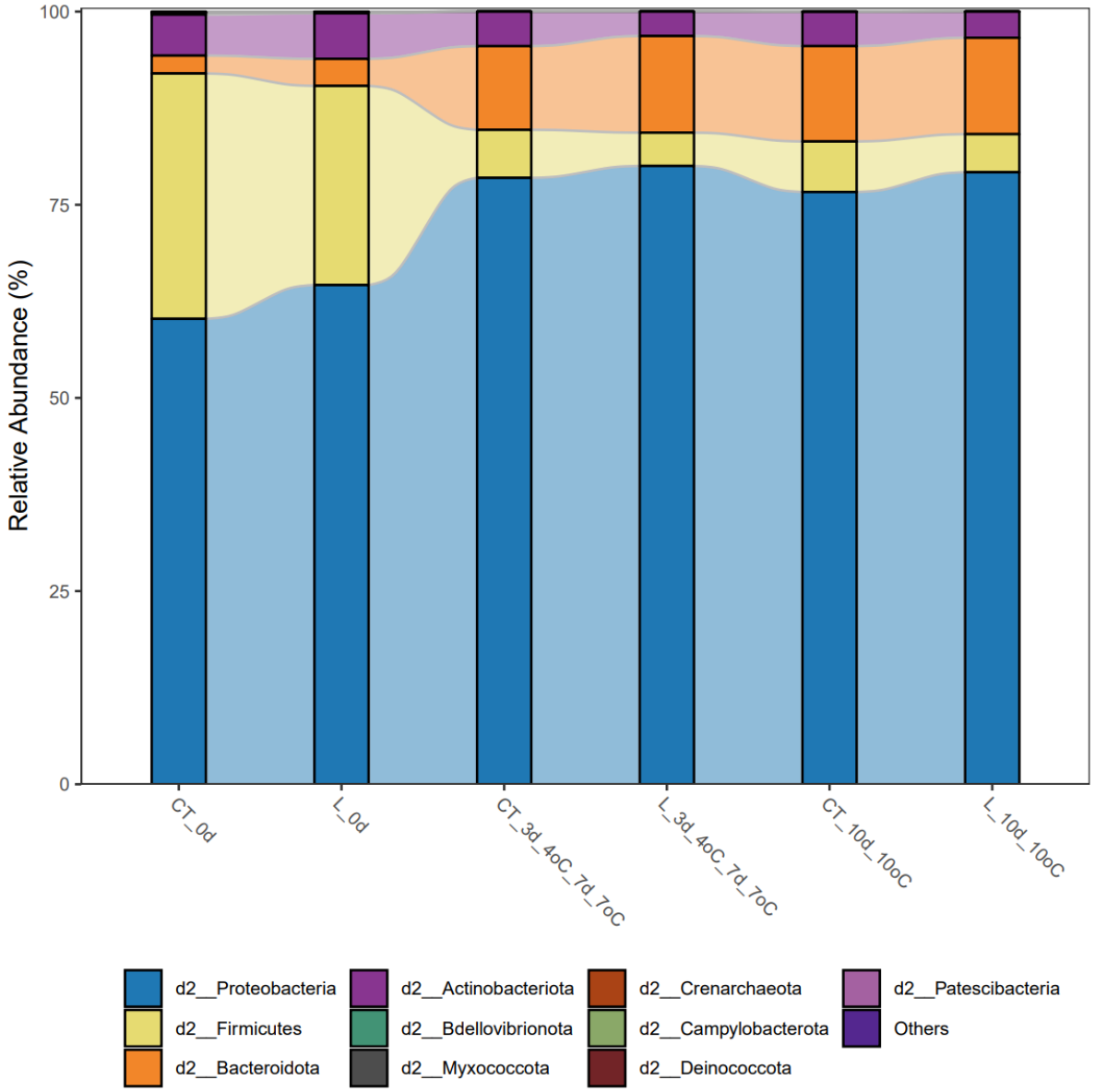


Figure 15. Barplots showing the mean relative abundance of top 10 bacterial phyla of control (CT) and Listex™ (L) treated baby spinach. Bacterial community is the average of 30 individual samples. Data shown are phyla that comprised at least 1% of the average sequences.

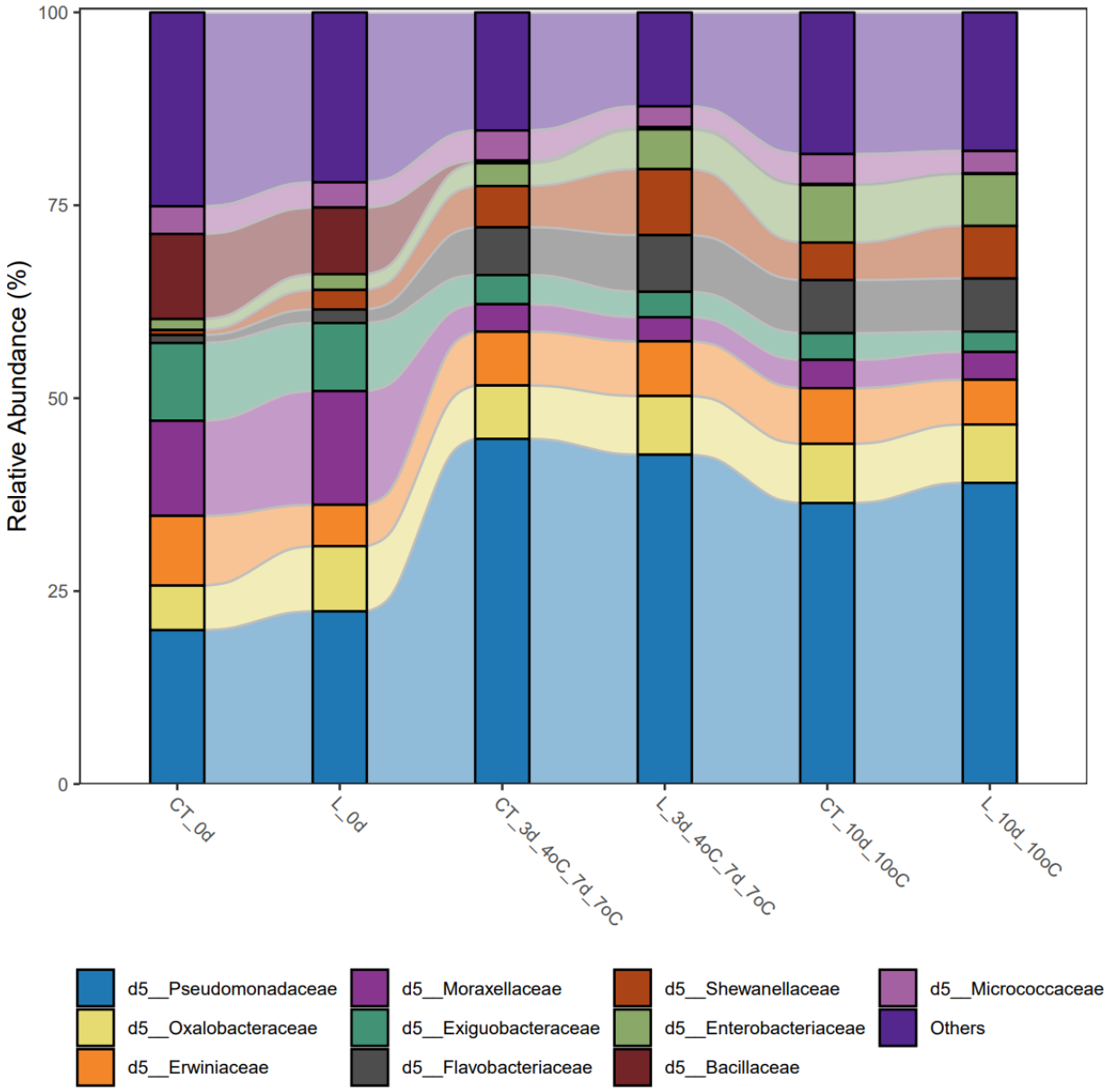


Figure 16. Barplots showing the mean relative abundance of top 10 bacterial family of control (CT) and Listex™ (L) treated baby spinach. Bacterial community is the average of 30 individual samples. Data shown are families that comprised at least 1% of the average sequences.

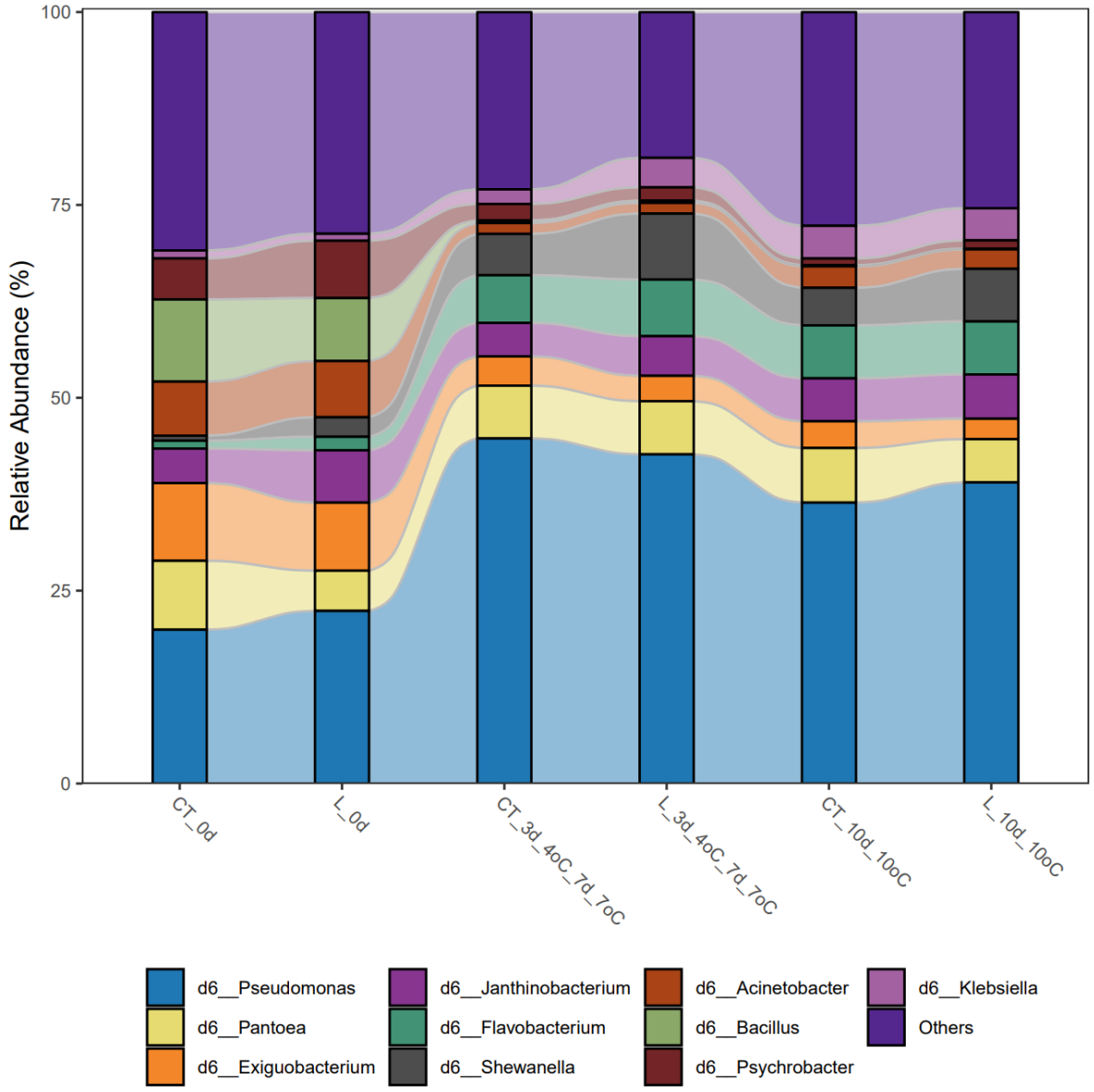


Figure 17. Barplots showing the mean relative abundance of top 10 bacterial genus of control (CT) and Listex™ (L) treated baby spinach. Bacterial community is the average of 30 individual samples. Data shown are genus that comprised at least 1% of the average sequences.

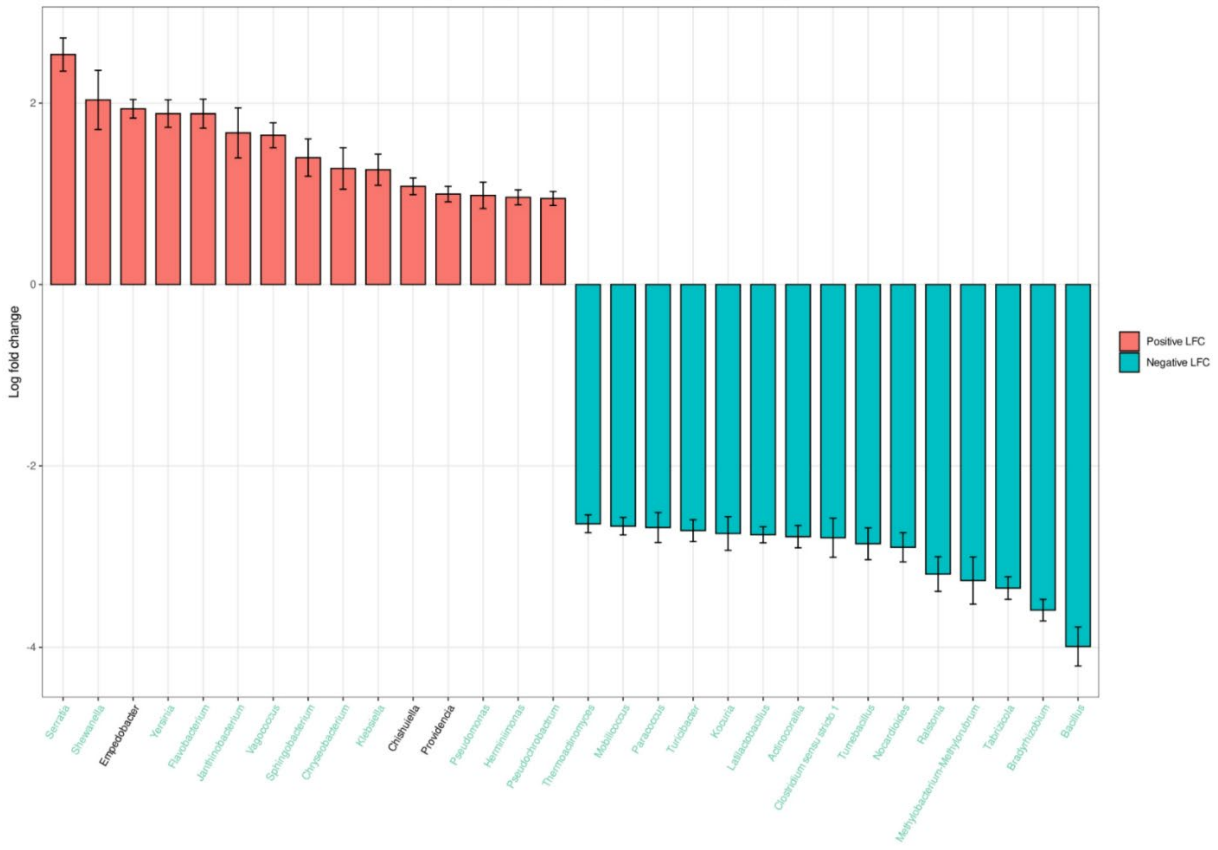
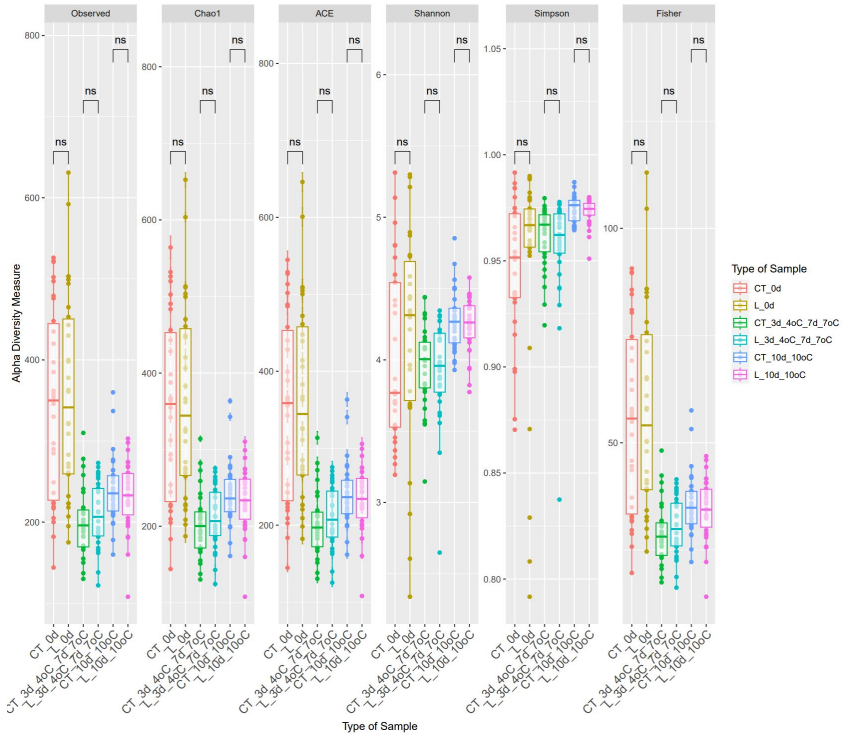


Figure 18. Bar plot representing of differentially of ten more abundant genera due to sampling day obtained from ANCOM-BC analysis. Data are represented by log-fold change (LFC) on the *y*-axis. A positive LFC (red color) indicates that the corresponding taxon is more abundant in baby spinach storage 10 days compared baby spinach at day 0. A negative LFC (blue color) indicates that the corresponding taxon is more abundant in baby spinach at day 0 compared with baby spinach at day 10. All genera listed in the figure had a significant LFC with p-value < 0.001. Green letters indicated that. Taxa in Green were also significant after multiple testing correction was applied at FDR < 0.05.

A)



B)

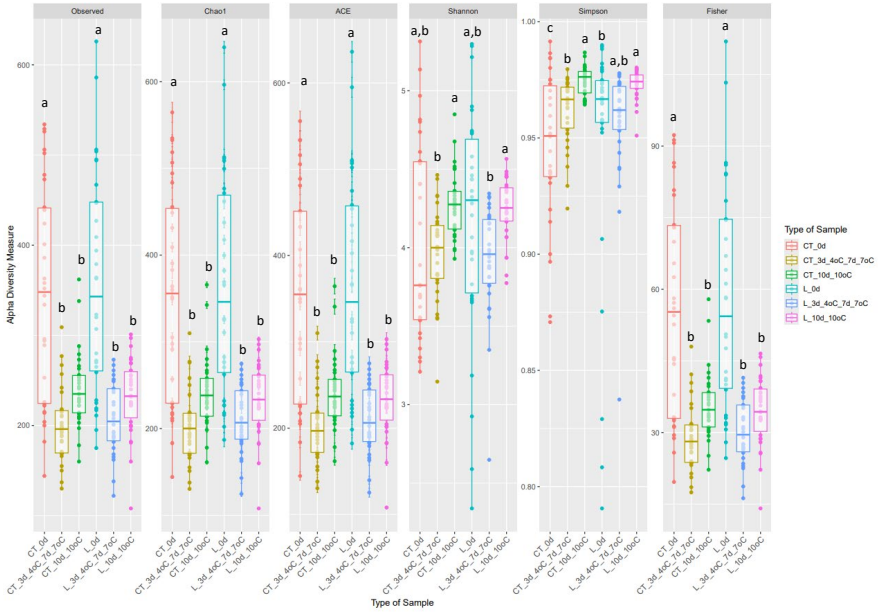


Figure 19. Boxplots for the observed features (ASVs), Pielou's evenness index, Shannon index, and Faith's phylogenetic diversity index comparing the microbial diversity of baby spinach Control (CT) and Listex™ (L) treated samples. The median value is shown as a horizontal line within the box. The inter-quartile range (from lower to upper quartile) represents the middle 50% of values for each group. A) The *p*-values were calculated using Kruskal–Wallis test (****p-value < 0.0001, ***p-value < 0.001, *p-value < 0.05, ns: not significant). B) The *p*-values were calculated using Kruskal–Wallis test. B) Box-plots labelled with different letters indicate significant difference at P < 0.05, according to Tukey's honest significant difference (HSD) test.

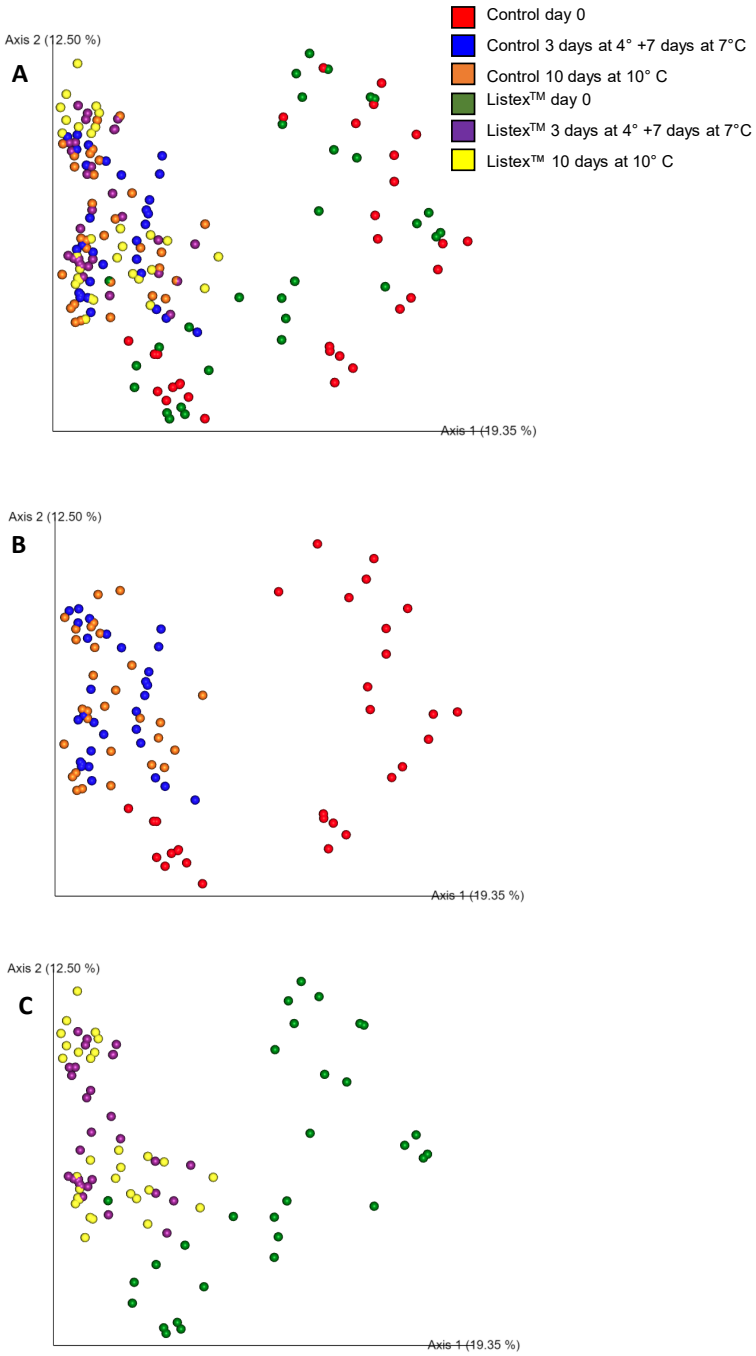


Figure 20. Principal coordinates analysis (PCoA) visualization. The PCoA based on Bray–Curtis distance matrices obtained from taxonomic profiles (n=180). Axes represent percentage of data explained by each coordinate dimension. All samples (A), control samples (B) and Listex™ samples (C). Each dot represents microbiota composition in a single sample.