CENTER for PRODUCE SAFETY

CPS 2017 RFP
FINAL PROJECT REPORT

Project Title

The effects of storage conditions and the microbiome of non-traditional salad ingredients on the fate of *Listeria monocytogenes*

Project Period

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Objectives

1. *Determine the ability of Listeria monocytogenes to grow, survive or die-off in fresh-cut broccoli stalk, Brussels sprouts, kale, and beet greens at recommended and abusive storage temperatures*
2. *Determine if the growth characteristics of L. innocua on the above products are equivalent to L. monocytogenes.*
3. *Determine the diversity and dynamics of microbial communities (microbiome) present on the selected produce during storage conditions, and determine correlates of community structure with changes in L. monocytogenes populations.*
4. *Determine the growth potential of L. innocua on the selected products under simulated storage and distribution conditions.*

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Abstract

Consumer demand for bagged salad has moved beyond shredded iceberg and chopped romaine to more nutritionally dense greens with bold flavors. New salad ingredients include kale, Brussels sprouts, broccoli stalk, and beet greens. Many of these ingredients have not normally been consumed raw or may not have even been widely consumed. The purpose of this project was to determine the fate of *Listeria monocytogenes* on non-traditional salad ingredients under ideal, abusive, and "real-world" storage conditions and to understand the influence of the produce microbiome on *L. monocytogenes* behavior. Kale, Brussels sprouts, broccoli stalk, and beet greens were inoculated with *L. monocytogenes* and incubated at 4, 12, 22, and 35°C. *L. monocytogenes* populations and the microbiome were monitored over the incubation period. All products supported the growth of *L. monocytogenes* when held at abusive temperatures, with nearly all vegetables showing significant ($p < 0.05$) growth after 3 d, 8 h, and 4 h at 12, 22, and 35°C, respectively. At 4°C, *L. monocytogenes* still grew but depending on the vegetable type, growth occurred before (broccoli stalk, 10 d), around (kale, beet greens, 17 d) or after (Brussels sprouts, 25 d) the typical shelf-life. To further assess *L. monocytogenes* growth risk, the products were tested under simulated storage and distribution conditions using *L. innocua* as a surrogate. Exposing product to simulated storage and distribution abuses had no significant effect on *L. innocua* growth regardless of vegetable or incubation temperature ($p > 0.05$). This result indicated that for the tested products, exposure to physical abuse during storage and distribution was not found to significantly increase the risk beyond what was observed as a result of chopping, slicing, or shredding.

Background

To prevent disease and promote health, increasing the consumption of fruits and vegetables is the target of many health and wellness campaigns (US HHS, 2010). Consumption of fruits and vegetables, particularly in the minimally processed or fresh form, has increased because of health awareness and increased convenience and availability. With the industry's fresh-cut and value-added produce sector reaching a staggering $7.6 billion in annual sales, the demand for bagged salads and other ready-to-eat products is at an all-time high (Cook, 2016). Consumer demand for bagged salad has moved beyond shredded iceberg and chopped romaine to more nutritionally dense greens with bold flavors. Additionally, the interest in sustainability amongst both the consumer and the food industry has caused producers to evaluate by-products and waste streams. So to meet consumer demands for "superfoods" and to create sustainable products by utilizing more of the whole vegetable, new salad blends have been developed from non-traditional fresh-cut salad ingredients. New salad ingredients include beet greens, kale, Brussels sprouts, and broccoli stalk. Many of these ingredients have not normally been consumed raw or may not have even been widely consumed. Because of this change in the way these products are consumed the potential risk associated with foodborne pathogens, such as *Listeria monocytogenes*, should be reassessed. The purpose of this project was to determine the fate of *L. monocytogenes* on beet greens, kale, Brussels sprouts, and broccoli stalk under ideal, abusive, and "real-world" storage conditions, and to understand the influence of the produce microbiome on *L. monocytogenes* behavior.

There has been an increase in concern for and awareness of fresh produce as a vehicle for foodborne pathogens and subsequent outbreaks (Lynh et al., 2009). Over 630 produce-related

outbreaks were recorded in the United States between 1990 and 2007 (Hoelzer, 2012). While *Salmonella* and *E. coli* O157:H7 have been the cause of the majority of these outbreaks, *L. monocytogenes* has been implicated more frequently in recent years. Within the last decade, *L. monocytogenes* linked produce outbreaks involved the consumption of celery, stone fruit, cantaloupe, apples, sprouts, and salad products (CDC, 2012, 2015, 2016a, 2016b; Gaul et al., 2013; Jackson, Salter et al., 2015). In 2016 and early 2017, seven fresh-cut/value-added produce products were recalled in the U.S. (FDA, 2017). In produce, prevention and control of *L. monocytogenes* can be challenging since the product can become contaminated anywhere from harvest to the production environment. Fresh produce receives minimal processing and outside of washing, which yields slight reductions in pathogen loads, no additional lethality steps are applied. After processing and packaging, refrigeration is the only control mechanism for microbial growth; however, *L. monocytogenes* is particularly problematic since it not only survives at refrigeration temperatures but can grow (Olaimat, 2012). Understanding if a produce product supports the growth or no growth of *L. monocytogenes* is important so that companies can utilize the information to make the best risked-based decisions for their products.

While many researchers have investigated the growth of *L. monocytogenes* on common fresh-cut salad ingredients, like spinach, romaine, and iceberg lettuce, research on non-traditional salad ingredients is incomplete. A review of the current literature shows that research on broccoli, Brussels sprouts, and kale is limited and is non-existent for beet greens. Previous researchers have established the potential for *L. monocytogenes* growth in broccoli and kale. Berrang et al. (1989) studied the growth of *L. monocytogenes* on broccoli florets at 15 and 4°C: from an initial inoculation level of 10^4–10^5 CFU/g; at 15°C a 3 log CFU/g increase in *L. monocytogenes* populations was observed within 6 days, and at 4°C, populations decreased slightly in the first few days and by the end of 21 days, a less than 1 log CFU/g increase was observed. Similar results on broccoli florets were reported by Alvarez et al. (2013), who reported that *L. monocytogenes* concentrations remained constant when produce was held at 5–7°C for 7 days. For kale (with only one replicate), growth (1.2 log CFU/g increase) of *L. monocytogenes* was observed on kale held at 7°C for 10 days (Lokerse, 2016). At a high inoculation level of 6 log CFU/g, Mansur et al. (2015) also detected similar log increases of *L. monocytogenes* on kale when held at both 4 and 7°C. A reduction in the *L. monocytogenes* population was reported in trimmed Brussels sprouts stored at 7°C for 7 days (Jacxsens et al., 1999). While these earlier studies provide insight into the behavior of *L. monocytogenes*, this project helps address some of the gaps in the literature. For example, some of the studies were conducted at very high initial inoculation levels which could influence growth. Additionally, the produce samples were relatively intact (i.e. broccoli florets, kale leaves, and trimmed Brussels sprouts) but subsequent size reduction like shredding or chopping could potentially increase growth. Finally, limited temperature conditions were studied.

This project evaluated multiple temperature parameters and also storage and distribution simulated conditions. Most fresh produce commodities, whole or processed, face numerous challenges in the distribution environment. The typical challenges these commodities are exposed to during distribution include temperature and humidity variations; dynamic and static physical abuse elements (shocks, vibration, compression, etc.); the presence of gasses (O_2 and CO_2); microbial activity; and the presence of volatile gases. Numerous studies documenting temperature abuses throughout the produce supply chain have been done (Brown et al., 2016; Kou et al., 2015; McKeller et al., 2012; Zeng et al., 2014). Temperature data collected during commercial transport and retail storage and display showed retail storage had the most abusive temperature conditions. When fresh-cut romaine mix was challenged with *L. monocytogenes* using the collected temperature data, populations increased by ≤ 0.6, 3.0, and 1.1 log CFU/g under transport, retail storage, and display, respectively (Zeng et al., 2014). While other studies used collected data to develop predictive models for pathogen growth, Zeng's work was the only

study found that conducted an actual challenge study to compare model results. Temperature is a prime factor influencing pathogen growth, but the previous research did not consider the physical damage that may occur during transport, which might increase the produce's susceptibility to microbial growth.

Beyond temperature and product damage, factors like pH, water activity, and background microbiota influence pathogen growth and survival. During challenge studies, researchers typically test product for background microbiota, and it is often hypothesized that these organisms could have contributed to limited growth or a reduction in the pathogen population. This project took the typical challenge study a step further and investigated the microbiome associated with each produce commodity. Several prior studies document microbiome structure on produce and leafy greens, and reviews by Jackson et al. (2015) and Gorni et al. (2015) are quite comprehensive. Briefly, current studies using high throughput 16S-rRNA-based methods reveal a microbiome on fresh-cut vegetables that, while possibly soil-derived, is less diverse than in most soils. No consensus community has been identified and a great deal of variation is reported, even within samples of the same vegetables. While genera containing human pathogenic species were identified in the microbiome, these methods for assessing microbiomes do not give unequivocal species-level identification. The effects of storage conditions on the microbiome include decreased diversity overall, as well as relative increases in specific subpopulations, most notably psychrophilic genera (Jackson et al., 2015). Minimal data exists on changes to the microbiome of leafy greens over storage time, with spinach and lettuce being the focus of a few studies (Gorni et al., 2015). In other studies, storage-related increases in Enterobacteriaceae were noted (Jackson et al., 2015), and packaging material appeared to affect microbiome structure (Di Carli et al., 2016). However, no record of microbiome studies performed in concert with a pathogen challenge was found. There is reason to believe that the microbiome on some fresh-cut vegetables could serve to exclude human pathogens. In results reminiscent of the protective effect of probiotic bacteria in animal digestive systems, studies in a variety of crop plants report correlations between rhizosphere microbiome structure and pathogen incidence (Mendes et al., 2013). Thus, by collecting information on the growth and survival of *L. monocytogenes* on fresh-cut vegetables while monitoring microbiome structure, this project may be a first step toward identifying microbiome components with similar protective capacity.

Research Methods

Objectives 1 and 2 – L. monocytogenes and L. innocua Challenge Study

Produce Samples. Chopped kale, chopped beet greens, sliced Brussels sprouts, and shredded broccoli stalk were obtained in bulk from our industry collaborator, a fresh-cut packing facility within 30 miles of Cal Poly. After being transported to Cal Poly the produce was held at 4°C for less than 24 h prior to inoculation.

Listeria Preparation. Five strains each of *L. monocytogenes* and *L. innocua* were used in this study (Table 1). For long-term storage, strains were stored frozen in trypticase soy broth (TSB) with 15% glycerol at −70°C. Prior to use, cultures were streaked onto trypticase soy agar (TSA) and incubated at 37°C for 18–24 h. An isolated colony was then transferred to TSB and incubated at 37°C for 18–24 h, two consecutive times. After incubation, cultures were harvested by centrifugation, washed twice with 1% peptone, and pooled to form a cocktail and then diluted to the targeted concentration prior to inoculation on the produce.

Produce Inoculation. Produce samples were bulk inoculated with the *Listeria* cocktails at a targeted concentration of 2–3 log CFU/g. Inoculation methods varied by vegetable to accommodate product volume, weight, and sensitivity to handling. Kale (500 g) was added to a sterile Whirl-Pak® bag and inoculated with 25 ml of the *Listeria* cocktail. The inoculum was dispersed by manually agitating the bag. To ensure even distribution of the inoculum five 500-g portions were combined in a sanitized container and held under a biosafety cabinet for 20–25 min to dry. A similar method was repeated with Brussels sprouts and broccoli stalk, using 25 ml of inoculum added to 1250 g of sample per bag, and two portions were combined before drying under the biological safety cabinet. Beet greens were too tender to handle by the above inoculation procedure, so samples were prepared by spray inoculation. Under a biosafety cabinet, 1250 g of beet greens were laid on sterile polypropylene film and sprayed with 30 ml of inoculum. The beet greens were then lightly tossed using sterile tongs and sprayed with an additional 30 ml of inoculum before drying for 25–30 min. Then a second batch of beet greens was inoculated, allowed to dry, and combined with the first batch in a sanitized container. After inoculation, produce was weighed into polypropylene bags and heat sealed. Product weight, bag size, and the number of pinholes varied based on respiration rates and was determined based on guidance from our industry collaborator (Table 2).

Product Storage, Sampling Times and Testing. All vegetable samples were incubated at 4, 12, 22, or 35°C and held for up to 25 days, 7 days, 48 hours, or 12 hours, respectively. At each sampling time, two inoculated samples (25 g) were enumerated for *L. monocytogenes*. Uninoculated control samples (25 g) were enumerated for aerobic and psychrotrophic microorganisms (APC, PPC) and lactic acid bacteria (LAB), and also plated on Modified Oxford Agar (MOX) to test for interference of background microorganisms. Also, at each pull time, the pH, water activity, and headspace gases (O_2/CO_2) of the uninoculated samples were measured. Samples for microbiome analysis were taken from *L. monocytogenes*–inoculated samples and from uninoculated controls at the beginning, middle, and end of incubation for all four tested temperatures. Twenty-five milliliters were taken from the initial 10^{-1} dilution and centrifuged, supernatants were discarded, and pellets were stored at −80°C until DNA extraction.

Incubation temperatures and sampling times:

4°C: 0, 5, 10, 17, and 25 d
12°C: 0, 12 h, and 1, 3, 5, and 7 d
22°C: 0, 4, 8, 16, 24, and 48 h
35°C: 0, 2, 4, 8, and 12 h

Enumeration parameters:

L. monocytogenes: Modified Oxford Agar (MOX), 35°C for 24 h
Aerobic plate count: Tryptic Soy Agar (TSA), 35°C for 24 h
Psychrotrophic plate count: Tryptic Soy Agar (TSA), 7°C for 10 days
Lactic acid bacteria: Man, Rogosa and Sharpe (MRS) Agar, 35°C for 48 h

Statistical Analysis. For each vegetable and temperature combination observed, a mixed-factor ANOVA, with trial as a random effect and time as a fixed effect, was used to pairwise compare *L. monocytogenes* growth (log CFU/g) across time. Paired Tukey's HSD method was used to determine time points of significant differences in growth ($p < 0.05$) when ANOVA effect tests showed a significant difference in growth by time. To determine the similarity of growth patterns between *L. innocua* and *L. monocytogenes* on each vegetable, over three trials growth values (log CFU/g) from duplicate bags were averaged to obtain a total of three data values for each time and temperature combination observed. The mean *L. monocytogenes* values were linearly regressed on *L. innocua* values to evaluate the strength of the relationship between log counts. To examine whether *L. innocua* and *L. monocytogenes* had statistically significant ($p < 0.05$)

non-equivalent growth rates, a t-test was used to compare the regression slope to 1, the value expected if growth rates were equivalent.

Objective 3 – Microbiome Testing and Analysis

Total DNA Extraction for Microbiota Analysis. DNA was extracted from approximately 0.25 g of each sample using DNeasy PowerSoil DNA extraction kit (Qiagen) following the manufacturer's protocol, adding a 10-min vortex in extraction buffer. The bench and pipets used in each extraction experiment were also swabbed and run through a DNA extraction, to serve as a negative control to confirm the absence of microbial DNA contaminants in the extraction processes. The concentration of DNA in each test sample and in the negative control sample was determined fluorometrically using Qubit 3 (Invitrogen) and Qubit dsDNA High Sensitivity Assay Kit. DNA samples were stored at −80°C until further use.

16S rRNA V4 Sequence Amplification. Bacterial community composition was determined by targeted metagenomic sequencing of the PCR-amplified V4 domain of the 16S rRNA gene. Briefly, V4 region of the 16S rRNA gene sequence was amplified using GoTaq PCR Polymerase (Promega), a forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3'), and a reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Unique Illumina i7 and i5 index adaptors were added to the 16S amplicon primers, as well as Illumina Sequencing adaptors, to create a single-round PCR protocol that adds both index barcodes and targets amplification of the V4 region. ZymoBIOMICS Microbial Community DNA Standards were included as a positive control. PCR thermal cycling for amplification of the 16S rRNA gene V4 region was conducted as follows: initial denaturation at 95°C for 3 min, 29 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and the final extension at 72°C for 5 min, and final hold at 4°C. PCR amplicons were visualized by running gel electrophoresis using a 2% agarose gel to confirm successful amplification of target sequences. Bands of the appropriate size were excised and purified via ZymoClean Gel Recovery Kit, a final elution volume of 10 µl. Concentrations of a subset of normalized libraries were verified using a high sensitivity double-stranded DNA kit with Qubit 3 and Bioanalyzer DNA 1000. Estimated amplicon lengths of 359 bp were used in the calculation of molar concentration and normalization. The library pool was denatured by diluting 5 µl of 4 nM library pool with 5 µl freshly prepared 0.2 N NaOH. The denatured library pool was diluted with a pre-chilled HT1 buffer to 7.5 pM with 10% PhiX internal control library. A total of 500 µl of denatured library pool spiked with PhiX was loaded onto the Illumina Miniseq MidOutput reagent cartridge. A maximum of 96 unique samples were pooled and loaded onto one Mid-Output cartridge, all with unique barcodes to allow for demultiplexing.

Sequence Analyses and OTU Normalization. Demultiplexing was carried out with bcl2fastq2 software (Illumina v2.20) to identify unique samples from the pooled libraries. Sequences were analyzed with Ribosomal Database Project (RDP) Classifier, a naïve Bayesian classifier to rapidly classify 16S rRNA sequences using a well-supported taxonomy database (RefSeq RDP 16S v4). Chimera were detected and discarded using UCHIME algorithm. Operational taxonomic units (OTUs) were calculated using opticlust with 97% similarity threshold.

Diversity of Microbiota. Alpha and beta diversity indices were calculated based on rarefied OTUs. For alpha diversity, Shannon and Inverse Simpson indices were calculated for both bacterial and fungal communities. For beta diversity, the weighted UniFrac distance between either bacterial or fungal communities was calculated in R using package Phyloseq. Principal coordinates analysis (PCoA) was used to visualize the beta diversity of microbiota with reduced dimensionality. Chi-square and Fisher's exact tests with Bonferroni correction were used to test

the significance of differences in *L*. *monocytogenes* occurrence among sample conditions. Pairwise permutational multivariate analysis of variance (PERMANOVA) test was carried out using package Pairwise Adonis and Bray-Curtis dissimilarity matrices to test the significance of differences in microbiota composition. Pairwise comparisons were carried out by greens type, *Listeria* inoculation, and storage temperature and duration.

Objective 4 – Storage and Distribution Testing

Produce Samples. Fresh packed 12-oz packages of sliced Brussels sprouts, chopped beet greens, shredded broccoli stalk, and chopped kale were obtained from our collaborator. Produce samples were packaged in retail film and packed in commercial transport cases to replicate packaging during storage and distribution (Table 3). Product was transported to Cal Poly, stored at 4°C and used within 24 h.

Listeria innocua Preparation and Product Inoculation. The same five strains of *L. innocua* were prepared as described above in the challenge study section. Under a biosafety cabinet using a sterile needle and syringe, 2 ml of inoculum was delivered at two locations (~1 ml each) at the top front and bottom back of the bag. As in the previous experiments, the targeted inoculum load was 2–3 log CFU/g. The puncture sites were sealed using masking tape and then the samples were manually agitated to disperse the inoculum.

Simulated Storage and Distribution Abuse. Immediately following inoculation, samples were transferred to Cal Poly's Packaging Dynamics Lab and held at 4°C. Simulated storage and distribution testing followed the International Safe Transit Association (ISTA) 3F *(Packaged product for a distribution center to retail outlet shipment 100 lb (45 kg))* standard. Six sample cases were used for one cycle of testing per experiment, meeting standard requirements and providing enough samples for desired incubation times. The duration of testing cycles was approximately 45 min and simulated one-way transportation and movement of RTE items from distribution sites to retail establishments (Table 25). Each simulation step is detailed below.

> Conditioning: Directly after inoculation samples were placed in a temperature and humidity-controlled chamber for 12–18 h conditioning at 4°C with 90% relative humidity. After conditioning, the ISTA 3F testing schedule was followed: cases were subject to drop, compression, vibration, and a final drop abuse series, in that order, at room temperature. The sequence of abuses was done consecutively without any time delay between abuses. Between sequences, individual treatment and control cases were kept in the 4°C chamber.
>
> Drop testing: Cases were dropped on the bottom (face 3) of the case from both a 12-inch and 30-inch height using a free-fall precision drop tester (Lansmont Corporation, Monterey, CA, USA) (Figure 33).
>
> Compression testing: Using a fixed platen on a compression tester (Lansmont Corporation) each case was subject to increasing compression force (0.5"/min) until a maximum deflection rate of the case was reached. Maximum test force was calculated based on case dimensions and known constants to take into account untested factors and average freight density.
>
> *Eq 1: Compression test force = 0.007 x (108-H) x L x W x H*
>
> Vibration testing: Random vibration was used to simulate packages being transported in a spring-loaded truck. The programmed profile (ISTA Steel Spring Truck) included random changing of frequencies between 1–200 Hz and required a root mean square

acceleration (Grms) of 0.54. Cases labeled one through three were stacked vertically, with case one at the base of the stack and case three at the top. Stacked cases had the bottom of the case (face 3) parallel to the vibration table. Case four tested with the bottom parallel with the table, case five with the side of the case (face 4) on the table and case six with the end of the case (face 6) on the table. Cases four through six were not stacked and all cases were restrained to prevent horizontal movement but not to restrict vertical movement. The vibration duration was 30 min.

Final drop testing: The abuse series concluded with a second drop series. Each case was dropped from 18 in on the bottom of the case (face 3). One additional 24-in drop tested different orientations of the six cases. Cases were dropped on different faces, corners, or edges of the case (Table 26).

Storage and Incubation. After abuse testing, cases were randomly assigned between two incubation temperatures using Excel's randomization function (Microsoft Office, Redmond, WA). Three of the six abused cases were transferred to chambers set for either 4 or 8°C, with ~90% relative humidity. Control (non-abused) sample cases were included in the chambers. Samples were stored in the chambers throughout shelf-life testing (16 days).

Product Testing. Samples were tested after inoculation, after conditioning, post-abuse treatment, and after abuse on days 2, 6, 11, and 16 or on days 2, 4, 6, and 11 when incubated at 4 or 8°C, respectively. For inoculated products, the entire sample was used for enumeration. Approximately 15 g of uninoculated sample was removed for pH and water activity testing, and the remainder was used for enumeration. The same testing was done on inoculated and uninoculated samples as described in the above challenge study section. Testing included enumeration of *Listeria*, APC, LAB, and PPC as well as pH, water activity, and headspace gases (O_2/CO_2).

Statistical Analysis. For each vegetable and temperature observed, sample growth values (log CFU/g) from duplicate bags were averaged to retain one mean value at each time observed, across three trials. To analyze the difference in growth patterns of *L. innocua* between treated and untreated samples, ANOVA was used to compare *L. innocua* values with main effects of time, treatment, and their interaction ($p < 0.05$). Additionally, Paired Tukey's HSD method was used to determine time points of significant growth ($p < 0.05$), in both temperatures and treatments, when ANOVA effect tests showed a significant difference in growth by time.

Research Results

Objective 1 and 2 – L. monocytogenes and L. innocua Challenge Study

All products showed significant ($p > 0.05$) increases in *L. monocytogenes* populations during the tested incubation period regardless of ideal (4°C) or abusive (12, 22, 35°C) temperature exposure. Kale supported the growth of *L. monocytogenes* by 17 d, 3 d, 8 h, and 4 h at 4, 12, 22, and 35°C, respectively (Table 4, Figures 1, 3, 5, 7). Brussels sprouts were the least supportive of *L. monocytogenes* growth, with growth observed by 25 d, 3 d, 8 h, 4 h at 4, 12, 22, and 35°C, respectively (Table 5, Figures 9, 11, 13, 15); at 4°C, it is interesting to note that by day 5 (120 h) *L. monocytogenes* counts dipped below original inoculation levels, and by the end of the 25-d incubation period only a 0.6 log CFU/g increase from time 0 was observed. On broccoli stalk incubated at 4, 12, 22, and 35°C, *L. monocytogenes* growth was observed by 10 d, 3 d, 8 h, and 4 h, respectively (Table 6, Figures 17, 19, 21, 23). On beet greens, growth of *L.*

monocytogenes was observed by 17 d, 3 d, 16 h, and 8 h at 4, 12, 22, and 35°C (Table 7, Figures 25, 27, 29, 31).

L. innocua challenge studies were run concurrently with *L. monocytogenes* testing. Results indicated that the tested *L. innocua* strains did not behave significantly different ($p > 0.05$) than the *L. monocytogenes* strains (Table 8). Therefore *L. innocua* was used in the storage and distribution testing study.

Objective 3 – Microbiome Testing and Analysis

Microbial DNA was successfully extracted from all 790 samples (four greens types, all storage temperatures, all time courses, plus positive and negative controls). Average DNA concentrations extracted were 0.01 µg/µl, with a range from 0.06–0.005 µg/µl, sufficient for generating multiple sequencing experiments from each extraction.

Illumina MiniSeq Mid-Output reagents were utilized, generating on average 5.62GB of data per run, with average quality scores of 92.7% of reads greater than or equal to Q30 and 87% of clusters passing filter (PF). This amount of high quantity data per run allows multiplexing of up to 96 samples per sequencing run, bringing the cost down to only $15 per sample from extraction to sequencing analysis.

To combine 96 individual samples into one sequencing run, index barcodes were added to the forward and reverse primers. During the PCR amplification, unique barcodes are added to either end of the sample, while the 16S V4 region is sequenced for all samples. A specific algorithm (bcl2fastq2) was used to assign barcodes to the original sample id. Each sample generated 32MB of sequencing data on average, with 95% identification to a known bacterial order.

Raw sequencing files are stored both locally on a 24TB external hard drive, and on AWS supported cloud compute resource (Illumina BaseSpace). BaseSpace also provides the infrastructure to build cloud-based analysis pipelines, allowing quick comparison across different 16S taxonomy databases to build confidence in community assignments.

While much of this objective was completed, the team experienced execution challenges as a result of the robustness of the study design and the novelty of the sequencing methodology. The team was establishing a brand-new sequencing facility at Cal Poly, which required building computational and molecular biology resources. Protocols had to be created to deal with the nearly 800 individual microbiome samples, all of which were critical for replication confidence and reproducibility but which stretched metadata and barcoding capacities. Because of these challenges, completion of objective 3 was not met by the end of the project term (Dec 2019). However, the team is committed to completing this objective and is currently optimizing statistical analysis to determine changes in microbiome community across the multi-variate dataset but we are confident this detailed level of analysis will improve our understanding of the conditions permissive or restrictive to growth of pathogenic bacteria and potentially identify strains that are prohibitive to *L. monocytogenes* expansion.

Objective 4 – Storage and Distribution Testing

Simulated storage and distribution abuse had no significant effect ($p > 0.05$) on *L. innocua* or APC, LAB or PPC growth regardless of vegetable or incubation temperature (Tables 35–37, Figures 34–41).

Outcomes and Accomplishments

- Determined the ability of *L. monocytogenes* to grow in kale, Brussels sprouts, broccoli stalk, and beet greens at 4, 12, 22, and 35°C.
- Showed that *L. innocua* can be used as a surrogate for *L. monocytogenes* in kale, Brussels sprouts, broccoli stalk, and beet greens.
- Microbiome analysis (DNA extraction, 16S amplification, multiplexing, and sequencing protocols and analysis pipelines) has been established in-house.
- Showed that simulated physical abuse during storage and distribution of the tested products did not increase the risk of *L. innocua* growth.

Summary of Findings and Recommendations

These findings support the need for close temperature control of RTE salads that may contain kale, Brussels sprouts, broccoli stalk, and/or beet greens. These data can be used to help assess the potential for increased risk of *L. monocytogenes* growth when loss of temperature control occurs. In addition, the data showed that even in the presence of ideal storage temperature (4°C), *L. monocytogenes* can still grow in these products. Depending on the vegetable type, growth occurred before (broccoli stalk, 10 d), around (kale and beet greens, 17 d) or after (Brussels sprouts, 25 d) the typical shelf-life. Storage and distribution results indicated that for the tested products, exposure to physical abuse during storage and distribution did not significantly increase the risk beyond what was observed as a result of chopping, slicing, or shredding.

APPENDICES

Publications and Presentations

No publications to date, although manuscripts are currently in preparation (to be submitted by late 2020).

Preliminary findings were presented at the annual CPS Research Symposium in 2018 and 2019 and final results will be presented at the Symposium in June 2020. An abstract has been submitted to the International Association of Food Protection (IAFP) annual meeting to be held in August 2020.

Budget Summary

The total funds awarded to this project were $358,058. The team expects to spend the majority of the project funds. Funds were enough to complete the project, and excess funds were a result of personnel changes from a postdoc to staff and students.

Tables and Figures (see below)

Tables 1–37 and Figures 1–41

Table 1. *Listeria* strains used in the study

***Listeria* Strain**	**Isolate Source**	**Provided by**
L. monocytogenes		
FSL J1-108	Coleslaw	Cornell[1]
FSL J1-031	Human Sporadic Case	Cornell
R9 -0506	Cantaloupe (2011)	Cornell
R9-5411	Caramel Apple	Cornell
R9-5506	Packaged Salad	Cornell
L. innocua		
B-33003	California	ARS[2]
B-33314	Turkey, Ham, Cheese Deli Sticks	ARS
B-33554	Food Isolate	ARS
B-33192	Wheat Processing Plant	ARS
C2-0008	Fish Processing Plant	Cornell

[1]Cultures provided by Cornell University were obtained from the Institute of Life Sciences of North America or the CPS culture collection.
[2]Agriculture Research Services, Peoria, IL

Table 2. Produce sample packing parameters for *L. monocytogenes* and *L. innocua* challenge studies

Produce	**Sample Size (g)**	**Bag Size[1]**	**Pinholes[2]**
Kale	50	7” x 9”	1
Brussels Sprouts	200	7” x 8”	4
Broccoli Stalk	200	7” x 8”	6
Beet Greens	50	7” x 8”	1

[1]Bag material was polypropylene
[2]Pinhole size was 33 gauge

Table 3. Produce sample packing parameters for the *L. innocua* storage and distribution testing

Produce	**Sample Size**	**Bag Size[1]**	**Box Dimension, Bags per Box**
Kale	12 oz	8" x 10" x 3"	16.25" x 11.75" x 5.25", 6
Brussels Sprouts	12 oz	8" x 10" x 3"	16.25" x 11.75" x 5.25”, 6
Broccoli Stalk	12 oz	7" x 9" x 3"	16.25" x 11.75" x 5.25", 6
Beet Greens	12 oz	8"x 10" x 3"	16.25" x 11.75" x 5.25", 6

[1]Bag material was polypropylene

Summary of *Listeria monocytogenes* Challenge Study Results

Table 4. Growth of *L. monocytogenes* on kale.
Time when statistically significant ($p < 0.05$) growth and a ≥1 log growth was observed.

Temp (°C)	Statistically Significant Time (log CFU/g)	≥1 log growth Time (log CFU/g)
4	17 d (1.2)	17 d (1.2)
12	3 d (1.8)	3 d (1.8)
22	8 h (1.1)	8 h (1.1)
35	4 h (0.9)	8 h (2.7)

Table 5. Growth of *L. monocytogenes* on Brussels sprouts.
Time when statistically significant ($p < 0.05$) growth and a ≥1 log growth was observed.

Temp (°C)	Statistically Significant Time (log CFU/g)	≥1 log growth Time (log CFU/g)
4	25 d (0.6)	*
12	3 d (1.1)	3 d (1.1)
22	8 h (0.6)	16 h (2.0)
35	4 h (0.4)	8 h (2.0)

*At the end of incubation *L. monocytogenes* had only increased by 0.6 log CFU/g.

Table 6. Growth of *L. monocytogenes* on broccoli stalk.
Time when statistically significant ($p < 0.05$) growth and a ≥1 log growth was observed.

Temp (°C)	Statistically Significant Time (log CFU/g)	≥1 log growth Time (log CFU/g)
4	10 d (0.9)	17 d (2.0)
12	3 d (2.6)	3 d (2.6)
22	8 h (0.7)	16 h (2.1)
35	4 h (0.6)	8 h (2.1)

Table 7. Growth of *L. monocytogenes* on beet greens.
Time when statistically significant ($p < 0.05$) growth and a ≥1 log growth was observed.

Temp (°C)	Statistically Significant Time (log CFU/g)	≥ 1 log growth Time (log CFU/g)
4	17 d (1.1)	17 d (1.1)
12	3 d (1.6)	3 d (1.6)
22	16 h (1.1)	16 h (1.1)
35	8 h (1.9)	8 h (1.9)

Table 8. *L. monocytogenes* and *L. innocua* growth comparison linear regression and ANOVA statistics.

Vegetable	R^2	F ratio	p-value
Broccoli	0.9512	1248.4	<0.0001
Brussel Sprouts	0.9596	1498.1	<0.0001
Kale	0.9442	1438.8	<0.0001
Beet Greens	0.8515	458.98	<0.0001

KALE
L. monocytogenes and *L. innocua* Challenge Study Results

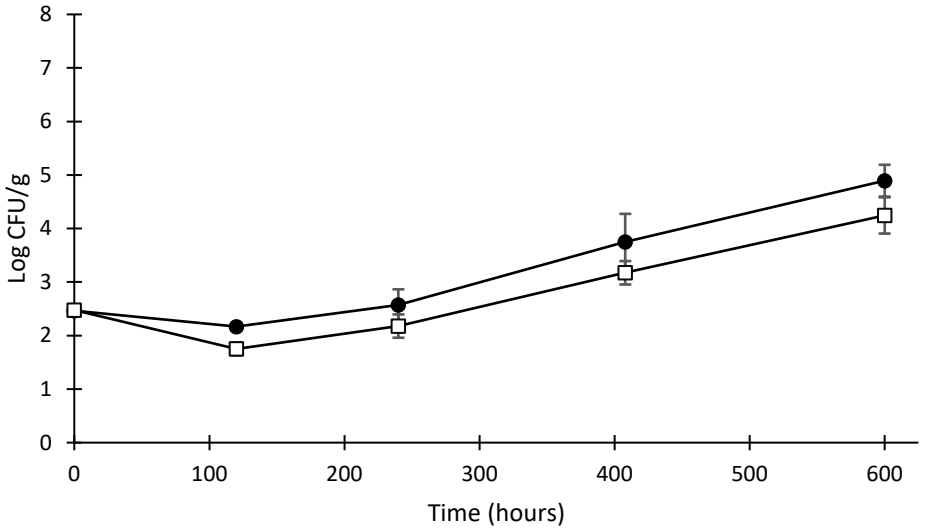

Figure 1. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on kale held at 4°C. Error bars represent standard error of growth means.

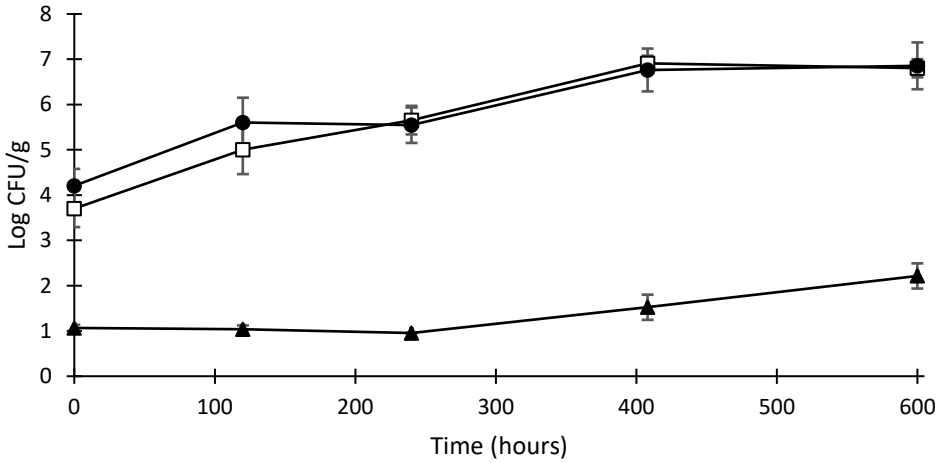

Figure 2. APC(□), PPC(●) and LAB(▲) growth on kale held at 4°C. Error bars represent standard error of growth means.

Table 9. Average headspace, water activity and pH for kale at 4°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	20.5 ± 0.4	0.3 ± 0.3	0.994 ± 0.003	6.7 ± 0.2
120	12.6 ± 4.7	6.7 ± 3.7	0.994 ± 0.003	7.0 ± 0.3
240	12.5 ± 5.0	6.4 ± 3.4	0.993 ± 0.004	7.0 ± 0.3
408	13.6 ± 4.4	5.7 ± 3.8	0.993 ± 0.003	7.3 ± 0.2
600	10.0 ± 5.4	7.5 ± 3.3	0.994 ± 0.003	7.4 ± 0.2

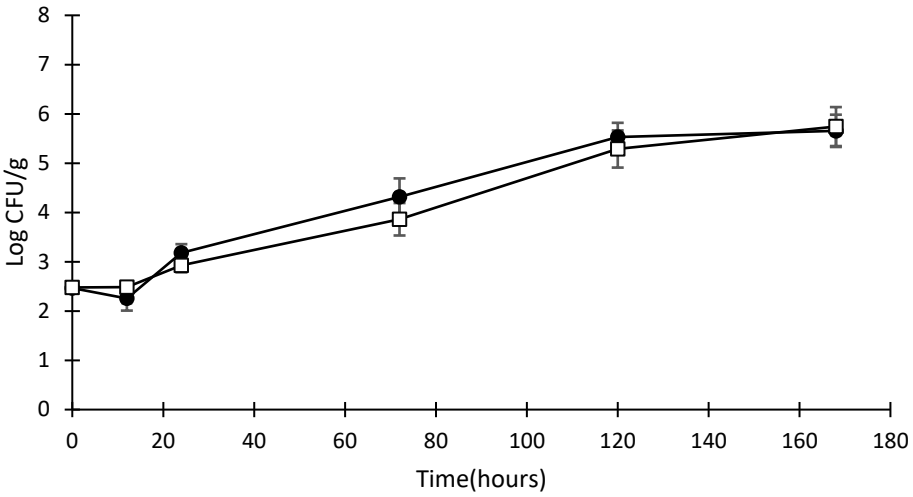

Figure 3. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on kale held at 12°C. Error bars represent standard error of growth means.

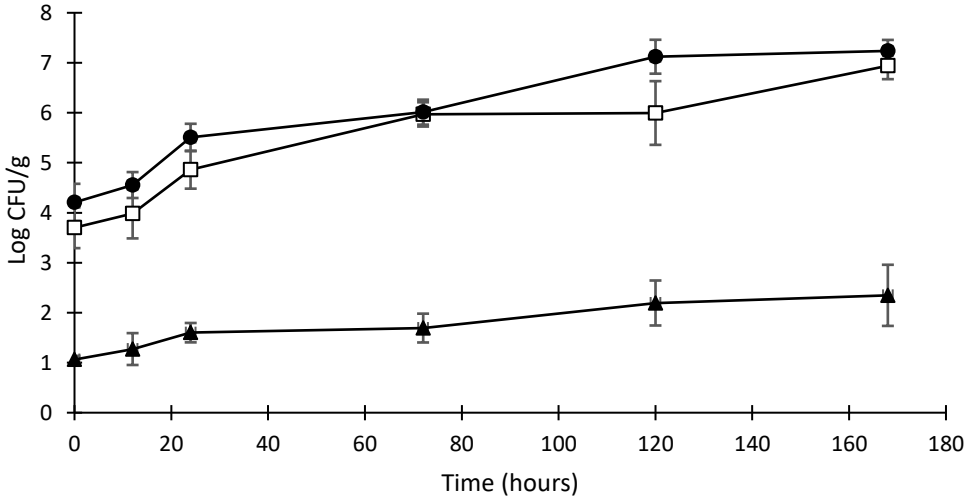

Figure 4. APC(□), PPC(●) and LAB(▲) growth on kale held at 12°C Error bars represent standard error of growth means.

Table 10. Average headspace, water activity and pH for kale at 12°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	20.5 ± 0.4	0.3 ± 0.3	0.994 ± 0.003	6.7 ± 0.2
12	15.7 ± 2.3	4.4 ± 2.0	0.991 ± 0.005	6.8 ± 0.3
24	13.1 ± 4.0	6.4 ± 3.4	0.990 ± 0.004	6.9 ± 0.1
72	7.58 ± 5.5	10.4 ± 3.8	0.995 ± 0.003	7.0 ± 0.2
120	6.8 ± 4.9	10.2 ± 2.7	0.989 ± 0.005	7.1 ± 0.2
168	5.1 ± 4.7	11.0 ± 2.3	0.993 ± 0.005	7.2 ± 0.3

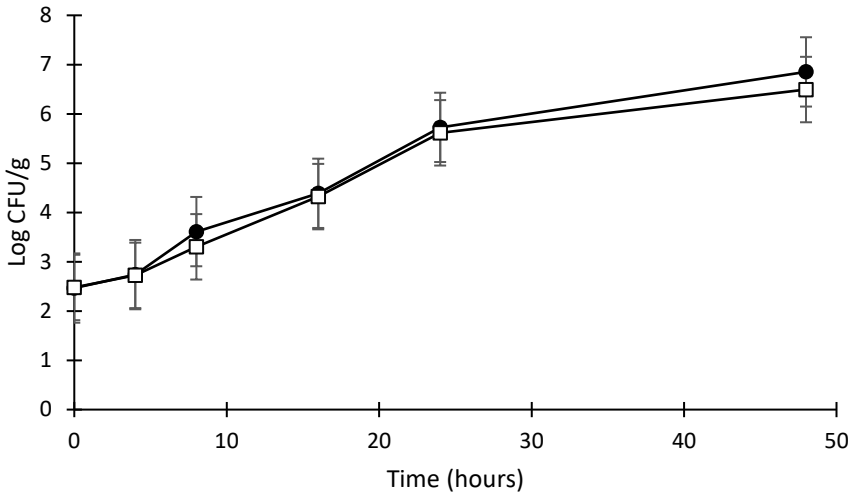

Figure 5. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on kale held at 22°C. Error bars represent standard error of growth means.

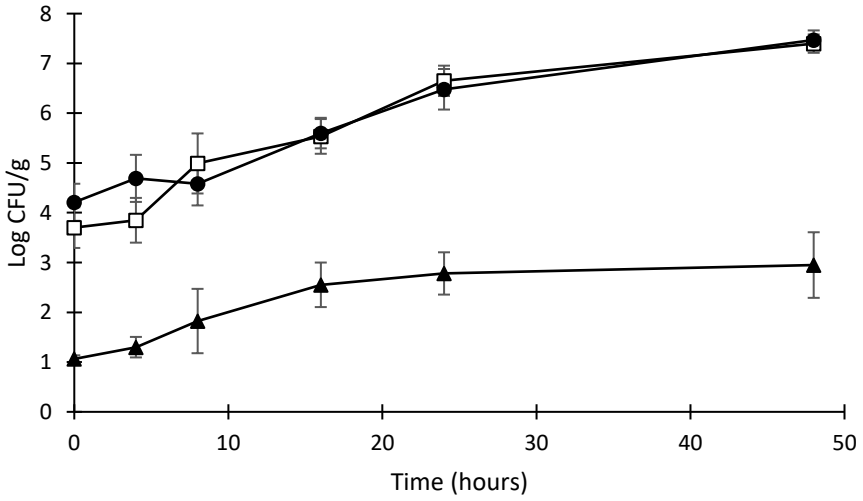

Figure 6. APC(□), PPC(●) and LAB(▲) growth on kale held at 22°C Error bars represent standard error of growth means.

Table 11. Average headspace, water activity and pH for kale at 22°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	20.5 ± 0.4	0.3 ± 0.3	0.994 ± 0.003	6.7 ± 0.2
4	14.5 ± 2.3	5.5 ± 2.2	0.993 ± 0.006	6.6 ± 0.3
8	11.9 ± 3.6	7.7 ± 3.2	0.993 ± 0.003	6.7 ± 0.2
16	8.39 ± 5.3	10.4 ± 4.3	0.992 ± 0.003	6.9 ± 0.3
24	5.74 ± 4.3	12.3 ± 3.7	0.993 ± 0.003	6.9 ± 0.2
48	3.47 ± 3.7	13.5 ± 2.7	0.991 ± 0.004	7.0 ± 0.2

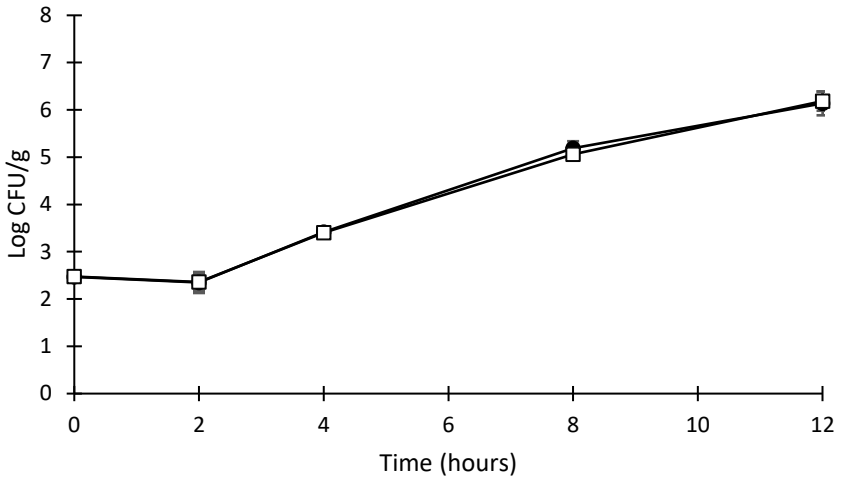

Figure 7. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on kale held at 35°C. Error bars represent standard error of growth means.

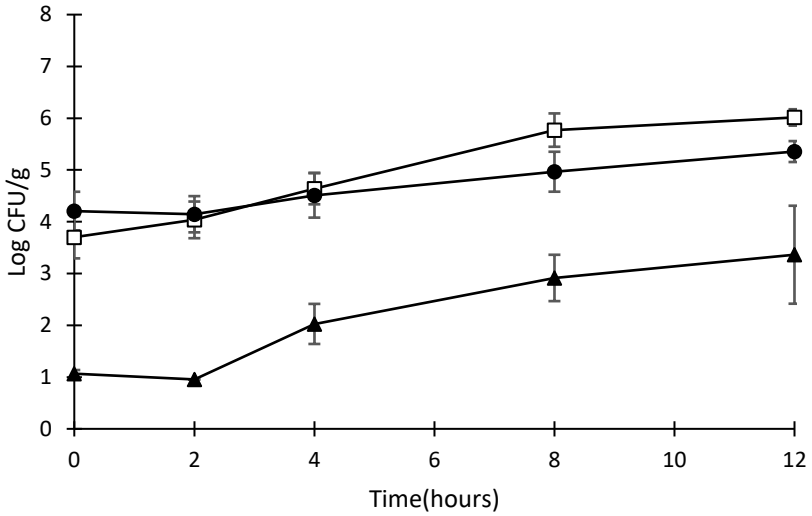

Figure 8. APC(□), PPC(●) and LAB(▲) growth on kale held at 35°C Error bars represent standard error of growth means.

Table 12. Average headspace, water activity and pH for kale at 35°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	20.5 ± 0.4	0.3 ± 0.3	0.994 ± 0.003	6.7 ± 0.2
2	13.4 ± 2.8	6.8 ± 2.7	0.994 ± 0.005	6.7 ± 0.2
4	8.8 ± 4.5	10.8 ± 4.2	0.988 ± 0.007	6.5 ± 0.5
8	4.7 ± 3.9	14.4 ± 4.4	0.993 ± 0.002	6.8 ± 0.2
12	2.4 ± 2.0	15.6 ± 2.7	0.993 ± 0.004	6.8 ± 0.2

BRUSSELS SPROUTS
L. monocytogenes and *L. innocua* Challenge Study Results

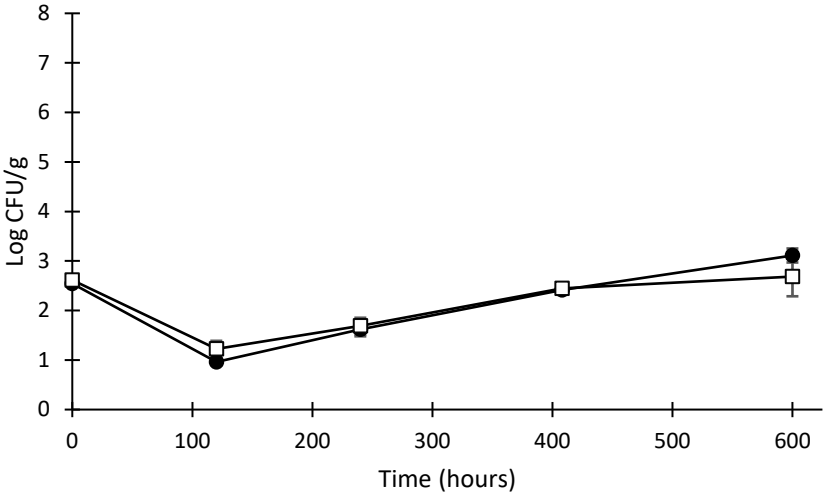

Figure 9. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on Brussels sprouts held at 4°C. Error bars represent standard error of growth means.

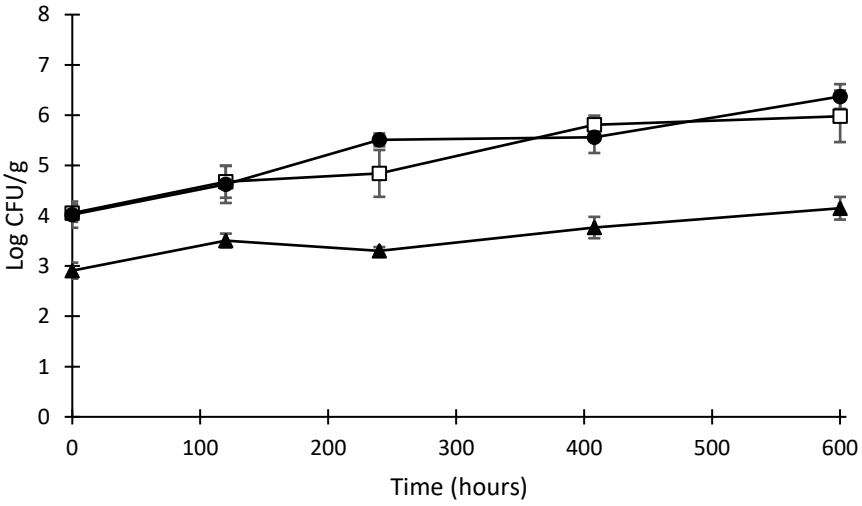

Figure 10. APC(□), PPC(●) and LAB(▲) growth on Brussels sprouts held at 4°C. Error bars represent standard error of growth means.

Table 13. Average headspace, water activity and pH for Brussels sprouts at 4°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	18.6 ± 0.4	2.5 ± 0.4	0.990 ± 0.002	6.7 ± 0.3
120	7.2 ± 1.8	15.0 ± 2.1	0.995 ± 0.002	6.7 ± 0.2
240	8.4 ± 4.5	13.7 ± 3.8	0.989 ± 0.003	6.5 ± 0.2
408	11.0 ± 4.3	10.6 ± 4.7	0.992 ± 0.004	6.7 ± 0.2
600	10.8 ± 2.6	11.1 ± 3.1	0.992 ± 0.005	6.2 ± 1.2

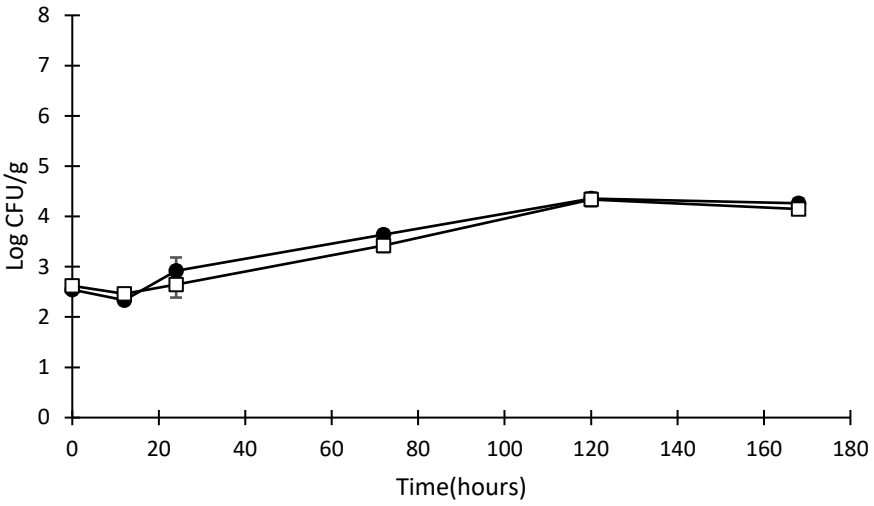

Figure 11. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on Brussels sprouts held at 12°C. Error bars represent standard error of growth means.

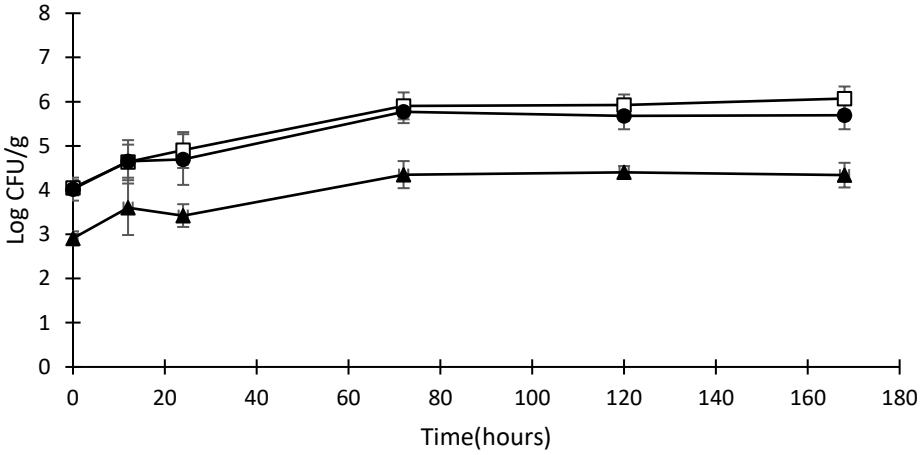

Figure 12. APC(□), PPC(●) and LAB(▲) growth on Brussels sprouts held at 12°C. Error bars represent standard error of growth means.

Table 14. Average headspace, water activity and pH for Brussels sprouts at 12°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	18.6 ± 0.4	2.5 ± 0.4	0.990 ± 0.002	6.7 ± 0.3
12	5.4 ± 1.8	15.2 ± 3.1	0.992 ± 0.002	6.7 ± 0.2
24	2.6 ± 1.1	18.1 ± 0.7	0.991 ± 0.004	6.8 ± 0.1
72	5.0 ± 2.5	16.7 ± 2.1	0.992 ± 0.001	6.9 ± 0.2
120	4.0 ± 1.0	17.1 ± 1.2	0.991 ± 0.004	6.9 ± 0.2
168	8.3 ± 1.1	13.0 ± 0.9	0.994 ± 0.003	6.9 ± 0.1

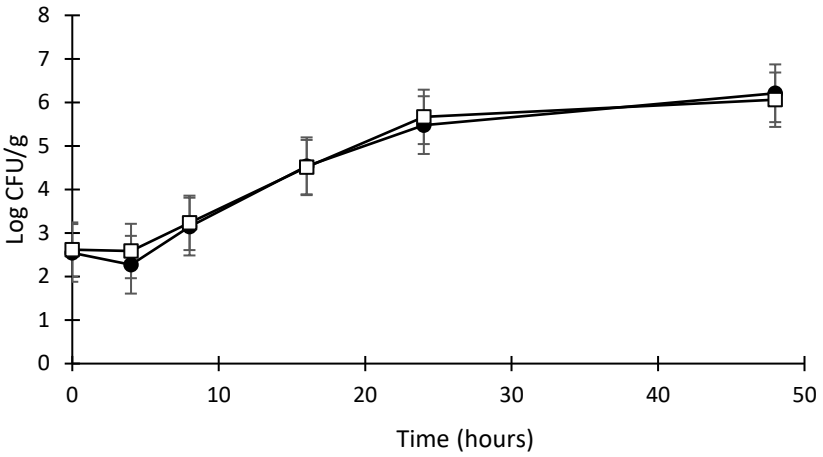

Figure 13. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on Brussels sprouts held at 22°C. Error bars represent standard error of growth means.

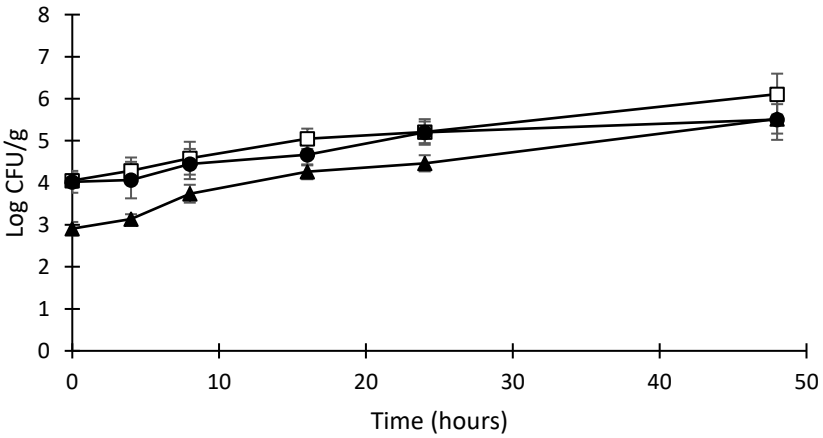

Figure 14. APC(□), PPC(●) and LAB(▲) growth on Brussels sprouts held at 22°C. Error bars represent standard error of growth means.

Table 15. Average headspace, water activity and pH for Brussels sprouts at 22°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	18.6 ± 0.4	2.47 ± 0.4	0.990 ± 0.002	6.7 ± 0.3
4	2.4 ± 2.1	17.3 ± 2.1	0.989 ± 0.003	6.7 ± 0.2
8	0.8 ± 0.9	21.9 ± 2.7	0.990 ± 0.006	6.9 ± 0.1
16	1.3 ± 1.8	23.4 ± 4.0	0.992 ± 0.002	6.8 ± 0.2
24	1.3 ± 1.1	21.9 ± 2.7	0.990 ± 0.002	6.9 ± 0.2
48	0.9 ± 0.3	21.2 ± 2.1	0.993 ± 0.003	7.1 ± 0.1

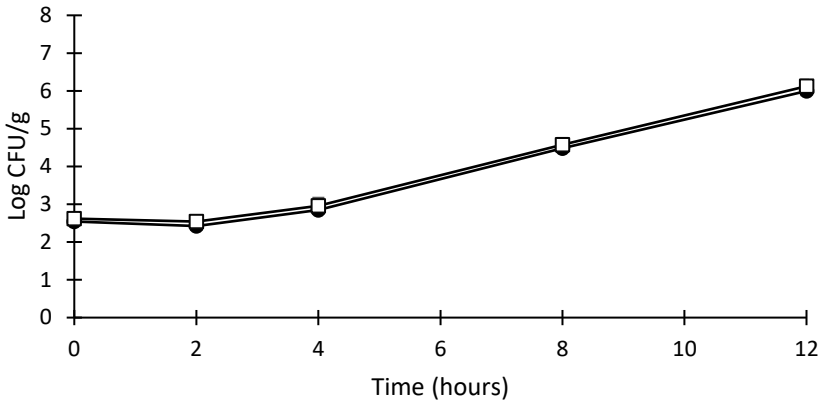

Figure 15. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on Brussels sprouts held at 35°C. Error bars represent standard error of growth means.

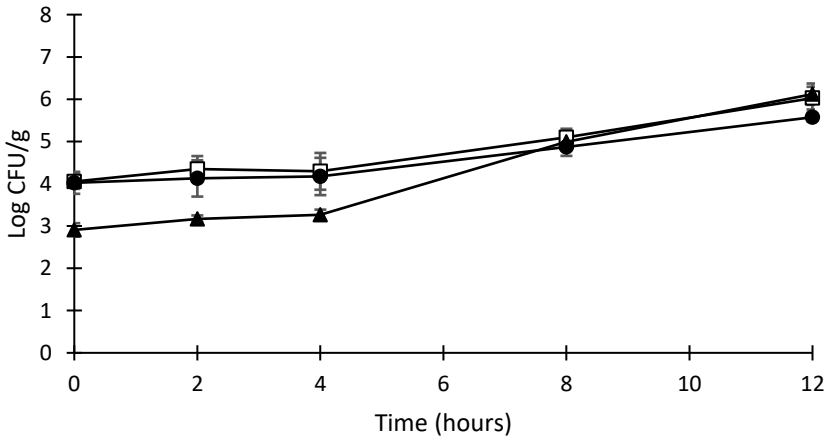

Figure 16 APC(□), PPC(●) and LAB(▲) growth on Brussels sprouts held at 35°C. Error bars represent standard error of growth means.

Table 16. Average headspace, water activity and pH for Brussels sprouts at 35°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	18.6 ± 0.4	2.5 ± 0.4	0.990 ±0.002	6.7 ± 0.3
2	5.9 ± 1.3	15.2 ± 1.0	0.994 ± 0.002	6.8 ± 0.2
4	0.9 ± 1.5	22.4 ± 11.0	0.990 ± 0.005	6.8 ± 0.1
8	0.8 ± 1.1	39.8 ± 7.1	0.991 ± 0.005	6.8 ± 0.1
12	0.3 ± 0.2	52.5 ± 5.8	0.992 ± 0.003	6.8 ± 0.1

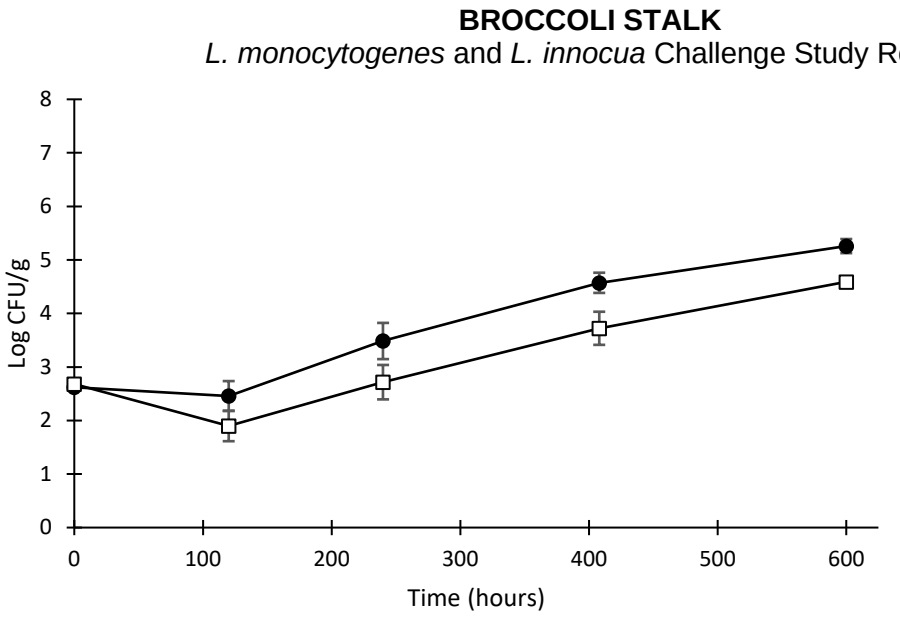

Figure 17. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on broccoli stalk held at 4°C. Error bars represent standard error of growth means.

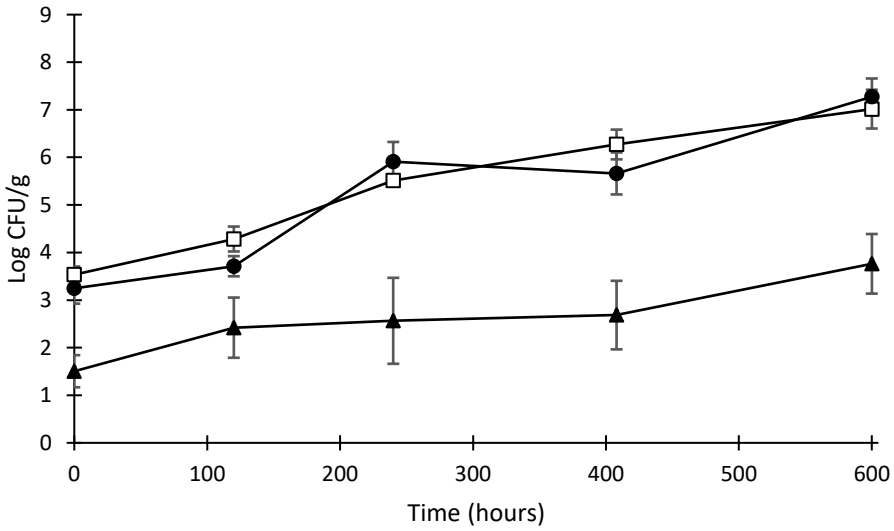

Figure 18. APC(□), PPC(●) and LAB(▲) growth on broccoli stalk held at 4°C. Error bars represent standard error of growth means.

Table 17. Average headspace, water activity and pH for broccoli at 4°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	19.6 ± 1.7	1.8 ± 1.7	0.996 ± 0.001	6.7 ± 0.2
120	8.1 ± 4.2	12.1 ± 2.9	0.996 ± 0.001	7.0 ± 0.1
240	11.1 ± 3.9	9.5 ± 3.2	0.996 ± 0.002	6.9 ± 0.2
408	10.6 ± 5.2	9.2 ± 3.3	0.995 ± 0.001	6.9 ± 0.2
600	13.2 ± 2.3	7.3 ± 2.0	0.995 ± 0.002	6.6 ± 0.4

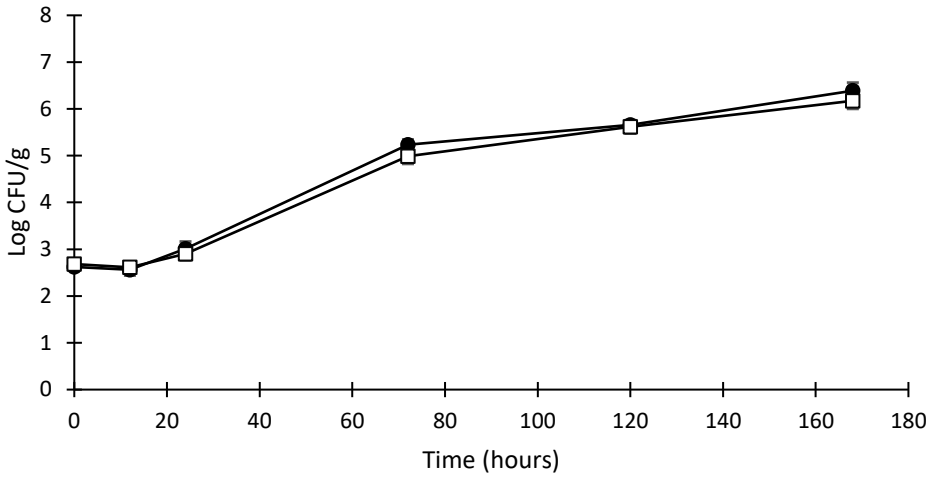

Figure 19. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on broccoli stalk held at 12°C. Error bars represent standard error of growth means.

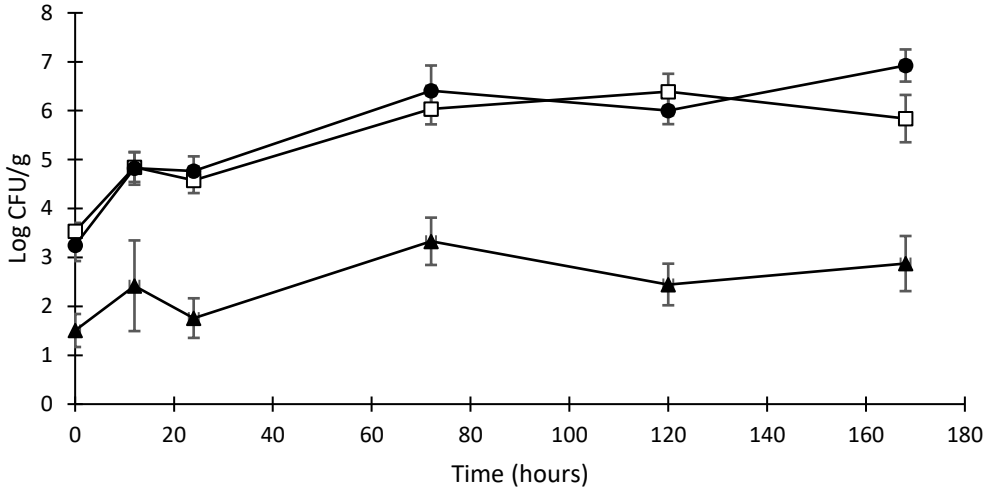

Figure 20. APC(□), PPC(●) and LAB(▲) growth on broccoli stalk held at 12°C. Error bars represent standard error of growth means.

Table 18. Average headspace, water activity and pH for broccoli at 12°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	19.6 ± 1.7	1.8 ± 1.7	0.996 ± 0.001	6.7 ± 0.2
12	4.2 ± 1.2	13.6 ± 0.7	0.996 ± 0.002	6.8 ± 0.2
24	3.6 ± 1.2	37.7 ± 35	0.997 ± 0.002	6.8 ± 0.3
72	4.8 ± 2.2	14.7 ± 1.4	0.995 ± 0.002	7.0 ± 0.4
120	7.1 ± 2.1	12.5 ± 1.2	0.995 ± 0.002	7.1 ± 0.1
168	8.6 ± 1.5	11.3 ± 1.0	0.993 ± 0.002	7.0 ± 0.2

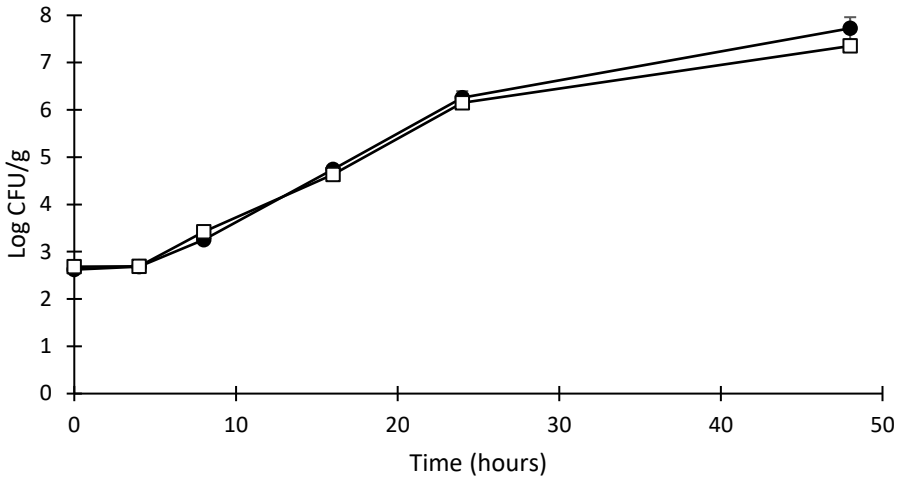

Figure 21. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on broccoli stalk held at 22°C. Error bars represent standard error of growth means.

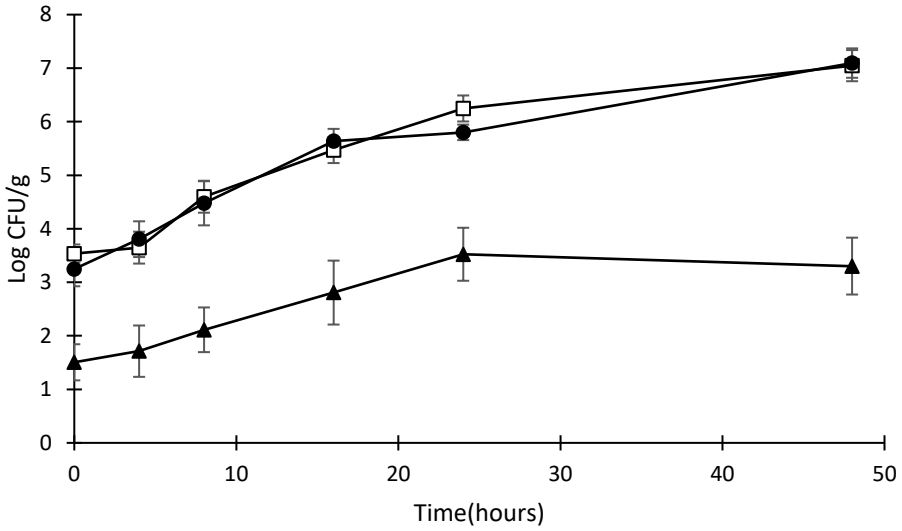

Figure 22. APC(□), PPC(●) and LAB(▲) growth on broccoli stalk held at 22°C. Error bars represent standard error of growth means.

Table 19. Average headspace, water activity and pH for broccoli at 22°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	19.6 ± 1.7	1.8 ± 1.7	0.996 ± 0.001	6.7 ± 0.2
4	4.6 ± 1.3	14.7 ± 1.0	0.991 ± 0.010	6.8 ± 0.4
8	1.0 ± 0.6	19.6 ± 1.2	0.997 ± 0.002	6.8 ± 0.3
16	0.5 ± 0.1	21.7 ± 1.6	0.996 ± 0.002	6.8 ± 0.3
24	1.0 ± 0.6	19.8 ± 0.6	0.995 ± 0.001	6.8 ± 0.3
48	1.3 ± 0.8	19.3 ± 1.4	0.995 ± 0.001	7.1 ± 0.4

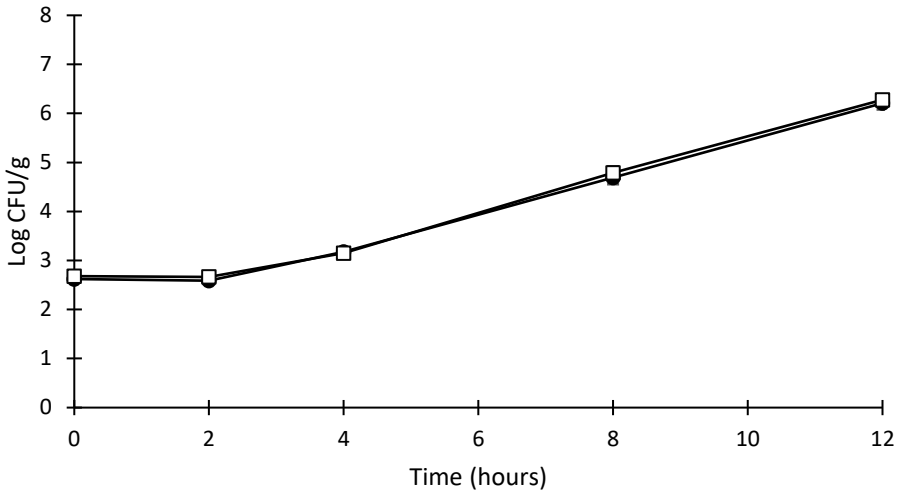

Figure 23. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on broccoli stalk held at 35°C. Error bars represent standard error of growth means.

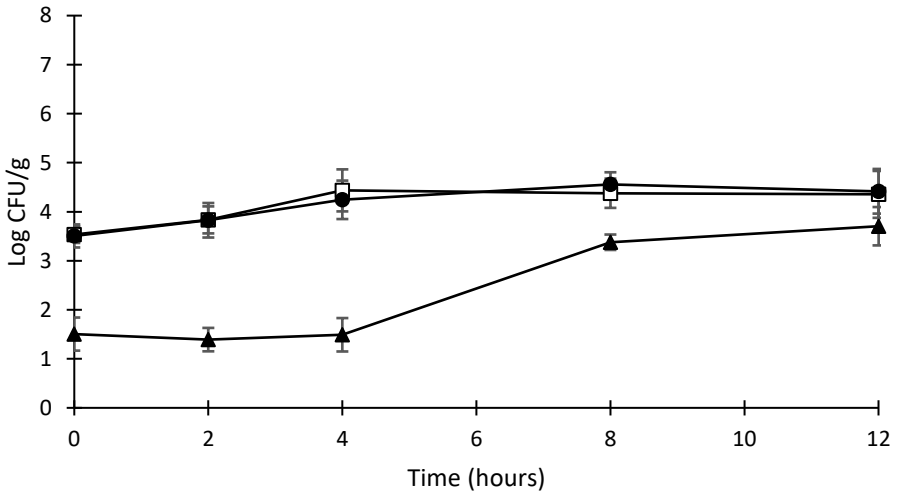

Figure 24. APC(□), PPC(●) and LAB(▲) growth on broccoli stalk held at 35°C. Error bars represent standard error of growth means.

Table 20. Average headspace, water activity and pH for broccoli at 35°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	19.6 ± 1.7	1.8 ± 1.7	0.996 ± 0.001	6.7 ± 0.2
2	6.9 ± 1.4	13.9 ± 1.6	0.994 ± 0.001	6.7 ± 0.2
4	0.6 ± 0.3	21.9 ± 0.5	0.995 ± 0.003	6.8 ± 0.4
8	1.2 ± 0.5	36.2 ± 2.3	0.996 ± 0.003	6.8 ± 0.3
12	1.5 ± 0.9	46.9 ± 4.7	0.994 ± 0.001	6.9 ± 0.2

BEET GREENS
L. monocytogenes and *L. innocua* Challenge Study Results

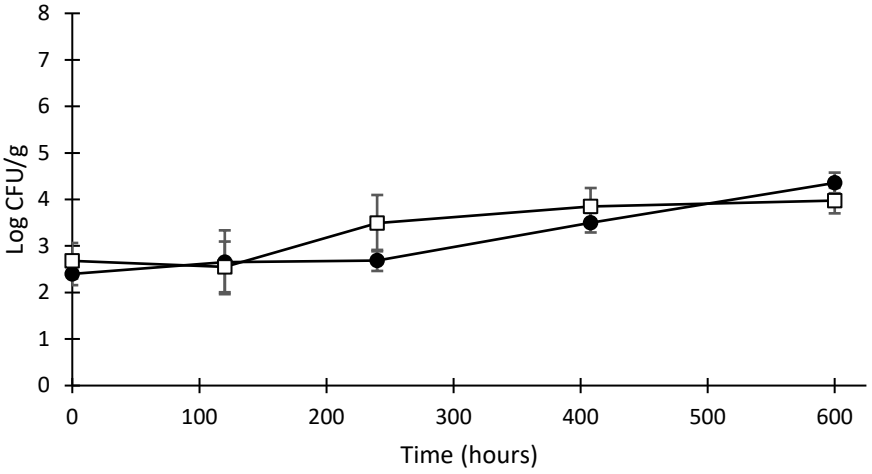

Figure 25. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on beet greens held at 4°C. Error bars represent standard error of growth means.

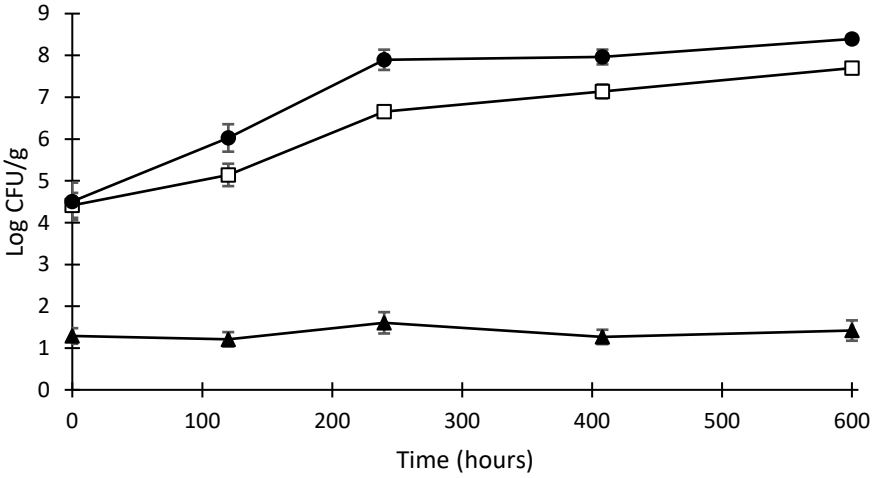

Figure 26. APC(□), PPC(●) and LAB(▲) growth on beet greens held at 4°C. Error bars represent standard error of growth means.

Table 21. Average headspace, water activity and pH for beet greens at 4°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	19.7 ± 0.4	1.0 ± 0.4	0.989 ± 0.005	6.6 ± 0.7
120	17.1 ± 0.9	3.0 ± 0.7	0.992 ± 0.004	7.1 ± 0.5
240	15.8 ± 1.7	3.7 ± 0.7	0.988 ± 0.002	7.3 ± 0.4
408	15.8 ± 1.0	3.7 ± 0.5	0.987 ± 0.003	7.6 ± 0.2
600	16.0 ± 0.8	3.6 ± 0.6	0.989 ± 0.003	8.3 ± 0.2

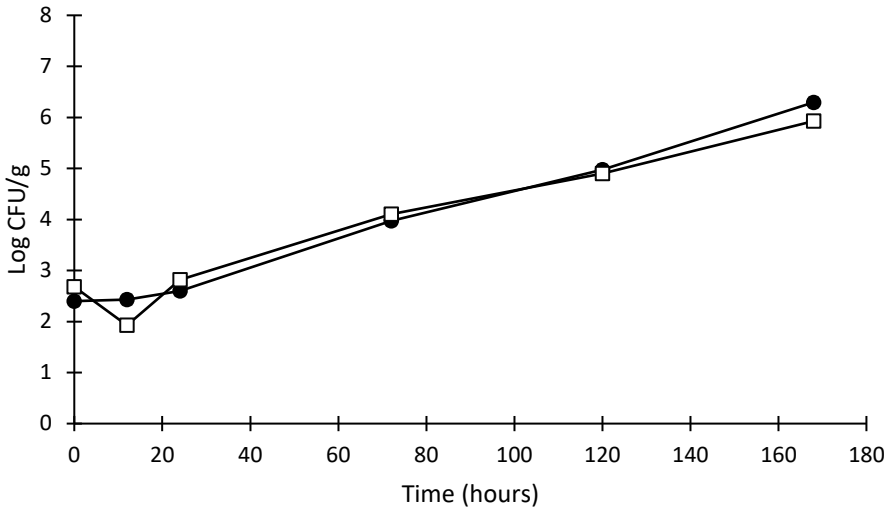

Figure 27. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on beet greens held at 12°C. Error bars represent standard error of growth means.

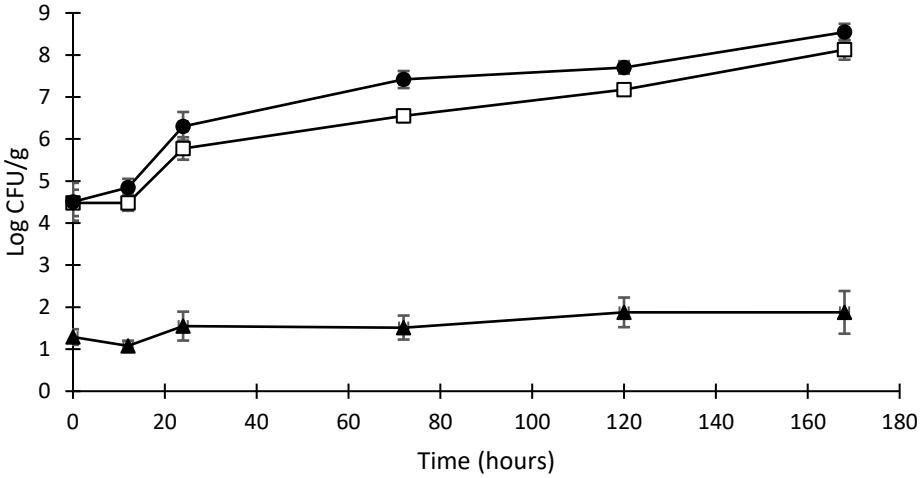

Figure 28. APC(□), PPC(●) and LAB(▲) growth on beet greens held at 12°C. Error bars represent standard error of growth means.

Table 22. Average headspace, water activity and pH for beet greens at 12°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	19.7 ± 0.4	1.0 ± 0.4	0.989 ± 0.005	6.6 ± 0.7
12	16.2 ± 2.0	3.7 ± 1.5	0.987 ± 0.007	6.5 ± 0.3
24	11.7 ± 5.4	7.2 ± 5.8	0.988 ± 0.007	6.9 ± 0.4
72	13.3 ± 2.0	5.8 ± 1.2	0.990 ± 0.004	7.2 ± 0.4
120	11.5 ± 1.7	6.8 ± 1.2	0.990 ± 0.003	7.3 ± 0.2
168	10.0 ± 2.9	7.1 ± 2.6	0.988 ± 0.005	7.8 ± 0.2

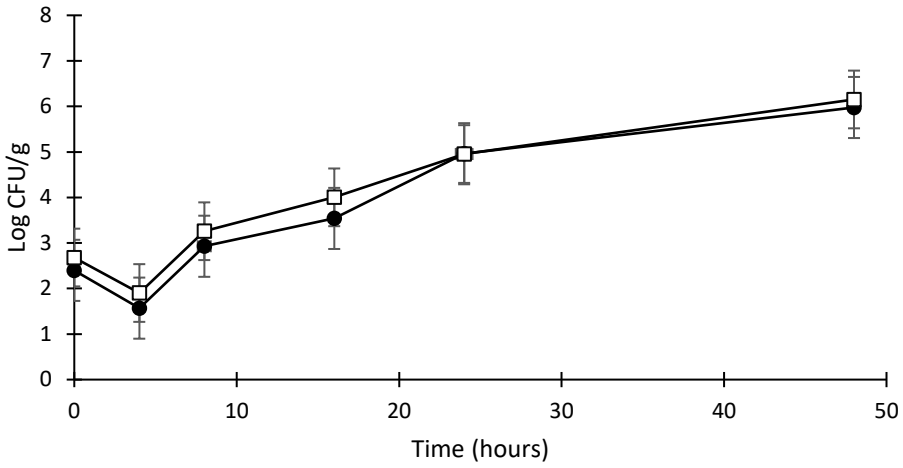

Figure 29. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on beet greens held at 22°C. Error bars represent standard error of growth means.

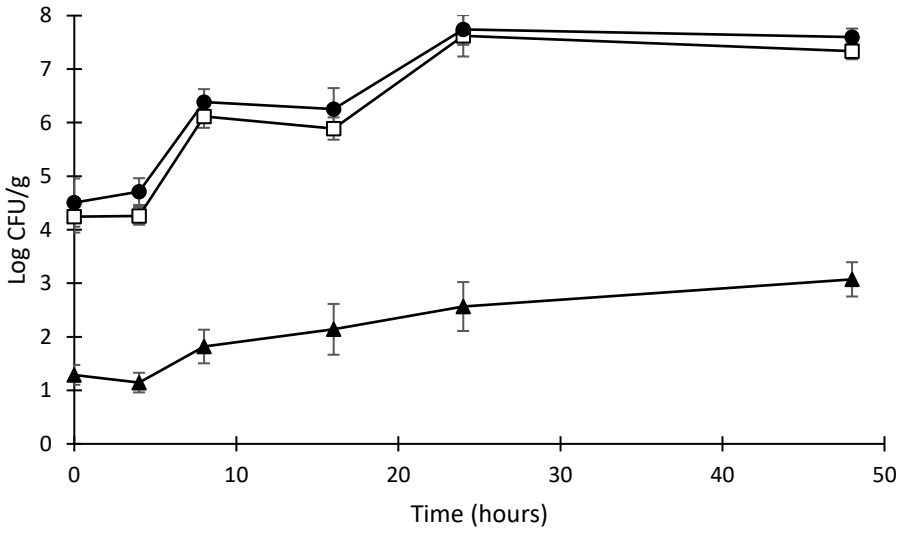

Figure 30. APC(□), PPC(●) and LAB(▲) growth on beet greens held at 22°C. Error bars represent standard error of growth means.

Table 23. Average headspace, water activity and pH for beet greens at 22°C

Time(hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	19.7 ± 0.4	1.0 ± 0.4	0.989 ± 0.005	6.6 ± 0.7
4	15.9 ± 1.2	3.8 ± 1.1	0.987 ± 0.005	6.5 ± 0.3
8	13.4 ± 1.4	5.8 ± 1.5	0.989 ± 0.004	6.8 ± 0.6
16	10.1 ± 1.5	8.6 ± 0.9	0.987 ± 0.004	6.9 ± 0.4
24	9.6 ± 1.5	8.1 ± 2.4	0.986 ± 0.008	7.0 ± 0.2
48	4.4 ± 2.9	11.2 ± 0.8	0.989 ± 0.004	7.3 ± 0.3

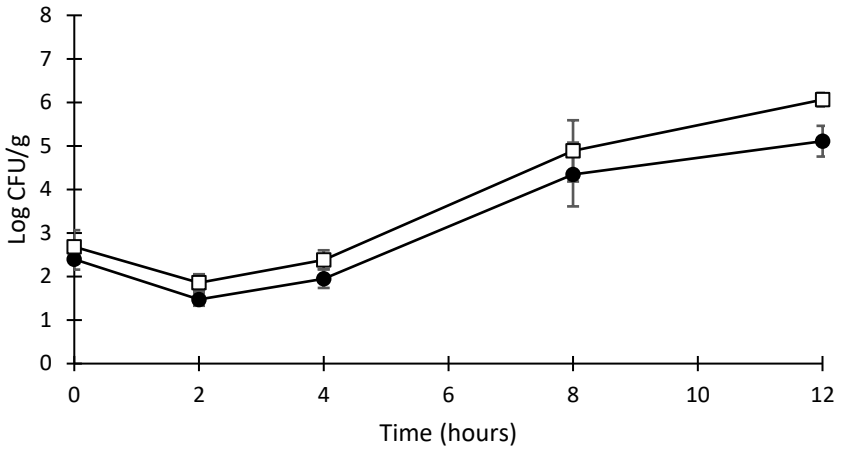

Figure 31. *Listeria monocytogenes* (●) and *Listeria innocua* (□) growth on beet greens held at 35°C. Error bars represent standard error of growth means.

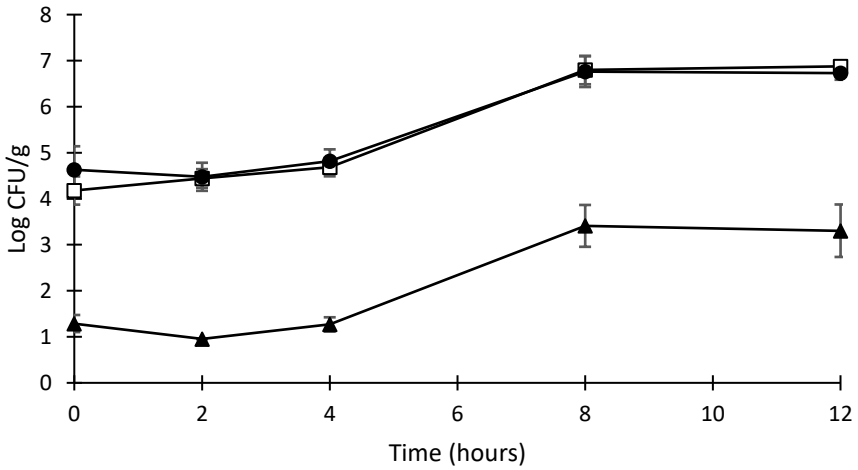

Figure 32. APC(□), PPC(●) and LAB(▲) growth on beet greens held at 35°C. Error bars represent standard error of growth means.

Table 24. Average headspace, water activity and pH for beet greens at 35°C

Time (hours)	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	19.7 ± 0.4	1.0 ± 0.4	0.989 ± 0.005	6.6 ± 0.7
2	16.2 ± 1.4	3.7 ± 1.1	0.993 ± 0.003	6.7 ± 0.4
4	12.2 ± 2.1	6.9 ± 2.1	0.990 ± 0.004	6.8 ± 0.4
8	7.9 ± 2.7	9.8 ± 2.4	0.991 ± 0.003	7.1 ± 0.3
12	4.2 ± 2.7	12.4 ± 2.3	0.984 ± 0.010	7.0 ± 0.2

***Listeria innocua* Simulated Storage and Distribution Study**

Table 25. Physical abuse simulation stresses in sequential order

Test	Conditions
Atmospheric Conditioning	4°F, 90% RH, 12–18 hours
Shock Testing	12" drop(x3), 30" drop(x3)
Compression Testing[1]	12 lb/ft3 load on top of case in 108" trailer, Test force = 0.007 x (108-H) x L x W x 5
Vibration Testing[2]	Grms = 0.54, table stroke = 1.777 in peak-peak, 30 minutes, triple stacks(x2): Face 3 of case on bottom (x3) + face 3, 4, and 6 on bottom
Shock Testing[3]	18" drops on face 3. Following drops for each of 6 packages from 24": 3, 3-4-5, 3-6, 2-3-5, 2-3 and 2-5

[1]Table 1: 0.007 lb/in3 = average density of freight, 5 = a compensating factor to account for effects not tested, such as stacking pattern, long-duration loading, etc.
[2]The root mean square acceleration (Grms) is the square root of the area under the acceleration spectral density curve in the frequency domain.
[3]Sequence of numbers are the sides or corners of a commercial case to receive initial impact of the drop.

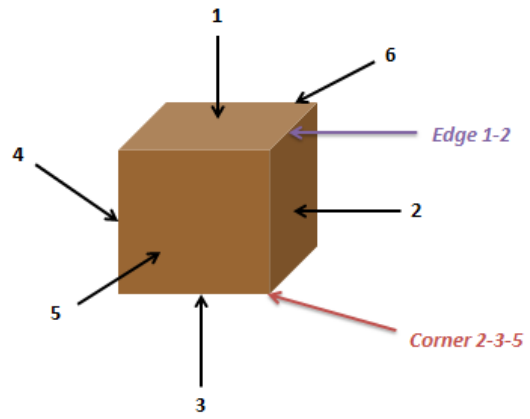

Figure 33. Face(black), edge(purple) and corner(red) identification of a typical product case.

Table 26. Case orientation for final 24" shock

Case Number	Drop Orientation*
1	Face 3
2	Corner, 3-4-5
3	Edge, 3-6
4	Corner, 2-3-5
5	Edge, 2-3
6	Edge, 2-5

*Numbers indicate face intersections (Figure 33)

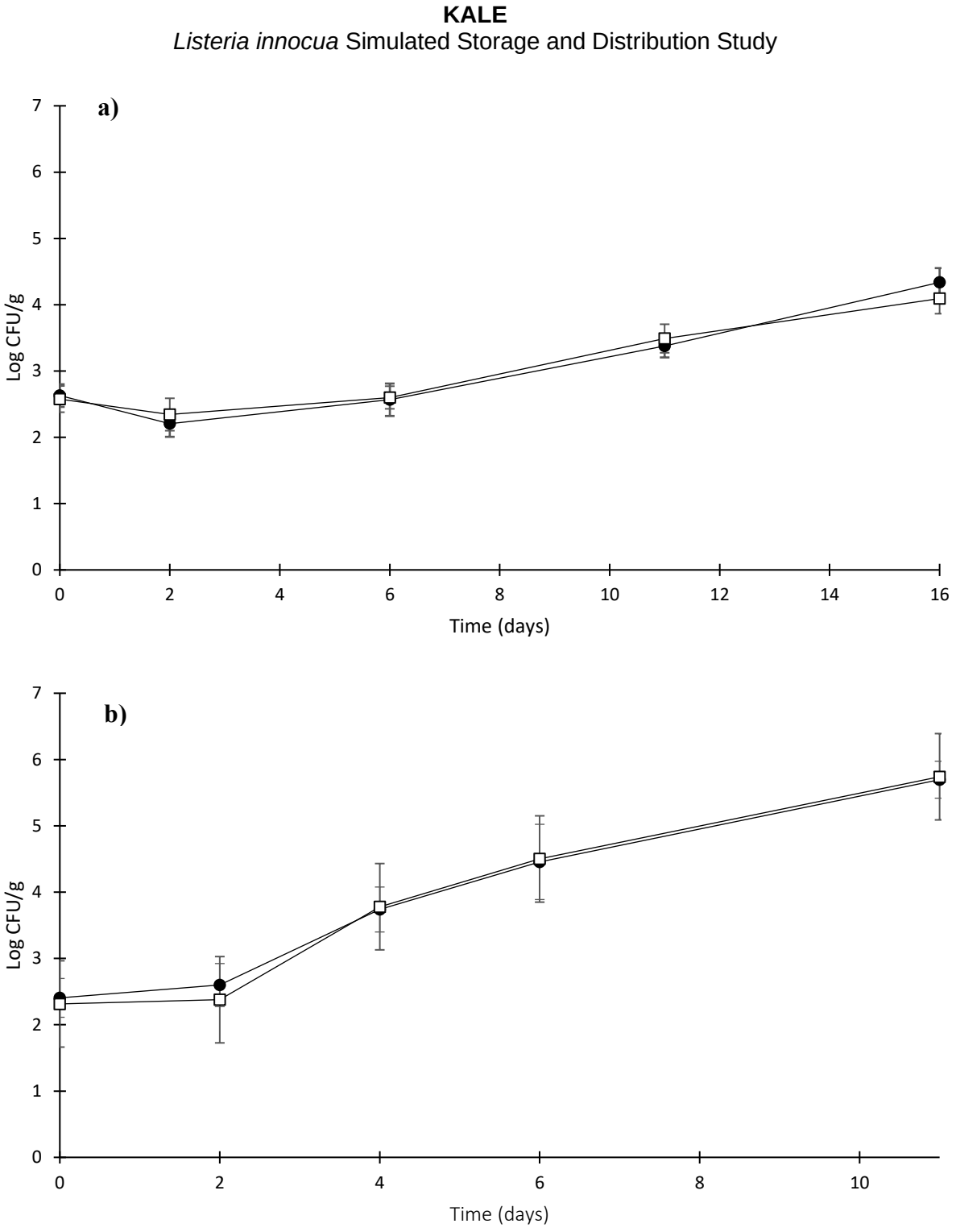

Figure 34. *L. innocua* growth in treated (□) and nontreated (●) kale when stored at 4°C (a) and 8°C (b). Error bars represent the standard error of *L. innocua* growth means.

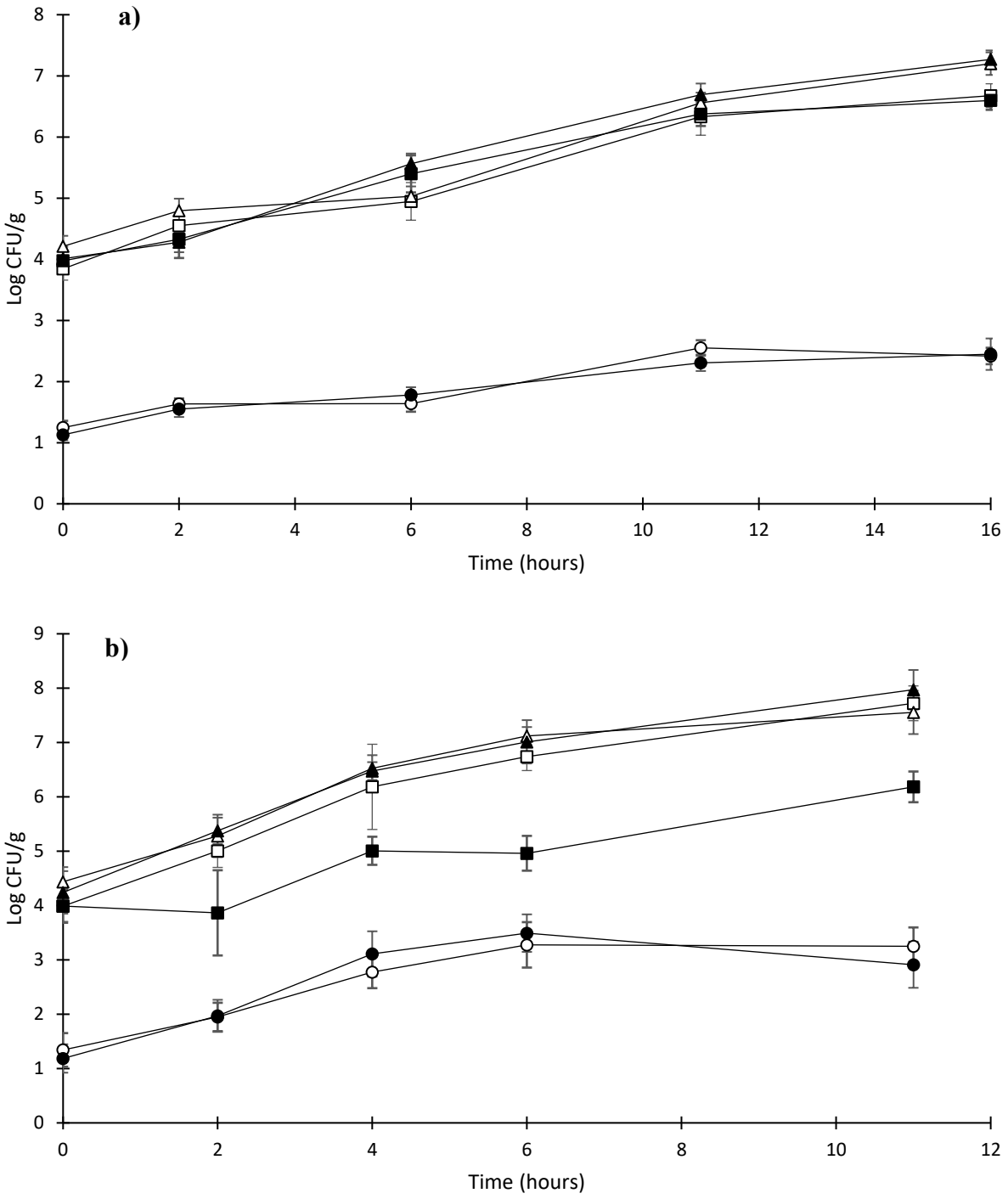

Figure 35. Growth of APC treated (■), nontreated (□), PPC treated (▲), nontreated (△), and LAB treated (●) and nontreated (○) in kale by temperatures 4°C (a) and 8°C (b). Error bars represent the standard error of growth means.

Table 27. Mean headspace, water activity and pH values for kale by time and treatment at 4°C

Time (days)	Simulated Distribution	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	N	13.2 ± 1.6	6.2 ± 1.5	0.993 ± 0.003	6.4 ± 0.1
2	N	10.5 ± 2.3	8.5 ± 1.5	0.993 ± 0.006	6.4 ± 0.1
6	N	11.4 ± 4.4	7.4 ± 3.8	0.994 ± 0.003	6.5 ± 0.1
11	N	10.1 ± 2.9	8.4 ± 2.4	0.995 ± 0.004	6.5 ± 0.2
16	N	8.1 ± 3.8	9.3 ± 3.6	0.995 ± 0.003	6.6 ± 0.2
0	Y	12.9 ± 1.5	6.3 ± 1.3	0.993 ± 0.002	6.4 ± 0.1
2	Y	12.3 ± 3.0	7.0 ± 2.3	0.991 ± 0.005	6.4 ± 0.1
6	Y	13.4 ± 5.6	6.7 ± 4.0	0.994 ± 0.002	6.5 ± 0.3
11	Y	11.5 ± 3.3	7.2 ± 2.4	0.995 ± 0.003	6.6 ± 0.2
16	Y	11.8 ± 1.8	7.4 ± 2.8	0.995 ± 0.003	6.6 ± 0.2

Table 28. Mean headspace, water activity and pH values for kale by time and treatment at 8°C

Time (days)	Simulated Distribution	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	N	13.2 ± 1.6	6.2 ± 1.5	0.993 ± 0.002	6.4 ± 0.1
2	N	9.9 ± 4.0	9.2 ± 3.7	0.992 ± 0.004	6.3 ± 0.1
4	N	8.1 ± 5.5	9.5 ± 3.5	0.997 ± 0.002	6.6 ± 0.2
6	N	8.3 ± 4.8	9.5 ± 3.3	0.994 ± 0.001	6.7 ± 0.1
11	N	6.1 ± 5.7	10.3 ± 4.0	0.992 ± 0.002	6.7 ± 0.3
0	Y	12.9 ± 1.5	6.3 ± 1.3	0.993 ± 0.002	6.4 ± 0.1
2	Y	8.2 ± 1.7	10.6 ± 1.7	0.991 ± 0.005	6.3 ± 0.2
4	Y	8.4 ± 3.6	9.2 ± 2.6	0.996 ± 0.002	6.4 ±0.1
6	Y	9.7 ± 3.0	8.6 ± 1.9	0.994 ± 0.002	6.6 ± 0.2
11	Y	8.5 ± 5.6	8.8 ± 4.7	0.994 ± 0.003	6.7 ± 0.3

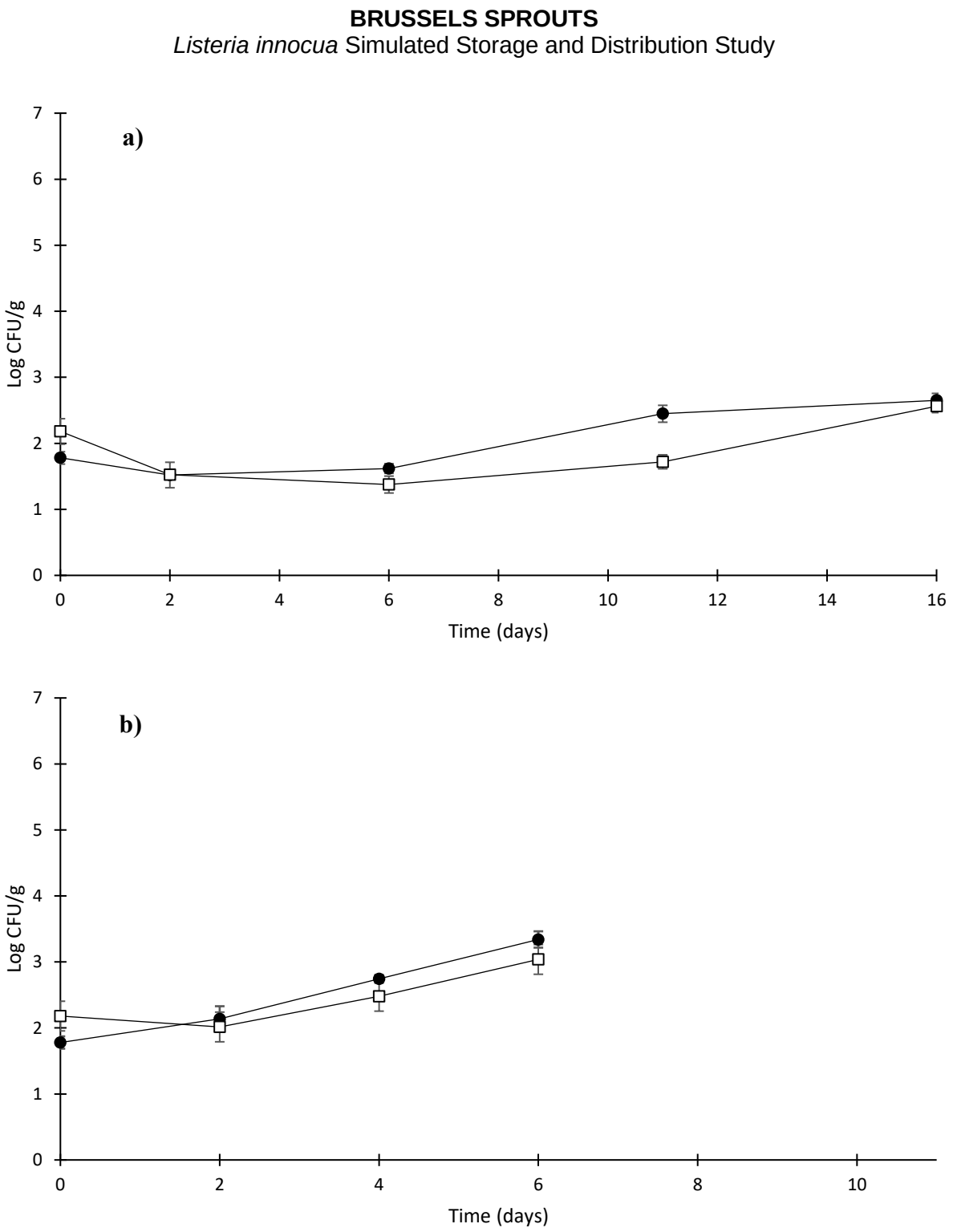

Figure 36. *L. innocua* growth in treated (□) and nontreated (●) Brussels sprouts when stored at 4°C (a) and 8°C (b). Error bars represent the standard error of *L. innocua* growth means.

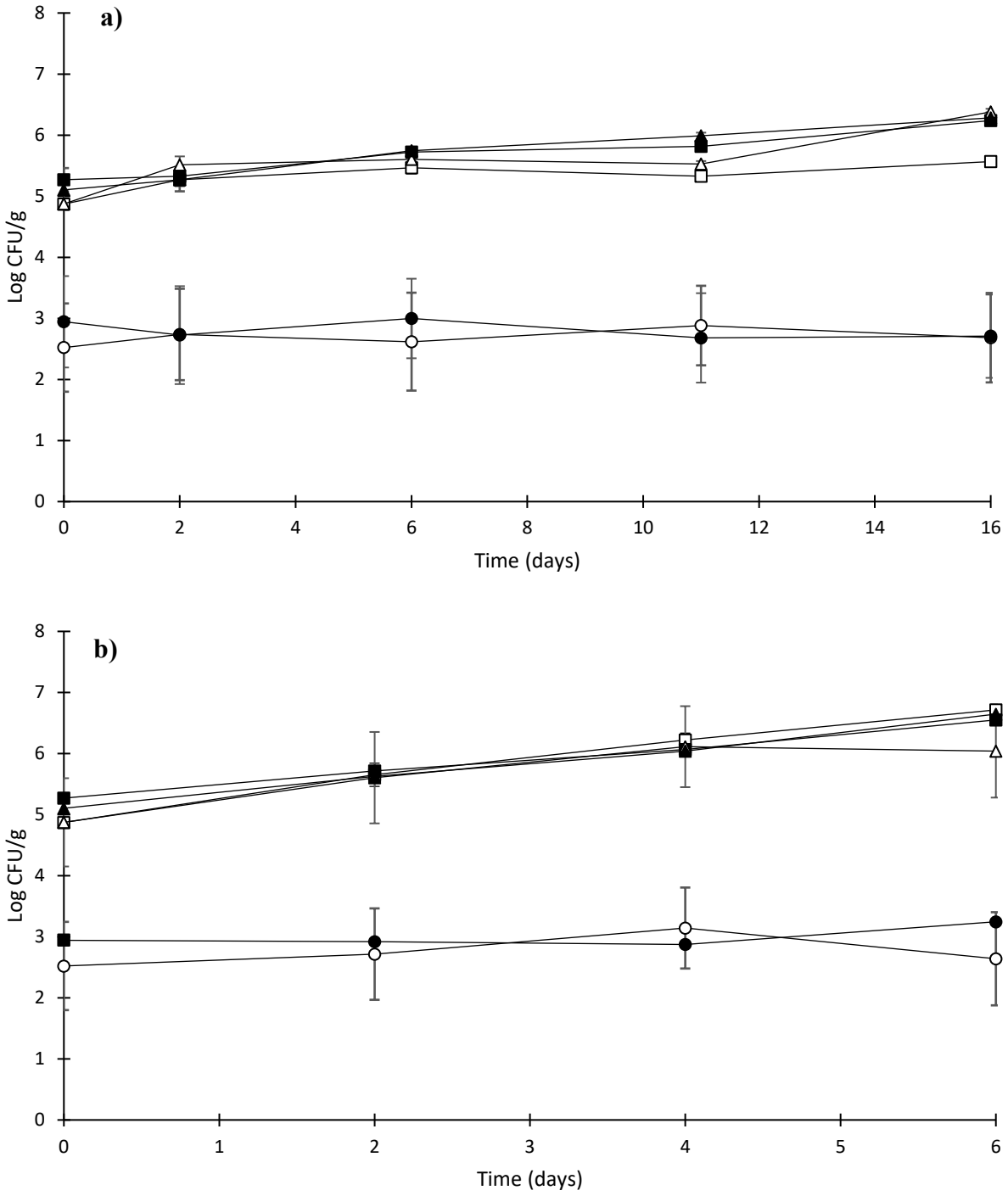

Figure 37. Growth of APC treated (■), nontreated (□), PPC treated (▲), nontreated (△), and LAB treated (●) and nontreated (○) in Brussels sprouts by temperatures 4°C (a) and 8°C(b). Error bars represent the standard error of growth means.

Table 29. Mean headspace, water activity and pH values for Brussels sprouts by time and treatment at 4°C

Time(days)	Simulated Distribution	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	N	5.2 ± 1.2	12.1 ± 1.3	0.993 ± 0.004	6.5 ± 0.2
2	N	1.4 ± 0.6	16.7 ± 0.6	0.991 ± 0.003	6.6 ± 0.4
6	N	1.0 ± 0.5	18.4 ± 1.0	0.991 ± 0.003	6.5 ± 0.2
11	N	1.4 ± 0.4	17.5 ± 0.8	0.992 ± 0.003	6.7 ± 0.2
16	N	3.9 ± 5.1	15.9 ± 4.3	0.992 ± 0.001	6.7 ± 0.1
0	Y	5.3 ± 2.1	12.0 ± 1.9	0.992 ± 0.003	6.6 ± 0.3
2	Y	1.5 ± 0.7	16.8 ± 1.0	0.993 ± 0.003	6.5 ± 0.2
6	Y	1.3 ± 0.4	18.5 ± 1.0	0.993 ± 0.002	6.5 ± 0.2
11	Y	1.1 ± 0.8	20.4 ± 3.6	0.993 ± 0.003	6.6 ± 0.1
16	Y	2.1 ± 2.2	17.2 ± 2.5	0.991 ± 0.003	6.6 ± 0.2

Table 30. Mean headspace, water activity and pH values for Brussels sprouts by time and treatment at 8°C

Time(days)	Simulated Distribution	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	N	5.2 ± 1.2	12.1 ± 1.3	0.993 ± 0.004	6.5 ± 0.2
2	N	0.5 ± 0.1	18.9 ± 0.5	0.992 ± 0.003	6.4 ± 0.2
4	N	0.8 ± 0.3	18.3 ± 1.0	0.992 ± 0.003	6.4 ± 0.1
6	N	1.3 ± 0.7	17.5 ± 0.5	0.989 ± 0.003	6.5 ± 0.2
0	Y	5.3 ± 2.1	12.0 ± 1.9	0.992 ± 0.003	6.6 ± 0.3
2	Y	5.7 ± 8.2	14.5 ± 7.6	0.991 ± 0.003	6.6 ± 0.4
4	Y	4.2 ± 7.0	15.1 ± 7.0	0.994 ± 0.001	6.7 ± 0.3
6	Y	3.8 ± 4.9	15.7 ± 3.6	0.992 ± 0.002	6.6 ± 0.2

BROCCOLI STALK
Listeria innocua Simulated Storage and Distribution Study

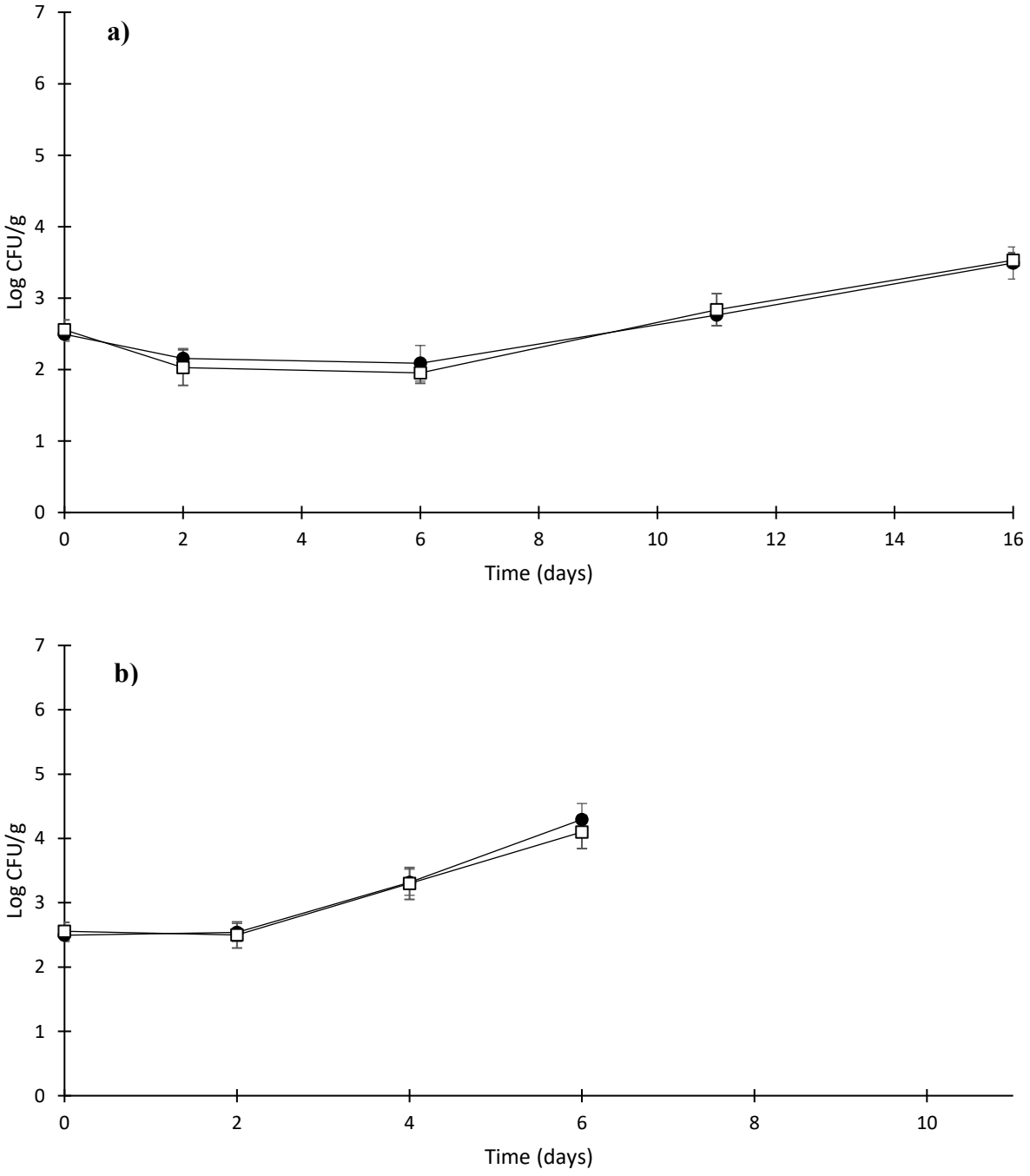

Figure 38. *L. innocua* growth in treated (□) and nontreated (●) broccoli stalk when stored at 4°C (a) and 8°C (b). Error bars represent the standard error of *L. innocua* growth means.

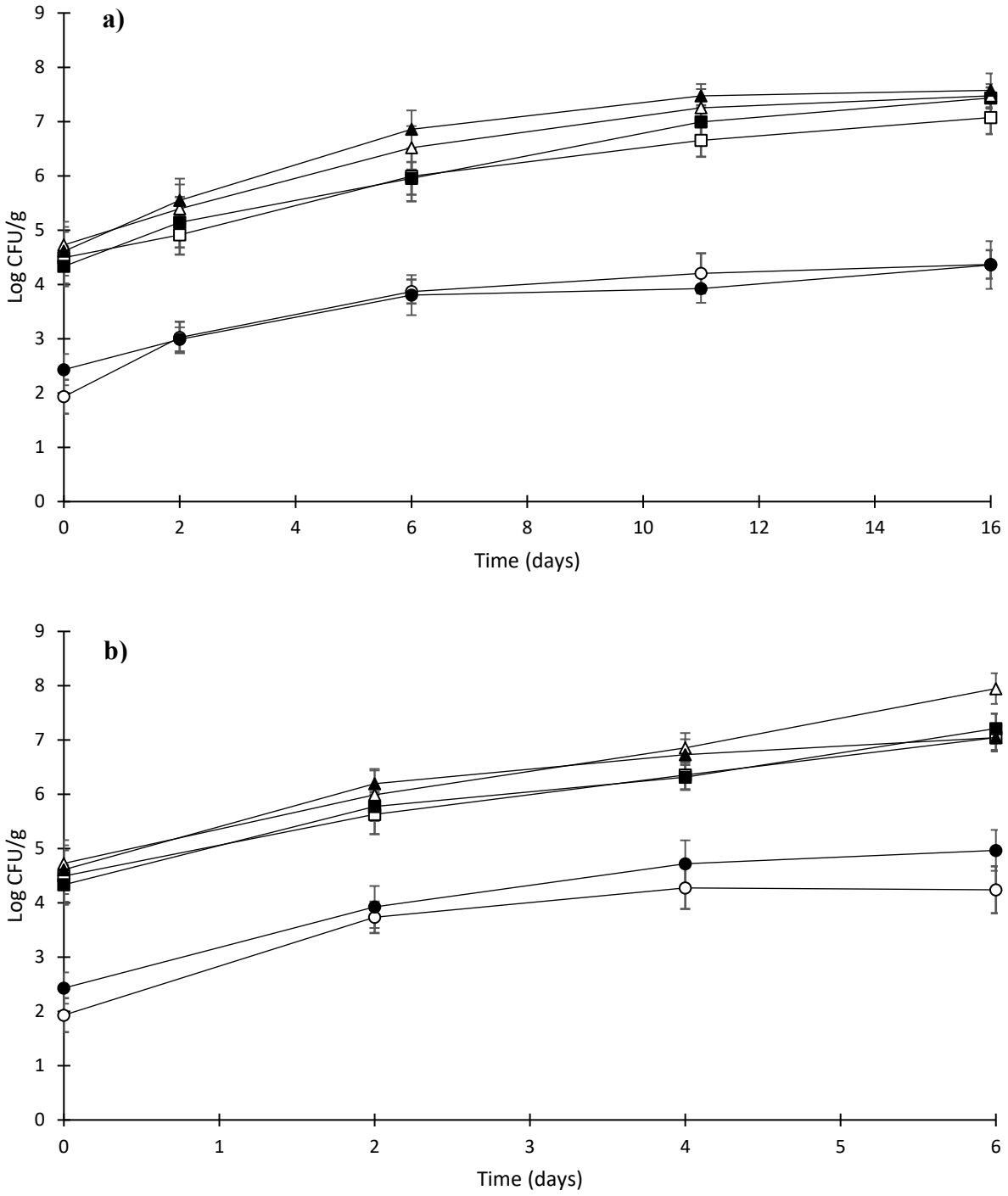

Figure 39. Growth of APC treated (■), nontreated (□), PPC treated (▲), nontreated (△), and LAB treated (●) and nontreated (○) in broccoli stalk by temperatures 4°C (a) and 8°C(b). Error bars represent the standard error of growth means.

Table 31. Mean headspace, water activity and pH values for broccoli stalk by time and treatment at 4°C

Time (days)	Simulated Distribution	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	N	3.0 ± 2.6	15.4 ± 4.0	0.997 ± 0.002	6.7 ± 0.1
2	N	1.5 ± 0.7	18.0 ± 3.0	0.995 ± 0.002	6.6 ± 0.1
6	N	1.6 ± 1.5	19.8 ± 3.5	0.995 ± 0.002	6.8 ± 0.3
11	N	1.9 ± 1.2	18.5 ± 3.1	0.995 ± 0.002	6.7 ± 0.2
16	N	4.1 ± 2.4	17.2 ± 3.6	0.996 ± 0.002	7 ± 0.2
0	Y	3.0 ± 2.5	15.8 ± 4.3	0.999 ± 0.004	6.7 ± 0.2
2	Y	2.0 ± 1.4	17.7 ± 3.4	0.997 ± 0.003	6.5 ± 0.1
6	Y	1.8 ± 1.3	19.6 ± 3.7	0.996 ± 0.001	6.7 ± 0.3
11	Y	5.8 ± 6.0	14.9 ± 6.3	0.996 ± 0.002	6.8 ± 0.2
16	Y	4.9 ± 3.9	16.5 ± 4.9	0.996 ± 0.003	6.9 ± 0.3

Table 32. Mean headspace, water activity and pH values for broccoli stalk by time and treatment at 8°C

Time (days)	Simulated Distribution	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	N	3.0 ± 2.6	15.4 ± 4.0	0.997 ± 0.002	6.7 ± 0.1
2	N	1.2 ± 1.6	21.5 ± 5.4	0.995 ± 0.002	6.7 ± 0.2
4	N	1.1 ± 0.7	20.3 ± 4.2	0.996 ± 0.002	6.9 ± 0.1
6	N	1.4 ± 0.6	19.5 ± 3.7	0.995 ± 0.001	6.9 ± 0.1
0	Y	3.0 ± 2.5	15.8 ± 4.3	0.999 ± 0.004	6.7 ± 0.2
2	Y	0.5 ± 0.2	22.1 ± 5.2	0.996 ± 0.001	6.7 ± 0.2
4	Y	1.9 ± 2.5	20.8 ± 6.4	0.996 ± 0.001	6.7 ± 0.3
6	Y	1.3 ± 0.5	20.0 ± 4.3	0.994 ± 0.003	6.8 ± 0.2

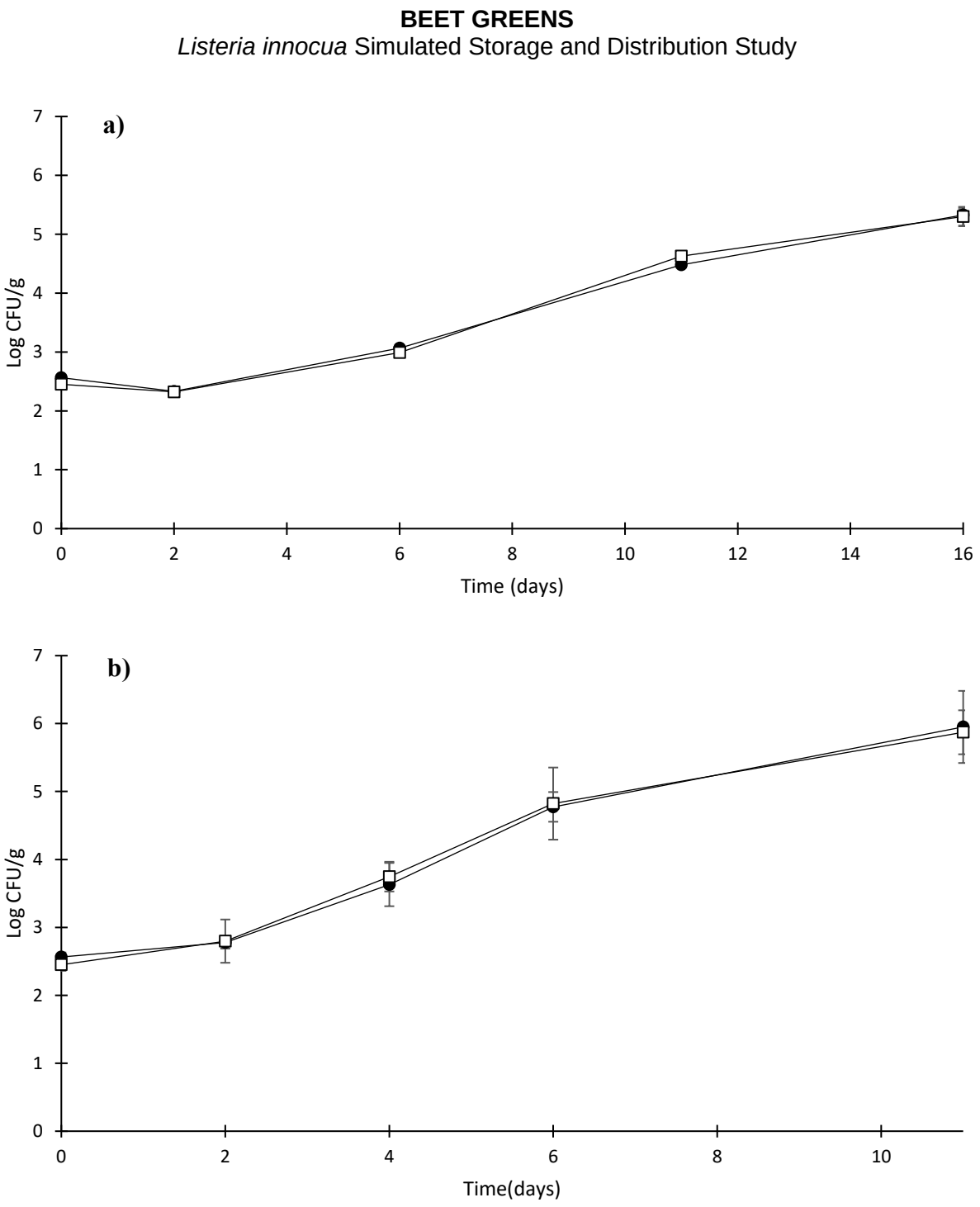

Figure 40. *L. innocua* growth in treated (□) and nontreated (●) beet greens when stored at 4°C (a) and 8°C (b). Error bars represent the standard error of *L. innocua* growth means.

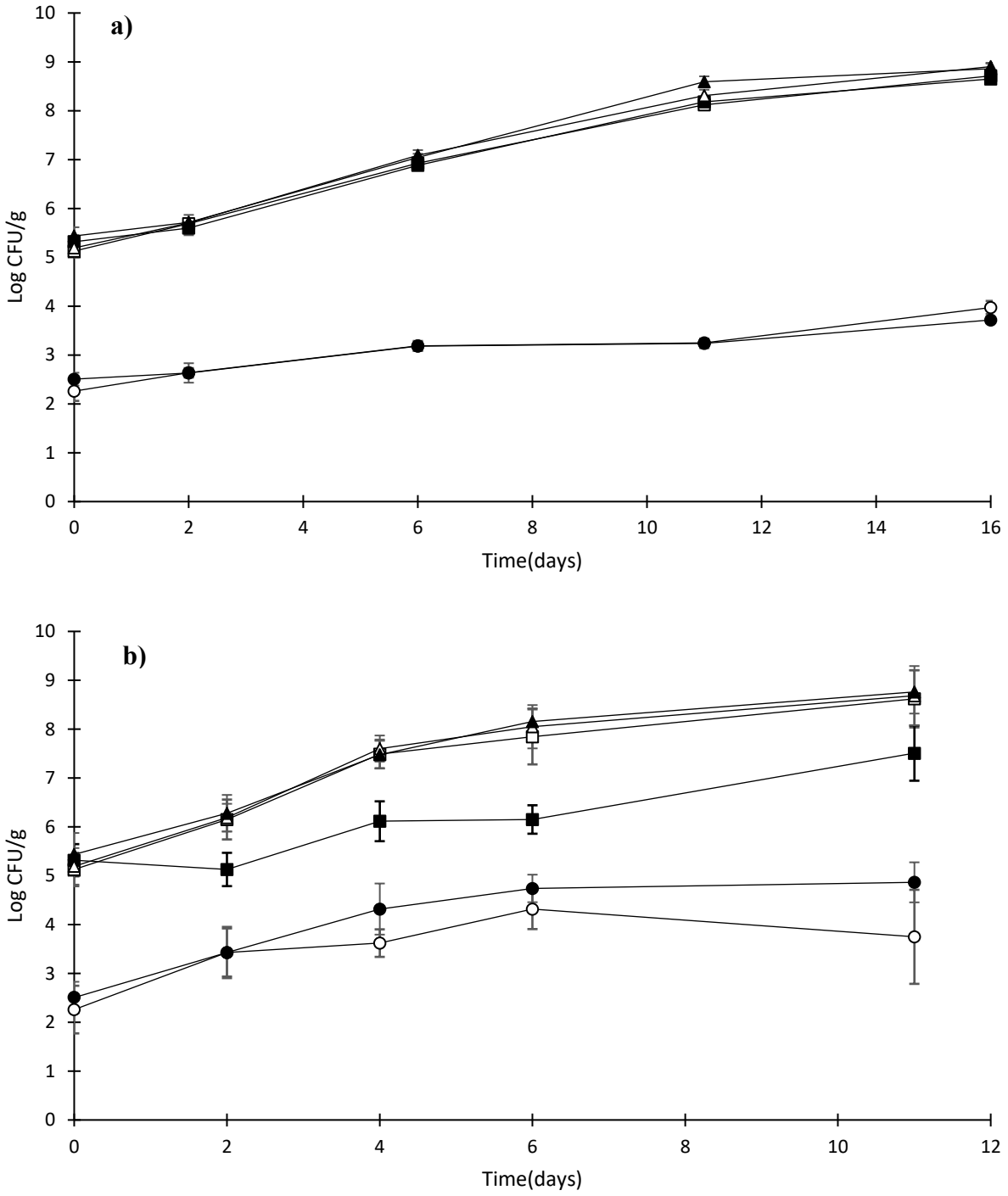

Figure 41. Growth of APC treated (■), nontreated (□), PPC treated (▲), nontreated (△), and LAB treated (●) and nontreated (○) in beet greens by temperatures 4°C (a) and 8°C(b). Error bars represent the standard error of growth means.

Table 33. Mean headspace, water activity and pH values for beet greens by time and treatment at 4°C

Time (days)	Simulated Distribution	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	N	11.6 ± 1.1	6.7 ± 1.0	0.996 ± 0.001	6.6 ± 0.1
2	N	9.2 ± 1.7	9.5 ± 1.5	0.992 ± 0.004	6.5 ± 0.2
6	N	7.6 ± 0.9	10.5 ± 0.4	0.994 ± 0.003	6.9 ± 0.1
11	N	6.4 ± 3.4	11.2 ± 2.3	0.999 ± 0.006	7.3 ± 0.1
16	N	4.5 ± 2.9	12.5 ± 2.0	0.991 ± 0.003	7.4 ± 0.1
0	Y	12.2 ± 1.0	6.4 ± 1.0	0.995 ± 0.003	6.5 ± 0.1
2	Y	10.3 ± 4.5	8.2 ± 3.7	0.995 ± 0.003	6.7 ± 0.1
6	Y	10.0 ± 4.5	8.8 ± 3.7	0.995 ± 0.001	7.0 ± 0.3
11	Y	6.7 ± 1.4	11.0 ± 1.2	0.992 ± 0.003	7.2 ± 0.2
16	Y	6.3 ± 5.6	11.0 ± 3.8	0.992 ± 0.002	7.4 ± 0.1

Table 34. Mean headspace, water activity and pH values for beet greens by time and treatment at 8°C

Time (days)	Simulated Distribution	O_2% ± SD	CO_2% ± SD	Aw ± SD	pH ± SD
0	N	11.6 ± 1.1	6.7 ± 1.1	0.996 ± 0.001	6.55 ± 0.1
2	N	7.4 ± 1.4	10.8 ± 1.3	0.999 ± 0.004	6.61 ± 0.3
4	N	7.0 ± 3.4	11.0 ± 2.4	0.992 ± 0.004	6.86 ± 0.2
6	N	3.5 ± 1.1	12.6 ± 0.5	0.990 ± 0.002	7.06 ± 0.1
11	N	2.9 ± 0.6	13.6 ± 0.2	0.991 ± 0.004	7.31 ± 0.1
0	Y	12.2 ± 1.0	6.4 ± 1.0	0.995 ± 0.003	6.53 ± 0.1
2	Y	8.0 ± 2.4	10.4 ± 1.7	0.993 ± 0.002	6.69 ± 0.2
4	Y	5.9 ± 2.1	12.5 ± 1.0	0.996 ± 0.001	6.88 ± 0.2
6	Y	6.2 ± 7.4	10.3 ± 6.1	0.992 ± 0.003	6.93 ± 0.1
11	Y	5.2 ± 6.1	11.7 ± 3.9	0.999 ± 0.005	7.34 ± 0.1

Storage and Distribution Statistical Analysis

Table 35. ANOVA treatment effect statistics on *L. innocua* growth for all vegetables at 4°C

Vegetable	F - ratio	p-value
Kale	0.0034	0.9536
Brussels sprouts	1.1858	0.2981
Broccoli stalk	0.0094	0.9239
Beet greens	0.0983	0.7575

Table 36. ANOVA treatment effect statistics on *L. innocua* growth for all vegetables at 8°C

Vegetable	F - ratio	p-value
Kale	0.0643	0.8024
Brussels sprouts	0.1673	0.6880
Broccoli stalk	0.0255	0.8752
Beet greens	0.9212	0.4732

Table 37. ANOVA treatment effect statistics on APC, LAB, and PPC growth for all vegetables at 4°C

Vegetable		F - ratio	p-value
Kale			
	APC	0.1401	0.7108
	LAB	0.1635	0.6888
	PPC	0.0194	0.8903
Brussels sprouts			
	APC	1.1125	0.3041
	LAB	0.0258	0.8740
	PPC	1.0059	0.3285
Broccoli stalk			
	APC	0.1861	0.6713
	LAB	0.0041	0.9498
	PPC	0.1678	0.6867
Beet Greens			
	APC	0.0247	0.8766
	LAB	0.0025	0.9607
	PPC	0.9258	0.3470

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