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CPS 2021 RFP
FINAL PROJECT REPORT

Project Title
Assessing Romaine lettuce “Forward Processing” for potential impacts on EHEC growth, antimicrobial susceptibility, and infectivity

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Objectives

1. *Comprehensive assessment of forward processing practice under routine operation conditions for product integrity and microbiological quality.*
2. *Assessment of microbiome dynamics on Romaine lettuce from harvest to retail for products being forward and source processed.*
3. *Comparative assessment of E. coli O157:H7 outbreak strains and laboratory strains, and the impacts of different practices on EHEC outbreak strains, on cell physiology that may affect their growth potential, susceptibility to antimicrobial treatment, or virulence.*
4. *Improvement of forward processing management by applying findings from comprehensive assessment and simulation.*

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Abstract

This project addressed research needs outlined in **CPS 2021 Research Priority #2 Harvest, b. Pathogen risk increase during pre-processing time delay**. Romaine lettuce forward processing, which is an industry practice of transporting raw commodity to distant "forward" facilities for processing and regional marketing, was used as a model of "pre-processing time delay" to examine the impacts of such delay on fresh produce quality, safety, and microbial dynamics, including potential for enterohemorrhagic *Escherichia coli* (EHEC) growth/survival.

Objective 1 aimed to define forward processing operational parameters and assess its potential impacts on romaine lettuce quality and on microbial activity. Under the conditions used in this project, forward processing was typified by pre-processing delays of approximately 1 wk under consistent refrigeration and high relative humidity, and also exposure to significant atmospheric pressure fluctuations during a major segment of the transportation. These conditions seemed to favor significant microbial proliferation and hasten post-processing product aging. ***Objective 2*** aimed to describe microbiome dynamics on raw commodity over the entire process from harvest to fresh-cut, and on commercial fresh-cut product during the distribution and retail display. The significance of microbiome dynamics on the potential of foodborne pathogen growth or survival has yet to be determined. ***Objective 3*** included comparative studies to better define the underlining fitness of the outbreak EHEC strains, and to better understand why these closely related outbreak strains persisted in the lettuce production/processing niche. The outbreak EHEC strains were inoculated onto romaine lettuce and exposed to simulated forward and source processing conditions, to examine the impacts of the practices on the growth and physiology of the outbreak EHEC strains. The outbreak strains appeared to have better fitness in lettuce production environments, as exemplified by enhanced biofilm forming capacity, which was associated with decreased susceptibility to sanitizer treatment. However, data from this project do not support the proposition that the practice of forward processing increases the risks of EHEC growth or survival. Data from multiple experiments also did not support that forward processing conditions significantly impact EHEC cell physiology to increase cell susceptibility to sanitizer treatment, although an increased percentage of persister cells appeared to develop under forward processing conditions. Transcriptomic analyses of EHEC subjected to the forward and source processing conditions detected a shift in gene transcription on lettuce that could enhance persistence and survival. However, data did not reveal significant changes in gene expression between EHEC subjected to source and forward processing that would suggest any altered survival attributed to the duration of the pre-processing delay.

Background

Romaine lettuce forward-processing. The Salinas area in California and Yuma area in Arizona supply most of the leafy green vegetables consumed in the US and Canada, although the major consumer demands are from the densely populated eastern part of the continent. To improve the freshness of fresh-cut products, processing facilities are strategically built near population centers for regional marketing by processing lettuce sourced from the Salinas or Yuma regions, a process termed "Forward Processing", in contrast to fresh-cut production near the growing regions, termed "Source Processing". Although these forward processing facilities typically observe the same processing and quality control procedures as those of source processing, the practice of romaine forward processing, which involves multi-day cross-continental transportation of highly perishable raw commodity under varied conditions, is considered by industry leaders as a potential

differentiating factor. During this long-distance transportation, Romaine lettuce can potentially endure great temperature volatility, sharp aerial pressure fluctuation, and increased physical pressure due to highway bumpiness for extended periods of time. The main focus of this project was to examine the potential effects of these factors on romaine lettuce integrity and on bacterial physiology.

Recent EHEC outbreaks associated with romaine lettuce. Fresh-cut romaine lettuce has been implicated in multiple outbreaks of enterohemorrhagic *Escherichia coli* (EHEC), primarily *E. coli* O157:H7. They include recent outbreaks in 2016, 2017, 2018, 2019, and 2020. In 2019, three outbreaks involving different genotypes of *E. coli* O157:H7 occurred in the US and Canada, with all implicated products traced back to ranches in the Salinas, CA region. These outbreaks often exhibit a pattern of seasonality, with infection cases peaking in later fall. There have been several large-scale multistate outbreaks, with confirmed cases distributed across the country. However, in some cases the outbreaks are correlated to the regional distribution patterns of Romaine products from forward processing facilities. Whole genome sequencing showed that the *E. coli* O157:H7 strains in the largest 2019 outbreak constituted a cluster which was closely related to the outbreak strains of 2016, 2017, and 2018.

EHEC and fitness. Recent romaine outbreaks often involved closely related EHEC isolates that share great genomic sequence similarity with bovine fecal isolates from the same region. The frequent resurgence of those closely clustered isolates in outbreaks suggests they have developed fitness for the environment of leafy green production. Despite industry-wide efforts to prevent on-farm contaminations by focusing on irrigation water, soil amendments, and animal intrusions, the outbreak strains seemed able to contaminate and survive on romaine lettuce, posing a great challenge for post-harvest processing. *E. coli* O157:H7, a zoonotic pathogen with cattle and other ruminants as prominent reservoirs, is capable of survival in the produce growing environment especially in soil and irrigation water. *E. coli* O157:H7 environmental isolates generally had higher fitness and exhibited slower decline than clinical isolates in aerosolizable soil. Interestingly, clonal variations with mutations in the *rpoS* gene were more frequently observed with the clinical isolates, which resulted in varied expression of fitness related genes. Survival of *E. coli* O157:H7 on lettuce phyllosphere typically follows a biphasic pattern in which the pathogen population decays rapidly at the initial stage, which in turn is followed by a slow and steady decay.

EHEC cell physiology, persisters, and VBNC. A sub-population of cells in an *E. coli* O157:H7 culture, termed "persisters", is capable of long-term survival. It was estimated that the fraction of persister populations can reach 0.2% when *E. coli* O157:H7 cells were inoculated on lettuce leaves, depending on the conditions permitting or inhibiting growth. The mechanisms for the occurrence of persister cells are not clear. *E. coli* O157:H7 populations grown from persister cells showed similar biphasic decay as the parental strains, arguing against an inheritable genetic modification. Therefore, it may reflect a certain physiological state of the cells in a population. On the other hand, *E. coli* O157:H7, like many other bacterial species, can lose cultivability under stressful environments such as starvation, desiccation, or chemical stress. But these cells retain some residual metabolism and are deemed viable, entering a physiological state referred to as viable but nonculturable (VBNC). Like persisters, bacterial cells in the VBNC state also exhibit enhanced tolerance or resistance to antimicrobial treatment. The induction of VBNC state in foodborne pathogens is a critical concern for food not subjected to a "kill step" before consumption, such as lettuce and other leafy greens, since the VBNC state can be reverted (resuscitation) under certain circumstances. The conditions for the long-haul transportation of freshly harvested romaine lettuce required for forward processing, with or without temperature abuse, could have profound effects on the physiology of EHEC cells. It could result in enhanced growth or decreased susceptibility to antimicrobial treatment. Due to the lack of cultivability,

bacterial cells in VBNC state are a challenge for detection. However, culture-independent methods, including various qPCR after selective sequestration of DNA from dead cells by the application of membrane impermeable dye propidium monoazide (PMA), have proven an effective approach to quantify VBNC cells in various food matrices.

Fresh produce microbiome. The surfaces of fresh produce such as leafy greens harbor a diverse and dynamic microbiome. A large-scale microbiome survey with romaine lettuce from the Salinas and Yuma production areas showed that the phyllosphere carried 10^{5-6} CFU/g of bacteria that are dominated by *Proteobacteria, Firmicutes, Bacteroidetes* and *Actinobacteria* at the phylum level and *Pseudomonas*, *Bacillus*, *Massilia*, *Arthrobacter* and *Pantoea* at the genus level as core romaine phyllosphere microbiota. Changes in environmental conditions could also significantly alter the microbiome dynamics on lettuce, such as that Enterobacteriaceae sequences were often overrepresented in summer instead of winter lettuce samples. Biotic stress could also shift the abundance of Enterobacteriaceae on lettuce leaves. It can be expected that shifts in lettuce leaf microbiome could, in turn, influence EHEC survival and physiological changes, including the expression of genes involved in stress responses and tolerance, biofilm formation, aggregation and virulence.

Research Methods and Results

Objective 1. Comprehensive assessment of forward processing practice under routine operation conditions for product integrity and microbiological quality.

Operational parameters of "forward-processing". Two approaches are used to determine the operational parameters for romaine lettuce forward processing: analyzing existing industry transportation data and actively collecting transportation data during routine industrial operations. Time/temperature profiles for 32 randomly selected truck recordings over 1 year to a forward processing facility on the East Coast showed that the trans-continental transportation took 3-4 days under stably maintained temperature control. The temperature in delivery trucks during romaine lettuce transportation (after trailer loading at 0 h) of each analyzed trip was below 4°C, while brief abusive temperature above 5 °C was rarely recorded (**Fig. 1.1**). The transported romaine lettuce is typically held in inventory for an additional 2-4 days at the forward facilities before being processed.

Data on forward and source processing operational parameters was also collected in conjunction with microbial dynamics data collection. In both the summer and fall trials, romaine lettuce from a one-acre commercial lot was harvested as per normal industry practice and evenly split for destinations to a source processing facility in the Salinas area and a forward processing facility on the East Coast. Three sets of paired bins, each embedded with a RC-51H PDF temperature and humidity data logger and a barometric Track-it logger to record time and range of air pressure changes, were randomly placed in the cargo trunks destined for source and forward processing facilities (**Fig. 1.2**). Following initial precipitous temperature and air pressure drop during vacuum cooling, temperature (<5°C) and humidity (>90%) were stably maintained during transportation and inventory holding time prior to processing for both source and forward processing destinations (**Fig. 1.3**). However, the processing date of lettuce at the forward processing facility was about one week later than that at the source processing facility (1 day after harvest). Notably, decreases in atmospheric pressure of up to 20 kPa were recorded during the transportation of lettuce to the forward processing destination, which matched with altitude changes when driving through mountainous regions. Similar trends in physical parameters were observed for both summer and fall samplings.

Microbial dynamics on romaine lettuce during transportation and post-processing storage. Samples of whole head romaine lettuce retrieved from the tracked bins prior to initial vacuum cooling and after delivery/inventory storage in source or forward processing facilities were collected and manually cut in the lab to 1-inch pieces for quality and microbial analyses. The lots of remaining raw romaine lettuce were subjected to cutting, washing with chorine-based sanitizer, and modified atmosphere packaging processes at the designated industrial facilities following common established procedures. Afterward, bags of packaged fresh-cut lettuce from both source and forward processing facilities were collected and stored at 4°C for up to two weeks, with bags of fresh-cut lettuce taken as samples on days 0, 1, 4, 7, and 14 for quality and microbial analyses. Note that the storage time of a bagged product from source or forward facilities reflected its post-processing age instead of post-harvest age, for which the product from the forward facility was about 1 wk older than the corresponding product from source.

For microbiological analyses, microbial cells were recovered from lettuce into saline by ultrasound sonication and plated on 3M AC, EC, and YM Petrifilm plates for total aerobic bacteria (TAB), total coliform (TC), yeast and mold (YM) enumerations. AC, EC, and YM Petrifilm plates were incubated at 30°C, 37°C, and 24°C for 72 h, 48 h, and 5 d, respectively. Colonies were enumerated using a Petrifilm Plate Reader.

In general, populations of TAB, TC, and YM increased during delivery for forward processing, declined after washing, and rebounded during storage for both source and forward processed products (**Fig. 1.4, Table 1.1**). Populations of TAB and TC of both whole head lettuce before processing and fresh-cut lettuce after storage from forward processing facilities were significantly higher than those from source processing facilities in both summer and fall production seasons. However, there was no obvious difference for YM counts in the summer trial, especially after storage. YM populations were about 1 log higher on processed fresh-cut lettuce after storage from forward processing facilities than that of source processing. In both summer and fall trials, the reduction of TAB after processing on source processed products was significantly higher ($p < 0.01$) than the reduction of forward processed products. The microbial populations including TAB, TC, and YM of field harvested lettuce (F) and forward processed products after cold storage (D1 to D14) were higher in the fall trial than in those in the summer trial ($p < 0.05$), while there was no significant difference for most source processed products between the two trials (**Table 1.1**).

Produce quality impacted by the forward processing conditions. Changes in romaine lettuce quality were assessed by measuring electrolyte leakage and by visual scoring. Electrolyte leakage, measured as increase in electrical conductivity, reflects lettuce tissue integrity. The loss of tissue integrity as measured by the increased electrolyte leakage, especially after 2-week storage, was significantly higher for forward processed than source processed products in both summer and fall trials (**Fig. 1.5**).

Produce visual quality was assessed by an in-house sensory panel of 10 trained judges for the fall trial. Due to time and location constraints in panel availability, high resolution digital images of cut romaine lettuce taken at time of sampling were used for visual assessment. Average quality ratings over time for all lettuce sections and overall visual quality (OVQ) scores followed similar patterns. Correlations between OVQ and leaves with veins, leaves without veins, rib, and heart sections are shown in **Table 1.2**. For source processed products, average OVQ scores for images from different timepoints were not significantly different up to D14 (**Fig. 1.6, Table 1.3**). For forward processed products, average OVQ scores significantly declined by D7. The forward processed product at D14 had the lowest average OVQ score. Browning was the major blemish diminishing the OVQ scores of the forward processed products.

Objective 2. Assessment of microbiome dynamics on Romaine lettuce from harvest to retail for products being forward and source processed.

Quantitative real-time PCR (qPCR) and 16S rRNA gene high-throughput sequencing were performed to analyze microbial communities on the romaine lettuce samples. Bacterial DNA of each sample was extracted using the DNeasy PowerLyzer PowerSoil Kit and qPCR targeting a highly conserved 180 bp portion of 16S rDNA gene was performed to estimate the total 16S rRNA gene copy numbers. Standard curves were generated in every qPCR run using serial dilutions of chromosomal DNA extracted from *E. coli* of known concentration using the same method. Cycle threshold numbers and baseline were determined automatically using the Noiseband algorithm. All qPCR amplifications were performed in triplicate. The growth of total bacteria estimated by qPCR and microbiome analysis followed the same trend as plate count: bacterial levels of fresh-cut lettuce from forward processing facilities were significantly higher than those from source processing facilities (**Fig. 2.1**).

High-throughput (HT) sequencing targeting 16S rRNA gene was carried out using both Illumina MiSeq and Nanopore MinION. The library preparation and sequencing run of Illumina sequencing were conducted using barcoded primer sets, 515F - 806R, and MiSeq Reagent Kit v3 (600-cycle). For Nanopore sequencing, DNA from the six replicates of each type of samples were combined and the 18 composite DNA samples were used for library preparation using the 16S barcoding kit SQK-16S024, according to the manufacturer's protocol of version 16S_9086_v1_revT_14Aug2019. The 16S rRNA gene sequencing data generated by Illumina MiSeq and Nanopore MinION have been submitted to NCBI (Accession No.: PRJNA939835).

The compositions of microbial communities analyzed by Illumina MiSeq and Nanopore MinION were comparable, and were significantly affected by romaine lettuce production season, post-harvest transportation, and processing condition (**Figs. 2.2** and **2.3**). Illumina sequencing of the 108 romaine lettuce samples produced 3,858,726 paired reads matching with bacterial 16S rRNA genes, which yielded 1,910 amplicon sequence variants (ASVs) by querying the SILVA database. At least 84.4% and 98.6% of the identified bacterial ASVs were assigned to genus and family levels, respectively. Proteobacteria, Firmicutes and Actinobacteria were the top three phyla on sampled romaine lettuce, with average relative abundance (RA) of 73.9%, 13.7%, and 9.4%, respectively (**Fig. 2.2A**). At the genus level, *Pseudomonas* (36.4%), *Pantoea* (17.0%), and an unassigned genus of family *Yersiniaceae* (4.6%) were the most abundant bacterial genera on romaine lettuce (**Fig. 2.3A**). Nanopore sequencing of the 18 composite DNA samples produced 952,462 long reads of the bacterial 16S rRNA gene. Querying the NCBI database yielded 2,232 species, 458 genera, and 15 phyla. Similar to Illumina sequencing results, Proteobacteria (82.4%) and Firmicutes (11.3%) were the most dominant bacterial phyla on romaine lettuce (**Fig. 2.2B**). The dominant native bacterial genera were identified as *Pantoea* (24.4%), *Pseudomonas* (21.0%), *Xanthomonas* (5.3%), *Erwinia* (4.7%), and an unassigned genus of the family *Yersiniaceae* (4.4%) (**Fig. 2.3B**). The shifting patterns of certain dominant taxa were differently impacted by production seasons, processing type and storage: RA of phylum Firmicutes was higher on source processed samples than that on forward processing; *Xanthomonas* was identified as a top genus on forward processed fresh-cut lettuce in fall, but not in summer or source processed samples; RA of *Pseudomonas* and *Pantoea* increased after cold storage, while the RA of *Bacillus* decreased.

Alpha diversity (Shannon index, accounting for both richness and evenness) of romaine lettuce microbiota was significantly higher in the fall production season (4.27 ± 0.88) than that in summer (3.09 ± 0.78) (**Fig. 2.4**). The diversity of bacterial communities on fresh-cut lettuce decreased after one week of storage (Fig. 2.4). Relatedness of the microbiome on romaine lettuce was significantly affected by production season, processing type and storage (**Fig. 2.5**). The initial bacterial communities on summer- and fall-grown lettuce were clearly separated. Based on the

lumped data of two seasons, lettuce microbiota was not significantly changed after source processing delivery and inventory storage, while the shift in forward processed samples before processing (B) was significantly relative to field samples (F). The relatedness of lettuce microbiota on fresh-cut products became closer after cold storage: the difference between D7 and D14 samples were not significant.

The absolute abundances of total and dominant bacteria were inferred based on the qPCR enumeration of 16S rRNA gene copy numbers and taxa specific relative abundance (**Figs. 2.6** and **2.7**). The levels of dominant bacteria on lettuce determined by Illumina MiSeq and Nanopore MinION were comparable. The shifts in most abundant bacteria (>4 log 16S copies/g) on lettuce were similar to that of total bacterial populations in both summer and fall seasons: increased during transportation especially for forward processing, reduced after washing, and proliferated during storage. For B samples (whole head lettuce collected before processing), the levels of most identified bacteria were higher on lettuce from the forward processing facilities than those from source processing. However, several interesting exceptions were observed with both sequencing datasets: levels of *Massilia* obviously decreased after processing and storage in summer and on source processed products in fall, but proliferated on forward processed products in fall; *Bacillus* populations declined after processing and storage on both source and forward processed products in both seasons; *Weissella* increased after storage for source processed fresh-cut lettuce, but did not proliferate on forward processed products; during cold storage, populations of *Acinetobacter* on fresh-cut lettuce decreased in summer, but increased in fall. Illumina sequencing data also showed that *Curtobacterium*, *Stenotrophomonas* and a genus of family *Microbacteriaceae* on fresh-cut forward processed products in summer decreased during cold storage but increased in the other conditions. Based on Nanopore sequencing, a genus in the family *Bacillaceae* decreased during cold storage in summer but increased in fall.

Objective 3. Comparative assessment of E. coli O157:H7 outbreak strains and laboratory strains, and the impacts of different practices on EHEC outbreak strains, on cell physiology that may affect their growth potential, susceptibility to antimicrobial treatment, or virulence.

E. coli O157H7 romaine outbreak strain characterization. Two *E. coli* O157:H7 (EcO157) strains, 2705C and 2705D isolated from lettuce during the outbreak in 2019, and one reference strain, EDL933, were adapted for rifampin resistance (RifR). The adapted RifR strains showed the same growth and survival trends as the original EcO157 strains in liquid media and on romaine lettuce. Sensititre™ Gram Negative NARMS Plates were used for antimicrobial susceptibility tests of the three EcO157 strains including 14 common antibiotics. Both outbreak and reference EcO157 strains were susceptible to all tested antimicrobials (**Table 3.1**).

Biofilm formation and susceptibility to sanitizer. Lettuce outbreak EcO157 strains, 2705C and 2705D, were evaluated and compared to a reference strain, EDL933, and a 2006 spinach outbreak strain, FS4157, for biofilm formation. Biofilm mass was measured by light absorbance after crystal violet staining. The biomass of lettuce outbreak strains (2705C and 2705D) was 0.91 and 0.88, respectively, which was significantly higher than that of the reference strain (EDL933) and the spinach outbreak strain (FS4157), with OD values of 0.12 and 0.12, respectively (**Fig. 3.1A**). Although the numbers of planktonic cells were similar for all tested strains, with an average value of 8.68 log CFU/mL (**Fig. 3.1B**), the biofilm cells of 2705C and 2705D were more than 2 logs higher than those of EDL933 and FS4157. The results indicate that lettuce outbreak strains were more potent in forming biofilms in 1/10 TSB than the reference strain and the spinach outbreak strain, which might benefit their persistence in the environment.

Biofilm resistance to sanitizer QAC was evaluated for the outbreak and reference strains after biofilm formation on stainless steel coupons in different media including M9, 10% TSB, and 10%

lettuce juice (LJ). Coupons were treated with 200 mg/L of QAC for 1 min, and the biofilm cells on coupons were recovered for enumeration by selective plating. Significantly higher biofilm populations were found in 2705C and 2705D in M9 medium than those in EDL933 and FS4157 (**Fig. 3.2A**). Biofilms formed by the outbreak strains in M9 and in 10%TSB were more resistant to QAC treatment compared to those by the reference strains, with average log reductions of 0.04 and 4.57 in M9, and 0.44 and 4.37 in 10%TSB, respectively (**Fig. 3.2B**). When observed under a confocal laser scanning microscope, biofilms formed by 2705C were dense and thick, while individual cells or small clusters of cells were observed in biofilms formed by EDL933 (**Fig. 3.3**). More cellulose (indicated by the blue color) was produced by 2705C than by EDL933. The complex biofilm structure and the thicker layer of cellulose might explain the stronger QAC resistance of biofilms formed by the outbreak strains.

Comparative genomics. Roary, a software that provides detailed information on gene presence/absence in each genome, was used to compare the whole-genome sequences of seven EcO157 strains, including four from our biofilm formation analysis (2705C, 2705D, EDL933, and FS4157) and three from previous lettuce outbreaks (FS4365 from 2018, and RM4406 and RM4688 from 2006). The aim was to identify genes that could be involved in the persistence and virulence of the recent outbreak strains. These analyses identified 14 genes of hypothetical proteins with unknown functions in the recent outbreak strains (2705C and 2705D) that were missing in the other strains. In addition, extra copies of genes for 18 proteins were also identified with the outbreak strains, which included genes related to antibiotic resistance (LdcC), stress tolerance (MscM and CutA), RNA metabolism (Orn), nutrient transport and utilization (PepD, PhoE, AspA, and DcuA), polypeptide biosynthesis (PrfB), proline synthesis (ProA and ProB), phospholipid metabolism (Psd), protein synthesis (RsgA and DsbD), toxin production (StxA and StxB), curli formation (Crl), and nucleic acid synthesis (Gpt) (**Table 3.2**). The presence of these extra genes with yet unidentified functions and the redundant copies of genes could contribute to the enhanced survival and fitness of the recent outbreak strains.

Survival of EcO157 and microbiome shift on romaine lettuce under simulated conditions. The survival of EcO157 and the microbial dynamics on romaine lettuce were examined under simulated conditions for source processing and forward processing (**Fig. 3.4**) using freshly harvested romaine lettuce. The whole head romaine lettuce was inoculated with EcO157 outbreak strain 2705C or reference strain EDL933. Inoculated leaf samples were tested on day 0 (IN, initial sampling conducted immediate after vacuum cooling), day 1 (SP, source processing simulation), and day 8 (FP, forward processing simulation with air pressure fluctuation; AT, alternative test without air pressure change) post inoculation. Temperature, humidity, and air pressure parameters were set up based on the recorded SP and FP conditions under Objective 1 (**Fig. 3.5**). EcO157, aerobic bacteria (AC), yeast and mold (YM) counts were enumerated, and microbial DNAs were extracted for microbiome analyses targeting bacterial 16S rRNA gene and fungal conserved ITS sequences.

The populations of both EcO157 strains (2705C and EDL933) were higher on lettuce exposed to SP conditions than those to FP and AT conditions (**Fig. 3.6A**). There were no significant differences between SP and FP samples for AC and YM populations (**Figs. 3.6B** and **3.6C**). Proteobacteria was the most dominant bacterial phylum on inoculated romaine lettuce (**Fig. 3.7**). In addition to inoculated EcO157, *Pantoea* and *Pseudomonas* were identified as two major bacterial genera on romaine lettuce. These findings were in concurrence with previous results on lettuce microbiomes (**Fig. 3.8**). Alpha diversity of FP and AT bacterial communities significantly increased after an additional one-week of storage (**Fig. 3.9**). Principal co-ordinates analysis (PCoA) indicated bacterial communities on FP and AT samples were separated from IN (day 0) and SP (day 1) romaine lettuce (**Fig. 3.10**). The dominant fungal phyla on romaine lettuce tested in this study were Basidiomycota and Ascomycota (**Fig. 3.11**), and the major fungal genera were

identified as *Filobasidium*, *Sporobolomyces*, and *Vishniacozyma* (**Fig. 3.12**). Different from bacterial communities, the diversity of fungal communities was not significantly changed under different conditions (**Fig. 3.13**).

EcO157 persister cell development under simulated forward processing conditions. The effect of forward processing on the formation of EcO157 persister cells on inoculated romaine lettuce was assessed by enumerating the surviving cells after exposure to bactericidal antibiotics (**Fig. 3.14**). EcO157 strains inoculated on romaine lettuce and exposed to source and forward processing conditions were recovered in TSB and subjected to ciprofloxacin and ampicillin treatments for 2 h at 37°C. Surviving populations of EcO157 were enumerated by both selective and overlay plating methods. Compared to the initial population of EcO157, the percentage of persister cells detected on overlay plates was significantly higher from lettuce under forward processing conditions than from the other samples (**Fig. 3.15**). Compared to the EcO157 population in the control media without antibiotic treatment, the percentage of persister cells from lettuce under forward processing conditions was significantly higher, except the ones detected on overlay plates after ampicillin treatment (**Fig. 3.16**).

EcO157 sanitizer susceptibility under simulated forward processing conditions. Freshly harvested commercially grown romaine lettuce was inoculated with rifampicin-adapted EcO157 strains EDL933 or 2705C at ~7 log CFU/leaf. Inoculated leaves were air-dried and stored inside a refrigerated vacuum desiccator under controlled temperature, humidity, and air pressure for different durations to simulate forward and source processing conditions. Afterward, in one set of experiments, EcO157 cells were recovered by sonication in 0.9% saline and treated with sanitizers including free chlorine (Cl), peroxyacetic acid (PAA), or quaternary ammonium compounds (QACs) for 1 min. In another set of experiments, the lettuce leaves inoculated with EcO157 strains were cut and treated with sanitizers in simulated washing, followed by recovering the surviving EcO157 population via sonication in saline within 1 h of the washing treatment or after 7 days of storage at 3°C. The cell suspension was plated on MacConkey (MAC) agar supplemented with rifampicin, with or without a TSA-overlay to enhance the recovery of injured cells. Cell counts differential on plates with and without TSA overlay indicated the level of cell injury. DNA was extracted after propidium monoazide treatment, and qPCR targeting *stx2* gene was used to estimate total viable cell counts. Theoretical viable-but-non-culturable (VBNC) cell counts were estimated by subtracting total culturable counts from qPCR-estimated total viable counts.

When cells were recovered from inoculated lettuce and treated with sanitizers, the susceptibility of EcO157 was affected by lettuce processing condition, bacterial strain and sanitizer (**Table 3.3** and **Fig. 3.17**). Average survival of EcO157 recovered from lettuce under source processing conditions was higher than that for forward processing. While no difference in injury level was detected between bacteria on lettuce under source and forward processing conditions, the average theoretic VBNC level in rinse water was higher for EcO157 on lettuce from forward processing than source processing ($p < 0.01$). The overall survival of EcO157 strain EDL933 was lower than strain 2705C after the sanitizer treatment. The average theoretic VBNC level in EDL933 was higher than 2705C, despite finding no difference in injury level.

During the simulated washing with sanitizers, the susceptibility of EcO157 2705C on lettuce was affected by lettuce processing conditions, sanitizer and post-washing storage (**Table 3.4** and **Fig. 3.18**). Overall, bacterial levels recovered from lettuce under source processing conditions were higher than those under forward processing. Injury levels did not differ by processing condition, but the average theoretic VBNC level on lettuce under forward processing conditions was higher than under source processing. The average theoretic VBNC level from the washing with 50 ppm Cl was lower than 80 ppm PAA, while no difference in injury was detected between these two sanitizer treatments. The survival of EcO157 on lettuce exposed to forward processing

conditions after washing in 50 ppm Cl decreased ~1.1 log during storage, without affecting injury or theoretic VBNC cell estimates at the same conditions. The overall injury level among EcO157 on lettuce decreased after the 7-day storage, while the average theoretic VBNC level increased after the storage.

Transcriptomics. Romaine lettuce was grown for 7 weeks, inoculated with EcO157 EDL933 or 2705C strains 24 h prior to harvest, and subjected to simulated source (SP) or forward processing (FP). Lettuce-associated bacterial cells before (BP) or after the simulated processing, as well as bacteria from the fresh inoculum culture (FC) were recovered for RNA extraction and RNA sequencing. The data was uploaded to the Galaxy web platform and analyzed using the public server at usegalaxy.org. Strain-specific alignment was performed against EcO157 EDL933 or 2705C genome with Bowtie2. Gene-counts computation and annotation were carried out with featureCounts using the annotation of the respective reference genome. Differential expression (DE) analyses were performed using DESeq2 for each strain to determine DE genes (DEG) between different samples. The Gene Ontology (GO) enrichment analyses and Omics data analyses were further conducted on the identified DEG using the OMICS Dashboard from BioCyc.

The sequencing run yielded ~533M high quality reads with a quality score between 30-35 (**Fig. 3.19**). Principal component analysis suggested that the overall gene expression pattern for both EcO157 strains shifted before and after inoculation onto lettuce and under different storage conditions (**Fig. 3.20**). Transcriptomic responses differed for BP, SP and FP in 2705C, and BP differed from SP or FP in EDL933. When compared to FC, more than half of the differentially expressed genes (DEG, $p < 0.05$ and Abs ($\log_2$ (fold change)) ≥ 1) were the same among BP, SP and FP samples for both strains, suggesting that EcO157 responded more strongly to the shift to the phyllosphere, than to the various environmental conditions of SP or FP (**Fig. 3.21**). The functions of DEG in EcO157 on lettuce were mainly related to stress tolerance and chemotaxis/motility changes. Compared to FC, DEG functions in EDL933 before lettuce processing were enriched in, e.g., transport, motility, chemotaxis, while response to stimulus was enriched after SP and FP (**Fig. 3.22**). DEG functions in 2705C were predominantly enriched in response to stress, catabolic processes and response to stimulus on BP lettuce, while transport and SOS response were also enriched after SP or FP, respectively (**Fig. 3.23**).

EcO157 cells on lettuce before processing exhibited upregulation of genes needed for oxidative stress response, osmotic stress, biofilm formation, detoxification, antibiotic resistance (especially efflux pump related). Most of these functions remained upregulated in lettuce subjected to source or forward processing (e.g., oxidative stress), while others increased further in storage conditions. When compared to BP, genes related to stress response, antimicrobial resistance and cold shock were upregulated and chemotaxis and bacterial motility genes were downregulated in EcO157 on lettuce after SP, suggesting enhanced stress tolerance and surface attachment. However, with extended storage time, the FP conditions did not induce further upregulation of these genes. Instead, gene expression of some stress-response or bacterial motility pathways in EcO157 on FP lettuce was reversed when compared to SP, suggesting that bacterial susceptibility to sanitizer/washing treatment could potentially be higher in FP lettuce after several days of storage.

Objective 4. Improvement of forward processing management by applying findings from comprehensive assessment and simulation.

Current findings from the project have been reported and communicated at the 2023 CPS Research Symposium, ASM 2023 annual meeting, IAFP 2023 annual meeting, ASHS annual meeting, and local meetings. Information has been shared with industry partners to improve production strategies to ensure food safety and quality of fresh-cut romaine lettuce.

Outcomes and Accomplishments

- Examining past transportation records from an East Coast forward processing facility and onsite monitoring of forward processing transportation revealed that forward processing is typified by a pre-processing delay of ~1 week. Temperatures recorded during forward processing transportation and ensuing inventory holding did not provide evidence of any temperature abuse during the course of this project. However, prolonged exposure to high relative humidity and extended atmospheric pressure fluctuations were characteristic of forward processing transportation.
- Significant microbial proliferation occurred during forward processing transportation and inventory holding, even when temperature abuse had not occurred. Antimicrobial washing efficacy was lower at the forward processing facility than at the source processing facility running identical washing procedures. Higher microbial proliferation was observed on product from the forward processing facility than that from the source facility during post-processing storage.
- In comparing bagged fresh-cut romaine products from the same lot of raw material and with identical post-processing ages for visual quality, those from the source facility significantly outscored those from the forward facility at the late stage of their shelf life. Browning was a major contributor to the observed visual quality scores for fresh-cut romaine products from the forward facility.
- No evidence was obtained that supports enhanced risk of EHEC proliferation during forward processing transportation and inventory holding in the absence of temperature abuse. In laboratory simulations of forward and source processing transportation and inventory holding with the outbreak associated EHEC strains, lower EHEC recovery was obtained from lettuce subjected to forward processing than that exposed to source processing conditions.
- There was no consistent evidence supporting EHEC cell physiology changes under forward processing conditions favoring its survival that would be any different than under source processing. No significant difference was observed for EHEC strains subjected to forward and source processing conditions in susceptibility to sanitizer treatments. Marginally higher rates of persister cells and theoretical VBNC induction were observed for EHEC strains exposed to forward processing conditions.
- The EHEC strains associated with recent romaine lettuce outbreaks showed higher biofilm formation and resistance to sanitizer treatment under laboratory conditions, suggesting enhanced fitness to survive in fresh produce production environments. Comparative genomics analyses suggested genetic evidence underlying this enhanced fitness.
- Transcriptomic analysis revealed that the most remarkable shift in gene expression occurred when EHEC bacteria transitioned from culture to the lettuce niche, as they adapted to the plant environment. Another shift occurred in cells after processing, most notably genes needed to adapt to cold shock and genes needed to extrude antimicrobials, suggesting adaptation to leaf and storage conditions. The shift in gene expression detected in cells recovered from source and forward processed lettuce was less remarkable than the shift detected between fresh culture to lettuce before processing and subsequently to the first day in storage. The shift from before to after processing (regardless of source or forward conditions) suggested adaptation to cold and stressful environmental conditions.

Summary of Findings and Recommendations

Forward processing of romaine lettuce or other leafy greens is an effective strategy for ensuring freshness and access to fresh-cut products for consumers far away from the commercial leafy green growing regions. This project demonstrated that the practice of forward processing, with its significant pre-processing delays due to long distance transportation and inventory control logistics, permitted significant microbial proliferation even without recorded evidence of temperature abuse, and negatively impacted product post-processing shelf life. In laboratory simulation of romaine lettuce source and forward processing transportation and inventory holding events with EHEC outbreaks strains, no evidence was obtained to support significantly increased risk of EHEC growth or EHEC cell physiology changes leading to decreased antimicrobial susceptibility or increased expression of virulence related genes under conditions of forward processing, except slightly high rates of EHEC persister and theoretical VBNC cell induction were observed after exposure to such conditions. Upregulation of EHEC detoxification genes, efflux pumps, and antimicrobial resistance genes were detected in both source and forward processed lettuce samples, compared to EHEC not exposed to lettuce surface. The prevalent EHEC strains in recent outbreaks also showed stronger potential for biofilm formation, indicating increased fitness for survival in fresh produce production environments.

Based on the above summary findings, the following are proposed for the fresh produce industry and research community in considering their current and future operational recommendations:

- The "pre-processing delay" (as exemplified by romaine lettuce forward processing) is unlikely a significant cause for "pathogen risk increase", in the absence of temperature abuse.
- The pre-processing time delay such as that in forward processing can be reflected as produce quality loss.
- Foodborne pathogen environmental fitness and the underlying genomic and transcriptomic mechanisms, along with development of persister cells and the VBNC state detected under processing conditions require further research.
- The finding that significant bacterial proliferation is not reflected in EHEC growth under forward processing conditions suggests a significant role of the lettuce microbiome in balancing the growth or survival of foodborne pathogens, and the need for deeper microbiome analyses to better understand the microbial dynamics.

APPENDICES

Publications and Presentations

Publications:
Two manuscripts have been submitted to peer-reviewed journals to report partial data derived from objectives 1, 2, and 3 of this project. Another four manuscripts are in preparation.

- Ding Q., Gu G., Nou X., Micallef S. (2024). Cultivar was more influential than bacterial strain and other experimental factors in recovery of *Escherichia coli* O157:H7 populations from inoculated live Romaine lettuce plants. *Microbiology Spectrum* (early online) e03767-23. https://doi.org/10.1128/spectrum.03767-23
- Gu G., Ding Q., Redding M., Yang Y., O'Brien R., Gu T., Zhang B., Zhou B., Micallef SA., Luo Y., Fonseca J., Nou X. (2024). Differential microbiota shift on whole romaine lettuce subjected to source or forward processing and on fresh-cut products during cold storage. Dynamics of microbial profile and quality evaluation of fresh-cut romaine lettuce under source and forward processing conditions. *International Journal of Food Microbiology* 416: 110665. https://doi.org/10.1016/j.ijfoodmicro.2024.110665

In preparation:
- Sanitizer susceptibility of *Escherichia coli* O157:H7 from inoculated romaine lettuce after simulated source or forward processing.
- Genome-wide transcriptome response of *Escherichia coli* O157:H7 on Romaine lettuce subjected to simulated source or forward processing.
- Survival and persister cell development of *Escherichia coli* O157:H7 and microbiome shift on romaine lettuce under source and forward processing conditions.
- *Escherichia coli* O157:H7 strains associated with reoccurring lettuce outbreaks display strong biofilm-forming ability and low sanitizer susceptibility.

Meeting Presentations:
2023
- Gu G., Redding M., Yang Y., Ding Q., Gu T., Zhou B., Luo Y., Shirley M., Zhang B., Nou X. (2023). Evaluation of romaine lettuce quality and microbial ecology under source processing and forward processing conditions. IAFP annual meeting. P3.198.
- Ding Q., Gu G., Luo Y., Nou X., Micallef SA. (2023). Comparison of the recovery efficiency of epiphytically associated *Escherichia coli* O157:H7 on lettuce leaves using different sample preparation methods. IAFP annual meeting. P2.182.
- Ding Q., Gu G., Luo Y., Nou X., Micallef SA. (2023). Comparing *Escherichia coli* O157:H7 cell count recovery from inoculated store-bought lettuce using sonication or stomaching. IAFP annual meeting. P2.183.
- O'Brien R., Gu G., Yang Y., Park E., Fonseca J., Luo Y., Nou X. (2023). Visual quality evaluation of fresh-cut romaine lettuce during post-processing storage as affected by forward processing and source processing. American Society for Horticultural Science (ASHS) annual meeting.
- Gu G., Redding M., Yang Y., Gu T., Zhang B., Nou X. (2023). Shift in microbial communities of romaine lettuce from farm to storage for both source and forward processing. American Society of Microbiology (ASM) Microbe annual meeting. 4446.
- Ding Q., Gu G., Luo Y., Nou X., Micallef SA. (2023). Assessing *Escherichia coli* O157:H7 recovery from lettuce plants using different sample processing approaches under diverse conditions. Postdoctoral Research Symposium at the University of Maryland, 22 Sept. 2023, College Park, Maryland, United States.

2024 (submitted)

- Gu G., Ding Q., Yang Y., Micallef S., Luo Y., Nou X. (2024). Survival of *Escherichia coli* O157:H7 and microbiome shift on romaine lettuce under source and forward processing conditions. IAFP annual meeting. (submitted)
- Ding Q, Gu G, Yishan Y, Luo Y, Nou X, Micallef SA. (2024). Sanitizer Solution Susceptibility of *Escherichia coli* O157:H7 Recovered from Inoculated Romaine Lettuce after Simulated Source or Forward Processing Conditions. IAFP annual meeting. (submitted)
- Ding Q, Gu G, Yishan Y, Luo Y, Nou X, Micallef SA. (2024). Sanitizer Susceptibility of Leaf-Associated *Escherichia coli* O157:H7 during Washing of Inoculated Romaine Lettuce after Simulated Source or Forward Processing Conditions. IAFP annual meeting. (submitted)
- Ding Q, Gu G, Luo Y, Nou X, Micallef SA. (2024). Genome-Wide Transcriptomic Responses of *Escherichia coli* O157:H7 Inoculated to Live Romaine Lettuce followed by Harvesting and Simulated Source or Forward Processing Conditions. IAFP annual meeting. (submitted)
- Ding Q, Gu G, Luo Y, Nou X, Micallef SA. (2024). Gene Expression Changes in *Escherichia coli* O157:H7 Inoculated to Live Romaine lettuce Plants and Following, Harvesting and Simulated Source or Forward Processing Conditions. IAFP annual meeting. (submitted)
- Yang Y., Redding M., Gu G., Luo Y., Nou X. (2024). *Escherichia coli* O157:H7 strains associated with reoccurring lettuce outbreaks display strong biofilm-forming ability and low sanitizer susceptibility. IAFP annual meeting. (submitted)

Budget Summary

All funds awarded for the project ($399,743) are spent or committed as budgeted (for 2024 CPS Research Symposium travel costs).

The project budget allocated $174,586 (43.67%) for a subaward contract with University of Maryland, which was expended on hiring one full time postdoc for 2 years and on purchasing lab supplies. The remaining funds for USDA ARS (56.33%) were mainly used on salary and benefits for one full-time postdoc (37.87%) and lab supplies (12.47%), including bacterial growth media, chemical regents, DNA extraction, qPCR, DNA sequencing kits, data loggers and various consumables. Travel accounts for 5.05% of the spending, which covered (and will cover) travel costs for research team members to California for sample collection and conducting onsite data collection and to CPS Research Symposia in 2022-2024. Other costs (0.94%) were used for and reserved for CPS Research Symposia registration in 2022-2024.

Figures 1.1 – 3.23 and Tables 1.1 – 3.4 (see below)

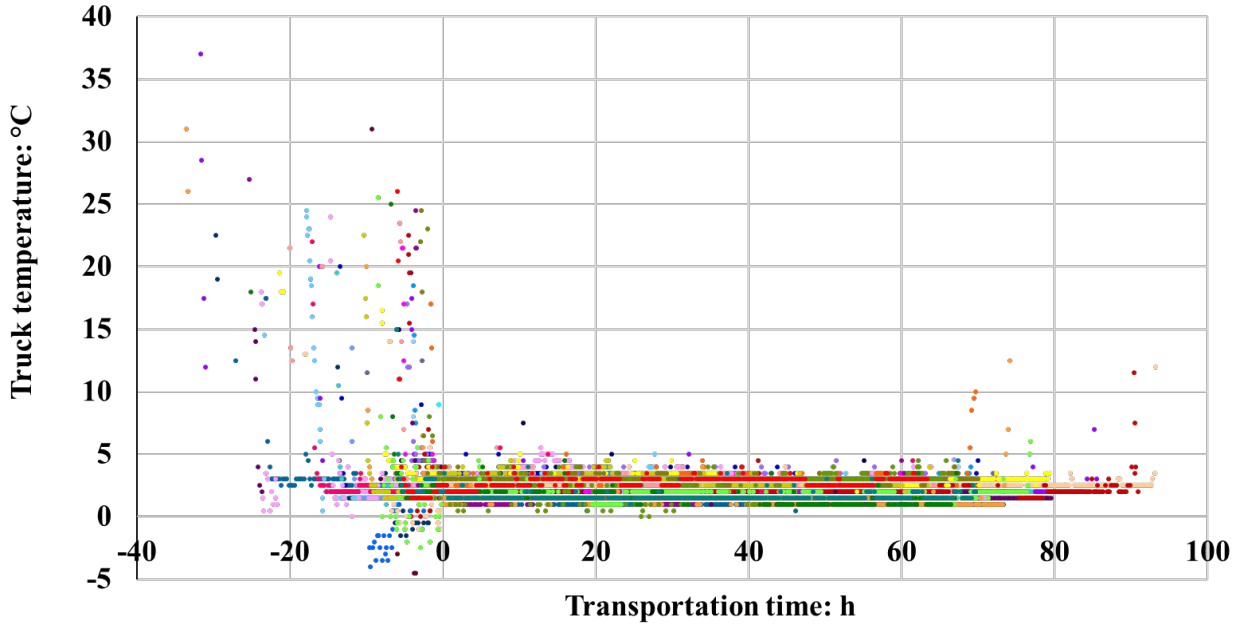

Figure 1.1. Temperature profiles from the fresh produce industry of randomly selected romaine lettuce delivery trailers (n=33) from Salinas, CA, to Mid-Atlantic processing facilities. Temperature was measured at air return. Hour 0 designates the time point of product loading. The average temperature in delivery trucks during romaine lettuce transportation (after trailer loading at 0 h) of each analyzed trip was below 4°C, while abusive temperature above 5°C was recorded sporadically at certain timepoints. Each color denotes one individual delivery.

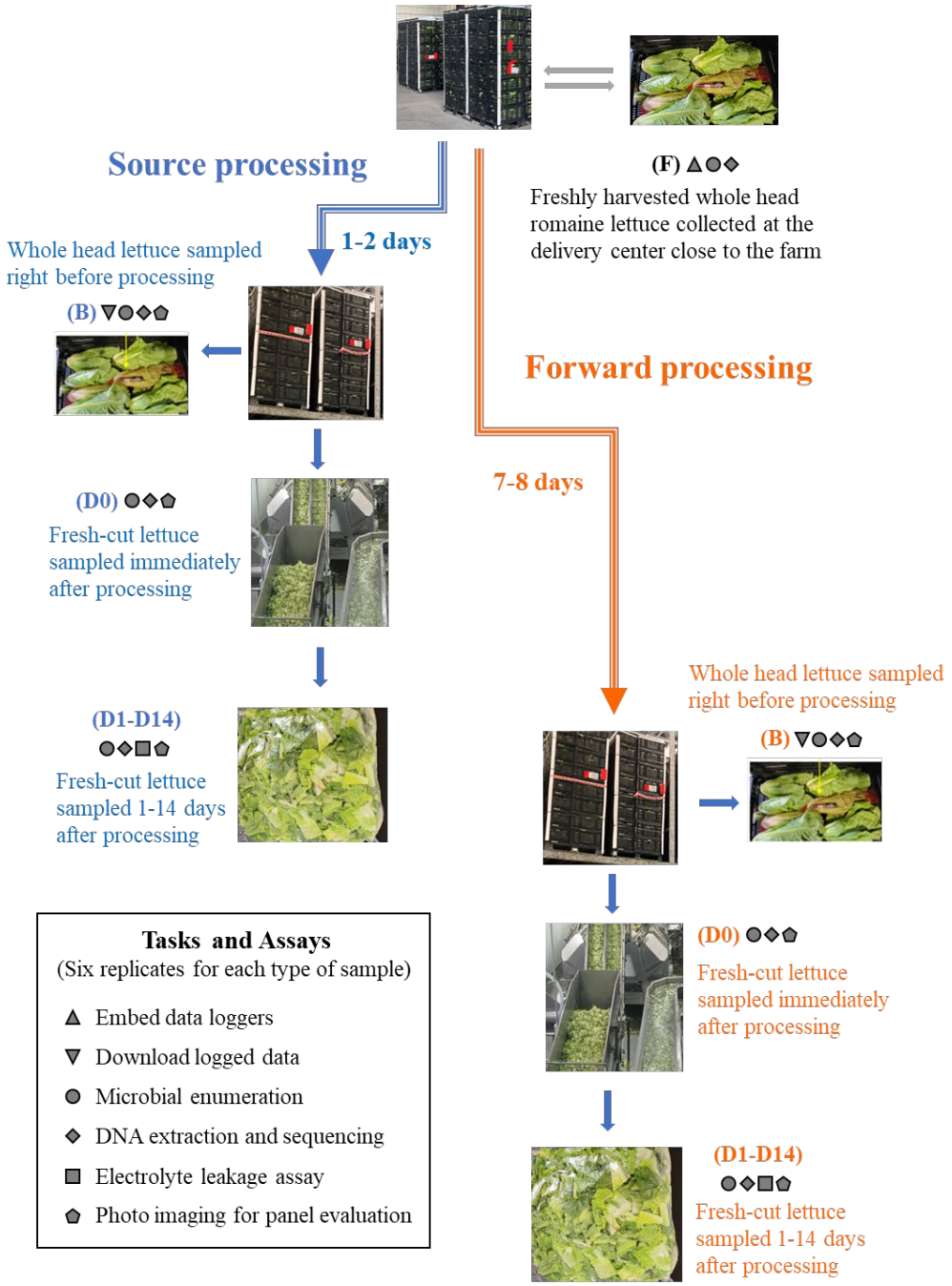

Figure 1.2. Schematic presentation of lettuce sampling. Blue and orange arrows indicate source (source processing, 1.2 days post-harvest) and forward (forward processing, 7-8 days post-harvest) processing related steps, respectively. Field harvested whole head romaine lettuce is marked as sample F, whole head lettuce retrieved from processing floors (prior to processing) as B, fresh-cut product as D0, and bagged fresh-cut lettuce for storage evaluation as D1 to D14 corresponding to the storage time. Tasks and assays for each type of samples (n = 6) are marked by different symbols. Photos provided are for schematic presentation. Lettuce samples were randomly collected with six replicates for each type of samples at each sampling point in both summer and fall trials.

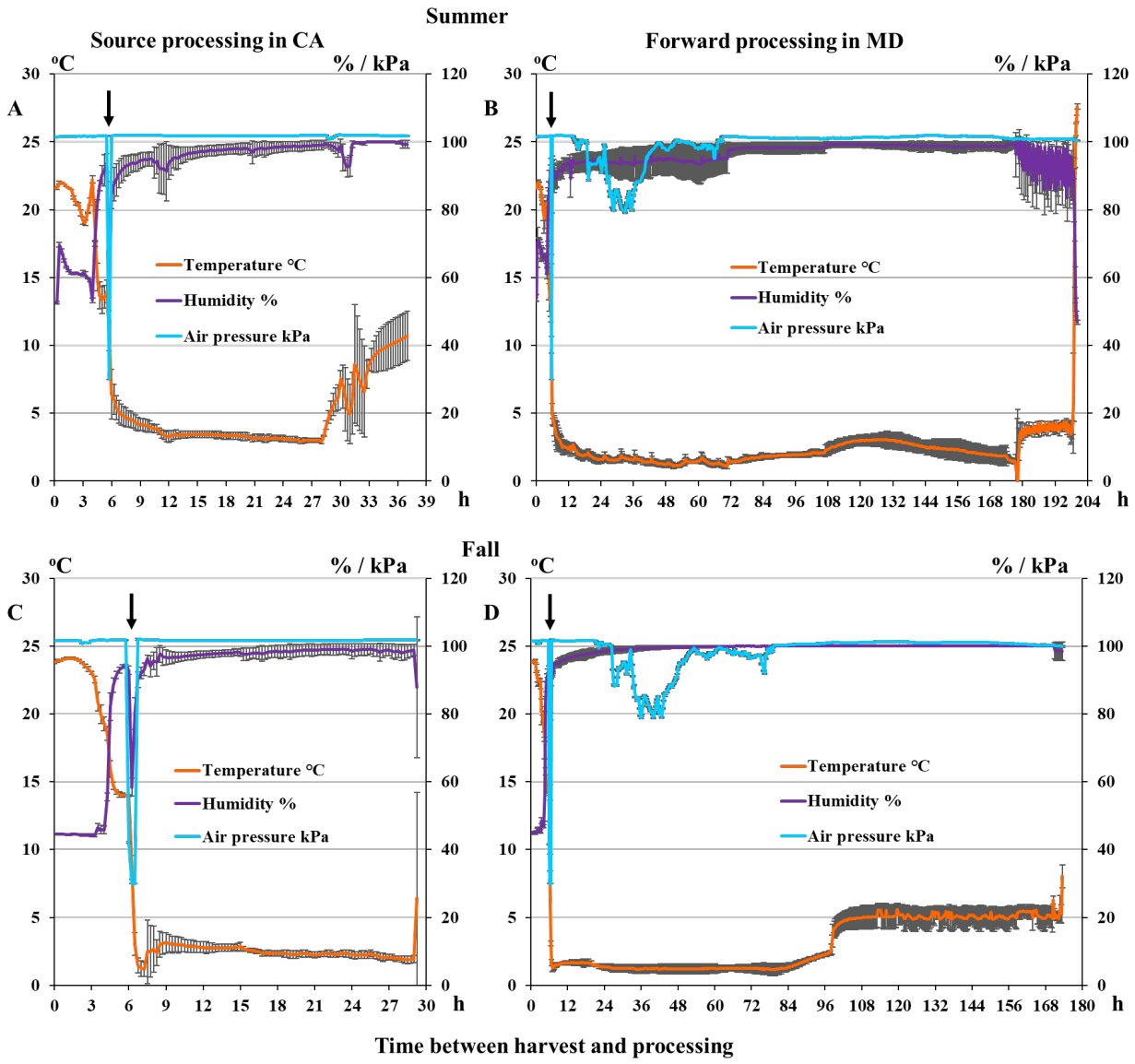

Figure 1.3. Data logger monitoring of romaine lettuce in source and forward processing production chains. The temperature, humidity, and air pressure of romaine lettuce were tracked for the whole process from field harvest to processing at source (A, C) and forward (B, D) processing facilities in summer (A, B) and fall (C, D) production seasons (n=6). Arrows point out the vacuum cooling points.

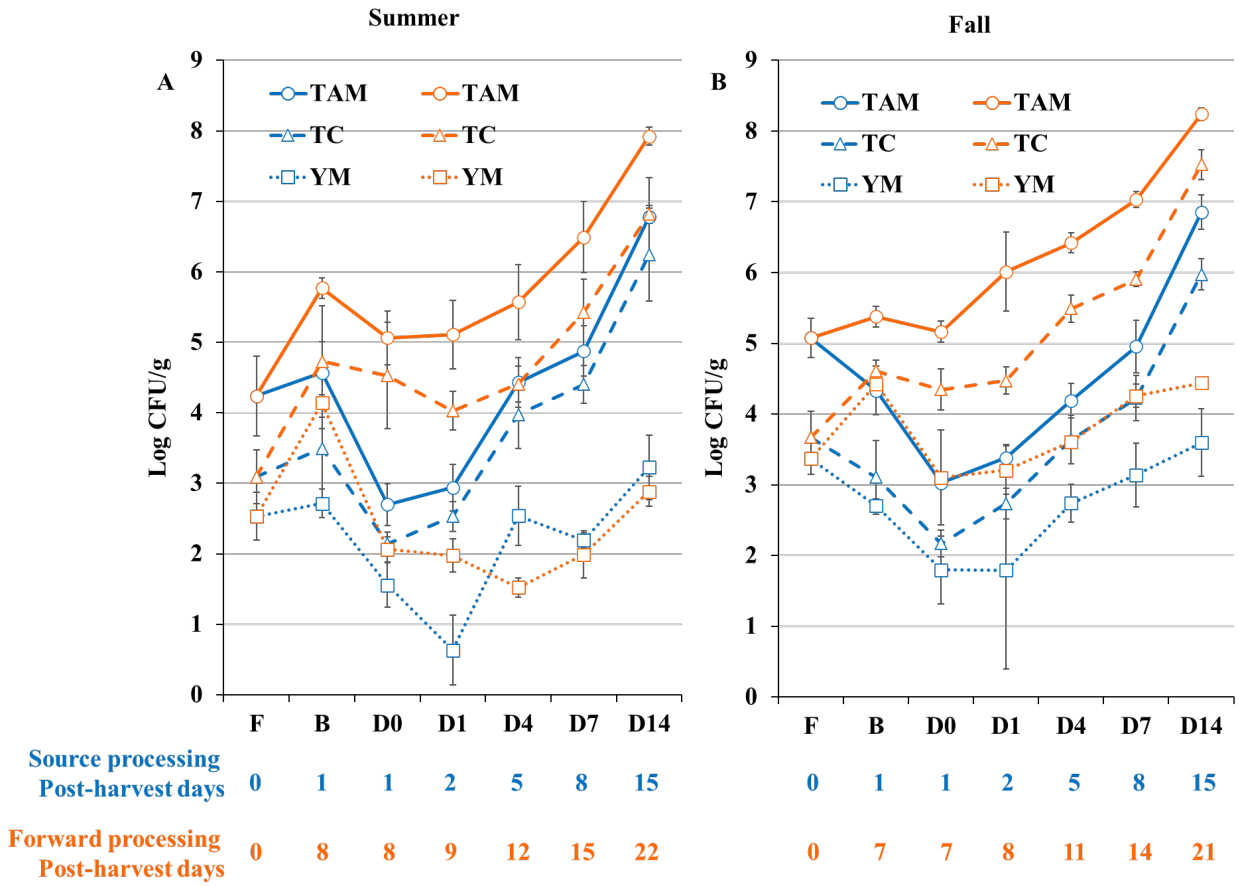

Figure 1.4. Dynamics of microbial populations on romaine lettuce under FP and SP production conditions. Populations of total mesophilic bacteria (TAB, solid line with round marker), coliform (TC, dash line with triangle marker), and yeast and mold (YM, dot line with square marker) in forward processing (FP, orange line) and source processing (SP, blue line) lettuce samples freshly harvested from field (F), collected on the processing date before processing (B), after processing on days 0 (D0, 3 h after processing), 1 (D1), 4 (D4), 7 (D7), and 14 (D14) in the summer (left) and fall (right) seasons were enumerated. The sampling days post-harvest (DPH) of both SP and FP products were listed below the post-processing dates (D0-14). Bars denote standard deviations (n=6). Limit of detection: 0.6 log CFU/g.

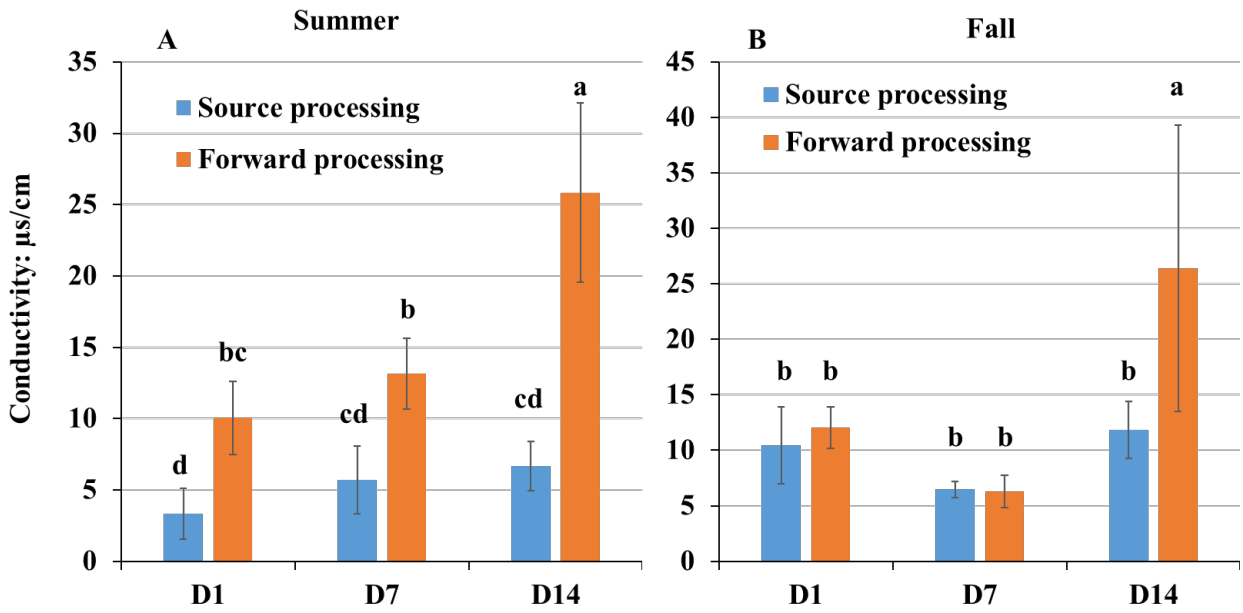

Figure 1.5. Electrolyte leakage of forward (FP) and source (SP) processed lettuce in summer (A) and fall (B) seasons after cold storage of 1 (D1), 7 (D7), and 14 (D14) days. Conductivity was measured at room temperature. Bars denote standard deviations (n=6). Lettering represents significant difference levels ($p < 0.05$).

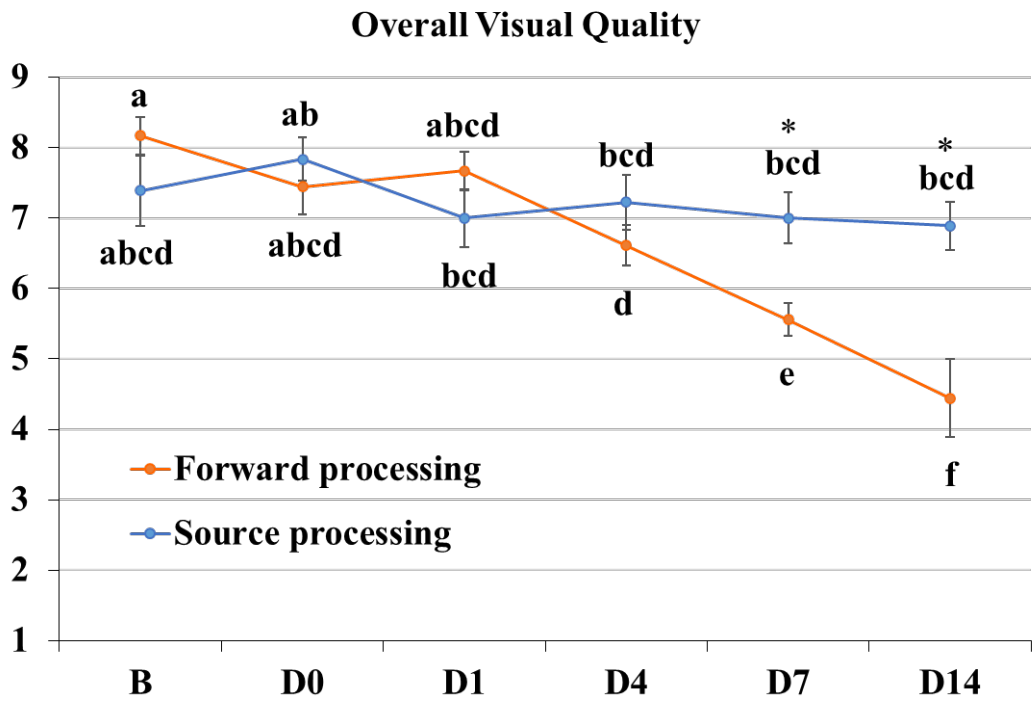

Figure 1.6. Average quality ratings for FP and SP lettuce images. Evaluated lettuce images were taken on the processing date before processing (B) and at 0, 1, 4, 7, and 14 days post-processing. Lettering represents significant difference levels between all samples. Starring represents significant differences between processing methods at each timepoint.

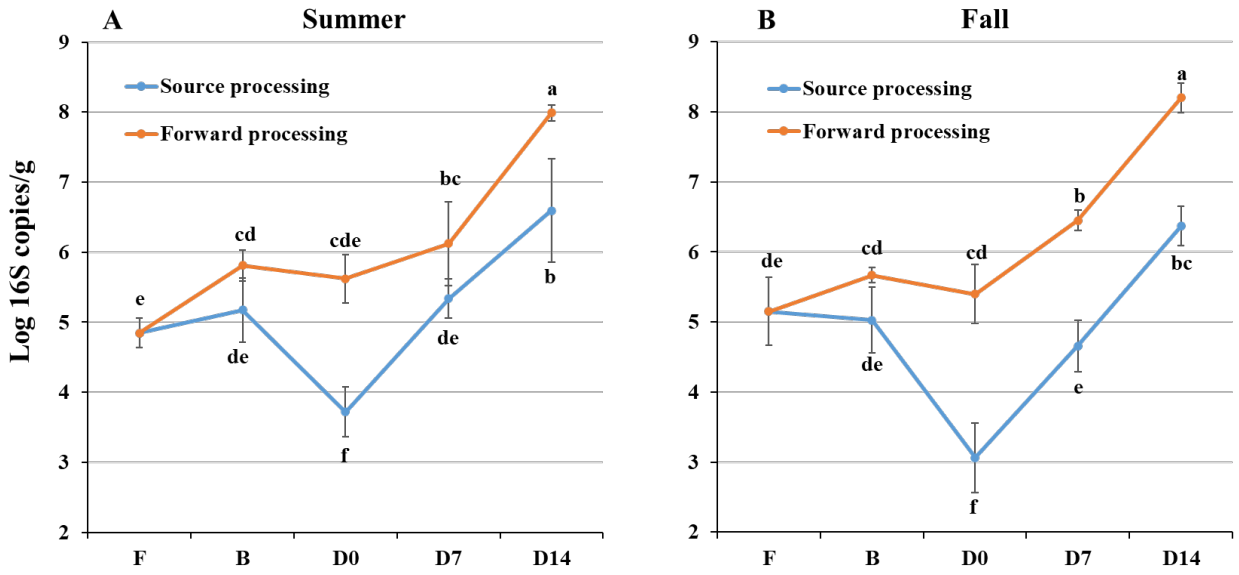

Figure 2.1. qPCR estimation of total bacterial levels on romaine lettuce freshly harvested from field (F), same day before processing (B), after forward (FP) and source (SP) processing on days 0 (D0), 7 (D7), and 14 (D14) during summer and fall production seasons. Bars denote standard deviations (n=6).

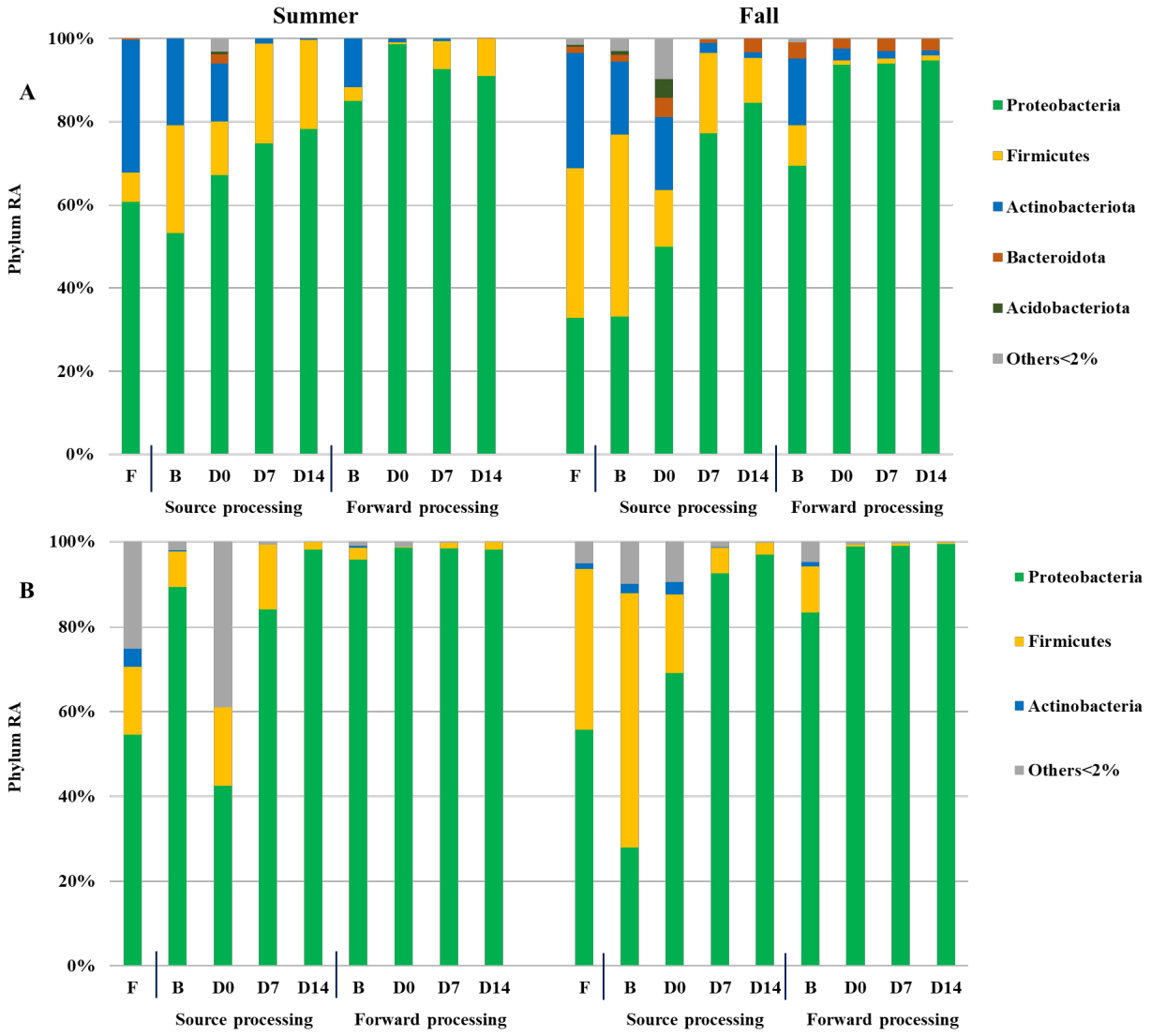

Figure 2.2. Relative abundance (RA) of dominant bacterial phyla on romaine lettuce. Lettuce samples were collected after field harvest (F, whole head), on the processing date before processing (B, whole head), after processing on days 0 (D0, 3 h after processing, packaged fresh-cut products), 1 (D1, fresh-cut), 4 (D4, fresh-cut), 7 (D7, fresh-cut), and 14 (D14, fresh-cut) in the summer (left) and fall (right) trials. (A) Illumina MiSeq sequencing, (B) Nanopore MinION sequencing. Only phyla >2 % (average RA) of the bacteria identified in at least one type of samples were included.

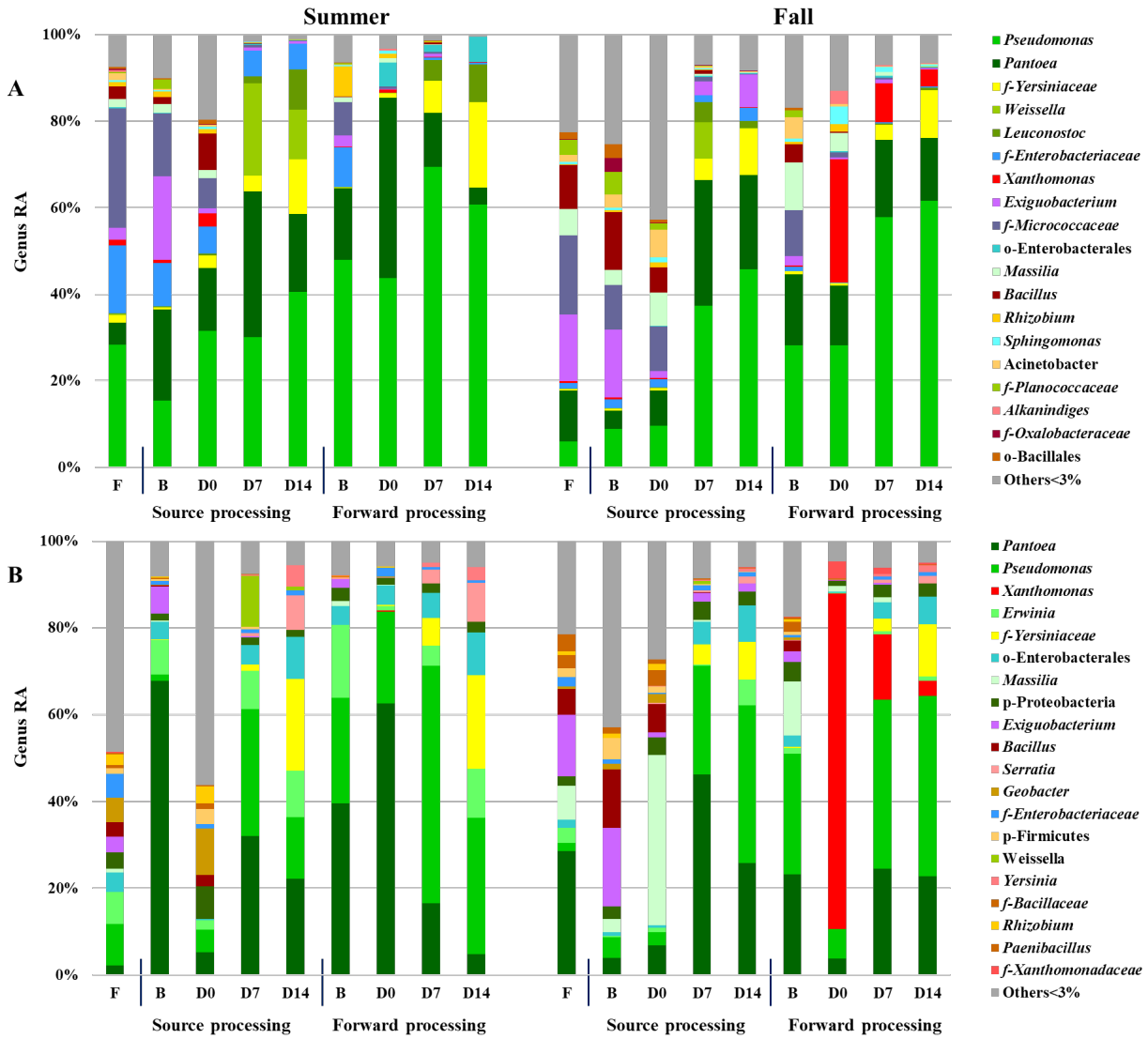

Figure 2.3. Relative abundance (RA) of dominant bacterial genera on romaine lettuce freshly harvested from field (F), collected on the processing date before processing (B), after forward (FP) and source (SP) processing on days 0 (D0), 7 (D7), and 14 (D14) during summer and fall production seasons. (A) Illumina MiSeq sequencing, (B) Nanopore MinION sequencing. Only genera >3 % (average RA) of the bacteria identified in at least one type of samples were included. The bacterial taxa identified at the higher taxonomic levels (f-, family; o-, order; p-, phylum) indicate unknown genera belonging to those taxa.

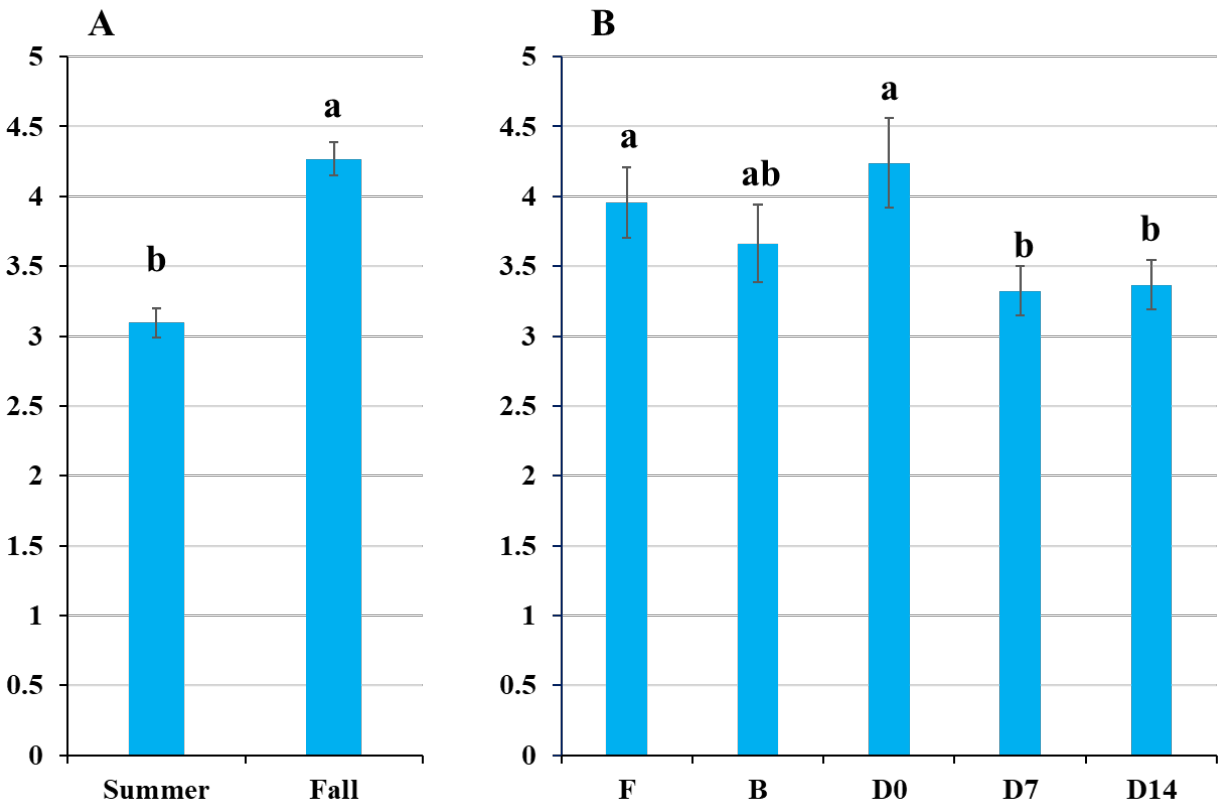

Figure 2.4. Alpha diversity (Shannon index) of bacterial communities on lettuce samples. (A) Comparison between summer and fall trials; (B) Comparison of samples freshly harvested from field (F), collected on the same day before processing (B), after processing on days 0 (D0), 7 (D7), and 14 (D14).

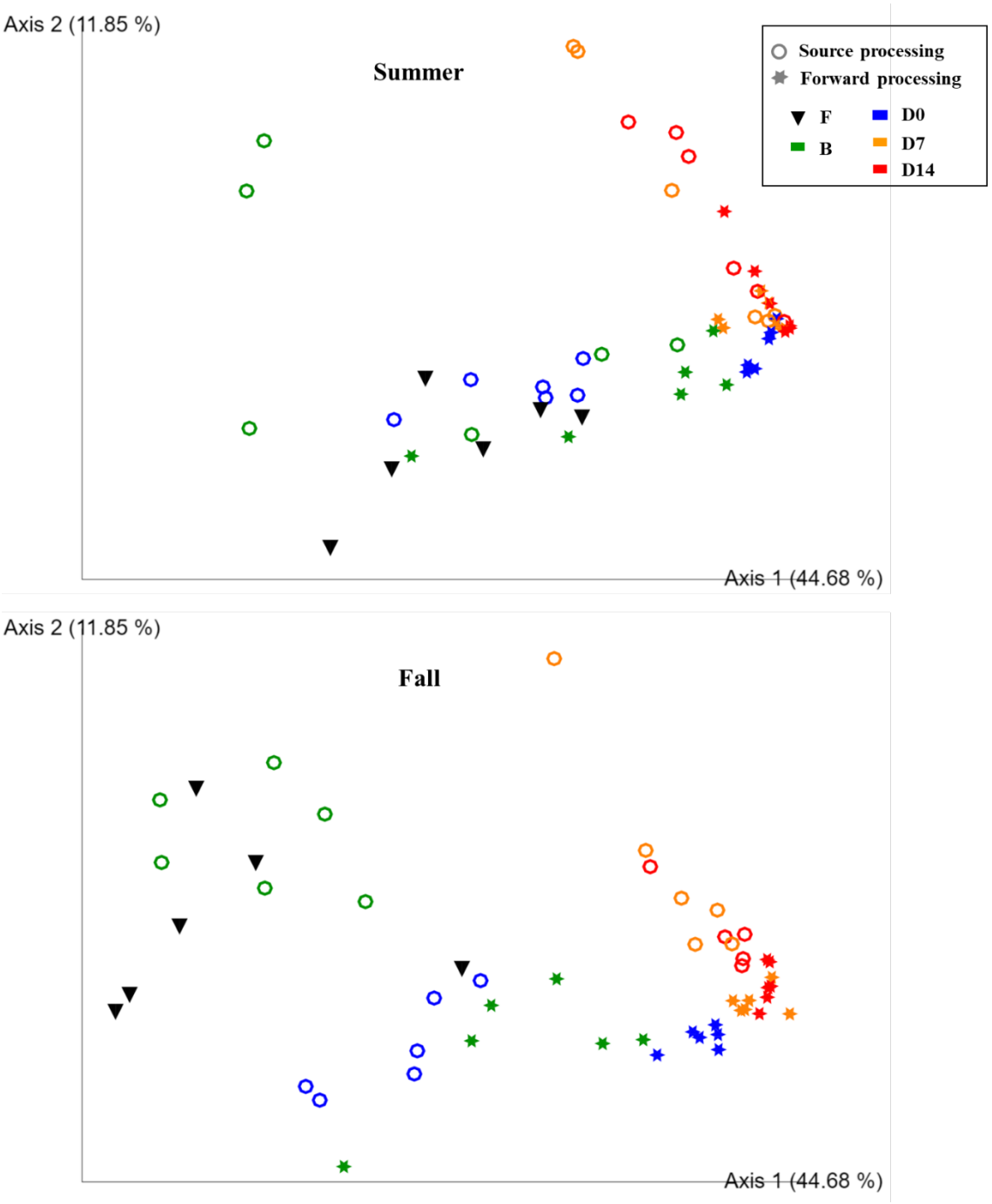

Figure 2.5. Relatedness of bacterial communities on romaine lettuce in summer (top) and fall (bottom) trials. Lettuce samples were collected after field harvest (F, whole head), on the processing date before processing (B, whole head), after processing on days 0 (D0, 3 h after processing, packaged fresh-cut products), 1 (D1, fresh-cut), 4 (D4, fresh-cut), 7 (D7, fresh-cut), and 14 (D14, fresh-cut). Principle coordinate analysis (PCoA) was performed based on Weighted Unifrac Distance to compare bacterial communities on romaine lettuce.

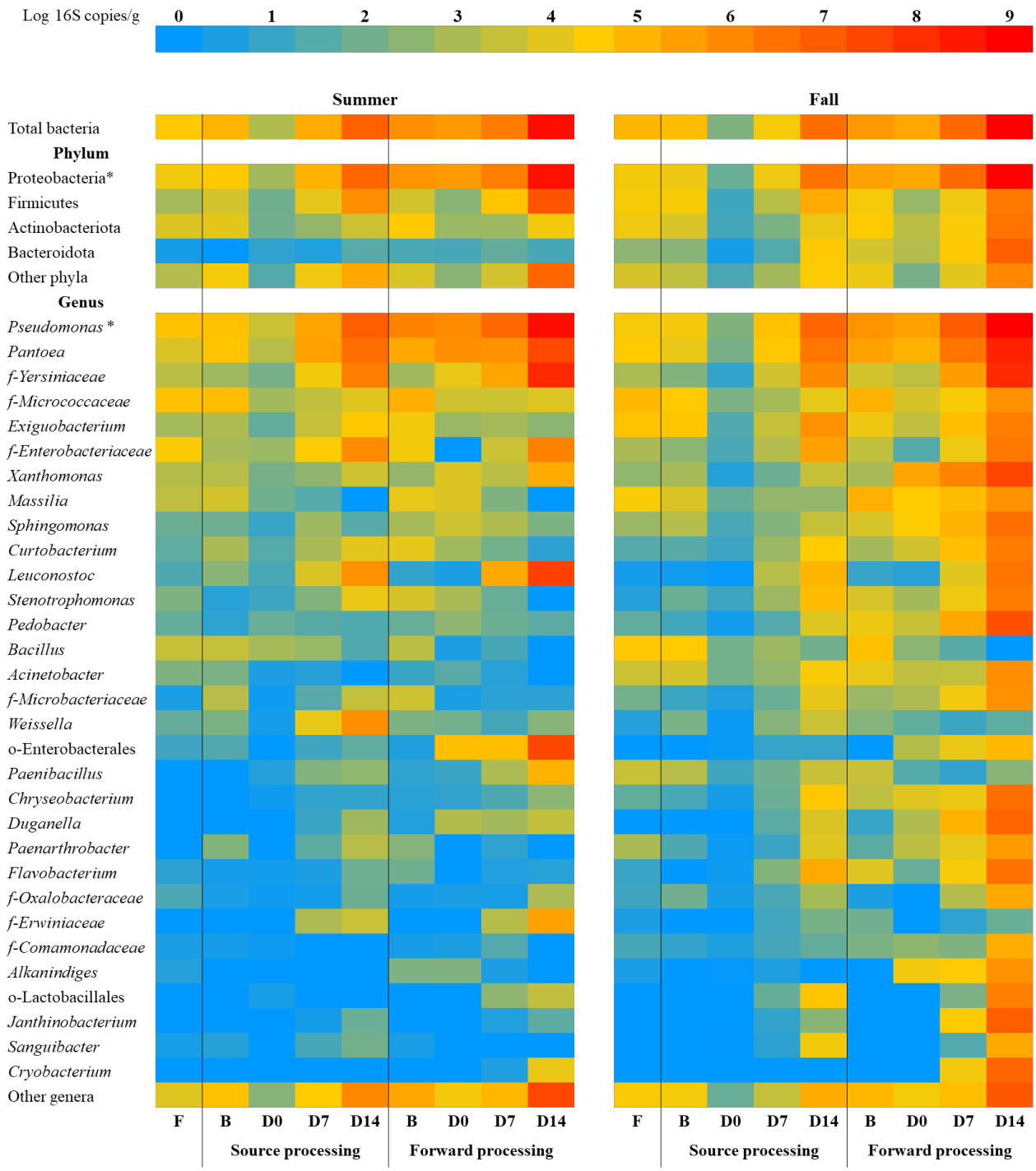

Figure 2.6. Shift in bacterial populations on romaine lettuce freshly harvested from field (F), collected on the processing date before processing (B), after forward (FP) and source (SP) processing on days 0 (D0), 7 (D7), and 14 (D14) during summer and fall production seasons revealed by Illumina sequencing. *, dominant bacterial taxa (> 4 log 16S rRNA amplicons/g in at least one type of samples) at phylum and genus levels were listed. The bacterial taxa identified at the family (f-) and order (o-) levels indicate unknown genera belonging to those taxa.

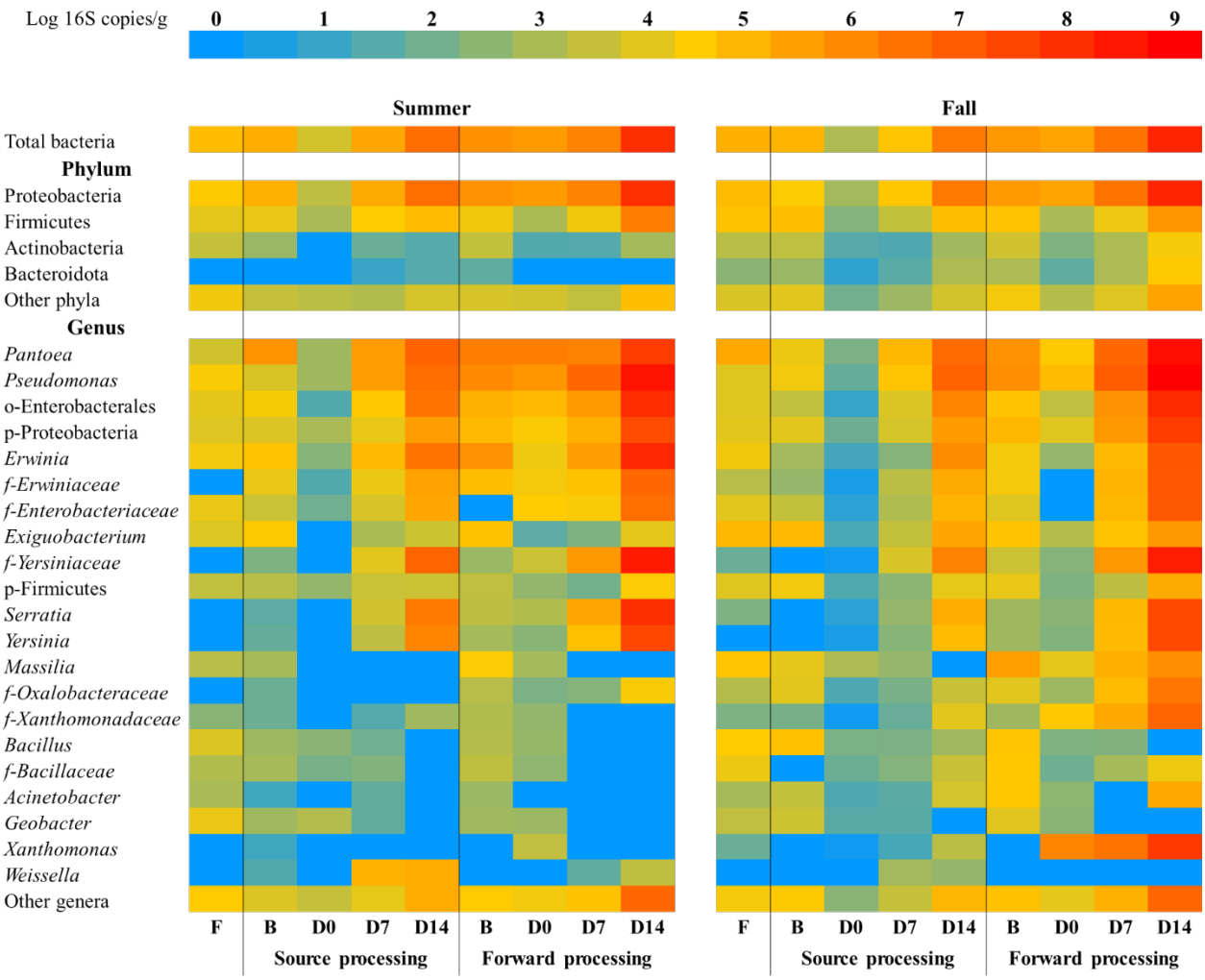

Figure 2.7. Shift in bacterial populations on romaine lettuce revealed by Nanopore sequencing. Lettuce samples were collected after field harvest (F, whole head), on the processing date before processing (B, whole head), after processing on days 0 (D0, 3 h after processing, packaged fresh-cut products), 1 (D1, fresh-cut), 4 (D4, fresh-cut), 7 (D7, fresh-cut), and 14 (D14, fresh-cut) in the summer (left) and fall (right) trials. *, dominant bacterial taxa (> 4 log 16S rRNA amplicons/g in at least one type of samples) at phylum and genus levels were listed. The bacterial taxa identified at the family (f-) and order (o-) levels indicate unknown genera belonging to those taxa.

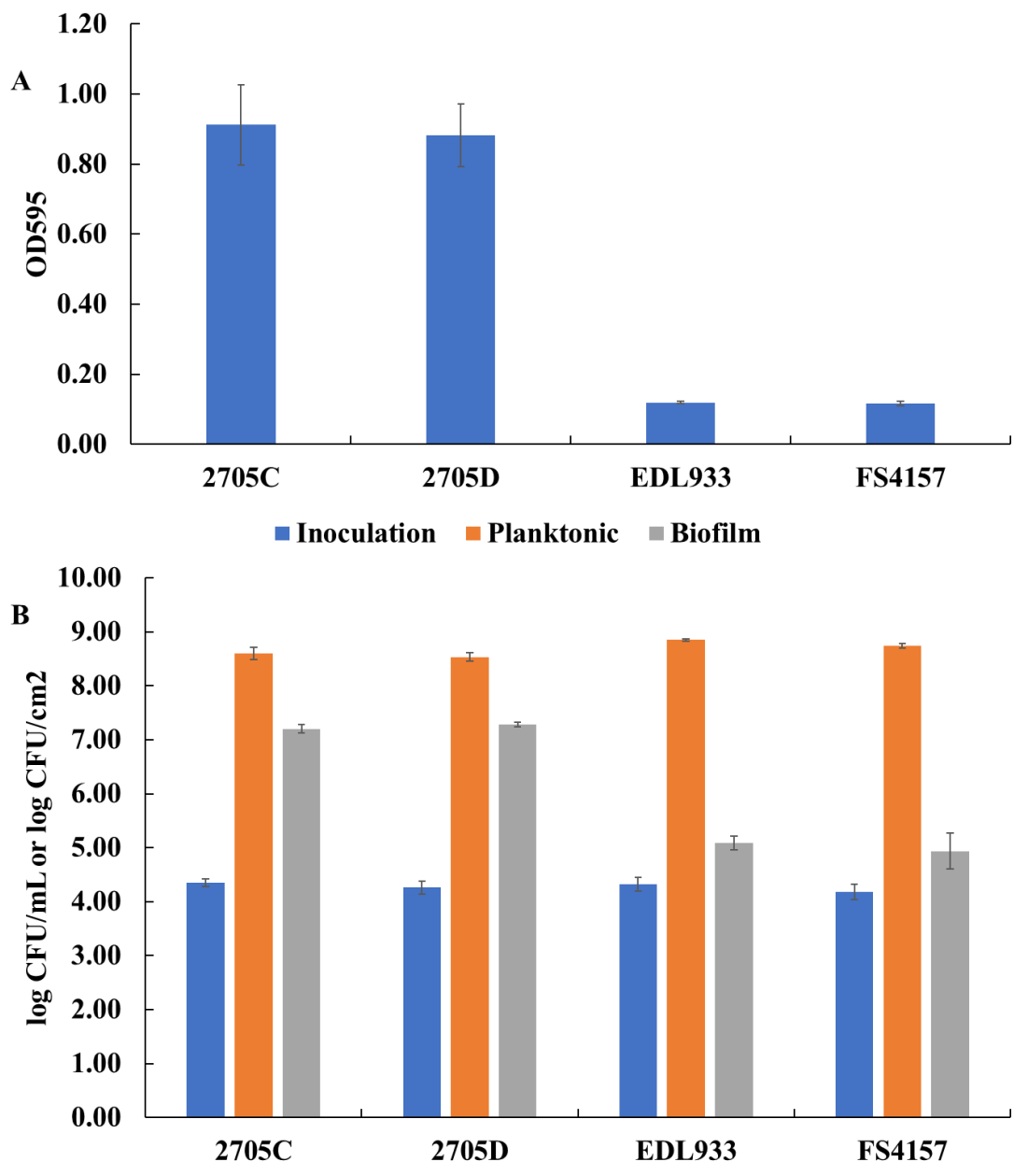

Figure 3.1. Biofilm formation of *E. coli* O157:H7 2705C, 2705D, EDL933, and FS4157 in 1/10 TSB at 25°C for 48 h in a 48-well plate measured by the crystal violet staining assay (A) and in a 48-well block quantified by the plate counting method (B). The populations of the inoculums, planktonic cells, and biofilm cells on stainless steel coupons were determined at 0, 24, and 48 h.

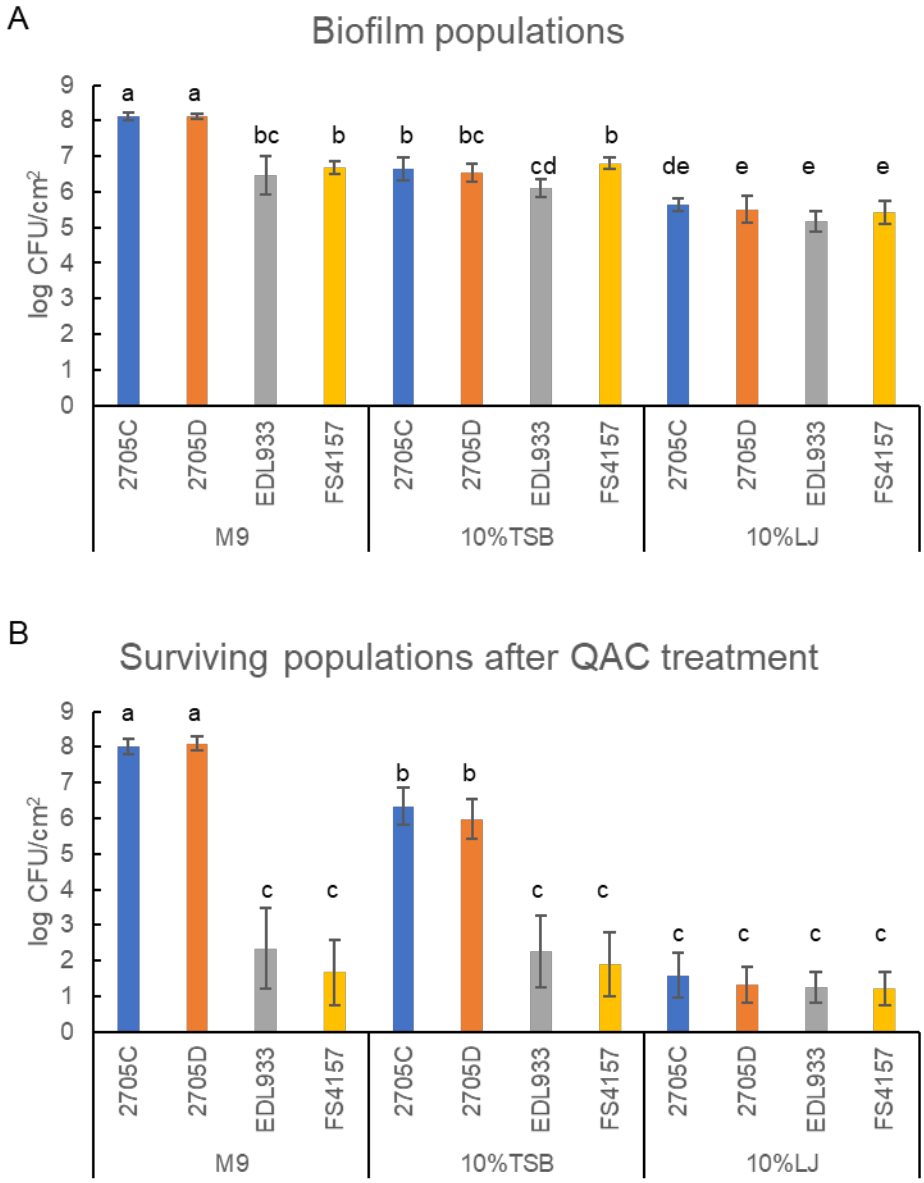

Figure 3.2. Biofilm populations of *E. coli* O157:H7 2705C, 2705D, EDL933, and FS4157 on stainless steel coupons (304, Ø=2.54 cm) in M9 medium, 10% tryptic soy broth (TSB), and 10% lettuce juice (LJ) at 25°C for 48 h (A) and survival of biofilms after quaternary ammonium compounds treatment at 200 ppm for 1 min (B).

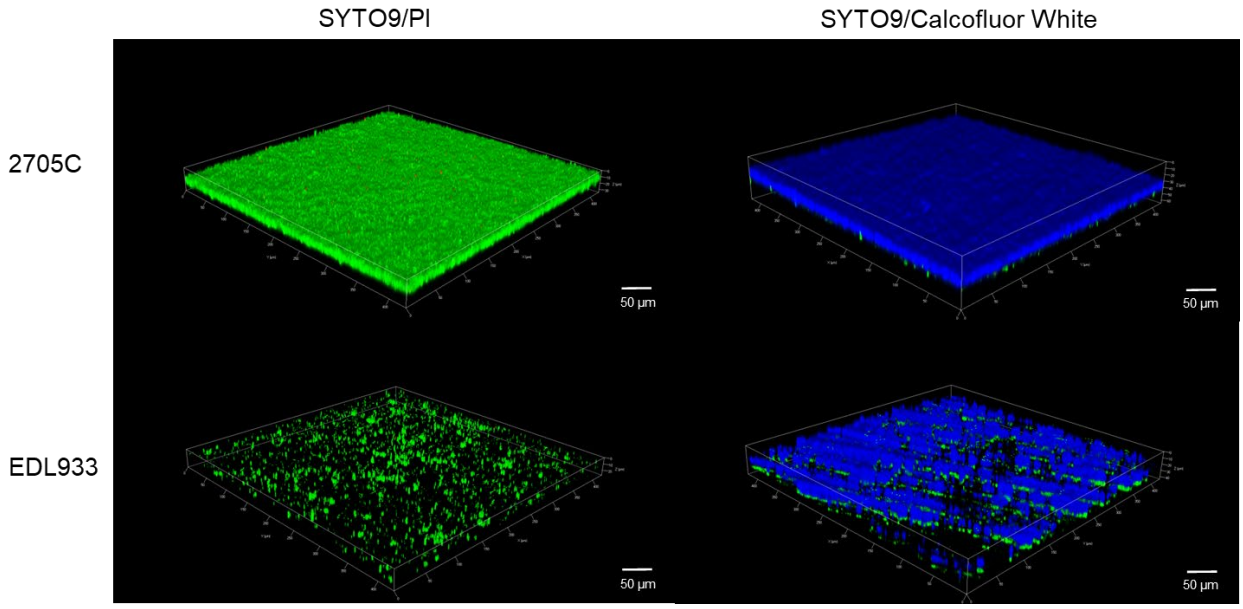

Figure 3.3. SYTO9/PI and SYTO9/calcofluor white staining of biofilms formed by *E. coli* O157:H7 2705C and EDL933 on stainless steel coupons (304, Ø=2.54 cm) in M9 medium at 25°C for 48 h. Biofilms were observed under 20× objective lens and scale bars represent 50 µm.

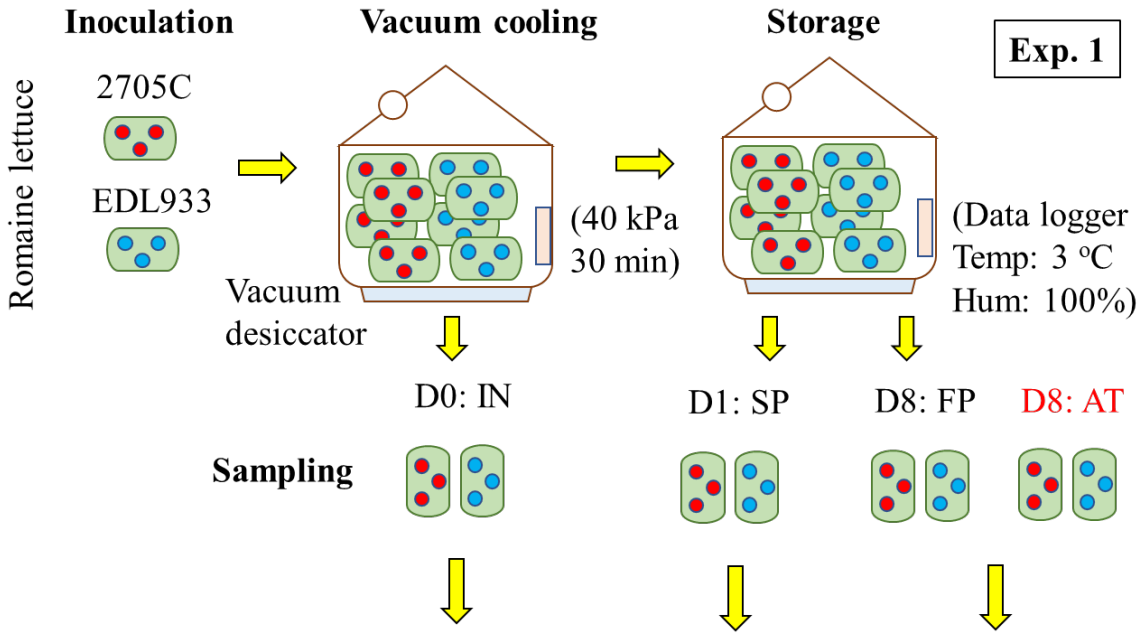

Figure 3.4. Schematic presentation of experiment flow and sample assignment about EcO157 survival on inoculated romaine lettuce.

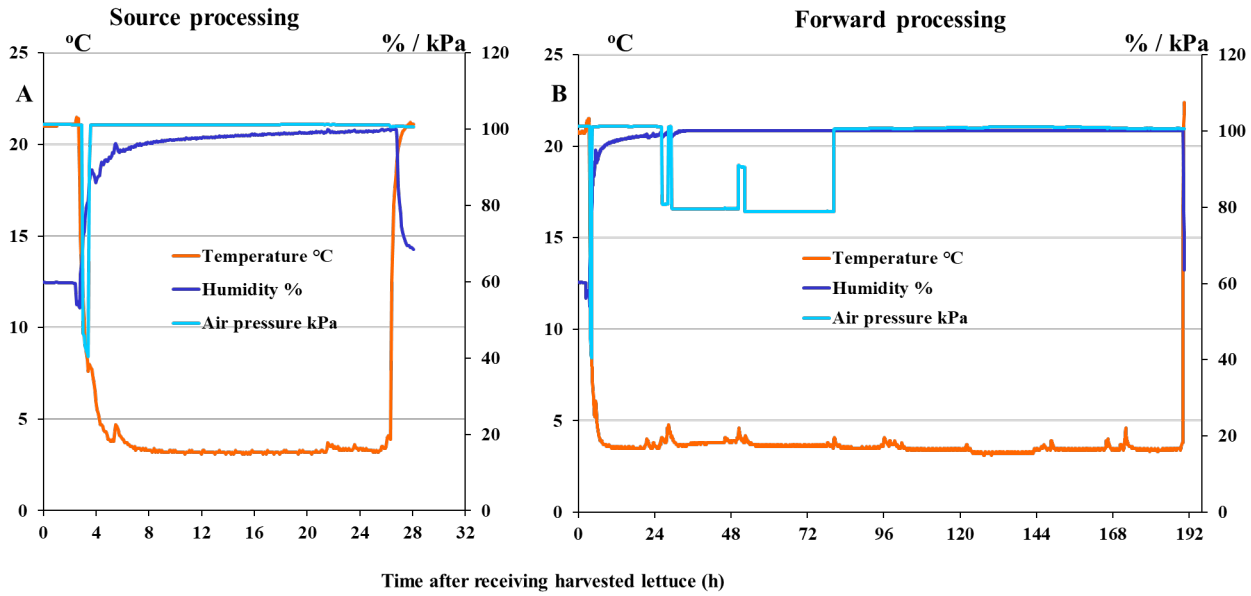

Figure 3.5. Temperature, humidity, and air pressure setup for simulated SP and FP romaine lettuce sampling.

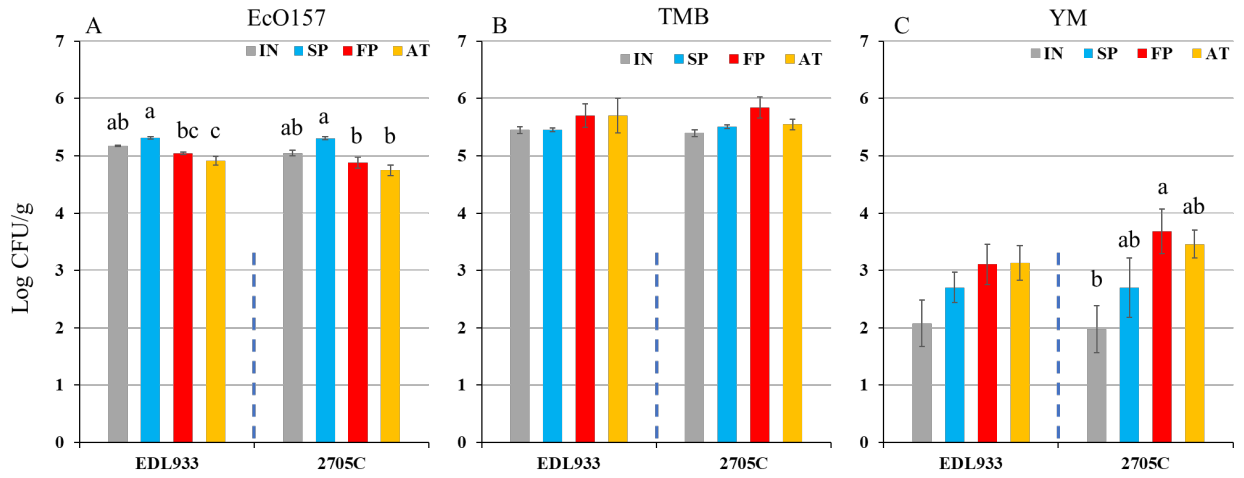

Figure 3.6. Dynamics of microbial populations on romaine lettuce under FP and SP production conditions. Inoculated leaf samples were manually cut and tested on Day 0 (IN, initial sampling conducted immediate after vacuum cooling), Day 1 (SP simulation), and Day 8 (FP, simulation with air pressure fluctuation; AT, alternative simulation test without air pressure change). EcO157, *Escherichia coli* O157:H7; TMB, total mesophilic bacteria; YM, yeast and mold; 2705C, EcO157 2019 lettuce outbreak strain; EDL933, EcO157 control strain. Letters above histogram bars denote significance levels (n = 4).

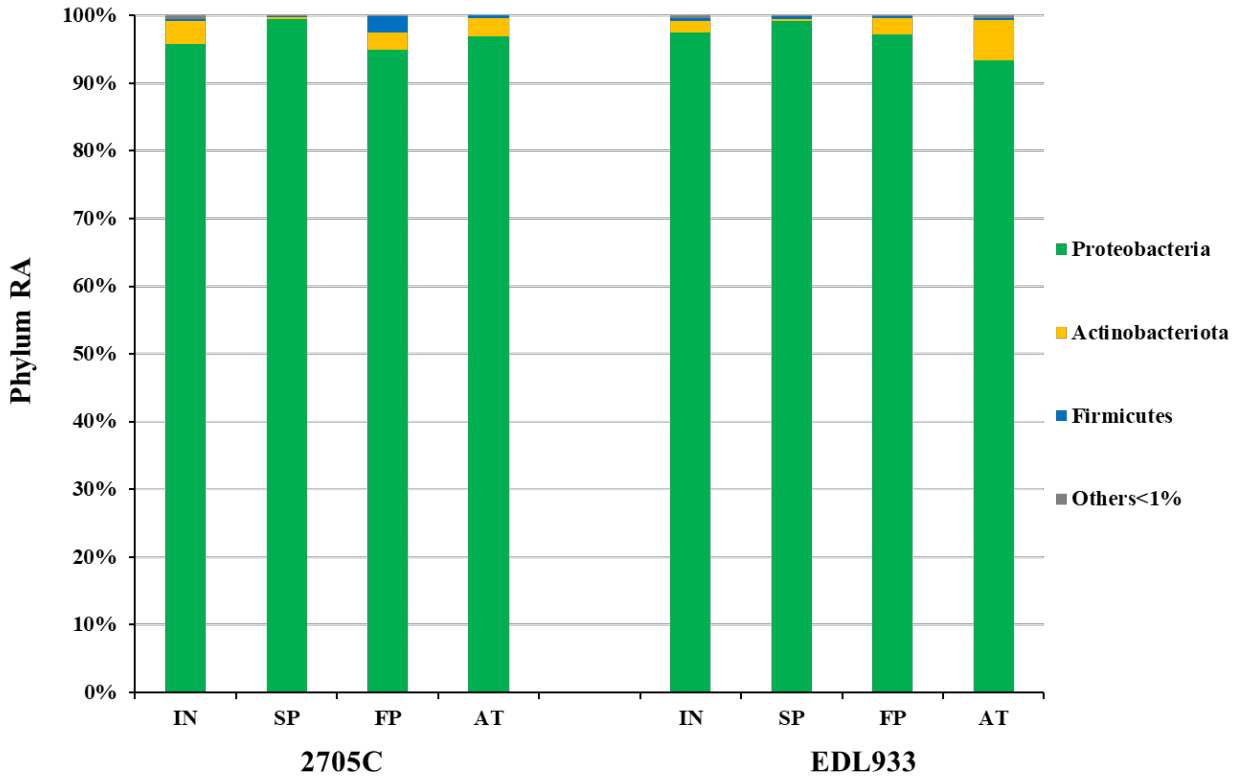

Figure 3.7. Relative abundance (RA) of dominant bacterial phyla on romaine lettuce under simulated FP and SP production conditions. Inoculated leaf samples were manually cut and tested on D0 (IN, initial sampling conducted immediate after vacuum cooling), D1 (SP simulation), and D8 (FP, simulation with air pressure fluctuation; AT, alternative simulation test without air pressure change). 2705C, *Escherichia coli* O157:H7 2019 lettuce outbreak strain; EDL933, control strain. Only phyla > 1% (average RA) of the bacteria identified in at least one type of samples were included (n = 4).

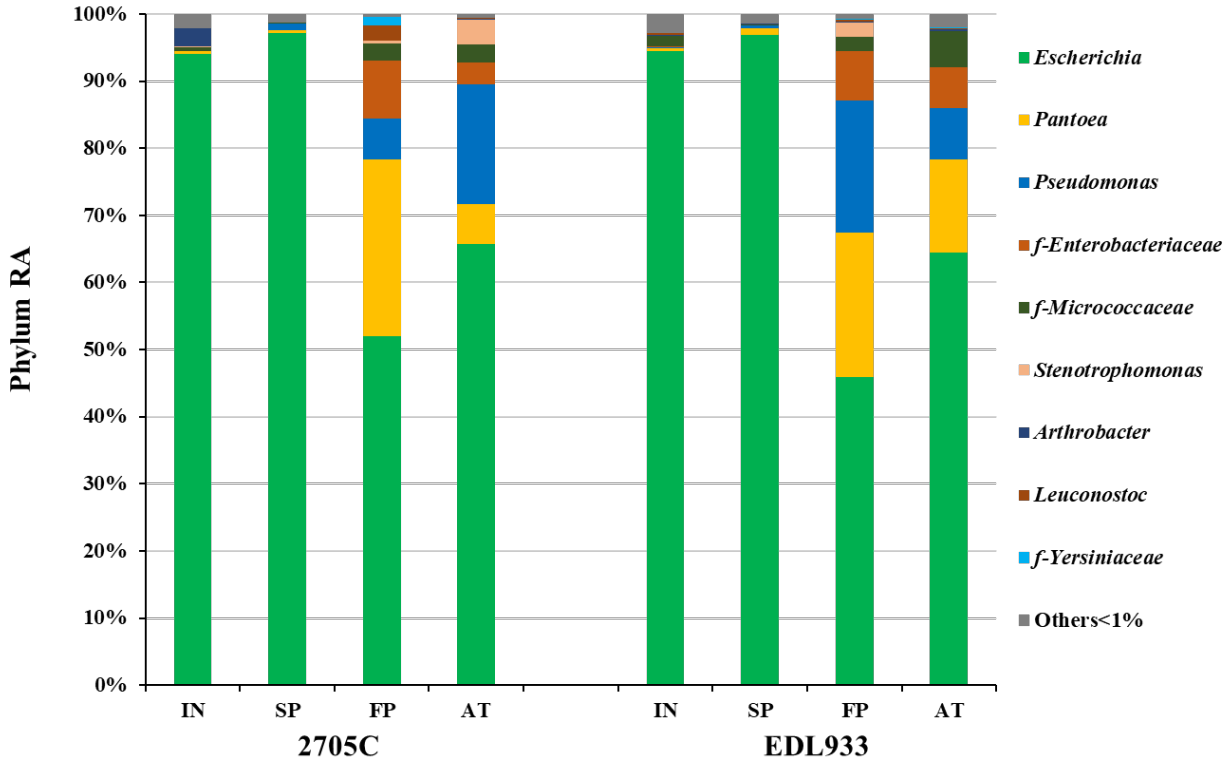

Figure 3.8. Relative abundance (RA) of dominant bacterial genera on romaine lettuce under simulated FP and SP production conditions. Inoculated leaf samples were manually cut and tested on D0 (IN, initial sampling conducted immediate after vacuum cooling), D1 (SP simulation), and D8 (FP, simulation with air pressure fluctuation; AT, alternative simulation test without air pressure change). 2705C, *Escherichia coli* O157:H7 2019 lettuce outbreak strain; EDL933, control strain. Only genera > 1% (average RA) of the bacteria identified in at least one type of samples were included (n = 4). The bacteria identified at the family level (f-, family) indicate unknown genera belonging to those families.

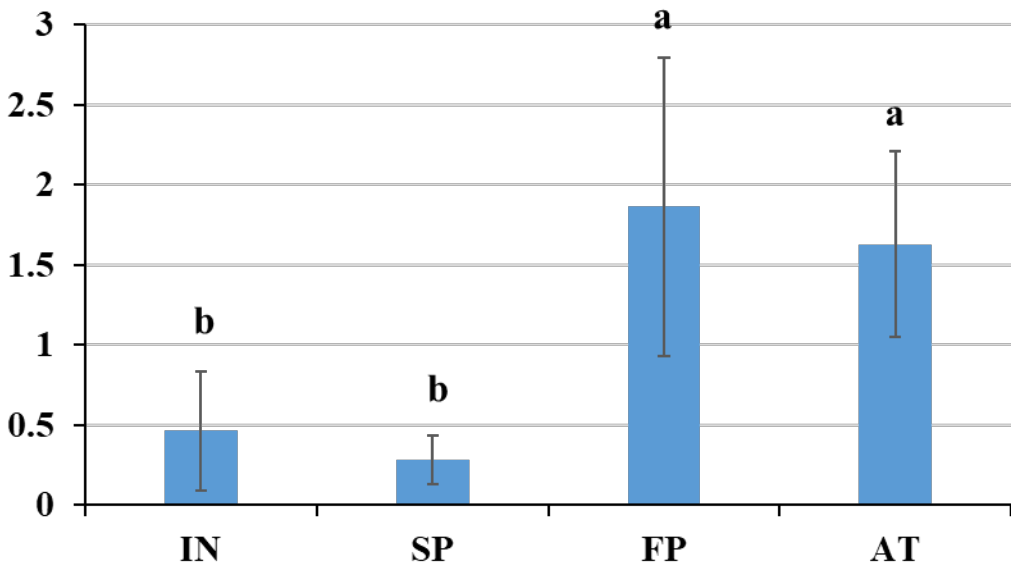

Figure 3.9. Alpha diversity (Shannon index) of bacterial communities on lettuce samples. Inoculated leaf samples were manually cut and tested on D0 (IN, initial sampling conducted immediate after vacuum cooling), D1 (SP simulation), and D8 (FP, simulation with air pressure fluctuation; AT, alternative simulation test without air pressure change).

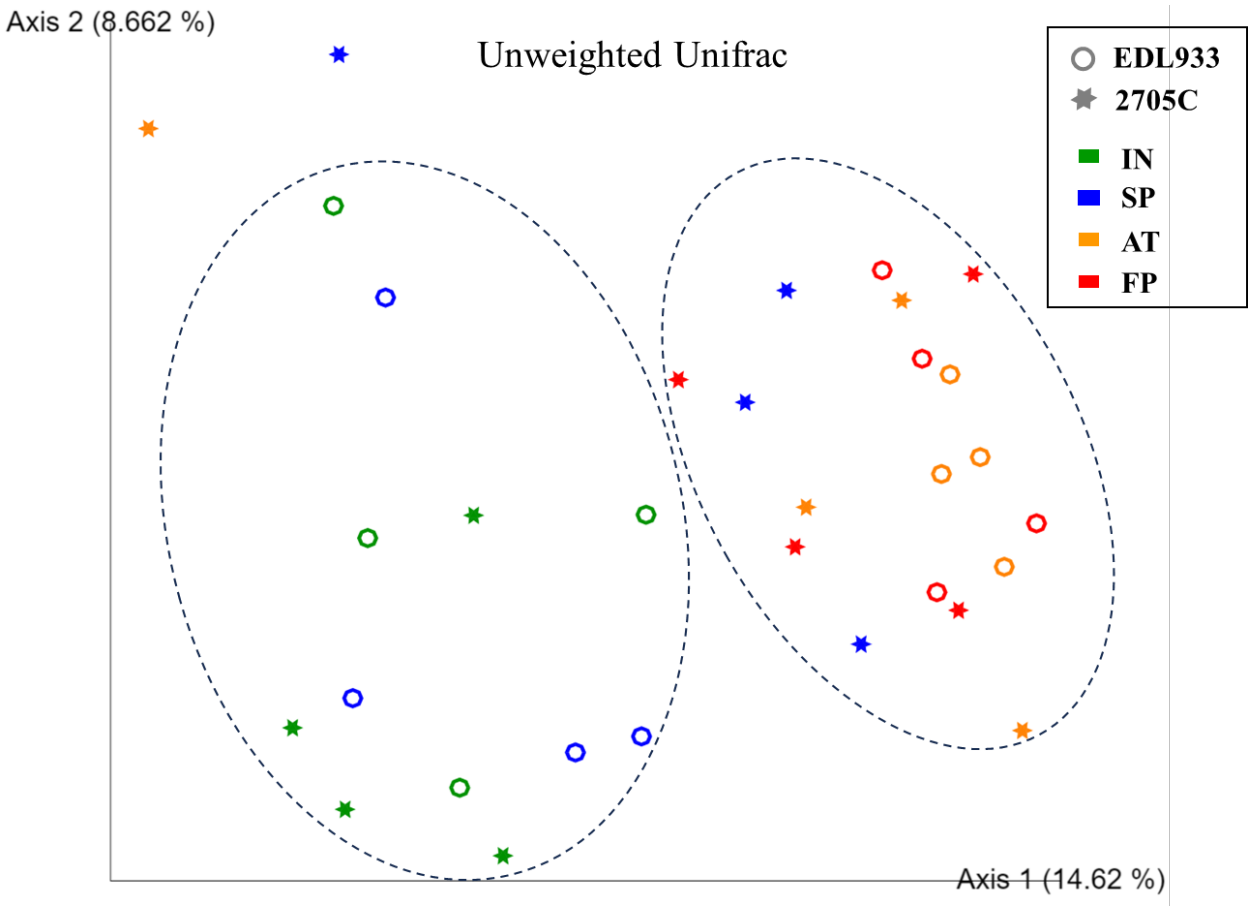

Figure 3.10. Relatedness of bacterial communities on romaine lettuce under simulated FP and SP production conditions. Inoculated leaf samples were manually cut and tested on D0 (IN, initial sampling conducted immediate after vacuum cooling), D1 (SP simulation), and D8 (FP, simulation with air pressure fluctuation; AT, alternative simulation test without air pressure change). Lettuce was inoculated with 2705C, *Escherichia coli* O157:H7 2019 lettuce outbreak strain; or EDL933, control strain. Principle coordinate analysis (PCoA) was performed based on Unweighted Unifrac Distance to compare bacterial communities on romaine lettuce.

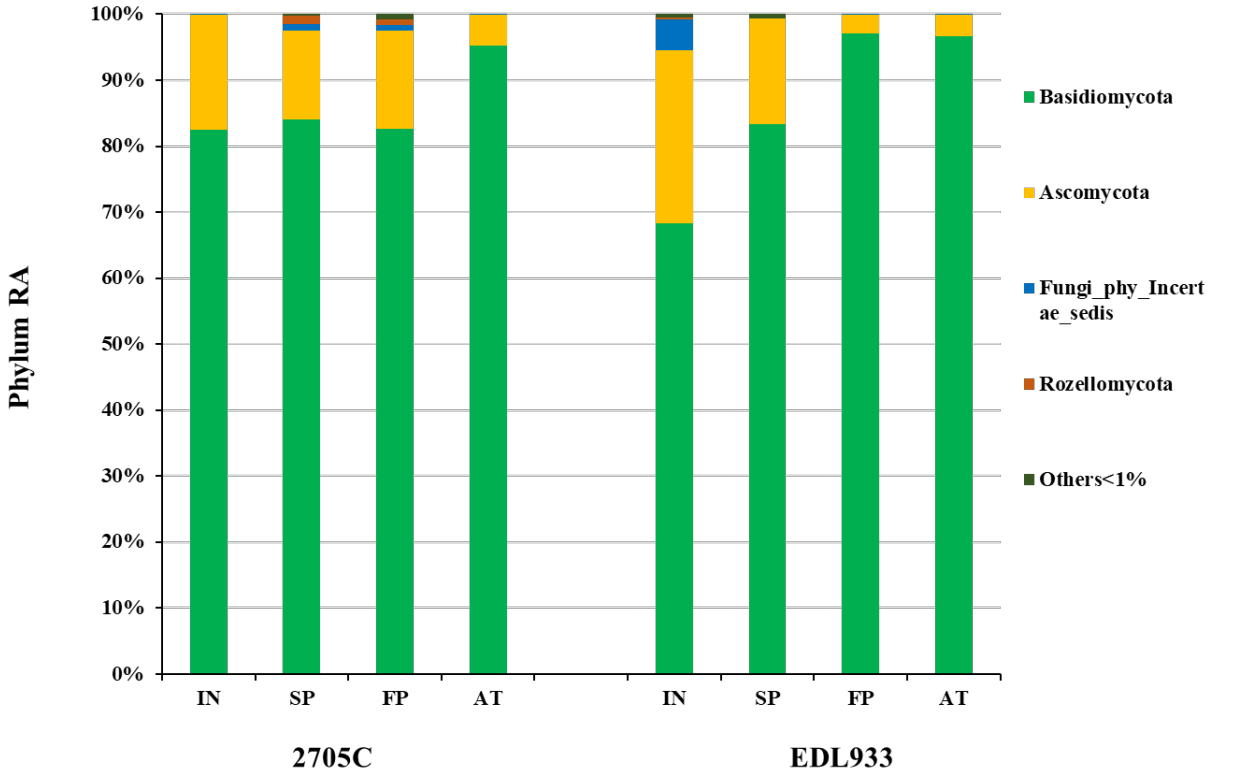

Figure 3.11. Relative abundance (RA) of dominant fungal phyla on romaine lettuce under simulated FP and SP production conditions. Inoculated leaf samples were manually cut and tested on D0 (IN, initial sampling conducted immediate after vacuum cooling), D1 (SP simulation), and D8 (FP, simulation with air pressure fluctuation; AT, alternative simulation test without air pressure change). 2705C, *Escherichia coli* O157:H7 2019 lettuce outbreak strain; EDL933, control strain. Only phyla > 1% (average RA) of the bacteria identified in at least one type of samples were included (n = 4).

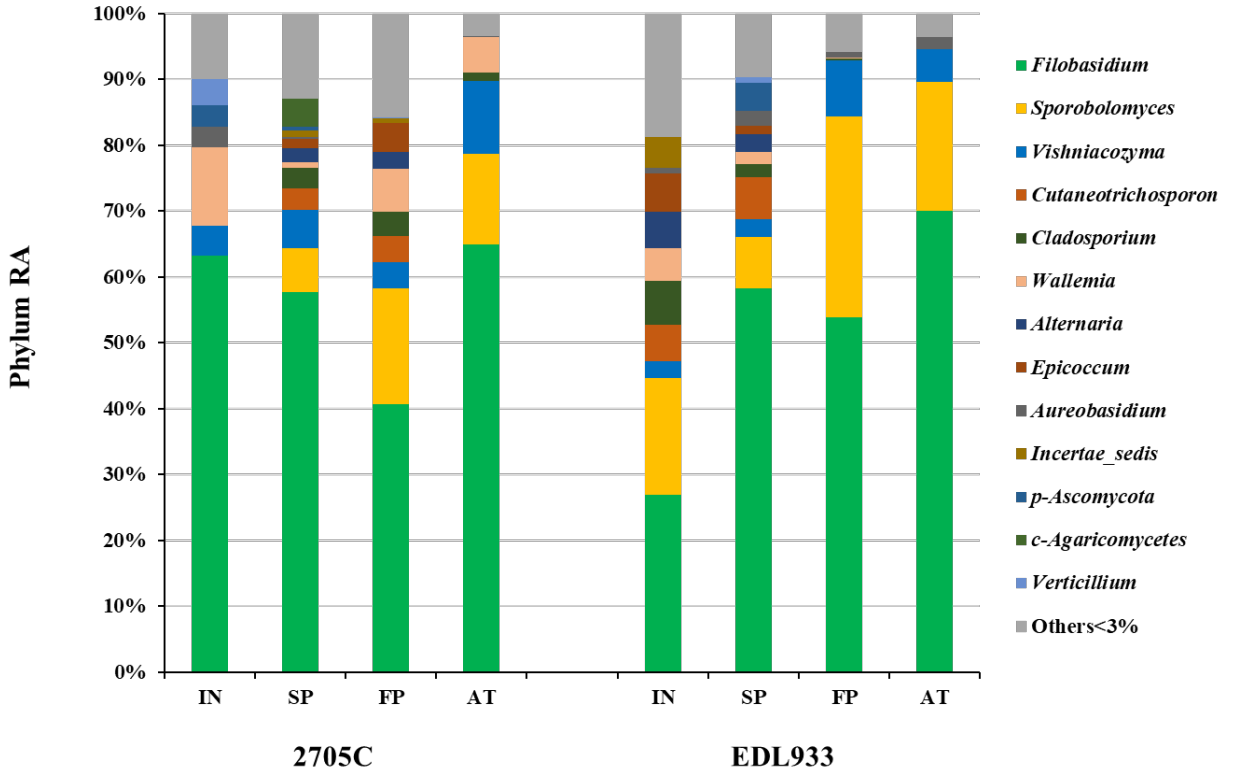

Figure 3.12. Relative abundance (RA) of dominant fungal phyla on romaine lettuce under simulated FP and SP production conditions. Inoculated leaf samples were manually cut and tested on D0 (IN, initial sampling conducted immediate after vacuum cooling), D1 (SP simulation), and D8 (FP, simulation with air pressure fluctuation; AT, alternative simulation test without air pressure change). 2705C, *Escherichia coli* O157:H7 2019 lettuce outbreak strain; EDL933, control strain. Only phyla > 1% (average RA) of the bacteria identified in at least one type of samples were included (n = 4).

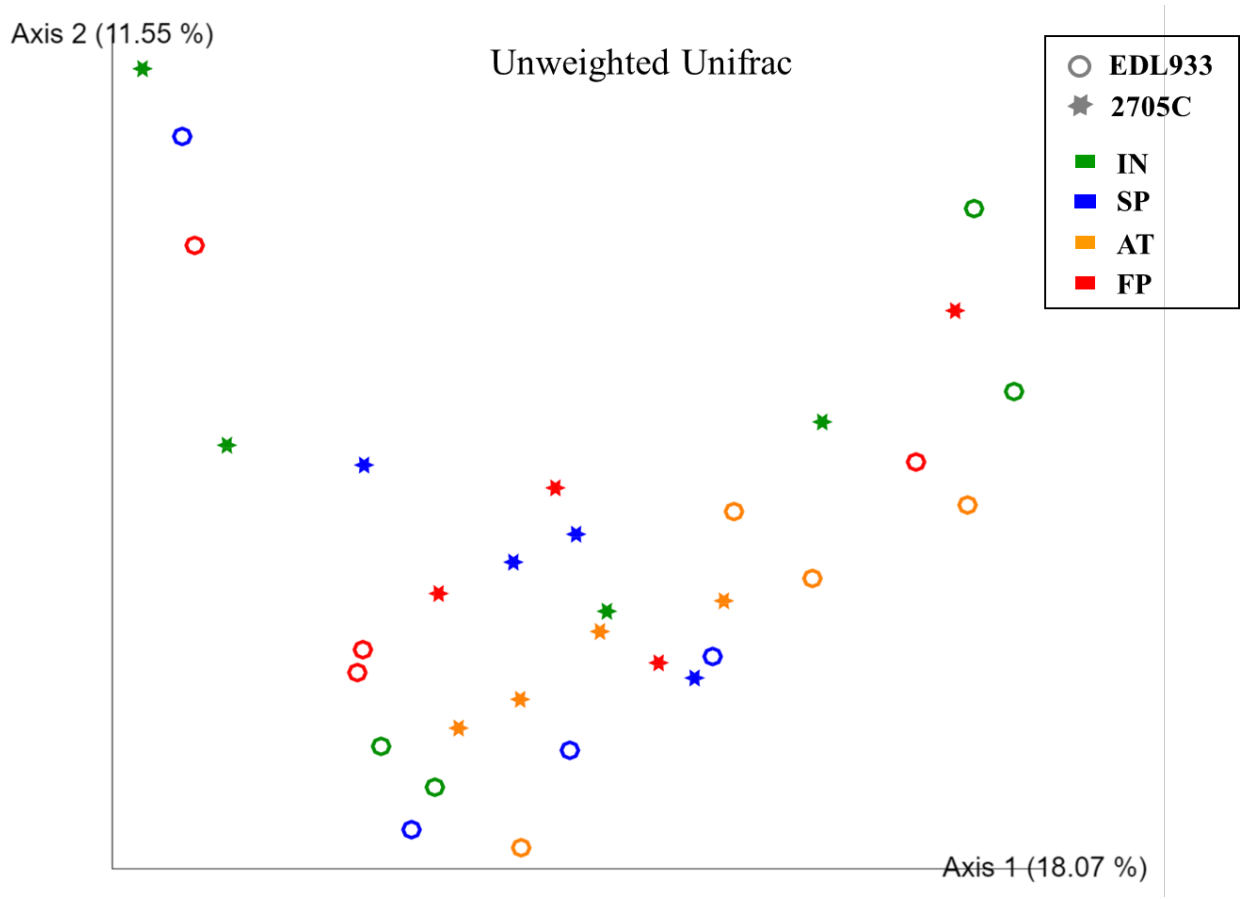

Figure 3.13. Relatedness of fungal communities on romaine lettuce under simulated FP and SP production conditions. Inoculated leaf samples were manually cut and tested on D0 (IN, initial sampling conducted immediate after vacuum cooling), D1 (SP simulation), and D8 (FP, simulation with air pressure fluctuation; AT, alternative simulation test without air pressure change). Lettuce was inoculated with 2705C, *Escherichia coli* O157:H7 2019 lettuce outbreak strain; or EDL933, control strain. Principle coordinate analysis (PCoA) was performed based on Unweighted Unifrac Distance to compare fungal communities on romaine lettuce.

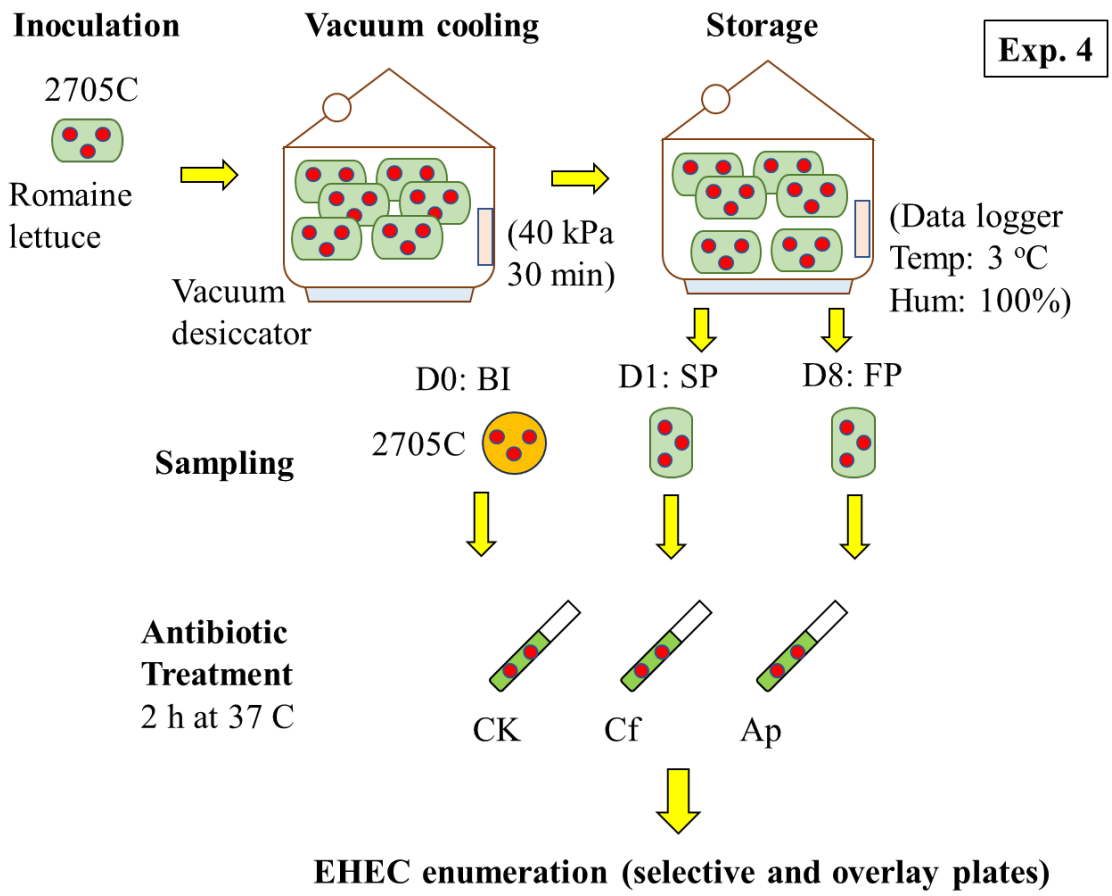

Figure 3.14. Schematic presentation of experiment flow and sample assignment about persister cells of EcO157 from inoculated romaine lettuce. BI, before lettuce inoculation; SP, source processing; FP, forward processing. CK, control without antibiotics; Cf, 3 µg/mL Ciprofloxacin; Ap, 100 µg/mL Ampicillin.

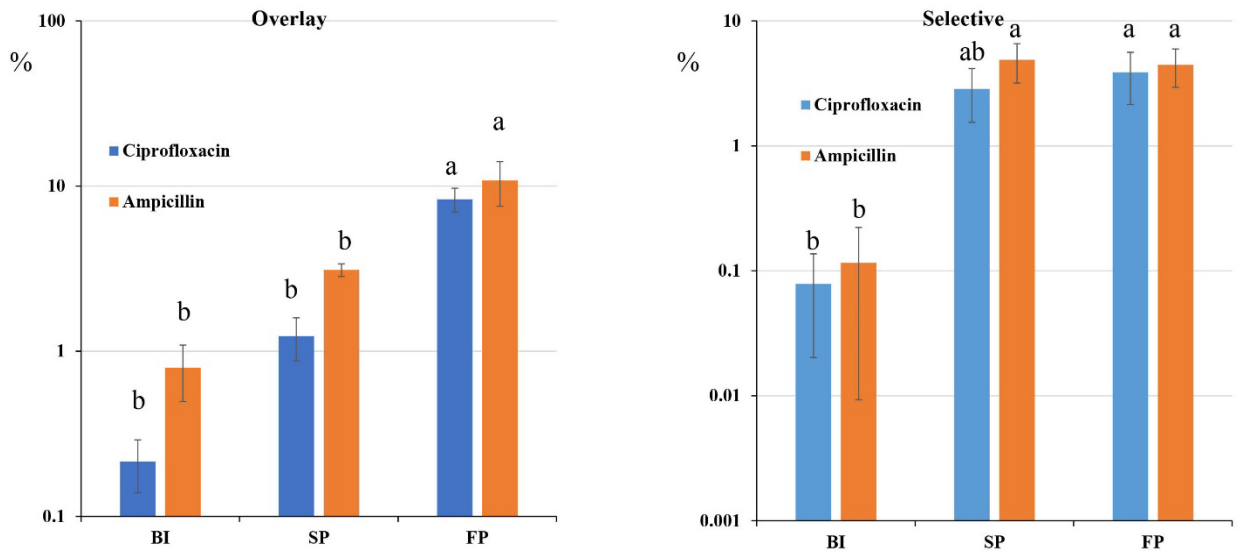

Figure 3.15. Persister cell percentage based on initial population before 2 h antibiotic treatment. BI, before lettuce inoculation; SP, source processing; FP, forward processing.

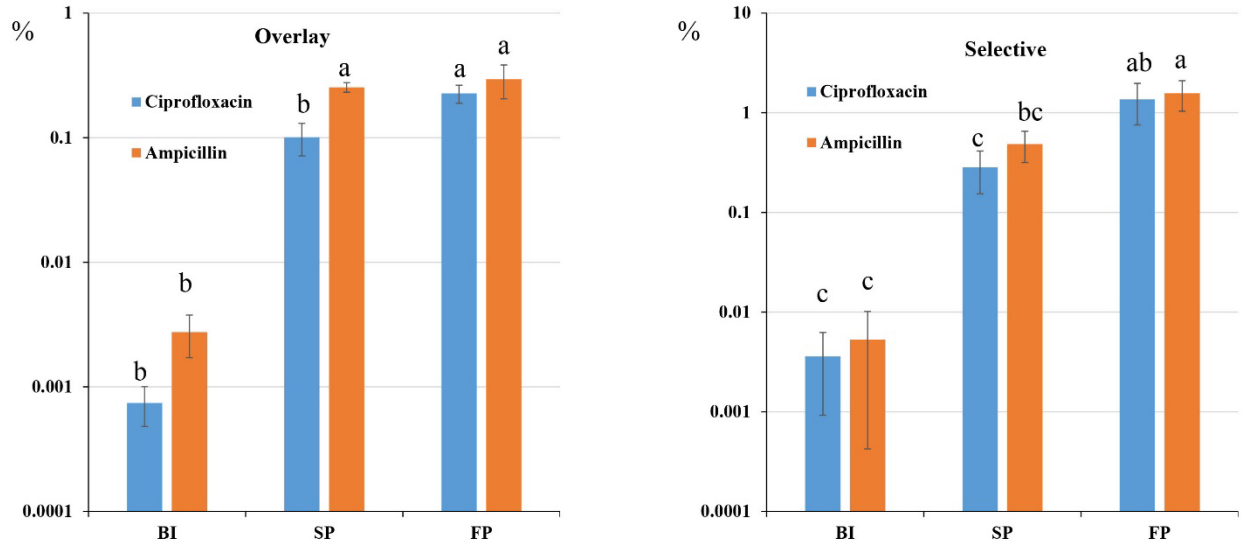

Figure 3.16. Persister cell percentage based on control without antibiotic treatment. BI, before lettuce inoculation; SP, source processing; FP, forward processing.

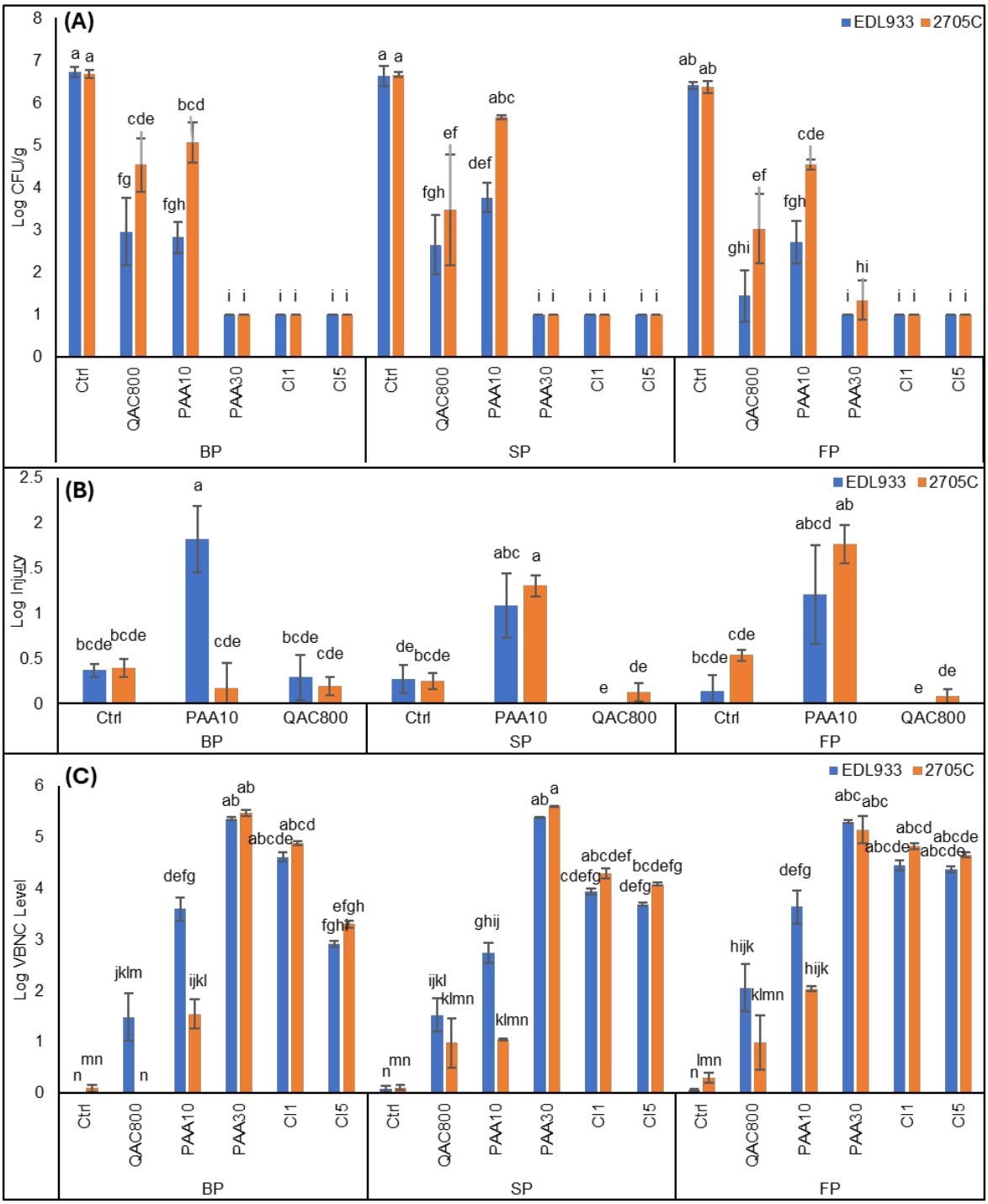

Figure 3.17. The total recovery, injury, or VBNC levels of EcO157 in rinse water affected by lettuce with source (SP) or forward processing (FP) conditions, treatments with different sanitizers (free chlorine (Cl), peroxyacetic acid (PAA), quaternary ammonium compounds (QAC), or DI water (Ctrl)), and different EcO157 strains (EDL933 or 2705C). Different letters indicate statistically significant differences ($p < 0.05$).

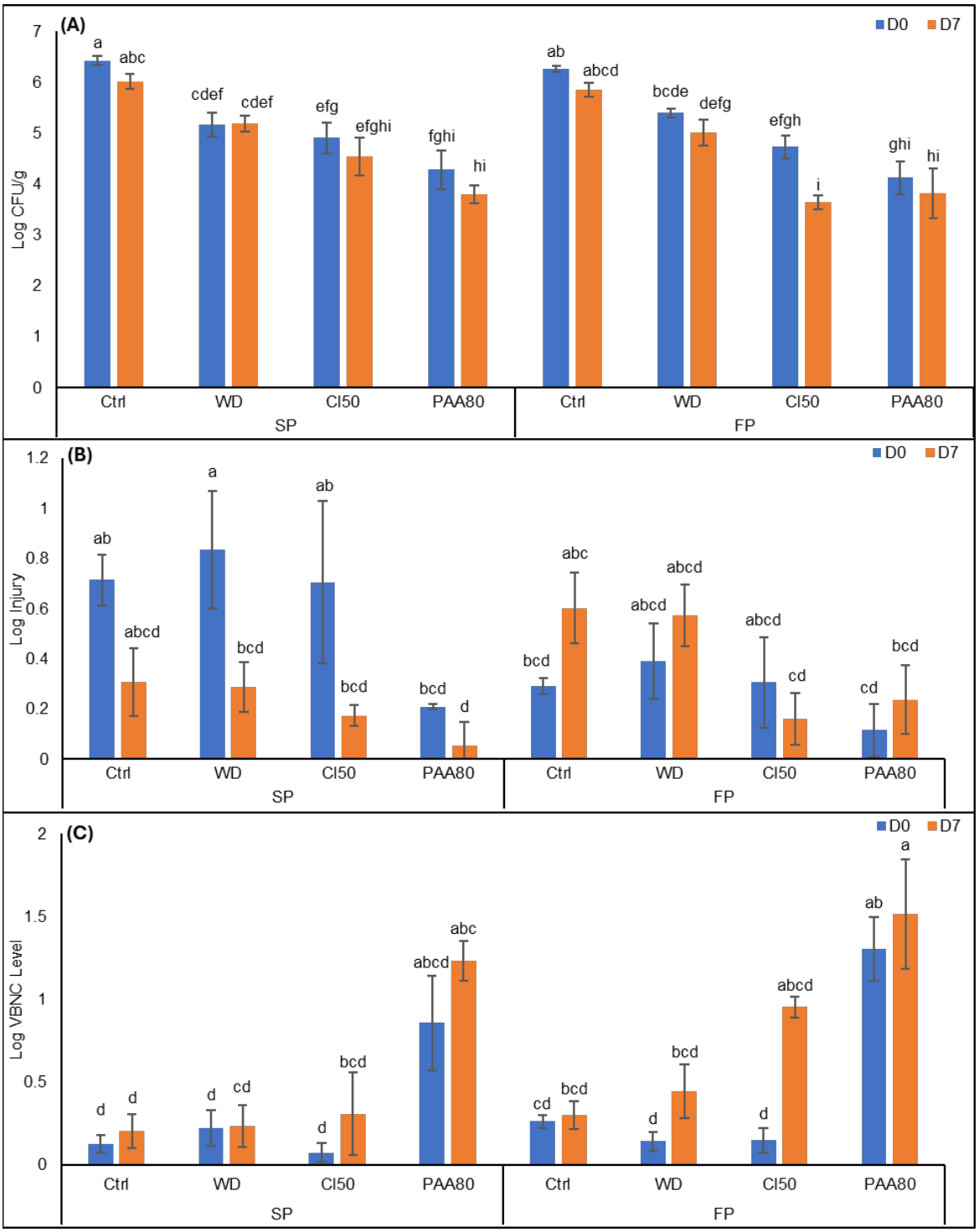

Figure 3.18. The total recovery, injury, or VBNC levels of EcO157 on lettuce after simulated washing affected by lettuce with source (SP) or forward processing (FP) conditions, before (Ctrl) or after washing with different sanitizers (free chlorine (Cl), peroxyacetic acid (PAA), DI water (WD)), and storage. Different letters indicate statistically significant differences ($p < 0.05$).

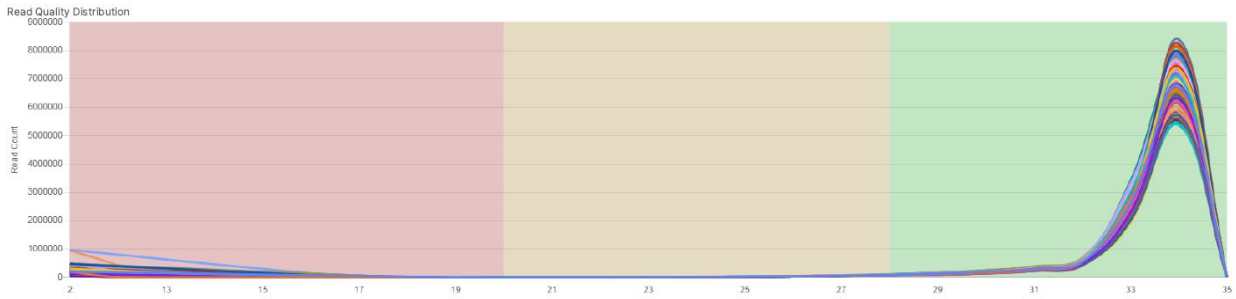

Figure 3.19. Mean quality distribution of the RNA-Seq data.

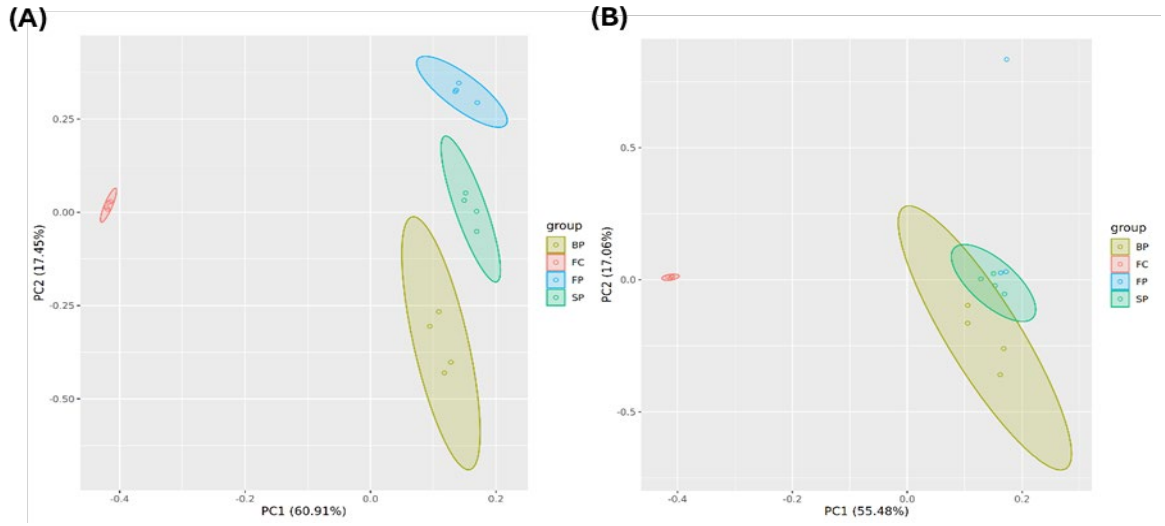

Figure 3.20. Principle component analysis of *E. coli* O157:H7 strain 2705C (A) and EDL933 (B) samples by transcriptome profiles.

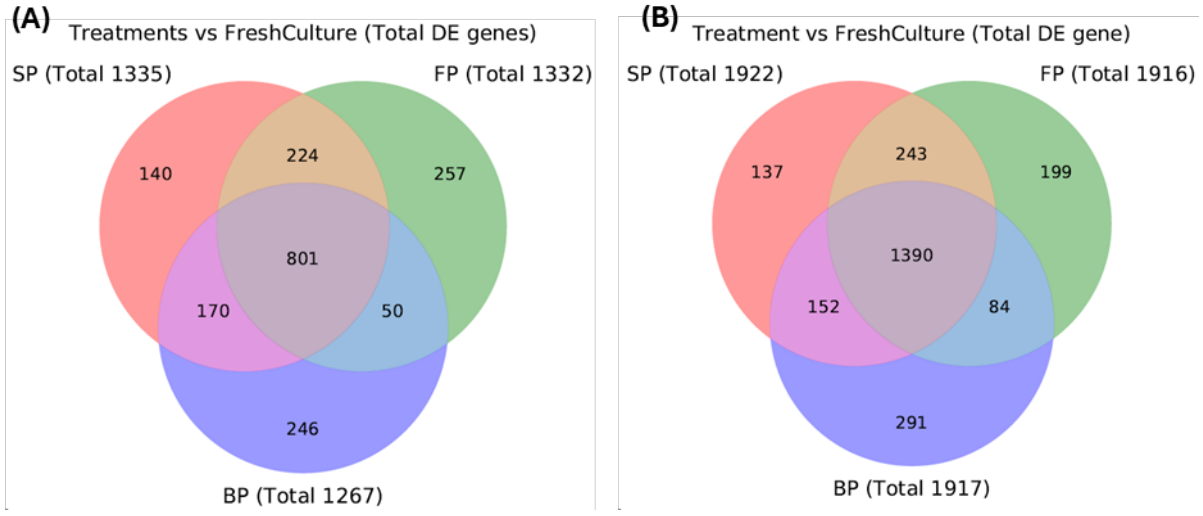

Figure 3.21. Venn Diagrams showing DEGs of *E. coli* O157:H7 strain 2705C (A) and EDL933 (B) recovered from inoculated lettuce before (BP) or after SP or FP compared to FC.

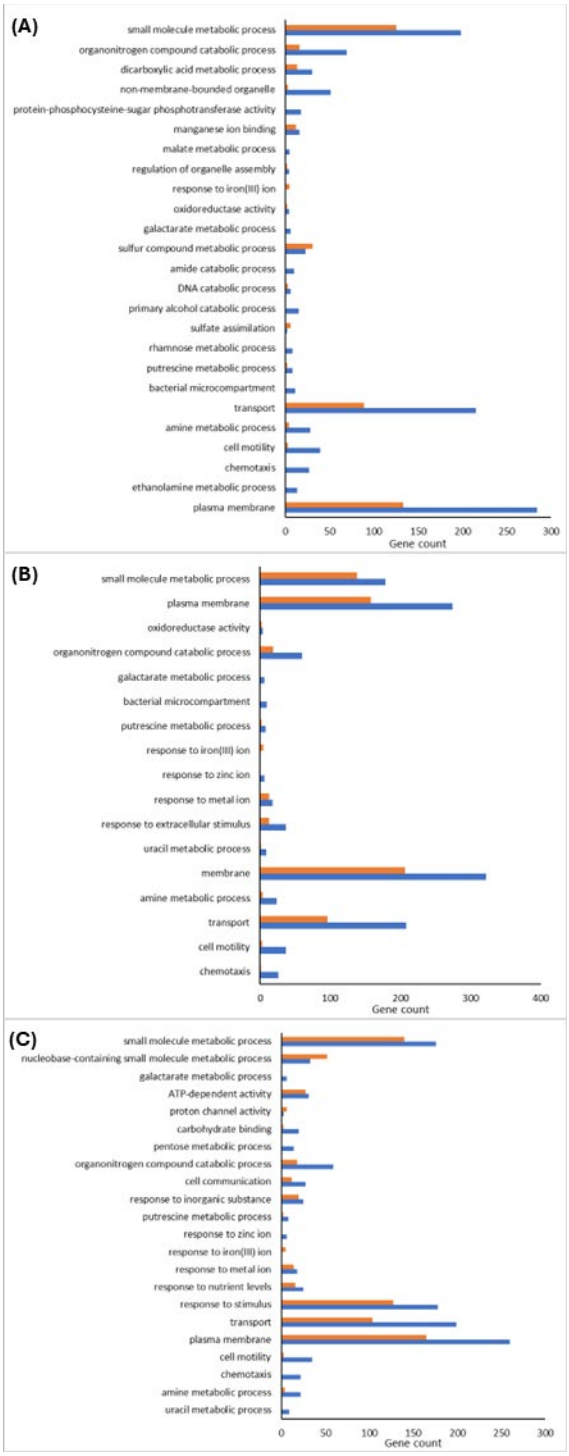

Figure 3.22. Enriched GO functional groups (P_{adj} < 0.05) and the associated differentially expressed genes of *E. coli* O157:H7 strain EDL933 between the lettuce-inoculated group (BP (A)/ SP (B)/ FP (C)) and FC. Blue or orange bars represent up- or down-regulated genes.

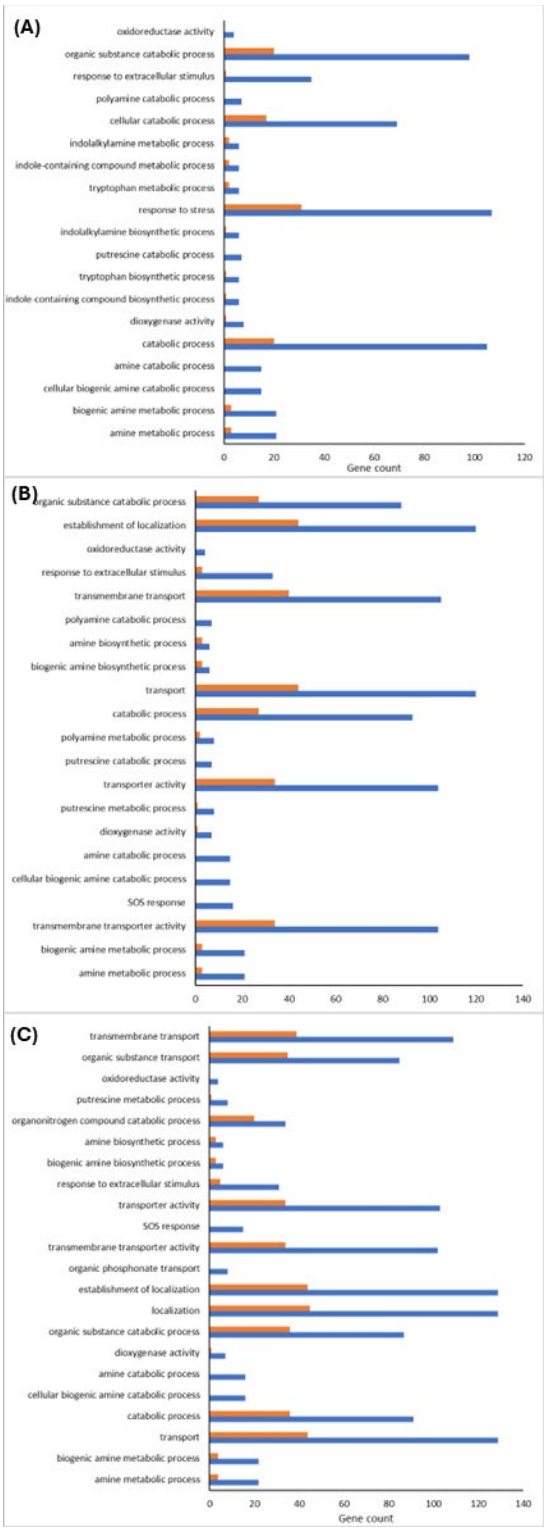

Figure 3.23. Enriched GO functional groups (P_{adj} < 0.05) and the associated differentially expressed genes of *E. coli* O157:H7 strain 2705C between the lettuce-inoculated group (BP (A)/ SP (B)/ FP (C)) and FC. Blue or orange bars represent up- or down-regulated genes.

Table 1.1. Populations of total aerobic bacteria (TAB), coliform (TC), and yeast and mold (YM) in source and forward processing lettuce samples freshly harvested from field (F), collected on the processing date before processing (B), after processing on days 0 (D0), 1 (D1), 4 (D4), 7 (D7), and 14 (D14) in summer and fall trials.

		Summer			Fall		
		TMB	TC	YM	TMB	TC	YM
	F	4.24 ± 0.56 g , B	3.09 ± 0.38 fg , B	2.53 ± 0.34 cde , B	5.08 ± 0.28 e , A	3.67 ± 0.37 d , A	3.37 ± 0.22 bc , A
Source processing	B	4.57 ± 0.44 fg , A	3.50 ± 0.76 ef , A	2.71 ± 0.2 bcd , A	4.33 ± 0.34 f , A	3.10 ± 0.52 ef , A	2.70 ± 0.09 cd , A
	D0	2.70 ± 0.29 h , B	2.14 ± 0.17 g , A	1.56 ± 0.32 f , A	3.03 ± 0.07 g , A	2.17 ± 0.19 g , A	1.80 ± 0.48 d , A
	D1	2.94 ± 0.33 h , B	2.53 ± 0.21 g , A	0.63 ± 0.49 g , A	3.38 ± 0.19 g , A	2.74 ± 0.22 f , A	1.79 ± 1.4 d , A
	D4	4.43 ± 0.35 fg , A	3.98 ± 0.48 def , A	2.54 ± 0.42 cde , A	4.19 ± 0.25 f , A	3.64 ± 0.34 de , A	2.74 ± 0.27 cd , A
	D7	4.88 ± 0.36 efg , A	4.41 ± 0.27 de , A	2.19 ± 0.1 de , B	4.96 ± 0.37 e , A	4.22 ± 0.32 c , A	3.14 ± 0.45 c , A
	D14	6.78 ± 0.56 b , A	6.24 ± 0.66 ab , A	3.23 ± 0.45 b , A	6.85 ± 0.24 bc , A	5.97 ± 0.22 b , A	3.59 ± 0.48 abc , A
Forward processing	B	5.77 ± 0.15 cd , A	4.73 ± 0.79 cd , A	4.14 ± 0.37 a , A	5.38 ± 0.15 e , B	4.61 ± 0.15 c , A	4.43 ± 0.14 a , A
	D0	5.07 ± 0.38 def , A	4.53 ± 0.75 cd , A	2.06 ± 0.18 ef , B	5.16 ± 0.15 e , A	4.35 ± 0.29 c , A	3.10 ± 0.67 c , A
	D1	5.11 ± 0.48 def , B	4.03 ± 0.27 def , B	1.97 ± 0.23 ef , B	6.01 ± 0.56 d , A	4.48 ± 0.2 c , A	3.21 ± 0.34 c , A
	D4	5.57 ± 0.53 de , B	4.41 ± 0.25 de , B	1.52 ± 0.14 f , B	6.42 ± 0.14 cd , A	5.49 ± 0.19 b , A	3.61 ± 0.08 abc , A
	D7	6.49 ± 0.5 bc , B	5.43 ± 0.47 bc , B	1.99 ± 0.33 ef , B	7.03 ± 0.12 b , A	5.91 ± 0.1 b , A	4.26 ± 0.16 ab , A
	D14	7.92 ± 0.13 a , B	6.82 ± 0.13 a , B	2.88 ± 0.21 bc , B	8.24 ± 0.09 a , A	7.53 ± 0.21 a , A	4.44 ± 0.08 a , A

Lower-case lettering represents significant difference levels across different types of samples (in each column); capital letters denote significant differences of microbial populations between summer and fall trials ($p < 0.05$).

Table 1.2. Results from Pearson's correlation between section quality ratings (1.9) and overall visual quality ratings.

Cut Section	r	*p* value
Leaf without Veins	0.65	<0.001
Leaf with Veins	0.88	<0.001
Rib	0.87	<0.001
Heart	0.86	<0.001

Table 1.3. Rank orders for level of browning from least apparent browning to most browning among rib and heart sections. FP, forward processing; SP, source processing. Lettering represents significant differences in rank order between samples ($p < 0.05$).

Rank	Rib Order	Rib Sample	Rank Total for Rib Ranking	Differences	Heart Order	Heart Sample	Rank Total for Heart Ranking	Rank Total for Heart Ranking
Least	1	FP-B	89	a	1	FP-B	96	a
	2	SP-D7	80	a	2	FP-0	74	ab
	3	FP-0	76	a	3	SP-D7	71	ab
	4	SP-B	71	ab	4	SP-B	68	abc
	5	SP-4	61	ab	5	SP-D4	64	abcd
	6	SP-0	59	abc	6	SP-14	50	bcd
	7	SP-14	52	abcd	7	SP-D0	47	bcd
	8	FP-D4	32	bcd	8	FP-D4	32	bcd
	9	FP-14	18	d	9	FP-7	26	cd
Most	10	FP-7	12	d	10	FP-14	22	d

Table 3.1. Antimicrobial susceptibility test of two EHEC outbreak strains (2705C and 2705D) and one reference strain (EDL933).

Antimicrobial	Resistance breakpoint (µg/ml)	EDL933 Resistance (µg/ml)	2705C Resistance (µg/ml)	2705D Resistance (µg/ml)
Amoxicillin/clavulanic acid	32/16	**2/1**	**2/1**	**2/1**
Ampicillin	32	**1**	**1**	**1**
Azithromycin	32	**0.5**	**0.5**	**0.5**
Cefoxitin	32	**4**	**4**	**4**
Ceftiofur	8	**0.25**	**0.25**	**0.25**
Ceftriaxone	4	**<0.25**	**<0.25**	**<0.25**
Chloramphenicol	32	**4**	**4**	**4**
Ciprofloxacin	0.12	**<0.015**	**<0.015**	**<0.015**
Gentamicin	16	**0.25**	**0.25**	**0.25**
Nalidixic acid	32	**1**	**1**	**1**
Streptomycin	32	**<2**	**2**	**2**
Sulfisoxazole	512	**32**	**32**	**32**
Tetracycline	16	**<4**	**<4**	**<4**
Trimethoprim/sulfamethoxazole	4/76	**<0.12/2.38**	**<0.12/2.38**	**<0.12/2.38**

Table 3.2. Unique genes in the seven *E. coli* O157:H7 outbreak strains.

Gene	Annotation	2705C	2705D	EDL933	FS4157	FS4365	RM4406	RM4688
group_1061	hypothetical protein	Y[#]	Y	N[#]	N	N	N	N
group_149	hypothetical protein	Y	Y	N	N	N	N	N
group_227	hypothetical protein	Y	Y	N	N	N	N	N
group_286	hypothetical protein	Y	Y	N	N	N	N	N
group_531	hypothetical protein	Y	Y	N	N	N	N	N
group_539	hypothetical protein	Y	Y	N	N	N	N	N
group_5490	hypothetical protein	Y	Y	N	N	N	N	N
group_6	hypothetical protein	Y	Y	N	N	N	N	N
group_6675	hypothetical protein	Y	Y	N	N	N	N	N
group_6915	hypothetical protein	Y	Y	N	N	N	N	N
group_7137	hypothetical protein	Y	Y	N	N	N	N	N
group_7479	hypothetical protein	Y	Y	N	N	N	N	N
group_892	hypothetical protein	Y	Y	N	N	N	N	N
group_988	hypothetical protein	Y	Y	N	N	N	N	N
ldcC_2	Constitutive lysine decarboxylase	Y	Y	N	N	N	N	N
mscM_2	Miniconductance mechanosensitive channel MscM	Y	Y	N	N	N	N	N
orn_2	Oligoribonuclease	Y	Y	N	N	N	N	N
pepD_2	Cytosol non-specific dipeptidase	Y	Y	N	N	N	N	N
phoE_2	Outer membrane porin PhoE	Y	Y	N	N	N	N	N
prfB_3	Peptide chain release factor 2	Y	Y	N	N	N	N	N
proA_3	Gamma-glutamyl phosphate reductase	Y	Y	N	N	N	N	N
proB_2	Glutamate 5-kinase	Y	Y	N	N	N	N	N
psd_2	Phosphatidylserine decarboxylase proenzyme	Y	Y	N	N	N	N	N
rsgA_2	Small ribosomal subunit biogenesis GTPase RsgA	Y	Y	N	N	N	N	N
aspA_2	Aspartate ammonia-lyase	Y	Y	N	N	N	N	N
crl_2	Sigma factor-binding protein Crl	Y	Y	N	N	N	N	N
cutA_2	Divalent-cation tolerance protein CutA	Y	Y	N	N	N	N	N
dcuA_2	Anaerobic C4-dicarboxylate transporter DcuA	Y	Y	N	N	N	N	N
dsbD_2	Thiol:disulfide interchange protein DsbD	Y	Y	N	N	N	N	N
gpt_2	Xanthine phosphoribosyltransferase	Y	Y	N	N	N	N	N
stxB_2	Shiga toxin subunit B	Y	Y	N	N	N	N	N
stxA_2	Shiga toxin subunit A	Y	Y	N	N	N	N	N

[#] Y and N indicate presence and absence, respectively.

Table 3.3. The total recovery (Unit: log CFU/g), injury (Unit: log), or VBNC levels (Unit: log) of EcO157 in rinse water affected by lettuce with source (SP) or forward processing (FP) conditions, treatments with different sanitizers (free chlorine (Cl), peroxyacetic acid (PAA), quaternary ammonium compounds (QAC), or DI water (Ctrl)), and different EcO157 strains (EDL933 or 2705C). Different letters indicate statistically significant differences within the variable ($P < 0.05$).

Measurement	Variable	Effect on recovered population (P-value)	Level	Least Sq Mean	Std Error	Significance within the variable
Recovery	Processing	< 0.0042	BP	2.90	0.376	A
			SP	2.90	0.345	A
			FP	2.57	0.380	B
	Sanitizers	< 0.0001	Ctrl	6.58	0.047	A
			PAA10	4.09	0.280	B
			QAC800	3.01	0.304	C
			PAA30	1.05 (< LOD)	0.055	D
			Cl1	1 (< LOD)	0	D
			Cl5	1 (< LOD)	0	D
	Strains	< 0.0001	EDL933	2.50	0.281	A
			2705C	3.07	0.311	B
Log Injury	Processing	> 0.05	BP	0.54	0.150	A
			SP	0.50	0.132	A
			FP	0.56	0.197	A
	Sanitizers	< 0.0001	Ctrl	0.32	0.042	B
			PAA10	1.22	0.156	A
			QAC800	0.05	0.092	B
	Strains	> 0.05	EDL933	0.53	0.147	A
			2705C	0.53	0.114	A
Log VBNC	Processing	< 0.001	BP	2.77	0.344	B
			SP	2.79	0.325	B
			FP	3.15	0.324	A
	Sanitizers	< 0.001	PAA30	5.38	0.059	A
			Cl1	4.50	0.084	B
			Cl5	3.84	0.147	C
			PAA10	2.43	0.254	D
			QAC800	1.17	0.234	E
			Ctrl	0.10	0.032	F
	Strains	< 0.001	EDL933	3.06	0.249	A
			2705C	2.74	0.288	B

Table 3.4. The total recovery (Unit: log CFU/g), injury (Unit: log), or VBNC levels (Unit: log) of EcO157 on lettuce after simulated washing affected by lettuce with source (SP) or forward processing (FP) conditions, before (Ctrl) or after washing with different sanitizers (free chlorine (Cl), peroxyacetic acid (PAA), DI water (WD)), and storage. Different letters indicate statistically significant differences within the variable ($P < 0.05$).

Measurement	Variable	Effect on recovered population (P-value)	Level	Least Sq Mean	Std Error	Significance within the variable
Recovery	Processing	0.0419	SP	5.04	0.177	A
			FP	4.85	0.193	B
	Washing	< 0.0001	Ctrl	6.14	0.074	A
			WD	5.19	0.071	B
			Cl50	4.46	0.168	C
			PAA80	4.01	0.124	D
	Storage	< 0.0001	D0	5.16	0.172	A
			D7	4.73	0.190	B
Log Injury	Processing	> 0.05	SP	0.41	0.067	A
			FP	0.33	0.044	A
	Washing	< 0.001	Ctrl	0.48	0.065	A
			WD	0.52	0.079	A
			Cl50	0.34	0.088	AB
			PAA80	0.15	0.037	B
	Storage	0.0077	D0	0.45	0.063	A
			D7	0.30	0.045	B
Log VBNC	Processing	0.0249	SP	0.41	0.100	B
			FP	0.63	0.120	A
	Washing	< 0.001	Ctrl	0.22	0.043	B
			WD	0.26	0.070	B
			Cl50	0.37	0.127	B
			PAA80	1.23	0.147	A
	Storage	0.0120	D0	0.39	0.100	B
			D7	0.65	0.118	A