CPS 2018 RFP
FINAL PROJECT REPORT

Project Title

Listeria monocytogenes growth potential, kinetics, and factors affecting its persistence on a broad range of fresh produce

Project Period

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Objectives

1. *Determine Listeria monocytogenes (Lm) growth potential and kinetics on major classes of whole and fresh-cut produce under normal storage/retail display conditions.*
2. *Determine Lm growth potential and kinetics under temperature abuse conditions, and develop an indexing system for quantifying temperature abuse.*
3. *Determine produce nutritional and physiochemical characteristics on Lm growth potential and kinetics.*
4. *Evaluate the effects of the indigenous microbial community from produce on Lm growth.*

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Abstract

Foodborne listeriosis outbreaks and product recalls associated with potential *Listeria monocytogenes* contamination in recent years have heightened the awareness of and concerns about *L. monocytogenes* survival and growth on various fresh produce commodities well beyond the traditional "ready-to-eat" product sectors. In this project, we selected a large number of fruits and vegetables representing a broad range of fresh produce categories to assess the growth potential and growth kinetics of *L. monocytogenes* under conditions reflecting commercial practices for storage and distribution and conditions reflecting sustained temperature abuse. A total of 20 types of fresh produce, including both whole and fresh-cut products, were obtained from a variety of commercial sources and were inoculated with *L. monocytogenes* strains to observe the survival and growth up to 25 days, depending on the projected commercial storage or retail shelf life. *L. monocytogenes* populations showed a sustained decrease on all tested whole fruits, including avocado, blueberry, grape, mango, peach, green pepper, and tomato. Under "normal" storage conditions (defined as those reflective of common commercial practices), significant *L. monocytogenes* growth was observed only on fresh-cut cantaloupe (~0.8 log) and fresh-cut mango (~0.6 log). Exposure to temperature abuse conditions did not change the trends of *L. monocytogenes* survival on the whole fruits but resulted in significant growth on several fresh-cut products, including celery, cauliflower, mango, onion, romaine lettuce, and cantaloupe. *L. monocytogenes* was also tested for growth in sterile juices extracted from selected fresh produce commodities to examine its maximal growth potential and the effect of factors intrinsic to the commodity, including pH and sugar content. Observations of *L. monocytogenes* growth in sterile juices, in general, corroborated those on fresh produce surfaces, with a few exceptions. While most juices from vegetables and cantaloupe supported significant *L. monocytogenes* growth, those from acidic fruits often rendered *L. monocytogenes* counts dropping to below the limit of detection. These observations, along with the finding that the neutralization of acidic juices supported limited *L. monocytogenes* growth, re-affirm the pH of produce as the primary determinant for *L. monocytogenes* growth. Assays of *L. monocytogenes* growth in sterile juices also provided evidence for the presence of potential anti-listerial substances in some types of fresh produce. The microbiome shifts on selected fresh produce commodities were examined using 16S rDNA high-throughput sequencing, the findings of which suggest that *L. monocytogenes* growth on fresh produce was also affected by indigenous microbiota. *L. monocytogenes* antagonist bacteria (LAB) strains were isolated from romaine lettuce and identified as *Lactococcus lactis, Leuconostoc mesenteroides*, and *Weissella cibaria*.

Background

Listeria monocytogenes is a foodborne bacterial pathogen characterized as "ubiquitously present" in the environment, including soil and water. It is one of the leading causes of hospitalization and death from foodborne illness and poisoning in the United States. Although historically *L. monocytogenes* was most frequently detected in ready-to-eat food products, including fresh-cut leafy greens, it was believed that consumption of a high infectious dose was necessary to cause listeriosis. Recent outbreaks have cast strong doubts on such a hypothesis and highlighted the need for a concerted effort to effectively control this pathogen in fresh produce druing production and distribution. In recent years, outbreaks and product recalls have greatly heightened the awareness of and concerns over food safety risks associated with *L.*

monocytogenes growth on fresh produce commodities by the industry and consumers. These widespread concerns are well beyond the products of ready-to-eat sectors.

A unique characteristic of *L. monocytogenes*, which makes the control of this foodborne pathogen more difficult, is its ability to grow at refrigeration temperatures. Several studies have indicated that *L. monocytogenes* survives better and proliferates faster on fresh produce at refrigeration temperatures than other common foodborne pathogens such as *Salmonella enterica*. Nevertheless, temperature abuse is widely recognized as a dangerous practice facilitating the rapid proliferation of foodborne pathogens, including *L. monocytogenes*.

L. monocytogenes growth depends on the type of produce and its intrinsic characteristics, including nutritional profiles. All *L. monocytogenes* strains are believed to be deficient in the synthesis of multiple vitamins and amino acids and are capable of using only a few types of sugars. Therefore, certain fresh produce commodities may not meet the nutritional requirement for supporting *L. monocytogenes* growth. On the other hand, many plants produce natural antimicrobials that could significantly impact *L. monocytogenes* survival or growth. Although the growth and survival potential of *L. monocytogenes* has been extensively studied on a number of whole and fresh-cut produce commodities under different storage conditions, knowledge gaps regarding *L. monocytogenes* growth characteristics on fruit and vegetable surfaces (both intact and fresh-cut) remain unaddressed for a wide variety of fresh produce commodities in relation to current regulatory policies for environmental monitoring requirements and expectations.

Foodborne pathogens are more likely to grow on fresh-cut produce than on intact produce surfaces, due to the lack of protective tissues and the presence of readily accessible nutrients on cut surfaces. Therefore, fresh-cut surfaces would be an appropriate model for *L. monocytogenes* risk assessment purposes, as physical damages can frequently occur during the packing and distribution of fresh produce. Sterile juices from fresh produce also can be an appropriate model for assessing the maximal growth potential of foodborne pathogens by eliminating the interference from indigenous microbial populations. Fresh produce juice is also more amenable for analyzing the effects of nutrients and chemical components on the survival and growth of *L. monocytogenes* on fresh-cut produce.

In addition to the intrinsic characteristics of fresh produce and other environmental factors, the metabolic activities of the contextual microbiota may influence *L. monocytogenes* survival and growth by supplementing required nutrients or producing antimicrobials. Multiple *Lactococcus lactis* strains have been isolated from vegetal sources that show the ability to control *L. monocytogenes* in cheese and on fresh-cut iceberg lettuce. However, little is known of the interactions between *L. monocytogenes* and indigenous microbiota on various types of fresh produce during storage at refrigeration and abusive temperatures. The rapid development of high-throughput DNA sequencing technology has provided powerful microbiome analysis tools for the improved characterization of such interactions. A better understanding of microbial interactions at the community level can lead to the development of effective and environment-friendly intervention strategies for mitigating the risks of *L. monocytogenes* contamination on fresh produce.

Research Methods and Results

Methods:

Fresh produce commodities and sample preparation: Seventeen types of fresh fruits and vegetables were obtained from various sources (**Table 1**), including commercial grower/packers, fresh-cut processors, importers/distributors, local wholesalers, and retail establishments. The commodities were selected to include a broad range of fresh produce

commodities while also considering factors such as market demand, availability, and lack of prior data. All acquired produce was stored at temperatures reflecting those in commercial storage or retail display until use, as suggested by the providers. In instances where whole commodities were obtained for fresh-cut experiments, produce was processed into experimental samples in portion cups or Whirl-Pak bags on the day of inoculation using clean, disinfected utensils. Selected commodities also were used for juice extraction. Crude juice was centrifuged to remove pulp, then filtered through 0.2 µm filters to obtain sterile juice. The pH and sugar contents of each sterile juice were determined and compared to previously reported values for the corresponding commodity. Acidic juices were adjusted to a neutral pH as needed using 1.0 M sodium hydroxide.

L. monocytogenes strains and inoculation: Three strains (FS 2025, FS 2030, FS 2061) of *L. monocytogenes* were used in various experiments for this project. These strains were associated with the 2011 outbreaks implicating cantaloupe from Colorado. Each strain was grown in tryptic soy broth (TSB) overnight at 37 °C. Cells harvested by centrifugation were subjected to starvation by incubating overnight in M9 minimal media without sugar supplementation at room temperature to deplete residual nutrient carry-over and cellular internal nutrient reserves. The starved cells were harvested by centrifugation and resuspended and diluted in phosphate-buffered saline (PBS) or distilled water to optical density of OD_{600} ~0.1, corresponding to approximately 8 log CFU/ml. The cocktail inoculum was prepared by mixing equal portions of the three strains with further dilution as needed.

In most cases, the fresh produce samples (whole and fresh-cut) were surface inoculated with multiple micro-droplets of the strain cocktail targeting 6 log CFU/sample. In some instances, the fresh produce samples were inoculated by submersion (parsley), injection into bags through an adhesive self-sealing foam septum (fresh-cut apple, fresh-cut romaine lettuce), or direct dilution into sterile juice. The inoculated whole commodities were left at room temperature in a biosafety cabinet to dry for up to 2 hours. The inoculated fresh-cut commodities were kept at room temperature in a biosafety cabinet until the inoculum had sufficiently absorbed or dispersed (~ 30 min). One set of replicates was processed to obtain the starting inoculation values (Day 0) , while uninoculated samples were tested to confirm the absence of *L. monocytogenes*.

Fresh produce storage at normal and abusive temperatures: The fresh fruit and vegetable samples for each set of experiments were stored in cooling incubators set to the desired storage temperatures. The “normal” storage temperature and storage duration for each commodity was determined based on industry practices for ripening, transportation, or retail display. Selected commodities were stored sequentially at different temperatures (isothermal storage) in a simulation of practices, e.g., refrigerated distribution followed by retail display at a higher temperature. Abusive temperatures were arbitrarily selected to reflect storage at sustained elevated temperatures as indicated. Whole produce commodities were placed on disinfected aluminum trays, and an open container of water was placed inside the incubator to maintain natural humidity. Temperature data loggers with external probes programmed to collect temperature and humidity data at selected intervals (~15 min) during storage were placed inside the incubators. Fresh-cut cantaloupe was also stored at incremental temperatures up to 216 h to collect data to develop a predictive model of *L. monocytogenes* growth potential.

Enumeration of fresh produce indigenous bacteria and *L. monocytogenes*: Samples were collected at each timepoint in commodity-dependent replicates of three, four, or five. The bacteria present on the samples were retrieved by placing each replicate in an appropriate volume of 10% buffered peptone water or PBS in a Whirl-Pak bag and subjecting the samples to a given retrieval treatment (sonication, pulverizing, or vortexing) for an indicated length of

time (**Tables 2** and **3**). The rinsate was spiral-plated or drop-flow plated onto both selective and non-selective agar with or without dilution, depending on the expected density of the *L. monocytogenes* population. The selective and differential Brilliance *Listeria* agar or Harlequin chromogenic agar was used to enumerate *L. monocytogenes* populations, and tryptic soy agar (TSA) was used for the enumeration of viable mesophilic aerobic bacteria (MAB) populations. Plates for *L. monocytogenes* were incubated for 48 hours at 37 °C prior to counting colonies, and the TSA plates were incubated at 30 °C for 48 hours before colony counting. When direct plate counts approached the limit of detection (~10 CFU/mL rinsate), a 5-tube 3-dilution most probable number (MPN) procedure was used for estimating *L. monocytogenes* populations. In experiments related to microbiome analyses, the total bacteria load and *L. monocytogenes* were also quantified using real-time PCR targeting bacterial 16S rRNA and *L. monocytogenes hly* genes.

L. monocytogenes growth model fitting (fresh-cut cantaloupe): The CFU values for each set of technical replicates within each temperature condition were averaged, and the mean values from the biological replicates (from different melons on different days) were compiled according to temperature. These pooled data sets for the eight temperature conditions were each fitted to growth models using the 'nlsMicrobio' package, and the RMSE statistic was used as an indicator of best fit. The parameters from each isothermal model (μ_{max}, Lag, $Log10N_0$, $Log10N_{max}$) were used to build secondary models describing the dependence of growth rate, lag phase duration, and maximum log CFU on temperature, to which biexponential curves were fit.

The empirical data from all isothermal curves were compiled into a single data set, and the log increase values from each were used to illustrate the exponential relationship between storage temperature and duration of storage (time-temperature curve). Biexponential curves were fitted to the data that fell within sequential one-log increase increments to define the boundaries of time/temperature combinations likely to result in a given log increase in the population of *L. monocytogenes* (see Appendix for equations).

Fresh produce microbiome analyses by 16S rDNA sequencing: The total microbial populations on select fresh produce (avocado, fresh-cut cantaloupe, fresh-cut romaine lettuce), with or without *L. monocytogenes* inoculation, were recovered in PBS as described above. The rinsate was sequentially centrifuged at different G-forces to removed plant material and to concentrate bacterial cells. The collected bacterial cells were treated with the membrane-impermeable DNA-modifying dye propidium monoazide to eliminate DNA from dead or damaged bacterial cells. DNA was extracted from the PMA treated cells and used for quantitative real-time PCR (qPCR) and microbiome analyses. Primers targeting a highly conserved 180 bp portion of the 16S rRNA gene and the *hly* gene of *L. monocytogenes* were used to quantify the total bacterial and *L. monocytogenes* populations using SYBR green-based qPCR. The Earth Microbiome Project protocol was used for high-throughput 16S sequencing targeting the hypervariable V4 region using the Illumina MiSeq platform. The Quantitative Insights Into Microbial Ecology (Qiime2) pipeline was used in conjunction with the Greengene database to quantitatively assign from the sequencing data relative abundance values for each detected microbial taxa from species to phylum and to determine microbiome diversity indeces.

Isolation and identification of *L. monocytogenes* antagonist bacteria (LAB) from lettuce: Diluted sterile lettuce juice (10%, 0.9 ml) inoculated with *L. monocytogenes* was incubated with small pieces (0.1 g) of lettuce leaf at 30 °C for 2 days in deep-well culturing blocks. Cultures from the wells showing >2-log inhibition of *L. monocytogenes* growth were selected to investigate further the potential presence of LAB. Randomly picked colonies from these cultures were screened by co-incubation with *L. monocytogenes* in sterile lettuce juice and on agar plates. Colonies with confirmed anti-listerial activity were then subjected to 16S rDNA sequencing for species

identification and further analyzed for the strength of this activity by co-culturing with *L. monocytogenes* at different inoculation ratios ranging from 1:8 to 8:1 log CFU/ml. The minimal inoculation ratios that resulted in >2-log inhibition of *L. monocytogenes* growth and to complete growth inhibition/inactivation were used to measure the strength of antagonist activity.

Statistical analysis: All statistical analyses were conducted in R version 4.1, SigmaPlot 13.0, or SAS (SAS release 9.3, SAS Institute Inc., Cary, NC). Bacterial CFU values and gene copy numbers were log transformed and analyzed using the analysis of variance (ANOVA) method with Tukey's HSD test. The Spearman correlation test was performed for analysis of the correlation between pH or sugar content (% Brix) with *L. monocytogenes* growth. Except when stated otherwise, *P* values of <0.05 were considered statistically significant. The differences of alpha diversity indexes, including evenness and Shannon index and beta diversity among the different types of samples, were analyzed using the Kruskal–Wallis H test and the permutational multivariate analysis of variance (PERMANOVA) test, respectively, in Qiime 2.

Results:

Growth and survival of *L. monocytogenes* during storage of fresh produce at normal and abusive temperatures (Objectives 1 and 2): The kinetics of *L. monocytogenes* growth and survival during storage of the 20 tested (nine whole and 11 fresh-cut) produce commodities are shown in **Figure 1**. The *L. monocytogenes* populations decreased on all whole commodities, including avocado, blueberry, grape, mango, parsley, peach, green pepper, and tomato (grape and round), at both normal and abusive temperatures, potentially due to lack of nutrients or abundance of competition with background microbiota. The *L. monocytogenes* populations also declined on certain fresh-cut products, including apple and pineapple, during storage at all tested temperatures, which might be associated with the low pH (≤4) level of these fruits, as discussed below. In contrast, significant *L. monocytogenes* growth was observed on several other fresh-cut products, including cantaloupe and mango at both normal and abusive temperatures, and cauliflower, celery, onion, and romaine lettuce only at abusive temperatures. Growth trends of *L. monocytogenes* on fresh-cut celery and lettuce, but not fresh-cut cauliflower or carrot, were consistent with the data acquired using sterile juices (discussion below).

The *L. monocytogenes* population densities at final shared sampling timepoints are presented in **Figure 2**. Generally, greater reductions in *L. monocytogenes* populations on whole products and higher rates of *L. monocytogenes* growth on fresh-cut products were observed, except for fresh-cut apple, carrot, and pepper. Abusive temperatures during storage did not significantly change the *L. monocytogenes* survival patterns for the whole products (mango, peach, green pepper, or tomato) but affected those of some fresh-cut products, as evidenced by significant increases in *L. monocytogenes* populations on cantaloupe, cauliflower, celery, mango, onion, and romaine lettuce, and significant decreases on apple, carrot, and pineapple. Between the initial and final timepoints during temperature abuse, there were increases of 1 to 2.5 log on fresh-cut cantaloupe, cauliflower, mango, onion, celery, and romaine lettuce (**Figure 3**), while 1- to 2.5-log reductions were observed on fresh-cut apple, carrot, pineapple, whole avocado, blueberry, grapes, tomato, and parsley. Even greater decreases were seen on whole mango and peach. The average daily changes in *L. monocytogenes* levels are presented in **Figures 4** and **5**. Most products showed an average daily change in the *L. monocytogenes* population of >0.5 log, with the exception of fresh-cut carrot, which showed a more rapid *L. monocytogenes* reduction. In general, a greater rate of *L. monocytogenes* population change was observed under abusive temperatures.

Quantified prediction of *L. monocytogenes* growth on fresh-cut cantaloupe (Objective 2): Based on the results of Objective 1 and its association with listeriosis outbreaks, fresh-cut cantaloupe

was selected as a high-risk product model for the quantified prediction analysis. Growth curves of *L. monocytogenes* on fresh-cut cantaloupe at different storage temperatures were generated by fitting to the Baranyi model (**Figure 6** and **Table 4**), without the maximum log or lag phase parameters as deemed appropriate by the goodness-of-fit statistics. Higher storage temperatures resulted in a shorter lag phase and a shorter time-to-stationary phase. The shortest lag phase (1.71 h) was observed at 20 °C. The Baranyi model with the inclusion of the lag parameter failed to provide adequate fits at temperatures above 20 °C; for temperatures of 24 °C, 28 °C, and 32 °C, the Baranyi model without the lag phase parameter was used. The highest maximum log increase estimated for *L. monocytogenes* growth on cut cantaloupe was at 16 °C, reaching 6.2 log after 28 h. The highest growth rate was detected at 32 °C (μ_{max} 0.83 log CFU/g/h), but the maximum log increase was lower (5.2 log after 48 h). These observations indicate that the maximum *L. monocytogenes* increase might occur on fresh-cut cantaloupe at medium-warm temperatures. The length of the lag phase and the rate of bacterial growth are critical for the evaluation of microbial safety of foods and establishing shelf-life. Thus, the short lag phase of *L. monocytogenes* in fresh-cut cantaloupe at 16 °C, as well as the high growth rate of *L. monocytogenes* at 32 °C indicate a higher risk for *L. monocytogenes* contamination when in contact with fresh-cut cantaloupe and under temperature abuse.

To better visualize the effect of storage temperatures on *L. monocytogenes* growth, *L. monocytogenes* growth curves were plotted as a function of incubation time and temperature (**Figure 7**). As expected, *L. monocytogenes* growth was positively correlated with incubation temperature and time. To predict *L. monocytogenes* growth under different conditions (in combination with storage temperature and time), population data were fitted to linear and exponential models to generate equations for *L. monocytogenes* lag phase and growth rate predictions, respectively (**Figures 8** and **9**, Appendix **Formulas**). Lag phase and growth rate (μ_{max}) were negatively and positively correlated with storage temperature, respectively.

Physiochemical characteristics of fresh produce juices and growth/survival of *L. monocytogenes* (Objective 3): Sterile juices from each tested produce commodity were measured for their pH and sugar content (% Brix) and compared to those for the corresponding fruit or vegetable as reported in the literature (**Table 5**). For all products that are generally considered to be fruits (Fuji and Gala apple, blueberry, cantaloupe, grape, mango, peach, pineapple), the juices had an acidic pH (pH 3.25–3.91) and a high sugar content (11.0–15.6% Brix), except for cantaloupe, which had a nearly neutral pH of 7.66. In contrast, for those products generally considered to be vegetables (carrot, cauliflower, celery, green pepper, parsley, lettuce, tomato), a less acidic or near-neutral pH (pH 5.60–7.71) and a low sugar content (2.1–5.1% Brix) were observed, except for tomato (pH 4.1) and carrot (9.3% Brix). Comparison to data for the corresponding fruit or vegetable indicated that the sterile juices, in general, reflected the nutritional characteristics of the whole product. *L. monocytogenes* growth measured in juices from carrot, cantaloupe, celery, green pepper, parsley, and romaine lettuce ranged from 1.74 to 2.31 log (**Figure 10**). *L. monocytogenes* growth was inhibited in juice extracts from all other produce tested. While mango and tomato juice slightly reduced the *L. monocytogenes* populations, by 0.12 and 0.59 log, respectively, cauliflower juice strongly reduced survival by 2.54 log after 24 h incubation. Juices from apple, blueberry, grape, peach and pineapple reduced *L. monocytogenes* populations below the limit of detection, resulting in a total reduction of more than 5.3 log.

The inactivation kinetics of *L. monocytogenes* were further analyzed for apple, blueberry, cauliflower, grape, mango, peach, pineapple, and tomato juices during a 24 h incubation (**Figure 11**). *L. monocytogenes* populations remained at the initial inoculation levels (7.0–7.4 log CFU/mL) during the first 6 h for juices extracted from mango, tomato, cauliflower, and blueberry; populations thereafter decreased at various rates to 6.80, 6.70, 4.77 log CFU/mL and to an undetectable level, respectively, by 24 h. When *L. monocytogenes* was incubated for 6 h in

pineapple and Fuji apple juice, the populations significantly declined by 1.89 and 2.46 log (P<0.001), respectively, and to an undetectable level after 24 h. In contrast, *L. monocytogenes* showed rapid inactivation in juice extracts from Gala apple, grape, and peach, in which populations were below the detection limit of 2 log CFU/mL throughout the 24 h incubation.

Intrinsic factors affecting *L. monocytogenes* growth (Objective 3): Plotting *L. monocytogenes* growth in fresh produce juices against the juice pH and sugar content (**Figure 12**) showed that *L. monocytogenes* growth is primarily dependent on the pH of juices (r=0.838, P<0.001). No viable *L. monocytogenes* cells were detected in juices with pH ≤3.78 (apple, blueberry, pineapple, grape, and peach), and significant reductions of *L. monocytogenes* counts were observed for juices with pH 3.91–4.14 (mango and tomato). Significant *L. monocytogenes* growth was observed for all juices with pH ≥5.60 (green pepper, parsley, celery, romaine lettuce, carrot, and cantaloupe), except for cauliflower, which failed to support *L. monocytogenes* growth despite having a nearly neutral pH. The acidic fruit juices possessed a high sugar concentration, ranging from 11.1 to 15.6% Brix (**Table 5**). In contrast, the sugar content of juices that supported *L. monocytogenes* growth was generally low (2.1–4.1% Brix for parsley, celery, green pepper, and romaine lettuce juice), except for cantaloupe and carrot juices (8.4–11% Brix) (**Figure 12 B**). Thus, the pH of produce juice seemed to be primarily associated with *L. monocytogenes* growth which favored a near-neutral pH and was less affected by the sugar content of juices (r=-0.660, P=0.004) (**Figure 12**). Produce pH as a primary determinant for *L. monocytogenes* was also demonstrated by its growth in acidic juices following pH neutralization (**Figure 13**). However, neutralized juices from peach and Gala apple supported no or very limited growth by *L. monocytogenes*, indicating the role of other factors.

L. monocytogenes growth inhibition in cauliflower juice was mitigated in diluted juice (**Figure 14**), indicating the presence of weakly anti-listerial substances. In addition, dilution of the non-acidic juices reduced *L. monocytogenes* growth as the nutrient level decreased, while dilution of the acidic juices lessened the inactivation effect but did not result in *L. monocytogenes* growth.

Carrot anti-listerial activity and *L. monocytogenes* cultivability (Objective 3): Multiple previous studies have demonstrated the anti-listerial activities of carrot. It was observed that inoculation on the surface of fresh-cut carrot or in carrot soaking water rapidly rendered *L. monocytogenes* unrecoverable by plating; however, this inactivation was not observed when the carrot was lightly cooked. Both raw and sterile carrot juice supported robust *L. monocytogenes* growth. In fact, despite a long lag phase, sterile carrot juice supported the highest *L. monocytogenes* growth among all produce tested in this study. Therefore, the carrot anti-listerial substances seem volatile. Although carrot-exposed *L. monocytogenes* tended to become unculturable, it exhibited no difference from untreated cells when examined by PMA-qPCR or by bacterial cell vitality staining, suggesting a viable but non-culturable (VBNC) state. Interestingly, when examined by transmission electron microscopy, carrot-exposed cells exhibited progressing invagination of the cell membrane (termed mesosome formation) (**Figure 15**). The importance of mesosome formation for *L. monocytogenes* VBNC will be further investigated.

Microbiome and dynamics on avocado, cantaloupe, and lettuce during storage (Objective 4): Mesophilic aerobic bacteria (MAB) population densities on non-inoculated and *L. monocytogenes*-inoculated whole avocadoes were 5.24 ± 0.02 and 5.27 ± 0.09 log CFU/fruit, respectively. The population decreased 0.8–1.6 log at all three storage temperatures of 5, 10, and 20 °C (**Figure 16**). MAB levels on fresh-cut cantaloupe and romaine lettuce significantly increased after storage, especially at abusive temperatures. PMA-qPCR estimation of total bacteria levels showed similar growth and survival trends, as measured by plate counts. *L. monocytogenes* levels on inoculated produce samples followed similar growth and survival

trends as the MAB, except for lettuce samples stored at 4 °C, on which the *L. monocytogenes* increase was lower compared to MAB growth (**Figure 17**).

After 16S rRNA gene high-throughput sequencing merging and quality control analyses using Qiime2, paired reads (each pair is composed of reads generated by the forward and reverse primers) were generated for the 48 avocado (5,447,339), 32 fresh-cut cantaloupe (4,074,598), and 48 romaine lettuce samples (6,505,974). Queries to the Greengene database using these sequences identified a total of over 2000, ~200, and ~500 OTUs in avocado, cantaloupe, and lettuce samples, respectively. At least 18.9%, 63.8%, and 87.1% of the identified bacteria and archaea OTUs from the produce samples were assigned to species, genus, and family levels, respectively.

Fresh produce microbiome composition is shown as relative abundance (RA) of the dominant bacterial phyla (**Figure 18**) and genera and species (**Figure 19**). Proteobacteria was the phylum with the highest abundance in all avocado samples, accounting for 67.7% of the total reads. Proteobacteria, Firmicutes, and Actinobacteria were the top three phyla on fresh-cut cantaloupe, with average RAs of 53.8%, 39.6%, and 6%, respectively. On sampled fresh-cut romaine lettuce, Proteobacteria (54.4%) and Firmicutes (45.5%) were the two dominant bacterial phyla. At the genus and species level, *Sphingomonas* sp. (average RA=16.7%), a species of family Enterobacteriaceae (RA=15.1%), and an unidentified bacterium (RA=14.1%) were the top species on the avocado surface. Several species of *Methylobacterium*, which are commonly found in soil and water, were also identified as major indigenous microbes on avocado. In addition to the inoculated *L. monocytogenes*, *Pseudomonas* sp. (24.3%), *Cupriavidus* sp. (12.3%), and an unidentified species of family Enterobacteriaceae (9.4%) were the most abundant bacteria on fresh-cut cantaloupe. On fresh-cut romaine lettuce, the dominant native bacteria were *Pseudomonas* sp. (21.9%), *Leuconostoc mesenteroides* (17.7%), a species of family Leuconostocaceae (11.3%), Enterobacteriaceae (10.5%), Lactobacillaceae (7.3%), *Weissella* sp. (9.5%), *Pantoea* sp. (6.5%), and *Pseudomonas veronii* (5.3%). *Listeria* was detected on all inoculated produce samples (**Figure 19**).

The 16S rDNA copy numbers of the most abundant bacterial species on tested samples, which reflected shifts in estimated population size instead of RA for major indigenous microbes and inoculated *L. monocytogenes* on produce during storage, are listed in **Tables 6–8**. The dominant phyla on both non-inoculated and *L. monocytogenes*-inoculated avocado were not obviously changed during storage at different temperatures, except for the inoculated samples stored at 5 °C after 17 days, which showed an average Proteobacteria RA of over 96% (**Figure 18A**). This change is mainly attributed to the striking increase of an unidentified species of family Enterobacteriaceae (**Figure 19A**), which increased by about 1 log while most other bacteria decreased during storage at 5 °C. Similar to the results of the qPCR analysis, the population and RA of *L. monocytogenes* on avocado decreased during storage. The shift in the avocado microbiome was mainly affected by the storage temperature (P<0.05) but not by *L. monocytogenes* inoculation (P=0.118) (**Figure 20A**).

Proteobacteria was the dominant phylum on non-inoculated fresh-cut cantaloupe, with RA above 60%, particularly for samples stored at 5 °C after 7 days (99.5%). In contrast, on inoculated cantaloupe, Firmicutes became the most abundant after storage at all three tested temperatures, with RA over 80%; this dramatic change was solely due to the rapid growth of *L. monocytogenes*, indicating that fresh-cut cantaloupe provides better proliferation conditions for *L. monocytogenes* than for indigenous microbes. The final population of *L. monocytogenes* after storage was at least 1 log higher than the indigenous bacteria on fresh-cut cantaloupe (**Table 7**). The relatedness of the microbiome on fresh-cut cantaloupe was mainly affected by inoculation but not storage temperatures (**Figure 20B**).

The RA of *L. monocytogenes* on fresh-cut romaine lettuce remained at similar levels when stored at abusive temperatures but was reduced at refrigeration temperatures (**Figure 19C**). This is in agreement with the significant increase of the MAB but a minimal change of *L. monocytogenes* population at 4 °C, as measured by plate count and PMA-qPCR results. There was a high variance for the sampled lettuce microbiome. Both storage temperature and *L. monocytogenes* inoculation affected a shift in the lettuce microbiome (**Figure 20C**).

Among the four biological replicates of romaine lettuce samples, multiple bacterial taxa, including *Leuconostoc* sp., Leuconostocaceae sp., and *Weissella* sp., showed a negative correlation to the RA of *Listeria*, especially after storage at 12 °C. The potential effect of *Weissella* and *Leuconostoc* spp. on *L. monocytogenes* suppression was supported by the bioassay described below.

L. monocytogenes growth in lettuce juice and LAB isolation from lettuce (Objective 4): Although *L. monocytogenes* was capable of significant growth both on fresh-cut romaine lettuce and in sterile romaine lettuce juice, it decreased to undetectable levels in raw lettuce juice after overnight incubation, suggesting that this growth inhibition/inactivation was likely due to the indigenous microbiota instead of intrinsic factors of the romaine lettuce. Co-culturing of *L. monocytogenes* inoculated into 10% sterile lettuce juice with small pieces of romaine lettuce resulted in *L. monocytogenes* growth inhibition by >2 log in approximately 10% (14 of 144) of the samples. At least a portion of randomly picked colonies from these cultures also showed *L. monocytogenes* growth inhibition, confirming their LAB activity.

These presumable LAB were identified at the species level by 16S rDNA sequencing as *Lactococcus lactis, Leuconostoc mesenteroides*, and *Weissella cibaria* (**Table 9**). When co-cultured in a liquid medium, they exhibited varying strengths of *L. monocytogenes* growth inhibition at different initial inoculation ratios. Most of these LABs produced moderate growth inhibition of *Listeria*, resulting in >2 log growth inhibition of *L. monocytogenes* at inoculation ratios between 10:1 and 1:10 and total growth inhibition at inoculation ratios larger than 1000:1. However, strain LAB7, a *L. lactis* isolate, resulted in >2 log *L. monocytogenes* growth inhibition at an initial inoculation ratio of 2.0:7.4 log CFU/mL (1:250,000) and complete growth inhibition at an inoculation ratio of 3.0:6.4 log CFU/mL (1:2,500).

Outcomes and Accomplishments

Determination of *L. monocytogenes* growth potential on a broad range of fresh produce. The growth and survival kinetics of *L. monocytogenes* on 20 types (whole or fresh-cut) of commonly consumed fresh produce commodities at both normal and abusive temperatures during storage (0–22 days) were determined. A significant decline in the *L. monocytogenes* population was observed on all whole products, likely because of restrictions in nutrient availability. *L. monocytogenes* was also reduced significantly on certain fresh-cut products such as apple and pineapple, which might be associated with the low pH (≤4) level of the fruits. On the other hand, *L. monocytogenes* increased on fresh-cut cantaloupe and mango, which indicates a higher contamination risk for food safety considerations.

Effect of temperature abuse on *L. monocytogenes* survival and growth. The *L. monocytogenes* growth kinetics on the above fresh produce commodities at one or two abusive temperatures were determined and compared to those at the corresponding normal storage/display temperature. Temperature abuse did not significantly change *L. monocytogenes* growth patterns for the whole products but affected those of some fresh-cut vegetables (e.g. cauliflower, celery, onion, and romaine lettuce).

Quantitative prediction analysis for fresh-cut cantaloupe as a high-risk product. Intensive tests on *L. monocytogenes* growth at various storage temperatures (ranging from 4 to 32 °C at 4-degree intervals) were performed to fit and parameterize models for growth prediction. *L. monocytogenes* growth was positively correlated with incubation temperature and time. The fastest growth rate was detected at the highest tested temperature (32 °C), while the maximum *L. monocytogenes* population increase on fresh-cut cantaloupe was observed at a moderate abuse temperature (16 °C).

L. monocytogenes growth in fresh produce juice. Sterile juices prepared from 14 different fresh produce commodities were used to determine the maximal potential for supporting *L. monocytogenes* growth under optimal growth conditions. *L. monocytogenes* growth in sterile juice generally reflected that on fresh produce surfaces, except for cauliflower (growth on the fresh-cut surface but not in sterile juice) and carrots (growth in sterile juice but not on the fresh-cut surface).

Intrinsic characteristics of fresh produce as determinates for *L. monocytogenes* growth. The primary intrinsic characteristic as an *L. monocytogenes* growth determinant is the pH of the produce, which is consistent with current federal food safety guidance in defining time-temperature controlled foods vs low-risk products. In most cases, neutralizing the acidic fruit juices to pH 7 resulted in *L. monocytogenes* growth, further demonstrating the critical role of produce pH in determining *L. monocytogenes* growth potential. However, incubating *L. monocytogenes* in neutralized juice from acidic fruits often resulted in only limited growth, indicating the possible role of undissociated organic acids in preventing *L. monocytogenes* growth. Another intrinsic characteristic of fresh produce affecting *L. monocytogenes* growth is the existence of potential anti-listerial substances, exemplified by *L. monocytogenes* growth in diluted but not 100% cauliflower juice, despite its neutral pH. Indeed, previous studies have demonstrated the antimicrobial activity of glucosinolates and their hydrolysis products, such as isothiocyanates, in plants of the *Brassicaceae* family.

Microbiome dynamics on selected fresh produce and potential interactions with *L. monocytogenes*. Microbiome shifts on avocado, fresh-cut cantaloupe, and fresh-cut romaine lettuce, with or without *L. monocytogenes* inoculation during storage at “normal” and abusive temperatures were determined by 16S rDNA sequencing. Fresh-cut cantaloupe, which featured a very simple initial microbiome, supported *L. monocytogenes* growth to become the dominant microbiome component. The outcome of *L. monocytogenes* growth on fresh-cut romaine lettuce, which has a moderately complex microbiome, was more varied. In contrast, *L. monocytogenes* failed to grow on the avocado surface. These observations suggested that, in addition to the intrinsic characteristics of fresh produce, the background microbota also play an important role in determining *L. monocytogenes* growth.

Isolation of *Listeria* antagonist bacteria (LAB) from romaine lettuce. LAB strains were isolated from romaine lettuce, and have been identified as *Lactococcus lactis, Leuconostoc mesenteroides*, and *Weissella cibaria*. Although most of these isolates showed moderate growth inhibition against *L. monocytogenes*, one isolate exhibited very strong growth inhibition or inactivation against *L. monocytogenes* when co-inoculated in a liquid medium. The mechanisms of this growth inhibition and potential use for mitigation of *L. monocytogenes* growth on fresh produce will be further explored in future studies. More LAB can also be isolated from diverse sources using the simple screening procedure described here.

Summary of Findings and Recommendations

L. monocytogenes growth potential and kinetics on a broad range of fresh produce at normal and abusive temperatures. Rapid declines in *L. monocytogenes* counts were observed on all whole fruits tested, including green pepper and tomato, likely caused by a lack of accessible nutrients on the fruit surface. *L. monocytogenes* growth and survival varied for the fresh-cut products. Fresh-cut cantaloupe and most fresh-cut vegetables but none of the fresh-cut acidic fruits supported significant *L. monocytogenes* growth. Most of these observations were also corroborated when corresponding sterile juices were used for assessing the effect of produce intrinsic characteristics on *L. monocytogenes* growth and survival. *Therefore, all fresh-cut produce with higher pH, such as cantaloupe and vegetables, should be considered highly conducive to L. monocytogenes growth, especially at abusive temperatures.*

Low pH is a primary deterrent for *L. monocytogenes* growth. Acidic fruits did not support *L. monocytogenes* either on commodity surfaces or in sterile juices. Exposing inoculated produce to abusive temperatures did not change the trend of *L. monocytogenes* growth on whole fruits. *Effective strategies for mitigating L. monocytogenes risks on such commodities should focus on preventing the initial contamination instead of temperature control.*

L. monocytogenes cultivability. A large proportion of *L. monocytogenes* cells rapidly lose cultivability after inoculation onto the surface of whole fruits, including non-acidic fruits such as avocado. The same was observed when *L. monocytogenes* was exposed to the surface of carrot and carrot-soaking water. It is not clear whether this large portion (99%) of unrecoverable *L. monocytogenes* cells die or survive in a VBNC state. Transmission electron microscopy showed that carrot-exposed *L. monocytogenes* cells form progressive membrane invagination. *Although these observation were beyond the scope of the current project, more research should be conducted to assess the food safety implications of foodborne pathogens with reduced cultivability.*

Intrinsic characteristics of fresh produce and effects on *L. monocytogenes* growth. In addition to pH as a primary determinant for *L. monocytogenes* growth, other fresh produce intrinsic characteristics potentially affecting *L. monocytogenes* growth include the presence of potential anti-listerial substances or the absence of essential micronutrients. We observed preliminary evidence of such substances playing a role in determining *L. monocytogenes* growth in neutralized peach juice (likely undissociated organic acids), in cauliflower juice (likely glucosinolates or degradation products), and fresh-cut carrots. We did not observe evidence of *L. monocytogenes* failing to grow due to a lack of micronutrients. *These naturally occurring anti-listerial substances can be further explored for mitigating L. monocytogenes risks.*

Microbiome of fresh produce and *L. monocytogenes* growth. In addition to the intrinsic factors of fresh produce, *L. monocytogenes* growth on a given product can be strongly affected by its microbiome. This was consistent with our observations of the overwhelming growth of *L. monocytogenes* on fresh-cut cantaloupe and the strong growth inhibition in raw romaine lettuce juice. We also isolated a *Lactococcus lactis* strain that strongly inhibited *L. monocytogenes* growth when co-cultured in a liquid medium at a low initial inoculation ratio. *Further research needs to be conducted to explore the use of such antagonist isolates for manipulating fresh produce microbiomes.*

Microbiome research. Fresh produce microbiome research can contribute to a better understanding of the interactions between foodborne pathogens and indigenous microbiota on produce. Such efforts are often discouraged due to limited access to fresh produce growth, processing, and distribution systems. *Closer industry collaboration and expanded access will greatly help such research for finding more useful tools against foodborne pathogens.*

APPENDICES

Publications and Presentations

Publications – *Manuscripts under review:*

Lichtenwald, M., Bolten, B., Luo, Y., Micallef, S., Millner, P., and Nou, X. Growth and survival of *Listeria monocytogenes* in sterile extracts of fruits and vegetables: impact of intrinsic factors. Manuscript under review by *Food Microbiology*.

Gu, G., Kroft, B., Lichtenwald, M., Luo, Y., Millner, P., Patel, J., and Nou, X. Dynamics of *Listeria monocytogenes* and microbiome on fresh-cut cantaloupe and romaine lettuce during storage at refrigerated and abusive temperatures. Manuscript under review by *International Journal of Food Microbiology*.

Publications – *Manuscripts in preparation:*

Kroft, B., Bolten, S., Micallef, S., Luo, Y., Millner, P., and Nou, X. Effects of temperature abuse on the growth and survival of *Listeria monocytogenes* on a wide variety of whole and fresh-cut fruits and vegetables during simulated storage. Manuscript in preparation for submission to *Food Microbiology*.

Kroft, B., Micallef, S., Luo, Y., Millner, P., and Nou, X. Prediction of *Listeria monocytogenes* growth on fresh-cut cantaloupe during non-isothermal storage using an empirically validated model. Manuscript in preparation for submission to *Food Control*.

Bolten, S., Gu, G., Mowery, J., Lichtenwald, M., Luo, Y., and Nou, X. *Listeria monocytogenes* loss of culturability on carrot is associated with the formation of mesosomes. Manuscript in preparation for submission to *Food Science and Technology*.

Liu, X., Bollinger, C., Deaver, W., Nou, X., and Micallef, SA. Association between phytochemical traits of cantaloupe, honeydew melon and watermelon and the foodborne pathogens *Listeria monocytogenes* and *Salmonella enterica*. Manuscript in preparation for submission to *Food Microbiology* or equivalent.

Presentations:

Gu G, M Lichtenwald, Y Luo, X Nou. 2021. Survival of *Listeria monocytogenes* in romaine lettuce juice and isolation of anti-*Listeria*l bacteria. International Association for Food Protection (IAFP) Annual Meeting. Virtual, July 18-21, 2021.

Liu X, C Bollinger, W Deaver, X Nou, SA Micallef. 2021. Melon phytocompounds may impact foodborne pathogen persistence in melon juice. International Association for Food Protection (IAFP) Annual Meeting. Virtual, July 18-21, 2021.

Kroft B, P Millner, Y Luo, S Micallef, X Nou. 2021. Effects of short-term temperature abuse during storage of fresh-cut cantaloupe on *Listeria monocytogenes* growth. International Association for Food Protection (IAFP) Annual Meeting. Virtual, July 18-21, 2021.

Lichtenwald M, S Micallef, P Millner, Y Luo, X Nou. 2020. Impact of nutrients and physiochemical characteristics on *Listeria monocytogenes* growth in fruit and vegetable juices. International Association for Food Protection (IAFP) Annual Meeting. Virtual, October 26-28, 2020.

Bolten S, G Gu, P Millner, Y Luo, X Nou. 2020. Determining the viability of Listeria monocytogenes following exposure to carrot surface. International Association for Food Protection (IAFP) Annual Meeting. Virtual, October 26-28, 2020.

Kroft B, S Bolten, P Millner, Y Luo, S Micallef, X Nou. 2020. Impact of simulated storage conditions on L. monocytogenes survival on whole avocado and mango. International Association for Food Protection (IAFP) Annual Meeting. Virtual, October 26-28, 2020.

Budget Summary

This project was awarded with a total contractual budget of $360,199, including $294,982 for personnel (subcontractual to University of Maryland), $53,891 for supplies, $6,025 for travels to CPS annual symposia, and $5,300 for other costs including registrations at CPS meetings and publication fees. All budgeted funds have been spent as July 31, 2021, in accordance to the guidelines, as shown in the table bellow. All funds for Personnel were spent through University of Maryland for salaries and fringe benefits for postdocs, students, and the lab technician working on this project during the period supported by this fund. Due to the Covid-19 pandemic that resulted in closure of the laboratory or limited access, our request for no-cost extensions were granted by CPS. A requested budget line shift was approved to transfer unspent funds for Travel and Other (due to changing of CPS symposia to virtual) to Supplies to cover the extra cost laboratory supplies and experimental materials. Final expenditures include $62,584 for Supplies, $1,383 for Travel, and $1,250 for Other.

	Initial Budget	Budget Line shift	Revised Budget	Total Expenditure
Personnel	$294,983.00	-$1.00	$294,982.00	$294,982.00
Supplies	$53,891.00	$8,693.44	$62,584.44	$62,584.44
Travel	$6,025.00	-$4,642.44	$1,382.56	$1,382.56
Other	$5,300.00	-$4,050.00	$1,250.00	$1,250.00
Indirect Cost	$0.00	$0.00	$0.00	$0.00
Total	$360,199.00	$0.00	$360,199.00	$360,199.00

Tables, Figures, and Formulas (see below)

Tables 1–9, Figures 1–20, and Formulas

Table 1. Fresh produce used for assessing *L. monocytogenes* growth on produce surfaces.

Commodity	Source	Prior Treatment	Conditions tested
Fresh-cut			
Apple, Fuji	Fresh-cut Processor	Anti-browning treatment	Slices in mini MAP bags
Broccoli	Local grocery	Unknown	fresh-cut, bagged
Cantaloupe	Packer/local grocery	Unknown	Cubes in portion cups
Carrot, Cello	Fresh-cut Processor	Chlorine washed	Slices in portion cups
Cauliflower,	Fresh-cut Processor	Chlorine washed	fresh-cut, bagged
Celery,	Fresh-cut Processor	Chlorine washed	fresh-cut, bagged
Lettuce	Fresh-cut Processor	Chlorine washed	fresh-cut, bagged
Mango, Atkins	Importer	Hot-water treated	Cubes in portion cups
Onion, Yellow	Local grocery	Unknown	Slices in bags
Parsley,	Local grocery	Unknown	Bunched
Pepper, Green	Grower & Packer	Chlorine washed	Fresh-cut, sealed trays
Pineapple, Golden	Local wholesaler	None	Fresh-cut in portion cups
Whole			
Avocado, Dass	Importer	None	Individual fruit in trays
Blueberry	Local wholesaler	Unknown	Multiple fruits in portion cups
Grape, Table red	Local wholesaler	Unknown	Multiple fruits in portion cups
Mango, Atkins	Importer	Hot-water treated	Individual fruit in trays
Peach, Yellow	Grower/Packer	Chlorine washed	Individual fruit in trays
Pepper, Green	Grower/Packer	Chlorine washed	Individual fruit in trays
Tomato, Grape	Grower/Packer	Chlorine washed	Multiple fruits in portion cups
Tomato, Round	Grower/Packer	Chlorine washed	Individual fruit in trays

Table 2. Experiment setup for fresh produce under isothermal storage conditions.

Commodity	Type	Temp (°C)	Storage Duration (day)	Sample Size (g)	Buffer Volume (mL)	Lm Recovery at Time 0 (log CFU/sample)
Carrot	fresh-cut	4 10 15	14	25	50	1.92
Grape	whole	4 10 15	7	25	50	2.90
Blueberry	whole	4 10 15	16	25	50	5.39
Apple	fresh-cut	4 10 15	10	70	50	2.39
Pineapple	fresh-cut	4 10 15	7	25	50	4.94
Mango	fresh-cut	4 10 15	7 7 5	25	50	3.97
Celery	fresh-cut	4 10 15	14 14 7	20	50	4.61
Cauliflower	fresh-cut	4 10 15	14 14 7	25	50	4.62
Cantaloupe	fresh-cut	4 10 15	8	25	50	2.49
Parsley	sprigs	0 4 8 12 16	10 10 8 5 5	25	100	6.32
Romaine	fresh-cut	4 8 12 16	10 10 10 8	15	60	1.76
Onion	fresh-cut	4 10	7 7	25	50	5.56
Broccoli	fresh-cut	4 10	7 7	25	50	5.43
Pepper	fresh-cut	4 10 15	10 10 5	25	25	2.82
Pepper	whole	10 20	14 10		300	5.50

Table 3. Experiment setup for fresh produce under non-isothermal storage conditions.

Commodity	Type	Stage 1		Stage 2		Stage 3		Storage Duration (day)	Sample Unit	Lm Recovery at Time 0 (log CFU/ sample)
		Temp (°C)	Time (day)	Temp (°C)	Time (day)	Temp (°C)	Time (day)			
Tomato (Round)	whole	20	14	12.8 20	6	22	5	25	fruit	3.66
Tomato (Grape)	whole	12.8 20	4	22	6	NA	NA	10	100	2.66
Mango	whole	12 20	16	20	3	22	3	22	fruit	5.62
Avocado	whole	5 12 20	10	22 22 22	10 8 0	NA	NA	20 18 10	fruit	4.6
Peach	whole	1.5 12 22	7	22 22 22	7 7 0	NA	NA	14 14 7	fruit	4.7

Table 4. Baranyi model parameters of isothermal growth curves for *L. monocytogenes* growth on fresh-cut cantaloupe.

Temp (°C)	Value and statistic significance (Mean + Std.Error (P value))			
	Lag (Hour)	Log10N0	Log10Nmax	μmax
4	41.4 ± 36.34 (0.28)	-0.12 ± 0.13 (0.38)	--	0.03 ± 0.01 (0)
8	52.22 ± 3.9 (0.00)	-0.02 ± 0.04 (0.57)	--	0.16 ± 0.02 (0)
12	5.93 ± 2.75 (0.06)	-0.16 ± 0.12 (0.22)	5.6 ± 0.18 (0)	0.17 ± 0.01 (0)
16	1.49 ± 3.89 (0.71)	-0.19 ± 0.25 (0.46)	6.16 ± 0.22 (0)	0.23 ± 0.02 (0)
20	1.71 ± 4.01 (0.69)	-0.05 ± 0.35 (0.89)	4.2 ± 0.25 (0)	0.4 ± 0.07 (0)
24	---	-0.43 ± 0.19 (0.05)	4.6 ± 0.18 (0)	0.52 ± 0.05 (0)
28	---	-0.22 ± 0.16 (0.20)	5.43 ± 0.25 (0)	0.64 ± 0.05 (0)
32	---	-0.34 ± 0.17 (0.07)	5.24 ± 0.11 (0)	0.83 ± 0.1 (0)

Lag, Lag phase (hours);

Log10N0, Initial cell density in log CFU;

$Log10N_{max}$, maximum cell density in log CFU;

μ_{max}, maximum growth rate in log CFU/gram/hour.

Table 5. Characterization of sterile juices from a variety of fresh produce.

Produce	Variety	Source	Ripening stage	pH		Sugar Content		Total Organic Acids
				CS*	Ref [1,2]	CS* (% Brix)	Ref [3] (% WT)	Ref [3] (% WT)
Apple, sliced	Fuji	Processor	--	3.78	--	12.6	--	N/A
Apple	Gala	Retail	2	3.72	3.33 – 3.84 [2]	15.6	11.4	0.46
Blueberry		Retail	3	3.25	3.12 - 3.33 [2]	12.9	6.05	1.37
Cantaloupe		Retail	3	7.66	6.17 – 7.13 [2]	11	12.4	0.08
Carrot	Baby	Processor	--	7.71	--	8.4	--	N/A
Carrot	Cello	Retail	--	6.82	5.88 - 6.40 [1]	9.3	4.8	0.26
Cauliflower		Processor	--	7.64	5.6 [1]	5.1	2.34	0.22
Celery		Packer	--	6.4	5.70 - 6.00 [2]	3.3	2.18	-
Grape	Table	Retail	3	3.38	2.90 - 3.81 [2]	15.3	15.2	0.35
Green Pepper	Bell	Packer	3	5.6	5.20 - 5.93 [2]	3.7	2.91	0.32
Mango	Atkins	Wholesale	3	3.91	3.40 - 4.63 [2]	11.1	12.5	0.34
Parsley		Retail	--	5.9	5.62 - 6.03 [2]	2.1	7.38	-
Peach	Yellow	Packer	2	3.75	3.30 - 4.05 [2]	13.9	8.89	0.57
Pineapple	Gold	Retail	3	3.29	3.20 – 3.64 [2]	15.4	12.4	0.72
Lettuce	Romaine	Retail	--	6.42	5.70 - 6.13 [2]	4.1	1.06	0.13
Tomato	Round	Retail	3	4.14	3.99 – 4.75 [2]	3.2	2.6	0.39
TSB control	--		--	7.11		3.5	--	N/A

* CS: Value measured in Current Study in sterile juice.

Reference 1 (USFDA/CFSAN, 2003)
Reference 2 (Bridges and Mattice, 1939)
Reference 3 (Souci et al., 2008)

Table 6. List of the most abundant bacterial species* from whole avocado (>3 log 16S copies/fruit in at least one sampling point) during storage at refrigerated and abusive temperatures.

	Non-inoculated							Inoculated					
Whole avocado		D10			D17				D10			D17	
	Initial	5[a]	12	20	5	12		Initial	5	12	20	5	12
Sphingomonas	4.31	3.13	3.62	3.89	3.79	4.13		4.39	2.92	3.86	3.00	2.36	3.79
unknown bacterium	4.60	3.02	3.55	3.98	3.39	3.89		4.60	3.04	3.36	3.03	2.47	3.68
Methylobacterium	4.24	2.69	3.37	3.85	3.40	3.55		4.18	2.65	3.26	2.82	2.07	3.49
Methylobacterium komagatae	4.15	2.92	3.31	3.18	3.39	3.35		3.82	2.75	3.20	2.54	2.13	3.38
f-Enterobacteriaceae	4.86	3.47	2.45	4.24	2.24	2.49		3.91	1.74	1.85	1.76	4.85	2.28
f-Methylobacteriaceae	3.81	2.50	3.51	3.17	2.64	3.32		4.06	2.49	2.95	2.20	2.15	2.97
f-Blattabacteriaceae	3.79	2.53	3.21	3.16	2.85	3.55		3.97	2.17	2.53	1.77	1.33	2.88
Pseudomonas	2.59	1.25	1.21	3.91	2.41	1.77		3.07	1.01	1.84	1.10	3.55	2.04
Listeria	0.00	0.00	0.00	0.00	0.00	0.00		3.90	1.98	3.59	1.46	2.00	2.42

Table 7. List of the most abundant bacterial species* from fresh-cut cantaloupe (>3 log 16S copies/g) during storage at refrigerated and abusive temperatures.

	Non-inoculated					*L. monocytogenes* inoculated			
Cut cantaloupe		D7					D7		
	Initial	4	10	15		Initial	4	10	15
f-Enterobacteriaceae	2.31	1.52	3.21	8.92		2.44	1.64	5.45	5.96
Curtobacterium	1.16	0.45	4.99	8.18		1.07	1.21	5.69	8.29
Pseudomonas sp.1	1.47	4.50	4.91	1.58		1.23	1.30	6.70	5.68
Listeria	0.00	0.00	0.00	0.00		2.58	4.02	9.05	9.47
Cupriavidus	3.14	2.43	1.92	5.33		3.02	2.58	3.71	2.59
Sphingomonas	1.59	0.57	1.51	5.92		2.03	0.56	2.69	5.95
Bacillus	0.00	0.00	3.54	8.10		0.00	0.00	0.00	6.56
Pseudomonas sp.2	0.00	0.00	3.98	4.84		0.00	0.33	1.29	6.85
Stenotrophomonas	0.00	0.19	1.59	7.10		0.49	0.00	3.19	2.73
f-Blattabacteriaceae	0.40	0.35	1.36	4.99		0.45	0.74	2.39	3.82
Methylobacterium	1.06	0.83	0.31	2.81		0.36	0.68	3.91	2.87
Paenibacillus amylolyticus	0.00	0.00	3.51	7.44		0.00	0.00	1.48	0.00
Bacillus	0.00	0.00	2.58	6.85		0.00	0.00	0.00	1.51
Unknown bacterium	0.82	1.09	0.83	4.08		0.91	1.08	0.00	1.35
Acinetobacter johnsonii	0.00	0.00	2.93	6.79		0.00	0.00	0.00	0.00
Acinetobacter	0.00	0.00	2.84	6.57		0.00	0.00	0.00	0.00
Terribacillus	0.98	0.00	1.16	6.39		0.00	0.17	0.00	0.00
Renibacterium	2.18	1.54	0.72	0.00		1.98	1.64	0.00	0.00
Methylobacterium komagatae	0.19	0.00	1.43	2.77		1.68	0.53	0.00	1.31
Ralstonia	1.52	1.32	0.74	0.00		2.13	1.58	0.00	0.00

Table 8. List of the most abundant bacterial species* fresh-cut romaine lettuce (>5 log 16S copies/g) during storage at refrigerated and abusive temperatures.

	Non-inoculated							*L. monocytogenes* Inoculated					
Fresh-cut romaine lettuce		D4			D8				D4			D8	
	Initial	4	12	24	4	12		Initial	4	12	24	4	12
Leuconostoc mesenteroides	3.79	5.96	7.70	8.28	6.07	8.54		2.95	6.03	7.43	8.43	5.74	8.54
Pseudomonas	4.56	6.38	7.26	7.59	6.57	7.96		4.92	6.65	7.58	4.98	7.21	7.47
f-Enterobacteriaceae	3.99	6.18	7.66	8.36	5.93	7.95		4.04	6.19	7.17	7.34	6.35	7.39
Pantoea	4.07	6.41	7.60	7.76	5.68	7.78		4.08	6.08	7.24	6.75	5.61	7.23
Pseudomonas veronii	2.36	5.77	7.04	7.56	5.79	7.87		3.42	5.72	7.05	5.28	6.59	7.62
f-Leuconostocaceae	4.66	5.24	6.41	5.19	5.29	6.85		1.27	6.41	6.99	6.60	1.34	7.10
Pseudomonas	2.81	5.03	6.41	7.25	5.36	7.28		3.72	5.36	6.00	2.00	5.88	5.24
f-Lactobacillaceae	2.55	3.93	5.42	7.29	3.80	6.68		1.69	4.74	6.13	8.62	3.87	7.51
Weissella	4.56	6.22	6.38	5.25	3.33	5.36		0.85	5.31	6.95	6.59	2.79	7.07
Leuconostoc	2.69	3.13	6.22	3.24	2.89	6.82		0.55	3.00	6.40	6.77	3.48	7.15
Listeria	2.00	3.80	2.27	2.76	1.82	1.22		4.08	4.78	7.10	7.49	5.27	7.76
Pseudomonas fragi	1.74	5.14	4.30	5.72	5.05	2.96		2.73	3.47	2.57	1.39	5.16	3.06
Pseudomonas umsongensis	0.00	0.00	3.19	5.03	4.17	6.44		2.38	1.17	4.09	1.85	4.58	1.64
Janthinobacterium lividum	0.68	3.19	3.96	1.53	4.22	4.23		1.40	2.04	3.47	1.51	5.22	2.81
Erwinia chrysanthemi	0.00	0.84	5.72	7.32	0.00	7.13		0.89	0.00	2.89	3.24	1.53	3.17
Stenotrophomonas retroflexus	0.00	0.73	2.55	4.83	1.69	5.62		1.46	0.97	3.60	2.75	1.76	4.49
Lactococcus	0.00	0.00	2.90	4.84	0.00	6.17		0.00	0.86	2.39	4.52	0.95	4.20
Aeromonas	2.05	1.91	2.60	0.00	2.33	5.44		0.28	1.11	3.92	2.62	0.89	2.82
Enterobacter	0.00	0.00	5.63	7.26	0.00	6.33		0.00	0.00	1.37	1.95	0.00	1.84
Morganella	0.00	1.02	4.31	7.04	0.00	6.46		0.00	0.00	0.00	1.88	0.00	1.50
Enterococcus casseliflavus	0.00	0.00	0.00	3.33	0.00	3.01		0.00	0.00	3.78	6.22	0.00	2.93
Lactobacillus brevis	0.00	0.00	0.00	3.07	0.00	0.00		0.00	0.00	0.00	5.84	0.00	0.00

* 16S rDNA copies of identified taxa; [a] storage temperatures at 4–24 °C.

Table 9. Potential *L. monocytogenes* antagonist bacterial strains (LAB) isolated from romaine lettuce.

ID	Species	LAB : Lm Ratio at inoculation (log CFU/mL : Log CFU/mL)	
		2 log Inhibition	**LDL***
LAB1	*Lactococcus lactis*	4.1 : 5.4	7.1 : 2.4
LAB2	*Leuconostoc mesenteroides*	4.9 : 4.4	6.9 : 2.4
LAB3	*Lactococcus lactis*	4.1 : 5.4	6.1 : 3.4
LAB4	*Leuconostoc mesenteroides*	4.9 : 4.4	5.9 : 3.4
LAB5	*Lactococcus lactis*	4.2 : 5.4	7.2 : 2.4
LAB6	unidentified	NI	NI
LAB7	*Lactococcus lactis*	2.0 : 7.4	3.0 : 6.4
LAB8	*Lactococcus lactis*	5.2 : 3.4	6.2 : 2.4
LAB9	*Weissella cibaria*	5.3 : 3.4	6.3 : 2.4
LAB10	*Leuconostoc mesenteroides*	3.7 : 4.4	5.7 : 2.4
LAB11	*Lactococcus lactis*	3.4 : 4.4	5.5 : 2.4
LAB12	*Weissella cibaria*	ND	ND
LAB13	*Weissella cibaria*	ND	ND
LAB14	*Lactococcus lactis*	ND	ND

* LDL, lower than detection limit; NI, no inhibition; ND, not determined.

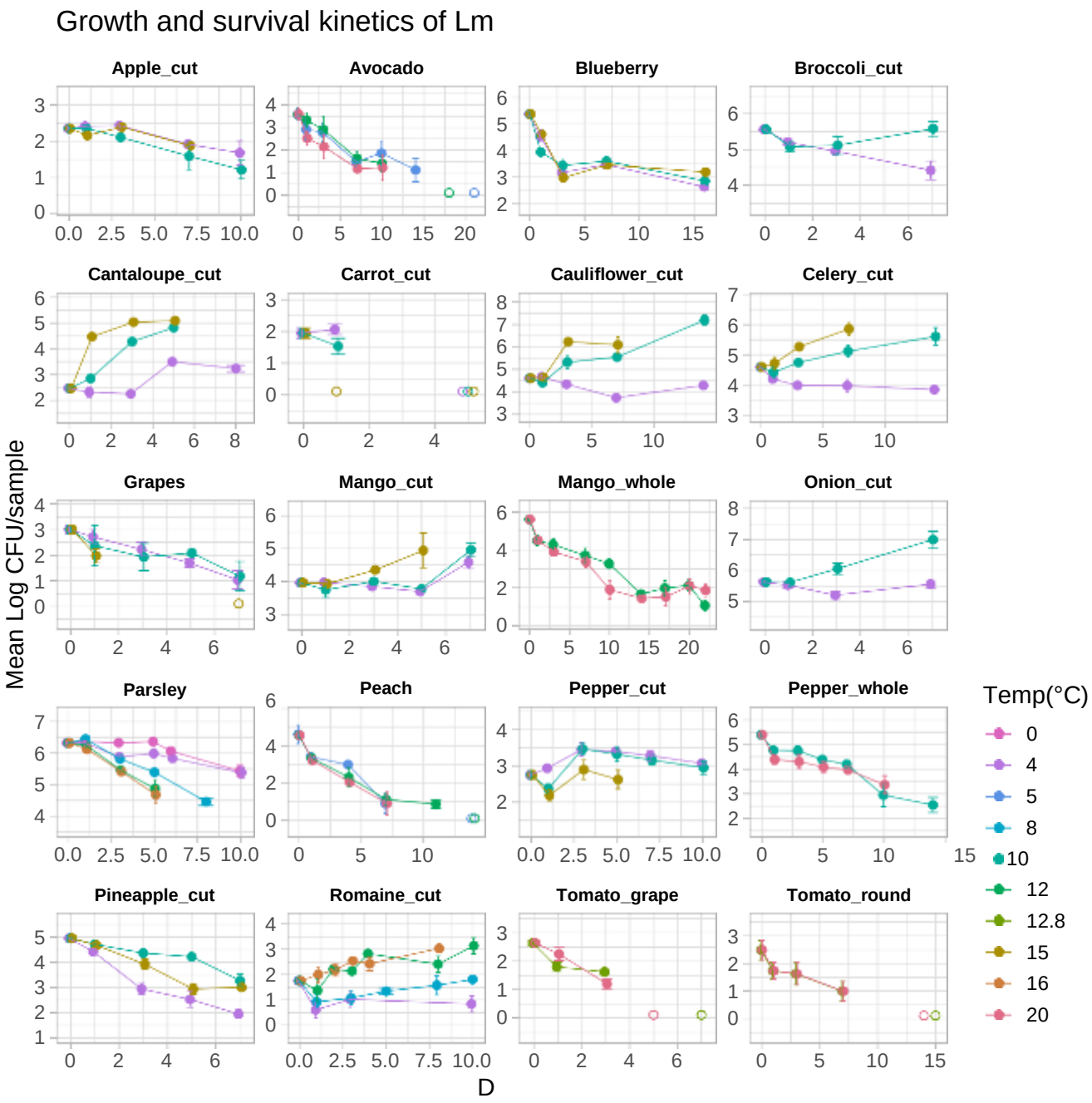

Figure 1. Growth and survival kinetics of *L. monocytogenes* during storage at normal and abusive temperatures. Each dot is the mean of a set of replicates, and the error bars indicate the calculated standard error. Unfilled dots indicate that the population was below the limit of detection at the corresponding timepoint. Normal and abusive storage temperatures are indicated with corresponding colors.

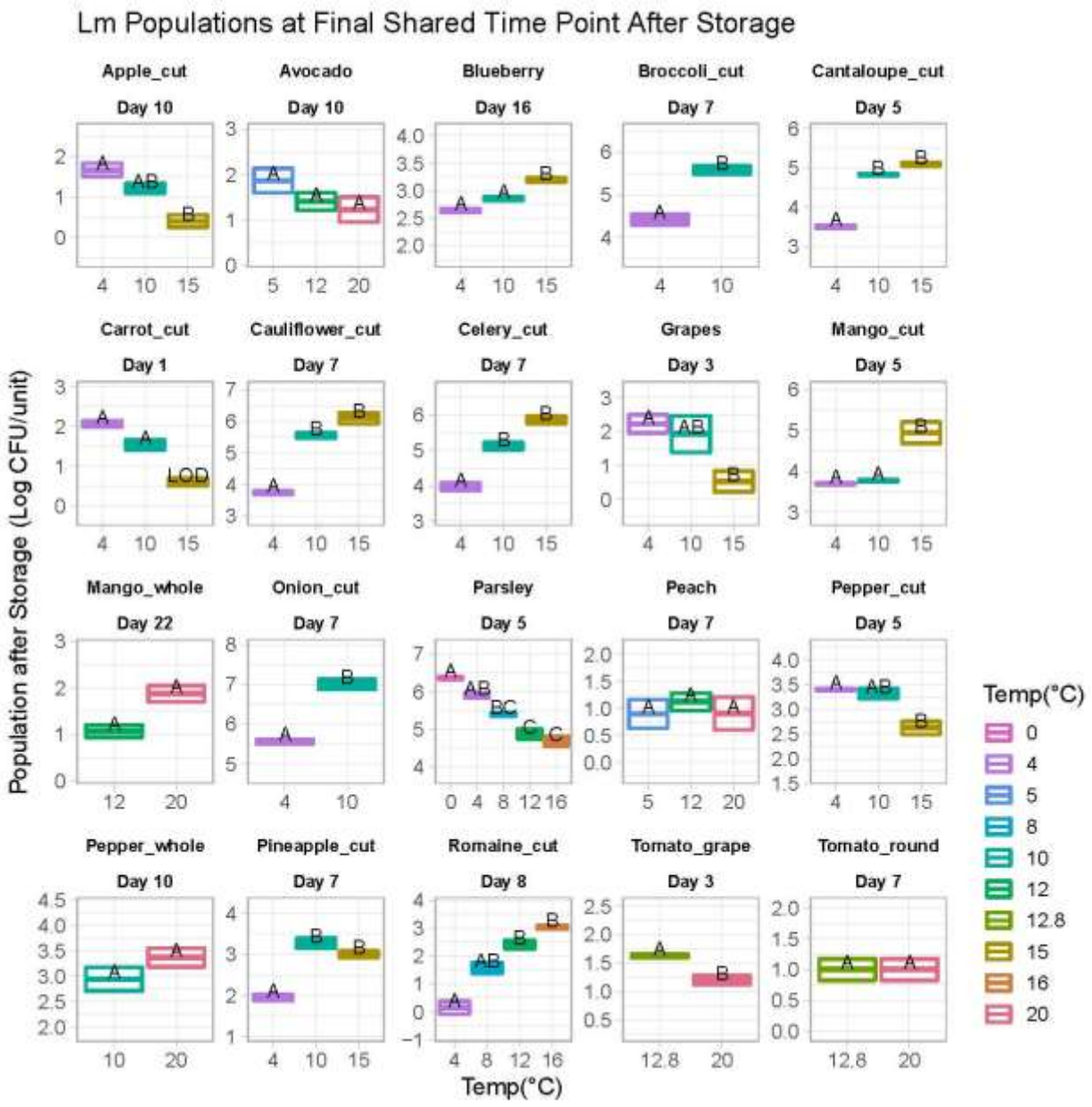

Figure 2. Comparison of *L. monocytogenes* populations at selected timepoints during storage at different temperatures. In instances where the shelf life of a commodity was decreased by storage at an elevated (abusive) temperature, the length of the shortest duration of storage for a commodity was selected to compare significant differences between the surviving *L. monocytogenes* populations at that timepoint. The timepoint is indicated above each plot. Colored rectangles represent the mean (horizontal bar) and standard error of each set of replicates at each temperature. Different letters within each commodity plot indicate a statistically significant difference in the surviving population at the indicated timepoint. 'LOD' signifies that the quantifiable population had reached or exceeded the limit of detection for a given temperature condition.

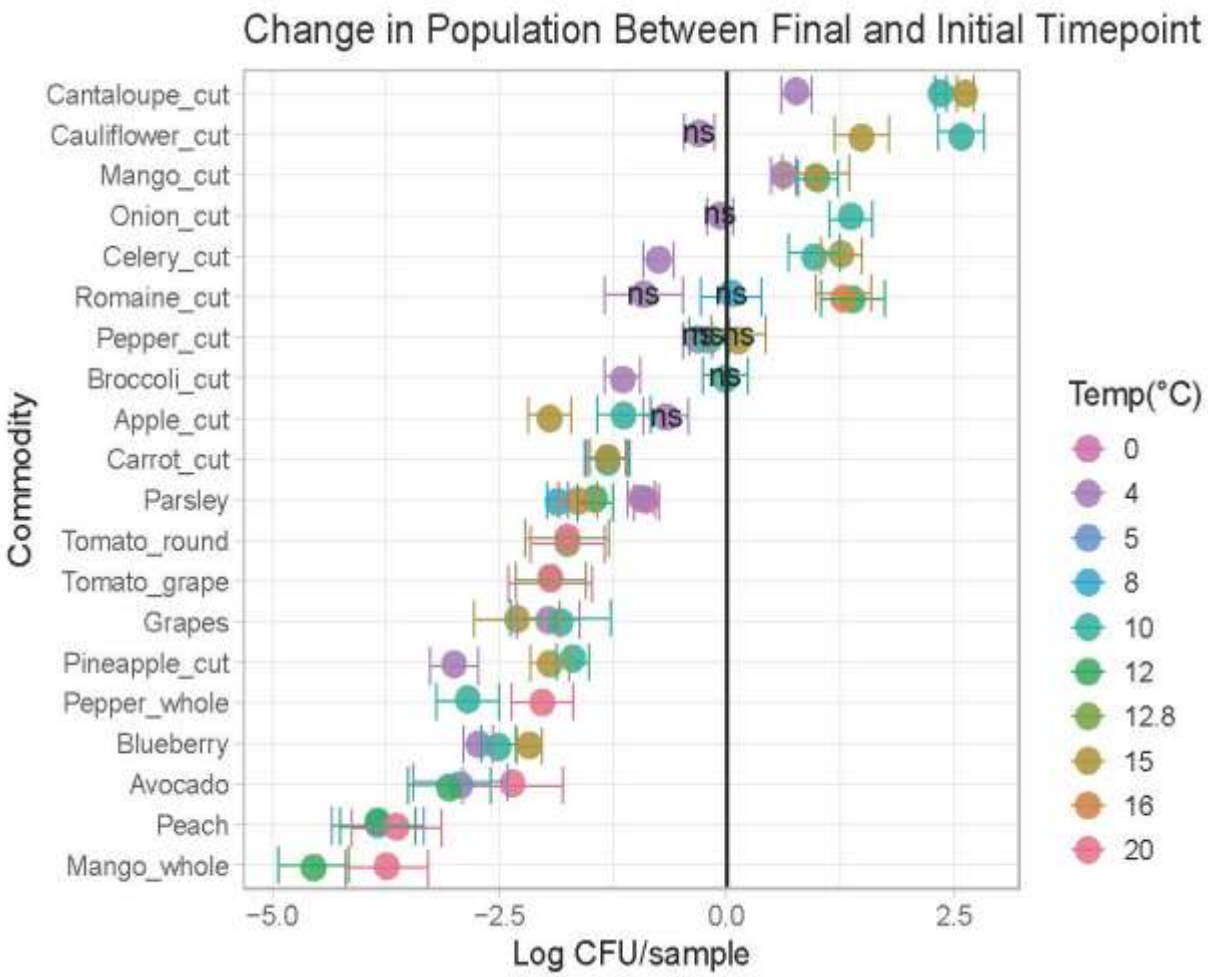

Figure 3. Change in *L. monocytogenes* population between final and initial timepoint. The difference between the mean population of *L. monocytogenes* remaining at the end of each storage period and the mean initial population on Day 0 is indicated by the colored dots, with error bars indicating the standard error of the difference. The letters 'ns' over dots denote that the quantified change in population during the course of storage was not statistically significant.

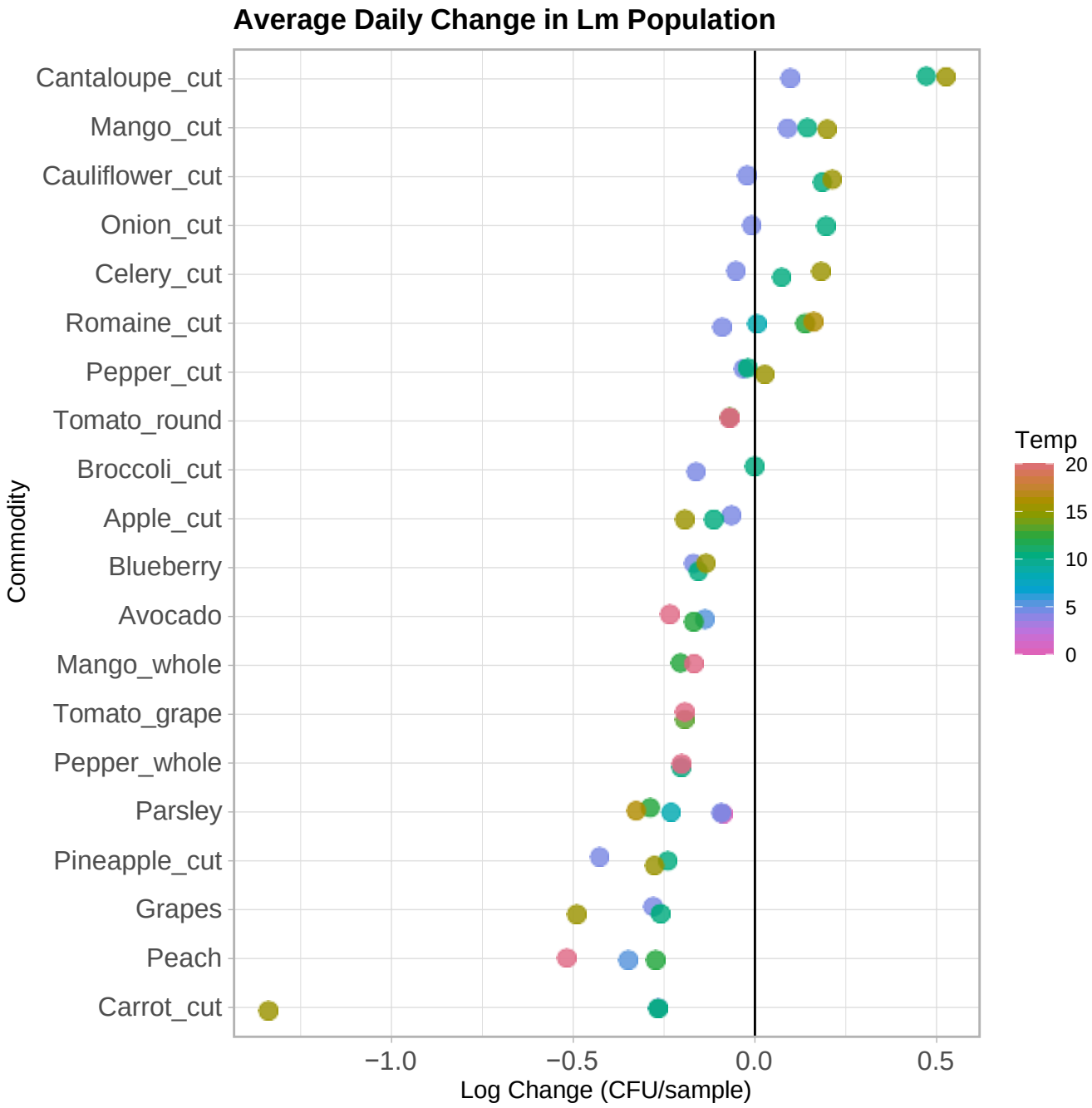

Figure 4. Rate of *L. monocytogenes* population change during storage at different temperatures (log/day). The average log change per day in the *L. monocytogenes* population for each commodity and storage condition is indicated by colored dots, with the color representing the storage temperature for each commodity.

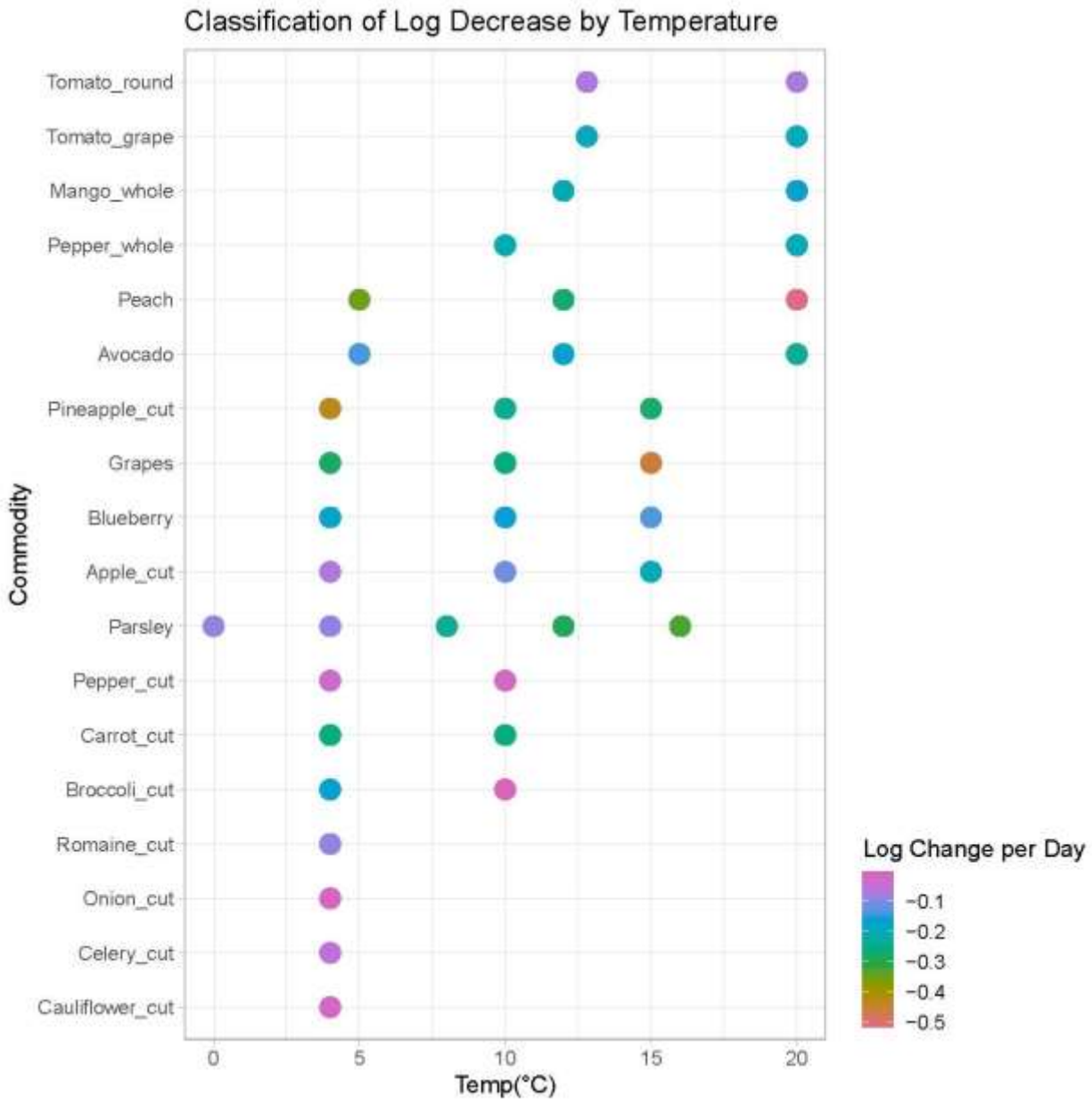

Figure 5. Average log decrease by temperature. The average log decrease of *L. monocytogenes* population for each commodity at different storage conditions is indicated by colored dots, with the color representing the average log change per day.

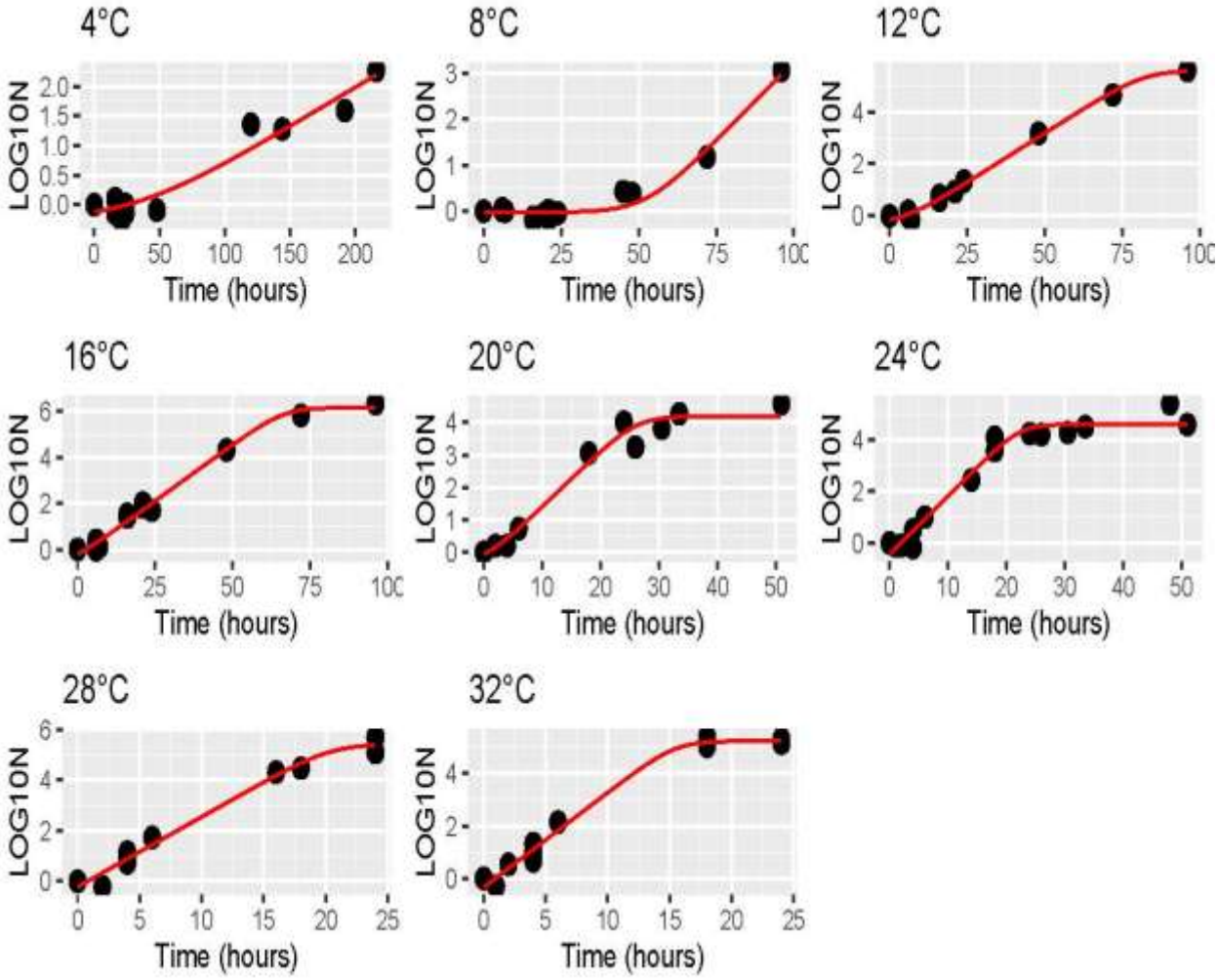

Figure 6. Fitted Baranyi growth curves of *L. monocytogenes* on fresh-cut cantaloupe at different storage temperatures.

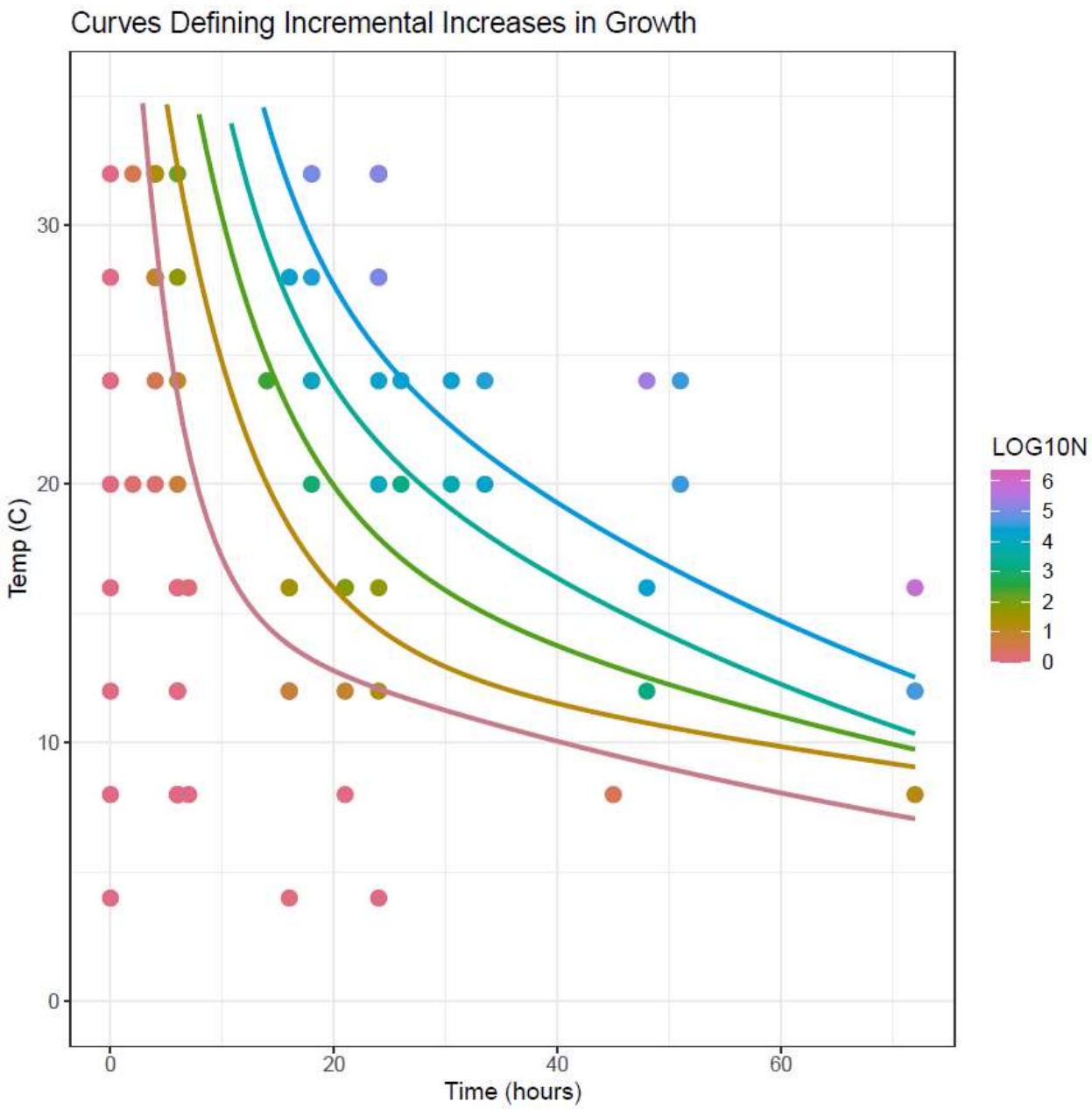

Figure 7. *L. monocytogenes* growth curves plotted as a function of incubation time and temperature. The colors of the curves indicate the boundary defining the combination of storage times and temperatures predicted to result in the indicated *n*-log increases on fresh-cut cantaloupe.

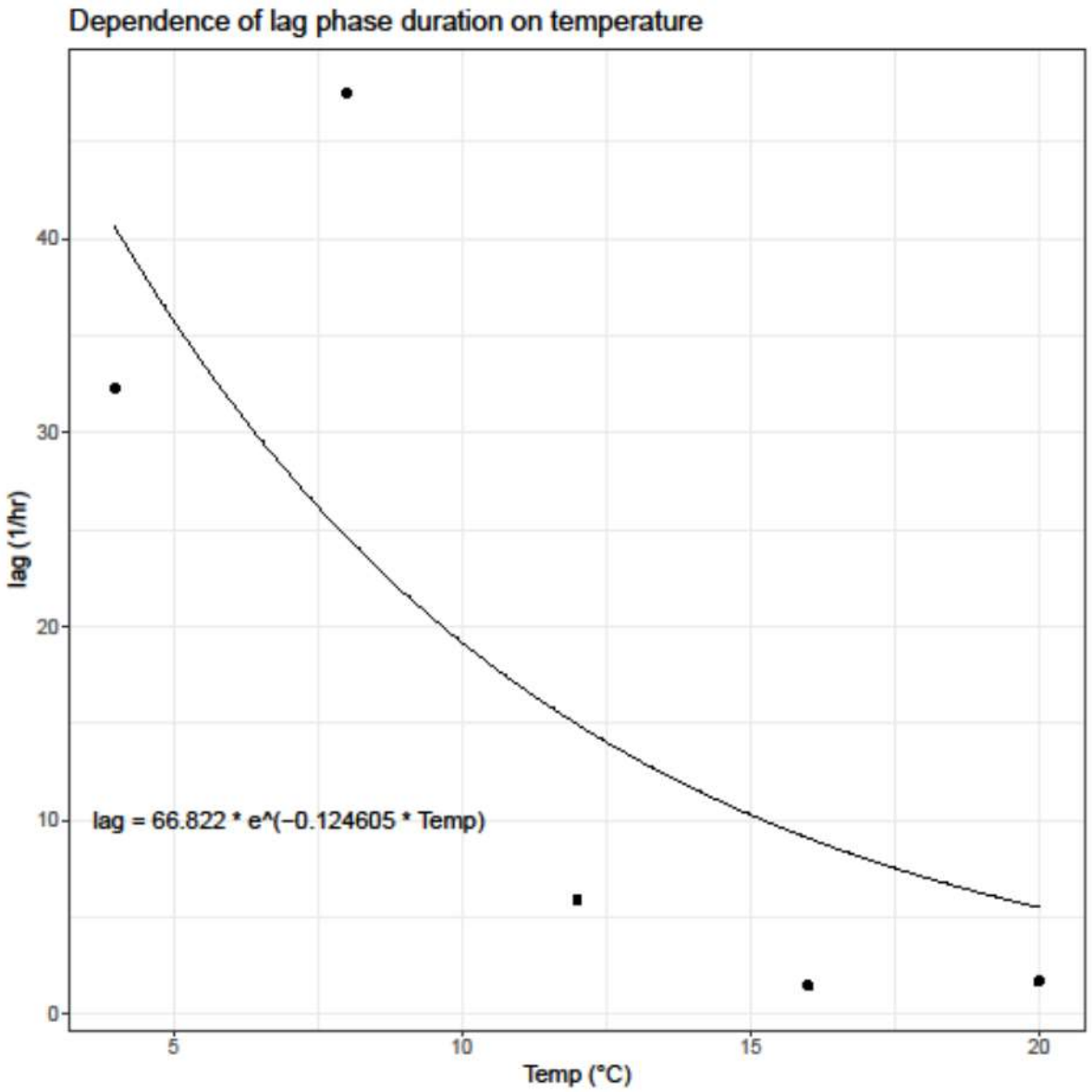

Figure 8. Dependence of the lag phase duration on temperature for *L. monocytogenes* on fresh-cut cantaloupe.

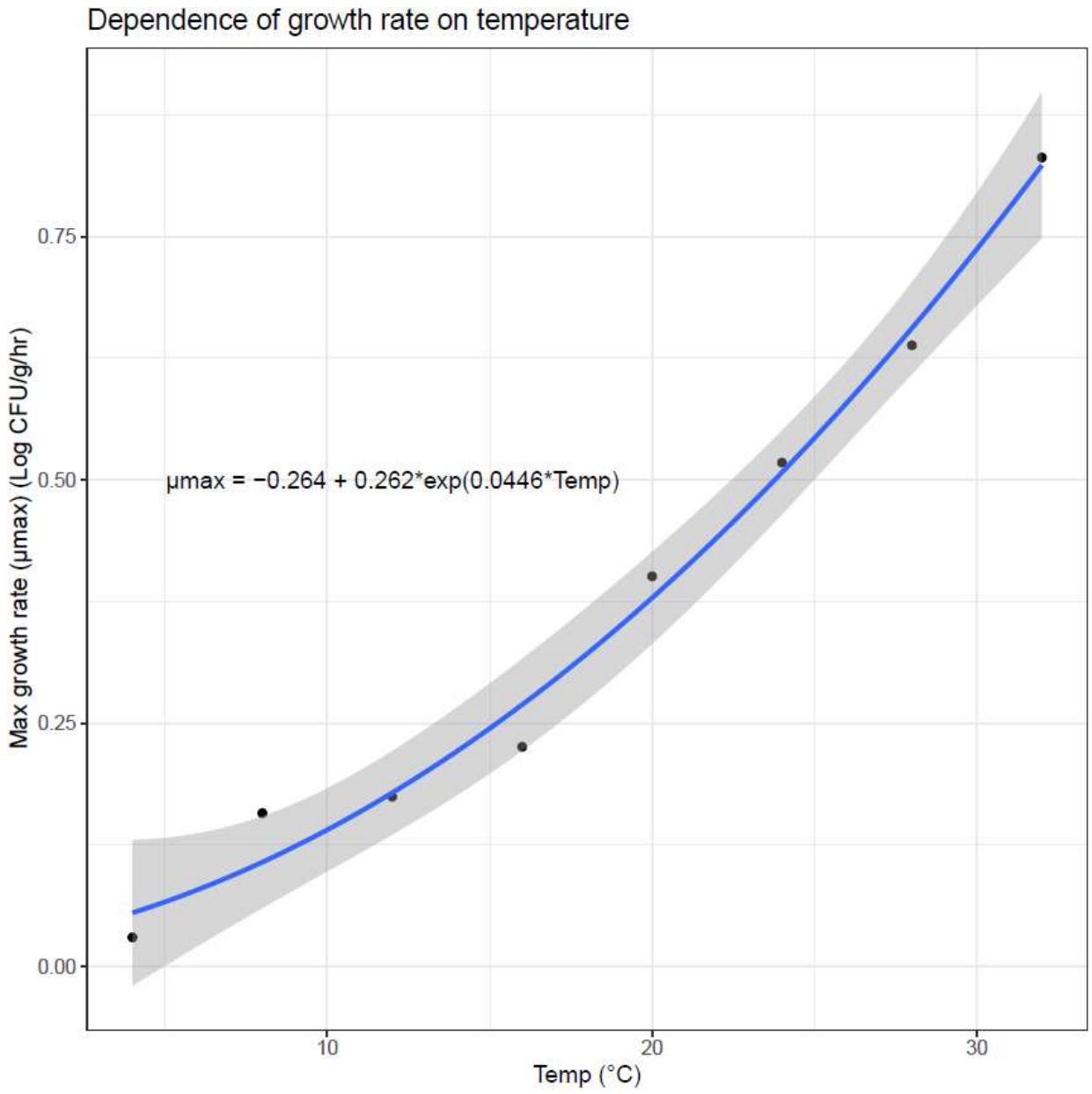

Figure 9. Dependence on temperature of *L. monocytogenes* growth rate μ_{max} on fresh-cut cantaloupe.

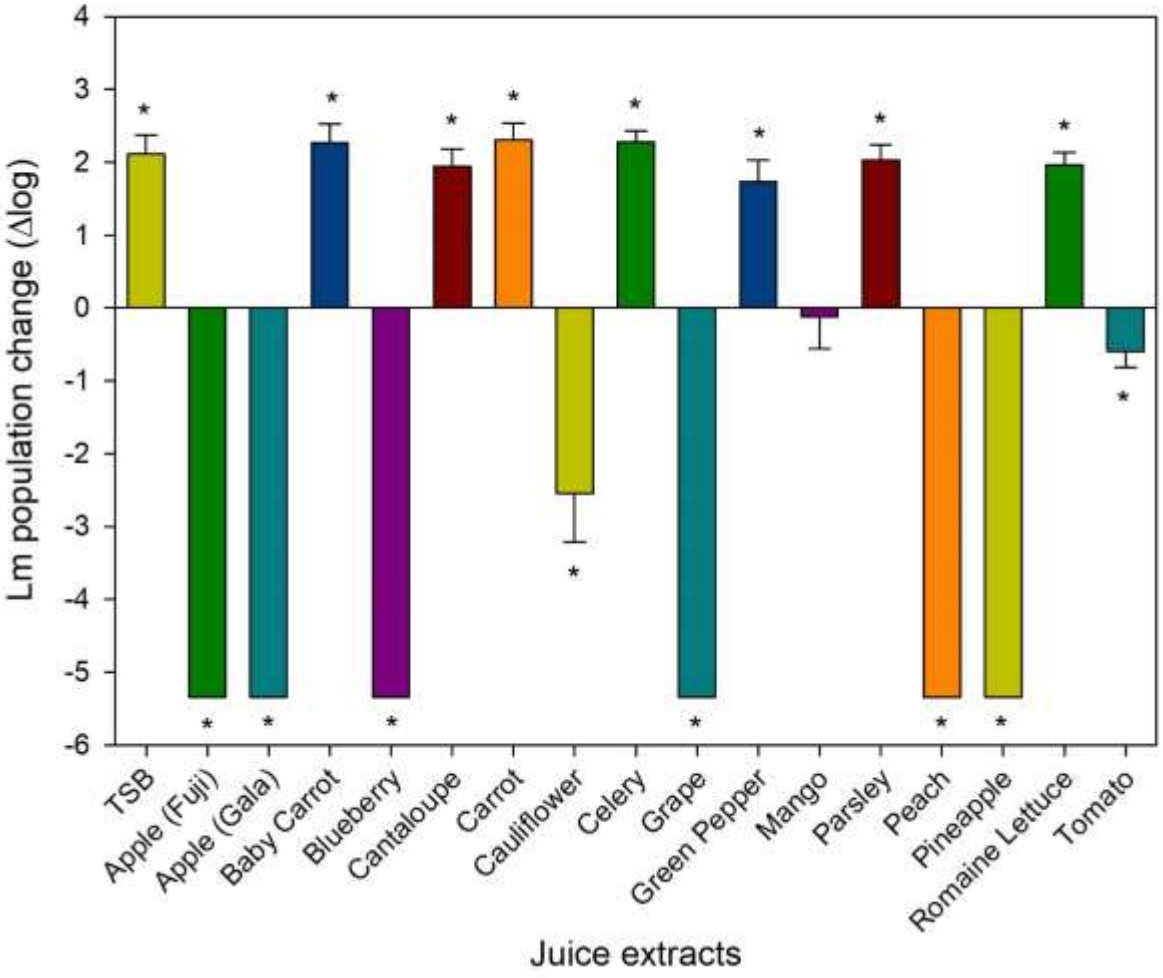

Figure 10. Change in plate counts of *L. monocytogenes* after 24 hours incubation in different juice extracts at 37 °C compared to inoculation level. Data shown represent the mean of three independent experiments with standard deviation (n=3). The differences between the means compared to the inoculation were considered significant if $P<0.05$ and were marked with an asterisk.

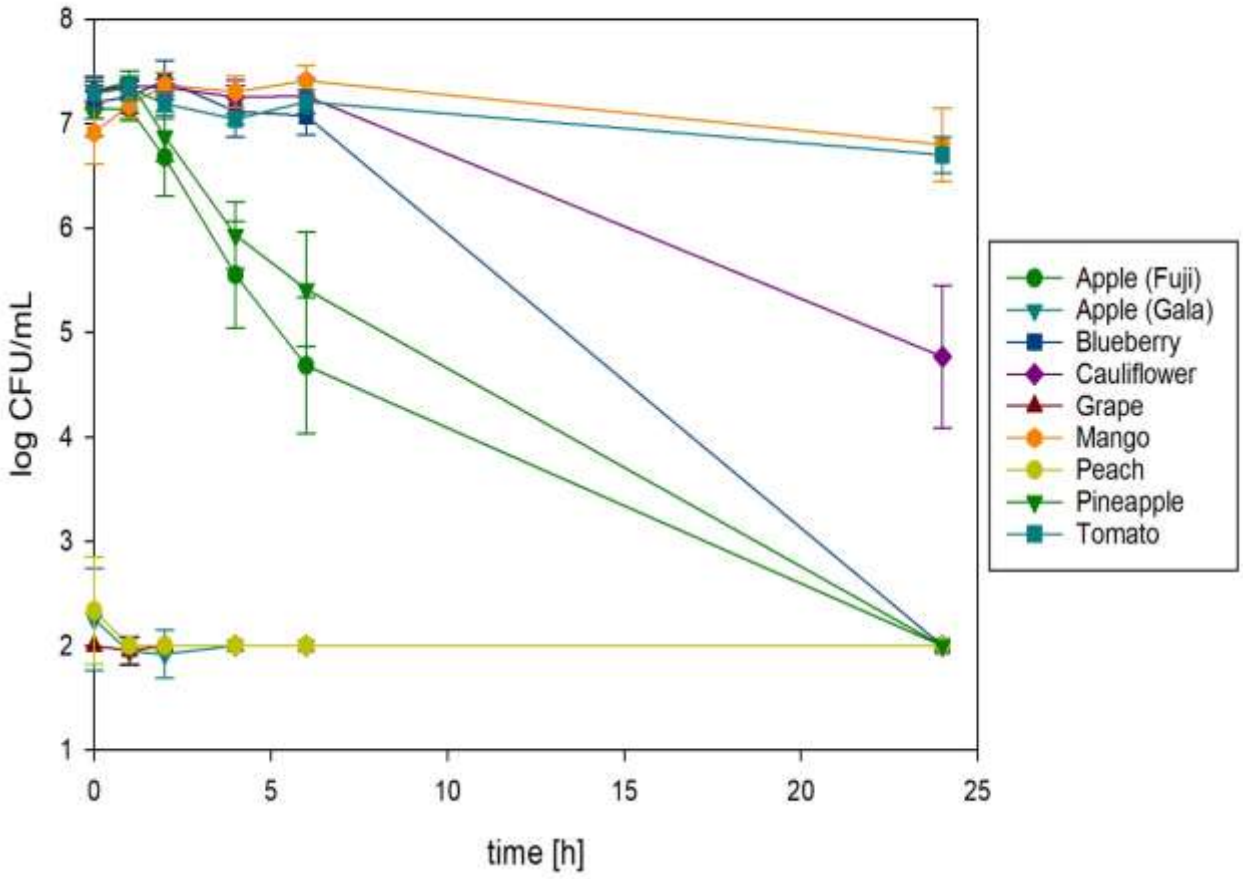

Figure 11. Survival of *L. monocytogenes* during 24 hours of incubation at 37 °C in selected juice extracts. Data shown represent the mean of three biological replicates with standard deviation (n=3). Samples with undetectable plate counts were set to 2 log CFU/mL which represents the lower detection limit.

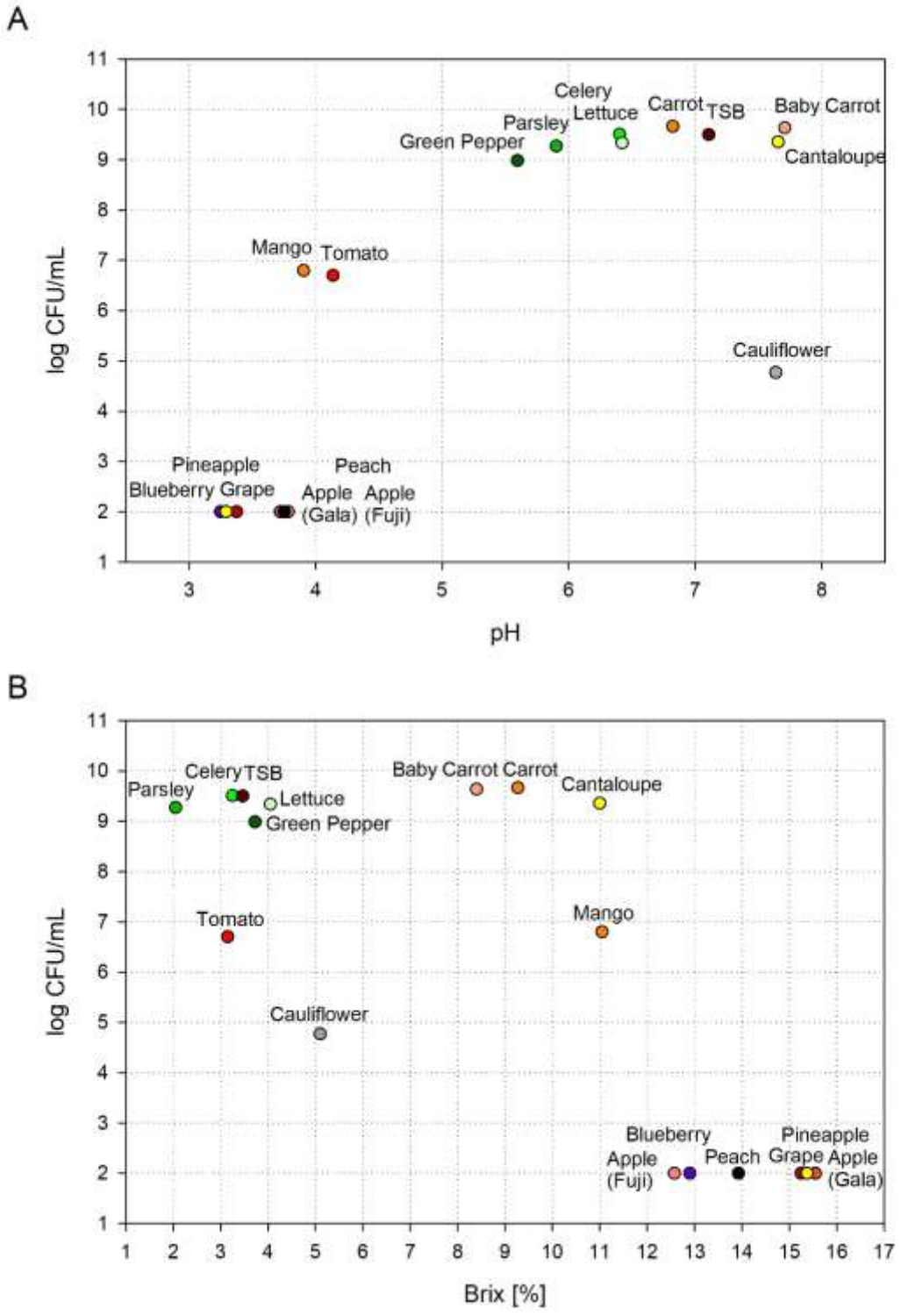

Figure 12. *Listeria monocytogenes* plate counts in log CFU/mL after 24 h incubation at 37 °C plotted against A) pH, and B) Sugar content (% Brix) of 100% juice extracts.

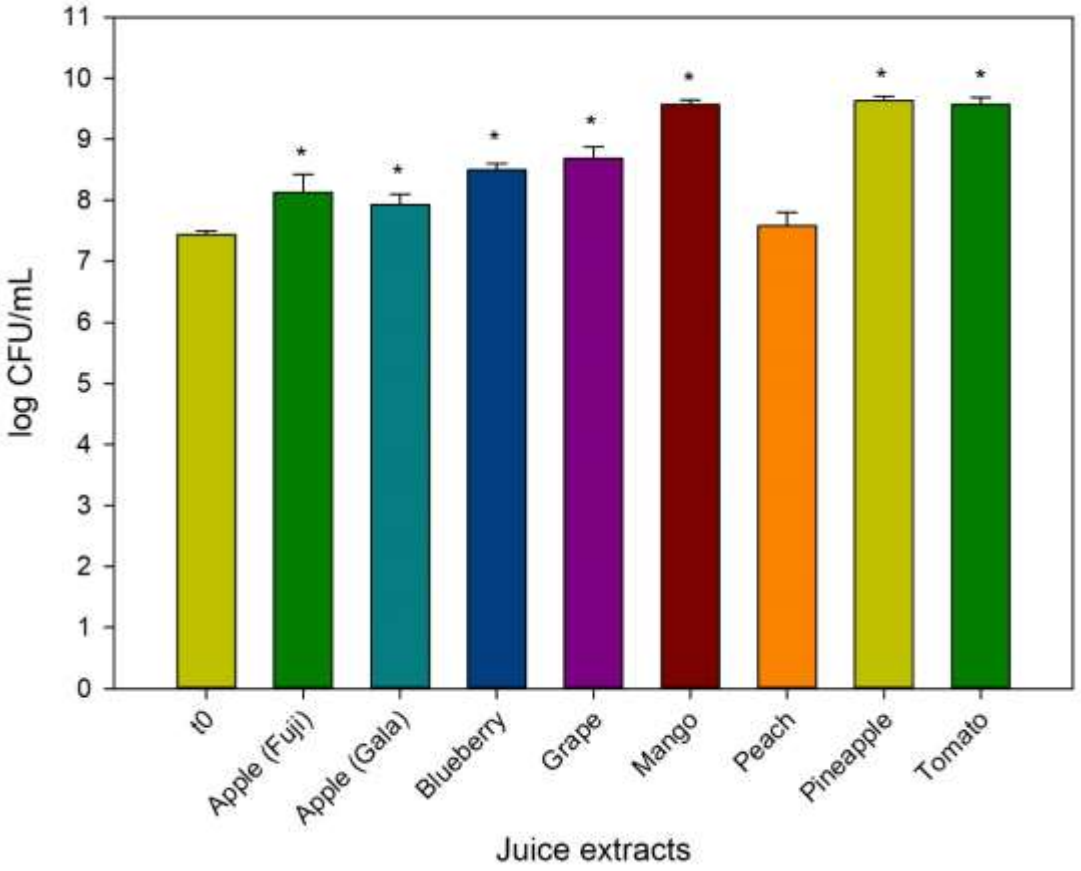

Figure 13. Plate counts of *L. monocytogenes* after 24 hours incubation in different juice extracts with an adjusted pH 7 at 37 °C. Data shown represent the mean of three biological replicates with standard deviation (n=3). The differences between the means compared to inoculation at timepoint zero (t0) were considered significant if $P<0.05$ and were marked with an asterix.

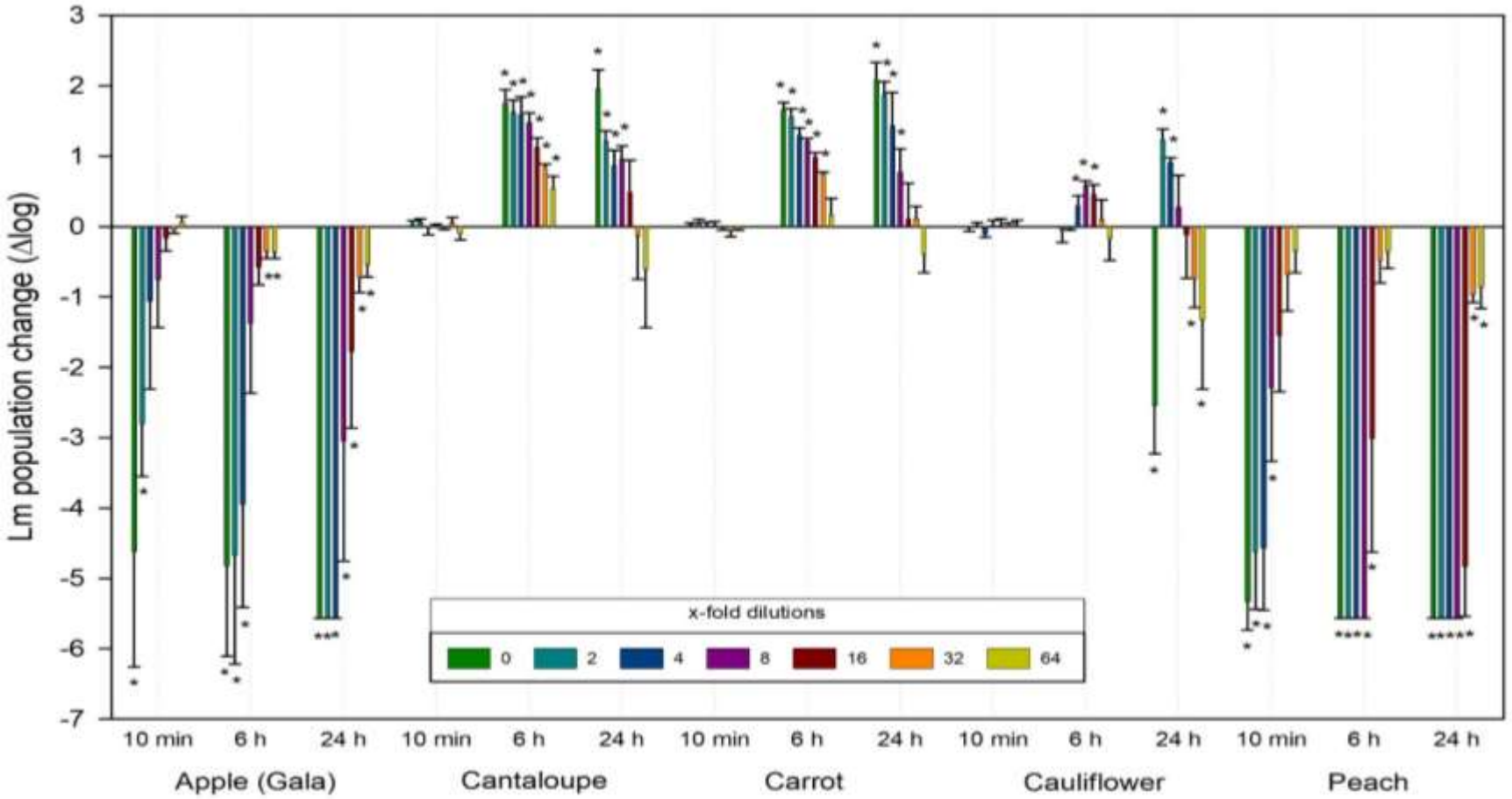

Figure 14. *L. monocytogenes* growth and survival at 37 °C in selected juices following 2-fold serial dilutions.
L. monocytogenes populations were determined immediately after inoculation (<10 min), and after 6 and 24 h incubation. Data shown represent the mean of three independent experiments with standard deviation (n=3). The differences between the means compared to the inoculation level were considered significant if $P<0.05$ and were marked with an asterix.

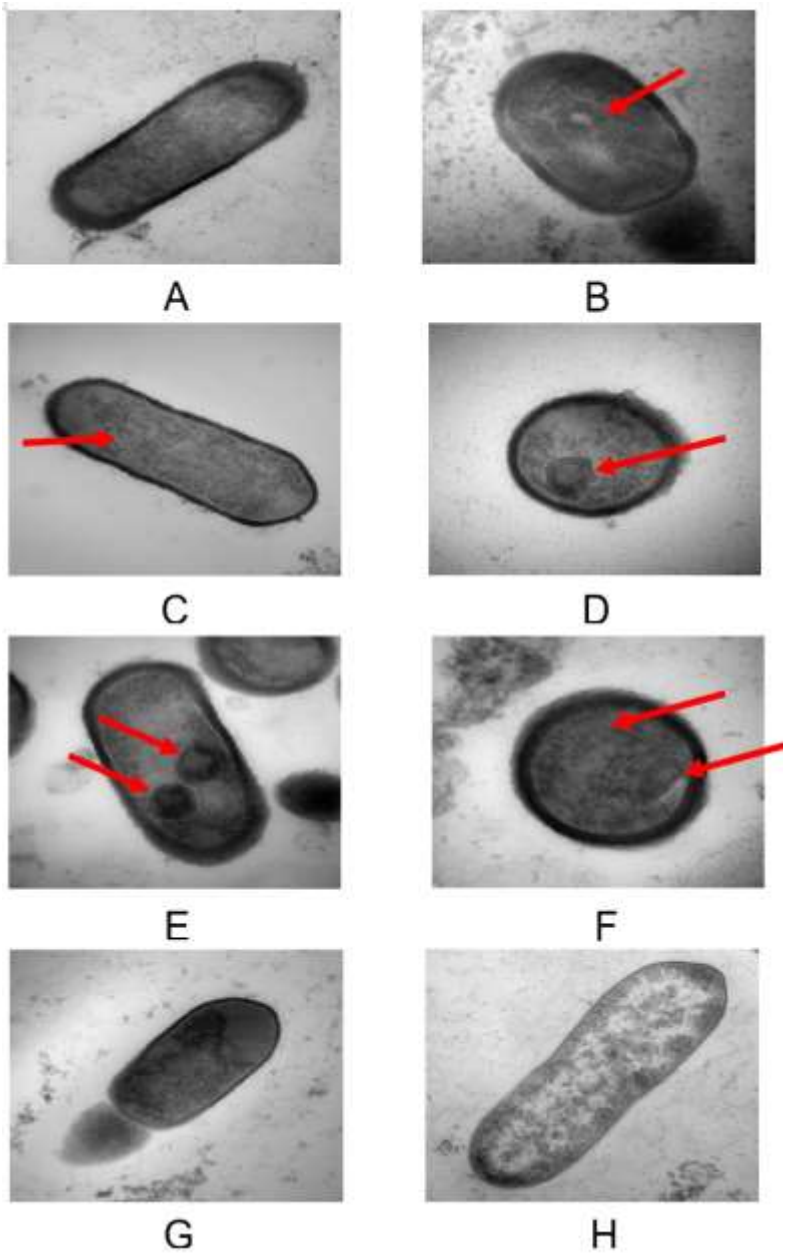

Figure 15. Transmission electron microscopy showing mesosome formation in *L. monocytogenes* exposed to carrot surface for defined time intervals. For all exposure time intervals <24 h, carrots were stored at 22 °C. Red arrows point to membrane invaginations (mesosomes). A: *L. monocytogenes* exposed to carrot for 1 min; B: *L. monocytogenes* exposed to carrot for 5 min; C: *L. monocytogenes* exposed to carrot for 10 min; D: *L. monocytogenes* exposed to carrot for 30 min; E: *L. monocytogenes* exposed to carrot for 2 h; F: *L. monocytogenes* exposed to carrot for 24 h at 4 °C; G: *L. monocytogenes* exposed to boiled carrot (carrot that had been held in 100°C distilled water for 10 min) surface for 30 min; and H: *E. coli* MG1655 exposed to carrot for 30 min.

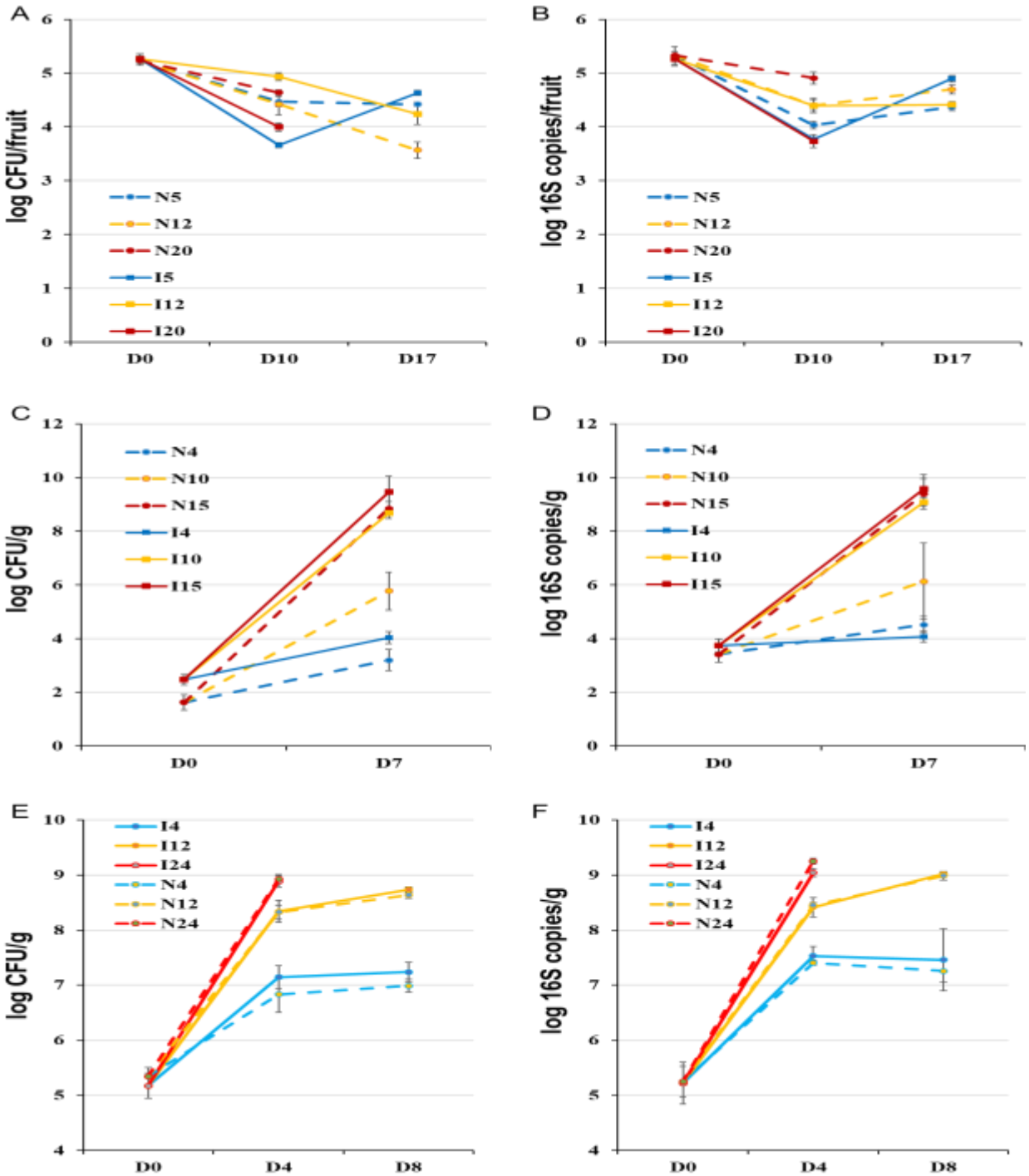

Figure 16. Total mesophilic bacteria (MAB) populations on whole avocado (A, plate count; B, 16S rDNA gene copies estimated by PMA-qPCR), fresh-cut cantaloupe (C, plate count; D, 16S rDNA gene copies), and fresh-cut romaine lettuce (E, plate count; F, 16S rDNA gene copies). N, non-inoculated samples; I, inoculated samples; numbers behind N and I denote storage temperatures at 4 - 24 ºC; D represents sampling date after inoculation on Days 0–17. Bars denote standard errors (n=4).

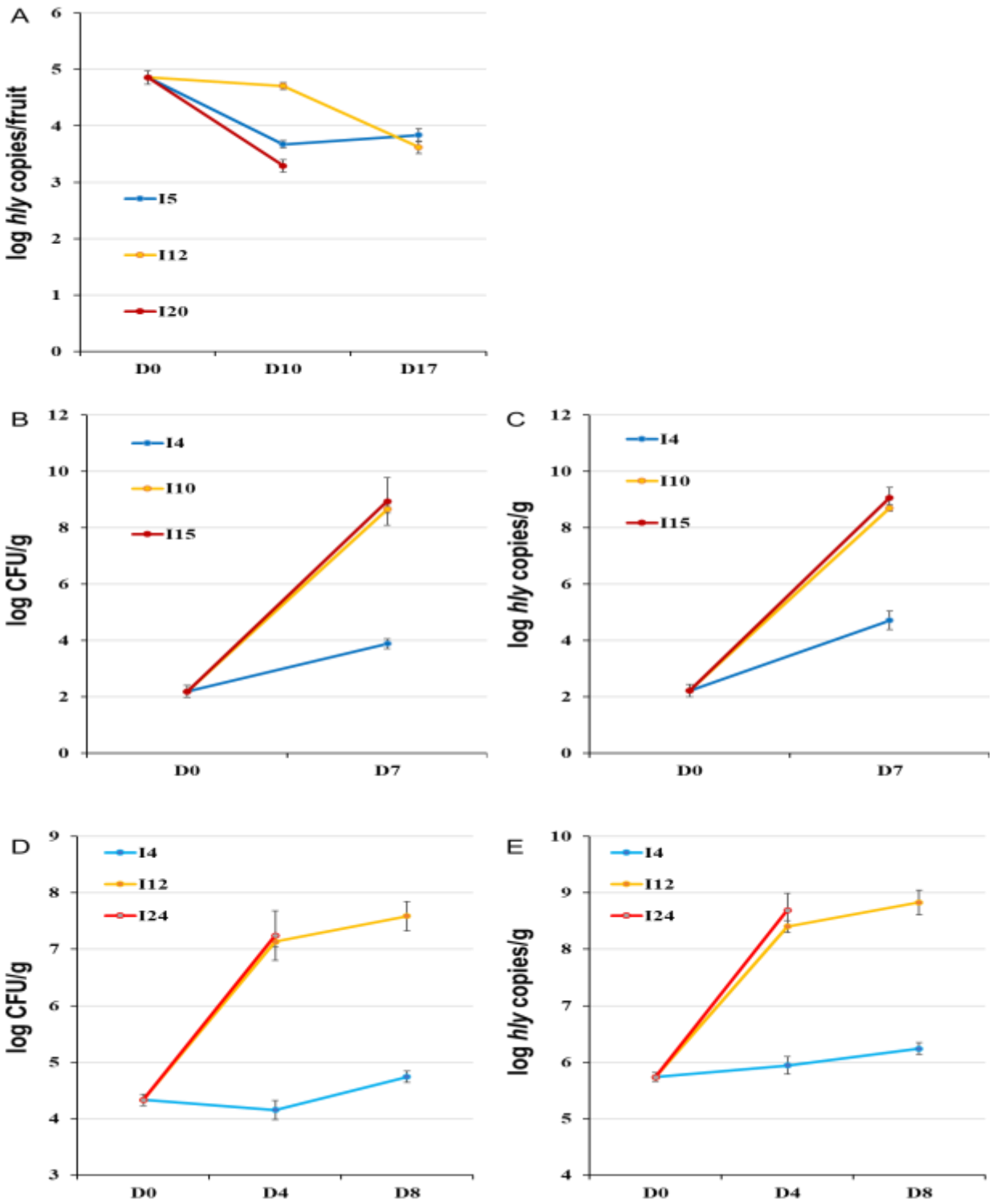

Figure 17. *L. monocytogenes* levels on inoculated avocados (A, *hly* gene copies estimated by PMA-qPCR), fresh-cut cantaloupe (B, plate count; C, *hly* gene copies), and fresh-cut romaine lettuce (D, plate count; E, *hly* gene copies). Bars denote standard errors (n=4).

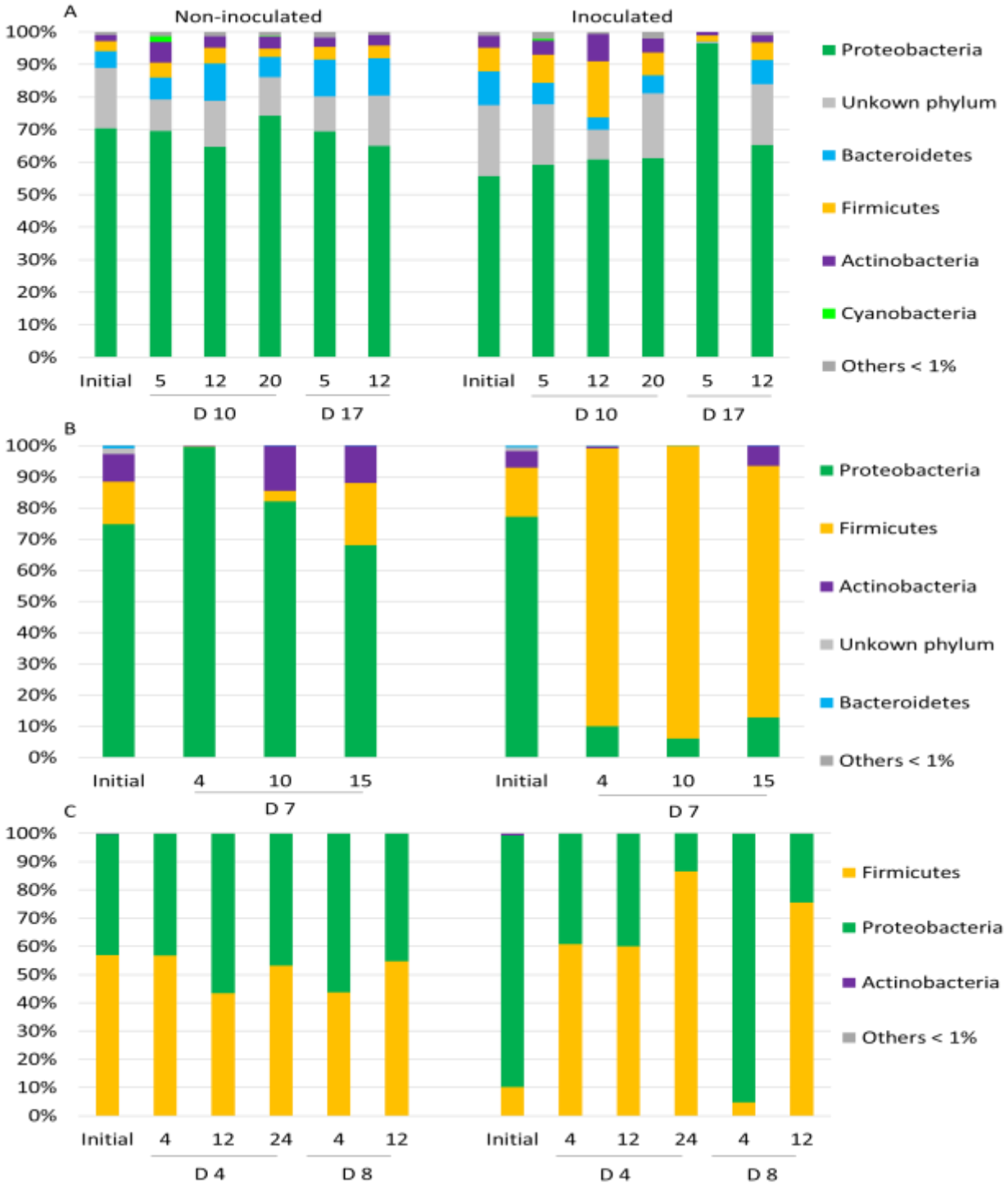

Figure 18. Relative abundance (RA) of the dominant bacteria phyla (>1% RA) on non-inoculated and *L. monocytogenes* inoculated whole avocado (A), fresh-cut cantaloupe (B), and fresh-cut romaine lettuce (C). Initial, sampling on the inoculation day; D denotes sampling days 0–8 after inoculation; numbers above sampling dates represent storage temperatures at 4–24 °C.

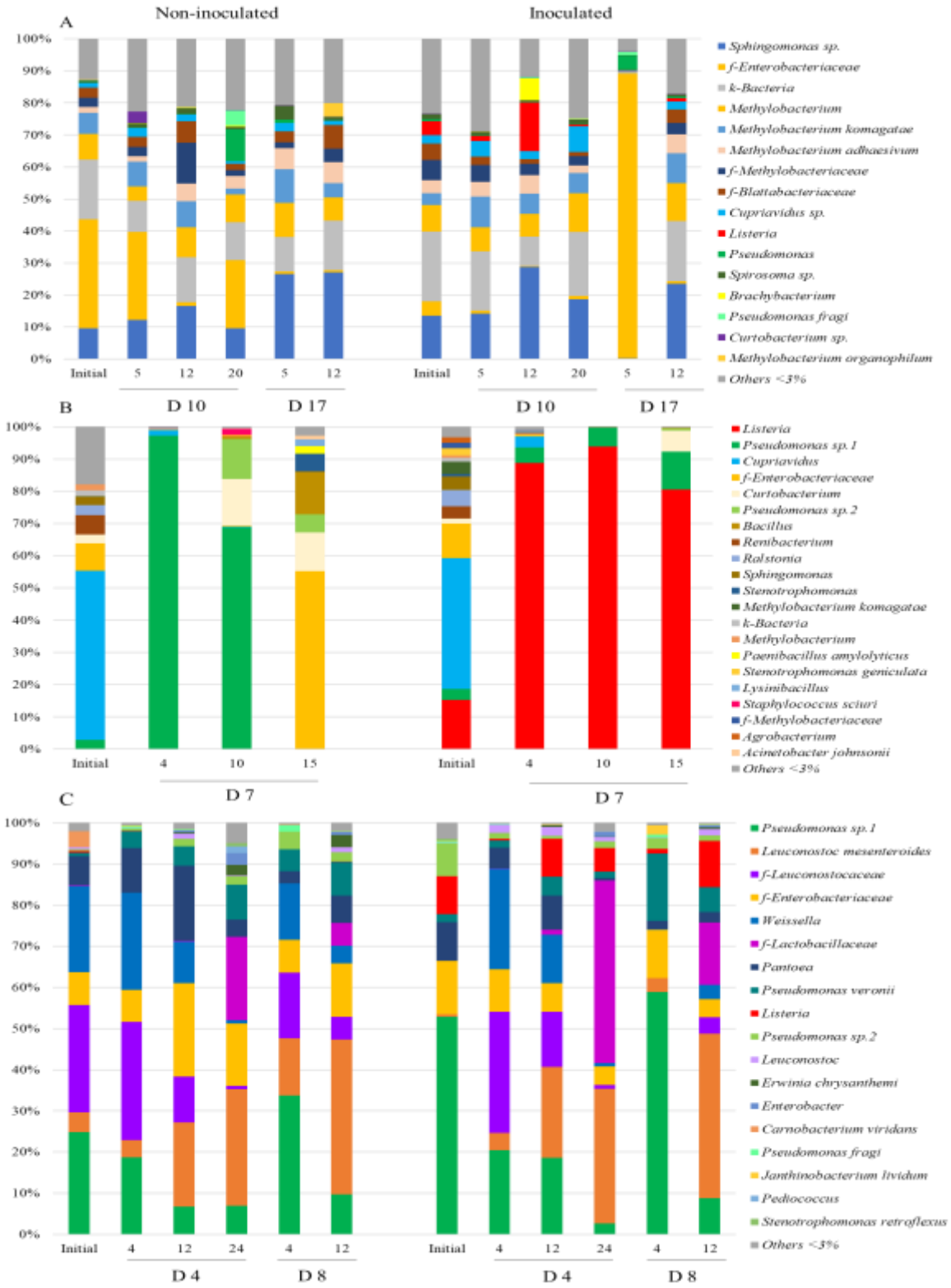

Figure 19. Relative abundance (RA) of the dominant bacteria genera and species (>3% RA) on non-inoculated and *L. monocytogenes* inoculated whole avocado (A), fresh-cut cantaloupe (B), and fresh-cut romaine lettuce (C).

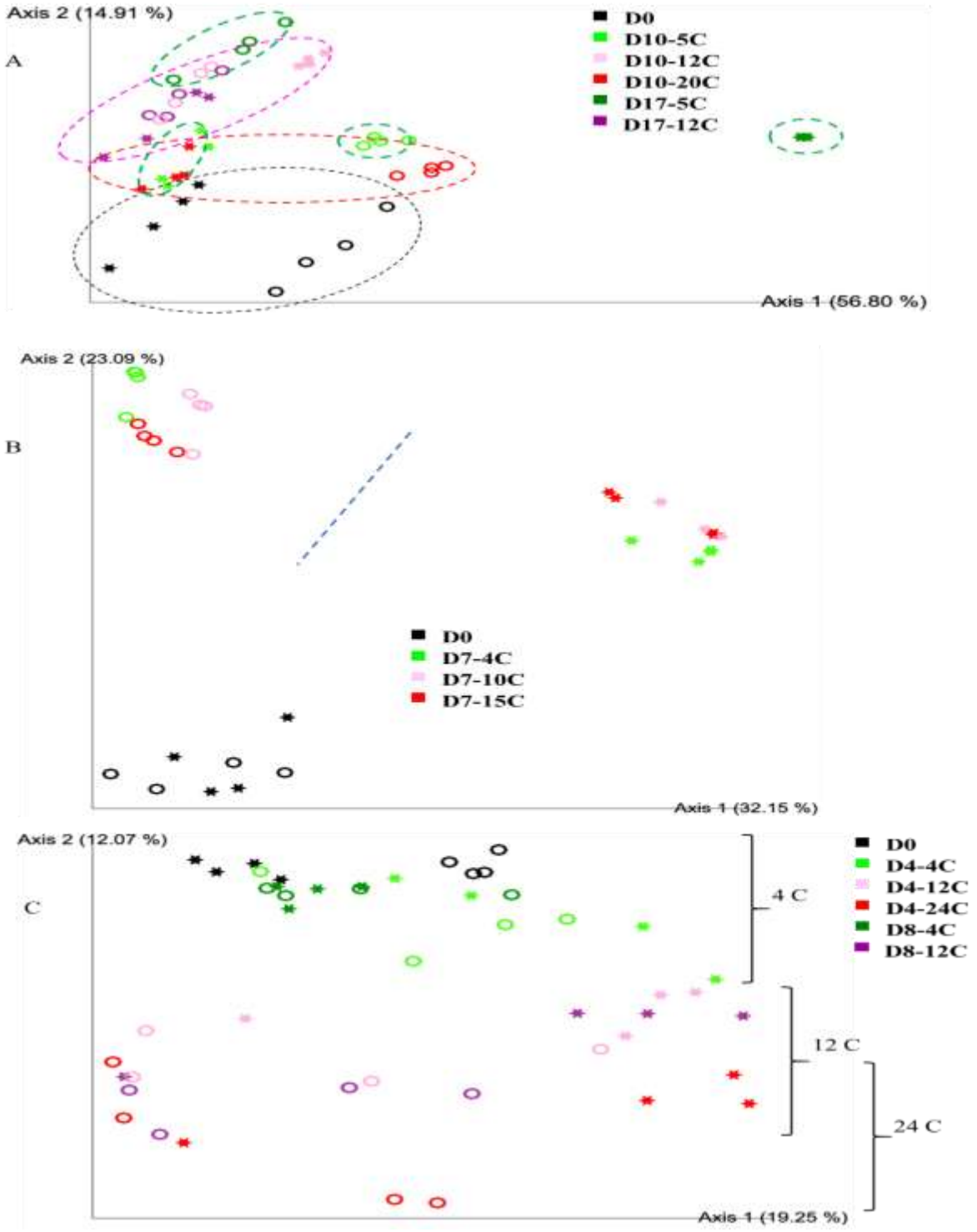

Figure 20. Microbiome relatedness of whole avocado (A), fresh-cut cantaloupe (B), and fresh-cut romaine lettuce (C) during storage at refrigerated and abusive temperatures. Principle coordinate analysis (PCoA) was performed to compare bacterial communities on non-inoculated produce samples (denoted by ring) and *L. monocytogenes* inoculated samples (represented by star). Different colors of rings and stars denote the tested sampling date (D0–D17) and storage temperature at 4–24 °C.

Formulas

Baranyi model, full:
$\log N = \log N_{max} + \log(-1 + e(mu_{max} * lag) + e(mu_{max} * t))/(e(mu_{max} * t) - 1 + e(mu_{max} * lag) * 10(\log N_{max} - \log N_0))$,

where logN is log CFU/g at time t, N_0 is the initial cell concentration, N_{max} is the maximum cell concentration, mu_{max} is the maximum specific growth rate, t is time in hours, and lag is the length of the lag phase.

Exponential model for secondary parameters:
μmax units: (Log CFU/grams/hour)
$\mu_{max} = -0.264 + 0.262*\exp(0.0446*Temp)$
lag = 66.822 * e^(-0.124605 * t),

where t is time in hours.