ABSTRACT

BLOUIN, BENJAMIN BATTISTO. Control of Microbiota during Fresh-Cut Leafy Green Washing and Low Salt Cucumber Fermentation. (Under the direction of Dr. Lynette Johnston and Dr. Ilenys M. Pérez-Díaz).

The microbiota of fresh vegetables are diverse, some populations of bacteria cause human disease while others can be exploited for desirable functions. Two independent studies, both involving the control of post-harvest vegetable microbiota, were conducted: 1) the exploration of peracetic acid (PAA) to control wash water quality during fresh-cut processing of leafy greens and 2) the evaluation of *Lactococcus lactis* starter culture to reduce bloater defects during low salt cucumber fermentation.

Peracetic acid is used as a sanitizer for preventing pathogen cross-contamination in fresh-cut leafy green wash water systems. While various factors, such as pH, temperature, and organic load, may affect the efficacy of PAA in wash water systems, PAA residual concentration is thought to be the primary metric for prevention of cross-contamination. The purpose of the study was to explore the efficacy of a recirculated wash water system using PAA in a fresh-cut leafy green facility based on relationships between physicochemical variables and microbial loads. The operation sequentially washed produce in two wash tanks continuously dosed with PAA prior to cutting, packaging, and packing. Wash water physicochemical variables were measured directly in the tanks (temperature, pH, oxidation-reduction potential) or with water samples (PAA residual concentration, turbidity, chemical oxygen demand). Microbial loads (aerobic plate count, total coliforms, *E. coli*) were estimated for water samples (100 mL, N=63), pre-wash and post-wash leafy greens (25 g, n=54). Average PAA concentrations were 19.5 ± 28.3 ppm in tank 1 and 22.9 ± 16.7 ppm in tank 2. *E. coli* was greater than 0.3 log CFU/100mL in tank 1 when the PAA concentration was below 2 ppm. There was no significant difference between pre-wash and

post-wash leafy green microbial loads for all indicator organisms. Results suggested dosing amounts may be optimized to maintain adequate PAA concentrations based on water temperature and expected microbial loads.

Lc. lactis, capable of a shorter doubling time than *Lactiplantibacillus pentosus*, is proposed as a starter culture for cucumber fermentation brined with low salt to prevent bloater defect. The metabolic activity of the cucumber intrinsic *Enterobacter* and *Leuconostoc*, and starter cultures are hypothesized to produce sufficient carbon dioxide (CO_2) to induce bloater defect. Model and starter culture-assisted cucumber fermentation were evaluated for bloater defects and metabolic activity in response to *Lc. lactis* single culture inoculation or mixed with *Lb. pentosus*. Model fermentations of cucumber juice medium (CJM) in vacutainers revealed that growth of *Lc. lactis* and *Leuconostoc* or *Enterobacter* was concomitant with reductions in production of acetic acid and ethanol from heterofermentation. *Leuconostoc* produced trace amounts of butyric acid (0.7 mM). The *Enterobacter* population density was reduced when co-inoculated with *Lc. lactis* in CJM. Model fermentations of pasteurized cucumbers confirmed a reduction of CO_2 production in the presence of *Lc. lactis* and a minimum attainable pH of 4.0 ± 0.2. Fresh cucumber fermentations brined with low salt and assisted by a *Lc. lactis* resulted in reduced bloater defect but produced butyric acid (40 mM). A metagenomic analysis shows *Leuconostoc* spp. was present and may have contributed to butyric acid production. *Lc. lactis* can reduce bloater index in the initial stage of cucumber fermentation brined with low salt, but an additional strategy is needed to control butyric acid production and the defect during the middle fermentation stage.

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Control of Microbiota during Fresh-Cut Leafy Green Washing and Low Salt Cucumber Fermentation

by
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BIOGRAPHY

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CHAPTER 1: Literature review

Part 1. Wash water for fresh-cut leafy green vegetables

1.1 Microbial contamination of fresh produce

Fresh produce has been the source of foodborne disease outbreaks caused by multiple human pathogens, including *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. (Mercanoglu Taban & Halkman, 2011; Singh et al., 2018). Multiple multi-state foodborne outbreaks between 2023 and 2024 have been attributed to fresh produce (CDC, 2024). Approximately 9% of foodborne illnesses associated with an identified pathogen are attributed to leafy greens; 2.3 million illnesses are associated with leafy greens and an identified or unknown pathogen annually in the United States (Yang & Scharff, 2024).

Shiga toxin-producing *E. coli* (STEC) linked to leafy greens, which are the second most common source of foodborne STEC O:157 outbreaks, caused 40 outbreaks between 2009 and 2018 in the United States and Canada (Marshall et al., 2020). “Leafy vegetables” were attributed to 606 outbreaks between 1973 and 2012 in the United States alone, of which 55% were norovirus, 18% STEC, and 11% *Salmonella* spp. (Herman et al., 2015). In a systematic literature review, 11 of 14 articles attributed water with an unclear contamination route (including field contamination and washing) to leafy greens and raw sprouts STEC contamination (Kintz et al., 2019). Due to the short shelf life of leafy greens, the approximately three-week lag in identifying outbreaks, and the short duration of most outbreaks, most STEC outbreaks are suspected to be caused by leafy greens rather than confirmed (Marshall et al., 2020). In 2006, multistate outbreaks of *E. coli* O157:H7 were traced to prepackaged baby spinach with 199 illnesses and shredded iceberg lettuce with 71 illnesses (Smolinski et al., 2018). A multi-state listeriosis outbreak in 2015-2016 was attributed to packaged salads (Self et al., 2019). In the United States,

invasive *Listeria monocytogenes* infections (listeriosis) was found to be the third leading cause of death from foodborne illness and cause an estimated 250 deaths per year (Scallan et al., 2011).

Leafy greens can become contaminated with human pathogens during field production, harvest, processing, or distribution (Gil et al., 2015). However, field contamination via contaminated irrigation water has been suspected to be the primary source of pathogen contamination (Berry et al., 2015; Mogren et al., 2018). Cross-contamination can then occur as field contamination becomes redistributed during washing or processing, which likely exacerbates the severity of outbreaks (Gil et al., 2009; Gombas et al., 2017; Smolinski et al., 2018).

1.2 Wash systems

Aqueous wash of vegetables such as leafy greens removes soil, pesticide residues, microorganisms, and plant cell exudates that support microbial growth (Gil et al., 2009). Various wash methods are commercially used for fresh produce; however, the general process has been standardized to the following order: cut/process, wash, and de-water; some operations wash before processing (Luo et al., 2014). Various systems are used in industry, including single-pass and recirculating water systems. Single-pass systems do not reuse water, as they maintain consistent water quality and a reduced risk of cross-contamination (Bornhorst et al., 2018). However, single-pass immersion-free systems likely do not remove field debris from produce with large surface areas (i.e. leafy greens) as effectively as recirculating systems with immersion. Therefore, single-pass systems may be better for produce with small surface areas such as melons or tomatoes.

Recirculating water systems treat process water during washing and are ideal for minimizing cost and overall water use (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2023). Recirculating water systems commonly have one to three sequential wash tanks in which the water quality is maintained separately in each tank. The first wash tank in multi-tank recirculating water systems is expected to require the greatest antimicrobial control as field debris and contamination becomes suspended in its wash water. Vegetables may be fully immersed in open flume tanks or closed wash systems; alternatively, wash water can be dispensed via shower or waterfall. Various types of produce may be best suited to different wash systems. For example, leafy greens may be best suited to open recirculating water systems, durable produce such as carrots may be best suited for closed recirculating water systems and produce with gas pockets such as peppers may be best suited to recirculating water systems employing showers/waterfalls (López-Gálvez et al., 2021). A final rinse of untreated potable water may be included depending on the antimicrobial agent used and its concentration (Gombas et al., 2017).

Recirculated wash water systems pose a higher risk of microbial transfer without controls for prevention of cross-contamination as process water can accumulate organic matter and microorganisms during reuse (Gil & Selma, 2006). Contaminated produce can release microorganisms into the recirculated wash water which can then become adhered to non-contaminated produce that is washed in the same water (Gombas et al., 2017). The main risk with these systems is cross-contamination of human pathogens via the wash water, which is affected by insufficient sanitizer activity (Gil et al., 2009). Transfer of pathogens in wash water can theoretically occur very quickly with direct leaf-to-leaf contact, but proper use of chemical sanitizers prevents water microbial buildup (Murray et al., 2017). Fresh-cut processing is

particularly susceptible to microbial transfer as process water can diffuse into the cut vegetables (Raffo & Paoletti, 2022).

The limitations of removing microbial contamination are well established, thus the microbial quality of raw produce entering a processing facility is of great importance (Gil et al., 2009). Produce surface texture also plays an important role in pathogen removal (Singh et al., 2018). Microorganisms can adhere strongly on the surface of the vegetable and become embedded into inaccessible parts of irregular surfaces (Ölmez & Kretzschmar, 2009; Reina et al., 2002). Such microorganisms are not expected to be inactivated or removed by process wash water; therefore, the food safety focus relies on prevention of cross-contamination rather than sanitation of produce surfaces (Gil et al., 2009).

Microbial attachment to produce is distinct from microbial adhesion as some microorganisms can use filaments and biofilms to attach to produce. Enterohemorrhagic *E. coli* can exploit filaments used for gut attachment for attachment to vegetable leaves. Such attached microorganisms are not transferred to other leaves during washing or processing (Shaw et al., 2008; Smolinski et al., 2018). The formation of biofilms also contributes to microbial attachment to leafy greens and resistance to removal via washing. STEC, *Salmonella enterica*, and *Bacillus cereus* can form biofilms on leafy greens (Elhariry, 2011; Hussain et al., 2019; Jahid et al., 2015; Litt et al., 2020). Perhaps most of the leafy green microbiota is aggregated with biofilm and protected from sanitizers, which may be why PAA in wash water does not have an apparent effect on the levels of total heterotrophic bacteria in lettuce (J. L. Banach et al., 2020; Gil et al., 2009).

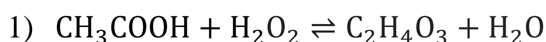
Microbial internalization into leafy green vegetables makes the microorganisms inaccessible during washing. Microbial internalization into leafy green vegetables can occur

during growth (Hou et al., 2013; Warriner et al., 2003). Infiltration is internalization caused by processes such as hydrocooling, vacuum cooling, washing, and processing (Ansah, Amodio, & Colelli, 2018; Li et al., 2008; Vonasek & Nitin, 2016). Microbial infiltration is thought to occur mostly via hydrocooling or via washing with a produce temperature that is substantially warmer than the wash water (Ansah, Amodio, De Chiara, et al., 2018, p. 201; Murray et al., 2017). Infiltration caused by a temperature differential may be due to the cooling of gases inside structures near the produce surface concomitant with a decrease in pressure inside the structure; this creates a negative pressure differential that pulls water with suspended microorganisms below the surface (Gómez-López et al., 2013; Warning et al., 2016). Microorganisms that have been internalized or infiltrated prior to fresh-cut processing (e.g. during field production or hydrocooling) are likely unaffected by the wash water and therefore have a minimal effect on water quality.

Chemical sanitizers (antimicrobial agent/pesticide) can be used in recirculated wash water to prevent pathogen cross-contamination, which can occur when insufficient concentrations of sanitizer are used (Gombas et al., 2017). The microbial load of washed produce after storage is similar when washed with tap water or sanitizing solution, supporting cross-contamination prevention as the sanitizer’s primary function. It has even been suggested that antimicrobial solutions allow rapid microbial growth that exceeds initial population during extended storage, which may be explained by a reduction in microbial competition against antimicrobial-resistant populations that can then grow uninhibited (Gil et al., 2009).

1.3 Peracetic acid in recirculated wash systems

Peracetic acid (PAA), also known as peroxyacetic acid, is stored and used as a mixture of PAA, acetic acid (AA), and hydrogen peroxide (HP). PAA is an organic acid formed from the combination of AA and HP as shown in equation 1 (Warriner & Namvar, 2014). Peracetic acid is produced via a reaction of AA and HP:



1) $CH_3COOH + H_2O_2 \rightleftharpoons C_2H_4O_3 + H_2O$

Peracetic acid inactivates bacteria by oxidizing a variety of membrane and intracellular organic molecules. Peracetic acid's peroxide constituent oxidizes disulfide and sulfhydryl bonds in cell wall proteins. Oxidized membrane proteins consequently lose structural integrity, disrupting the cell wall and affecting cell membrane permeability. Peracetic acid, a small molecule, can then enter the cell. The formation of the hydroxyl radical may be catalyzed by transition metal centers naturally present in the cell, and the radical species oxidize intracellular enzymes, membrane proteins, and DNA, consequently compromising metabolic pathways and cellular function (J. Banach et al., 2015; Gombas et al., 2017; Singh et al., 2018).

Peracetic acid has slower inactivation kinetics against *E. coli* compared to chlorine-based sanitizers, and a higher residual PAA concentration is necessary to achieve rapid disinfection of process wash water (Van Haute et al., 2015). However, free chlorine is rapidly depleted by organic load and requires substantial use of the sanitizer to maintain desired residual concentration (10 mg/L free chlorine), potentially leading to equipment degradation and carcinogenic by-products (Gil et al., 2015; Luo et al., 2018). Haloacetic acids (HAAs) and trihalomethanes (THMs) can exceed US-EPA standards in a dynamic (involving changes in water quality) chlorinated wash water system (Shen et al., 2016). Furthermore, chlorine is banned for vegetable washing in Germany and Denmark (Botondi et al., 2016). A recent study

found that sodium hypochlorite wash (20 mg/L) increased the respiration rate of fresh-cut iceberg lettuce and caused more tissue browning than a PAA wash (80 mg/L) which did not significantly affect lettuce respiration rate (Pahariya et al., 2022). Indeed, peracetic acid seems to be a superior substitute for free chlorine.

E. coli has been selected for multiple PAA efficacy studies due to its prominence in outbreaks. Peracetic acid at 30 mg/L in a laboratory-scale wash of lettuce inoculated with vegetative *E. coli* O157:H7 to 5.6 log CFU per piece, with 10% organic load, resulted in 0.07 log CFU per uninoculated lettuce piece while PAA at 20 mg/L or less resulted in 0.68 log CFU per uninoculated lettuce piece (G. Zhang et al., 2009). Peracetic acid at 85 mg/L in a laboratory-scale simulated wash of lettuce inoculated with vegetative *E. coli* O157 almost entirely prevented cross-contamination except for one uninoculated lettuce which was suggested to have direct contact with the inoculated lettuce (Singh et al., 2018). Peracetic acid at 75 mg/L in an industrial-scale simulated wash of lettuce inoculated with vegetative *E. coli* 12-123.2 effectively sanitized wash water and aided in preventing cross-contamination (J. L. Banach et al., 2020). These studies suggest that *E. coli* cross-contamination can be controlled but not completely prevented by PAA when residual concentration is at least 30 mg/L in wash water.

Bacterial spores, fungi, and viruses are typically less of a concern compared to vegetative bacteria during fresh-cut processing. Peracetic acid at 500 mg/L takes one hour at room temperature to cause ~1-2 log CFU/mL reduction in *Bacillus subtilis* spores, thus PAA at concentrations relevant to produce washing are not expected to affect bacteria spores (Leggett et al., 2016). Peracetic acid at 10 mg/L can decrease viability of *Aspergillus* fungal spores; however, research on peracetic acid efficacy for fungal spores is performed in the context of wastewater treatment rather than fresh-cut processing (Zuo et al., 2022). Fruit and vegetable

surface-attached viruses are more difficult to inactivate than suspended viruses, and the efficacy of PAA varies significantly by virus. Peracetic acid can permeate through some virus capsids and the permeability of virus capsids varies. However, both rotavirus and Tulane virus can be compromised by PAA via capsid oxidation, binding damage, and genome damage (Fuzawa et al., 2020). Although PAA has some degree of antimicrobial activity against other microorganisms, its primary function is to control cross-contamination of human pathogenic bacteria.

PAA concentration in process wash water is recommended at 30-50 ppm but is typically used at 5-30 ppm because of cost (Barrera et al., 2012). Evidence suggests PAA does not negatively affect produce taste characteristics (Alvaro et al., 2009; Nicolau-Lapeña et al., 2019). However, PAA at 80 ppm was detected by panelists with post-wash produce (J. Banach et al., 2015). Experiments have been conducted with PAA concentration of 60-80 ppm, thus poorly representing realistic residual concentrations in industrial operations (Baert et al., 2009; J. L. Banach et al., 2020; López-Gálvez et al., 2009). For example, the average wash water PAA concentration at minimally processed spinach operation was observed to be 6.2 ppm where 57% of water samples tested positive for *E. coli* (Barrera et al., 2012). Peracetic acid sanitizers may be a substantial expense for fresh-cut operations, thus low residual concentrations of PAA in process wash water seem to be more realistic than excessively high concentrations.

The control of cross-contamination in a wash process utilizing PAA is primarily based on monitoring of sanitizer residual concentration (Davidson et al., 2017; Van Haute et al., 2015). PAA residual concentration is normally quantified in ppm or mg/L, which are equivalent. The two part iodometric titration method first uses ceric sulfate as a titrant for the hydrogen peroxide present and second uses potassium iodide solution followed by sodium thiosulfate as a titrant for

liberated iodine (Greenspan & MacKellar, 1948). The two part iodometric titration method in simulated wash water experiments with high organic loads (via the addition of blended spinach) and 0 ppm sanitizer added resulted in high PAA measurements, suggesting an interference from the organic load (Ghostlaw et al., 2020). This two-part iodometric titration method was not designed for such applications with abundant biological materials. It may be the use of sodium thiosulfate as a titrant that is sensitive to the organic load. Commercially available PAA test kits using a dropper method are iodometric titration methods with proprietary titrants that may be less sensitive to organic loads and more accurate for PAA measurement during fresh-cut processing. Test strips for measuring PAA concentration can be subjective and inaccurate; however, they have been used in conjunction with an automatic strip reader for quantitative results (Petri et al., 2021). The N,N-diethyl-p-phenylenediamine (DPD) method is a highly accurate colorimetric method for determining PAA residual concentration (Van Haute et al., 2015). Potassium iodide is added to samples and PAA oxidizes the iodide to iodine, which subsequently oxidizes the DPD to form a pinkish color that can be quantified at a 530 and 550 nm wavelengths (Domínguez-Henao et al., 2018). Chronoamperometric sensors were found to have good selectivity for PAA with minimal interference but are more expensive than titration or colorimetric methods (Albolafio et al., 2021). Other titration methods and sensors for PAA measurements are less feasible for fresh-cut processing due to interferences.

Residual sanitizer in wash water samples can be neutralized prior to microbial analyses. Sodium thiosulfate can first be used to reduce all PAA, then catalase can be added to reduce residual HP. Catalase is added after PAA is reduced because PAA can react with catalase (Van Haute et al., 2015). Sodium thiosulfate without catalase has also been used (Baert et al., 2009). Difco Neutralizing Buffer has been used (Davidson et al., 2017). A solution of peptone, mean

extract, lecithin, NaCl, and sodium thiosulfate has been used (Petri et al., 2021). Maximum recovery diluent has been used (Singh et al., 2018). Some studies did not use a neutralizer prior to microbial analyses (J. L. Banach et al., 2017, 2020; Barrera et al., 2012; Ghostlaw et al., 2020).

1.4 Regulatory considerations for PAA in fresh produce wash systems

Regulations of fresh produce are driven by their natural state, as well as the degree of value-added processing. Raw agricultural commodities (RAC) include produce in their raw or natural state, whether they have been stripped of their outer leaves, waxed, or prepared into fresh salads, for example. RACs also include fresh fruits, grains, nuts, eggs, raw milk, meats, and similar agricultural produce (40 CFR 180.1). A processing facility is an operation in which fresh produce is chopped, sliced, cut, peeled, or subjected to another form of processing. Operations are not considered processing facilities if only RACs are handled, and the use of antimicrobial pesticides for washing does not change the RAC status. The regulation of antimicrobial pesticides in water that contacts vegetables depends on location (farm or facility) and status of food (RAC or processed) (Gombas et al., 2017). Farms and handling facilities, such as packing houses, are subject to 21 CFR 112, Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption. Within this regulation for harvest and postharvest water, farms must use potable water that does not contain *E. coli* in a 100 mL water sample and must maintain the quality of water throughout its use. Activities such as washing with recirculated water may compromise the water's microbial quality as vegetables are introduced; therefore, antimicrobial pesticides are a method to treat the water and maintain adequate microbial quality. Processing facilities are subject to 21 CFR 117, Current Good Manufacturing Practice, Hazard

Analysis, and Risk-Based Preventive Controls for Human Food. The addition of sanitizers in produce processing water is often considered a process preventive control and must be validated and routinely monitored to assure its effective use according to the Environmental Protection Agency (EPA)-approved label.

The EPA registers and regulates antimicrobial pesticides to protect public health and the environment under the statutory authority of the Federal Insecticide, Fungicide, and Rodenticide Act. As the pests are invisible disease-causing microbes, the EPA requires special testing of antimicrobial pesticides and provides guidelines to label such pesticides accordingly. The EPA considers PAA an antimicrobial pesticide, and it has been registered to provide a 3-log reduction of pathogenic *E. coli*, *Salmonella* spp., and *L. monocytogenes* in wash water when used at 30 to 80 ppm with a dwell time of 90 seconds (Gombas et al., 2017). For food processing facilities, the Food and Drug Administration (FDA) regulates PAA differently than the EPA because it considers PAA a secondary food additive when used in fresh produce washing (21 CFR 173.315). The following substances may be used on fruits and vegetables that are not RACs: hydrogen peroxide, not to exceed 59 ppm in wash water; PAA, not to exceed 80 ppm in wash water; 1-Hydroxyethylidene-1,1-diphosphonic acid (etidronic acid) may be used only with PAA, not to exceed 4.8 ppm in wash water (21 CFR 173.315).

As part of 21 CFR 117, process preventive controls must be validated. Validation means obtaining and evaluating scientific and technical evidence that a control measure, combination of control measures, or the food safety plan, when properly implemented, is capable of effectively controlling identified hazards (21 CFR 117.3). Validation is required to ensure important hazards such as microbial cross-contamination are controlled by processing facilities. Methods for validation described by Gombas et al. include 1) the use of a microbial surrogate to demonstrate

prevention of cross-contamination, 2) the use of sensors and a demonstration that antimicrobial levels are maintained under worst-case conditions, and 3) the placement of sensors in processing equipment to demonstrate antimicrobial levels are maintained at all locations under all conditions (Gombas et al., 2017). Major challenges of validation include understanding worst-case conditions of a fresh-cut operation and providing scientific proof that control measures are effective.

1.5 Variables impacting the efficacy of PAA

The efficacy of PAA for inactivating microorganisms and controlling cross-contamination in process wash water can be impacted by multiple variables. Variables that are known to substantially affect peracetic acid efficacy include, but are not limited to, organic load, water temperature, and the temperature differential between process water and produce. In some cases, variables are referred to as parameters; however, this is technically incorrect as the term parameter generally refers to a quantity/quality that remains constant during the run of a model (e.g. wash tank size/material) (Altman & Bland, 1999).

Organic and inorganic loads can be important variables that directly impact the quality of the wash water. Organic load is a metric for total organic material present in wash water, including plant material and soil. Microbial load contributes to organic load. An increased organic load diminishes residual PAA concentration as PAA oxidizes both organic load and microorganisms present (Ghostlaw et al., 2020). PAA is more resistant to organic load than chlorine-based sanitizers (J. L. Banach et al., 2020; Barrera et al., 2012; G. Zhang et al., 2009). Process wash water with 50 ppm residual PAA was rarely impacted by organic loads simulated with blended lettuce, indicating that PAA has generally consistent efficacy when residual

concentration is maintained (Davidson et al., 2017). The level of sanitizer sensitivity is dependent on the chemical nature of the load. For example, PAA is most sensitive to antioxidants such as vitamins A & E and sulfur-rich produce such as onions (Gombas et al., 2017). It seems that organic load is more of a concern for quenching PAA and decreasing residual concentration than impacting PAA's efficacy of inactivating microorganisms when it is held at desired concentrations.

Chemical oxygen demand (COD) is the amount of oxygen required to oxidize organic matter present in water. Chemical oxygen demand corresponds with organic load and has been used as a metric for organic load in simulated wash water processes (Davidson et al., 2017; Ghostlaw et al., 2020; Van Haute et al., 2015). COD has been quantified using Hach Reactor Digestion Method 8000 (Davidson et al., 2017; Ghostlaw et al., 2020). PAA and byproduct acetic acid contribute to COD because they can be oxidized (J. L. Banach et al., 2020; López-Gálvez et al., 2009). Reported increases of COD are between 1.9 and 4 mg O_2/L per 1 mg/L of PAA dosed; however, decreased COD has also been observed due to oxidation of organic material (Luukkonen & Pehkonen, 2017). PAA may be an ideal sanitizer for the disinfection of *E. coli* 0157 in water with high COD when low sanitizer refreshing rate (dosing rate) is applied (Van Haute et al., 2015). However, 750 mg/L COD interferes with sanitizer oxidizing capacity and antimicrobial performance (Ghostlaw et al., 2020). Therefore, high COD may be an indicator for when some degree of water replenishment is necessary instead of increasing PAA dosing rate.

Turbidity is a measure of the light absorbed through a sample of water. It can be measured with a turbidimeter or spectrophotometer (Barrera et al., 2012; Van Haute et al., 2015). Water samples may be passed through a filter to remove suspended solids prior to turbidity

measurement (Davidson et al., 2017). Turbidity was not measured in multiple past studies involving PAA efficacy in process wash water (J. L. Banach et al., 2020; Ghostlaw et al., 2020).

Microbial load impacts efficacy of PAA by contributing to organic load and quenching residual PAA. The abundance of enzymes, cations, and other large organic molecules is expected to react strongly with PAA, especially if cell membranes are compromised and PAA enters the cell. The viable but nonculturable (VBNC) state of human pathogens such as *L. monocytogenes* and *E. coli* O157:H7 has been associated with antimicrobial agents used in produce washing. In the VBNC state, pathogens become unculturable using conventional cultural methods; however, they may grow in a suitable environment (Arvaniti et al., 2021). There is evidence to suggest that the efficacy of antimicrobial agents such as PAA have been overestimated due to the use of cultural methods and that the efficacy of antimicrobial agents in conjunction with optimal washing practices may need revision (Truchado et al., 2021). A study by Gu et al. suggested that the use of PAA as a sanitizer in produce rinse water (0.5-100 mg/L) can result in the substantial presence of potentially VBNC human pathogen populations (STEC, *Salmonella*, and *L. monocytogenes*). Methods for VBNC quantification include 1) fluorescence microscopy and carboxyfluorescein diacetate /propidium iodide double staining and 2) propidium monoazide and quantitative real-time polymerase chain reaction (PMA-qPCR) (Arvaniti et al., 2021; Gu et al., 2020).

pH does not typically affect PAA efficacy in produce wash systems. PAA has an acid dissociation constant (pKa) of 8.2, thus it is effective at a pH range of 0 to 7.5 (Gombas et al., 2017). Acetic acid has a pKa much lower than PAA (4.8 vs 8.2), thus pH is expected to drop as PAA reacts and residual acetic acid increases. pH has been observed to decrease as organic load

increases, which may be due to the additional sanitizer added at higher organic loads to maintain the targeted residual sanitizer concentration (Davidson et al., 2017).

Temperature affects both microbial adhesion to produce and PAA reactivity, thus it is an important consideration for control of cross-contamination during fresh-cut processing. The process wash water temperatures of previous studies were 4-19 °C (J. L. Banach et al., 2020; Barrera et al., 2012; Davidson et al., 2013, 2017; Ghostlaw et al., 2020; Van Haute et al., 2015). One of three facilities observed by Barrera et al. had water temperatures of 0.2-2.1 °C (Barrera et al., 2012). Bacterial adhesion to produce is less extensive at lower wash temperatures (J. L. Banach et al., 2017, 2020; Reina et al., 2002). However, in the presence of PAA, higher water temperatures cause a greater decrease in *E. coli* concentration over time (Davidson et al., 2013; Ghostlaw et al., 2020). Peracetic acid concentration is more rapidly quenched at higher temperatures by the organic and microbial loads (Ghostlaw et al., 2020). This explains the greater decrease in *E. coli* concentration over time but is concomitant with a greater decrease in residual concentration and an increased demand for dosing to maintain desired concentration. Evidence suggests that higher produce temperatures decreases the efficacy of the wash process, which may be explained by increased microbial adhesion and/or infiltration (Barrera et al., 2012). The relationship between water temperature and control of cross-contamination using PAA is complex; however, monitoring produce and water temperatures may aid in better understanding water quality trends in fresh-cut operations.

Oxidation-reduction potential (ORP) is the intensity of oxidizing or reducing conditions and expresses the concentrations of mobile electrons in a medium. ORP is commonly used to monitor hypochlorite and hypochlorous acid concentrations in a medium. At a neutral pH, PAA has a standard redox potential of 1.385 V, greater than many disinfectants. At pH 0 and 14, PAA

has a standard redox potential of 1.748 and 1.005, respectively (C. Zhang et al., 2018). ORP is generally a variable to assess microbial kill-rate such as with free chlorine; however, ORP may not be a reliable metric for PAA efficacy in wash water because PAA undergoes a two-step reaction, thus ORP does not directly represent PAA residual concentration. Therefore, it seems ORP cannot be used to monitor antimicrobial efficacy in a wash system employing PAA.

Decomposition of PAA is affected by concentration, temperature, and pH. Decomposition generally occurs via one of the following mechanisms: 1) Two PAA molecules spontaneously decompose into two AA molecules and oxygen, 2) hydrolysis into AA and HP, or 3) decomposition via transition metal ions through a radical chain mechanism, breaking it down into AA, oxygen, and other products (Gombas et al., 2017; Yuan et al., 1997). Hydrogen peroxide reacts with Selenoproteins and certain thiol proteins; HP reacts slowly with low-weight thiol and most cysteine residues (Winterbourn, 2013). Although PAA can react with other molecules, it reacts at a slower rate than aqueous chlorine with the proteins, phenolic compounds, organic acids, and sugars leached from produce during washing or processing (T. Zhang et al., 2022).

Other variables that may also affect PAA quenching and/or antimicrobial activity of water include, but are not limited to, water mineral hardness, soluble and insoluble solids, product to water ratio, filtration, microbial attachment or adhesion to produce, and rate of water replenishment (J. L. Banach et al., 2020; Gombas et al., 2017).

Part 2. Low salt cucumber fermentation

2.1 Fundamentals of cucumber fermentation

Vegetables can be submerged in cover brines, generally with electrolytes and acetic acid, to facilitate desirable fermentations by bacteria that vastly extend shelf life and reduce pH to an inhibitory level for human pathogens and vegetable spoilage organisms. Desirable fermentations are the results of controlled imbalance of microbial populations. Cucumber sizes include 1A (≤19 mm diameter), 2A (25-32 mm), 2B (32-38 mm), 3A (38-44mm) and 3B (45-51mm) (Lu et al., 2002; Pérez-Díaz et al., 2015). Larger cucumbers (size 3) tend to have a mildly acidic juice (pH 5.8-5.9) with less malic acid (8.7 mM) and more carbohydrates (35.3 mM glucose, 38.2 mM fructose) compared to smaller cucumbers (size 1; pH 6.2-6.3, 11.6 mM malic acid, 24.4 mM glucose, 29.0 mM fructose) (Lu et al., 2002).

Organic acids normally contain one or more carboxylic acid groups, which can be protonated or unprotonated depending on the pH (Ottosson et al., 2011; Schmidt et al., 2021). The proportion of an organic acid that is protonated varies between each organic acid and can be calculated using the Henderson-Hasselbalch equation (Lucy, 2023). Protonated organic acids preserve fermented cucumbers via bacteriostatic activity (Carpenter & Broadbent, 2009). Protonated (undissociated) organic acids can diffuse through microbial membranes and enter cells while unprotonated counterparts are not membrane soluble. Once the acid enters the cell, it undissociated due to the neutral pH of the cytoplasm and the proton acidifies a different molecule. The quality of proteins decreases at low intracellular pH due to altered function, and eventually the metabolism of the cell cannot persist due to the energy demands of the acid stress (Cohen et al., 2015). Understanding how microorganisms respond to acid pH is central to their control and successful exploitation (Lund et al., 2020).

Temperature is an important factor in many fermentation industries (Deed et al., 2017; Feng et al., 2024; Karlsson, 2008). The ideal temperature for the growth of lactic acid bacteria (LAB) varies by species; furthermore, the optimal temperature for growth may differ from the optimal temperature for lactic acid production. The optimal temperate range for different strains of *Lactococcus lactis* was observed to be 27-33 °C (Novak et al., 1997). *Lc. lactis* 1387 has maximum growth rate at 32 °C and maximum lactic acid production at 28 °C. *Lactobacillus bulgaricus* Lb12 and *Lactobacillus acidophilus* La5 have maximum growth rates and lactic acid production at 41-42 °C (Adamberg, 2003). 30 °C may be the ideal temperature for cucumber fermentation assisted by *Lc. lactis*. Understanding how temperature affects the growth of populations relevant to cucumber fermentation may lead to a rational method for selecting biocontrol of starter cultures (Dougherty et al., 2002).

2.2 Microbial populations

Cells need to generate energy for energy-requiring processes. This energy is available in phosphate bond intermediates and electrochemical energy stored in ion gradients such as protons (Endo & Dicks, 2014). Fresh vegetables contain diverse microbiota often harboring *Pseudomonadaceae*, *Enterobacteriaceae*, and LAB (Rothwell et al., 2022). Many microorganisms belonging to the bacterial genera *Enterobacter*, *Enterococcus*, *Pantoea*, *Weissella*, *Leuconostoc*, *Lactiplantibacillus, Levilactobacillus*, and *Pediococcus* are naturally present in cucumber fermentations (Zhai & Pérez-Díaz, 2020). Some LAB such as *Lb. plantarum* achieve dominance due to their genetic content (Makarova et al., 2006; Pérez-Díaz et al., 2020). *Lactiplantibacillus pentosus* and *Lactiplantibacillus plantarum*, which are closely related, are capable of complete fermentation due to their high acid tolerance (*Lb. plantarum* can grow to pH

3.3 and produce acid to pH 3.1) (Anekella & Pérez-Díaz, 2020; McDonald et al., 1990; Pérez-Díaz et al., 2017).

Lc. lactis is a strictly homolactic fermentative bacteria which completely converts pyruvate into L-lactic acid through an efficient lactate dehydrogenase enzyme (Hols et al., 1999). *Lc. lactis* has shown mitigated transcriptomic responses for the main mechanisms shown to confer acid resistance: 1) the proton-translocating enzyme involved in the expulsion of protons from the cell cytoplasm, 2) the arginine deiminase, and 3) the citrate decarboxylation pathways (Cretenet et al., 2011). ATP yield is lower at pH 4.4 than at pH 6.6 due to energy drain from acidic conditions (Dougherty et al., 2002). *Lc. lactis* has shown to produce methyl butyric acid during carbohydrate starvation (Ganesan et al., 2006). *Lactococcus* spp. is typically grown on M17 which contains disodium-β-glycerophosphate, an inhibitor of *Lactobacillus* growth. Reports of broad spectrum, bacteriocin-producing LAB indicate that antimicrobial proteins play a role in the ecology of fermented foods. Nisin, a broad-spectrum bacteriocin with stability at low pH, is produced by some strains of *Lc. lactis* subsp. lactis (Nip+) (Harris et al., 1992).

Leuconostoc spp. are non-acidophilic and prefer to grow at pH 6.5, although their growth may proceed at pH 4.5. These species are also sensitive to high salt concentrations, so their growth and production of carbon dioxide (CO_2) is of importance in low salt fermentation (Zhai & Pérez-Díaz, 2020). *Ln. mesenteroides* metabolism stops at an intracellular pH of 5.4-5.7 (McDonald et al., 1990). *Leuconostocaceae* produce lactic acid, acetic acid, ethanol, and some succinic acid; however, they produce no more than 28 mM acetic acid and 25 mM ethanol in cucumber juice medium, suggesting these compounds minimally contribute to fermentation (Zhai & Pérez-Díaz, 2020). *Leuconostoc* spp. likely can ferment glucose and fructose naturally present in cucumbers to a mixture of formate, acetate, lactate, and succinate, anaerobically, by

converting the central intermediate phosphoenolpyruvate into oxaloacetate. Because acidic succinic acid production is a CO_2 fixing pathway, the production of such acid would reduce the CO_2 concentrations contributed by *Leuconostocaceae* (Zhai & Pérez-Díaz, 2020). Succinate is produced via the reductive tricarboxylic acid cycle, which is affected by pH, temperature, the concentration of H_2, CO_2, carbon and nitrogen sources, and metal ions available in the growth medium (Agarwal et al., 2007; Andriani et al., 2019). In addition to classical heterofermentation, *Leuconostoc* can use a variety of reactions including the fermentation of malate, pyruvate, and citrate and the production of erythritol and glycerol (Zaunmüller et al., 2006). *Leuconostoc lactis* and *Lc. lactis* are capable of diacetyl production (Hugenholtz & Starrenburg, 1992; Jordan et al., 1996).

Pantoea, *Citrobacter*, *Serratia*, *Providencia*, *Leclercia*, *Enterobacter*, and *Kluyvera* are present in early (day 1-3) cucumber fermentation. *Pantoea* spp. is in fresh cucumbers in a relative abundance of 9.25%. Organic acids such as lactic acid are hurdles for the metabolism of proteobacteria. Potassium sorbate and acetic acid in cucumber juice medium negatively affected the ability of ɣ-proteobacteria to proliferate, so it is assumed that sorbic and acetic acid negatively affect enteric growth in natural cucumber fermentation (Rothwell et al., 2022).

2.3 Primary fermentation

During equilibration, after cucumbers are initially submerged in a cover brine, the indigenous microbiota are likely diluted and community structures disrupted (Rothwell et al., 2022). The little to no residual sugar remaining at the end of a fermentation indicates completeness. Fermentation substrates and products (malic acid, glucose, fructose, lactic acid, acetic acid, ethanol, and others) have been quantified using two liquid chromatographic

procedures (McFeeters, Thompson, et al., 1984). A novel cover brine reformulation employs 342 mM NaCl, 18 mM $CaCl_2$, 18 mM calcium hydroxide, and concentrated acetic acid (20%, to adjust starting pH) to form a potent pH-buffered low NaCl cover brine that facilitates primary fermentation (Zhai & Pérez-Díaz, 2017). Calcium chloride ($CaCl_2$) added to the fermentation cover brine may maintain the crisp texture of the fermented cucumbers and inhibit the action of softening enzymes (Buescher et al., 2011; Jen, 1989; McFeeters & Fleming, 1990). Differences in bacterial populations of cucumber fermentations brined with no salt, 99 mM $CaCl_2$, and 1.03 M NaCl suggest low salt cucumber fermentations result in enhanced microbial diversity (Pérez-Díaz et al., 2020).

Bloater defect is an undesirable quality of fermented cucumbers in which cucumber endocarps become damaged and/or softened by gas production during primary fermentation. In 1939, CO_2 was identified as the main contributor to bloater defect by Veldhuis and Etchells. In 1973, Fleming demonstrated that bloater defect is caused by the accumulation of CO_2. CO_2 is less dissolvable in acidic solutions, so rapid acidification of fermented cucumbers is an important aspect of preventing bloater defect (Zhai & Pérez-Díaz, 2020). Malic acid can be significant during cucumber fermentation as it can be decarboxylated by some populations to form carbon CO_2 (Zhai et al., 2018). Microbes associated with bloater defect include 1) *Leuconostocaceae* and 2) *Enterobacter* spp. in combination with yeasts and facultative heterofermentors such as *Lb. plantarum* and *Lb. pentosus* able to decarboxylate malic acid (McFeeters et al., 1982; McFeeters, Fleming, et al., 1984; Zhai et al., 2023; Zhai & Pérez-Díaz, 2020). In low salt cucumber fermentations, *Leuconostocaceae* and *Enterobacteriaceae* were identified as causative agents for bloater defects (Zhai & Pérez-Díaz, 2021). *Enterobacteriaceae* can induce CO_2-mediated bloater defect by its uncontrolled growth (Zhai et al., 2023). The use of malic acid decarboxylase-

deficient starter culture in NaCl-free cucumber fermentations was evaluated as a strategy to reduce bloater incidence. However, NaCl-free fermentations may be less stable than 342-513 mM NaCl fermentations (Zhai et al., 2018).

2.4 Secondary fermentation and spoilage

Characteristics of spoilage-associated secondary cucumber fermentation include lactic acid degradation (often by yeast), decline in redox potential, increase in pH, and production of malodorous butyric and propionic acids (Franco et al., 2012). During secondary fermentation, spoilage yeasts can consume lactic acid and increase pH while *E. cloacae* can consume lactic acid to produce acetic and propionic acid (Franco & Pérez-Díaz, 2013). Catabolite repression is a phenomenon relevant to secondary fermentation because it is the catabolites of primary fermentation that repress further metabolic activity. The ability of some LAB such as *L. buchneri* to catabolize lactic acid may depend on their ability to metabolize residual carbohydrates after primary fermentation (Johanningsmeier & McFeeters, 2015). Further understanding of metabolic activity associated with secondary fermentation could lead to improved technologies that prevent lactic acid degradation during bulk storage and identification of metabolites, or indicator compounds, to be monitored for the initiation of spoilage.

Yeast is responsible for fermentation in other industries but is generally undesirable in cucumber fermentation. Acid-tolerant yeast cell walls with chitin limit diffusional entry of undissociated acid (Piper et al., 2001). *Pichia kudriavzevii* has demonstrated co-aggregation with bacteria. *Pichia kudriavzevii* NG7 is able to grow in extremely acidic and hot environments (Chelliah et al., 2016; Park et al., 2018). The ability of acid-tolerant yeast to metabolize the

organic acids that preserve fermented cucumbers means control of their growth and activity is relevant to novel cucumber fermentation methods.

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CHAPTER 2: Wash water physicochemical variables and microbial load for fresh-cut leafy greens

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Abstract

Peracetic acid (PAA) is used as a sanitizer for preventing pathogen cross-contamination in fresh-cut leafy greens wash water systems. While various factors, such as pH, temperature, and organic load, may affect the efficacy of PAA in wash water systems, PAA residual concentration is thought to be the primary metric for prevention of cross-contamination. The purpose of the study was to explore the metrics of a recirculated wash water system using PAA in a fresh-cut leafy greens facility based on relationships between physicochemical variables and microbial loads. The operation sequentially washed produce in two wash tanks continuously dosed with PAA prior to cutting and packaging. Wash water physicochemical variables were measured directly in the tanks (temperature, pH, oxidation-reduction potential) or with water samples (PAA residual concentration, turbidity, chemical oxygen demand). Microbial loads (aerobic plate count, total coliforms, *E. coli*) were estimated for water samples (100 mL, N=63), and pre-wash and post-wash leafy greens (25 g, n=54). Average PAA concentrations were 19.5 ± 28.3 ppm in tank 1 and 22.9 ± 16.7 ppm in tank 2. *E. coli* levels were greater than 0.3 log CFU/100mL in tank 1 when the PAA concentration was below 2 ppm. There was no significant difference between pre-wash and post-wash leafy green microbial loads for all indicator organisms. Results suggested dosing amounts may be optimized to maintain adequate PAA concentrations based on water temperature and expected microbial loads. Findings underscore the significance of monitoring process wash water as a potential cross-contamination point in fresh-cut processing.

1. Introduction

Fresh produce has been associated with foodborne outbreaks of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. (Herman et al., 2015; Mercanoglu Taban & Halkman, 2011; Scallan et al., 2011; Self et al., 2019; Singh et al., 2018; Yang & Scharff, 2024). Shiga toxin-producing *Escherichia coli* (STEC) linked to leafy greens, which are the second most common source of foodborne STEC O:157 outbreaks, caused 40 outbreaks between 2009 and 2018 in the United States and Canada (Marshall et al., 2020). As fresh produce is commonly consumed raw, there is a lack of a practical lethality step that removes pathogens without degrading the overall quality. Fresh-cut produce is particularly susceptible to contamination during processing, as microbial transfer from wash water systems can spread contamination (Raffo & Paoletti, 2022).

The industry often utilizes a wash water step to remove soil, pesticide residues, plant exudates, and some microorganisms on produce surfaces (Gil et al., 2009). However, microbial contamination of pathogens that may occur during fresh produce preharvest and harvesting operations can be cross-contaminated via washing and further processing activities (Kintz et al., 2019; Smolinski et al., 2018). Various wash systems, such as recirculating water systems with open flume tanks, are commercially employed based on the type of produce (Bornhorst et al., 2018; López-Gálvez et al., 2021; Luo et al., 2014). While wash systems with recirculating water might utilize less water than a single-pass system, the reused water poses an increased risk for cross-contamination if not properly monitored or maintained. Recirculated wash water that becomes contaminated with a pathogen and does not have sufficient sanitizer can contaminate washed produce and further spread contamination (Gil et al., 2009).

In fresh-cut leafy greens operations, chemical sanitizers such as peracetic acid (PAA) are added to wash water systems to prevent pathogen cross-contamination between produce during washing (Gombas et al., 2017). The use of PAA has increased recently due to concerns with chlorine-based sanitizers (Botondi et al., 2016; Shen et al., 2016; Singh et al., 2018). Furthermore, organic load in wash water affects PAA less than chlorine-based sanitizers, making PAA an attractive antimicrobial option (Gil et al., 2015; Luo et al., 2018). According to the Food and Drug Administration (FDA), PAA residual concentration should not exceed 80 ppm in process wash water (21 CFR 173.315). Despite recommendations to use PAA to at least 30 ppm residual concentration to prevent cross-contamination, it is typically used below 30 ppm due to cost (Baert et al., 2009; J. L. Banach et al., 2020; Barrera et al., 2012; Warriner & Namvar, 2014; Zhang et al., 2009). Maintaining adequate residual sanitizer concentration with a constant-flow pump can be difficult with variables such as microbial load and temperature affecting residual concentration (J. L. Banach et al., 2017; Ghostlaw et al., 2020; Petri et al., 2021).

Wash systems, particularly those utilizing recirculating water, can serve as a vehicle for widespread contamination if antimicrobial methods are not monitored and controlled, thus maintaining their quality is of utmost importance. The purpose of this study was to evaluate wash water variables (oxidation-reduction potential (ORP), turbidity, temperature, chemical oxygen demand (COD)) and microbial loads (aerobic plate count, total coliforms, *E. coli*) and their relationships with observed PAA residual concentrations during washing of kale and collard greens in a commercial operation. Leafy greens and wash water samples were collected from a fresh-cut processing facility employing a recirculating water system with two separate, open flume tanks in which the product was consecutively washed.

2. Materials and Methods

2.1 Facility and Wash System Description

The observed facility was in North Carolina. Kale or collard greens were sequentially washed in water from two separate wash systems. Each wash system consisted of a reservoir tank that was continually dosed with Perasan A (Enviro Tech, Modesto, CA) using a peristaltic pump and a wash tank in which the leafy greens were fully immersed. Water from reservoir tanks was constantly pumped into the wash tanks and recirculated. Leafy greens were washed prior to cutting, centrifugation, and packaging.

2.2 Sample collection

Produce and wash water samples were collected from four different production days. Samples were collected hourly over a seven-hour production period. During each time point, two pre-wash and two post-wash (collected after centrifugation to remove water) vegetable samples were collected and stored at 4 °C. Water samples (400 mL, n=21 from each tank per day) were collected from the reservoir tanks using a sterile dipper and Nalgene bottle and processed immediately after sample collection (Nelson Jameson, Turlock, CA). Directly after sample collection of water, 100 mL from each water sample was aliquoted into 120 mL capacity sterile bottles (IDEXX, Westbrook, ME) containing 10 mL of sodium thiosulphate solution (8 g/L) immediately followed by 1 mL of concentrated catalase solution. Solutions were briefly mixed and stored at 4 °C until analysis.

2.3 Water physicochemical analyses

Wash water temperature, pH, and ORP were measured directly in both tanks using a Multiparameter Water Meter (Hanna, Woonsocket, RI). Immediately after sample collection, PAA residual concentrations were measured using a dropper titration method at 6 ppm

sensitivity (Peracetic Acid Test Kit, LaMotte) and the DPD method at 0.1 ppm sensitivity using a DR900 colorimeter (Hach, Loveland, CO). One mL of water was added to 9 mL deionized water prior to DPD measurement to adjust PAA measurement range from 0.1-10 mg/L (ppm) to 1-100 ppm. Two 10 mL aliquots were collected from each tank followed by the addition of 20 µL sulfuric acid solution to each sample for preservation and stored at 4 °C for COD measurement within 14 days using Hach Method 8000 (Hach, Loveland, CO). Thirty mL of each water sample was stored at 4 °C for off-site turbidity measurements using DR900 within 48 h of sample collection.

2.4 Microbiological analyses

Microbiological analyses were performed within 24 h of sample collection. Water and leafy green samples were enumerated for total aerobic bacteria (Aerobic Plate Count (APC)), coliforms, and *E. coli*. Twenty-five grams of each produce sample was added to 225 mL Butterfield's diluent and stomached on high for 90 s (Stomacher® 400 Circulator, Seward, Hamilton, NJ). Serial dilutions were performed using 9 mL Butterfield's diluent blanks. Aerobic Plate Counts were estimated using plate count agar (PCA) and spiral plated onto petri dishes followed by incubation at 35 °C for 48 hours (easySpiral Dilute, Interscience, Saint Nom la Bretèche, France). Colonies were counted using a plate counter (Scan 300, Interscience, Saint Nom la Bretèche, France). Total coliforms and *E. coli* concentrations were estimated for both water and vegetable samples using Petrifilm™ as directed by manufacturer (3M, St. Paul, MN). Water samples were also analyzed using Colilert Quanti-tray 2000 as directed by manufacturer to lower water coliform and *E. coli* lower detection limits (LDL) to 0.04 log MPN/100mL (IDEXX, Westbrook, ME).

2.5 Statistical analysis

Pearson correlation coefficients, linear regression models, and comparison of means by day using Student's t-test were analyzed using JMP© Pro 17.0.0 (JMP Statistical Discovery, Cary, NC). Enumerative results below the LDL were assigned a value halfway between zero and the detection limit to avoid overrepresentation or underrepresentation of sample counts. The lower detection limit for produce *E. coli* concentration was <1 log CFU/g, samples below detection limit were analyzed as 0.7 log CFU/g. However, *E. coli* concentrations from water samples were recorded as the LDL when *E. coli* was not detected (0.04 log MPN/100mL), as Colilert provides a low LDL. Linear regressions were performed in Microsoft® Excel® for Microsoft 365. A significance level (α) of 0.05 was used for statistical testing.

3. Results

Microbial loads of produce and water varied significantly by day. Leafy green microbial loads were highest on day 1 (Table 1). Pre-wash leafy green coliforms were highest on day 1 (3.7 log CFU/g), significantly higher than the other sampling days (1.9-2.5 log CFU/g). Average microbial loads did not significantly vary between pre-wash and post-wash samples, but all leafy green microbial loads increased after washing on day 1.

The highest water total coliform concentration mean was also on day 1 (5.9 log CFU/100 mL in tank 1, 3.1 log CFU/100 mL in tank 2; Table 2). In tank 1, the average PAA residual was lowest (0.4 ppm) and the APC was significantly highest (7.8 log CFU/100 mL) on day 1 compared to other processing days. *E. coli* was observed at higher concentrations in tank 1 (max of 1.8 log CFU/100 mL) compared to tank 2 (max of 0.34 log MPN/100 mL). Water temperature

was significantly warmer in both tanks on day 1 (19.2-20.2 °C) compared to other processing days.

pH, ORP, and COD were strongly correlated with PAA residual concentration in both tanks (Table 3a). All the water microbial load measurements (APC, coliforms, and *E. coli*) had significant negative correlations with PAA residual concentration except for *E. coli* values in tank 2, which was likely due to less detection of a smaller data range. As expected, turbidity was not correlated with PAA concentration. Combined data from tank 1 and tank 2 yielded significant correlations between PAA concentration and all variables except for turbidity. APC, ORP, and COD had a significant correlation with PAA concentration on all sampling days in tank 2 (Table 3b). In tank 1, most correlation coefficients were insignificant when analyzed by day.

E. coli was not detected in wash water when PAA residual concentration was in the desired concentration (30-80 ppm) for control of cross-contamination (Figure 1). APC and coliform levels appeared to be similar when PAA residuals were between 30 and 80 ppm, suggesting 80 ppm PAA residual does not substantially decrease microbial load of wash water when compared to 30 ppm residual.

As expected, regressions of pH, ORP, or COD cannot be used to accurately predict PAA residual concentration (Figure 2). Data with high ORP (>400 mV) and low PAA residual concentration (<20 ppm) were observed in both tanks, confirming ORP cannot accurately predict PAA residual concentration. Interestingly, ORP had a strong negative correlation with pH. As expected, turbidity was significantly correlated with COD.

Although the convenient titration method was not as sensitive as the DPD method, they provided similar results (Figure 3). According to the slopes of the linear regressions comparing

the two methods (<1), the titration method slightly underestimated PAA concentrations compared to the DPD method.

4. Discussion

Temperature affects both microbial adhesion to produce and PAA reactivity, thus it is an important consideration for control of cross-contamination during fresh-cut processing. PAA has increased reactivity with the microbial and organic loads at higher temperatures, thus microbial inactivation and PAA quenching occurs more rapidly (Ghostlaw et al., 2020; Van Haute et al., 2015). To maintain desired residual concentration, greater sanitizer dosing is likely necessary in warmer washes. Peracetic acid dosing was a confounding variable that likely affected the relationship between water temperature and PAA residual concentration. The rate of PAA dosing into the wash systems was not observed during this study and the difference in dosing rates between sampling days is unknown; however, it was reported to increase with each sampling day because data was shared with the wash system operator after each sampling day. This explains the increase in average PAA residual concentration between each sampling day, especially in tank 1. Peracetic acid dosing was assumed to be lowest on sampling day 1 while the water temperature was warmest, therefore it cannot be estimated whether the low PAA concentration in tank 1 was attributable to the low dosing rate or the warmer water temperature given neither variable remained constant throughout the study.

Differences in temperature, PAA dosing, and the organic load from produce likely affected correlations between PAA concentration and other water variables by day. In tank 1, the insignificance of almost all correlations with PAA concentration on days 1-3 may be due to the increased organic load compared to tank 2 and lower PAA dosing compared to day 4. It was

assumed that PAA dosing into both tanks was highest on day 4 compared to other days, this may be why pH, ORP, COD, and turbidity were significantly correlated with PAA concentration in both tanks on day 4. The strongest physicochemical variable correlations with PAA concentration in tank 1 were on day 4, during which the average PAA concentration was highest.

In colder washes, bacteria are less adhesive to produce and more likely to transfer to the wash water (J. L. Banach et al., 2017, 2020; Reina et al., 2002). Although warmer washes with adequate PAA residual appear to have greater antimicrobial activity, colder washes with 30-80 ppm residual PAA concentration may be ideal for flume tank washing of leafy green vegetables because of 1) reduced PAA reactivity with the non-microbial organic load and 2) reduced microbial adhesion to the vegetables. However, microbial infiltration into the leaves is thought to occur when washing with a produce temperature that is substantially warmer than the wash water (Ansah et al., 2018; Murray et al., 2017). Therefore, colder washes should only be used when the pre-wash produce is colder or about the same temperature as the process wash water.

Multiple methods can be used for measuring PAA, including test strips, titration kits such as the dropper method, colorimetric methods, and in-line sensors. While the cost and labor of different methods varies significantly, titration kits and colorimetric methods are affordable and accurate. Test strips are sensitive to subjectivity and does not provide a quantitative metric for residual concentration unless an automatic reader is used (Petri et al., 2021). Therefore, titration or colorimetric methods should be used instead of test strips, or to validate test strips.

Large ranges in PAA residual concentration from both tanks provided ideal data to analyze the relationships between physicochemical variables and microbial loads of the process wash water. Tank 1 *E. coli* concentration was likely significantly higher on day 1 due to increased leafy green microbial loads and insufficient PAA dosing to maintain desired residual.

A similar study described a minimally processed spinach operation in which average PAA residual was 6.2 ppm and 57% of spent wash water samples were positive for *E. coli*, thus the control of PAA residual requires attention (Barrera et al., 2012).

For all sampling points during which *E. coli* was observed on pre-wash samples, *E. coli* was also observed on post-wash samples; this suggests *E. coli* that adhered to leafy green samples persisted throughout the wash system. The limitations of removing microbial contamination from leafy greens are well established, as bacteria can strongly adhere to vegetables within irregular surfaces (Murray et al., 2017; Ölmez & Kretzschmar, 2009; Reina et al., 2002; Warriner et al., 2003). Microbial attachment is distinct from adhesion; enterohemorrhagic *E. coli* are able to utilize filaments to strongly attach to the vegetable surface, rendering it difficult to remove via washing (Shaw et al., 2008).

PAA residual was not correlated with *E. coli* load in tank 2 because *E. coli* was only observed above the detection limit once, all other microbial loads were negatively correlated with PAA residual. Negative relationships between sanitizer residual and wash water microbial loads are supported by literature, where residual PAA oxidizes cell membranes and can irreparably compromise metabolic function (J. Banach et al., 2015; Ghostlaw et al., 2020; Singh et al., 2018; Zhang et al., 2009). Multiple studies evaluating the efficacy of PAA during simulated washing of leafy greens maintain a PAA residual concentration of 60 ppm or greater (Baert et al., 2009; J. L. Banach et al., 2020; López-Gálvez et al., 2009; Singh et al., 2018). In this study, APC and coliform loads in the wash water did not noticeably increase when PAA

residual was closer to the upper limit of 80 ppm. Therefore, 35-50 ppm residual may provide the optimal balance between water microbial loads and overall sanitizer use.

Peracetic acid and its acetic acid byproduct can be oxidized, thus contributing to higher COD levels. Reported increases in COD are between 1.9 and 4 mg O_2/L per 1 ppm of PAA dosed (J. L. Banach et al., 2020; López-Gálvez et al., 2009; Luukkonen & Pehkonen, 2017). Therefore, COD by itself cannot be used to estimate organic load that is not from PAA. Turbidity and COD had a significant positive correlation, so the measurement of turbidity may be a rapid and affordable method for estimating organic load. Peracetic acid at 50 ppm is not significantly affected by organic load; however, PAA can be sensitive to high organic loads, particularly those with PAA-sensitive proteins, antioxidants, and vitamins (Davidson et al., 2017; Ghostlaw et al., 2020). Therefore, knowledge of organic load is not imperative for the use of PAA but it may be useful for optimization of water replenishment.

The combination of kale and collard green data, which may have had significantly different pre-wash microbial loads, is a limitation of this study. Pre-wash leafy green temperatures were not measured, thus the temperature differential between produce and water is unknown. Furthermore, leafy green microbial loads between tank 1 and tank 2 are unknown. Water replenishment data was not collected in this study. Microbial loads estimates were based on cultural methods; therefore, information regarding viable but non-culturable (VBNC) bacteria was unknown (Arvaniti et al., 2021; Truchado et al., 2021). There is a concern with VBNC pathogens because cultural methods for estimating microbial loads may overestimate sanitizer efficacy if pathogens become VBNC in the presence of the sanitizer.

5. Conclusion

PAA residual concentration appears to be a reliable estimator for control of cross-contamination when using PAA based sanitizers. Optimal PAA residual concentration is 35-50 ppm. Temperature and turbidity may be useful water physicochemical variables for estimation of optimal sanitizer dosing amounts to maintain PAA residual concentration. Control of water temperature may provide benefits for maintaining desired PAA residual. Relationships between water physicochemical variables and PAA residual concentration should be observed in all wash operations.

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Table 1. Leafy green microbial loads per day (log CFU/MPN/100mL). For *E. coli*, (time points detected/total time points). Comparisons of means across sampling days were performed by row, different letters represent significantly different loads. (n=12 of each pre-wash and post-wash per day).

	Day 1	Day 2	Day 3	Day 4	Average
			APC		
Pre-wash	7.2 ± 0.9^{A}	5.5 ± 0.9^{B}	6.2 ± 0.6^{AB}	6.3 ± 0.1^{AB}	6.3 ± 1.0
Post-wash	6.7 ± 0.6^{A}	5.4 ± 0.7^{B}	6.1 ± 0.3^{A}	6.5 ± 0.1^{A}	6.1 ± 0.7
			Coliforms		
Pre-wash	3.7 ± 0.3^{A}	2.1 ± 0.8^{B}	2.4 ± 1.3^{B}	1.9 ± 0.7^{B}	2.5 ± 1.0
Post-wash	3.8 ± 0.4^{A}	2.1 ± 0.4^{B}	2.6 ± 0.5^{C}	2.1 ± 0.5^{BC}	2.6 ± 0.8
			E. coli		
Pre-wash	1.2 ± 0.7^{A} (5/6)	0.7 ± 0.0^{B} (0/6)	0.7 ± 0.0^{B} (0/6)	0.7 ± 0.0^{B} (0/6)	0.8 ± 0.4 (5/24)
Post-wash	1.4 ± 0.4^{A} (5/6)	0.7 ± 0.0^{B} (0/6)	0.8 ± 0.1^{B} (1/6)	0.7 ± 0.0^{B} (0/6)	0.9 ± 0.3 (6/24)

Mean ± standard deviation.

Table 2. Wash water PAA residual concentration (ppm), temperature (ºC), and microbial loads by day (log MPN and CFU/100mL). Comparisons of means across sampling days were performed by row, different letters represent significantly different loads. (n=21 for each tank per day. N=84).

	Day 1	Day 2	Day 3	Day 4	Average
			Tank 1		
PAA residual	0.4 ± 0.4^{A}	4.1 ± 3.3^{A}	11.3 ± 4.3^{A}	62.3 ± 25.9^{B}	19.5 ± 28.3
Temperature	19.2 ± 1.3^{A}	13.0 ± 0.5^{B}	11.0 ± 0.6^{C}	11.3 ± 0.8^{C}	13.6 ± 3.5
APC	7.8 ± 0.2^{A}	6.6 ± 0.6^{B}	6.6 ± 0.5^{B}	4.5 ± 0.3^{C}	6.4 ± 1.3
Coliforms	5.9 ± 0.2^{A}	3.2 ± 0.4^{B}	2.8 ± 0.4^{B}	1.2 ± 0.9^{C}	3.3 ± 1.8
E. coli	1.3 ± 0.5^{A}	0.2 ± 0.5^{B}	0.1 ± 0.1^{B}	0.0 ± 0.0^{B}	0.4 ± 0.6
			Tank 2		
PAA residual	17.6 ± 14.0^{A}	18.7 ± 11.2^{A}	15.3 ± 7.0^{A}	40.2 ± 20.4^{B}	22.9 ± 16.7
Temperature	20.2 ± 1.0^{A}	15.1 ± 0.4^{B}	13.1 ± 1.0^{C}	13.3 ± 1.2^{C}	15.4 ± 3.1
APC	5.5 ± 0.9^{AB}	5.2 ± 0.4^{BC}	6.1 ± 0.4^{A}	4.8 ± 0.5^{C}	5.4 ± 0.7
Coliforms	3.1 ± 1.2^{A}	2.5 ± 0.6^{A}	3.3 ± 0.2^{A}	3.0 ± 0.4^{A}	3.0 ± 0.8
E. coli	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	0.1 ± 0.1^{A}	0.0 ± 0.0^{A}	0.1 ± 0.1

Mean ± standard deviation.

Table 3a. Water ranges and correlations with PAA residual concentration by tank.

Variable	Range	Units	PAA Correlation Coefficient	*p*-value
Tank 1				
PAA	0-98	ppm	--	--
APC	4.1-8.1	CFU /100mL	-0.84	<0.001*
Coliforms	0.1-6.1	CFU /100mL	-0.75	<0.001*
E. coli	0.1-1.8	MPN/100mL	-0.41	0.03*
Temp	9.9-20.7	°C	-0.43	0.02*
pH	4.1-8.2	n/a	-0.71	<0.001*
ORP	149-447	mV	0.64	<0.001*
COD	20-628	mg/L	0.67	<0.001*
Turbidity	3-92	FAU	0.14	0.47
Tank 2				
PAA	1-58	ppm	--	--
APC	4.0-6.9	CFU/100mL	-0.79	<0.001*
Coliforms	1.3-5.1	CFU/100mL	-0.39	0.04*
E. coli	0.1-0.3	MPN/100mL	-0.06	0.76
Temp	10.9-21.0	°C	-0.08	0.66
pH	4.4-7.4	n/a	-0.75	<0.001*
ORP	260-434	mV	0.76	<0.001*
COD	27-393	mg/L	0.70	<0.001*
Turbidity	2-49	FAU	0.31	0.11
Combined				
PAA	0-98	ppm	--	--
APC	4.0-8.1	CFU/100mL	-0.77	<0.001*
Coliforms	0.1-6.1	CFU/100mL	-0.67	<0.001*
E. coli	0.1-1.8	MPN/100mL	-0.36	0.007*
Temp	9.9-21.0	°C	-0.28	0.039*
pH	4.1-8.2	n/a	-0.72	<0.001*
ORP	149-447	mV	0.66	<0.001*
COD	20-628	mg/L	0.68	<0.001
Turbidity	2-92	FAU	0.15	0.25

<0.05 *p*-value are considered significant correlations and marked (*).

Table 3b. Water correlations with PAA residual concentration by tank and day.

Variable	Day 1	Day 2	Day 3	Day 4
		Tank 1		
APC	-0.32	-0.78*	-0.78	-0.30
Coliforms	-0.02	-0.68	0.10	-0.58
E. coli	-0.4	-0.54	0.34	0
Temp	-0.17	-0.10	-0.45	0.65
pH	-0.22	0.46	-0.69	-0.87*
ORP	0.71	-0.03	0.72	0.92*
COD	0.14	0.32	0.61	0.99*
Turbidity	0.04	-0.64	0.49	0.82*
		Tank 2		
APC	-0.82*	-0.96*	-0.91*	-0.79*
Coliforms	-0.77*	-0.68	-0.65	-0.32
E. coli	0	0	0.15	0
Temp	0.61	0.26	0.92*	0.68
pH	-0.74	-0.94*	-0.55	-0.99*
ORP	0.77*	0.96*	0.75*	0.99*
COD	0.90*	0.96*	0.86*	0.96*
Turbidity	0.51	0.77*	0.59	0.80*

<0.05 *p*-value are considered significant correlations and marked (*). Significant positive correlations are filled in green and significant negative correlations are filled in red.

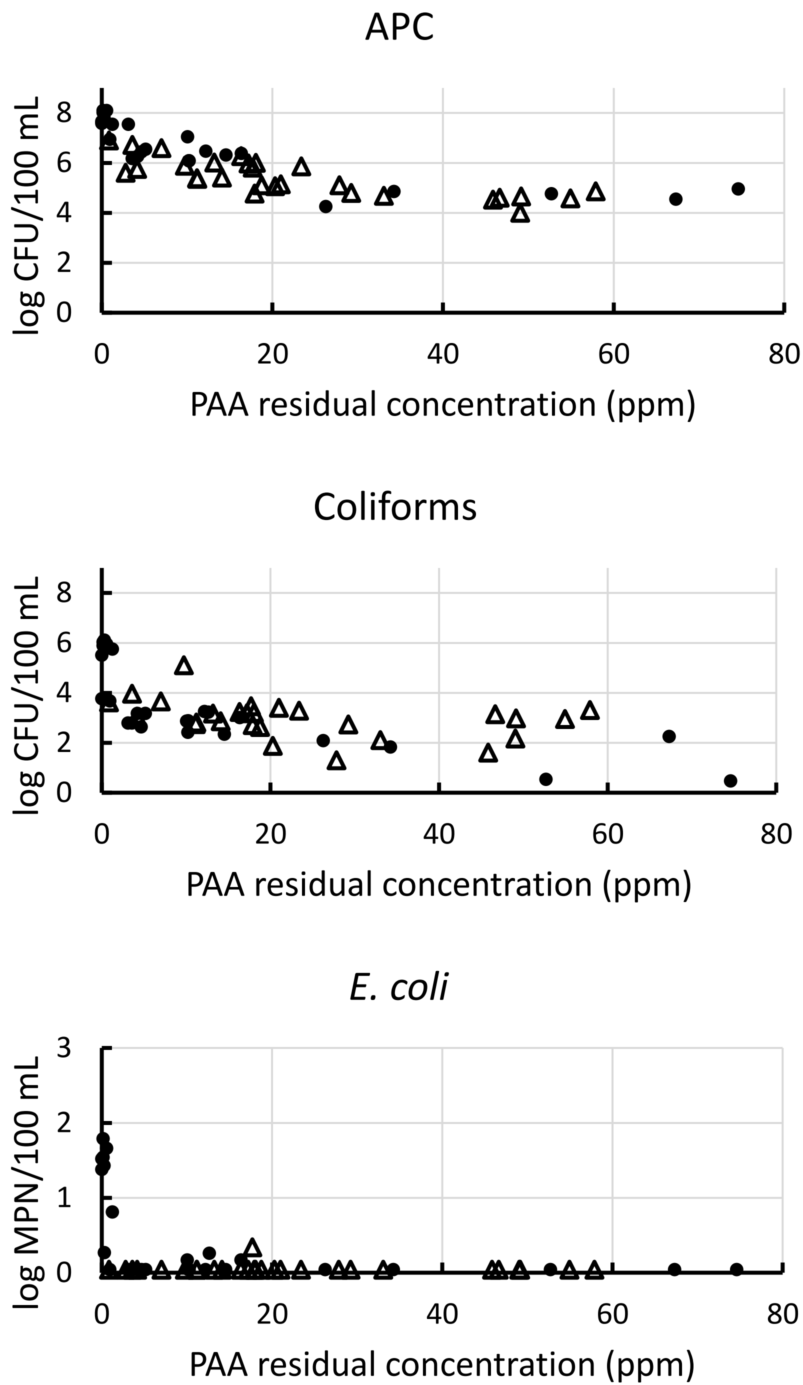

Figure 1. Water microbial loads (log CFU and MPN/100mL) by PAA concentration (ppm) for (●) tank 1 and (Δ) tank 2.

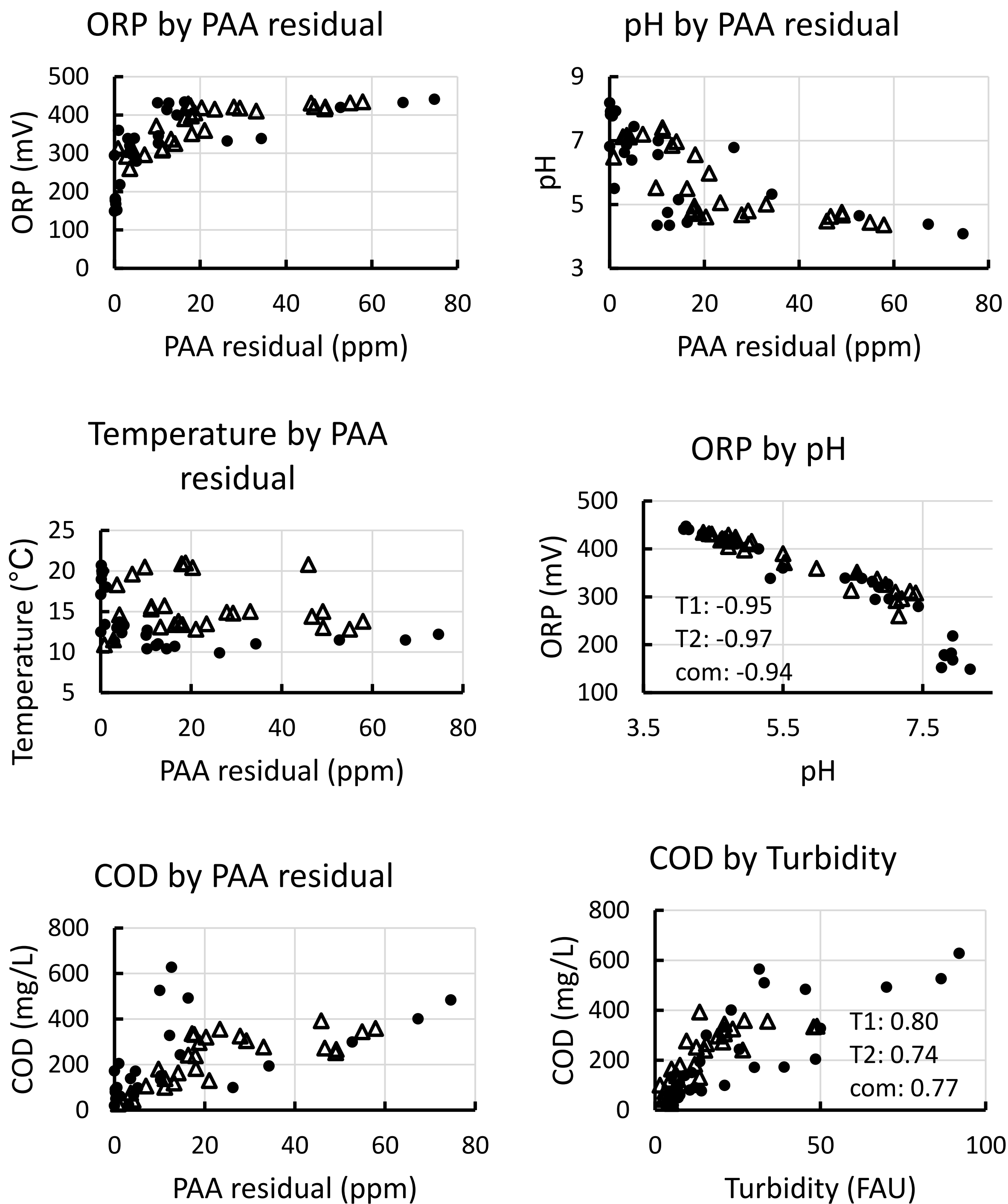

Figure 2. Water physicochemical biplots for (●) tank 1 and (Δ) tank 2 with correlation coefficients for (T1) tank 1, (T2) tank 2, and (com) combined. The six correlation coefficients had *p*-values <0.001*.

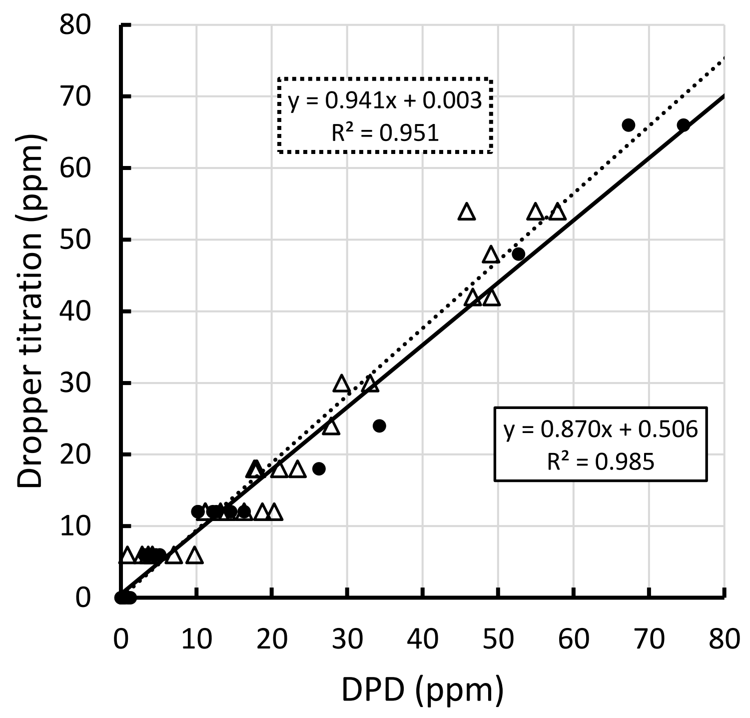

Figure 3. Comparison of PAA concentration measurement methods for (●) tank 1 and (Δ) tank 2 with linear regressions for (solid) tank 1 and (dashed) tank 2.

CHAPTER 3: *Lactococcus lactis* starter culture reduces bloater defect in cucumber fermentations brined with low salt

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Abstract

Lactococcus lactis, capable of a shorter doubling time than *Lactiplantibacillus pentosus*, is proposed as a starter culture for cucumber fermentation brined with low salt to prevent bloater defect. The metabolic activity of the cucumber intrinsic *Enterobacter* and *Leuconostoc*, and starter cultures are hypothesized to produce sufficient carbon dioxide (CO_2) to induce bloater defect. Model and starter culture-assisted cucumber fermentation were evaluated for bloater defects and metabolic activity in response to *Lc. lactis* single culture inoculation or mixed with *Lb. pentosus*. Model fermentations of cucumber juice medium (CJM) in vacutainers revealed that growth of *Lc. lactis* and *Leuconostoc* or *Enterobacter* was concomitant with reductions in production of acetic acid and ethanol from heterofermentation. *Leuconostoc* produce trace amounts of butyric acid (0.7 mM). *Enterobacter* population density was reduced by 4.7 log CFU/ml in CJM after 22 h when co-inoculated with *Lc. lactis*. Model fermentations of pasteurized cucumbers confirmed a reduction of CO_2 production in the presence of *Lc. lactis* and a minimum attainable pH of 4.0 ± 0.2. Fresh cucumber fermentations brined with low salt and assisted by a *Lc. lactis* resulted in reduced bloater defect but produced butyric acid (40 mM). A metagenomic analysis shows *Leuconostoc* spp. was present and may have contributed to butyric acid production. *Lc. lactis* can reduce bloater index in the initial stage of cucumber fermentation brined with low salt, but an additional strategy is needed to control butyric acid production and the defect during the middle fermentation stage.

Significance:

Prevention of bloater defect in commercial cucumber fermentation brined with low salt can reduce economical losses for processors and manufacturers. Bloater defect can affect up to

60% of a fermentation vessel. The control of such defects in cucumber fermentations using a *Lc. lactis* starter culture significantly reduces bloater defect and additionally enables the expanded implementation of environmentally compatible low salt systems.

1. Introduction

Fresh cucumbers, preserved by fermentation, contain diverse microbiomes often harboring endophytes belonging to *Pseudomonadaceae*, *Enterobacteriaceae,* and several lactic acid bacteria (LAB) (Pérez-Díaz et al., 2017, 2019; Rothwell et al., 2022). Bloater defect is an undesirable quality in fermented cucumbers, induced by the accumulation of the microbially produced CO_2 (Zhai, Pérez-Díaz, Diaz, et al., 2018). Larger fruits brined with low salt are particularly susceptible to bloater defect (Lu et al., 2002; McMurtrie & Johanningsmeier, 2018; Zhai, Pérez-Díaz, & Diaz, 2018). The *Enterobacteriaceae*, capable of metabolizing glucose, fructose, malic acid and lactic acid to acetic acid and CO_2 in CJM, are inhibited by the production of organic acids, the declining pH at values below 4.2, the depletion of dissolved oxygen, and the high sodium chloride (NaCl) concentration in unequilibrated cover brines (Dupree et al., 2019; Pérez-Díaz et al., 2019; Pérez-Díaz et al., 2020; Rothwell et al., 2022). However, low salt cucumber fermentations (<500 mM NaCl) are particularly susceptible to CO_2-induced bloater defect due to the production of the gas in the early stage by the indigenous *Enterobacteriaceae* as well as *Leuconostocaceae* (Pérez-Díaz et al., 2015; Rothwell et al., 2022; Zhai & Pérez-Díaz, 2021). Heterofermentative LAB, such as *Leuconostoc* spp., employ the phosphoketolase pathway which favors the production of CO_2 (Zaunmüller et al., 2006; Zhai et. al., 2018; Zhai & Pérez-Díaz, 2020) and present accelerated growth rates in cucumber juice (μ_{max}=0.33 to 0.77; Zhai & Pérez-Díaz, 2020). Thus, growth and the metabolic activity associated with *Enterobacteriaceae* and *Leuconostocaceae* is undesirable in low salt cucumber fermentation. Additionally, the production of malic acid by selected *Enterobacteriaceae*, such as *Enterobacter nimipressuralis* is undesirable, as the organic acid can be decarboxylated by

several fermentative lactic acid bacteria including the prevalent *Lactiplantibacillus pentosus* (Zhai et al., 2023).

We propose the use of *Lactococcus lactis*, a homolactic fermentative bacteria, as a functional starter culture to reduce bloater defect in cucumber fermentation brined with low salt. *Lc. lactis* is a homofermentor expected to outcompete *Enterobacteriaceae* and *Leuconostocaceae* in cucumber fermentations brined with low salt given its short doubling time and rapid growth rate in the CJM model (Table 2). *Lb. pentosus*, a candidate for a robust starter culture for cucumber fermentation brined with low salt, undergoes a long lag phase and present an extended doubling time (2.6 to 3.0 h; Rothwell et al., 2022) to reach maximum growth as compared to *Enterobacteriaceae* (Rothwell et al., 2022; Zhai et al., 2023). However, the final pH of a cucumber fermentation is more acidic than the minimum expected pH tolerance of *Lc. lactis*. Thus, the growth of a *Lc. lactis* starter culture in early fermentation was expected to induce a desirable microbial succession in cucumber fermentation and cease at pH 4.0 ± 0.2, prior to the end of sugar conversion. It was conceptualized that cucumber fermentations assisted by a *Lc. lactis* starter culture would be completed by the intrinsic and more aciduric LAB, such as *Lb. pentosus* or an added culture of such species (Breidt & Fleming, 1998; McDonald et al., 1990).

Experiments were conducted to analyze the growth of *Lc. lactis* in cucumber juice medium (CJM) and the effects of *Lc. lactis* LA0312 starter culture on an efficiently replicable model for cucumber fermentation. Competitive *Enterobacter* and *Leuconostoc* species were selected based on CO_2 production to assess competition with *Lc. lactis* (Rothwell et al., 2022; Zhai & Pérez-Díaz, 2020). CO_2-producing *Enterobacteriaceae* and *Leuconostocaceae* species were observed to produce less metabolites associated with CO_2-production when co-inoculated with *Lc. lactis*, suggesting that the starter culture has potential in low-salt cucumber

fermentations. *Lb. pentosus* LA0445 was selected to complete fermentation after *Lc. lactis* and co-inoculated with *Lc. lactis* in fresh cucumber fermentations (Anekella & Pérez-Díaz, 2020; Zhai & Pérez-Díaz, 2017). Cucumber fermentations were conducted in open-top 18 L and brined with low salt and no potassium sorbate to reduce the environmental impact of the process to be commercialized. *Lc. lactis* reduced bloater index in all the fresh cucumber fermentations evaluated in this study.

2. Materials and Methods

2.1 Cultures and inocula preparation

All cultures used in this study were isolated from cucumber fermentation brine and maintained in the USDA-ARS Food Science & Market Quality and Handling Research Unit located in Raleigh, North Carolina, USA. Lactic acid bacteria, *Enterobacteriaceae*, and *Leuconostocaceae* were streaked from frozen stocks onto M17, Lactobacilli DeMan Rogosa Sharpe (MRS), Violet Red Bile-Glucose (VRBG) or Brain Heart Infusion (BHI) agar plates as indicated on Table 1. All cultures were incubated at 30 °C under aerobic and static conditions. After incubation, plates were stored at 4 °C to minimize bacterial metabolism and preserve colonies for up to 14 days. Isolated colonies on agar plates were transferred to 5 mL of the respective media broth (Table 1) and incubated at 30 °C under aerobic and static conditions (16 to 24 h) for the preparation of inocula.

2.2 Growth trends of *Lc. lactis* in CJM

Broth cultures were spun down at 12,900 *g* using an Eppendorf Centrifuge 5810R (Fisher Scientific, Fremont, CA, USA) and washed twice with saline solution (145 mM NaCl) using the same initial volume to obtain the inocula in late exponential phase. CJM was prepared as

described by Zhai et al. (2023) with size 2B cucumber sourced from the local farmer's market. 20 µL of inocula were combined with 180 µL of CJM in each 200 µL well in a 96-well plate. Five different inoculation levels (2-6 log CFU/mL) were assessed. Two independent CJM lots were inoculated with biological replicates of each strain. Three wells were assigned to each independent biological replicate, for a total of 6 replicates. The 96-well plates were incubated at 30 °C and absorbance was measured at 630 nm wavelength, immediately after plate was shaken for 10 s on medium, in 1-h intervals for 24 h (BioTek ELx808 Microstation, Agilent, Santa Clara, CA). Growth rate (µ) and doubling time (T_d), or generation times, were estimated based on the equations (1) and (2) below, respectively. The μ_{max} is the maximum growth rate of each run.

1) $$\mu\ (h^{-1}) = \ln(10) * \left(\text{absorbance}_{(\text{time})} - \text{absorbance}_{(\text{time}-1\text{ h})}\right)$$

2) $$T_d\ (h) = \frac{\ln(2)}{\mu_{max}}$$

2.3 Determination of viable colony counts

Colony counts for presumptive LAB, enteric bacteria, and yeasts were determined by plating on Lactobacilli MRS agar supplemented with 10 mL/L of a 0.1% cycloheximide solution to inhibit yeast growth, VRBG agar, and Yeast and Mold agar (YMA) supplemented with 0.04% chloramphenicol and 0.04% chlortetracycline to inhibit bacterial growth, respectively. Spiral plating was done using an Eddy Jet 2 automated spiral plater (IUL Instruments, Barcelona, Spain). Inoculated VRBG and MRS and YMA plates were incubated at 30 °C for 24 h, and 48 h, respectively. An Acolyte 3 HD plate reader was used to enumerate colonies from agar plates (Synbiosis, Frederick, MD).

2.4 Model CJM fermentations

CJM batches of 21 mL were inoculated, depending on treatment with *Lc. lactis*, *Enterobacter* spp. (*E. cancerogenus* and *E. nimipressuralis*), or *Leuconostoc* spp. (*Ln. fallax* and *Ln. lactis*) to 6 log CFU/mL. 5 mL aliquots from inoculated CJM batches were injected into 10-mL sterile vacutainers (BD, Franklin Lakes, NJ, USA) and incubated at 30 °C for 48 h. At 6, 22, and 48 h of fermentation, aliquots of 3 mL were removed from the vacutainers using a needle and aseptic techniques. Such samples were used for the enumeration of colonies from Lactobacilli MRS and VRBG agar plates as described in section 2.3, for pH measurements taken as described in section 2.5, and for High Performance Liquid Chromatography (HPLC) analysis performed as described in section 2.6.

2.5 pH measurements

1.5 mL microcentrifuge tubes were aseptically filled with 1 mL of culture medium or cover brine and spun down for 5 min at 12,900 *g* using Eppendorf Centrifuge 5810R, the supernatant was transferred to another microcentrifuge tube for pH measurement using a calibrated Thermobrand Accumet AR25 (Thermo Fisher Scientific, Waltham, MA) equipped with a thin probe (model 13-620-290).

2.6 HPLC analysis

1 mL of culture medium or cover brine samples were collected, spun down at 12,900 *g* for 10 min, and 0.9 mL of supernatants were stored at -20 °C until processed. Samples were thawed at room temperature and spun down at 18,500 *g* for 10 min. 300 µL of supernatants were transferred to HPLC vials and refrigerated until the analysis was performed. An Aminex HPX-87H column (Biorad, Hercules, CA) was used to separate metabolites. The operating conditions of the system included a column temperature of 65 °C and a 0.01 N H_2SO_4 eluent set to flow at

0.9 mL/min. A SPD- 20A UV–Vis detector (Shimadzu Corporation, Canby, OR) was set at 210 nm at a rate of 1 Hz to quantify organic acids. A RID-10A refractive index detector (Shimadzu Corporation) connected in series with the UV-Vis detector was used to measure glucose, fructose and ethanol. The external standardization of the detectors was done using eight gradient concentrations of the standard compounds (data not shown). The compound concentration for the samples was calculated based on the height peak of each compound in the chromatogram as compared to the corresponding compounds on the standard curves at specific retention times using the LabSolutions Workstation (Shimadzu Corporation, Kyoto, Japan). The estimated limit of detection was 0.001 mM.

2.7 Fermentation of pH-adjusted pasteurized cucumbers to evaluate the induction of bloater defect

Commercially available Kosher dill pickles (Mount Olive Pickle Company, Mt. Olive, NC, USA) were used for this experiment following the protocol described by Zhai et al. (2023). Briefly, the cover liquor was supplemented with sufficient glucose and 5.0 N sodium hydroxide to equilibrate at 20 mM and pH 6.0 ± 0.5, respectively. Jars were refrigerated at 4 °C for 3 days with sporadic shaking to enable microbial stability while the glucose and sodium hydroxide equilibrated with the fruits. To test the ability of *Lc. lactis* to outcompete bloater defect-inducing-*Enterobacteriaceae*, the jars contents were aseptically transferred to sterile 3.8 L jars to create an aerobic headspace. The negative control treatment consisted of pickle jars that remained uninoculated. The positive control jars were inoculated with *Lc. lactis* LA0312 or *Enterobacter cancerogenus*. *E. cancerogenus* was selected because it was found to produce the most CO_2 (171 mM) in CJM fermentation after 48 h of incubation relative to other

Enterobacteriaceae (Zhai et al., 2023). The treatment jars were co-inoculated with *Lc. lactis* LA0312 (5 log CFU/g) and *E. cancerogenus* (4 log CFU/g).

Two *Leuconostocaceae*, *Leuconostoc lactis* and *Leuconostoc fallax*, were chosen to test the ability of *Lc. lactis* to prevent bloater defect given their ability to produce CO_2 in CJM (Zhai and Pérez-Díaz, 2020). In this experiment, the fermentations were conducted in the original 946 mL jars. The negative control consisted of jars that remained uninoculated. The positive controls were inoculated with *Lc. lactis* LA0312, *Ln. lactis* or *Ln. fallax*. *Leuconostoc* species were selected based on fermentation biochemistry in CJM (Zhai & Pérez-Díaz, 2020). *Lc. lactis* and *Ln. lactis* were inoculated to 4 log CFU/g of the total mass. *Ln. fallax* was inoculated to 5 log CFU/g to compensate for its slower growth rate (Zhai & Pérez-Díaz, 2020).

The inocula were prepared using the culture broth identified in Table 1. The bacterial cells were washed twice with the same volume of physiological saline solution prior to inoculation. The inocula were plated as described in section 2.3 on the media type described in Table 1 to confirm inoculation levels. After inoculation, the jars were gently shaken for 30 s prior to resealing and incubating at 30 °C for 72 h. Samples were collected at the end point for determining CO_2 amounts produced, viable counts, pH measurements, and for HPLC analysis, which were conducted as described in sections 2.8, 2.3, 2.5 and 2.6, respectively. Bloater index was calculated as described by Zhai et al. (2023).

2.8 Measurement of dissolved gases in fermentation cover brines

Aliquots of 2 ml of cover brine were collected from jars using a 25 cm needle and deposited in vacutainers . Aliquots of 3 mL 200 grains vinegar (20 % acetic acid) were injected in the vacutainers to release CO_2 into the headspace for detection. The vacutainers were gently shaken 20 times, avoiding the entrapment of liquid into the septa, after injecting the vinegar and

prior to detection. The gases were measured by injecting the needle of the Map-Pak Combi gas analyzer (AGC Instruments, Shannon, Ireland) through the rubber septa of the vacutainer. The gas analyzer was purged between sample measurements.

2.9 Evaluation of cucumber fermentations assisted by a *Lc. lactis* starter culture

Cucumber fermentations were conducted in open-top, 18 L buckets outdoors to observe differences in bloater index in response to a *Lc. lactis* starter culture. Size 2B-3B cucumbers were packed using a 70:30 ratio (w/w; fruit: cover brine) and brined to equilibrate at 342 mM NaCl, and 18 mM calcium hydroxide ($Ca(OH)_2$). The pH of the fermentation batch was adjusted to 6.0 ± 0.1 with 3.8 M acetic acid added as vinegar. The amount of vinegar added was determined from a titration of 100 g of cucumber slurry and cover brine mixed to 70% fruit and 30% cover brine (w/w). Plastic discs, with a diameter slightly smaller than the interior diameter of the buckets and drilled with holes, were placed on top of brined cucumbers and weighed down using bags of sand at beginning of fermentation to ensure cucumbers were fully submerged during fermentation. The *Lc. lactis* LA0312 starter culture was pre-adapted in 100 mL of CJM, which was inoculated to 6 log CFU/mL and incubated at 30 °C for 3.5 h to reach 7 log CFU/mL. A lyophilized culture of *Lb. pentosus* LA0445 (11 log CFU/g) was used for inoculation to replicate industry practice. 1 g of lyophilized culture was suspended in 9 mL saline solution and diluted to 7 log CFU/mL. Cucumbers were brined and both cultures were inoculated to 4 log CFU/g of the total mass. Buckets were allowed to equilibrate indoors at room temperature for 2 days prior to transportation outdoors. Cover brine samples were aseptically collected 3 inches below the surface for colony enumeration and biochemical analyses as described in sections 2.3, 2.5, and 2.6. Buckets were covered during heavy rainfall. Two 10 mL cover brine samples were collected from each fermentation after 13 days for total DNA extraction performed as described

in section 2.10 for subsequent metagenomic analysis. Bloater index was assessed as described by Zhai et al. (2023). Half of the fermentations were assessed for bloater index after 4 days of fermentation and the other half after 13 days of fermentation at pH of 4.2 ± 0.2 and 3.6 ± 0.1, respectively.

2.10 Total DNA extractions and metagenomic analysis

10 mL brine samples were spun down at 12,900 *g* for 10 min and the supernatants decanted. The pellets were resuspended in 490 µL sterile saline solution and transferred to microcentrifuge tubes. 10 µL of 2.5 mM propidium monoazide (PMA) was added to each sample to render extracellular DNA unreadable by metagenomic sequencing (Pan & Breidt, 2007). Suspensions were briefly vortexed and placed in the dark for 5 min at room temperature to allow for PMA equilibration. Samples were placed on chipped ice and exposed to a 650-W halogen lamp placed 20 cm above the tubes for 5 min to cross-link PMA to DNA. Suspensions were spun at 12,900 *g* for 5 min, the supernatants were removed, and pellets were stored at -20 °C until DNA extraction.

To lyse bacterial membranes, 120 µL of a 10 mg/mL lysozyme solution was added to pellets suspended in 480 µL of saline solution and incubated at 37 °C for 15 min prior to DNA extraction, which was performed using the MasterPure™ Yeast DNA Purification Kit as directed by the manufacturer (Biosearch Technologies, Middleton, WI). Purified DNA samples were submitted for whole genome sequencing on Illumina NovaSeq platform 2 x 150 bp followed by bioinformatics analysis via CosmosID-HUB methods, resulting in relative abundance estimates separated by taxonomic classification (CosmosID, Germantown, MD).

2.11 Statistical analyses

Statistical analyses were performed on HPLC, pH, and colony count data from model CJM fermentations, pH-adjusted pasteurized cucumber fermentations, and cucumber fermentations assisted by *Lc. lactis*. Multiple comparisons of all pairs using Tukey-Kramer HSD procedure was performed using JMP© Pro 17.0.0 (JMP Statistical Discovery, Cary, NC). A significance level (α) of 0.05 was used for statistical testing. No significant differences were observed for cucumber fermentations assisted by *Lc. lactis*.

3. Results and Discussion

3.1 *Lc. lactis* growth in CJM

A challenge in preventing bloater defect in cucumber fermentation brined with low salt is the slow growth rate of the predominant lactic acid bacterium, *Lb. pentosus,* relative to the culprit bacteria of the *Enterobacter* and *Leuconostoc* species (Zhai et al., 2023; Zhai & Pérez-Díaz, 2020 & 2021). The observed average growth rates and doubling times for *Lc. lactis* in CJM (0.7928 ± 0.075 to 0.5886 ± 0.0182, and 0.878 to 1.188, respectively; Table 2) suggested that it could serve as a more efficient starter culture than *Lb. pentosus* (0.4234 ± 0.0412, and 1.6516, respectively; Table 2). *Lc. lactis* LA0312 could additionally outcompete *Ln. fallax* (μ_{max}=0.33, T_d = 2.07 h), *Ln. lactis* (μ_{max}=0.77, T_d=0.89 h). While *Lc. lactis* doubling times are shorter than those of *E. cancerogenus* 3.2.13E (1.92 h) and *E. nimipressuralis* 1.2.7E (8.20 h), it is longer than that for several strains of *E. cloacae* (0.25 h), a species that dominates early in cucumber fermentations brined with low salt and capable of producing significant amounts of CO_2 (Rothwell et al., 2022). However, a combination of cover brine acidification and a *Lc. lactis* starter culture is a promising strategy to control CO_2 production by *E. cloacae* (Zhai et al., 2023).

It is relevant to note that the informed selection of a *Lc. lactis* starter culture is needed for the proposed strategy to reduce bloater defect as slightly different growth rates are observed among strains. Faster doubling times were observed for *Lc. lactis* LA0312 (0.878 h) than for *Lc. lactis* 1.8.12 (1.188 h), both isolated from commercial cucumber fermentations. In this study, *Lc. lactis* LA0312 was selected for all remaining experiments given its accelerated growth relative to *Lc. lactis* 1.8.12 (Table 2).

3.2 Model CJM fermentations

CJM fermentations performed in vacutainers suggested that, *Lc. lactis* can compete with mixed cultures of *Enterobacteriaceae* and *Leuconostocaceae* by reducing production of metabolites associated with CO_2 production (Tables 3 and 4). In this experiment the glucose and fructose concentrations present in CJM agreed with previously reported values (Zhai & Pérez-Díaz, 2020). However, malic acid of non-inoculated control was observed to be significantly higher than previous experiments (Rothwell et al., 2022; Zhai et al., 2023). Most of the fructose was consumed within 22 h by *Leuconostoc* spp., which metabolized more glucose and malic acid than *Lc. lactis* and the *Enterobacter* spp. Less glucose and fructose was metabolized when *Leuconostoc* spp. were co-inoculated with *Lc. lactis* LA0312. Significantly less acetic acid production was observed when *Leuconostoc* spp. were co-inoculated with *Lc. lactis*. Less ethanol and butyric acid production was observed when the *Enterobacteriaceae* and *Leuconostocaceae* competed with *Lc. lactis*. The end point pH of 5.14 for *Enterobacter* spp. agrees with previously estimated endpoints of 5.5 and 4.9 for *E. cancerogenus* and *E. nimipressuralis*, respectively (Zhai et al., 2023). The difference in endpoint pH between these two enteric bacteria is likely due to acetic and lactic acid production by *E. nimipressuralis* (Zhai et al., 2023). The end point pH of 4.0 for *Leuconostoc spp.* agrees with previously estimated endpoints of 3.9 and 3.8 for *Ln. lactis*

and *Ln. fallax,* respectively (Zhai & Pérez-Díaz, 2020). *Leuconostoc* spp. divert sugar carbons to diacetyl, butanediol, acetoin, and formate, which were not measured, to maintain a higher pH as compared to acid production alone, and maximize sugar utilization prior to self-inhibition (Hugenholtz & Starrenburg, 1992; Jordan et al., 1996; Zhai & Pérez-Díaz, 2020). Reduction in acetic acid and ethanol production by mixed cultures suggests less CO_2 production when competing with *Lc. lactis*. Previously studied ethanol-producing bacteria were found to have an aldehyde dehydrogenase enzyme that catalyzes the conversion of acetaldehyde to ethanol while regenerating cofactor NADH in CJM with low salt brine constituents (Rothwell et al., 2022). *Lc. lactis* facilitates homolactic primary fermentation and reduced production of metabolites associated with CO_2 production.

3.3 Fermentation of pH-adjusted pasteurized cucumbers with *Enterobacteriaceae* and *Leuconostocaceae*

The minimum pH tolerance of *Lc. lactis* agrees with final pH of fermentations inoculated with *Lc. lactis* (Table 5). *Enterobacteriaceae* produced significantly less succinic and ethanol when competing with *Lc. lactis,* which agrees with results from the CJM experiment (Table 6). Model fermentations performed with pH-adjusted fermented cucumbers supplemented with glucose suggest *Lc. lactis* LA0312 can reduce bloater defect induced by *Enterobacter cancerogenus* 3.2.13E and *Ln. lactis* 1.2.28. However, *Lc. lactis* increased bloater index induced by *Ln. fallax* 1.2.22 (Table 7). *Lc. lactis* produced less lactic acid when co-inoculated with *Ln. lactis*, suggesting the *Leuconostoc* species was not completely restricted. Significantly more glucose was consumed, and slightly more butyric acid was produced when *Ln. fallax* and *Lc. lactis* were co-inoculated. Bloater index of fermentations inoculated with *Ln. lactis* was reduced

when also inoculated with *Lc. lactis* LA0312. *Lc. lactis* LA0312 and *Ln. fallax* may have had a microbial interaction and/or competition that resulted in higher bloater index.

3.4 Cucumber fermentation assisted by *Lc. lactis* starter culture

Cucumber fermentations in 18 L buckets were performed outdoors in open sunlight without potassium sorbate during the summer months (July-August) with temperatures ranging from 17 to 38 °C, which likely affected microbial activity (Dougherty et al., 2002). Calcium chloride was used to maintain cucumber firmness and crispness (Buescher et al., 2011; McFeeters & Fleming, 1990). Calcium hydroxide and acetic acid form calcium acetate buffer and prolong onset of acid stress (Zhai & Pérez-Díaz, 2017). Microbial growth (possibly surface yeasts) was observed across all treatments within 5 days of fermentation, which may have been due to an abundance of oxygen at the surface of fermentation. pH was not significantly different between treatments at sampling time points, suggesting the brine formulation and environmental conditions supported growth of autochthonous LAB and concomitant drop in pH.

Fresh cucumber LAB, enteric bacteria, and yeast populations were at 3.9 ± 1.2, 6.1 ± 1.0, 3.5 ± 0.2 log CFU/g, respectively. Significant differences in colony counts between treatments were not observed on any media at any time point (Figure 1). VRBG counts of cucumber fermentations with higher salt consistently drop below detection levels by day 10 (Rothwell et al., 2022). *E. cloacae* is not expected to grow at pH below 4.25 (Rothwell et al., 2022). pH above 4.5 and reduced conditions are necessary for *E. cloacae* to utilize lactic and acetic acids contained in fermented brines and produce butyric and propionic acids (Franco et al., 2012). Organic acid utilization by yeasts such as *P. manshurica* and *I. occidentalis* causes chemical reduction in the brine and initiates secondary fermentation (Franco & Pérez-Díaz, 2013). *Pichia kudriavzevii* was the prevalent fungi after 13 days of fermentation (>96% relative abundance in 7

out of 8 buckets). *P. kudriavzevii* is known to have high acid tolerance and likely co-aggregated with many bacteria, it limits diffusion entry of undissociated acids particularly well and/or has a metabolism adapted to acid stress (Chelliah et al., 2016; Park et al., 2018; Piper et al., 2001).

Glucose and malic acid were depleted after 4 days (data not shown). Significant differences were not observed for any metabolite (Figure 2). Succinic acid concentration did not significantly vary between treatments and remained low throughout the fermentations (data not shown). Acetic acid concentration decreased throughout fermentation, it may have been metabolized by spoilage yeasts and/or converted into ethanol by ɣ-proteobacteria (Franco & Pérez-Díaz, 2012; Rothwell et al., 2022). Propionic acid may have been produced by *E. cloacae* (Franco & Pérez-Díaz, 2013). Butyric acid production has been associated with chemically reduced environments with a negative redox potential, which is strongly correlated with high yeast populations (Franco & Pérez-Díaz, 2013). Acetic acid is assumed to be a precursor for butyric acid, which may partially explain the decrease in acetic acid throughout fermentation (Bourriaud et al., 2005) Furthermore, the presence of acetic acid has been suggested to be a factor supporting lactic acid conversion into butyric acid by anaerobic organisms (Franco & Pérez-Díaz, 2013). According to the preceding experiments, *Leuconostoc* spp. autochthonous to cucumbers may have contributed to butyric acid production during fermentation. Further study is required to assess the effects of yeast presence on acetic and lactic acid conversion into butyric acid by anaerobic and/or microaerophilic bacterial populations.

Lc. lactis was dominant (67% relative abundance) in one of four inoculated buckets after 13 days of fermentation, and the bloater index of this fermentation was comparable to the non-inoculated fermentation of the sample fresh cucumber lot (Figure 3). *Lc. lactis* was not present after 13 days fermentation in lot A fermentations, suggesting the age of cucumber and/or

autochthonous populations substantially affect starter culture activity. Indeed, low salt fermentations results in enhanced microbial diversity (Pérez-Díaz et al., 2020). *Leuconostoc* spp. and *L. brevis* were observed in all fermentations. Lot B of cucumbers appeared visually older than lot A and had higher initial VRBG counts. However, it is interesting that the average bloater index for lot A was higher than lot B despite starter culture treatment. *Weissella* spp., which have a substantially different NaCl sensitivity than *Leuconostoc* spp. and *Lactococcus* spp., were observed in lot B fermentations without *Lc. lactis* inoculation, suggesting *Lc. lactis* activity negatively affects *Weissella* growth (Pérez-Díaz et al., 2020).

4. Conclusion

Lc. lactis starter culture reduced bloater index in all natural fermentations. The starter culture can rapidly convert glucose to lactic acid in early low salt cucumber fermentation while reducing CO_2 production by autochthonous populations. *Lc. lactis* has potential as a microbial technology for low salt cucumber fermentations but may need to be used with other methods. Further experiments are needed to understand interactions between *Lc. lactis* and *Ln. fallax* and determine if butyric acid was attributable to *Lc. lactis*.

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Table 1. Cultures used in this study, all isolated from commercial cucumber fermentations.

Genus, species, strain	Referred to as	Media
Lactococcus lactis LA0312	*Lc. lactis*	M17
Lactococcus lactis 1.8.12	*Lc. lactis* 1.8.12	M17
Lactiplantibacillus pentosus LA0445	*Lb. pentosus*	MRS
Enterobacter cancerogenus 3.2.13E	*E. cancerogenus*	BHI
Enterobacter nimipressuralis 1.2.7E	*E. nimipressuralis*	BHI
Leuconostoc fallax 1.2.22	*Ln. fallax*	MRS
Leuconostoc lactis 1.2.28	*Ln. lactis*	MRS

Table 2. Maximum growth rates (μ_{max}) and doubling times (T_d) of *Lactiplantibacillus pentosus* LA0445 and *Lactococcus lactis* strains at 30 °C and various inoculation levels (log CFU/mL) in Cucumber Juice Medium.

	Lc. lactis LA0312		*Lc. lactis* 1.8.12		*Lb. pentosus* LA0445 (Rothwell et al., 2022)	
Inoc.	μ_{max}	T_d (h)	μ_{max}	T_d (h)	μ_{max}	T_d (h)
2	0.694 ± 0.056	0.998	0.550 ± 0.040	1.259	0.395 ± 0.036	1.755
3	0.790 ± 0.036	0.877	0.515 ± 0.006	1.344	0.379 ± 0.030	1.829
4	0.810 ± 0.090	0.855	0.570 ± 0.030	1.217	0.399 ± 0.063	1.737
5	0.837 ± 0.103	0.828	0.660 ± 0.007	1.050	0.467 ± 0.023	1.484
6	0.833 ± 0.090	0.832	0.648 ± 0.008	1.070	0.477 ± 0.054	1.453

Table 3. Colony counts and pH measured from the model cucumber juice medium fermentations. Mean ± standard error of two lots of Cucumber Juice Medium are shown. Significant differences between rows are denoted by difference superscript letter (*p*-value≤0.05). BDL denotes below the detection limit.

	Time (h)	Not inoculated control	*Lc. lactis*	*Enterobacter* spp.	*Enterobacter* spp. and *Lc. lactis*	*Leuconostoc* spp.	*Leuconostoc* spp. and *Lc. lactis*
			Colony counts (log CFU/mL)				
MRS	22	$TFTC^{A}$	8.8 ± 0.2^{B}	n/a	7.9 ± 0.6^{B}	8.5 ± 0.1^{B}	8.6 ± 0.1^{B}
MRS	48	$TFTC^{A}$	8.0 ± 0.4^{A}	n/a	5.2 ± 3.2^{A}	7.4 ± 0.7^{A}	7.9 ± 0.1^{A}
VRBG	22	$TFTC^{A}$	n/a	8.6 ± 0.2^{B}	3.9 ± 1.9^{AB}	n/a	n/a
VRBG	48	$TFTC^{A}$	n/a	8.4 ± 0.1^{B}	4.2 ± 2.2^{AB}	n/a	n/a
			pH measurements				
	6	6.24 ± 0.42^{A}	6.07 ± 0.19^{A}	5.98 ± 0.46^{A}	5.92 ± 0.18^{A}	5.86 ± 0.35^{A}	5.81 ± 0.08^{A}
	22	6.27 ± 0.40^{A}	4.24 ± 0.05^{BC}	5.40 ± 0.05^{AB}	4.38 ± 0.27^{BC}	4.12 ± 0.14^{C}	4.20 ± 0.01^{C}
	48	5.62 ± 0.01^{A}	4.02 ± 0.01^{B}	5.14 ± 0.17^{A}	4.36 ± 0.27^{B}	4.01 ± 0.11^{B}	3.96 ± 0.01^{B}

*n/a not applicable

Table 4. Metabolites detected in model cucumber juice medium fermentations. Mean ± standard error of two lots of Cucumber Juice Medium are shown. Significant differences between rows are denoted by difference superscript letter (p-value≤0.05). BDL denotes below the detection limit.

	Time (h)	Not inoculated control	*Lc. lactis*	*Enterobacter* spp.	*Enterobacter* spp. and *Lc. lactis*	*Leuconostoc* spp.	*Leuconostoc* spp. and *Lc. lactis*
Substrates of fermentation (mM)							
Malic acid remaining	48	31.1 ± 5.0^{A}	8.8 ± 8.5^{A}	18.8 ± 5.8^{A}	16.1 ± 0.6^{A}	13.3 ± 3.5^{A}	13.8 ± 4.1^{A}
Glucose remaining	48	44.1 ± 16.7^{A}	24.3 ± 17.2^{A}	16.1 ± 1.1^{A}	26.3 ± 13.7^{A}	12.5 ± 12.5^{A}	16.6 ± 16.6^{A}
Fructose remaining	48	52.6 ± 13.4^{A}	48.5 ± 16.9^{A}	48.8 ± 4.3^{A}	48.5 ± 17.0^{A}	3.8 ± 3.8^{A}	29.7 ± 11.6^{A}
Catabolites of fermentation (mM)							
Lactic acid	48	0.2 ± 0.2^{A}	66.7 ± 10.6^{B}	7.7 ± 1.4^{A}	55.7 ± 17.6^{B}	63.9 ± 0.3^{B}	78.2 ± 1.2^{B}
Acetic acid	48	0.4 ± 0.4^{A}	1.8 ± 0.1^{A}	10.1 ± 6.2^{AB}	5.5 ± 1.9^{AB}	27.8 ± 8.3^{B}	14.3 ± 0.4^{AB}
Succinic acid	48	0.3 ± 0.3^{A}	1.7 ± 0.2^{A}	15.1 ± 3.7^{B}	2.3 ± 1.1^{A}	1.7 ± 0.6^{A}	1.9 ± 0.1^{A}
Butyric acid	48	BDL^{A}	BDL^{A}	BDL^{A}	BDL^{A}	1.2 ± 0.5^{B}	0.9 ± 0.1^{AB}
Ethanol	48	1.2 ± 1.2^{A}	1.2 ± 1.2^{A}	11.4 ± 8.6^{A}	1.1 ± 1.1^{A}	20.2 ± 6.9^{A}	4.3 ± 1.1^{A}

Table 5. Characteristics of fermentations of pH-adjusted pasteurized cucumbers inoculated with *Enterobacter cancerogenus* and *Lc. lactis* after 72 h fermentation at 30 °C. Mean ± standard error of two lots of cucumbers are shown. Significant differences between rows are denoted by difference superscript letter (p-value≤0.05). BDL denotes below the detection limit.

	Not inoculated control	*E. cancerogenus* 3.2.12	*Lc. lactis*	*E. cancerogenus* 3.2.12 and *Lc. lactis*
Colony Counts from Agar Plates (log of CFU/mL)				
MRS	7.1 ± 0.5^{A}	8.9 ± 0.4^{AB}	9.4 ± 0.2^{B}	9.5 ± 0.1^{B}
VRBG	$TFTC^{A}$	9.3 ± 0.3^{B}	$TFTC^{A}$	2.5 ± 2.5^{AB}
pH measurements				
Initial Equilibrated pH	6.04 ± 0.12^{A}	6.12 ± 0.12^{A}	6.03 ± 0.18^{A}	6.11 ± 0.01^{A}
Final pH (72 h)	5.47 ± 0.63^{AB}	6.29 ± 0.08^{A}	4.11 ± 0.04^{B}	4.05 ± 0.10^{B}
Change in pH	0.57	0.17	1.92	2.06
Measurements of Gases Produced (%) and Bloater Index				
Oxygen	14.7 ± 0.3^{A}	9.6 ± 2.3^{A}	16.0 ± 0.2^{A}	16.1 ± 0.3^{A}
Carbon Dioxide	10.2 ± 0.4^{A}	57.7 ± 8.9^{B}	7.2 ± 0.4^{A}	7.6 ± 0.1^{A}
Bloater Index	0.7	25.4	0.1	0.8

Table 6. Metabolites detected in fermentations of pH-adjusted pasteurized cucumbers inoculated with *Enterobacter cancerogenus* and *Lc. lactis* after 72 h at 30 °C. Mean ± standard error of two lots of cucumbers are shown. Significant differences between rows are denoted by difference superscript letter (p-value≤0.05). BDL denotes below the detection limit.

	Not inoculated control	*E. cancerogenus* 3.2.12	*Lc. lactis*	*E. cancerogenus* 3.2.12 and *Lc. lactis*
Substrates of fermentation (mM)				
Malic acid remaining	4.9 ± 1.6^{A}	BDL^{A}	3.4 ± 0.2^{A}	BDL^{A}
Glucose remaining	14.7 ± 4.2^{A}	BDL^{B}	BDL^{AB}	BDL^{AB}
Fructose remaining	14.0 ± 1.1^{A}	BDL^{A}	13.4 ± 4.1^{A}	11.9 ± 3.2^{A}
Catabolites of fermentation (mM)				
Lactic acid	11.6 ± 3.9^{A}	BDL^{A}	61.1 ± 0.5^{B}	63.5 ± 4.5^{B}
Acetic acid	65.5 ± 0.5^{A}	55.9 ± 2.9^{A}	64.4 ± 0.1^{A}	63.7 ± 1.9^{A}
Succinic acid	BDL^{A}	7.8 ± 0.6^{B}	BDL^{A}	BDL^{A}
Ethanol	BDL^{A}	16.3 ± 1.6^{B}	BDL^{A}	BDL^{A}

Table 7. Bloater index and metabolites detected in fermentations of pH-adjusted pasteurized cucumbers inoculated with *Leuconostoc* spp. and *Lc. lactis* after 72 h at 30 °C. Mean ± standard error of two lots of cucumbers are shown. Significant differences between rows are denoted by difference superscript letter (p-value≤0.05). BDL denotes below the detection limit.

	Lc. lactis	*Ln. lactis*	*Ln. lactis and Lc. lactis*	*Ln. fallax*	*Ln. fallax and Lc. lactis*
Bloater index	13.5	38.8	4.9	20.2	46.8
Substrates of fermentation (mM)					
Malic acid remaining	3.9 ± 2.0^{A}	15.1 ± 0.9^{A}	4.1 ± 2.2^{A}	15.9 ± 0.8^{A}	5.5 ± 3.8^{A}
Glucose remaining	20.2 ± 1.8^{AB}	BDL^{A}	15.2 ± 1.6^{AB}	23.7 ± 0.6^{B}	6.4 ± 3.3^{A}
Fructose remaining	22.6 ± 0.4^{A}	14.4 ± 2.5^{A}	16.6 ± 0.1^{A}	BDL^{B}	BDL^{B}
Catabolites of fermentation (mM)					
Lactic acid	80.7 ± 3.6^{A}	40.3 ± 9.1^{BC}	50.5 ± 2.4^{ABC}	27.3 ± 1.3^{C}	61.7 ± 7.7^{AB}
Acetic acid	97.6 ± 0.3^{A}	113.7 ± 0.4^{A}	113.6 ± 11.8^{A}	118.6 ± 3.9^{A}	110.9 ± 4.3^{A}
Succinic acid	2.9 ± 0.4^{A}	2.1 ± 0.1^{A}	2.6 ± 0.7^{A}	3.1 ± 0.6^{A}	2.4 ± 0.4^{A}
Butyric acid	BDL^{A}	BDL^{A}	BDL^{A}	1.1 ± 0.1^{B}	1.5 ± 0.1^{C}
Ethanol	5.0 ± 0.3^{A}	33.4 ± 6.1^{B}	18.0 ± 5.2^{AB}	14.9 ± 1.2^{AB}	25.3 ± 6.8^{AB}

*Not inoculated control fermentations developed unknown microbial activity characterized by turbidity and surface growth and were therefore discarded prior to analyses.

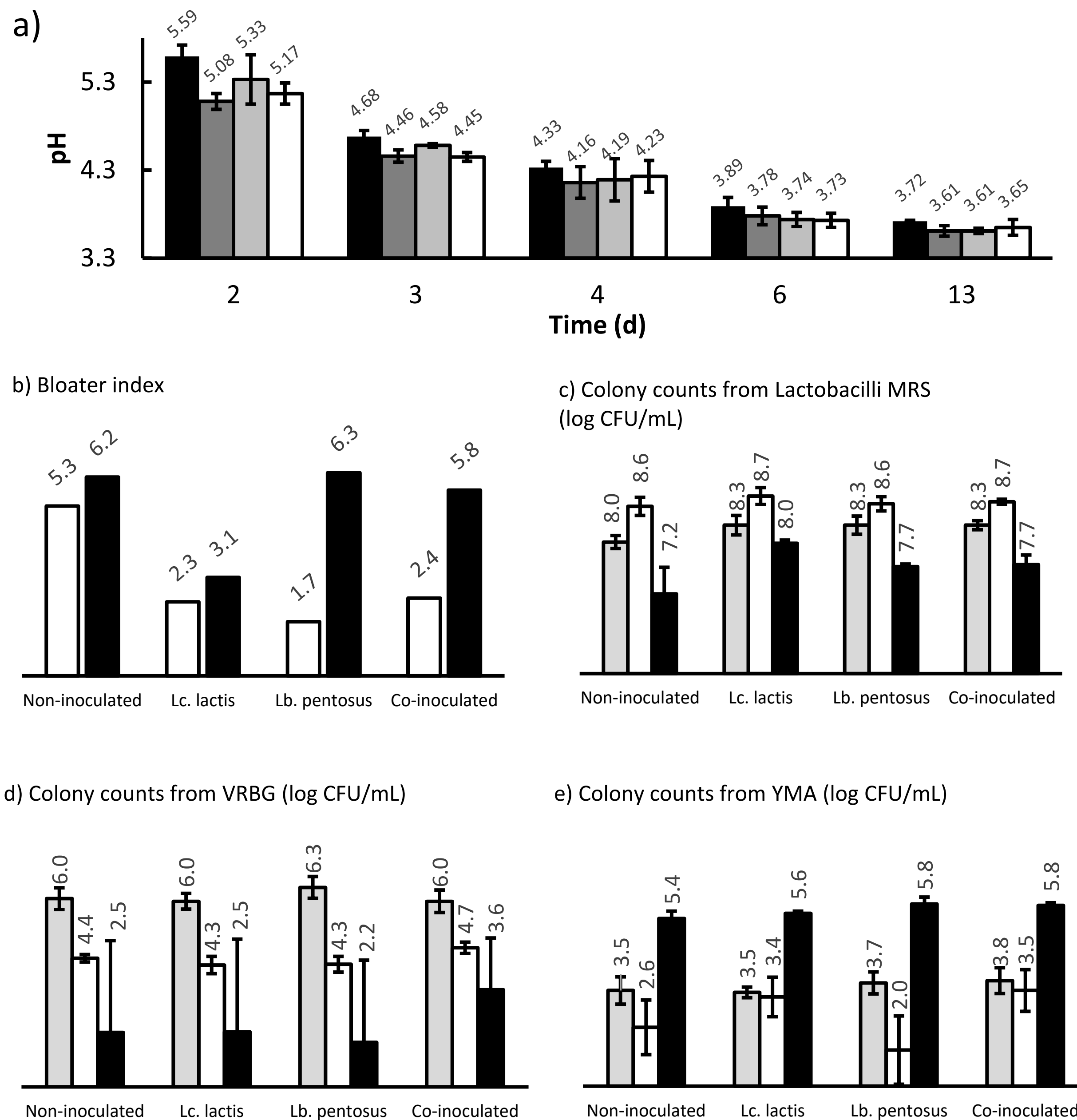

Figure 1. Cucumber fermentation (18 L) brined with low salt and assisted by *Lc. lactis.* (a) pH through fermentation time: (■) Non-inoculated, (■) *Lc. lactis*, (■) *Lb. pentosus* and (□) Co-inoculated. (b) Bloater index after (□) 4 and (■) 13 days of fermentation. Colony enumeration from (c) Lactobacilli MRS, (d) VRBG, and (e) YMA Colony counts after (■) 2, (□) 4, and (■) 13 days of fermentation, lower limit of detection was 2.4 log CFU/mL.

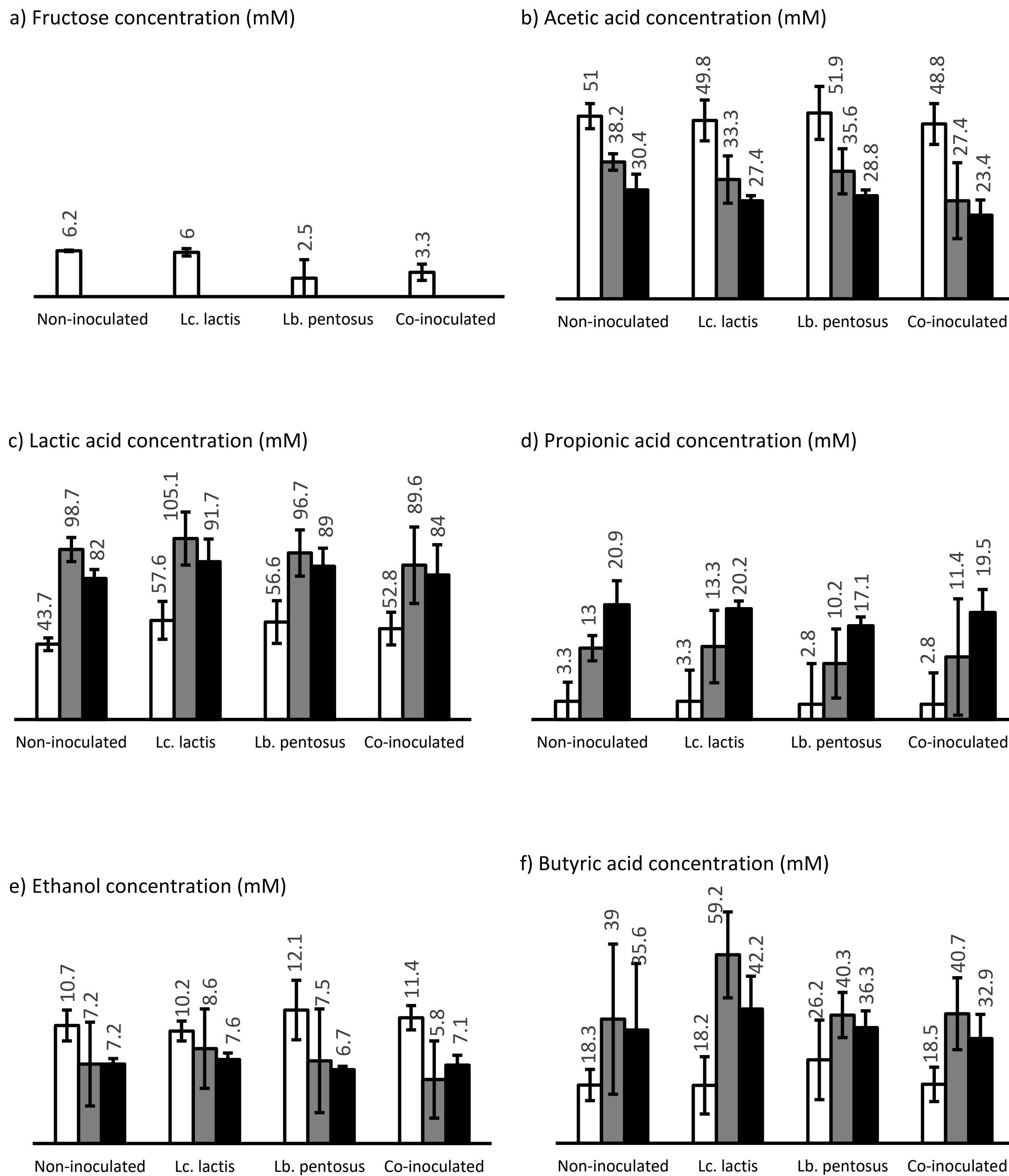

Figure 2. Cucumber fermentation brined with low salt and assisted by *Lc. lactis* in 18 L buckets (a) fructose, (b) acetic acid, (c) lactic acid, (d) propionic acid, (e) ethanol, and (f) butyric acid concentrations (mM) after (□) 4, (■) 8, and (■) 13 days of fermentation.

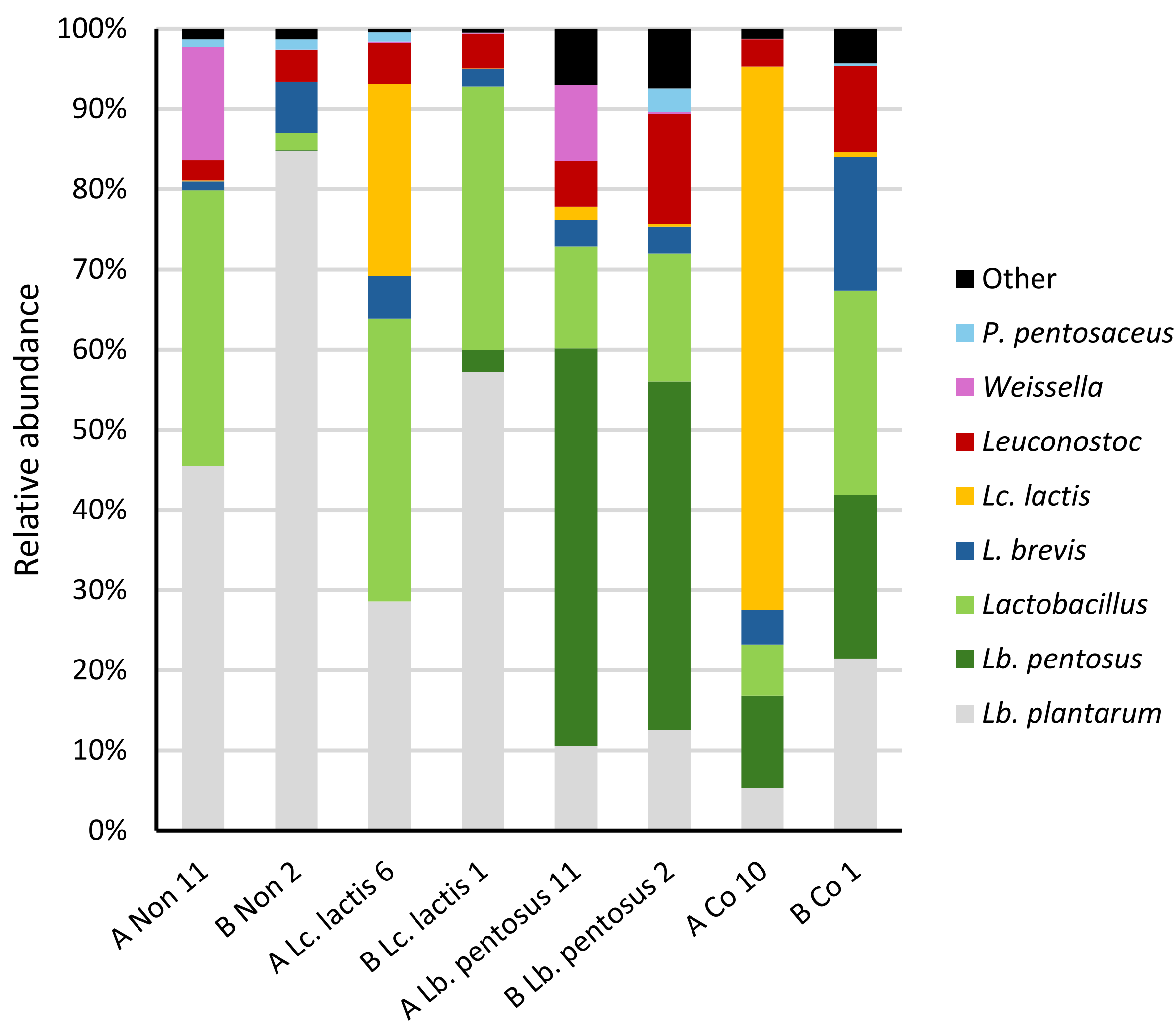

Figure 3. Relative abundance of bacteria grouped by species or genus in cucumber fermentations brined with low salt and assisted by *Lc. lactis* in 18 L buckets after 13 days fermentation. Sample names indicate cucumber lot, inoculation treatment, and bloater index, respectively.

CHAPTER 4: Conclusions and future work

4.1 Chapter 2 conclusions and future work

The observational study in Chapter 2 sought to improve understanding of the use of PAA and variables that impact its efficacy. As part of an extension and outreach program, the observed fresh-cut operation did not operate under adequate food safety conditions at the initial onset of the study; however, conditions improved over the course of four sampling days. This study indicates the need for increased education and outreach efforts that encourages fresh-cut operations utilizing PAA to optimize existing methods for the consistent maintenance of adequate PAA residual concentration (30-80 ppm) to meet federal food safety regulations. It is concluded that process wash water pH, ORP, temperature, or organic load cannot replace PAA concentration as the direct metric for control of cross-contamination. Assuming COD is not excessively high (<750 mg/L), PAA concentration is the only necessary variable for monitoring sanitizer efficacy. One important conclusion from our study is that although PAA test strips are commonly used throughout the industry, they do not accurately and objectively measure PAA concentration. Titration or colorimetric methods should be used instead.

A potential future direction is the development of a robust method for the optimization of dosing to maintain PAA residual concentration. It seems likely that process wash water temperature will be an important factor in the optimization of PAA dosing.

4.2 Chapter 3 conclusions and future work

Cucumber fermentation is inherently difficult to study due to the number of factors and amount of variance involved. Each natural fermentation with fresh cucumbers is different, and models such as cucumber juice medium cannot accurately represent natural fermentations. Although substantial trial and error was involved in experimentation with *Lc. lactis*, a

combination of model and natural fermentations provided comprehensive data on how *Lc. lactis* impacts cucumber fermentation. The original research question (Does *Lc. lactis* starter culture cause bloater defects during low salt cucumber fermentation?) was successfully answered. *Lc. lactis* has potential for reducing bloater low salt fermentation, but future work is needed to achieve a desirable and consistent product.

Further experiments are needed to understand interactions between *Lc. lactis* and *Ln. fallax* and determine if butyric acid was attributable to *Lc. lactis*. Furthermore, anaerobic fermentation may prevent the growth of problematic surface yeasts. Potential future directions in research include 1) the employment of bacteriophages to target undesirable microbial populations and 2) antibiotic production by cultures such as *Lc. lactis*.