POST-HARVEST DETECTION AND PATHOGEN CONTROL OF FOODBORNE PATHOGENS IN THE FRESH PRODUCE INDUSTRY

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Food Science – Master of Science

2024

ABSTRACT

Outbreaks of listeriosis associated with apples have led to increased research revealing the presence of *Listeria* species in apple processing environments. *Listeria* spp. were environmental sampled across separate facilities (A, B, and C) and from 9 controlled atmosphere (CA) apple storage rooms of apple processing facilities during 2021 and 2023. Out of f 300 samples, 6.3% (19) were positive for *Listeria* spp., with 65 isolates collected. The majority of positive samples came from floors below condenser units (9/19). Facility A had the highest number of positive samples - 11 total positives and the greatest diversity of *Listeria* spp. The highest rates of Listeria spp were recorded in Fall 2022 and Spring/Fall 2023. *L. monocytogenes* was the predominant species compromising 48 (74%) isolates. Three allelic types (AT) of *L. monocytogenes* were identified. Facility A had repeatedly sampled positive for *L. monocytogenes* contamination at one location in the same room but with different ATs, highlighting the utility of *sigB* allelic typing. These findings demonstrate, through the presence, distribution, and characterization of *Listeria* spp., the potential of CA apple storage rooms in spreading *Listeria* to the processing environment or on apples.

Ozone is a potential alternative to chlorine due to its high oxidative potential but has limitations due to its volatility, hazardous off-gassing, and inconsistency. Nanobubble (NB) technology was explored to stabilize ozone in wash water and increase its antimicrobial effects. Submerging 0.5 kg of shredded Romaine lettuce in 80 L of 6 ppm ozone NBs for 90 s reduced *E. coli* O157:H7 populations by 0.8±0.20 log colony forming units (CFU)/g. When examining the application of 6 ppm ozone NBs in the context of a pilot-scale flume system for production of shredded lettuce, the addition of ozone NB into the final overhead rinse water, after washing in 10 or 20 ppm of chlorinated flume water for 90 s, did not significantly enhance the reduction of *E. coli* O157:H7 compared to tap water ($p > 0.05$). Furthermore, the application of ozonated NBs during centrifugal drying of shredded lettuce did not significantly differ from tap water in reducing *E. coli* O157:H7 populations ($p > 0.05$). Although there was a significant difference observed in microbial reduction between ozonated water applied before centrifugal drying and tap water ($p < 0.05$), the overall magnitude of this difference was minimal. These results suggest the application of ozone NBs as a sanitizer during pilot-scale processing may not be effective at inactivating *E. coli* O157:H7 on shredded Romaine lettuce.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor Dr. Bergholz. Her patience, kindness, and guidance during my time as a graduate student has left a positive impact on me and is someone who I truly say is a role model. I would also like to thank my committee members Dr. Ryser and Dr. Mitchell for providing constructive feedback on my academic progress and serving as knowledgeable resources when called upon.

Special thanks to the team at En Solución, Alex and Dirk, for providing the commercial nanobubble and ozone generator equipment and the apple industry partners that allowed us to collect samples from their facilities. Also, I would like to express how much I appreciated and enjoyed being around my lab mates, Dimple, Hazel, Yawei, and Jun. To say that I "look up to them" would be an understatement and am forever grateful for their support. Then my undergraduate students Erik, Shania, and Avery. It was a pleasure mentoring them in the lab and knowing I made an impact on them during our time together.

Finally, I would also like to thank my family and friends who believed and supported me throughout my academic career.

The environmental monitoring study was supported by Michigan Tree Fruit Commission Michigan Department of Agriculture and Rural Development grant #220000000881 and the ozone nanobubble work was supported by FDA grant 2R44FD006565-02.

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LIST OF ABBREVIATIONS

µg ..... microgram(s)
µl ..... Microliter(s)
amp ..... Ampicillin
ANOVA ..... Analysis of Variance
AT ..... Alleic types(s)
BHI ..... Brain Heart Infusion
BLAST ..... Basic Local Alignment Search Tool
CA ..... Controlled atmosphere
CDC ..... Center for Disease Control
CFU ..... Colony forming units
DAEC ..... Eterotoxigenic diffusely adherent *E. coli*
EAEC ..... Enteroaggregative *E. coli*
EHEC ..... Enterohemorrhagic *E. coli*
EIEC ..... Enteroinvasive *E. coli*
EPEC ..... Enteropathogenic *E. coli*
ETEC ..... Enterotoxigenic *E. coli*
FDA ..... Food & Drug Administration
GRAS ..... Generally Recognized As Safe
h ..... Hour(s)
HC ..... Hemorrhagic colitis
HUS ..... Hemolytic uremic syndrome
kg ..... Kilogram(s)
L ..... Liter(s)
LEE ..... Locus of enterocyte effacement
LLO ..... Listeriolysin
ml ..... Mililiter(s)
NB ..... Nanobubble(s)
oz ..... Ounce(s)
PAA ..... Peracetic acid
ppm ..... Parts per million

QAC ..... Quaternary ammonia compounds

rif ..... Rifampicin

TSA ..... Tryptic Soy Agar

TSAYE..... Trypticase Soy Agar with 0.6 % Yeast Extract

TSB ..... Tryptic Soy Broth

TSBYE ..... Trypticase Soy Broth with 0.6 % Yeast Extract

w/v ..... Weight to volume

YE ..... Yeast extract

Introduction and Rationale

According to the 2020-2025 US Dietary Guidelines, Americans are recommended to consume 2 cups of fruit and 3 cups of vegetables daily (Lee et al., 2022). Depending on the source, fresh produce is nutritionally rich in fats, carbohydrates, and/or protein, minerals and vitamins A, $B_{6,}$ and C (Vincente et al., 2014). Furthermore, research suggests that fruit and vegetable consumption is inversely correlated to development of diseases such as cancer, diabetes, stroke and heart disease (Vincente et al., 2014). Despite only 12.3% of American adults meeting dietary intake (Lee et al., 2022) and the well-established nutritional and health benefits, consumption of fresh produce reported to be responsible for nearly half of all foodborne outbreaks (Painter et al., 2013).

Listeria monocytogenes, Enterohemorrhagic *E. coli* (EHEC), and *Salmonella* collectively contribute to an excess of more than a million reported illnesses, 28,000 hospitalizations, 700 fatalities, and substantial economic costs exceeding $6 billion dollars (CDC, 2022a). A notable vehicle for foodborne outbreaks is fresh produce. According to Carstens et al. (2019), fresh produce outbreaks were associated with 4949 illnesses, 895 hospitalizations, and 9 fatalities between 2004 to 2010 and 1797 foodborne outbreaks were associated with fresh produce between 2010 and 2017. This is exemplified by continued outbreaks of EHEC associated with leafy greens over the last decade (Irvin et al., 2021). Moreover, *L. monocytogenes* is frequently associated with outbreaks traced back to food processing environments, as exemplified by a multistate outbreak of listeriosis in 2014 linked to consumption of contaminated caramel apples resulting in 34 hospitalizations and 7 fatalities (Belias et al., 2022; Tan et al., 2019). Environmental sampling and whole genome sequencing of *L. monocytogenes* revealed the apple processing environment as the source of contamination (CDC, 2015).

Produce contamination transcends the supply chain from farm to fork. Given the absence of a kill-step during produce washing, sanitizers are used to decrease contamination from water. Among available wash sanitizers, chlorine compounds are most often used. However, their limitations as a sanitizer are well documented (Deng et al., 2020). Ozone's potent oxidative functionalities render it an appealing alternative to chlorine as a sanitizer (Bachelli et al., 2013). However, its volatility and reactivity make it difficult to use. Additionally, prior environmental sampling investigations have provided valuable insights into the presence of *Listeria* species on food and non-food contact surfaces in apple packing facilities (Ruiz-Llacsahuanga et al., 2021;

Tan et al., 2019). However, packing houses constitute just one segment of the apple harvesting continuum and the presence of *Listeria* spp. in CA storage rooms has been overlooked. Therefore, the overall objectives of these studies were to detect and evaluate a control measure for foodborne pathogens in the post-harvest environment of fresh produce facilities. The objective of the first study was to conduct environmental monitoring for *Listeria* spp. within CA apple storage rooms over a 2-year time span. Additionally, the objective of the second study was to evaluate the effect of applying novel ozone NBs at different stages of pilot-scale processing to reduce *E. coli* O157:H7 populations on shredded Romaine lettuce.

1. Literature Review

Shiga toxin producing *Escherichia coli* and *L. monocytogenes* are bacterial foodborne pathogens that cause more than 260,000 illnesses and more than 230 deaths annually (CDC, 2016, 2023b). Infections of either pathogen can cause GI distress. However, STEC and *L. monocytogenes* infections can cause more severe illnesses known as hemolytic uremic syndrome and listeriosis, respectively, which can be fatal (Mengistu & Mengesha, 2023; Radoshevich & Cossart, 2018). Multiple recalls and/or foodborne outbreaks of *E. coli* and *L. monocytogenes* in leafy greens and apples (CDC, 2023a; Fan et al., 2023), respectively, calls for the need to prevent and control the pathogens in the post-harvest environment.

1.1. *Escherichia coli*

Escherichia coli is a diverse species of Gram-negative, facultative anaerobes. One notable pathogenic group of *E. coli* is termed "diarrheagenic *E. coli*" due to diarrheal symptoms upon infection and are grouped by the presence of surface antigens, pathogenicity, virulence factors, and presentation of clinical symptoms (Matthews et al., 2017a; Scheutz, 2014). In total, there are 6 subgroups of pathogenic *E. coli*: diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (Matthews et al., 2017a). The EHEC subgroup is of particular importance because outbreaks are most likely to be associated with food (Gelalcha et al., 2022). The EHEC serotypes commonly associated with outbreaks and illness in the U.S. are O157:H7, O26, O103, O111, and O145 (Gelalcha et al., 2022).

1.1.1. Pathogenesis of EHEC

EHEC infections manifest in two clinical presentations: hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Matthews et al., 2017a; Mengistu & Mengesha, 2023). Hemorrhagic colitis is self-limiting, typically persisting for 12 days. The spectrum of HC symptoms includes bloody/non-bloody diarrhea, gastrointestinal pain, fever, dehydration, nausea, and vomiting (Matthews et al., 2017a; Mengistu & Mengesha, 2023). In cases where infection persists, less than 10% of infected individuals, predominantly children, will progress into the more severe condition of HUS. HUS is characterized by kidney failure, microangiopathic hemolytic anemia, and thrombocytopenia (Matthews et al., 2017a; Mengistu & Mengesha, 2023).

1.1.2. Virulence of EHEC

The process of EHEC infection starts with the pathogen adhering to and damaging the cellular structure of microvilli, and subsequently releasing toxins that cause kidney failure (Matthews et al., 2017b; Melton-Celsa, 2014). The key virulence factors of EHEC are the loci for enterocyte effacement (LEE) and Shiga toxin (ST). *E. coli* O157:H7 LEE allow the colonization of the small intestinal cells (Melton-Celsa, 2014). LEE encodes a multitude of genes responsible for synthesis of structural proteins within the type 3 secretion system (Gelalcha et al., 2022). Two noteworthy genes of LEE are *tir* and *eae*. The *eae* gene encodes for intimin, a protein that binds to *tir*, whereas *tir* encodes Tir, a bacterial protein that EHEC translocates into the host cell via the type 3 secretion system. Collaboratively, Tir and intimin facilitate colonization within the small intestines (Gelalcha et al., 2022; Matthews et al., 2017a; Stevens & Frankel, 2014). This mechanism of infection is distinctive with most pathogens utilizing pre-existing receptors rather than constructing their own (Matthews et al., 2017a).

EHEC's most virulent tool is STs. "Shiga-toxin" is immunologically similar to toxins produced by *Shigella dysenteriae* type 1 (Gelalcha et al., 2022; Scheutz, 2014). STs are divided into two groups known as ST1 and ST2 (Scheutz, 2014) . One hundred seven variations of STs differ between each other by one or more amino acids. ST1 exhibits diminished virulence compared to ST2 and is suppressed in the presence of iron (Melton-Celsa, 2014). ST2, compromising 5 subtypes, may appear individually or concurrently with ST1 subtypes during EHEC infections (Matthews et al., 2017a). Furthermore, ST2 is impervious to neutralization and associated with a greater severity of illness compared to ST1 (Matthews et al., 2017b; Melton-Celsa, 2014). The mode of action of ST starts with internalization using Gb3, a cholesterol receptor. Afterwards, endosomes mistakenly engulf the toxin, deliver it to the Golgi apparatus before final transport to the endoplasmic reticulum. Here, STs cleave the N-glycosidic bond of the 28S ribosome during transcription t0 halts protein synthesis, culminating in cellular death (Melton-Celsa, 2014).

1.1.3. Epidemiology

Leafy greens have emerged as common vehicles for EHEC O157 infections (Table 1). Irvin et al. (2021), investigated three *E. coli* O157:H7 outbreaks associated with Romaine lettuce during the spring of 2018, fall of 2018, and fall of 2019. The cumulative impact of these outbreaks resulted in 474 reported illnesses, 46 cases of HUS, and 5 deaths. Traceback investigations, utilizing whole-genome sequencing and purchase records implicated Romaine lettuce from 84 ranches

and 45 farms in California and Arizona. Similarly, Marshall et al. (2020), reviewed 40 STEC outbreaks involving both O157 and non-O157 EHEC serotypes linked to leafy greens between 2009 and 2018 in the US and Canada. During that period 1,212 fell ill, resulting in 420 hospitalizations, 77 cases of HUS, and 8 fatalities. Consumption of contaminated Romaine lettuce was the predominant cause of illness. Most outbreaks were attributed to EHEC serotype O157 (80%), with O145, O26, EHEC O111, and EHEC O126 contributing a smaller portion of cases. Additionally, serotypes O26 and O157 were jointly responsible for 1 outbreak. Traceback investigations highlight the complexity of identifying sources of foodborne outbreaks. US governmental agencies reported an average of 4 points of sale across an average of 2 states, resulting in a range of 1-9 illnesses. In parallel, Canadian traceback investigations reported a range of 2-30 point of sales, involvement of 1-11 leafy green handlers, and less than 21 suppliers. These epidemiological studies shed light on the adverse effects that leafy greens contaminated with EHEC have on public health. The situation is further complicated by bagged salad mixes, which stand as the primary culprits behind *E. coli* O157:H7 outbreaks associated with leafy greens (Stuart, 2011). This is due to their practice of sourcing leafy greens from various suppliers and centralized processing that encourages cross-contamination. Therefore, a step during post-harvest handling of leafy greens, such as washing with sanitizers, is useful to reduce EHEC.

Table 1. 16 multistate EHEC outbreaks documented between 2010-2022 in the U.S. and Canada (CDC, 2022b).

EHEC Serotype	Year	Source	# Infected
O145	2010	Romaine Lettuce	26
O157:H7	2011	Romaine Lettuce	58
O26	2012	Sprouts	29
O157:H7	2012	Spinach	33
O157:H7	2013	Salad	33
O121	2014	Sprouts	19
O157:H7	2016	Sprouts	11
O157:H7	2017	Romaine Lettuce	25
O157:H7	2018	Romaine Lettuce	210
O157:H7	2018	Romaine Lettuce	25
O157:H7	2019	Romaine Lettuce	167
O157:H7	2019	Salad Kits	10
O103	2020	Sprouts	51
O157:H7	2020	Leafy greens	40
O157:H7	2021	Baby Spinach	15
O157:H7	2021	Salad	10

1.1.4. Pre- and post-harvest contamination of leafy greens

The pre-harvest stage of produce production offers ample opportunity for the introduction of pathogens via contaminated water from nearby cattle farms, fecal contamination from domesticated and animals, and dust (Irvin et al., 2021; Kintz et al., 2019; Marshall et al., 2020). For example, Marshall et al. (2020), reported 2 EHEC outbreaks involving Romaine lettuce that were traced back to runoff water from a nearby farm and a water reservoir used to irrigate fields. Post-harvest operations including machinery designed to manipulate produce, hands of field laborers, shipment containers with leftover soil residue, and produce washing systems can also cross-contaminate fruits and vegetables (Gil et al., 2015). Washing systems serve a multipurpose role of removing dirt, debris, pesticides and reducing microbial populations on produce when combined with sanitizers (Chinchkar et al., 2022). Unfortunately, drawbacks of washing systems are their unintended potential of cross-contaminating produce and their limited efficacy in reducing microbial populations on the product (Davidson, 2013; Smolinski et al., 2018). For example, studies by Jensen et al. (2015) and Smolinski et al. (2018) collectively demonstrated that washing leafy greens, whether in small-scale or pilot-scale settings, can lead to spread of *E. coli* O157:H7 and *Salmonella* Typhimurium from contaminated to uncontaminated produce. Despite differences in scale and pathogen of interest, both studies showed that pathogens

remained in the wash water, leading to contamination of most, if not all, pieces of produce in the batch, highlighting concerns regarding the effectiveness of flume washing as a method to ensure produce safety.

1.1.5. Produce Wash Sanitizers

Fresh produce processing does not employ a kill step, such as heat, to eliminate pathogens. Instead, chemical sanitizers are introduced into the washing systems to reduce, not eliminate, microbial populations in the water. The ideal wash sanitizer will have the following characteristics: (1) non-harmful to the environment or humans, (2) effective against pathogens and spoilage microbes, and (3) maintains the nutritional value and sensory composition of the produce (Malka & Park, 2022). Chlorine is often used as a wash sanitizer, but other sanitizers such as peroxyacetic acid and ozone have been explored as solutions to reduce microbial contamination of produce (Table 2).

In the fresh produce industry, chlorine is the gold standard chemical sanitizer. It is relatively inexpensive and not influencing the quality of produce (Kang et al., 2020). Even widely used, chlorine has numerous drawbacks as well. Its efficacy is influenced by the organic load, pH, free chlorine, temperature, and contact time (Gil et al., 2015). For these reasons, the initial concentration of chlorine must be high, typically around 50-200 ppm (parts per million) (Chinchkar et al., 2022) Other disadvantages of using chlorine are that it can generate carcinogenic by-products and is most effective as an antimicrobial within a limited pH range and is hazardous to humans (Chinchkar et al., 2022; Fallik & Ilic, 2022).

Studies assessing the efficacy of chlorine as a produce sanitizer have reported varying results based on differences in well-established influential experimental factors. Keskinen et al. (2009), reported less than a log reduction of *E. coli* O157:H7 on 25 g pieces of shredded Iceberg and Romaine lettuce washed for 2 min in 500 ml of 20 and 200 ppm sodium hypochlorite. Similarly Li et al. (2001) observed only a 0.7 to 1.1 log CFU/g reductions of populations on shredded lettuce washed at 20°C or 50°C 20 ppm chlorinated water. A pilot scale study by Davidson (2013) reported only a 0.9 and 1 log CFU/g reduction of *E. coli* O157:H7 after washing in 30 and 50 ppm of chlorinated flume water for 90 s.

However, other leafy green washing studies reported higher reductions of pathogens. Ölmez (2010) reported a 2.57 log reduction of *E. coli* ATCC 25922 populations on 100 g of shredded lettuce when submerged in 2 L of 100 ppm free chlorine for 2 min. Rodgers et al. (2004) saw 5

log reductions or more of *E.coli* populations on shredded and whole leaf lettuce after exposure to 100 ml of 100 and 200 ppm of chlorinated water and Singh et al. (2018) reported a 2.1 log CFU/g reduction of *E. coli* O157:H7 on Romaine lettuce treated with 100 ppm of chlorine for 5 min.

Ozone is a molecule with three oxygen atoms; the third oxygen atom of ozone is highly unstable and has a strong oxidation potential. As a sanitizer, the FDA approved ozone as a GRAS (Generally Recognized As Safe) antimicrobial in 1997 and is allowed to be utilized to treat, store, and process foods (Botondi et al., 2021, Secondary Direct Food Additives Permitted In Food For Human Consumption, 2023). Previously, ozone has been used as a food, equipment, and water decontaminant (Botondi et al., 2021; Chinchkar et al., 2022). Ozone could potentially be an alternative to chlorine as a wash sanitizer because since it has a high oxidation potential and the fact that it does not lead to hazardous residues in produce after treatment (Botondi et al., 2021). However, ozone is incredibly volatile which can be harmful to handlers and requires specialized equipment for use.

Like chlorine, studies show varying efficacy of ozone as a produce sanitizer. By misting shredded lettuce with 3 ppm of ozone for 3 min, Rodgers et al. (2004) reduced *E. coli* O157:H7 and *L. monocytogenes* populations on shredded lettuce by 6 logs after 5 min post application. Wang et al. (2019) dipped 10^7 log CFU/g non-pathogenic *E. coli* O157:H7 inoculated shredded lettuce into 2 ppm of aqueous ozone at a 1 to 20 weight to volume ratio and observed only a 0.8 log CFU/g reduction.

Peracetic acid (PAA) is a strong oxidizing agent that is approved by the Food & Drug Administration (FDA) to be used at a maximum of 80 ppm (Chinchkar et al., 2022). For some produce processors, PAA is preferred because it is effective at killing fungi and biofilms, as well as bacteria, and poses no threat to the environment after disposal (Chinchkar et al., 2022). In a pilot plant scale study by Davidson (2013), 5.4 kg batches of shredded iceberg lettuce inoculated with an avirulent 4 strain *E.coli* O157:H7 cocktail at 10^6 log CFU/g were washed in an 890 L flume system for 90 s with multiple organic loads (0, 2.5, 5, and 10%) and different sanitizers including 50 ppm of peroxyacetic acid and 50 ppm mixed peracid. After processing, peroxyacetic acid and mixed peracid reduced microbial populations by an average of 1.0 to 1.7 and 1.4 log CFU/g respectively. Singh et al. (2018) reported an average of 2.1 log reductions of *E. coli* O157:H7 on 400 grams of Romaine lettuce using PAA at 45, 85, and 100 ppm. Rodgers et al.

(2004) reported a significantly higher 4.4 log and greater than 5.9 log reductions of *E. coli* O157:H7 and whole lettuce green leaf lettuce, respectively, after using 80 ppm via misting and washing in 100 ml of the sanitizer.

Table 2. Summary of different wash sanitizers and their efficacy on *E. coli* on leafy greens.

References	Sanitizer	ppm	Lettuce Type	Microbe	Washing Time	Log Reduction	Lab or Pilot Scale
(Keskinen et al., 2009)	Chlorine	20; 200	Romaine and Iceberg	*E. coli* O157:H7	2 min	0.52; 0.65	Lab
(Ölmez, 2010)	Chlorine	100	Loose Leaf	*E. coli* ATCC 25922	2 min	2.57	Lab
(Li et al., 2001)	Chlorine	20	Iceberg	*E. coli* O157:H7	90 s	0.7 to 1.1	Lab
(Rodgers et al., 2004)	Chlorine	100, 200	Green Leaf	*E. coli* O157:H7	5 min	5	Lab
(Singh et al., 2018)	Chlorine	100	Romaine	*E. coli* O157:H7	5 min	2.1	Lab
(Davidson, 2013)	Chlorine	30, 50	Iceberg	*E. coli* O157:H7	90 s	0.9, 1	Pilot Scale
(Rodgers et al., 2004)	PAA	80	Green Leaf	*E. coli* O157:H7	5 min	4.6, > 5.9	Lab
(Davidson, 2013)	PAA	30	Iceberg	*E. coli* O157:H7	90 s	1.4	Pilot Scale
(Singh et al., 2018)	PAA	45, 85, 100	Romaine	*E. coli* O157:H7	5 min	1.8, 1.9, 2.2	Lab
(Rodgers et al., 2004)	Ozone	3	Green Leaf	*E. coli* O157:H7	3 min	6	Lab
(Singh et al., 2002)	Ozone	10	Romaine	*E. coli* O157:H7	10 min	1.47, 1.5, 1.63	Lab
(Wang et al., 2019)	Ozone	2	Lettuce	*E. coli* O157:H7	2 min and 30 s	0.8	Lab
(Ölmez, 2010)	Ozone	1.5	Lettuce	*E. coli* O157:H7	2 min and 30 s	1.19	Lab

1.1.6. Current challenges

Fresh produce processing has no kill-step during operations. Instead, flume washing is used to reduce the microbial load on the surface of produce and decrease pathogens in wash water to prevent cross-contamination. Despite chlorine being the preferred produce sanitizer in the industry, foodborne outbreaks involving leafy greens have continued to be a public health problem, highlighting a need for an alternative wash sanitizer. Ozone is the most oxidative produce sanitizer on the market. However, its volatility, hazardous off-gassing, reactivity, and capital needed to apply in wash water systems dissuades ozone from being considered as a viable alternative to chlorine. Therefore, a method of generating ozone that stabilizes ozone's volatility during produce washing should be explored.

1.2. *Listeria monocytogenes*

L. monocytogenes is a gram-positive, psychrotrophic, facultative bacilli (Matthews et al., 2017b; Van Der Merwe & Pather, 2023). Of the 27 known species of *Listeria*, they are divided into two groups, *sensu lato* and *sensu stricto*, based on their relatedness to *L. monocytogenes* (Carlin et al., 2022; Zakrzewski et al., 2024). The *sensu stricto* group refers to *Listeria* species genetically and phenotypically like *L. monocytogenes* and (not including *L. monocytogenes*) includes *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri*, and *Listeria marthii* (Carlin et al., 2022). The *sensu lato* group includes the remaining *Listeria* spp. not as related to *L. monocytogenes* due to lacking characteristics such as cold-resistance and motility (Carlin et al., 2022). *L. monocytogenes* is more virulent and more often associated with human illness compared to the other species (Bagatella et al., 2022). *L. monocytogenes* causes a rare, but highly fatal, foodborne disease appropriately termed "listeriosis" (Matthews et al., 2017b). It is also a durable and ubiquitous pathogen; not only can *L. monocytogenes* grow at lower temperatures, high salinity, and in acidic environments, it is naturally found in soil, bodies of water, and sewage (Matthews et al., 2017b; Radoshevich & Cossart, 2018). *L. monocytogenes* also resides in food processing facilities, where it can become a post-processing contaminant, posing a serious threat to vulnerable populations (Matthews et al., 2017b; Ruiz-Llacsahuanga et al., 2021).

1.2.1. Pathogenesis

For most of the population, an infection of *L. monocytogenes* may manifest with no symptoms or gastroenteritis (Matthews et al., 2017b) However, vulnerable populations such as immunocompromised individuals, elderly, young, and pregnant populations are susceptible to a more severe manifestations of a *L. monocytogenes* infection known as listeriosis. In these groups of people, listeriosis can cause sepsis, meningitis, and spontaneous abortion (Ireton et al., 2021; Matthews et al., 2017b; Radoshevich & Cossart, 2018). Furthermore, while only causing around 1600 cases of illness each year, listeriosis is estimated to have a mortality rate as high as 30% (Bintsis, 2017), making it one of the deadliest foodborne diseases.

1.2.2. Virulence factors

L. monocytogenes employs multiple virulence factors to infect a host. The first stage of infection is cellular host entry. It employs proteins known as internalins to bind onto host receptors of cellular membranes to initiate endocytosis of *L. monocytogenes* (Radoshevich & Cossart, 2018). Following engulfment, *L. monocytogenes* directs the rearrangement of the host cell cytoskeleton, allowing mobility and persistent infection of tissue throughout the body (Radoshevich & Cossart, 2018). Another essential virulence factor is the *hly* gene that encodes listeriolysin (LLO) (Matthews et al., 2017b). LLO plays a crucial role in the pathogenesis of *L. monocytogenes* by creating pores in host cell cellular membranes to facilitate bacterial entry, disrupting the functionality of organelles such as the mitochondria, endoplasmic reticulum, and lysosomes (Matthews et al., 2017b).

1.2.3. Epidemiology

L. monocytogenes infections are rare, with approximately 1600 cases reported annually (CDC 2023). Recent *L. monocytogenes* outbreaks have been linked to fresh produce. Leafy greens and enoki mushrooms that sickened 19 and 5 individuals, respectively, in 2022. Furthermore, in the previous decade, 18 *L. monocytogenes* recalls were issued for apples (Table 3) (Fan et al., 2023). In some instances, these recalls were the aftermath of foodborne outbreaks. For example, between 2014 and 2017, two multistate outbreaks of listeriosis were traced back to caramel apples. The 2017 outbreak led to 3 hospitalizations across 3 states, while the 2014 outbreak spanned 12 states resulting in 35 illnesses, 7 deaths, 3 cases of meningitis and one case of spontaneous abortion (Marus, 2019; Ward et al., 2022).

Table 3. Recalls of apple products due to *L. monocytogenes* contamination (Fan et al., 2023). "LM" refers to *L. monocytogenes*.

Year	Product Type	Reason
2012	Apple Slices	Processing Equipment
2013	Apples Slices	LM on apples
2014	Fres-cut Apples	LM on apples
2014	Cut Apples	LM on apples
2014	Caramel Apples	LM on apples
2014	Caramel Apples	Associated with outbreak
2014	Caramel Apples	Associated with outbreak
2015	Caramel Apples	LM on apples
2015	Whole Apples	Associated with outbreak
2015	Apple slices with dip	Products positive
2015	Apple Slices	Environmental Samples
2015	Whole Apples	Possible LM
2016	Apple Slices	Products Positive
2017	Apple Slices	Products Positive
2017	Packaged products	LM on apples
2017	Whole Apples	LM on apples
2019	Whole Apples	LM on apples
2020	Apple Slices	Processing equipment

1.2.4. Pre- and post-harvest contamination of apples

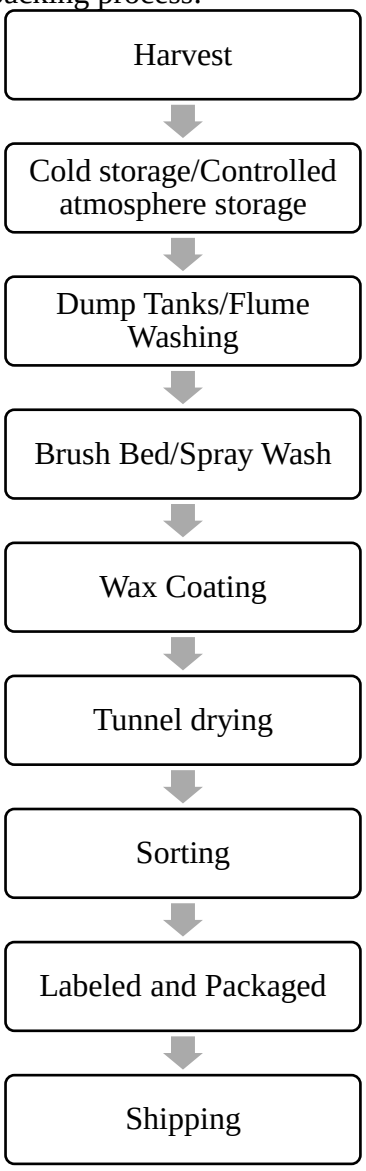
Apples are harvested from orchards and collected into bins, then either stored in cold storage for weeks or CA storage rooms for up to a year, sent to packing houses to be washed, waxed and dried, sorted, labeled, packaged, and finally transported to retailers for consumer purchase (Fan et al., 2023; Pietrysiak et al., 2019) (Figure 1). Since *L. monocytogenes* is ubiquitous, especially in agricultural environments, the hypothesis that contamination of whole apples begins at pre-harvest is plausible. (Pietrysiak et al., 2019). However, evidence of apples being contaminated by *L. monocytogenes* during pre-harvest is not supported by research or the FDA (Fan et al., 2023; Macarisin et al., 2019). Contrastingly, *L. monocytogenes* has been traced back to multiple post-harvest operations (Fan et al., 2023).

L. monocytogenes could potentially be introduced into CA storage rooms from bins and forklifts that track in soil and debris from harvest (Pietrysiak et al., 2019). Furthermore, CA storage rooms are operated under environmental conditions conducive to *Listeria* survival including temperatures as low as 0°C and modified atmosphere gases of oxygen, carbon dioxide, and nitrogen to delay apple ripening and the spread of fungi (Prange & Wright, 2023). Previous studies have demonstrated the survivability of *Listeria* spp. on different apple cultivars during

cold and controlled atmospheric storage conditions. Hamilton et al. (2022) found that *L. innocua* to survive on Gala and Cosmic Crisp apples during 3 months of CA storage conditions. Similarly, Sloniker et al. (2023) reported the survival of *L. monocytogenes* biofilms on the majority of apple cultivars used in the study after storage for 90 days in CA storage rooms. Meanwhile, Sheng et al. (2018) reported the presence of *L. innocua* on Fuji apples after 6 months under cold storage conditions.

Another potential source of contamination is through flume washing when, apples dumped into flume tanks, introduce large amounts of organic material that can hinder the efficacy of sanitizers leading to cross-contamination of apples subsequently entering the washing system (Fan et al., 2023; Pietrysiak et al., 2019). Given their unique physical shape of apples, stem and calyx in particular, apples are difficult to sanitize which can cause cross-contamination of other apples (Pietrysiak et al., 2019). Processing environments are extremely hospitable towards the growth of *Listeria* as well. Ambient temperatures, wet environments caused by standard processing operations, soil and debris brought into facilities by equipment, and structural designs of equipment and surfaces that make cleaning and sanitization problematic support *Listeria* growth (Fan et al., 2023; Pietrysiak et al., 2019). This is supported by three cases of *L. monocytogenes* contamination of sliced apples trace backed to processing equipment and environmental samples of apple packing facilities (Table 3). Furthermore, studies have shown *Listeria* introduced into apple packing houses can persist in both contact and non-food contact surfaces of rollers, dividers, tunnel dryers, floor and floor drains, and bed brushes for extended periods of time (Fan et al., 2023; Ruiz-Llacsahuanga et al., 2021).

Figure 1. Flow diagram of apple packing process.



1.2.5. Environmental Monitoring

Environmental monitoring is used to ensure that preventive controls are being implemented correctly such as cleaning and sanitization (Food Engineering, 2017). Environmental monitoring incorporates the zone concept: the division of food processing facilities into 4 conceptual zones (Zones 1-4) based on the likelihood of food being contaminated. Zone 1 being the most likely and 4 the least likely.

Zone 1 consists of surfaces in direct contact with food: slicers, utensils, workers hands, conveyor belts, bins, and brushes. Zones 2 and 3 are the most fluid categories of the zone concept and can change depending on the structure of the facility. Zone 2 areas are categorized as nonfood-

contact surfaces in proximity to zone 1 and may include equipment parts, screens and monitors, pipes, carts, tools, and air conditioners. Zone 3, in proximity to zone 2, may include restrooms, hallways, drains, forklifts, and storage rooms. Zone 4, the furthest of all zones, includes loading docks, warehouses, locker rooms and offices (Jones, 2016; MDARD, 2012; Penn State Extension, 2023). In the event that Zone 1 and 2 are positive for *Listeria*, these areas must be recleaned, sanitized, and retested. Products should be tested during the next production cycle, held and reprocessed or destroyed in case of repeat contamination. The source of the must be identified with operations halted until the problem is resolved (United States Fresh Produce Association, 2018).

Indicator organisms can help determine if an area, food source, etc. is capable of harboring associated pathogens when present (Matthews et al., 2017). A well-known indicator organism for *L. monocytogenes* is *L.* i*nnocua*. Both species are genetically and phenotypically similar except for the hemolytic virulence factor of *L. monocytogenes* (Mohan et al., 2019). Given their similarity, *L. innocua* can live in environments inhabited by *L. monocytogenes*. Therefore, the FDA and the United States Fresh Produce Association recommends testing for *L. innocua* and other *Listeria* species as it can indicate that an environment is suitable for the introduction of *L. monocytogenes* which could potentially lead to foodborne outbreaks (FDA, 2017; Mohan et al., 2019; United States Fresh Produce Association, 2018). For these reasons, Sullivan and Wiedmann (2020) and Ruiz-Llacsahuanga et al. (2021) included *L. innocua* and other *Listeria* species besides *L. monocytogenes* in their environmental monitoring plans of food and non-food contact surface of produce processing facilities.

1.2.6. Classifying *Listeria* isolates with *sigB* subtyping

Marker genes can be utilized to characterize microbes. Found in all species, marker genes allow for differentiation among species based on differences in sequence and can be amplified using PCR technology (Hollister et al., 2015). Once amplified, researchers can cross reference the nucleotide sequence of the isolate with the nucleotide sequences of other pathogens stored in online databases, such as Basic Local Alignment Search Tool (BLAST) (version 2.15.0+, National Center for Biotechnology Information, Bethesda, Maryland) (Hollister et al., 2015; Jagadeesan et al., 2019). In a study by Sullivan and Wiedmann (2020), *sigB*, which encodes the general stress response sigma factor in *Listeria*, was utilized to characterize 461 *Listeria* isolates collected from produce packing houses and fresh-cut facilities. Using this approach, 15, 4, 10,

and 11 distinct subtypes of *L. seeligeri, L. welshimeri, Listeria innocua,* and *L. monocytogenes* were identified, respectively. Similarly, Estrada et al. (2020) and Sullivan et al. (2022) utilized *sigB* allelic typing to characterize *L. monocytogenes, L. welshimeri, L. innocu*a, *L. ivanovii,* and *L. marthi* in zones 2, 3, and 4 from 11 produce packing houses and 16 fresh cut produce operations, respectively. Estrada et al. (2020) identified 40 *Listeria* allelic types (AT) among these 5 *Listeria* species, while Sullivan et al. (2022) identified *sigB* ATs from 276 representative isolates from nonfood contact surfaces. Using this subtyping tool along with whole genome sequencing, researchers can conclude the relatedness of *Listeria* isolates collected from different zones within produce processing operations as well as the virulence and stress response capabilities of isolates (Sullivan et al., 2022). Furthermore, *sigB* allelic typing can inform produce processors whether areas are contaminated with persistent or transient *Listeria,* and if their cleaning and sanitization plans should be reassessed to prevent a recall and/or foodborne outbreak.

1.2.7. Current challenges

L. monocytogenes has been isolated from apple packing operations in multiple studies and outbreaks investigations. However, CA storage facilities could also be a point of *Listeria* introduction. *Listeria* is a psychrotrophic and facultative anaerobe that grows well in environments with organic matter such as soil and water. CA storage rooms are wet environments held at about 4°C with decreased oxygen and increased carbon dioxide. Furthermore, equipment such as forklifts and bins can introduce organic matter from the outside environment, creating suitable conditions for *Listeria* to flourish. In a longitudinal study, Estrada et al. (2020) reported cold storage rooms with a high prevalence of *L. monocytogenes* and *L. innocua*. Previous studies (Ruiz-Llacsahuanga et al., 2021; Sullivan & Wiedmann, 2020) have highlighted the presence of *Listeria* contamination on food contact surfaces, materials, and equipment in produce operations. However, CA storage rooms as a potential source of *Listeria* have not been explored. This information could assist produce processing operations to make better informed decisions regarding cost-effective cold atmosphere storage room materials and equipment that contributes to *Listeria* growth, and the re-evaluate of sanitization practices.

2. Environmental Monitoring for *Listeria* Species in Controlled Atmosphere Apple Storage Facilities

2.1. Abstract

Studies have revealed the presence of *Listeria* species on food and non-food contact surfaces in apple packing facilities. However, CA storage facilities serving as potential sources of *Listeria* contamination of apples has not been explored. This two-year longitudinal study assessed the prevalence, diversity, and distribution of *Listeria* spp. within nine CA storage rooms of three different facilities (A, B, and C) in Michigan. A total of 300 swabs were collected, revealing a 6.3% (19) positivity rate for *Listeria* spp., predominantly on floors below condenser units. Of the 19 positive sample sites, 65 isolates were collected. *L. monocytogenes* emerged as the major species, accounting for 74% (48/65) of the isolates, followed by *L. seeligeri* at 15% (10/65), *L. welshimeri* at 6.2% (4/65), *and L. innocua* at 4.6% (3/65). The study uncovered three distinct strains of *L. monocytogenes*; AT 61 lineage 1 (30), AT 67 lineage 1 (17), and AT 58 (1) emphasizing the diversity and potential risk posed by this pathogen. During thr two-year period, none of the rooms sampled for positive for repeat contamination of *Listeria* spp. with identical ATs in the same location. Our findings underscore the necessity of preventive measures such as environmental monitoring and cleaning and sanitization programs to mitigate *Listeria* spp. contamination in CA storage environments, considering the microbes ability to survive in low-temperature, modified atmosphere conditions conducive to long-term apple storage.

2.2. Introduction

L. monocytogenes is gram-positive, psychrotrophic foodborne pathogen found in water, decaying organic matter, agricultural environments, and food processing environments (Belias et al., 2022; Pietrysiak et al., 2019; Radoshevich & Cossart, 2018). *L. monocytogenes* may be introduced into food processing environments by food products, soil from the agricultural environment, workers, and equipment such as forklifts or storage bins (Belias et al., 2022; Matthews et al., 2017b). Once introduced, *Listeria* may persist in food processing environments for years and potentially cause foodborne outbreaks (Belias et al., 2022; Matle et al., 2020; Pietrysiak et al., 2019). One multistate outbreak of listeriosis linked to caramel apples resulted in 35 infections, 34 hospitalizations, and 7 deaths (CDC, 2015). According to the CDC (2019), whole genome sequencing revealed that the two different strains of *L. monocytogenes* responsible for the illnesses were genetically similar to the environmental isolates of *L. monocytogenes* collected

from the apple packing facility. Multiple studies (Estrada et al., 2020; Ruiz-Llacsahuanga et al., 2021; Sullivan & Wiedmann, 2020) have assessed the diversity, distribution, and prevalence of *Listeria* spp. on food and non-food contact surfaces of apple packing facilities. For example Chen et al. (2022), conducted a comprehensive whole genome analysis of environmental isolates collected from non-food contact surfaces of three commercial tree fruit packinghouses. The study reported clonal differences among the environmental isolates of *L. monocytogenes*, the spread of different strains within and across areas of the facilities, the presence of sanitizer tolerant genes among isolates, and a relationship between diversity of *L. monocytogenes* and sanitary conditions within the facilities. Additionally, Tan et al. (2019) collected environmental samples from three different fruit processing facilities. Out of the 117 samples examined from each facility, *L. monocytogenes* was identified in 11, 16, and 39, samples with the last facility having a unique microbial environment compared to the other two.

Current food safety guidelines for apple production advise growers to minimize the contact between harvested apples and water sources, including condensate and standing water during storage, to mitigate the risk of *Listeria* contamination. This is particularly relevant in CA storage facilities, where floors can be flooded as part of the sealing process for long-term storage. Environmental sampling within apple packing houses has identified 1) standing water, 2) drains, and 3) cold storage rooms as those sites with higher prevalence of *Listeria* (Estrada et al., 2020; Tan et al., 2019). Despite this, the risk of *Listeria* contamination in CA storage rooms, where apples can be held for up to a year preserve apple quality (Guan et al., 2021), has not been explored. Introduction of *Listeria* spp. into CA storage rooms could occur through soiled bins and forklifts, and once present, *Listeria* can thrive, especially in areas where condenser units leak water, creating an ideal environment for its growth and replication (Guan et al., 2021). Consequently, apple processors have adopted cleaning and sanitization protocols, employing peracetic acid, quaternary ammonia compounds, or sodium hypochlorite to prevent the harborage and spread of *Listeria* within CA storage rooms (apple processor, CA storage room owner, personal communications). Considering the prevalence of *Listeria* in apple packing facilities, the association of apple products with listeriosis, and the gap in research on *Listeria* spp. in CA storage rooms —a setting conducive to *Listeria* proliferation— there is a clear need for focused investigation. This study assessed the prevalence of *Listeria* spp. in CA storage rooms over two years and characterized the isolates using *sigB* allelic typing. This information should assist

apple processers in making informed decisions about the cost-effectiveness of materials, sanitization practices, and equipment used in CA storage rooms to minimize the presence of *Listeria*.

2.3. Methods

CA storage facility selection. Apple processors and growers across Michigan were given a semi-structured interview via MSU Extension and invited to participate in the study. Three apple packing facilities (coded as A, B, and C for anonymity) were selected from the interview to assess *Listeria* during the 2021-2022 and 2022-2023 seasons. Within each facility, 3 different CA rooms were sampled. Facilities A and B were sampled over a two-year period, while facility C was enrolled later and was only sampled in year 2 of the study. Facility A was located next to an apple packing house while facilities B and C were off-site.

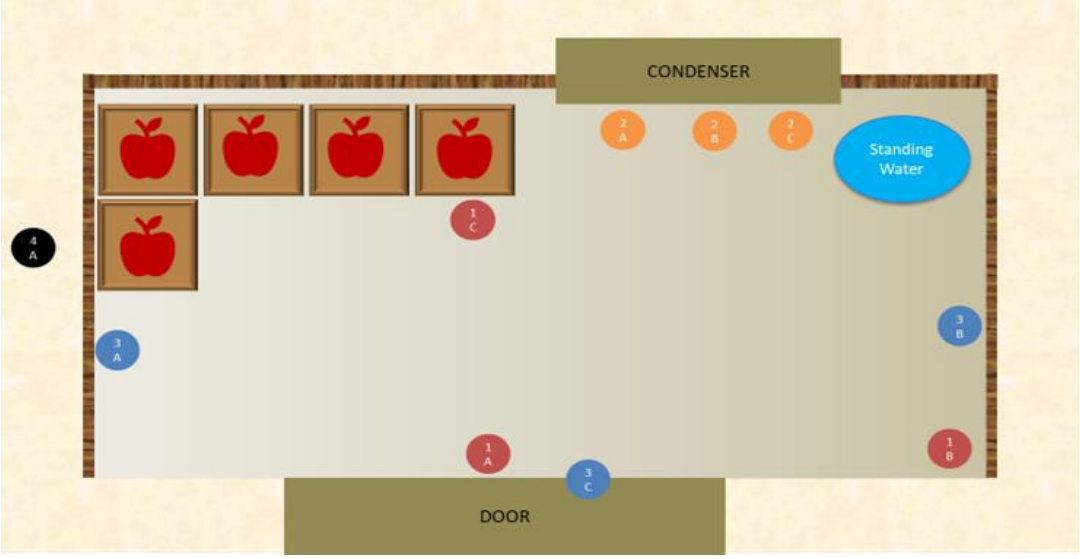
Sample Timing. For each year of the study, environmental samples were collected twice: first, in late summer, before apples were harvested and moved into CA storage rooms and prior to cleaning and sanitization. The second round of sampling took place in the spring, after apples were moved out of CA storage rooms for further processing and following cleaning and sanitization. Due to the large crop of apples from the Fall 2022 harvest season, some CA storage rooms were not emptied until early Fall 2023. Samples during Fall 2023 were taken after the CA storage rooms were emptied and were included with the results for Spring 2023.

Sample collection. Within each CA storage room, the following locations were sampled for *Listeria*: on the floor 30 cm away from the door (1A), floor in the corner near the door seal (1B), floor where stacked bins would be placed and forklifts would travel (1C), three floor locations below the condenser unit(s) (2A 2B, and 2C), two curb locations parallel to each other (3A and 3B), the wall two feet from the floor (4A), and the entire surface area of the door seal (3C). Standing water was also collected if present. The floor, wall, and curb material were recorded during the initial sample collection (Figure 2).

At least 3 samples per location were collected aseptically, using sterile gloves that were changed for each sampling location, in Whirl-Pak bags (Sigma-Aldrich, St. Louis, MO) using Kim wipes, as described by Vorst et al. (2004), hydrated with D/E broth (Neogen, Lansing, MI). If present, 10 ml of standing water was collected into conical tubes using serological pipettes. Samples were transported in an insulated container with cold paks, stored in cold room at 4°C, and processed

within 24 h of collection. Sampling locations and CA storage rooms were coded using alpha-numeric combinations (e.g., A1-1, A1-2, etc.) for identification.

Figure 2. Diagram of the locations sampled in the CA apple storage rooms.



Sample Processing. Samples collected were enriched in Less-Plus Media (Neogen, Lansing, MI) was at a 1:10 w/v ratio for 18-20 h at 37°C. Following overnight enrichment, detection of *Listeria spp*. was conducted using Neogen's ANSR system (Neogen, Lansing, MI) and the ANSR for *Listeria* kit (Neogen, Lansing, MI). Overnight Brain Heart Infusion (BHI) cultures of *L. innocua* 33090 and *L. monocytogenes* F6854 served as controls for the ANSR system. Presumptive positives were then streaked from the Less-Plus enrichment onto Harlequin plates and incubated for 44-52 hours at 37°C.

***Listeria* isolation.** From each Harlequin plate, up to 8 *Listeria* isolates were selected. On this medium, *L. monocytogenes* produces blue/green colonies with halos, whereas *L. innocua* produces blue/green colonies without a halo. If both *Listeria* spp. were present, 6 colonies of *L. monocytogenes* and 2 colonies of *L. innocua*, total of 8 colonies, were inoculated into individual 5 ml BHI tubes. If only colonies of either *L. monocytogenes* or *L. innocua* were present, then 8 colonies were selected and inoculated into 5 ml BHI tubes. Inoculated BHI tubes were incubated, while shaking in a shaking incubator, for 18-20 h at 37°C. After incubation, 500 µl of culture and 500 µl of 70% glycerol were added to cryovials, vortexed, and stored at -80°C for later subtyping.

***sigB* allelic typing.** Frozen stocks stored at -80°C were streaked onto Harlequin media and incubated for 24-48 h at 37°C. Individual colonies grown overnight were inoculated into 5 ml of BHI incubated with shaking for 18-20 h. DNA was extracted using the QIAGEN DNeasy Powerlyzer Microbial Kit (QIAGEN Sciences Inc., Germantown, MD). After extraction, DNA was quantified using a Qubit Flex Fluorometer (Thermo Fisher Scientific, Waltham, MA). Amplification of the 780 bp fragment of the *sigB* stress response gene was carried via hot start PCR using previously described methods (Cai et al. 2002) *L. monocytogenes* and *L. innocua* served as positive controls while qPCR Grade Water (Fisher Scientific, Hampton, NH) served as the negative control. The 780 bp fragment of the *sigB* gene was visualized by gel electrophoresis using 1% E-Gel Double Comb (Invitrogen, Waltham, MA) with the E-Gel 1 kb Plus DNA Ladder. Samples were considered positive for *Listeria* spp. if bands matched the 780 bp region of the ladder and the *L. monocytogenes* and *L. innocua* controls. PCR products were purified according to the Exosap-IT Express PCR Product Cleanup protocol (Thermo Fisher Scientific, 2017). Purified PCR products were submitted to the Genomics Depot at Michigan State University for Sanger Sequencing. Sequences were trimmed, assembled, and blasted against a reference *sigB* sequence, using GENEIOUS (version 2023.1.2, Biomatters Inc., Boston, MA). Food Microbe Tracker (http://www.foodmicrobetracker.com) was used to categorize sequences based on species, allelic type, and lineages (Vangay et al., 2013).

2.4. Results

Sampling locations and controlled atmosphere storage rooms most prevalent with *Listeria* spp. contamination. During the 2-year longitudinal study, 300 samples were collected from 9 CA storage rooms. Of the 300 samples, 19 (6.3%) were confirmed positive for *Listeria* spp. based on *sigB* allelic typing (Table 4). Of the 19, 9 (47.5 %) were collected from floors below condenser units, 3 (15.8%) were from floors where stacked bins were placed, 3 (15.8%) from curbs, 2 (10.5%) from the floor corner closest to the entrance door, and 2 (10.5%) from the floor near the entrance of the door. Facility A had the highest number of positives samples at 11 total over the 2-year period (57.9%) followed by 3 and 5 from facilities B and C, respectively. Facility A had the most diverse areas positive for *Listeria* spp. (floor near door, floor by stacked bins, floor of corner near door, and floor underneath condenser units). The Fall 2022 and Spring 2023/Fall 2023 season had the highest number of positives with 7 each (73.7%), followed by Spring 2022 with 4 (21.1%), and Fall 2021 with 1 positive (5.3%).

Prevalence and characterization of *Listeria* spp. in controlled atmosphere storage rooms. Of the 19 locations that were positive for *Listeria*, 65 isolates belonging to four different species were identified. *L. monocytogenes* was the predominant species with 48 (74%) isolates, followed by 10 *L. seeligeri* (15%), 4 *L. welshimeri* (6.2%), and 3 *L. innocua* (4.6%) (Table 5). Food Microbe Tracker revealed three different strains of *L. monocytogenes* based on *sigB* allelic typing which included 30 (63%) isolates of *L. monocytogenes* AT 61, 17 (35%) isolates of *L. monocytogenes* AT 67, and a single isolate (2.1%) of *L. monocytogenes* AT 58. *L. seeligeri* and *L. welshimeri* each belonged to AT 12 and AT 129, respectively. *L. innocua* had two ATs present: AT 31 and AT 23 (Table 5).

Repeated contamination of *Listeria* spp. over time. None of the sampling sites had repeated contamination during the 2-year study (repeated contamination defined as the same *Listeria sigB* AT collected in the same sampling site across storage seasons). Facility A was the only facility with consistent contamination of *Listeria* spp. in the same rooms (Room 1 and 3) over the storage seasons (Table A-1). In room 1, *L. innocua* AT 23 was recovered during Fall 2021 from the floor below the condenser unit, with *L. innocua* AT 31 recovered from the floor corner near the door in Spring 2022. Room 3 was the only room with repeated isolation of *Listeria* in the same location from the floor below the condenser units a year apart during the Fall 2022 and Fall 2023 seasons. Both samplings yielded different lineage 1 isolates of *L. monocytogenes* – AT 61 in Fall 2022 and AT 67 in Fall 2023. Furthermore, Room 3 yielded *L. monocytogenes* AT 61 lineage 1 (two from the floor below the condenser units and floor near door entrance (1A), and *L. seeligeri* AT 12 (floor of corner near door) during Fall 2022. *Listeria* was again found a year later during Fall 2023 in room 3 of Facility A with *L. monocytogenes* AT 67 Lineage 1 obtained from the floor near stacked bins and flooring below the condenser units. Room 2 of facility A only had one instance of *Listeria* contamination during Fall 2023 with *L. seeligeri* AT 12 recovered from the floor near stacked bins. Room 1 of facility B only had occurrences of *Listeria* contamination in Spring 2022 (Table A-2). *L. seeligeri* was isolated once from flooring below condenser units meanwhile *L. monocytogenes* AT 61 lineage 1 was isolated twice at two locations below condenser units. Facility C had 5 instances of *Listeria* contamination (Table A-3). Two samples were a co-occurrence of *L. monocytogenes* and *L. seeligeri* during Fall 2022 on the curb seal of Room 2. Then, during 2023, *L. welshimeri* AT 129 and *L. monocytogenes* AT 61

lineage 1 were collected from the floor near stacked bins and curb material, respectively, in room 1. *L. innocua* AT 31 (3A) was collected from flooring below the condenser units of room 3.

Table 4. Total *Listeria* spp. prevalence across facilities and seasons. "N/A" indicates no samples were collected during that storage season.

Location	Facility	F21	S22	F22	S23/F23	Total *L.* spp. /Total Location
Floor near door entrance	A	0	0	2	0	2/12
Corner near door	A	0	1	1	0	2/12
Floor of stacked bins	A	0	0	0	2	2/12
Floor below condensers	A	1	0	2	2	5/36
Curb	A	0	0	0	0	0/24
Curb seal	A	0	0	0	0	0/12
Wall	A	0	0	0	0	0/12
Standing water	A	N/A	N/A	N/A	0	0/2
Floor near door entrance	B	0	0	0	0	0/12
Corner near door	B	0	0	0	0	0/12
Floor of stacked bins	B	0	0	0	0	0/12
Floor below condensers	B	0	3	0	0	3/36
Curb	B	0	0	0	0	0/24
Curb seal	B	0	0	0	0	0/12
Wall	B	0	0	0	0	0/12
Standing water	B	N/A	N/A	N/A	N/A	0/0
Floor near door entrance	C	N/A	N/A	0	0	0/6
Corner near door	C	N/A	N/A	0	0	0/6
Floor of stacked bins	C	N/A	N/A	0	1	1/6
Floor below condensers	C	N/A	N/A	0	1	1/18

Table 4. (Cont'd)

Curb	C	N/A	N/A	2	1	3/12
Curb Seal	C	N/A	N/A	0	0	0/6
Wall	C	N/A	N/A	0	0	0/6
Standing Water	C	N/A	N/A	N/A	0	0/2
Total L spp.	-	1/19	4/19	7/19	7/19	19/300

Table 5. *Listeria* spp. *sigB* allelic typing, and lineage based on sanger/PCR positive isolates. "-"indicates no *sigB* allelic type or lineage was identified for that species.

Listeria spp.	Total *Listeria* spp.	*sigB* AT#	*sigB* AT#	*sigB* AT#
L. monocytogenes	48/65	61 (30)	58 (1)	67 (17)
L. seeligeri	10/65	12 (10)	-	-
L. welshimeri	4/65	129 (4)	-	-
L. innocua	3/65	31(2)	23(1)	-
Total	65/65			

2.5. Discussion

***L. monocytogenes* was the most prevalent *Listeria* spp. in controlled atmosphere storage rooms.** In the current study, *L. monocytogenes* represented nearly three-fourths of isolates characterized by *sigB* sequencing among the four species of *Listeria* in total which included *L. innocua*, *L. seeligeri*, and *L. welshimeri*. Murugesan et al. (2015) reported similar findings for samples collected from nonfood contact surfaces in a fresh mushroom processing plant at three time points. They reported *L. monocytogenes* predominated at 78.7% (48/61). Rückerl et al. (2014) also observed the dominance of *L. monocytogenes* in a cheese processing facility during a 3-year longitudinal study. Of the total *Listeria* spp. identified from environmental sampling, *L. monocytogenes* was the most prevalent at 88% (250/281). This is notable because this study differed by including both food and nonfood contact surfaces in its environmental monitoring, while the current study focused only on nonfood contact surfaces, which are generally viewed as

less risky for product contamination (Penn State Extension, 2023). Facility A accounted for most of the positive samples collected (11/19) during the study. This predominance might be due to facility A's CA storage rooms being the only sampling site located next to an apple packing facility. Previous environmental studies of produce packing houses (Estrada et al., 2020; Ruiz-Llacsahuanga et al., 2021) have identified the presence of *Listeria* spp. in these environments. The frequent movement of forklifts and workers between CA rooms and packinghouses at facility A could be facilitating the spread of *Listeria*.

Flooring below condenser units were areas most prevalent with *Listeria* spp. contamination. Condenser units lower the temperature of CA storage rooms to retard the ripening of produce such as apples and pears (Bartsch & Blanplied, 1984; Prange & Wright, 2023). However, condensate from these units could potentially harbor *Listeria* and leak into the CA storage room to contaminate apples. Given the psychrotrophic characteristics of *Listeria* spp., the addition of a water source in CA storage rooms creates a wet and cold environment ideal for *Listeria* to thrive during apple storage. According to Cutter (2016) and the FDA (2017) condenser units pose a legitimate risk of contaminating ready-to-eat foods, meat, and poultry with *Listeria* and the drip pans of these condensation units should be cleaned weekly and sanitized monthly as a result. An example of condenser units contaminating food occurred in 1999 with Sara Lee hot dogs. It is suspected that a *Listeria* positive condenser unit spread the pathogen across the food processing area while being removed. The result of this outbreak was the recall of 15 million pounds of meat products, 100 illnesses, 6 miscarriages, and 15 deaths. Additionally, in the current study, sealed concrete was the construction material of every floor location specified as "floor below condenser units." In a study by Dygico et al. (2020), sealed concrete inoculated with a seven strain cocktail of *L. monocytogenes* to form biofilms supported the growth of *L. monocytogenes* with 5.3 log CFU/cm^2 present on the coupon. However, compared to concrete without a sealing treatment, there was a 2.4 log CFU/ cm^2 difference on sealed concrete compared to non-sealed concrete.

None of the sampling locations had repeated contamination with the same *Listeria* spp. or allelic type. Only samples collected from floors below condenser units in room 3 of facility A yielded repeated isolations of *L. monocytogenes* from the same location during multiple seasons. However, the ATs were 67 and 61 and therefore would not qualify as repeated contamination. The food plant sanitizers used to clean and sanitize CA apple storage rooms post storage could

be preventing *Listeria* spp. from establishing a niche in the environment. The apple processors used peracetic acid, sodium hypochlorite, and/or quaternary ammonia compounds to clean and sanitize their CA storage rooms. In one study Hua et al. (2019) inoculated 15 mm x 7.5 mm coupons (stainless steel, rubber, polyester, polyvinyl chloride, and low-density polyethylene) with *L. monocytogenes* biofilms. Afterwards, the coupons were submerged in 160 and 200 peroxyacetic acid (PAA), 100 and 200 ppm chlorine, 2.5 and 5 ppm chlorine dioxide, 200 and 400 ppm quaternary ammonia compounds (QAC) for 1 or 5 min. Results showed average reductions of 4.32, 3.26. and 3.44 log CFU/coupon for *L. monocytogenes* biofilms using 200 ppm of PAA, 200 ppm chlorine, and 400 ppm of QAC after 5-minute contact time. This study is encouraging given their sizable reduction of *L. monocytogenes* biofilm populations and the sanitizers applied are identical to those used by the apple processors.

2.6. Conclusion

This two-year longitudinal study elucidated the prevalence and distribution of *Listeria* spp. in 9 controlled atmosphere storage rooms. The limited number of CA storage rooms selected in this study (9) are not generalizable to the entirety of CA storage rooms across Michigan. However, these results highlight potential critical areas of contamination and diverse species of *Listeria* that can survive during CA storage. Less than 10% of the 300 samples collected were positive for *Listeria* spp. Among the identified species—*L. monocytogenes, L. welshimeri, L. innocua,* and *L. seeligeri*—*L. monocytogenes* emerged as the most prevalent, constituting 74% of the *Listeria* found, underscoring the pathogenic potential within these environments. Furthermore, three distinct ATs of *L. monocytogenes* were characterized, demonstrating the genetic diversity of the pathogen in CA storage rooms. None of the sampling locations yielded repeated contamination of *Listeria* spp. of the same AT during the study. However, facility A had multiple occurrences of *Listeria* contamination across the storage seasons and accounted for most of the samples collected (11/19). A concerning trend is the consistent presence and diversity of *Listeria* spp. located on the floor below condenser units, indicating a need for more frequent cleaning and sanitization of condenser units and installing and/or monitoring the state of drip pans to prevent condensation drips on apples or the environment. Future studies should include CA apple storage rooms of large, medium, and small operations, across multiple regions of Michigan, and only sample those that distribute within the state. Including this information would enhance

generalizability and deepen our understanding of the dynamics between *Listeria* spp. and CA storage conditions.

Acknowledgements

This work was supported, in part, by the Michigan Tree Fruit Commission Michigan Department of Agriculture and Rural Development grant #220000000881. We thank Neogen for providing the environmental sampling tools and media necessary to complete the study and our anonymous industry partners for allowing us to collect samples from their CA storage facilities.

3. Evaluating Efficacy of Ozone Nanobubbles to Inactivate *E. coli* O157:H7 on Shredded Romaine Lettuce at Different Stages of Pilot-Scale Processing

3.1. Abstract

Ozone is an appealing alternative to chlorine as a produce sanitizer due to its high oxidation potential and decomposition without the generation of carcinogenic by-products. However, its volatility and reactivity prevent it from being a commonly used sanitizer. This study evaluated the efficacy of applying novel ozone NBs at different stages of pilot-scale processing to reduce *E. coli* O157:H7 populations on shredded Romaine lettuce. Submerging 0.5 kg of shredded lettuce in 6 ppm ozone NBs for 90 significantly reduced *E. coli* O157:H7 populations by 0.8±0.20 log CFU/g ($p < 0.05$). In a pilot-scale flume system, the application 6 ppm ozone NBs or tap water as a final rinse application after washing in 10 or 20 ppm chlorinated flume water did not significantly reduce *E. coli* O157:H7 regardless of chlorine ($p > 0.05$). The application of ozone NBs during centrifugal drying of shredded lettuce did not significantly differ from tap water in reducing *E. coli* O157:H7 populations ($p > 0.05$). Although there was a significant difference observed in microbial reduction between ozone NBs and tap water applied before spinning ($p < 0.05$), the overall magnitude of this difference was minimal. Overall, the efficacy of ozone NBs as a sanitizer on shredded lettuce varied during washing of Romaine lettuce. However, ozone NBs deserve continued exploration as a viable alternative to chlorine.

3.2. Introduction

Despite multi-governmental efforts, outbreaks of EHEC linked to leafy greens remain a food safety issue. Between 2010 and 2021, 16 EHEC outbreaks were documented in the United States resulting in 762 illnesses (CDC, 2022b). These outbreaks included 6 from Romaine lettuce, 4 from sprouts, 2 from spinach, 2 from salads, and 1 from an unspecified leafy green source. *E. coli* O157:H7 was responsible for most of these outbreaks (12/16), while *E. coli* O145, O26, O121, and O103 were each responsible for one outbreak.

In the field, leafy greens are exposed to potential microbial contaminants including runoff water from nearby livestock farms, wildlife, insects, and animal feces (Kintz et al., 2019; Marshall et al., 2020). Following harvest, leafy greens must be washed to reduce microbial populations, including pathogens (Botondi et al., 2021). According to Gil et al. (2013), vegetable washing is usually a multi-stage process to remove dirt and debris and reduce the microbial load of the wash water, using chemical sanitizers, to prevent cross-contamination. These processes include

floating produce in flume water to allow dirt and debris to sink and drain from the tank, filtering and replenishing fresh water, monitoring the wash water to produce ratio, and introducing wash sanitizers such as chlorine. Unfortunately, the unintentional cross-contamination of other produce can occur (Smolinski et al., 2018). Therefore, the selection of an effective produce sanitizer is crucial.

A variety of produce sanitizers are approved for fresh produce. Among the oxidizing agents, chlorine is the preferred sanitizer in the U.S. due to low cost, ability to kill a variety of microbes, and simple application (Chinchkar et al., 2022). However, chlorine has drawbacks. Chlorine is notoriously ineffective in the presence of an organic load and therefore must be monitored constantly and replenished, forms carcinogens, effective at a limited pH range, and can damage processing equipment (Chinchkar et al., 2022; Fallik & Ilic, 2022).

Ozone is the most potent oxidative FDA approved GRAS antimicrobial (Botondi et al., 2021; Ölmez, 2010; Wang et al., 2019). Compared to chlorine, ozone does not leave chemical residues and is non-corrosive to processing equipment (Ölmez, 2010; Rodgers et al., 2004; Wang et al., 2019). However, ozone's molecular structure is unstable, hazardous to individuals, and is costly generate on site (Botondi et al., 2021; Ölmez, 2010). Previous lab studies have yielded mixed results using aqueous ozone to sanitize produce. Selma et al. (2007) reported a 1.4 log reduction of *Shigella sonnei* after submerging inoculated shredded lettuce in 2 ppm aqueous ozone for 5 min at a 1:50 w/v ratio. Alexopoulos et al. (2013) observed a 3.04 log reduction of aerobic mesophilic bacteria on shredded lettuce after washing in 0.5 ppm of water continuously ozonated for 30 min. Rodgers et al. (2004) reported greater reductions utilizing aqueous ozone. *E. coli* O157:H7 and *L. monocytogenes* populations were reduced by 5.9 log CFU/g or greater when whole apples, lettuce, strawberries, and cantaloupes were submerged in 3 ppm of ozone for 5 min and when sliced apples and shredded lettuce were misted with 3 ppm ozone for 3 min.

Common washing systems in produce operations utilize three tanks to remove soil and debris from harvest, pesticides, and potential pathogenic microbes acquired during pre- or postharvest production (Chinchkar et al., 2022; Gil et al., 2015). Chemical sanitizers are introduced during the first and second stages of washing to reduce microbial load in the wash water and prevent cross-contamination (Chinchkar et al., 2022). Larger flume washing systems using tanks can be cost prohibitive for smaller produce operations. Submersion of produce in tanks or sinks and spraying with water are viable alternative washing methods (Howard et al., 2020; Mpanga et al.,

2020). Like large scale systems, these processes require sanitizers to prevent cross-contamination. Furthermore, another aspect of produce washing is "makeup water." Makeup water is fresh water replenished during produce washing after loss from leaks, phase changes such as humidification, evaporation and steaming, wind drift, blowdown and intentional discarding due to "build up" of microbial load (Chinchkar et al., 2022; Gil et al., 2015; Guyer, 2014).

NBs measuring less than 13 nm in diameter can be formed in aqueous solutions (Agarwal et al., 2011; Atkinson et al., 2019). Larger bubbles such as macro- or microbubbles are short lived in comparison to NBs, which are stable against internal pressure in liquids. This allows NBs to remain in aqueous solutions for weeks to months (Agarwal et al., 2011). Furthermore, when NBs burst, they burst in liquids instead of at the threshold of liquid and air. (Agarwal et al., 2011; Atkinson et al., 2019). As previously mentioned, the drawback of ozone as a sanitizer is its instability. However, ozone delivered through NBs reduces its reactivity and theoretically extends its stability in liquids. According to Agarwal et al. (2011), NBs can disrupt established biofilms on surfaces and effectively reduce *E. coli* populations in water such as baths and pools when combined with ozone. However, there is a lack of data on the application of ozone NBs and their efficacy when applied to commercial production of fresh produce. Therefore, the objective of this study was to assess the efficacy of ozone delivered via novel NB technology to inactivate *E. coli* O157:H7 on shredded Romaine lettuce by 1) submersion in ozone NBs, 2) application of ozone NBs as makeup water during pilot-scale flume washing, 3) and application of ozone NBs prior to and during pilot-scale centrifugal drying.

3.3. Methods

Strains and growth conditions. Four avirulent and ampicillin (amp) resistant green fluorescent protein-tagged *E. coli* O157:H7 (6980-2, 6982-2, CV2b7, and 43888) isolates originally sourced from Michael Doyle at the Center for Food Safety at the University of Georgia, Griffin, GA (Erickson et al., 2010, 2013) were streaked onto Tryptic Soy Agar plates (TSA) (Neogen, Lansing, MI) with 0.6% yeast extract (YE) (Thermo Fisher Scientific, Waltham, MA), 100 ppm of ampicillin (Sigma Aldrich, St. Louis, MO) (TSAYE + amp), and incubated at 37°C for 18-24 h. To provide an additional selective marker, strains were exposed to increasing concentrations of rifampicin (rif) to select for resistance. A colony from each plate grown overnight was inoculated into 5 ml of Tryptic Soy Broth (TSB) (Neogen, Lansing, MI) with 0.6% yeast extract,

100 µl/ml of ampicillin (TSBYE + amp) and rifampicin (4 µg/ml) (Tokyo Chemical Company, Portland, OR) and incubated in a shaking incubator at 37°C at 18-20 h. Two successive and identical inoculation methods for 18-20 h intervals were carried out by inoculating 100 µl of overnight culture into 5 ml TSBYE + amp tubes with 40 µg/ml and then a final 80 µg/ml of rifampicin (TSBYE + amp + rif). After confirmation of growth, overnight cultures were streaked onto TSAYE + amp plates with 80 µg/ml rifampicin (TSAYE +amp+ rif) then incubated overnight at 37°C. A single colony from each plate was inoculated into TSBYE+ amp+ rif and incubated in a shaking incubator for 18-20 h. Finally, 500 µl of each strain grown overnight and 500 µl of 70% glycerol were combined into freezer storage vials, labeled, and stored into a -80°C freezer for future use.

For each experiment, all isolates were streaked onto TSAYE +amp+ rif and incubated at 37°C for 18-24 h. Three colonies were inoculated into 10 ml of TSBYE +amp+ rif and incubated, static, for 18-20 h. Finally, 200 µl of the overnight culture was inoculated into 200 ml TSBYE +amp+ rif in culture flasks and incubated, static, for 18-20 h.

Inoculation of Romaine lettuce. The four cultures were combined to create an 800 ml cocktail and added to 80 L of tap water in a 121 L plastic container and mixed. Batches of 11.4 or 22.7 kg of Romaine hearts (D'Arrigo Brothers, Salinas, CA) stored in a 4°C walk-in cold room, obtained from Culinary Services at Michigan State University (East Lansing, MI), were placed in net baskets then submerged for 15 min in the 4-strain avirulent cocktail. After submersion, lettuce heads were drained/air dried by dumping residual inoculum from net baskets and sitting for 1 h at 22°C and stored for 24 h in a 4°C walk-in cold room before processing. On the day of the experiment, each batch was shredded using a TRS 2500 TransSlicer (Urschel, Chesterton, IN) and 3 x 25-gram samples were collected to assess initial inoculation. On the day of the experiment, inoculated Romaine lettuce batches were shredded using a TRS 2500 TransSlicer (Urschel, Chesterton, IN) and 3 x 25-gram samples were collected to assess initial inoculation.

Ozone generation. 6 ppm of ozone nanobubbles were produced by commercial NB and ozone generators (En Solución, Austin, Texas). The ppm of ozone was confirmed using a Thermo Scientific Orion AQUAfast AQ3700 portable multiparameter colorimeter (Thermo Fisher Scientific, Ann Arbor, Michigan). The size and density of nanobubbles were measured in previous diagnostic studies by En Solución using a Nanosight Pro nanoparticle analyzer

(Salisbury, United Kingdom). Diagnostics showed a mean diameter of 126 nm and density maximum of 1 x 10^7 bubbles per ml.

Submersion of shredded Romain lettuce. 80 L of 6 ppm ozone NBs, produced by commercial NB and ozone generators (En Solución, Austin, Texas), was collected in a 121 L plastic container. Shredded lettuce was gathered into a 0.5 kg batch, submerged for 90 s, removed, and loaded into a 22.7-kg capacity Model SD50-LT centrifugal Spin Dryer (Heinzen Manufacturing, Gilroy, CA) to remove excess liquid. Three trials were completed in total.

Flume washing of shredded Romaine lettuce. A small-scale commercial leafy green processing line was assembled consisting of a flume tank, shaker table equipped with three overhead rinse sprayers, and a centrifuge. The 1000 L capacity recirculation tank was filled with 700 L and connected to the 3.6-meter long Heinzen Manufacturing flume tank. Wash water in the recirculation tank was dosed with 10 or 20 ppm of free chlorine using XY-12 (Ecolab, Saint Paul, MN) at an average pH of 7.1±.06 and adjusted to 1% of organic load using 7 kg of shredded lettuce blended using a Waring Xtreme Blender with a 64 oz Raptor Container (McConnellsburg, PA). Free chlorine ppm and pH were constantly monitored using a Model Q46H-62 Free Chlorine Monitor (Analytical Technology Inc, Brown Deer, Wisconsin) and adjusted periodically if pH decreased or increased by 2 magnitudes throughout flume washing using XY-12 and 6 N hydrochloric acid. The average and standard deviation of free chlorine ppm was calculated using Microsoft Excel. Three separate 6 kg shredded lettuce batches were held in the flume tank for 90 s, dewatered on the shaker table, followed by application of an overhead curtain of tap water of 6 ppm ozone NBs flowing at 20 gal/min, and finally loaded into the 22.7-kg capacity centrifugal Spin Dryer. Furthermore, three technical replicates were collected from each of the 3 batches of shredded Romaine lettuce treated with the following flume and final rinse as makeup water: 10 ppm chlorine + tap water, 20 ppm chlorine + tap water, 10 ppm chlorine + 6 ppm ozone water, and 20 ppm chlorine + 6 ppm ozone water. Three trials were completed in total.

Application of ozone nanobubble water to lettuce during centrifugal drying. On the day of the experiments, each 22.7-kg batch of shredded lettuce was loaded into the centrifugal Spin Dryer. 6 ppm of ozone NBs was applied for 90 s either before or during centrifugal drying using a nozzle spray head at a flow rate of 1.8 L/min as recorded by a RESTMO digital water flow

meter (RESTMO, USA). Tap water applied for 90 s at either before or during centrifugal drying served as the control.

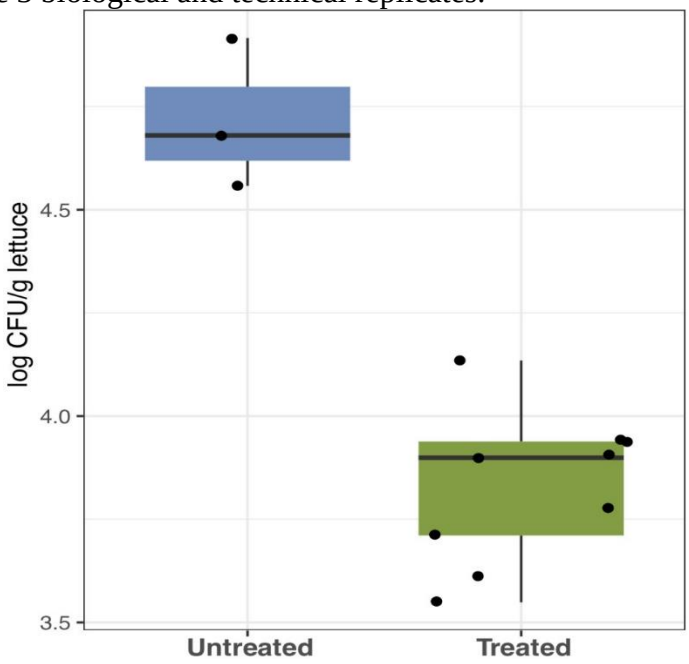
***E. coli* O157:H7 enumeration.** Lettuce samples (25g) were collected in triplicate after centrifugal drying, diluted in D/E broth (Neogen, Lansing, MI) to a 1:10 ratio, homogenized in a stomacher (Stomacher 400 Circulator, Thermo Fisher Scientific, Waltham, MA), serially diluted, and plated on TSA +amp+ rif. After overnight incubation at 37° C, fluorescing colonies under a handheld UV light (300-400 nanometers, BioRad, Ann Arbor, MI) were counted using an Interscience Scan 300 Colony Counter (Woburn, MA). Microbial plate counts of *E. coli* O157:H7 were converted to log CFU/g and averaged based on technical replicates using Microsoft Excel.

Data analysis. Statistical analysis was done in R (4.3.2, www.r-project,org). The data was analyzed using the students t-test and ANOVA (Analysis of Variance). The plots were made using ggplot2 (Hadley, 2016) (3.4.4).

3.4. Results

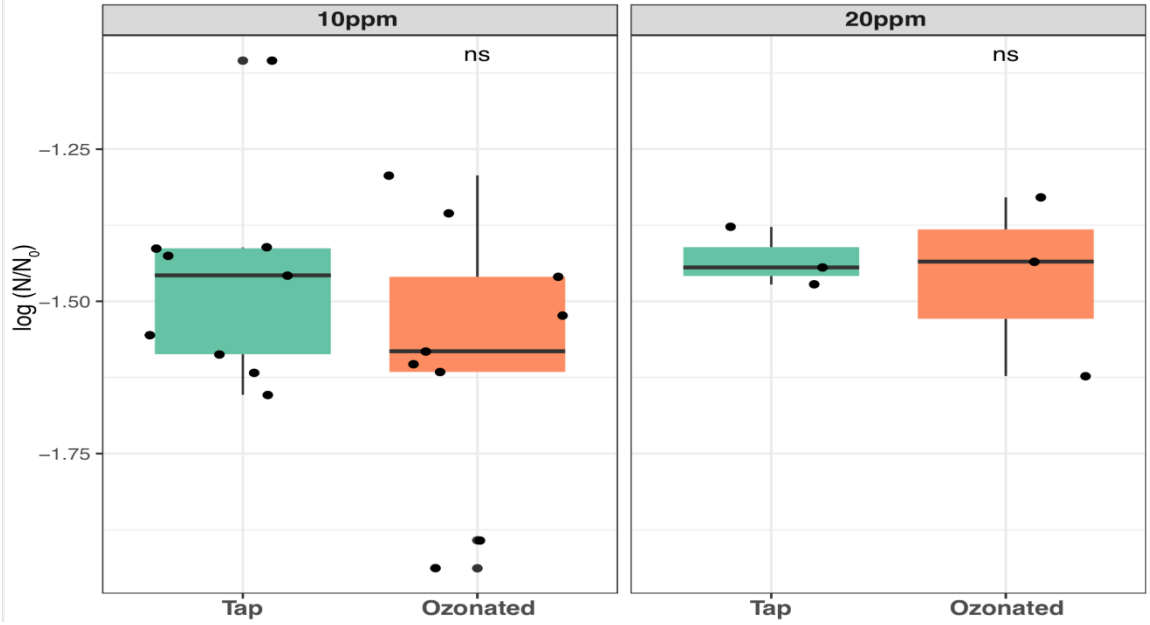
***E. coli* O157:H7 reduction on Romaine lettuce after submersion**. After inoculation, the average *E. coli* O157:H7 populations on shredded Romaine lettuce were 4.6±0.3 log CFU/g. In relationship to the untreated lettuce, shredded lettuce submerged in 6 ppm of ozone NBs for 90 s showed a significant reduction of 0.8±0.2 log CFU/g *E. coli* O157:H7 ($p < 0.05$) (Figure 4).

Figure 4. *E. coli* O157:H7 populations on Romaine lettuce untreated and treated via submersion in 80 L of 6 ppm ozone NBs for 90 s. Boxplots show the distribution of log CFU/g values of the 3 biological and technical replicates.



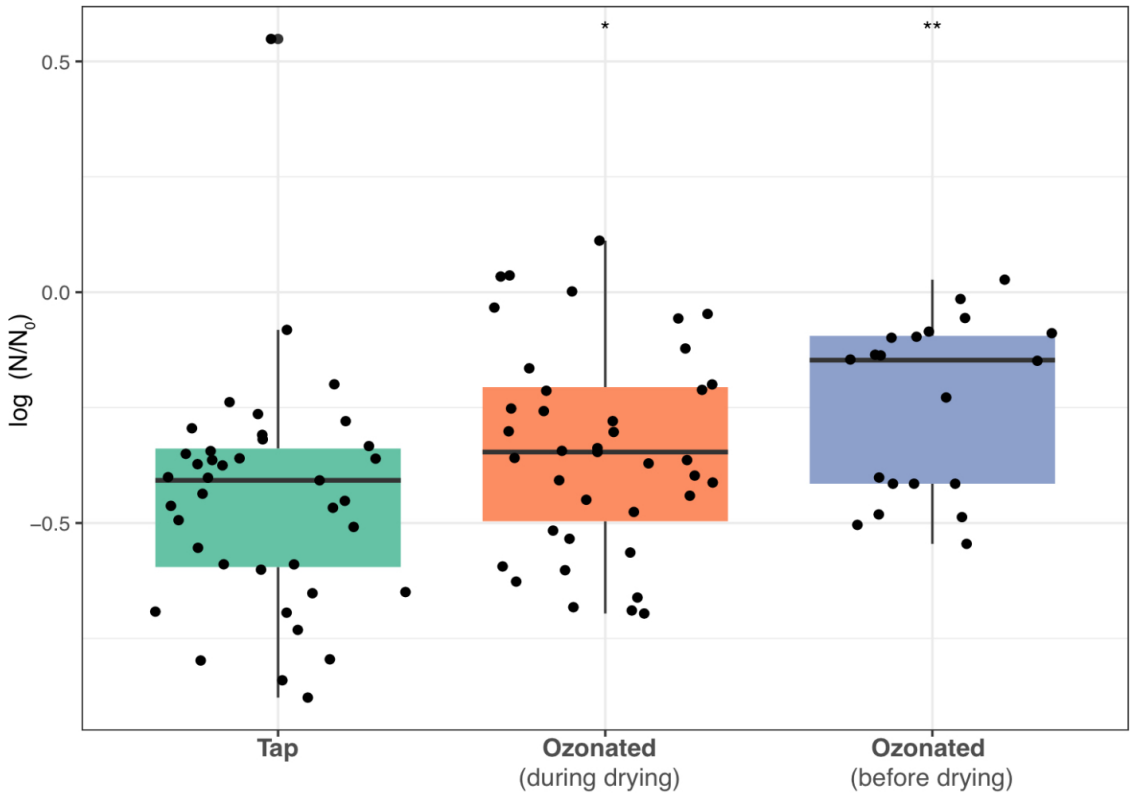
Inactivation of *E. coli* O157:H7 on shredded Romaine during pilot-scale flume washing. The average initial *E. coli* O157:H7 count on shredded lettuce was 4.94±0.32 log/CFU g. While the target free chlorine levels in the pilot-scale flume system were 10 and 20 ppm, the average and standard deviations of free chlorine were 10.84±0.54 and 19.54±0.83 ppm respectively, over the course of the experiment. Overall reductions of *E. coli* O157:H7 on shredded lettuce after processing in 10 ppm chlorine + tap water and 20 ppm chlorine + tap water were 1.46±0.06 and 1.4±0.07 log CFU/g, respectively ($p > 0.05$). Overall reductions of *E. coli* O157:H7 on shredded lettuce after processing in 10 ppm chlorine + 6 ppm ozone water and 20 ppm chlorine + 6 ppm water were 1.75±0.04 and 1.43±0.08 log CFU/g, respectively ($p > 0.05$) (Figure 5). The application of ozone NBs after flume washing with either 10 or 20 ppm free chlorine did not significantly impact the overall reduction of *E. coli* O157:H7 on shredded Romaine lettuce ($p > 0.05$).

Figure 5. Log reduction of *E. coli* O157:H7 on shredded Romaine lettuce after flume washing in 10 or 20 ppm of chlorinated flume water and spraying with tap water (tap) or 6 ppm ozone NBs (ozonated).



Reduction of *E. coli* O157:H7 during centrifugal drying with ozone nanobubbles. The average initial count of *E. coli* O157:H7 on shredded Romaine lettuce was 5.87±0.39 log CFU/g. Average count of *E. coli* O157:H7 on shredded lettuce after spraying with tap water during centrifugal drying were 5.33±0.45 log CFU/g. Counts on shredded lettuce after treatment with 6 ppm of ozonated NBs during centrifugal drying were 5.51±0.5 log CFU/g. Counts on shredded lettuce after treatment with 6 ppm of ozone NBs sprayed before centrifugal drying were 5.31±0.38 log CFU/g (Figure 6). The reduction due to 6 ppm of ozonated during centrifugal drying (0.32 log CFU/g) was not significantly different from (0.37 log CFU/g) or 6 ppm of ozonated applied before centrifugal drying (0.17 log CFU/g) ($p > 0.05$). Even though there was a significant difference between reductions of microbial populations of shredded lettuce treated with ozone NBs (before drying) and tap water, the magnitude of difference was not significant ($p < 0.05$).

Figure 6. Log reduction of *E. coli* O157:H7 on Romaine lettuce that was treated with tap water or 6 ppm ozone NBs during drying or 6 ppm ozone NBs before drying.



3.5. Discussion

Ozone NBs limited efficacy as a sanitizer. Ozone NBs is an attractive alternative sanitizer due to its ability to stabilize ozone's highly reactive third oxygen atom, prolonging its lifespan in aqueous solution, and thus increasing its antimicrobial potential. In these studies, ozone showed limited efficacy and/or no significant difference compared to tap water in reducing *E. coli* O157:H7 on shredded Romaine lettuce when applied at different stages of pilot-scale processing. Previous studies have shown the efficacy of ozone micro- and nanobubbles in reducing plant pathogens, spores, and pathogenic bacteria on different agricultural products (Seridou & Kalogerakis, 2021). For example, in a study by Phaephiphat & Mahakarnchanakul (2018), *Salmonella* Typhimurium populations were reduced by 2.6 log CFU/g on 100 g of sweet basil leaves after washing in 4 L of 1 ppm ozone microbubbles. Additionally, ozone NBs have been used to disinfect water. Nghia et al. (2021) reduced *Vibrio parahaemolyticus* populations by more than 6 log CFU/ml in 15% saline water after application of ozone nanobubbles for up to 6 minutes at 2-minute intervals. Consequently, the difference in efficacy between these studies and the current studies could be due to factors affecting ozone's efficacy. Similarly, Jhunkeaw et al.

(2021), reduced 50 L of water inoculated with *Streptococcus agalactiae* or *Aeromonas veroni* by more than 99.99% after a 15-minute treatment with ozone nanobubbles.

The type of produce and the attachment of microbes on produce should be considered when choosing ozone as a sanitizer. According to Glowacz & Rees (2016), produce with a physiology consisting of rough surfaces, such as lettuce, can shield pathogens from sanitization unlike fresh produce with smooth and exposed surfaces such as tomatoes. Additionally, *E. coli* O157:H7 may have escaped oxidation of ozone NBs by being internalized into the vascular network of the lettuce via damaged lettuce tissue and stomata (Esmael et al., 2023) after the 15 minute inoculation. This was also hypothesized by Davidson (2013) who used an identical inoculation method using iceberg lettuce heads. Furthermore, the efficacy of ozone is dependent on its distribution and contact on produce. During the centrifugal dryer studies, 22.7 kg of shredded Romaine lettuce had treatment applied before or after centrifugal drying. Notably, the application of ozone NBs before centrifugal drying was significantly different ($p < 0.05$) than tap water applied during centrifugal drying. The difference in reductions observed during centrifugal drying could be due to being ozone nanobubbles applied more evenly across the shredded lettuce as it was rotated.

Submersion of shredded Romaine lettuce into ozonated nanobubbles led to significant reduction of *E. coli* O157:H7. The efficacy of submerging produce in produce sanitizers has been explored in lab scale and pilot-scale studies. In a study by Allende et al. (2008), overall number of microbes decreased by submerging 20 kg of shredded lettuce in tap water, 100 ppm of free chlorine, 250 and 500 ppm of Sanova (acidified sodium chlorite) 10 and 20 ppm of Sanoxol 20 (hydrogen peroxide and peroxyacetic acid), 80 ppm of Tsunami 100 (peroxyacetic acid and hydrogen peroxide), 10 and 20 ppm of Purac FCC 80 (lactic acid), and 2.5 and 5 ppm of Citrox 14W (organic acids and flavonoids). After submersion, most sanitizers were able to reduce mesophilic populations about 1 log CFU/g. Coliform populations were reduced by 1.6 and 2.3 log CFU/g using Purac and 5 ppm of Citrox, respectively. All other sanitizers reduced coliform populations by 0.7-1.3 log CFU/g.

In the current study, shredded lettuce submerged in 6 ppm of ozone NBs for 90 s led to a 0.8 log/CFU g reduction of *E. coli* O157:H7. A study by Silveira et al. (2017) used laboratory scale equipment to investigate the efficacy of multiple combinations of washing and disinfection procedures on shredded lettuce inoculated with *Salmonella* Enteritidis SE86. After submersion of

shredded lettuce in a 500 ml 200 ppm sodium hypochlorite solution for 1 to 15 min, 1-3 log CFU/g reductions in *Salmonella* were observed. The largest reduction was achieved by pre-washing shredded lettuce with 250 ml of potable water, submersion it in chlorine for 15 min, and then rinsing with 250 ml of potable water. Generally, fruit and vegetable processors incorporate a washing step before sanitization to remove soil and debris that can interfere with the efficacy of sanitizers (Gil et al., 2015; Howard et al., 2020). However, the wash water appeared to have the unintended effect of removing *Salmonella* on shredded lettuce as well, resulting in an additional log reduction. This was also observed by Van Haute et al. (2013) during their washing process to reduce *E. coli* O157:H7 counts on shredded lettuce.

Rodgers et al. (2004) observed greater than 5.9 log CFU/g reduction of *E. coli* O157:H7 and *L. monocytogenes* on whole apples, lettuce, and strawberries after submersion in 100 ml of 3 ppm ozone. The substantial difference in log reduction could be due to the physiology of produce during sanitization. Exposed tissue of the shredded lettuce used in this study could have possibly reduced the efficacy of ozone NBs by allowing *E. coli* O157:H7 cell internalize into the vascular networks and escape sanitization (Gil et al., 2009). Furthermore, the produce to wash water ratio used by Rodgers et al. (2004) a 2:5 w/v, meanwhile the current study used a 1:160 w/v ratio. Hypothetically, a ratio using less produce in comparison to greater amounts of wash water would improve the efficacy of ozone given its reactivity to organic matter. However, using this study as a reference, our results contradict that.

Application of ozone via makeup water did not lead to additional reductions in pathogen levels on shredded lettuce during pilot-scale flume washing with chlorine. In this study, reduction of *E. coli* O157:H7 ranged from 1.4-1.7 log CFU/g after flume washing with and without ozone. Davidson (2013) observed smaller log reductions using the same flume washing system. Washing 5.4 kg of shredded iceberg lettuce for 90 s in flume water containing a 0.0006% organic load and 30 ppm of available chlorine (pH 7.85), 30 ppm available of chlorine with citric acid (pH 6.5), or 30 ppm of available chlorine with T-128 (pH 6.5) reduced *E. coli* O157:H7 by 0.8, 0.9, and 1 log CFU/g populations, respectively. Given the smaller organic load, similar product to wash water ratio, and higher ppm of chlorinated wash water used in this study, the current study suggests the hurdle effect of flume wash and a final water rinse can lead to larger pathogen reductions than standalone flume wash.

Application of ozonated nanobubbles as a final rinse spray before pilot-scale centrifugal drying lead to greater reductions than tap water applied during centrifugal drying. In a lab scale study by Pang and Hung (2016), two Romaine lettuce leaves inoculated on the outer and inner surface with a three strain nalidixic acid-resistant cocktail of *E. coli* O157:H7 were overhead spray sanitized with 0.5 ppm of ozonated water for 5, 10, and 15 min. After contact with ozonated water for 5 min, 2 and 3 log reductions were observed for the outer and inner surface leaves, respectively. Furthermore, a 10-minute overhead spray increased pathogen reduction by 0.7 log for outer leaves only and no further increase in treatment time resulted in greater reduction. Compared to the current study, the 2 and 3 log reductions observed by Pang and Hung (2016) could be due to the physiological state, scale of shredded lettuce and longer contact times. Ozone's efficacy as a sanitizer is affected by organic matter (Botondi et al., 2021). Therefore, the smaller log reductions, compared to log reductions observed by Pang and Hung (2016), could be due to the highly reactive oxygen atoms of ozone oxidizing the exposed tissue of shredded lettuce rather than the pathogen.

The scale of the shredded lettuce should also be considered. During the current study, a shower head attached to a centrifugal dryer applied ozone NBs to 22.7 kg of shredded lettuce. Meanwhile an overhead sprayer was used to sanitize only 2 pieces of lettuce. This could also explain why despite using 12 times the ppm of ozone, greater log reductions were observed using 0.5 ppm of ozone rather than 6 ppm. Finally, the treatment time of ozone could be a potential factor as well. While these studies did not use spraying methods, Chen et al. (2020) and Liu et al. (2021) observed higher log reductions among fresh cut yellow onions and fresh cut cabbage, respectively, after treatment with 1.4 ppm of ozonated water using their longest contact time 5 and 10 min.

3.6. Conclusions

Washing systems in produce operations are intended to remove soil and debris from harvest, pesticides, and potential pathogenic microbes acquired during pre- or postharvest production. The introduction of produce sanitizers is crucial to reduce the microbial load of the wash water and reduce microbial populations on produce if possible. The first objective assessed the efficacy of ozonated NBs via submersion of shredded lettuce. Submerging shredded lettuce in 80 L of 6 ppm of ozone NBs led to significant reductions of *E. coli* O157:H7 ($p < 0.05$). However, the log reduction was less than 1 log.

The second objective assessed the application of ozone NBs during pilot-scale flume washing. The application of 6 ppm of ozone NBs as a final rinse during pilot-scale flume washing did not lead to a significant reduction in *E. coli* O157:H7.

The third objective assessed the application of ozone NBs prior to and during pilot-scale centrifugal drying. The reduction of *E. coli* O157:H7 populations on shredded Romaine lettuce due to application of 6 ppm of ozone NBs during centrifugal drying (0.32 log CFU/g) was not significantly different from tap water (0.37 log CFU/g) or 6 ppm of ozonated applied before centrifugal drying (0.17 log CFU/g) ($p > 0.05$). A significant difference was seen between reductions of microbial populations on shredded lettuce treated with ozone NBs (before drying) and tap water. However, the magnitude of difference was not large ($p < 0.05$).

Overall, this research showed limited efficacy of ozone NBs as a sanitizer for shredded lettuce during simulated commercial production. Chlorine remains the gold standard sanitizer in terms of cost and ease of use. However, its drawbacks as a produce sanitizer are notable. Ozone NBs as a wash sanitizer should be continued to be explored as a viable alternative to chlorine.

Acknowledgements

This work was supported, in part, by FDA grant 2R44FD006565-02 and En Solución by providing the commercial NB and ozone generators needed to perform these experiments.

4. Conclusions and Future Directions

4.1. Overall Conclusions

CA storage rooms, integral to extending the shelf life of apples by delaying ripening and fungal decay through the manipulation of atmospheric gases and temperature, also present a unique environment where *L. monocytogenes* could survive. The first study, a two-year longitudinal study assessing the prevalence and distribution of *Listeria* spp. in CA storage rooms revealed a 6.3% (19) positivity rate among 300 samples. Among the 19 positive sample sites, a total of 65 *Listeria* isolates were recovered, consisting of *L. monocytogenes* (48/65), followed by *L. seeligeri* (10/65), *L. welshimeri* (4/65), and *L. innocua* (3/65). Notably, three distinct ATs of *L. monocytogenes* were identified, highlighting the genetic diversity within the species present in the storage rooms. The study identified floors below condenser units as the most prevalent (9/19) sites for *Listeria* spp., accounting for nearly half of the positive samples. Finally, none of the locations had repeated contamination of *Listeria* spp. during the 2-year study.

Following harvest, it is crucial to wash leafy greens as this process effectively removes dirt and reduces microbial populations. Chemical sanitizers are normally used during produce washing to reduce the microbial load in the wash water and on the produce. The second study investigated the efficacy of ozone NBs in inactivating *E. coli* O157:H7 on shredded Romaine lettuce during different stages of pilot-scale flume washing. Submerging 0.5 kg of shredded Romaine lettuce in 6 ppm of ozone NBs for 90 s achieved a significant reduction of ($p < 0.05$) 0.8±0.20 log CFU/g in *E. coli* O157:H7 populations, demonstrating the antimicrobial capability of ozone NBs. However, when examining the application of ozone NBs in the context of a pilot-scale flume system with shredded lettuce, the addition of ozone NB water as a final overhead rinse did not significantly enhance the reduction of *E. coli* O157:H7 compared to tap water, regardless of the chlorine concentration of the flume water ($p > 0.05$). This outcome suggests that while ozone NBs possess antimicrobial activities, their effectiveness can vary depending on the application method and the processing environment. Furthermore, the application of ozonated NBs during centrifugal drying of shredded lettuce did not significantly differ from tap water in reducing *E. coli* O157:H7 populations ($p > 0.05$) Although there was a significant difference observed in microbial reduction between ozonated water applied before spinning and tap water ($p <0.05$), the overall magnitude of this difference was minimal.

Overall, the findings from these studies contribute valuable insights into the prevalence and control of foodborne pathogens in different settings. With *L. monocytogenes* established as a commonly occurrence in apple packing houses in previous environmental monitoring studies, tracking the presence and diversity of *Listeria* spp. in CA storage rooms offers another means to identify possible routes of cross-contamination to other areas of the facility. Additionally, the potential of ozone NBs as a wash sanitizer warrants further exploration, particularly in optimizing application methods for enhanced efficacy.

4.1. Future Research Recommendations of Ozone Nanobubbles

The study presented in Chapter 2 focused on the presence, diversity, and persistence of *Listeria* spp. in CA storage rooms of apples. A notable limitation of this research was the limited number of CA storage rooms and facilities environmental sampled. Originally, it was intended that 16 CA storage facilities were to be sampled, which would have significantly increased our sample size and offered more validity to our findings. Unfortunately, the willingness of apple processing officials to participate diminished after learning of our intent to test for *Listeria* spp., resulting in fewer sampled locations. Therefore, future studies should broaden the scope to include CA apple storage rooms of large, medium, and small apple packing facilities across multiple locations in Michigan and only sample CA storage rooms of apples distributed in Michigan to increase generalizability. Additionally, future studies could incorporate whole genome sequencing to provide greater discrimination among isolates compared to the more limited discrimination when using only *sigB* allelic typing as used in this study.

The studies presented in Chapter 3 showed that the application of ozone NBs during pilot-scale leafy green processing varied in its efficacy. Ozone is a potent oxidative sanitizer and used in combination with NBs, could increase its efficacy against foodborne pathogens by prolonging its atomic structure in aqueous solutions. However, the results of Chapter 3 showed that its efficacy is inconsistent or not substantial during submersion, pilot-scale flume washing, and as a final rinse spray during centrifugal drying.

Studies could examine the efficacy of ozone NBs on different food sources or as a sanitizer on food and non-food contact surfaces. Recent unpublished work in our lab (data not shown) compared the efficacy ozone NBs, using identical equipment provided by our industry partners, against PAA in an apple packing facility to reduce native aerobic populations on apples and brush beds as an overhead final rinse spray after washing in chlorinated water. Results show that

in comparison to PAA, ozone NBs were able to reduce aerobic populations by an additional 0.21 CFU/apple and 1.59 log CFU/sponge on apples and brush beds, respectively. Using the pilot plant here on site, additional experiments could compare the antimicrobial efficacy of ozonated NBs against other common industry sanitizers on food and food contact surfaces inoculated with pathogens such as *L. monocytogenes* or the *E. coli* O157:H7 isolates used in the current study.

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APPENDIX

Table A-1. Presence of *Listeria* spp. based on *sigB* allelic type, sampling location, storage season, and room number in facility A. "-" indicates no presence of *Listeria* spp. N/A indicates no samples were collected at that time.

Location	Room	F21	S22	F22	S23/F23
1A (Floor near door)	1	-	-	-	-
1B (Floor of corner near door)	1	-	*L. innocua* AT 31	-	-
1C (Floor near Stacked Bins)	1	-	-	-	-
2A (Floor below condenser)	1	-	-	-	-
2B (Floor below condenser)	1	*L. innocua* AT 23	-		-
2C (Floor below condenser)	1	-	-	-	-
3A (Curb)	1	-	-	-	-
3B (Curb)	1	-	-	-	-
3C (Curb seal)	1	-	-	-	-
4A (Wall)	1	-	-	-	-
Standing Water	1	N/A	N/A	N/A	-
1A (Floor near door)	2	-	-	-	-
1B (Floor of corner near door)	2	-	-	-	-
1C (Floor near Stacked Bins)	2	-	-	-	*L. seeligeri* AT 12
2A (Floor below condenser)	2	-	-	-	-
2B (Floor below condenser)	2	-	-	-	-
2C (Floor below condenser)	2	-	-	-	-
3A (Curb)	2	-	-	-	-

Table A-1. (Cont'd)

3B (Curb)	2	-	-	-	-
3C (Curb seal)	2	-	-	-	-
4A (Wall)	2	-	-	-	-
Standing Water	2	N/A	N/A	N/A	-
1A (Floor near door)	3	-	-	*L. monocytogenes* AT 58; *L. monocytogenes* AT 61 Lineage 1	-
1B (Floor of corner near door)	3	-	-	*L. seeligeri* AT 12	-
1C (Floor near Stacked Bins)	3	-	-	-	*L. monocytogenes* AT 61 Lineage 1
2A (Floor below condenser)	3	-	-	-	*Listeria monocytogenes* AT 61 Lineage 1
2B (Floor below condenser)	3	-	-	*L. monocytogenes* AT 61 Lineage 1	-
2C (Floor below condenser)	3	-	-	*L. monocytogenes* AT 61 Lineage 1	*L. monocytogenes* AT 61 Lineage 1
3A (Curb)	3	-	-	-	-
3B (Curb)	3	-	-	-	-
3C (Curb seal)	3	-		-	-
4A (Wall)	3	-	-	-	-
Standing Water	3	N/A	N/A	N/A	N/A

Table A-2. Presence of *Listeria* spp. based on *sigB* allelic type, sampling location, storage season, and room number in facility B. "-" indicates no presence of *Listeria* spp. N/A indicates no samples were collected at that time.

Location	Room	F21	S22	F22	S23/F23
1A (Floor near door)	1	-	-	-	-
1B (Floor of corner near door)	1	-	-	-	-
1C (Floor near Stacked Bins)	1	-	-	-	-
2A (Floor below condenser)	1	-	*L. monocytogenes* AT 61 Lineage 1	-	-
2B (Floor below condenser)	1	-	*L. monocytogenes* AT 61 Lineage 2	-	-
2C (Floor below condenser)	1	-	*L. seeligeri* AT 1	-	-
3A (Curb)	1	-	-	-	-
3B (Curb)	1	-	-	-	-
3C (Curb seal)	1	-	-	-	-
4A (Wall)	1	-	-	-	-
Standing Water	1	N/A	N/A	N/A	N/A
1A (Floor near door)	2	-	-	-	-
1B (Floor of corner near door)	2	-	-	-	-
1C (Floor near Stacked Bins)	2	-	-	-	-
2A (Floor below condenser)	2	-	-	-	-
2B (Floor below condenser)	2	-	-	-	-
2C (Floor below condenser)	2	-	-	-	-
3A (Curb)	2	-	-	-	-
3B (Curb)	2	-	-	-	-
3C (Curb seal)	2	-	-	-	-
4A (Wall)	2	-	-	-	-
Standing Water	2	N/A	N/A	N/A	N/A
1A (Floor near door)	3	-	-	-	-
1B (Floor of corner near door)	3	-	-	-	-
1C (Floor near Stacked Bins)	3	-	-	-	-
2A (Floor below condenser)	3	-	-	-	-

Table A-2. (Cont'd)

2B (Floor below condenser)	3	-	-	-	-
2C (Floor below condenser)	3	-	-	-	-
3A (Curb)	3	-	-	-	-
3B (Curb)	3	-	-	-	-
3C (Curb seal)	3	-		-	-
4A (Wall)	3	-	-	-	-
Standing Water	3	N/A	N/A	N/A	N/A

Table A-3. Presence of *Listeria* spp. based on *sigB* allelic type, sampling location, storage season, and room number in facility C. "-" indicates no presence of *Listeria* spp. N/A indicates no samples were collected at that time.

Location	Room	F21	S22	F22	S23/F23
1A (Floor near door)	1	N/A	-	-	-
1B (Floor of corner near door)	1	N/A	N/A	-	-
1C (Floor near Stacked Bins)	1	N/A	N/A	-	*L. welshimeri* AT 129
2A (Floor below condenser)	1	N/A	N/A	-	-
2B (Floor below condenser)	1	N/A	N/A	-	-
2C (Floor below condenser)	1	N/A	N/A	-	-
3A (Curb)	1	N/A	N/A	-	*L. monocytogenes* AT 61 Lineage 1
3B (Curb)	1	N/A	N/A	-	-
3C (Curb seal)	1	N/A	N/A	-	-
4A (Wall)	1	N/A	N/A	-	-
Standing Water	1	N/A	N/A	N/A	N/A
1A (Floor near door)	2	N/A	N/A	-	-
1B (Floor of corner near door)	2	N/A	N/A	-	-
1C (Floor near Stacked Bins)	2	N/A	N/A	-	-

Table A-3. (Cont'd)

2A (Floor below condenser)	2	N/A	N/A	-	-
2B (Floor below condenser)	2	N/A	N/A	-	-
2C (Floor below condenser)	2	N/A	N/A	-	-
3A (Curb)	2	N/A	N/A	-	-
3B (Curb)	2	N/A	N/A	*L. monocytogenes* AT 61 Lineage 1; *L. seeligeri* AT 12	-
3C (Curb seal)	2	N/A	N/A	-	-
4A (Wall)	2	N/A	N/A	-	-
Standing Water	2	N/A	N/A	N/A	N/A
1A (Floor near door)	3	N/A	N/A	-	-
1B (Floor of corner near door)	3	N/A	N/A	-	-
1C (Floor near Stacked Bins)	3	N/A	N/A	-	-
2A (Floor below condenser)	3	N/A	N/A	-	-
2B (Floor below condenser)	3	N/A	N/A	-	-
2C (Floor below condenser)	3	N/A	N/A	-	-
3A (Curb)	3	N/A	N/A	-	-
3B (Curb)	3	N/A	N/A	-	-
3C (Curb seal)	3	N/A	N/A	-	-
4A (Wall)	3	N/A	N/A	-	-
Standing Water	3	N/A	N/A	N/A	N/A

Table A-4. TB#, storage season, room number, sampling location, *Listeria* spp., and allelic type of *Listeria* isolates.

TB#	Season	Room	Location	Species	AT
TB0857	F21	A1	2B (Floor Below Condenser)	*L. innocua*	23
TB1013	S22	B1	2C (Floor Below Condenser)	*L. seeligeri*	12
TB1014	S22	B1	2C (Floor Below Condenser)	*L. seeligeri*	12
TB1015	S22	B1	2C (Floor Below Condenser)	*L. seeligeri*	12
TB1017	S22	B1	2C (Floor Below Condenser)	*L. seeligeri*	12
TB1019	S22	B1	2C (Floor Below Condenser)	*L. seeligeri*	12
TB1020	S22	B1	2C (Floor Below Condenser)	*L. seeligeri*	12
TB1025	S22	A1	1B (Floor of Corner Near Door)	*L. innocua*	31
TB1042	S22	B1	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1043	S22	B1	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1044	S22	B1	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1045	S22	B1	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1046	S22	B1	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1047	S22	B1	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1048	S22	B1	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1049	S22	B1	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1050	S22	B1	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1051	S22	B1	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1052	S22	B1	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1053	S22	B1	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1054	S22	B1	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1055	S22	B1	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1056	S22	B1	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1057	S22	B1	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1070	F22	A3	1B (Floor of Corner Near Door)	*L. seeligeri*	12
TB1071	F22	A3	1B (Floor of Corner Near Door)	*L. seeligeri*	12
TB1076	F22	A3	1A (Floor Near Door)	*L. monocytogenes*	61
TB1077	F22	A3	1A (Floor Near Door)	*L. monocytogenes*	58
TB1078	F22	A3	1A (Floor Near Door)	*L. monocytogenes*	61
TB1079	F22	A3	1A (Floor Near Door)	*L. monocytogenes*	61
TB1080	F22	A3	1A (Floor Near Door)	*L. monocytogenes*	61
TB1081	F22	A3	1A (Floor Near Door)	*L. monocytogenes*	61
TB1082	F22	A3	1A (Floor Near Door)	*L. monocytogenes*	61
TB1083	F22	A3	1A (Floor Near Door)	*L. monocytogenes*	61
TB1084	F22	A3	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1085	F22	A3	2B (Floor Below Condenser)	*L. monocytogenes*	61
TB1097	F22	A3	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1099	F22	A3	2A (Floor Below Condenser)	*L. monocytogenes*	61
TB1109	F22	C2	3B (Curb)	*L. monocytogenes*	61
TB1110	F22	C2	3B (Curb)	*L. monocytogenes*	61

Table A-4. (Cont'd)

TB1111	F22	C2	3B (Curb)	*L. seeligeri*	12
TB1149	S23	C1	1C (Floor near Stacked Bins)	*L. welshimeri*	129
TB1150	S23	C1	1C (Floor near Stacked Bins)	*L. welshimeri*	129
TB1155	S23	C1	1C (Floor near Stacked Bins)	*L. welshimeri*	129
TB1156	S23	C1	1C (Floor near Stacked Bins)	*L. welshimeri*	129
TB1159	S23	C1	3A(curb)	*L. monocytogenes*	61
TB1309	S23	C3	2B (Floor Below Condenser)	*L. innocua*	31
TB1381	F23	A3	2C (Floor Below Condenser)	*L. monocytogenes*	67
TB1382	F23	A3	2C (Floor Below Condenser)	*L. monocytogenes*	67
TB1384	F23	A3	2C (Floor Below Condenser)	*L. monocytogenes*	67
TB1385	F23	A3	2C (Floor Below Condenser)	*L. monocytogenes*	67
TB1386	F23	A3	2C (Floor Below Condenser)	*L. monocytogenes*	67
TB1387	F23	A3	2C (Floor Below Condenser)	*L. monocytogenes*	67
TB1388	F23	A3	2C (Floor Below Condenser)	*L. monocytogenes*	67
TB1397	F23	A3	1C (Floor near Stacked Bins)	*L. monocytogenes*	67
TB1398	F23	A3	1C (Floor near Stacked Bins)	*L. monocytogenes*	67
TB1404	F23	A3	1C (Floor near Stacked Bins)	*L. monocytogenes*	67
TB1405	F23	A3	2A (Floor Below Condenser)	*L. monocytogenes*	67
TB1406	F23	A3	2A (Floor Below Condenser)	*L. monocytogenes*	67
TB1407	F23	A3	2A (Floor Below Condenser)	*L. monocytogenes*	67
TB1409	F23	A3	2A (Floor Below Condenser)	*L. monocytogenes*	67
TB1410	F23	A3	2A (Floor Below Condenser)	*L. monocytogenes*	67
TB1411	F23	A3	2A (Floor Below Condenser)	*L. monocytogenes*	67
TB1412	F23	A3	2A (Floor Below Condenser)	*L. monocytogenes*	67
TB1516	F23	A2	1C (Floor near Stacked Bins)	*L. seeligeri*	12