

## ABSTRACT

MCMURTRIE, ERIN KELLY. Quality of Cucumbers Fermented in Acidified and Non-Acidified Calcium Chloride Brines for Reduced Environmental Impact of Brining Operations. (Under the direction of Dr. Suzanne Johanningsmeier).

Commercial fermentation processes for the bulk preservation of cucumbers rely on natural microbiota and brines that equilibrate to 0.9-1.4 M sodium chloride (NaCl), producing large volumes of wastewater with high salt concentrations. An alternative fermentation process utilizing 0.1 M calcium chloride (CaCl<sub>2</sub>) as the only salt has been developed to eliminate NaCl from fermentation brines to reduce the environmental impact of brining operations. The objective of this study was to determine cucumber texture quality, color, and physical defects encountered during normal production routines using the two processes. This was accomplished by evaluating the mesocarp firmness, CIELAB colorimeter values and bloater indices for the two brining processes at the raw product, fermented and bulk stored, desalted, and finished product stages of commercial production. Fermentations with the current commercial NaCl process (n=27) used recycled brines with sufficient NaCl, CaCl<sub>2</sub>, and potassium sorbate to equilibrate with the cucumbers to 1 M, 0.04 M, and 2 mM, respectively as well as organic acids and volatile compounds carried over in the recycled brines. The fermentations with the CaCl<sub>2</sub> process (n=41) used fresh brines with sufficient CaCl<sub>2</sub> and potassium sorbate to equilibrate to 0.1 M and 6 mM, respectively, and was inoculated with a *Lactobacillus plantarum* starter culture at 10<sup>6</sup> CFU/mL. Cucumbers fermented with the NaCl process were significantly firmer by 1.7 N ( $P < 0.0001$ ) after fermentation and bulk storage. Longer bulk storage times were correlated with significantly less firm cucumbers in both processes by 0.74 N/100 days ( $P = 0.0030$ ). Cucumbers fermented with the CaCl<sub>2</sub> process had significantly decreased chroma (intensity) and

increased hue ( $P < 0.0269$ ). These differences first appeared after fermentation and bulk storage indicating that the quality changes were a result of a process occurring at that stage. Lactic and acetic acid concentrations were significantly higher in the NaCl process than in the CaCl<sub>2</sub> process, likely because of the acid incorporated into the fermentation by the reuse of spent brines. This resulted in a different fermentation environment as compared to the initial, neutral pH of the CaCl<sub>2</sub> process. Brine acidity has the potential to impact the microbial ecology, activity of pectinolytic enzymes, non-enzymatic softening, and preservative efficacy. Therefore, laboratory fermentations were conducted with size 2B cucumbers in 946 mL jars using a full factorial design for salt (CaCl<sub>2</sub> and NaCl) and acid (acetic acid, hydrochloric acid, and non-acidified) to determine the effect of brine acidification on the fermentation microbiota and texture quality of cucumbers fermented in each brine. Fermentations were inoculated with 10<sup>6</sup> CFU/mL *Lactobacillus plantarum* and included 4 mM potassium sorbate, a common yeast and mold inhibitor. Lactic acid bacteria grew faster and to greater cell counts in the CaCl<sub>2</sub> brines, resulting in increased utilization of fermentable sugars and production of more lactic acid than in NaCl brined fermentations ( $P < 0.0084$ ). Addition of 25 mM acetic acid to fermentation brines (but not the addition of HCl at the same pH) reduced *Enterobacteriaceae spp.* in brines and cucumbers during the initiation of fermentation for both brining salts ( $P < 0.002$ ). However, acidification had no effect on texture quality of fermented cucumbers for either brining salt ( $P = 0.8235$ ). Interestingly, cucumber firmness was 2.9 N higher in CaCl<sub>2</sub> brined cucumbers compared to those brined in NaCl and stored for 100 days ( $P < 0.0001$ ). These results differed from the commercial trials, indicating that there are production variables not present on the laboratory scale interacting with the CaCl<sub>2</sub> process to negatively affect texture quality. Of the

commercial fermentations using the  $\text{CaCl}_2$  process, 12% had an average mesocarp firmness below acceptable quality levels, indicating a need for further optimization of this process.

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Quality of Cucumbers Fermented in Acidified and Non-Acidified Calcium Chloride Brines  
for Reduced Environmental Impact of Brining Operations.

by  
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A thesis submitted to the Graduate Faculty of  
North Carolina State University  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

Food Science

Raleigh, North Carolina

2016

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## **DEDICATION**

I would truly love to thank my family for their love and support through this process. I dedicate this thesis to all of you, your constant pestering and encouragement to finish my research has been appreciated despite my gripes and allowed me to do my very best. Lastly and certainly not least, I would also like to thank my fiancé Korbin Bray for his never ending kindness and reassurance. He has always been my first call when something goes well and has always been there to listen when something is wrong. He is my best friend and I am looking forward to the day I finally get to marry him!

## **BIOGRAPHY**

Erin McMurtrie was born on October 7, 1991 to David and Jennifer McMurtrie. She has one older sister, Kristen, and one younger brother, Jonathan. Erin attended The Early College at Guilford for high school and continued her education at North Carolina State University studying Chemistry and Food Science. Erin completed undergraduate research projects in both departments as well as a 15 month internship in lab support at GlaxoSmithKline in Research Triangle Park. In 2013, Erin graduated magna cum laude with Bachelor of Science degrees in both Chemistry and Food Science. After graduation, she worked for the USDA-ARS Food Science Research Unit as a Biological Sciences Technician for 6 months before beginning a Master of Science degree in Food Science at North Carolina State University under the direction Dr. Suzanne Johanningsmeier. During both undergraduate and graduate school at NCSU, she participated actively in the Food Science Club at NCSU, and served as Secretary and Dairy Bar Chair during her time as a student.

## ACKNOWLEDGMENTS

I would like to thank Dr. Suzanne Johanningsmeier for the opportunity to work with her to complete my Master's Degree as well as her encouragement, advice, and patience throughout the course of this research. In addition to Dr. Johanningsmeier, I would like to thank my other committee members, Dr. Fred Breidt and Dr. E. Allen Foegeding, for serving on my committee, for their expert guidance, for their assistance throughout my research, and for reviewing my thesis.

I greatly appreciate the sample donations from Mt. Olive Pickle Company and all of the other processors. Janet Turner was incredibly helpful with organizing Mt. Olive's side of the commercial study when she was thrown into coordination of this project unexpectedly. Mt. Olive supplied the funding for my research assistantship, and I owe the project to them.

I would also like to thank all of the USDA-ARS FSRU, both current and former. There are far too many of you to name, but Sandy, Dr. Pérez-Díaz, Andrew, Janet, Summer, and Robert have all helped cart buckets and jars from Mt. Olive saving me numerous trips and hours of time. I would also like to thank Emily, Lisa, Robert, Rong, Seth, Summer, and anyone else who helped me with training and/or analysis. Additionally, Kat K., Jacques, Jenn, Sarah, Sean, Zuska, and everyone else who helped lift my spirit when I was having a bad lab day, you are awesome and I will/do miss seeing your faces every day. Special thanks to Kat D., my pickle duo 2 partner. We started graduate work under Dr. Johanningsmeier together, right as the first pickle duo was leaving, and I truly appreciated being a pair with you. From late night dairy bar excursions or really any ice cream-esque trips, Guasaca, coffee via Caribou/Starbucks/Port City Java, RBC, the rare gym trip (on my part at least), and any other occasion shared, I am glad I had someone else to go through graduate school



with. Also thank you to anyone else in the department and Food Science Club not listed thus far that has helped me in my research, classes, professional development, and improving my mood including but in no way limited to Andy, Angie, Claire, Kelsey, Kyle, Meg, and Yiwen.

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## CHAPTER 1 – Literature Review

### 1.1 Introduction to Cucumber Fermentation

Traditionally, the fermentation of cucumbers (*Cucumis sativus*) is accomplished using natural fermentations in brines containing high concentrations of sodium chloride (NaCl). Currently, commercial cucumber fermentations are natural fermentations, relying on microorganisms from the tanks, air, and incoming cucumbers for inoculation. The high NaCl concentration inhibits pathogenic and spoilage microorganisms by selecting for lactic acid bacteria (LAB) that rapidly ferment the cucumbers. These LAB include *Lactobacillus plantarum*, *Lactobacillus brevis*, *Pediococcus pentosaceus*, and *Leuconostoc mesenteroides* (Etchells and others 1974). The modern pickling industry still commonly ferments in brines that equilibrate with the cucumbers to 5-10% NaCl to inhibit spoilage microorganisms and maintain a crisp texture, and processors often add NaCl up to a total of 18% NaCl to prevent the fermented cucumbers in commercial tanks from freezing in the winter (McFeeters and Pérez-Díaz 2010, Pérez-Díaz and others 2015). Fermented cucumber pickles are desalted before packaging into finished products to create a more palatable pickle product of 2-2.5% NaCl. Desalting is performed by removing the fermentation brine and allowing the salt in the cucumbers to equilibrate with fresh water that may contain firming agents such as alum or calcium chloride ( $\text{CaCl}_2$ ). Both the fermentation brine and desalting water contain high NaCl concentrations that must be disposed of by the pickle processor. The high salt waste associated with this process has led to investigations into other processes that will maintain a crisp cucumber texture while minimizing the high chloride wastewater.

As the pickling industry grew, the high salt content of the wastewater became an environmental concern for the EPA (US Environmental Protection Agency 1987). The



desire to minimize high salt wastewater led to the development of brine filtration and recycling procedures (Geisman and Henne 1973, Palnitkar and McFeeters 1975, McFeeters and others 1978, Ratnani and others 1980) and efforts to develop low or no salt fermentation processes. Research on low salt fermentations has revealed several problems such as texture changes, hollow cavities inside the cucumber fruit (bloating damage), and microbial instability leading to spoilage. These challenges led to the goal of creating a low salt fermentation process that will produce firmer pickles, less bloater damage, and be stable during bulk storage for up to one year.

## **1.2 Development of Low Salt Fermentation Processes**

Fermentation brines used in the pickling industry typically range from 5-10% salt and more may be added to inhibit freezing during bulk storage in the winter (McFeeters and Pérez-Díaz 2010). Disposal of brines with this salt concentration is hazardous to the environment. In an effort to minimize salt, Fleming and others (1987) investigated firmness retention of pre-washed cucumbers brined in 0-5.8% NaCl with 0.2% CaCl<sub>2</sub> and a 65 mM acetate buffer (pH 4.9) with a *Lactobacillus plantarum* starter culture and a nitrogen purge cycle. They found normal fermentations could occur in all treatments and firmness was maintained during storage with 2.6% or higher salt in combination with 0.2% CaCl<sub>2</sub>. Fleming and others (1988) also tested cucumber fermentations with the same procedure as Fleming and others (1987) at 2.7% NaCl in anaerobic tanks. They found that a high quality product could be produced under these conditions with firm texture and minimal bloater defects throughout bulk storage. Fleming and others (2002) also tested a reduced salt fermentation process using bag-in-box technology. Blanched cucumbers were used with a starter culture and 4% salt brines. The fermentation was considered successful, but

financially impractical due to the high cost of blanching the cucumbers. However, the fermentation process also succeeded in reducing wastewater generated because a desalting step was not needed before the cucumbers were packed with fresh cover brine to obtain equilibrated concentrations of 2-2.5% salt in the finished product.

Guillou and others (1992) attempted to find a lower salt concentration that could be used to obtain high quality natural fermentations without the need for more costly anaerobic vessels or pretreatment such as blanching, pre-washing, or chlorination procedures. They tested 15 combinations of 0-10% NaCl, 0-0.4% CaCl<sub>2</sub>, and 0-0.4% potassium sorbate with 25 mM acetic acid in open top barrels under UV light. All of the 0% NaCl fermentations spoiled and lower potassium sorbate levels accelerated the spoilage. The cucumbers fermented in 5% NaCl, 0.2% CaCl<sub>2</sub> and 0.2% potassium sorbate with 25 mM acetic acid had excellent quality over the 21 weeks studied. Guillou and Floros (1993) took the data from the experiment in 1992 and applied response surface methodology and multivariate optimization to predict a formulation to minimize salt while maintaining acceptable pH, texture quality, and microbial stability. All four optimization methods utilized, produced similar formulations with the optimum, minimum salt concentration determined to be 3% NaCl, 0.28% CaCl<sub>2</sub>, 0.3% potassium sorbate and 25 mM acetic acid. These fermentation conditions were experimentally verified and shown to produce high quality fermented cucumbers.

Attempts to ferment cucumbers in the absence of NaCl have frequently failed due to spoilage and/or unacceptable quality (Fleming and others 1987, Guillou and others 1992). Fleming and others (1995) attempted fermentation without NaCl in an effort to further improve on the pickling industry's attempt to lower chloride concentrations in wastewater.

Cucumbers were blanched for 3 minutes at 77°C and then packed into a calcium acetate buffer (pH 4.7) and inoculated with a malolactic deficient (MDC<sup>-</sup>) *Lactobacillus plantarum* starter culture. They found that fermentation of blanched cucumbers without salt was possible under laboratory conditions. However, at the pilot scale using open-top barrels, the cucumbers had decreased firmness after seven months storage and were severely bloated due to microbial spoilage.

In light of the initial challenges observed in salt-free fermentation, McFeeters and Pérez-Díaz (2010) attempted a laboratory scale fermentation of cucumbers without NaCl using an elevated calcium chloride concentration. Naewbanij and others (1986) had already tested the growth of *L. plantarum* in cucumber extracts using 333 mM calcium chloride. They found that 60% of the viable counts in raw filtered cucumber extracts and 75% of the viable counts in cucumber extracts filtered after heating to 80°C were *Lactobacillus spp.* This showed that a *Lactobacillus* starter culture would not be inhibited by elevated CaCl<sub>2</sub> concentrations and would predominate the fermentation in CaCl<sub>2</sub> brines. McFeeters and Pérez-Díaz (2010) tested 100 mM calcium chloride brines with 25 mM acetic acid compared with the current commercial practice of 1.03 M NaCl with 40 mM calcium chloride brines. The fermentations progressed in a similar, but accelerated pattern compared to traditional fermentation for utilization of sugars and production of metabolites with safe pH values obtained. The fermentation proceeded more quickly in this calcium chloride brine because it offered little inhibition towards the LAB. Mesocarp firmness of cucumbers fermented in 100, 200, and 300 mM CaCl<sub>2</sub> brines were not significantly different from the fermentation using a commercial formulation of 1 M NaCl and 0.04 M CaCl<sub>2</sub>. The bitter flavor associated

with higher concentrations of calcium chloride was of concern, but additional desalting procedures could reduce the level of calcium chloride to undetectable levels.

Commercial scale ups were initiated after the success of the CaCl<sub>2</sub> brined fermentations by McFeeters and Pérez-Díaz (2010). Pérez-Díaz and others (2015) completed 6 commercialization trials of 100 mM CaCl<sub>2</sub> fermentation processes. Adjustments were made to the formulation between trials to troubleshoot problems as they were made apparent. The first trial was essentially according to McFeeters and Pérez-Díaz (2010) using 100 mM CaCl<sub>2</sub> and 20 mM acetic acid determining the importance of inoculation with a *Lactobacillus plantarum* starter culture under typical commercial production. The fermentations spoiled with and without a starter culture, but they determined that the starter culture was necessary and measures needed to be taken against spoilage yeasts. The use of potassium sorbate and sodium benzoate as preservatives was tested along with limiting the introduction of oxygen by reducing the rate and frequency of air purging to inhibit aerobic yeast spoilage. Parallel studies reported in Pérez-Díaz and McFeeters (2008) found that the preservative levels used were insufficient for yeast inhibition so potassium sorbate was used at double the previous concentration and acetic acid was eliminated from the formulation which was hypothesized to be acting as a substrate for secondary spoilage by yeast as had been observed by Franco and Pérez-Díaz (2012). This modified process with the additional changes of the elimination of acetic acid, double the potassium sorbate concentration, and an additional reduction in the air purging rate resulted in complete fermentations with expected utilization of sugars and production of lactic acid compared to the current commercial process. This process was tested using multiple cucumber sizes, fermentation temperatures, processing facilities, and times spent in bulk storage. A trained descriptive analysis panel evaluated hamburger dill

chips made from this fermentation process and found no significant flavor differences but hardness, fracturability, crispness, and crunchiness were significantly reduced. Hamburger dill chips from each process were also tasted by consumers and the texture of the hamburger dill chips from the current commercial process were liked significantly more than the texture of pickles fermented using the  $\text{CaCl}_2$  process (Wilson and others 2015).

### **1.3 Methods for Evaluating Texture Quality**

Various methods have been used for texture measurement in research experiments over the years. A fruit pressure tester (FPT) has been used as a simple, inexpensive method for many years and is still commonly used for quality assessments by the pickling industry. A FPT is a blunt rod with a tip of a known diameter (typically 5/16" is used for cucumbers) equipped with a spring that measures the resistance of a fruit to puncture (Jones and Etechells 1950). The quality of the fermented cucumber stock is measured semi-quantitatively with quality levels corresponding to a range of pounds of pressure resistance; >18 lbs. is very firm, 14-17 lbs. is firm, 11-13 lbs. is inferior, 5-10 lbs. is soft and <4 lbs. is mushy (Bell and others 1955). Jeon and others (1973) compared texture quality using a FPT with a 5/16" tip to sensorial evaluations of crispness and firmness by a 6 member trained panel and found that there was a strong enough correlation that the FPT would be sufficient for industry work, but because of insensitivity and high operational variability, recommended using other methods of texture analysis for measurements for research.

Jeon and others (1973) also compared sensorial evaluations of crispness and firmness with Texture Profile Analysis (TPA) for brittleness, hardness, and total work of compression on both a cucumber slice with skin included and the dissected cucumber mesocarp tissue. High correlations were found with sensory attributes for nearly every measurement

indicating that the main contributions to sensorial measurements were being captured in TPA measurements. Buescher and others (2011) compared a trained panel to other commonly used methods of texture analysis: mesocarp puncture test and sound analysis. A mesocarp puncture test measures the peak force required for a small blunt probe to penetrate through the fleshy area between the cucumber skin and seed cavity. Sound analysis uses a microphone to capture the noise emitted during the puncture test and calculate the magnitude of sounds at desired frequencies (2-5 kHz in this experiment). Both the mesocarp puncture test and the corresponding sound analysis were found to be highly correlated with the sensory scores for crispness.

Texture analysis using a mesocarp puncture test has become the standard instrumental method of analyzing fermented and raw cucumbers. Thompson and others (1982) compared instrumental texture tests of mesocarp and endocarp cucumber tissue with sensory evaluations of firmness. The endocarp is the botanical name for the seed cavity of the cucumber. They found a high correlation ( $r=0.88$ ) between the mesocarp puncture test and firmness measured by a 5 member trained panel. The use of a mesocarp puncture test has been found to be well correlated with trained panel measurements of firmness, crispness, hardness, and crunchiness in several studies since, showing the robustness of the method (Yoshioka and others 2009, Buescher and others 2011, Pérez-Díaz and others 2015).

#### **1.4 Texture Quality Changes Associated with Fermentation of Cucumbers**

Many factors have been associated with changes in texture quality of cucumbers during fermentation, bulk storage, desalting, processing, and shelf life. Some factors that have been shown to cause significant differences in firmness cannot be easily controlled by the processor such as cultivar or growing conditions, such as location and seasonal crop

differences (Jones and others 1941, Jones and Etchells 1950, Breene and others 1972, Jeon and others 1973, Suojala-Ahlfors 2005). Other variables, such as the addition of  $\text{CaCl}_2$  to fermentation brines, can be easily manipulated, have been well studied, and have been widely incorporated in the commercial production of cucumber pickles for improvement of texture quality. The effects of other variables, such as organic acids and alum, on mesocarp firmness are less understood and have shown contradicting results, but are still used in the production of pickles by many processors.

#### *1.4.1 Effects of Brine Composition on Firmness*

##### ***Calcium***

Calcium ions have been shown to improve firmness in many plant tissues (Kertesz 1939, Doesburg 1961, Van Buren 1979). The increase in firmness that occurs with use of calcium chloride as a firming additive in cucumber fermentations, fresh packed cucumber pickles, and pickled peppers has been well established in many circumstances (Etchells and others 1977, Howard and others 1994, Buescher and others 2011, Tang and McFeeters 1983). Naewbanij and others (1986) found that 0.33 M  $\text{CaCl}_2$  concentrations were slightly selective and non-inhibitory to *Lactobacillus spp.* (60% of isolates were *Lactobacillus spp.* compared to 78% in 1 M NaCl and 51% in the salt free control) indicating its addition at much lower levels should not prevent fermentation, but will also not be as selective as NaCl. The inclusion of 0.03 M  $\text{CaCl}_2$  as an additive has been tested in fermentation brines and does not affect fermentation rates or desalting rates, but does improve the firmness of the cucumber mesocarp tissue whether it is added in the fermentation brine or at desalting (Buescher and Burgin 1988).  $\text{CaCl}_2$  has been shown to result in firmer cucumbers in the presence of softening enzymes such as polygalacturonase and Cx-Cellulase (Buescher and others 1979,

Buescher and Hudson 1984) and slow the rate of softening in conditions favoring acid hydrolysis (pH = 2.6) and at temperatures of 65°C (McFeeters and others 1995).

The mechanism for the firming action of CaCl<sub>2</sub> has been the subject of debate (McFeeters and Fleming 1989). Grant and others (1973) proposed a mechanism to explain the strong gels that pectin forms in calcium solutions. This mechanism called the “egg-box” model consists of divalent cations, such as calcium or strontium, forming ionic bridges between two chains of negatively charged pectin. The egg-box mechanism is widely accepted for pectin gels and it has been assumed that the same mechanism explained the firming effect of calcium on pectin in plant cell walls (Walter 1991). However, evidence built against the same mechanism applying to the pectin in cell walls in the late 1980s and early 1990s. McFeeters and Fleming (1991) found calcium to be a very effective firming agent in low pH systems when the pectin would frequently be protonated and unable to make calcium bridges. McFeeters and Fleming (1989) compared the rate of cucumber mesocarp tissue softening using magnesium, strontium, barium, zinc, cobalt, and cadmium, divalent cations that should be capable of producing the same bridges as calcium. Only barium and strontium were able to lower the softening rate of cucumber mesocarp tissue like calcium, indicating they were not preventing softening by making salt bridges between the pectin in solution since the other ions did not prevent softening. Cadmium is a divalent cation with a higher binding affinity for pectin in solutions than calcium, but it did not prevent cucumber softening in the previous experiment. In a competition experiment between calcium and cadmium, the cadmium did not affect the softening rate at any concentration even though it should displace any bound calcium ions and remove the firming effect of calcium. This experiment showed there is no competition between cadmium and calcium ions, indicating



the cadmium cannot use the binding site calcium uses to inhibit softening. Additionally, McFeeters and Fleming (1990) determined the enthalpy and entropy of activations for various  $\text{CaCl}_2$  concentrations as well as the dissociation constant for calcium to the binding site. However, the dissociation constant was not consistent with the affinity of  $\text{CaCl}_2$  for a pectin solution; the dissociation constant implied a binding site with much greater affinity than would be found under the egg-box model. Additionally, Kohn and Furda (1967) pointed out that calcium has a lower binding affinity for high methoxy pectin and native plant pectin is highly methylated. Fermentation frequently lowers the methylation of pectin in plant tissue, but the use of  $\text{CaCl}_2$  has been shown to have a lessened firming capability if it is added to the desalting brine than if it was in the fermentation brine from the start (Buescher and Burgin 1988). This indicates its greatest firming action occurs before or during the fermentation process when the pectin has a higher degree of methylation.

### ***Alum***

Alum is the name for a class of hydrated chemical compounds containing two sulfate anions with both a monovalent cation, such as potassium, sodium, or ammonium, and a trivalent cation, usually aluminum. While it is not actually an alum, aluminum sulfate can be used interchangeably with the alum salts. Alum salts have been used in desalting brines after cucumber fermentation processes for many years because alum was thought by pickle processors to increase the crispness and firmness of pickled cucumbers (Fabian and Krum 1949). The firming capability of alum has been disputed by some, particularly in fresh pack cucumbers (Etchells and others 1977). Fresh pack cucumber pickles have been found to have cucumber firmness values 1.2-3.5 lb. less in the presence of 0.1% alum (Etchells and others 1972). Meanwhile, Buescher and Burgin (1988) found that the use of 0.3% alum in

the desalting process significantly improved the firmness of cucumbers compared to those desalted in the control treatment (water). However, fermented cucumber pickles desalted in 0.3% CaCl<sub>2</sub> had significantly firmer texture after 6 months shelf storage than both those desalted in alum and those desalted in water. McFeeters and Fleming (1989) likewise found calcium to be more effective than aluminum at lowering softening rates of mesocarp tissue brined in 1.5 M NaCl. Buescher and others (2011) also found elevated calcium concentrations to be more effective at retaining cucumber crispness than the use of alum in the desalting process. These studies suggest that alum is not needed if calcium has been introduced into the fermentation brine or desalting solution using at least 0.3% CaCl<sub>2</sub>. However, if insufficient CaCl<sub>2</sub> was included, 0.3% alum can increase the firmness of fermented cucumber pickles during desalting, but creates pickles that soften during shelf storage more quickly than those desalted in CaCl<sub>2</sub>.

### ***Acids and pH***

The presence of certain organic acids has been shown to affect the firmness of cucumber pickles. However, much of the work looking at organic acid effects has been done in fresh pack cucumber pickles which are acidified cucumbers that are pasteurized without undergoing a fermentation step. Fresh pack pickles have the benefit of eliminating enzyme softening effects because they are pasteurized after packing which should denature many enzymes.

Bell and others (1972) investigated the firmness of fresh pack cucumber pickles packed with brines intended to equilibrate to pH 4 using 2% acetic, 0.4% lactic, 0.4% citric, 0.4% malic, or 0.16% oxalic acid after 4 and 8 months storage. They found extensive softening that turned the cucumbers packed with oxalic acid to mushy texture quality (< 3 lb.

using a fruit pressure tester) while quality of the cucumbers packed in the other acids were of firm texture quality at 4 and 8 months (13.2-15.8 lb.). Doesburg (1961) also found oxalic acid softened plant tissue quite quickly, potentially by sequestering the natural calcium in the plants.

When Bell and others (1972) doubled the concentrations of the acids mentioned prior, the cucumbers packed in acetic acid retained inferior quality (10.4-12.5 lb.) while the cucumbers packed in lactic, citric, or malic acid were reduced to soft quality (5.5-9.5 lb.). These results showed less texture damage with acetic acid than the other acids and texture damage increased with time spent in the acid as well as greater concentrations of each acid. Howard and others (1994) studied softening in Jalapeno peppers with 7% salt and 0.9-4% acetic acid and found increased softening with greater concentrations of acetic acid. Fleming and others (1993), however, found no significant effect of acetic acid ranging from 2-4% in the presence of 2.5% salt and 0-0.4%  $\text{CaCl}_2$  when using the more sophisticated mesocarp puncture test by Thompson and others (1982) nor when testing Red Cherry or Jalapeno peppers. Calcium is a known firming agent, but when Howard and others (1994) tested the acetic acid concentrations with 0.2%  $\text{CaCl}_2$ , they found the  $\text{CaCl}_2$  was able to protect the fruit from the acid softening, which may minimize the acid softening, but did not eliminate the firmness difference.

Bell and others (1972) also tested the firmness of cucumbers packed with 2% NaCl brines using 0.2-1% lactic acid after 3 and 15 months to simulate bulk storage softening in fermentation brines since lactic acid is the primary product of fermentation. Increased lactic acid resulted in softer cucumber texture at both 3 and 15 months with texture at 15 months being soft or mushy (<9.2 lb.) for all levels of lactic acid. Etchells and others (1972) also

saw this same trend of decreasing firmness with higher lactic acid content in cucumbers packed with 3% NaCl and 0.18-0.54% lactic acid at 10 months storage. This showed that fermented cucumbers with high concentrations of lactic acid bulk stored for extensive time may undergo softening.

The pH of brines has also been found to affect the texture quality of cucumbers. McFeeters and Fleming (1991) looked at firmness and softening rates of blanched, dissected cucumber mesocarp tissue in 1.5 M NaCl brines buffered to pH 2.0-9.0 using a mesocarp puncture test developed by Thompson and others (1982). The softening rate increased as pH decreased below 3.5 and a large peak in softening rate occurred between a pH of 5.5 and 8.0. McFeeters and others (1995) looked at the pH effect of softening rates as well, but using cucumbers fermented for 24 days in 2% NaCl before dissecting the cucumber mesocarp tissue to pack into jars with pH ranging from 2.6-3.8. Softening rates were found to increase as pH decreased from 3.8 to 2.6. This softening pattern has also been observed in potatoes, beets, turnips, apples and cauliflower where products were less firm as they were stored in solutions with pH values decreasing from 4.0 to 3.0 and increasing from 5.0 to 6.5 (Doesburg 1961).

#### *1.4.2 Temperature*

Increased temperatures at any point during the fermented cucumber process have the ability to affect the texture properties of the final product. Softening rates have been shown to increase with temperature during cucumber bulk storage (McFeeters and others 1995). In this study, fermented cucumber slices with their endocarp removed were stored in brines of various pH levels and calcium concentrations at temperatures ranging from 25 to 65°C. Softening rate was determined by measuring firmness changes over time and a model was

developed to relate temperature, pH, and calcium levels to the rate of softening. Temperature effects on the softening rate were best modeled by a quadratic term in this study, showing the softening rate will increase more rapidly as the temperature increases. Rodrigo and Alvarruiz (1988) looked at how pasteurization temperatures affect texture using both instrumental and sensorial methods. Higher pasteurization temperatures were found to decrease firmness by a first order form of degradation for fermented cucumbers over longer pasteurization times.

Work has also been done on the texture changes associated with pasteurization temperatures and storage thereafter. Fleming and others (1993) found that  $\text{CaCl}_2$  had a protective effect against the firmness loss associated with pasteurization temperatures in pickled peppers as well as reducing softening during storage at 36.7 and 46.7°C. Nicholas and Pflug (1960) measured softening of fresh pack pickles stored at temperatures more indicative of warehouse storage (4, 22, 30, 32, and 38°C). They found the pickles stored at 38°C had softened considerably faster than at the other temperatures and were mushy less than 10 months into shelf-life using a fruit pressure tester. Pangborn and others (1959) also found firmness decreased at 37°C compared to 1, 21, and 30°C using measurements by both a trained panel and shear press.

#### *1.4.3 Non-Enzymatic Softening*

Non-enzymatic softening has been observed in blanched cucumbers with softening rates that can be manipulated by pH changes (McFeeters and Fleming 1991). Blanching was used to denature the enzymes to measure only the non-enzymatic softening rate changes. The cucumber samples with pH values below 3.5 had increased softening rates, likely due to acid hydrolysis of the pectin molecules. Acid hydrolysis of pectin is the addition of a water molecule to the glycosidic bond of pectin. Krall and McFeeters (1998) came to the same

conclusion that acid hydrolysis of pectin samples was occurring at pH ranges below 4 by measuring the rate of reducing sugar formation, the product of acid hydrolysis. Higher methylation of the pectin was found to have a lower rate of reducing sugar formation and therefore lower activity due to acid hydrolysis. Additionally, they found low methylated (<5%) pectin had its highest rate of reducing sugar formation at low pH and gradually tapered off as the pH rose. McFeeters and Fleming (1991) also found softening rates were accelerated between pH values of 5.5 to 8.0. This peak in softening rate was attributed to increased  $\beta$ -elimination reactions as the pH became less acidic and the decrease in the rate above pH 8 was associated with demethylation of the pectin substrate. Krall and McFeeters (1998) also found that degradation of pectin isolated from citrus could happen as the pH rose to neutral conditions, corroborating McFeeters and Fleming (1991). They attempted to find out if the degradation near neutral was from  $\beta$ -elimination reactions by absorbance at 235 nm, but above pH 4 a browning developed, interfering with the absorbance measurements. They also subjected polypectate to the same treatments and found that the increase in reducing group formation rate did not occur at higher pH values. Polypectate cannot undergo  $\beta$ -elimination because its degree of methylation (<5%) is too low. This provides greater evidence that  $\beta$ -elimination reactions are responsible for softening under neutral pH conditions. As the degree of methylation was lowered, the rate of acid hydrolysis at low pH values was higher and the rate of  $\beta$ -elimination reactions at neutral pH values was decreased. Therefore, with respect to pH, the degradation of polypectate followed an exponential decay curve with high softening at low pH and no softening at high pH values while the 35% methylated pectin had a U-shaped activity curve and the 70% methylated pectin had a J-shaped activity curve reaching a softening rate at pH 6 more than three times greater than its

activity at pH 2. Interestingly, calcium ions did not inhibit acid hydrolysis and the temperature did not have a large effect on acid hydrolysis rates indicating that these non-enzymatic softening methods are probably not occurring by the mechanism that calcium prevents and temperature accentuates.

Maruvada and McFeeters (2009) analyzed texture loss using blanched cucumbers in low salt fermentations to exclusively look at the non-enzymatic softening rates. They determined that at 0.3 M NaCl, enzymatic activity was partially responsible for observed softening over 12 weeks, but not entirely responsible as the blanched cucumbers were softer than the control fermentation using 1 M NaCl with 18 mM CaCl<sub>2</sub>. They hypothesized that the non-enzymatic softening was a result of noncovalent interactions such as conformational changes in cell wall components. However, their experimental design lacks the requisite treatments to make those conclusions because they did not include a treatment containing blanched cucumbers and CaCl<sub>2</sub> added to brine. CaCl<sub>2</sub> has been shown to act as a firming agent and its exclusion could explain the difference in firmness they attributed to non-enzymatic softening. Additionally, Cho and Buescher (2012) have shown since this publication was released that heat inactivation of polygalacturonase in brine requires longer and hotter treatments to be inactivated than was used by Maruvada and McFeeters. They found 90°C for 15 minutes completely inactivates PG and a 75°C heat treatment for 15 minutes would have 16% residual PG activity. Therefore, the 75°C for 6 minute treatment of Maruvada and McFeeters should not have been sufficient to inactivate all the enzymes. It is still possible the texture is affected by non-enzymatic softening, but the experiment by Maruvada and McFeeters (2009) was not designed to determine this.

#### 1.4.4 BLOATER DEFECTS

Physical defects known as bloaters cause serious economic losses for processors. Bloaters occur when gas pockets form inside cucumbers during fermentation. Bloaters are thought to form when naturally occurring intercellular air spaces of the cucumber experience increased pressure that ruptures the tissue to form larger gas pockets (Etchells and others 1968, Fleming and Pharr 1980). The increased pressure is hypothesized to come from the production of carbon dioxide (CO<sub>2</sub>) in the cucumber that is attempting to diffuse into the intercellular spaces of the cell to obtain equilibrium. CO<sub>2</sub> is produced by respiration of the cucumbers, heterofermentative yeasts, molds and bacteria, as well as malolactic fermentation by many organisms including the *Lactobacillus plantarum* and other homofermentative bacteria that carry out the primary fermentation (Etchells and Bell 1950, Etchells and others 1968, Fleming and others 1973a, Fleming and others 1973b, McFeeters and others 1984). Purging the CO<sub>2</sub> from the brines using nitrogen gas has been effective at producing fermented cucumbers with little to no bloater defects under controlled fermentation conditions (Fleming and others 1973a, Costilow and others 1977). The importance of purging can be seen in the much greater incidence of bloater damage found in non-purged fermentations, especially with higher fermentation temperatures, deeper locations in the tank, and higher cucumber to brine ratios (Etchells and others 1975). Bloating damage is also more frequent with larger cucumber sizes, increased salt concentration (from 5.3 to 21.1%), and lactic acid addition to the cover brine (Jones and others 1941, Etchells and Bell 1950, Jones and others 1940).

The expense of nitrogen purging led processors to look at a more cost effective purge process with air (Costilow and others 1977, Costilow and others 1980, Costilow and others



1981, Costilow and Uebersax 1982). However, air purging has resulted in softening of the fermenting cucumbers in the laboratory as well as commercially, so Gates and Costilow (1981) looked into factors influencing softening with high and low air flow rates. They found that high air purging resulted in very soft fermented cucumbers with high yeast growth. However, use of 0.035% (2.3 mM) potassium sorbate, nitrogen purging for at least two days followed by air purging, or pretreatment of the cucumber by blanching or immersion in hypochlorite solutions prevented the formation of soft pickles. The simplest and therefore more frequently implemented method is the addition of potassium sorbate. Potts and Fleming (1982) also looked into inhibition of mold induced softening in air-purged systems by the use of acetic acid. They found that 0.16% (28 mM) acetic acid reduced pectinase activity in the brines presumably by inhibiting mold growth. The lack of pectinase activity maintained cucumber firmness during fermentation. The explanation in both cases for the softening in air purged systems is that an aerobic microorganism producing a softening enzyme could be inhibited by potassium sorbate, acetic acid or other methods. Likewise, when Pérez-Díaz and others (2015) used air purged tanks, the fermentations spoiled until they used a purging routine one-eighth the normal routine to minimize the introduction of oxygen and prevent the proliferation of aerobic organisms. Using the minimal purging routine and potassium sorbate, they were successful in creating a controlled fermentation without sodium chloride.

### **1.5 Biochemical Mechanisms for Cucumber Tissue Softening**

The structure of most plant materials are affected by the plant cell wall polysaccharides with pectin, cellulose, and hemicellulose being the primary polysaccharide components (McFeeters 1985). Softening of plant tissue by pectinolytic enzymes has been

well studied in relation to the physiological maturation process produced by the plant during ripening and softening enzymes produced during infection by pathogenic organisms. One class of pectinolytic enzymes, polygalacturonases, has been extensively studied for the softening effects produced in fermented cucumbers as a result of hydrolysis of the pectin bonds. Other enzymes of interest include cellulases which break cellulose bonds, pectinesterase which modifies pectin by lowering the degree of esterification, and pectin lyases which break pectin bonds by trans-elimination reactions. Additionally, many of these enzymes have been examined to determine how calcium chloride interacts with the activity of the enzyme in a fermentation environment.

#### *1.5.1 Polygalacturonase Enzyme Softening*

Polygalacturonase (PG), also called pectinase, is an enzyme responsible for ripening processes in fruits (Payasi and others 2009, Van Buren 1979), but has also been found to be produced by species of fungi, yeast, and bacteria (Walter 1991). Polygalacturonase was first studied in cucumber fermentations by Bell and others (1950). PG hydrolyzes pectin and other galacturonans into galacturonic acid and oligosaccharides. There are two types of polygalacturonase enzymes, exo-polygalacturonase (exo-PG) and endo-polygalacturonase (endo-PG). Endo-PG is responsible for random hydrolysis of polygalacturonic acid while exo-PG specifically hydrolyzes polygalacturonic acid from the end of the polysaccharide producing monomers, dimers, and other small units. The difference in hydrolysis location means that endo-PG can produce a large change in pectin structure and function after only a few bonds have been hydrolyzed while exo-PG has a relatively small effect, even after breaking many bonds. This means that endo-PG can cause significant break down of pectin

resulting in physiological changes faster and with lower enzyme concentrations than exo-PG. Both types are produced by plants, pathogenic bacteria, fungi, and yeasts.

### ***Polygalacturonase Structure***

The structure of PG was hypothesized by comparing many sources of the enzyme and looking for conserved residues. Sequence conservation between different sources of the enzyme analyzed shows the importance of eight amino acid residues. These eight residues include three aspartate residues, one glycine, one lysine, one arginine, one histidine, and one asparagine (Kester and others 1996). The glycine is required because of the conformational angles required for the residue at that position. Glycine is very flexible due to its small size and is the only residue capable of occupying that position without changing the structure and resulting in loss of activity.

Through site-directed mutagenesis, van Santen and others (1999) examined the importance of six amino acid residues (the aspartates, histidine, arginine and lysine). By changing each residue and measuring the activity of the mutated enzyme, they determined that all of these residues are responsible for the activity of the enzyme through either substrate binding or catalysis. Mutation of the three aspartate residues or the histidine resulted in a diminished activity but did not affect the  $K_m$  value. Mutation of the lysine and arginine residues lowered the activity as well as the  $K_m$  value. Therefore, the histidine and aspartate residues are likely involved in catalysis while the lysine and arginine are involved in substrate binding. Based on location and water accessibility, two of the aspartate residues are hypothesized to bind to a water molecule activating it for nucleophilic attack of the  $\alpha(1\rightarrow4)$ -glycosidic bond while the last aspartate residue is a proton donor for the leaving group. The importance of the histidine residue in catalysis is not well understood; however,

there are hypotheses that it either facilitates the leaving group or that it is responsible for distorting the substrate.

### ***Polygalacturonase Sources***

Polygalacturonase has been isolated from a variety of yeasts, molds, plants, and bacterial sources. The most frequently studied bacterial source is *Erwinia caratovora*, but lactic acid bacteria including *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactococcus lactis* isolated from raw milk have also shown PG activity (Karam and Belarbi 1995). Additionally, yeasts such as *Saccharomyces cerevisiae* and molds such as *Aspergillus spp.* have been shown to exhibit PG activity (Visser and de Vries 2001).

The first investigation into sources of softening enzymes in cucumber fermentations came from Etchells and others (1958). They examined different parts of cucumbers for PG activity using the Bell and others (1950) method for viscosity changes. They found the highest activity came from the flowers of the plant. They also examined fungi populations from the plant for enzymatic activity and found many species that were capable of degrading pectin as well as cellulose. They determined the most common source of enzymatic activity came from the fungi that were introduced by the flowers of the plants. Pressey and Avants (1975) isolated a PG from cucumbers that was determined to be an exo-PG of 59 kDa in size that had an optimum pH of 5.5 and 0.4 mM CaCl<sub>2</sub>. McFeeters and others (1980) also used the same method to characterize the first endo-PG purified from cucumbers. This endo-PG was estimated to be 35 kDa in size which was smaller than other endo-PGs isolated from plants and the exo-PG previously isolated by Pressey and Avants. The endo-PG isolated by McFeeters and others was also dependent on pH and ionic strength with optimum conditions being ionic strength around 0.20 and a pH of 5.6.

Sakellaris and others (1988) identified a polygalacturonase enzyme produced by *Lactobacillus plantarum* strain BA11. They determined the optimum pH to be 4.5 and the optimum temperature to be 30°C. In 1989, the same group found there were actually two PG enzymes, PG I and PG II, after purification (Sakellaris and others 1989). The isomers were found to have the same mass (~32 kDa), isoelectric points (~5.5), pH (4.5) and temperature (35°C) optimums, and pH (3.5-6) and temperature (up to 50°C) stabilities with the only difference being variation in activities and  $K_m$  values. Cucumber fermentations are quickly acidified to a pH below 4 by the addition of an acidulant or by fermentation and result in a pH below 3.5. The optimum pH value of these PG enzymes are well above the pH of the fermented cucumbers, otherwise the fermented cucumbers would likely be very soft. Other LAB including *Lactobacillus casei*, *Lactococcus lactis*, and *Leuconostoc mesenteroides* have been identified as producing pectinolytic enzymes as well (Karam and Belarbi 1995, Juven and others 1985).

Costilow and others (1980) tested air-purged fermentations for molds that softened cucumbers by displaying pectinase activity. The softening observed frequently occurred localized to only a section of the fermentation rather than uniformly throughout the tank. The growth of the associated mold can be controlled by limiting the oxygen content introduced into the tank by purging with air or nitrogen.

### ***Polygalacturonase Inhibition***

Inhibition of polygalacturonase has been tested with a variety of possible inhibitors. Bell and Etchells (1961) tested the effect of different salt concentrations on texture over time of fresh-packed cucumbers inoculated with pectinase. They used a commercial source of pectinase isolated from a fungus, a purified pectinase enzyme, and four pectinase producing

fungi to test the effect of enzyme concentration and source. Higher salt concentrations were associated with firmer cucumbers, and higher enzyme concentration was associated with softer cucumbers. They also showed that if the cell free filtrate of a pectinase producing fungi was inoculated into fresh-pack cucumbers, the cucumbers retained a firmer texture if brined with higher salt concentrations.

Polygalacturonase has been found in many fruits but has been most studied in tomatoes. Wills and Rigney (1979) determined how calcium levels affected the activity of PG as well as another pectic enzyme called pectinesterase (PE) that lowers the methylation of the pectin for PG to work more efficiently (Jansen and MacDonnell 1945). Both enzymes were isolated from tomatoes. Wills and Rigney found that the highest activity for pectinesterase occurred at concentrations of calcium around 0.01 M and a pH between 7 and 8 while PG optimum conditions were no calcium and a pH of 4.5 with activity being very sensitive to pH and decreasing slowly as calcium was added.

Buescher and others (1979) noted calcium chloride is used commonly in the food industry for increasing firmness. To test if calcium chloride had an inhibitory effect on polygalacturonase, they tested the pickle firmness of a control fermentation and treatments with 1) calcium chloride added, 2) PG added, and 3) calcium chloride and PG added, all in low and high salt concentrations for a total of eight treatments. They found all the treatments with PG added had no real change in PG activity which determined that calcium was not irreversibly inhibiting the PG. Additionally, all treatments with CaCl<sub>2</sub> added were firmer than the control treatments despite the presence of PG or concentration of NaCl. Cucumbers fermented in low salt brines with PG added were significantly less firm than all other treatments followed by the cucumbers from the high salt PG fermentation.

Hudson and Buescher (1986) determined the effect of pectin esterification on the ability of PG to soften the cucumbers. Hudson and Buescher inhibited the pectinesterase to keep the esterification of the pectin high enough that polygalacturonase could not use the pectin. They found the degree of esterification (DE) greatly influenced the cucumber firmness if the DE was below  $12.3 \pm 1.2$  but firmness was unchanged above that value. They found treatments using calcium chloride in the brine or blanching kept the DE above the 12.3 cutoff value but all fermentation treatments lowered the DE.

### *Polygalacturonase Assays*

The first PG tests for cucumbers used viscosity changes (Bell and others 1955) but later tests were modified from a method by Nelson (1944) for reducing sugars to measure the production of galacturonic acid. Pressey and Avants (1973) compared the two methods using peaches and found that the results of these two tests together could be used to differentiate between endo-PG and exo-PG.

Since the development of the first PG assays using viscosity and a reducing sugar method, many other methods have been developed based on the same principles. Buescher and Burgin (1992) developed a qualitative diffusion plate assay using Ruthenium Red, an inorganic dye that binds to intact polygalacturonic acid. A clear zone results from the breakdown of polygalacturonic acid around the sample well, and the diameter of the clear zone was used to determine PG activity levels in a liquid sample such as spent fermentation brine. A regression line was generated with a correlation coefficient of 0.964 comparing the clear zone diameter to the % viscosity change using the Bell and others (1955) method. Torres and others (2011) also used ruthenium red dye to spectrophotometrically determine the endo-PG activity by measuring the concentration of residual ruthenium red after the

ruthenium red-polygalacturonic acid complex precipitated to the bottom of a cuvette. The use of these methods with a ruthenium red dye has greatly improved the throughput of polygalacturonase assays.

### *1.5.2 Cellulase*

Cellulase enzymes degrade cellulose, another structural polysaccharide found in the cell wall of plants. Cx-cellulase was first reported in cucumber fermentations in a paper presented at the annual IFT Meeting by Etchells and others (1955). The same research group developed a method for determining activity of both polygalacturonase and Cx-cellulase in 1955 (Bell and others 1955). Buescher and Hudson (1984) irreversibly destroyed the PG from a commercial source of Cx-cellulase using 7 N urea to look at the effect of Cx-cellulase on fermented cucumber softening without polygalacturonase because they are often found together. They also wanted to determine the effect of calcium on Cx-cellulase to see if that was the mechanism by which  $\text{CaCl}_2$  acts as a firming agent. Calcium treatments had firmer texture while Cx-cellulase created significant texture loss over long term storage, but the calcium in the combination treatment negated the Cx-cellulase and had a firmer texture than the control. Cx-cellulase is not frequently monitored by pickling processors because its softening effect is minimal during the beginning of storage (Buescher and Hudson 1984) and softening by Cx-cellulose is prevented by the addition of  $\text{CaCl}_2$ . Cx-cellulase activity has been found in fermentation brines and from fungi (Bell and others 1955, Etchells and others 1958), but now that  $\text{CaCl}_2$  and potassium sorbate are included in commercial fermentation brines, Cx-cellulase production by the fungi sources is believed to be strongly inhibited.



### *1.5.3 Pectinesterase*

Native plant sources of pectin typically have a high degree of esterification. Pectinesterase is a pectinolytic enzyme that deesterifies the pectin, lowering the degree of esterification. Bell and others (1951) were the first to study the presence of pectinesterase in cucumbers. Bell and others (1950) attempted to determine the effect of polygalacturonase activity on softening of fermented cucumbers and discovered pectinesterase. Prior studies (Jansen and MacDonnell 1945) had shown that pectin must be de-esterified before glycosidic hydrolysis could occur by polygalacturonase. This means that pectinesterase prepares the pectin for use by polygalacturonase which explains why they were found together. Bell and others (1951) analysis of the enzyme revealed that it is most commonly found in the leaves, flowers and small fruits of the plant. They also found the enzyme was consistently in higher concentrations in cucumber juice than in the brine after one week storage but smaller cucumbers had greater diffusion into the brine. However, after undergoing a fermentation period, pectinesterase activity was severely diminished due to low pH as they found the pectinesterase optimum pH is 7.5.

McFeeters and others (1985) examined the effect of thermal inactivation and calcium on pectinesterase in cucumber slices. They found  $\text{CaCl}_2$  concentration had no effect on pectinesterase, which is not the case with other sources of the enzyme. Concentrations of 0.05 M  $\text{Ca}^{2+}$  has been reported to inhibit PE activity in alfalfa while 0.01 M  $\text{Ca}^{2+}$  was reported to suppress tomato PE activity (Walter 1991). Since McFeeters and others (1985) only tested concentrations up to 0.04 M calcium chloride, it is uncertain whether they did not reach the level of inhibition or whether calcium does not inhibit PE from cucumbers. They also tested thermal inactivation using blanching treatments of 54, 66, 81 and 99°C for three

minutes. PE activity for the 81 and 99°C treatments showed significant loss of activity; however the 81°C treatment recovered some of its activity during storage resulting in lower pectin methylation. Calcium was associated with firmer texture over time, which is well established, but the calcium levels had no effect on enzyme activity, indicating that the calcium was able to retain firmness while demethylation of pectin occurred.

#### 1.5.4 Endo-Pectin Lyase

Endo-pectin lyase (endo-PNL) is an enzyme that can degrade highly esterified pectin and has increased activity with greater esterification (Walter 1991). Endo-PNL does not degrade polygalacturonic acid, which has a degree of esterification (DE) of 0. The optimum pH was determined to be between 4.9 and 6.5 depending on source organism and the level of pectin esterification. Calcium ions at concentrations between 0.5 and 1.0 mM increase activity but higher concentration are inhibitory. Magnesium and sodium ions can also promote activity of endo-PNL but with lesser effects. Juven and others (1985) analyzed a strain of *Leuconostoc mesenteroides* isolated from tomato juice for pectin degradation. They analyzed the supernatant of the strain in citric acid broth with pectin or carboxymethylcellulose added and found that the carboxymethylcellulose was not utilized while the pectin was used. Changing pectin sources to test different levels of methyl esterification, they found that the activity was increased with greater methyl esterification. The inability to use polygalacturonic acid, lack of a pectinesterase, and the increase in activity with greater esterification led Juven and others to the conclusion that the *Leuconostoc mesenteroides* isolate was producing an endo-pectin lyase.

By any method of texture changes that could occur, the variability in texture quality observed in the implementing of the CaCl<sub>2</sub> fermentation process on the commercial scale

must be confirmed and analyzed further. Research is still needed to determine if any production variables can be linked to the variability of the texture quality and the causes of the softening of cucumbers fermented and stored in 0.1 M CaCl<sub>2</sub>.

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**Table 1.1 – Changes in Cucumber Texture as a Result of Various Physical and Chemical Factors.**

<b>Factor</b>	<b>Effect</b>	<b>Use</b>	<b>References</b>
<b>Cultivar</b>	Variable	-	Jones and Etchells 1950 Jeon and others 1973 Suojala-Ahlfors 2005
<b>Growing Season</b>	Variable	-	Jones and others 1941 Suojala-Ahlfors 2005
<b>Calcium</b>	Firming	Other plants	Kertesz 1939 Van Buren 1979 Howard and others 1994
		Fresh Pack	Etchells and others 1977 McFeeters and Fleming 1991
		Fermentation and Bulk Storage	Buescher and others 1979 Tang and McFeeters 1983 Buescher and Hudson 1984 Buescher and Burgin 1988 Buescher and others 2011
		Desalting	Buescher and Burgin 1988
<b>Alum</b>	Softening	Fresh Pack	Etchells and others 1972 Etchells and others 1977
	Slight Firming	Desalting	Buescher and Burgin 1988 Buescher and others 2011
<b>Oxalic Acid</b>	Severe Softening	Fresh Pack	Doesburg 1961 Bell and others 1972
<b>Acetic Acid</b>	Slight Softening	Fresh Pack	Bell and others 1972 Howard and others 1994
	None	Fresh Pack	Fleming and others 1993
<b>Lactic Acid</b>	Moderate Softening	Fresh Pack	Bell and others 1972 Etchells and others 1972
<b>Low pH</b>	Softening	Other plants	Doesburg 1961
		Fresh Pack	McFeeters and Fleming 1991
		Bulk Storage	McFeeters and others 1995
<b>High Temperatures</b>	Softening	Bulk Storage	McFeeters and others 1995
		Pasteurization	Rodrigo and Alvarruiz 1988 Fleming and others 1993
		Finished Product Storage	Nicholas and Pflug 1960 Pangborn and others 1959

## **1.7 Overview of Proposed Research**

### *1.7.1 Hypothesis*

Mesocarp firmness values of cucumbers fermented using the CaCl<sub>2</sub> brining process developed by Pérez-Díaz and others (2015) will be significantly less firm than the typical NaCl brining process during routine commercial production of hamburger dill chip pickles due to a lack of acidulant in the initial brine resulting in a less selective environment during the initiation of fermentation.

### *1.7.2 Research Objectives*

1. To determine variability in mesocarp firmness encountered during normal commercial production using NaCl and CaCl<sub>2</sub> fermentation processes.
2. To determine the effects of brine acidification on the fermentation microbiota and texture quality of cucumbers fermented with the CaCl<sub>2</sub> fermentation process.

### *1.7.3 Approach*

Commercial cucumber fermentation samples originating from a local processor will be analyzed for mesocarp firmness, color, bloater defects, pH, and fermentation metabolites to determine the variability in quality for NaCl and CaCl<sub>2</sub> brining processes. These samples will include raw cucumbers, fermented and bulk stored cucumbers, desalted cucumbers, and finished product stored 6 months to determine if and when a change in texture is occurring. Information on commercial production variables such as cucumber size, fermentation start date, and fermentation vessel will be collected by the processor and recorded.

The effect of brine acidification on the firmness of cucumbers fermented using the CaCl<sub>2</sub> fermentation process will be accomplished using laboratory fermentations with brine

compositions according to the full factorial combination of CaCl<sub>2</sub>/NaCl with acidification using acetic acid, hydrochloric acid, or no acid. Enumeration of yeasts and molds, *Enterobacteriaceae*, and lactic acid bacteria as well as pH and fermentation metabolites will be monitored throughout the fermentation and texture will be monitored weekly during fermentation and after a simulated bulk storage time.

#### *1.7.4 Impact/Significance*

The pickling industry has been looking for ways to minimize their usage of sodium chloride because its release in wastewater is detrimental to the environment. Various methods have been tested over the years, but the fermentation process using 100 mM CaCl<sub>2</sub>, 6 mM potassium sorbate, a starter culture, and a reduced purge rate has received the greatest chance of implementation since the introduction of brine recycling. This new fermentation process has the potential to reduce the chloride content of the brines by more than 80% compared to the current method, 1 M NaCl and 30 mM CaCl<sub>2</sub> brines, but this process will not be successful unless quality of the product matches the current process. This project will determine the variability and quality differences between the CaCl<sub>2</sub> fermentation process and the current commercial fermentation process for cucumber tissue firmness as well as color, bloater defects, pH, and fermentation metabolites. Research will also focus on understanding the variability in firmness to provide understanding of how to create the highest quality products possible.

## **CHAPTER 2 – Quality of Cucumbers Commercially Fermented in Calcium Chloride Brine for Reduced Environmental Impact**

Target Journal: Journal of Food Quality

### **2.1 Abstract**

Commercial fermentation of cucumbers is conducted using brines that equilibrate to 0.9-1.4 M sodium chloride (NaCl), producing large volumes of wastewater with high salt concentrations. An alternative process using 0.1 M calcium chloride (CaCl<sub>2</sub>) has been developed to eliminate NaCl from fermentation brines, reducing the environmental impact of brining operations. This study evaluated the quality of fermented cucumbers produced commercially using the CaCl<sub>2</sub> brining process. Over the 2013 and 2014 production seasons, 41 fermentations conducted in brines prepared to equilibrate with the cucumbers to 0.1 M CaCl<sub>2</sub>, 6 mM potassium sorbate, and 10<sup>5</sup>-10<sup>6</sup> CFU/mL *Lactobacillus plantarum* starter culture with a minimal air-purging routine were compared to 27 fermentations using standard industry practices. Production variables included commercial processor (n=6), fermentation start date (June-September), fermentation vessel size (10,000-40,000 L), cucumber size (2.7-5.1 cm diameter), and bulk storage time before processing into finished products (55-280 days). Cucumber mesocarp tissue firmness was measured using a puncture test with a 3 mm probe on a 6.7 mm slice from 30 cucumbers/fermentation. Mesocarp color was measured using the L\*a\*b\* color scale, and bloater defects were scored on 25 cucumbers/fermentation. Brine samples were used to measure pH and quantify organic acids and residual sugars using High Performance Liquid Chromatography. Complete lactic acid fermentations were achieved with terminal fermentation pH values of 3.23 ± 0.09 and 3.30 ± 0.12 for CaCl<sub>2</sub> and NaCl brines, respectively. Fermented, CaCl<sub>2</sub> brined cucumbers had an average peak force

1.7 N lower than NaCl brined cucumbers, which remained significant in the finished product ( $P < 0.0001$ ). Bulk storage time decreased mesocarp firmness by 0.74 N after 100 days, regardless of fermentation process. Fermented cucumbers brined in  $\text{CaCl}_2$  had significantly increased hue values and decreased chroma values compared to those fermented in NaCl brines ( $P < 0.0269$ ). Longer bulk storage times were correlated with lower chroma values for cucumber mesocarp tissue ( $P = 0.0003$ ). Additional research is warranted to understand the tissue softening observed in the  $\text{CaCl}_2$  brined cucumber fermentations and identify process variables for quality improvements in this environmentally-friendly fermentation process.



## 2.2 Introduction

Commercial cucumber fermentation is a preservation method that depends on high sodium chloride (NaCl) concentrations. These fermentations are conducted in 10,000 to 40,000 L open-top tanks containing at least 0.86 M (5%) NaCl, equilibrated. This salt concentration enables a natural fermentation to occur by selecting for the lactic acid bacteria present on the cucumbers and inhibiting the salt sensitive spoilage bacteria. The fermented cucumbers are then desalted to palatable salt levels by pumping the fermentation brine out of the tanks and diluting the cucumbers in fresh water. This process reliably produces high quality, fermented pickle products but produces significant volumes of high salt wastewater with chloride concentrations above the EPA's Secondary Drinking Water Standards for suggested levels of less than 250 ppm or 7 mM chloride (U.S. Environmental Protection Agency 2009). To lessen the amount of salt in wastewater, the spent fermentation brines are now commonly stored and reused in future fermentations (McFeeters and others 1978). Despite extensive reuse, processors still routinely produce wastewater above the Secondary Drinking Water Standards. Pérez-Díaz and others (2015) estimated that the production of fermented cucumber pickles in the US resulted in 45 million liters of wastewater from desalting processes containing 0.58 M NaCl, 1.3 billion liters of wastewater with 79 mM chlorides, and 12,000 tons of salt sludge solid waste delivered to the landfill.

An alternative fermentation process utilizing 0.1 M calcium chloride ( $\text{CaCl}_2$ ), 0.025 M acetic acid, and a *Lactobacillus plantarum* starter culture was developed by McFeeters and Pérez-Díaz (2010) and found to produce a complete fermentation with fermented cucumbers that maintained firmness without using any NaCl and much lower chloride content. Since the development of this process, attempts to commercialize the technology have resulted in

slight adjustments of the brine formulation and process. These adjustments have included the elimination of the acetic acid, the addition of 6 mM potassium sorbate, and the introduction of a minimal purging routine (Pérez-Díaz and others 2015). A typical purging routine utilizes the flow of air or nitrogen gas through the tank during the active fermentation period to help encourage the removal of carbon dioxide (Fleming and others 1973a, Costilow and others 1977, Costilow and Uebersax 1982). Carbon dioxide (CO<sub>2</sub>) is produced as a product of heterofermentative fermentation, malolactic fermentation, and respiration (Etchells and Bell 1950, Etchells and others 1968, Fleming and others 1973a, Fleming and others 1973b, McFeeters and others 1984, Costilow and others 1977). An excess concentration of CO<sub>2</sub> can cause hollow cavities in the flesh of the cucumber that are physical defects called bloaters. Both air and nitrogen purging have been suggested for use and implemented in commercial fermentations to remove the CO<sub>2</sub> from the brine to minimize bloater damage. Nitrogen purging is ideal as air purging can introduce oxygen into the tank allowing aerobic microorganisms to flourish, lowering the quality of the product in the fermentation; however, nitrogen purging is too costly for most processors (Costilow and others 1980, Gates and Costilow 1981). During the early stages of commercialization, fermentations using the alternative process were found to spoil much more easily than the current commercial process. Therefore, the purging routine was altered to minimize the introduction of oxygen and potassium sorbate was added as a preservative against yeast spoilage (Pérez-Díaz and others 2015). Although the use of calcium chloride has been shown to protect against acetic acid softening (Howard and others 1994) and acetic acid is the least destructive organic acid with regard to cucumber firmness (Bell and others 1972), acetic acid can serve as a substrate for oxidative spoilage yeasts such as *Issatchenkia occidentalis* and *Pichia manshurica*

(Franco and Pérez-Díaz 2012). Acetic acid was thought to be a substrate for these spoilage microorganisms, so it was removed from the brine formulation (Pérez-Díaz and others 2015).

Firmness measurements of pickles produced commercially using this alternative fermentation process have shown variable results. In the laboratory development of the process, mesocarp firmness of cucumbers fermented in this  $\text{CaCl}_2$  brine was found to be insignificantly different from the cucumbers fermented in a commercial brine formulation (McFeeters and Pérez-Díaz 2010). However, cucumbers fermented in 12,500 L open-top tanks with the post-commercialization formulation changes, have been shown to have significantly different mesocarp firmness values as well as lower hardness, fracturability, crispness and crunchiness scores using a trained sensory panel (Pérez-Díaz and others 2015). Consumer testing has also shown a decreased texture liking for cucumber pickles fermented in the calcium chloride brines in some experiments (Wilson and others 2015).

The objective of this study was to determine the variability in physical, textural, and color quality of cucumbers fermented using the  $\text{CaCl}_2$  brining process and typical  $\text{NaCl}$  brining process during routine commercial production of hamburger dill chip pickles.

## **2.3 Materials and Methods**

### *2.3.1 Commercial Scale Cucumber Fermentations*

Pickling cucumbers grown throughout the United States over the 2013 and 2014 growing seasons were fermented in 10,000 to 40,000 L open-top tanks at six processing facilities between the months of June and September. Fermentation tanks contained size 2A (2.7-3.5 cm diameter), 2B (3.5-3.8 cm diameter), 3A (3.8-4.4 cm diameter) or 3B (4.4-5.1 cm diameter) cucumbers with a fermentation brine. Fermentation brines for the control fermentations were prepared using the current commercial process and were comprised of

recycled brines with additional sodium chloride, calcium chloride, and/or potassium sorbate such that the cucumbers equilibrated to approximately 1.03 M sodium chloride, 40 mM calcium chloride, and 2 mM potassium sorbate. Fresh brines for the calcium chloride brining process were created to equilibrate with the fresh cucumbers to concentrations of 100 mM calcium chloride and 6 mM potassium sorbate. Not all processors used every cucumber size for both brine treatments, but in total 41 fermentations were monitored using the CaCl<sub>2</sub> brining process and 27 control, NaCl brines underwent standard industry practices.

The tanking process began with the addition of cushion brines (18-20 inches deep) to the tanks to minimize damage as the cucumbers were added to the tanks. Cucumbers were added to the tanks and then wooden boards were placed over the top of the cucumbers to ensure they stayed submerged during fermentation. Brines were then added to the tanks and the calcium chloride brined tanks were inoculated with 10<sup>6</sup> CFU/mL *Lactobacillus plantarum* (LA0445 or other presumptive *Lactobacillus plantarum* isolates from cucumber fermentations unpublished, USDA-ARS, Food Science Research Unit, Raleigh, NC culture collection) starter culture prepared according to the kosher process described by Pérez-Díaz and McFeeters (2011). Sodium chloride brined cucumbers underwent a natural fermentation. Sodium chloride brined cucumbers at processor 1 were air-purged in cycles of eight hours followed by a four hour reprieve for the duration of fermentation. During the 2013 season, calcium chloride brined cucumbers at processor 1 received a minimal purge cycle of only two hours, twice a day, but followed the same purge routine as the sodium chloride tanks during the 2014 season. All other processors used a purging routine of 20 hours purging each day for all fermentations.

Fermented cucumbers were bulk stored in the fermentation brine between 55 and 280 days before processing into finished products. Prior to processing, a five gallon sample of cucumbers and brine was collected for analysis hereafter referred to as “Fermented” samples. Processing of the  $\text{CaCl}_2$  brined cucumbers utilized a two-step desalting procedure that entailed the pumped removal of the fermentation brine and replacement with fresh water. This was allowed to equilibrate for a day, during which the cucumbers were air-purged for 2 hours before the liquid was pumped out again. Freshly made 0.2% alum water was added to the tanks and allowed to equilibrate for a second day, again allowing 2 hours of purging. The  $\text{NaCl}$  brined tanks followed a one desalt procedure where the fermentation brine was removed and replaced with 0.2% alum water and allowed to equilibrate for one day. A five gallon sample of cucumbers and brine was collected for analysis just before packaging into finished product and will be referred to as “Desalted” samples. The alum water was then pumped out of the tank and the cucumbers were pumped to the packing line for cutting, packing, addition of cover liquor, capping, and pasteurization using standard practices. Cover liquors were formulated for each fermentation to create finished products that equilibrated to the processor’s desired product specifications such that cucumbers fermented in  $\text{CaCl}_2$  and cucumbers fermented in  $\text{NaCl}$  would be stored as finished product with the specified concentrations of  $\text{NaCl}$ ,  $\text{CaCl}_2$ , vinegar, and flavors. Finished products (at least 3/fermentation) were collected near the beginning, middle, and end of processing of each fermentation and stored for six months at ambient temperature before being analyzed as a “Finished Product” sample.

### *2.3.2 Bloater Defects*

Bloater severity and type of bloater were recorded for 25 cucumbers sliced longitudinally for the Fermented and Desalted samples (Etchells and others 1974). Bloater Indices were calculated as described by Fleming and others (1977).

### *2.3.3 Texture Analysis*

Instrumental firmness measurements were performed using a mesocarp puncture test. A  $6.7 \pm 0.1$  mm slice was taken from the center of 30 cucumbers for the raw, fermented, and desalted samples for testing. Finished products were crinkle cut using a commercial slicer before packing into jars, so 30 crinkle cut slices were collected evenly from the top, middle, and bottom of three jars 6 months after pasteurization. The mesocarp puncture test was conducted on a TA.XT Plus Texture Analyzer (Texture Technologies Corp, Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) using a 3 mm probe to puncture the mesocarp tissue at a test speed of 2.5 mm/sec (Thompson and others 1982, Yoshioka and others 2009). In turn, the mesocarp of one lobe of each slice was centered above a 3.1 mm hole on the base plate and the probe was lowered through the sample. The test was conducted and data analyzed using Texture Expert software (version 6.1.3.0, Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK). The peak force measured during the puncture test and the thickness of each slice were determined and recorded to create an average peak force and average thickness for each fermentation.

### *2.3.4 Color Analysis*

Cucumber mesocarp color was measured using a Minolta Chroma Meter model CR-300 (Minolta Co., Ltd., Osaka, Japan) to record the L\*, a\*, and b\* values of a different lobe of the 30 slices used for firmness measurements. The cucumber slices were placed on a

representative cucumber background at least 1.5 inches high to account for any translucency of the slices. Values of hue and chroma were calculated to convert the CIELAB color variables to the polar coordinate system CIELCH as follows:

$$\text{Hue} = \tan^{-1}(b^*/a^*) \quad [1]$$

$$\text{Chroma} = (a^{*2} + b^{*2})^{1/2} \quad [2]$$

where hue indicates the pigment of the color and chroma indicates the intensity. A hue of  $0^\circ/360^\circ$  represents red,  $90^\circ$  – yellow,  $180^\circ$  – green, and  $270^\circ$  – blue.

### 2.3.5 Detection of Fermentation Metabolites

Concentrations of organic acids, residual sugars, and some spoilage metabolites were quantified by High Performance Liquid Chromatography analysis using an Agilent 1260 Infinity HPLC (Agilent Technologies Inc., Santa Clara, California, USA) equipped with an HPX-87H ion exchange column for organic acid analysis (Bio-Rad Laboratories, Hercules, California, USA). Chromatography was conducted with 0.03 N sulfuric acid as an eluent at a flow rate of 0.6 mL/minute and using a column temperature of  $37^\circ\text{C}$ . Organic acids were quantified using an Agilent 1260 Infinity DAD detector (Agilent Technologies Inc., Santa Clara, California, USA) at 210 nm. Monomeric sugars and alcohols were quantified using an Agilent 1260 Infinity RI detector (Agilent Technologies Inc., Santa Clara, California, USA) connected in series with the DAD detector.

### 2.3.6 Chemical Analyses

A Fisher Accumet (model AR25) pH meter calibrated with standard pH 2, 4, and 7 buffers (Fisher Scientific) was used for pH measurement. Calcium concentrations were determined by titration with 0.025 N disodium ethylenediaminetetraacetate dihydrate (EDTA) with hydroxy naphthol blue as the indicator according to AOAC Method 968.31

(2000). Chloride concentrations were determined by titration (Fajans Method) with 0.171 N silver nitrate ( $\text{AgNO}_3$ ) and a dichlorofluorescein indicator Fleming and others (2001). Reported values for NaCl are calculated by subtracting the chloride contributed by  $\text{CaCl}_2$  from the total chloride content using the formula  $[\text{NaCl}] = [\text{Cl}^-] - 2*[\text{CaCl}_2]$  where the concentration of  $\text{CaCl}_2$  is presumed to be equal to the concentration of calcium.

### 2.3.7 Polygalacturonase Activity

Polygalacturonase (PG) activity was assayed using the diffusion plate assay developed by Buescher and Burgin (1992). Polygalacturonase activity was assayed for both brines and slurries of cucumbers. Slurries were produced by blending the cucumbers from texture and color analysis in a Waring Blender until homogenous. An aliquot of 30  $\mu\text{L}$  of each brine or slurry supernatant was pipetted into a well created by a size 4 cork borer and the PG plates were incubated at 38°C for 48 hours. After incubation, the plates were stained for 30 minutes using ruthenium red which binds to polygalacturonic acid. After staining, the plates were rinsed and destained in DI water for 20 hours. The diameter of the clear zone around each sample well was measured using a caliper. Clear zones indicated the activity of the enzyme as the dye will not bind to galacturonic acid residues.

### 2.3.8 Statistical Analysis

All results were analyzed using SAS statistical software (version 9.4, SAS Institute, Cary, NC, USA). A mixed model analysis of variance (ANOVA) was used with least square means. The fermentation process, cucumber size, and stage in processing were designated as fixed effects and the individual fermentations were designated as random effects. Statistical significance was indicated at  $P < 0.05$ . Values are presented as means  $\pm$  standard deviation. Figures are represented with error bars using standard error.



## 2.4 Results and Discussion

Raw cucumber samples, representative of those fermented, had initial mesocarp firmness of  $8.7 \pm 0.9$  N, sugar content of  $54.17 \pm 12.00$  mM glucose and  $65.21 \pm 14.14$  mM fructose, and calcium content of  $3.0 \pm 0.8$  mM. Average terminal pH values as well as CaCl<sub>2</sub>, NaCl, lactic acid, and acetic acid concentrations for CaCl<sub>2</sub> and NaCl brined fermentations after the combination of fermentation and storage can be found in Table 2.1. Average CaCl<sub>2</sub> concentrations were  $37.9 \pm 4.6$  mM in the current commercial process as expected and  $85.2 \pm 6.9$  mM in the CaCl<sub>2</sub> process, a little lower than the intended 100 mM. These values are also slightly inflated because they include the calcium naturally present in the cucumber which averaged  $3.0 \pm 0.8$  mM for the raw cucumbers in this study. It is possible that the calcium content was underestimated because the calcium was bound to the cucumber tissue and therefore became undetectable by simple titration procedures. Additionally, variability in the ratio of cucumbers to brine used in the fermentation and the purity of the CaCl<sub>2</sub> ingredient could contribute to the lower than targeted calcium content measured in the commercial fermentations. Cucumbers fermented and stored in 100 mM, 200 mM, and 300 mM CaCl<sub>2</sub> brines without NaCl have been shown to produce fermented cucumbers with mesocarp firmness insignificantly different from cucumbers fermented and stored in a 1.03 M NaCl/40 mM CaCl<sub>2</sub> brine over 8 months storage in a laboratory experiment, but to our knowledge no research has investigated lower concentrations of CaCl<sub>2</sub> without NaCl included in the brine formulation (McFeeters and Pérez-Díaz 2010). The minimum CaCl<sub>2</sub> concentration to retain firmness in the absence of NaCl is unknown, but the range observed in our commercial fermentations (70.5-98.3 mM CaCl<sub>2</sub>) was below anything tested in a controlled experiment thus far and therefore may have been at or below the

minimum concentration. However, the  $\text{CaCl}_2$  concentration was not significantly correlated with mesocarp firmness ( $P = 0.9917$ ) so there is likely another factor affecting the mesocarp firmness.

Fermentations achieved safe, insignificantly different pH values of  $3.23 \pm 0.09$  in the  $\text{CaCl}_2$  process and  $3.30 \pm 0.12$  in the current commercial process using NaCl. Lactic acid and acetic acid were significantly higher in the NaCl fermentation process despite the insignificant difference in pH values, likely because they came from recycled brines containing residual acid generated in previous fermentations. It is possible the terminal pH values are not significantly different from each other because of the different ratio of lactic to acetic acid. Lactic acid has a pKa of 3.86 which is much lower than the pKa of acetic acid, 4.76. Since the NaCl tank has a greater acetic acid to lactic acid ratio, the pH could be insignificant because of greater buffering. The presence of residual acid coming from recycled brines likely means the NaCl brines started with a lower initial pH than the initial pH of the calcium chloride brines which were not acidified prior to fermentation. Based on the quality control data collected by the commercial processors, the NaCl fermentations had an average pH of  $3.6 \pm 0.4$  on day 1 while the experimental tanks had an average pH of  $4.9 \pm 0.2$  on day 1.

A desalting process is used in both fermentation processes to lower the NaCl to palatable concentrations or to lower the  $\text{CaCl}_2$  concentration to below the legal limit of 36 mM in the finished product (21 CFR 184.1193). Table 2.1 shows how the average pH values,  $\text{CaCl}_2$ , NaCl, lactic acid, and acetic acid concentrations for  $\text{CaCl}_2$  and NaCl brined tanks were affected by the desalting process. During desalting, the pH rose to  $3.47 \pm 0.28$  for tanks with  $\text{CaCl}_2$  brines and  $3.40 \pm 0.19$  for tanks with NaCl brines as the lactic acid and

acetic acid concentrations were diluted along with the salts (Table 2.1). As mentioned earlier, the ratio of acetic acid to lactic acid may be contributing to greater buffering capacity by the acids. This would explain how the two treatments, NaCl brined and CaCl<sub>2</sub> brined, could have insignificantly different desalted pH values while the NaCl brined cucumbers have significantly more lactic acid and acetic acid than the CaCl<sub>2</sub> brined cucumbers. The lactic acid and acetic acid concentrations are also much lower in the cucumbers fermented in CaCl<sub>2</sub> brines because they were desalted two times while cucumbers fermented in NaCl brines were only desalted once.

In the production of finished pickle products, desalted cucumbers are separated from the desalting water before being cut and packed with freshly made cover liquor containing sufficient NaCl, CaCl<sub>2</sub>, vinegar, and flavors, such as dill, to equilibrate to each processor's desired formulation. Processors measure salt and acid concentrations in the desalting solutions to calculate the concentrations of ingredients for the cover liquor to equilibrate with the desalted cucumbers to the specifications for the product they are making. Because these specifications vary based on the type of pickle product made and between brands there was greater variability in the composition of the finished product samples. Because the cover liquors were made for each batch, the concentrations of acetic acid, NaCl, and/or CaCl<sub>2</sub> were able to equilibrate to the processors' target concentrations (Table 2.1).

#### *2.4.1 BLOATER DEFECTS*

BLOATER INDICES indicating the type and severity of bloaters were calculated for the balloon, lens, and honeycomb type bloaters as well as total bloaters according to Fleming and others (1977) and can be found in Figure 2.1. Brining process did not significantly affect lens, balloon, or total bloaters ( $P > 0.3878$ ), but it did affect the honeycomb bloaters ( $P =$

0.0226). Size 3B NaCl brined cucumbers had significantly higher honeycomb indices than any other treatment ( $P < 0.0006$ ). However, there were only a few fermentations of this type and an outlier severely skewed that treatment. Other bloater indices were highly variable and not significantly affected by brine type (Figure 2.1). The alternative purging routine used in the experimental tanks was anticipated to affect the incidence of bloaters because of lessened removal of carbon dioxide ( $\text{CO}_2$ ) from the fermentation brines. Overall, the fermentations in  $\text{CaCl}_2$  brines had greater bloater indices ( $\text{CaCl}_2$ :  $10.5 \pm 1.7$  NaCl:  $9.8 \pm 1.9$ ); however, this increase was not statistically significant. Brining season was found to be significantly correlated with the bloater index ( $P = 0.0019$ ). This could be a result of any number of uncontrolled growing conditions such as cultivar, farm location, rainfall, etc., but is more likely confounded with the modification in the purging routine between green seasons. In the 2013 season, processors observed more highly bloated tanks than usual using the  $\text{CaCl}_2$  fermentation process and increased the purge rate to match the current commercial process for the 2014 fermentations. The use of the minimal purging routine with the  $\text{CaCl}_2$  process was found to significantly affect the total bloater indices ( $P = 0.0258$ , Figure 2.2). Purging procedures were developed to minimize the accumulation of  $\text{CO}_2$  gas that can cause bloaters, but the introduction of oxygen into the nearly anaerobic fermentation environment was seen as an impediment to successful fermentation by LAB in the  $\text{CaCl}_2$  fermentation process. However, the use of a minimal purge routine appears to be a detriment to cucumber pickle quality. The quality variability associated with the use of the minimal purging routine indicates a need for optimization to obtain a high quality product with this fermentation process.

Bloater indices of desalted cucumbers were not found to be significantly different from bloater indices of the fermented cucumbers ( $P = 0.1601$ ). Bloaters are formed by an excess of  $\text{CO}_2$  in the cucumbers that builds pressure, breaking cell walls (Fleming and others 1973b). The desalting process involves replacing the fermentation brine, which may still contain higher  $\text{CO}_2$  concentrations, with fresh water that any  $\text{CO}_2$  remaining in the cucumber could easily equilibrate towards. It is sensible that little bloater damage would occur during the desalting process as any  $\text{CO}_2$  pockets would be less pressurized as the gas dissolved into the fresh brine minimizing the development of new bloater damage. However, if aerobic spoilage microorganisms were entering the fermentation during desalting, they could produce new  $\text{CO}_2$  increasing the bloater damage, since processors purge the cucumbers during the desalting process which allows oxygen to enter the vessel. The fact that  $\text{CaCl}_2$  brined cucumbers are not more susceptible to an increase in bloater defects during the desalting process is important for the future usage of the  $\text{CaCl}_2$  fermentation process. Additionally, once the bloater damage occurs, there is no way to ameliorate the cellular damage so it is highly unlikely for the desalting process to cause a change in bloater damage unless new spoilage organisms were introduced to the cucumbers during desalting allowing secondary fermentation and the production of new  $\text{CO}_2$ . This is usually well controlled for in industry because processors do not start the desalting process unless they are ready to process the product. Unfortunately, while the desalted cucumbers were not found to be significantly different from the fermented cucumbers, one reason this experimental design was unbalanced was that some of the fermentations with higher bloaters at the fermented stage were processed into relish rather than hamburger dill chips. These tanks that were designated for

relish production frequently used the  $\text{CaCl}_2$  process and were not measured at the desalted stage and could be artificially lowering the bloater indices of the desalted cucumbers.

#### 2.4.2 Texture Quality

Raw cucumber mesocarp firmness was  $8.7 \pm 0.9$  N and the firmness after fermentation and bulk storage was  $9.0 \pm 1.7$  when brined with  $\text{CaCl}_2$  or  $10.8 \pm 1.1$  after natural fermentation in recycled NaCl brines. Significant firmness differences between brine treatments were first detected at the fermented stage and were detected thereafter at the desalted and finished product stages. Fermented,  $\text{CaCl}_2$  brined cucumbers had an average peak force 1.7 N lower than NaCl brined cucumbers (Figure 2.3). Firmness was higher in smaller diameter cucumbers ( $P = 0.0187$ ). This is somewhat contrary to the finding by Lu and others (2002) that found smaller cucumbers had lower firmness after one year of storage. The difference found by them was only significant between size 1 cucumbers and sizes 2 and 3 cucumbers ( $P < 0.05$ ), but the trend was increasing firmness with increasing size. They were using a fruit pressure tester (FPT) to measure firmness which has been shown to be less sensitive to sensory differences than the mesocarp puncture test we used (Jeon and others 1973, Rosenberg 2013). Rosenberg (2013) found that size 2B cucumbers were significantly firmer than 3A cucumbers in both the NaCl and  $\text{CaCl}_2$  fermentation processes when using a mesocarp puncture test, but the difference was not significant when testing them with a FPT. This is consistent with our findings that cucumber size significantly affected mesocarp firmness ( $P = 0.0187$ )

The desalting process did not significantly affect the mesocarp firmness ( $P = 0.2891$ ). The inclusion of alum in desalting water has been shown to increase the firmness of fermented cucumbers, but this has been found to be insignificant if 0.3%  $\text{CaCl}_2$  is included in

the fermentation brine (Buescher and Burgin 1988). At least 0.3%  $\text{CaCl}_2$  was included in the fermentation brine of both treatments, so it is not surprising that the desalted cucumbers were not significantly different in mesocarp firmness from the fermented cucumbers.

The finished products for both brining treatments were considerably lower in mesocarp peak force (N) than the fermented and desalted samples. All raw, fermented, and desalted samples were obtained as whole cucumbers and cut manually by a hand operated food slicer to slices measured to be  $6.7 \pm 0.1$  mm. However, the finished products were cut into crinkle cut chips by commercial slicers as is sold on store shelves. The commercial slicers were less precise and produced thinner chips that were  $3.8 \pm 0.7$  mm thick. Thompson and others (1982) found that slices less than 3.2 mm had significantly reduced peak force measurements in cucumbers, but the slice thickness between 4.8-9.5 mm did not significantly affect the peak force measurement. Since most of the finished product slices were in between 3.2 mm, where the effect of thickness on firmness is known to be significant, and 4.8 mm, the range where the effect of thickness on firmness is known to be insignificant, it is reasonable to expect that the thickness may be influencing the decrease in instrumental firmness. Other explanations for the overall softening in finished product could include the pasteurization process and temperature at which the finished product is stored. Higher pasteurization temperatures and longer processes are correlated with softer finished product (Rodrigo and Alvarruiz 1988). However, insufficient pasteurization could result in survival of polygalacturonase enzyme (Cho and Buescher 2012). Despite the overall reduction in mesocarp firmness in the finished products, the cucumber pickles fermented in NaCl brine were still significantly firmer than those fermented with the  $\text{CaCl}_2$  process by 1.6 N. Instrumental mesocarp firmness has been well correlated with sensorial measurements of

firmness, crispness, hardness, and crunchiness (Thompson and others 1982, Yoshioka and others 2009, Buescher and others 2011, Pérez-Díaz and others 2015). Wilson and others (2015) found significant hedonic texture liking differences in the finished product of cucumbers from the NaCl and CaCl<sub>2</sub> fermentation processes that were measured to be 1.1 N different in firmness. Therefore, it is likely that a consumer would notice the 1.6 N difference between the NaCl and CaCl<sub>2</sub> fermentation processes.

Organic acids have been shown to cause softening of cucumbers in fresh pack pickles, so it was of interest if the different concentrations of lactic and acetic acid would be related to softening. Lactic acid (as little as 26 mM) has been shown to cause softening over time in low salt (2% NaCl) conditions with greater concentrations of lactic acid causing greater softening (Bell and others 1972). Since the fermentations in CaCl<sub>2</sub> were bulk stored with lactic acid concentrations of  $92.22 \pm 17.83$  mM, it was of concern whether firmness could be correlated with the concentration of lactic acid. Additionally, acetic acid (350 mM) has produced softening of fresh pack pickles, but at concentrations much higher than the  $14.98 \pm 6.35$  we found in fermentation brines (Bell and others 1972). The firmness of fermented cucumbers was not correlated to lactic acid concentration (CaCl<sub>2</sub>:  $P = 0.2071$ , NaCl:  $P = 0.4636$ ) or acetic acid concentration (CaCl<sub>2</sub>:  $P = 0.3647$ , NaCl:  $P = 0.1577$ ). CaCl<sub>2</sub> has been shown to minimize the softening induced by acetic acid and the concentration of acetic acid in the fermentation brines is much lower than has been used for studying acetic acid softening, so it makes sense that these concentrations would not be correlated with decreased firmness (Howard and others 1994). Lactic acid softening has been shown in fresh pack cucumbers at the concentrations found in the fermentation brines, but it is possible the



fermentation process, high NaCl concentration, or the inclusion of CaCl<sub>2</sub> to the fermentation brine minimized any softening by lactic acid.

Time spent in bulk storage was found to negatively correlate with fermented cucumber mesocarp firmness relating to a loss of  $0.74 \pm 0.24$  N over 100 days regardless of fermentation process (Figure 2.4). However, the effect of the time spent in bulk storage on texture became more discernible as the cucumbers were processed into desalted and finished product samples with  $0.83 \pm 0.21$  N and  $1.07 \pm 0.21$  N lower peak forces for every 100 days, respectively. Polygalacturonase (PG) activity was assayed to look for correlations with mesocarp firmness, but no strong correlations were found for fermented, desalted, or finished product samples.

#### 2.4.3 Color changes

Cucumber mesocarp color was evaluated by colorimetric analysis using L\*a\*b\* color space and converted to hue and chroma values. The differences in hue and chroma caused by brining treatment at different processing stages can be seen in Figures 2.5 and 2.6, respectively. Cucumbers fermented with the CaCl<sub>2</sub> process had significantly increased hue values than those fermented in NaCl brines at each stage in processing. The cucumbers shown in Figure 2.7 are from fermentations with average hue, chroma, and L\* values very close to the means for each treatment. Chroma was significantly more intense in the finished product for each treatment than in the fermented and desalted stages. Chroma measures the intensity of a color and the addition of yellow 5 dye to the cover liquor shows a marked increase in the intensity of the mesocarp color ( $P < 0.0001$ , Figure 2.6) that masked some of the variability in appearance of the fermented cucumbers. The cucumbers fermented in NaCl brine also had significantly higher chroma values than their counterparts in the CaCl<sub>2</sub> brine

( $P = 0.0269$ ); however, this difference was no longer significant after the desalting procedure ( $P = 0.1807$ ).

Color changes occurring during cucumber fermentation are primarily a result of the degradation of chlorophyll. The degradation of chlorophyll to pheophorbide has been proposed to occur in plant products by two pathways, through pheophytin as an intermediate or through chlorophyllide as an intermediate (Figure 2.8, adapted from Heaton and others 1996). Color changes in cucumbers exposed to acidic brines have been shown to occur by the production of a combination of pheophorbide and pheophytin pigments (Jones and others 1962). This degradation of brightly colored green chlorophyll to pheophorbide and pheophytin produces a dull olive-green or olive-yellow color in food products (Gupte and others 1964). Fermenting cucumbers have very small concentrations of chlorophyllide, but pheophytin accumulates in large concentrations, so it has been modeled that the accumulation of pheophorbide occurs predominantly using chlorophyllide as an intermediate while the pathway from pheophytin to pheophorbide is rarely used because of a high reaction rate (White and others 1963, Heaton and others 1996). This is in agreement with how fermentations using fresh brines occur. At the beginning of fermentation, the pH is neutral allowing the enzymatic conversion of chlorophyll to chlorophyllide by chlorophyllase to dominate the degradation reactions. Once the pH is lowered by fermentation, the acid causes the loss of the magnesium ( $Mg^{2+}$ ) from the center of the chlorin ring of the remaining chlorophyll or newly formed chlorophyllide to produce pheophytin and pheophorbide, respectively, to be highly favored. This results in the chlorophyll being converted to pheophytin directly or being converted to pheophorbide from the intermediate

chlorophyllide, but the high acid environment makes the final concentration of chlorophyllide minimal.

The proposed mechanism was for non-acidified brine; however, commercial production of fermented cucumber pickles currently utilizes recycled, acidic brines. This acid still must equilibrate into the cucumbers to degrade their chlorophyll molecules, but the slightly acidified brines would likely result in less product proceeding through the enzymatic process to form chlorophyllide before acid reactions became more favorable. The brines for the  $\text{CaCl}_2$  process were not acidified and would more closely follow the previously detailed mechanism. This could therefore result in a higher ratio of pheophorbide: pheophytin in the tanks undergoing  $\text{CaCl}_2$  fermentation process. It is worth noting that pheophytin can be converted to pheophorbide through the use of chlorophyllase enzyme, but this reaction has not been measurably observed in fermenting cucumbers (White and others 1963, Heaton and others 1996).

As Figure 2.9 shows, the chroma (intensity of the color) decreased with greater time spent in bulk storage and was lower in cucumbers fermented in  $\text{CaCl}_2$  brines. This decrease in chroma leads to the idea that the pigments may be degrading with greater exposure time to the sun and oxygen. While the opaque tanks and the use of wooden boards to submerge the cucumbers minimizes the exposure of the cucumbers to the sun, the cucumbers are exposed during the first day while tanking and the sunlight would penetrate the surface of the brine. Eisenstat and Fabian (1953a, 1953b) used salt stock of 16% and desalted stock of 3.8% salt to find that direct sunlight caused severe bleaching in a matter of hours unless the cucumber pickles were completely submerged in the 16% salt stock brine. The process of filling a fermentation tank with cucumbers would result in a short period of time exposed to the sun

and it may be enough to start the bleaching process. Eisenstat and Fabian also found exposure to  $\text{CaCl}_2$  could cause slight bleaching of cucumbers in jars exposed to diffused light which could explain the lower chroma value of the cucumbers fermented in  $\text{CaCl}_2$  brines (Figure 2.9). They also found increased bleaching as storage time was increased from 1 to 6 weeks in the 3.8% salt desalted cucumbers. This experiment was conducted before colorimetry was very sophisticated and they recorded bleaching results as a visual qualitative test, so it is uncertain how the observed bleaching would affect  $L^*$  value, hue, or chroma. However, Buescher and Hamilton (2000) found an increase in  $L^*$  value by 10 and a  $5^\circ$  ( $100$  to  $95^\circ$ ) change toward yellow in hue value for fermentations conducted under incandescent lights compared to fermentations in the dark with brines composed of 5% NaCl, 0.35%  $\text{CaCl}_2$ , 0.1% acetic acid, and 0.05% potassium sorbate. Additionally, Guillou and others (1992) found the hue of cucumbers fermented in 5% NaCl, 0.2%  $\text{CaCl}_2$ , and 0.2% potassium sorbate to be between  $105^\circ$  and  $125^\circ$  during 18 weeks (126 days) of storage under 254 nm UV lights while chroma decreased from 20 to 7. This hue is greener than the hue of these commercial fermentations but the chroma decrease matches our observed values fairly well (Figure 2.9). It's possible there are multiple color changing mechanisms occurring that affect the different parameters of colorimetry since Eisenstat and Fabian (1953a, 1953b) found differences in observed bleaching using direct sunlight, an IR lamp, and using a 210-328.7 nm UV lamp.

## **2.5 Conclusion**

The use of the  $\text{CaCl}_2$  fermentation process instead of the current commercial practice affects the color, physical, and texture quality. These quality changes are occurring during fermentation and bulk storage but are still evident in the desalted cucumbers and finished

pickles. It appears that no further degradation of quality takes place in the later processing steps with the possible exception of texture. Texture of finished pickles was significantly decreased compared to the texture in fermented or desalted stages. This is likely a result of different sample preparation causing the finished pickle chips to have a different thickness, but could also be a result of softening during pasteurization. The hue of cucumbers fermented in  $\text{CaCl}_2$  brine was significantly greater than cucumbers fermented in the  $\text{NaCl}$  brine and the chroma was less intense in cucumbers fermented in the  $\text{CaCl}_2$  brines, possibly because of the degradation pathway of chlorophyll or photooxidation reactions. Bloating defects were more common in the 2013 season, but were not found to be significantly affected by fermentation process. Increasing the purging routine with the  $\text{CaCl}_2$  fermentation process resulted in complete fermentation and stable bulk storage, so the minimal purging routine may not be necessary for future use of this process, but would need a controlled experiment to confirm this. Additional research is warranted to understand the tissue softening observed in the  $\text{CaCl}_2$  brined cucumber fermentations since we could not identify process variables that correlated with the reduced texture quality in the experimental environmentally-friendly fermentation process. Additionally, there is a need to identify the effect of concentrations lower than 100 mM  $\text{CaCl}_2$  brines on cucumber firmness to establish a minimum  $\text{CaCl}_2$  concentration for successful fermentations of this process. Color changes would also be an interesting avenue for researching such as identifying the mechanisms and other products of photooxidation reactions. The color changes resulting from the fermentation processes also introduce the possibility of manipulating chlorophyll degradations which could lessen or eliminate the use of yellow 5 in cucumber pickle products.

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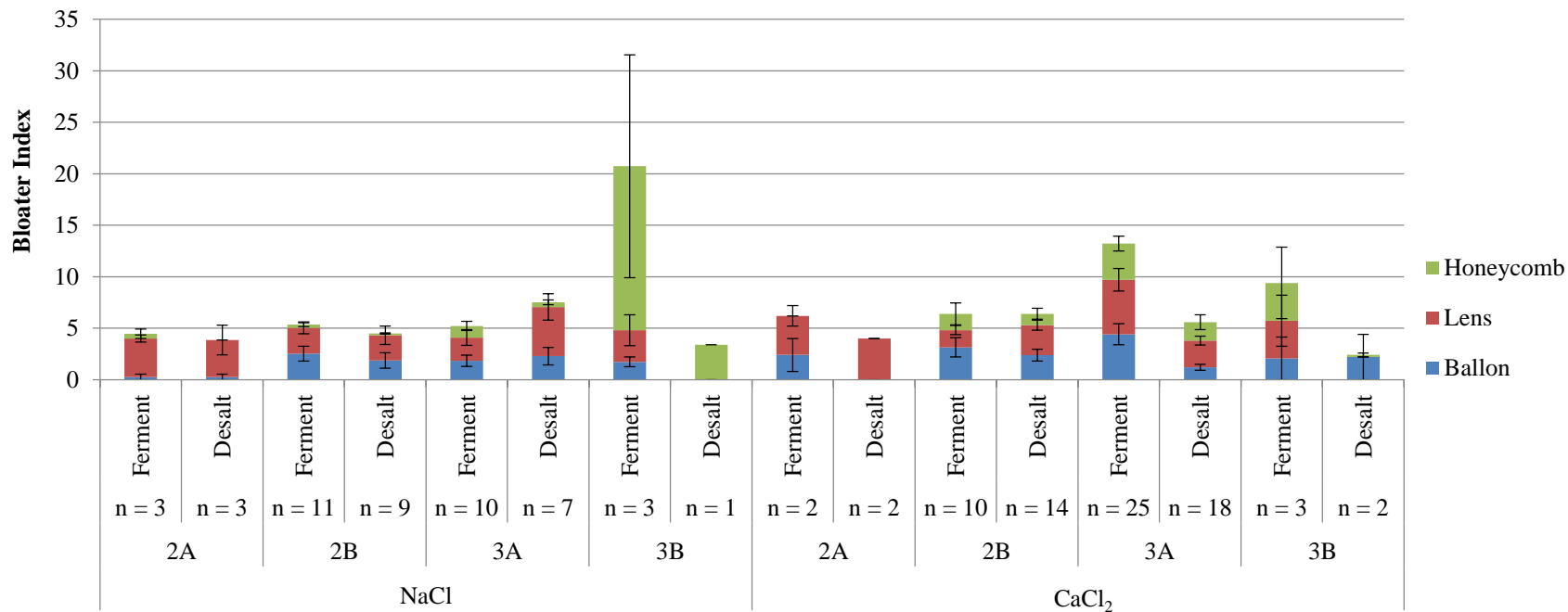
**Table 2.1 – Fermentation chemistry for cucumber pickles commercially fermented in either 1.03 M NaCl recycled brine or 100 mM CaCl<sub>2</sub> brine.**

Stage	Brine	No. of fermentations	Terminal pH <sup>1</sup>	CaCl <sub>2</sub> (mM) <sup>2</sup>	NaCl (mM) <sup>3</sup>	Lactic Acid (mM) <sup>1</sup>	Acetic Acid (mM) <sup>1</sup>
Fermented	NaCl	27	3.30 ± 0.12 <sup>bc</sup>	37.9 ± 4.6	1087.5 ± 63.5	111.7 ± 12.8 <sup>a</sup>	26.9 ± 14 <sup>a</sup>
	CaCl <sub>2</sub>	41	3.23 ± 0.09 <sup>c</sup>	85.2 ± 6.9	-	92.2 ± 17.8 <sup>b</sup>	15.0 ± 6.3 <sup>b</sup>
Desalted	NaCl	20	3.40 ± 0.19 <sup>ab</sup>	26.7 ± 3.3	738.5 ± 28.5	66.4 ± 21.5 <sup>c</sup>	11.7 ± 3.7 <sup>b</sup>
	CaCl <sub>2</sub>	36	3.47 ± 0.28 <sup>a</sup>	31.4 ± 4.9	-	26.7 ± 11.2 <sup>d</sup>	4.0 ± 2.0 <sup>c</sup>
Finished Product	NaCl	14	3.40 ± 0.08	24.8 ± 1.9	240.1 ± 176.0	45.4 ± 13.5	105.4 ± 36.7
	CaCl <sub>2</sub>	26	3.35 ± 0.05	28.4 ± 6.7	239.9 ± 43.6	33.7 ± 8.4	114 ± 36.7

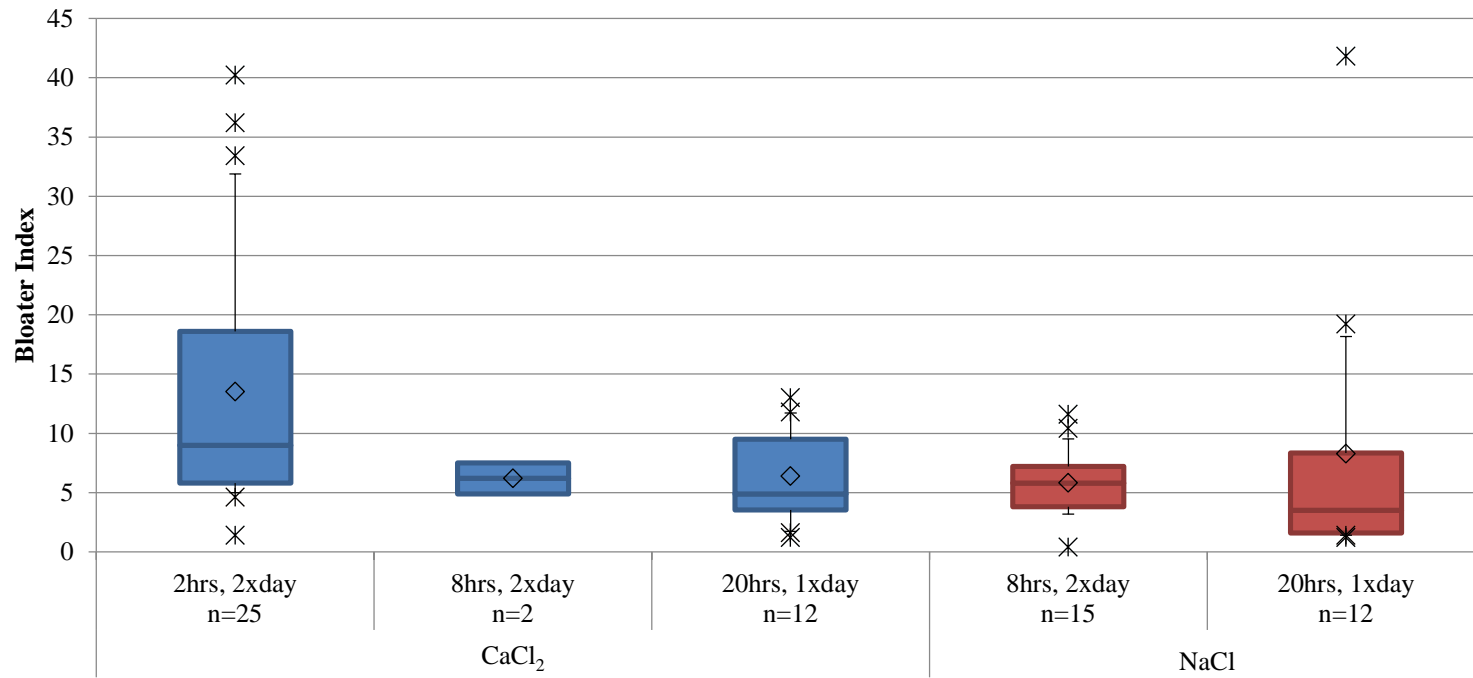
<sup>1</sup>Means for different treatments followed by different letters are significant at  $P < 0.05$ .

<sup>2</sup>CaCl<sub>2</sub> concentrations are calculated from Ca<sup>2+</sup> concentrations and are slightly inflated from natural cucumber calcium concentrations of 3.0 ± 0.8 mM.

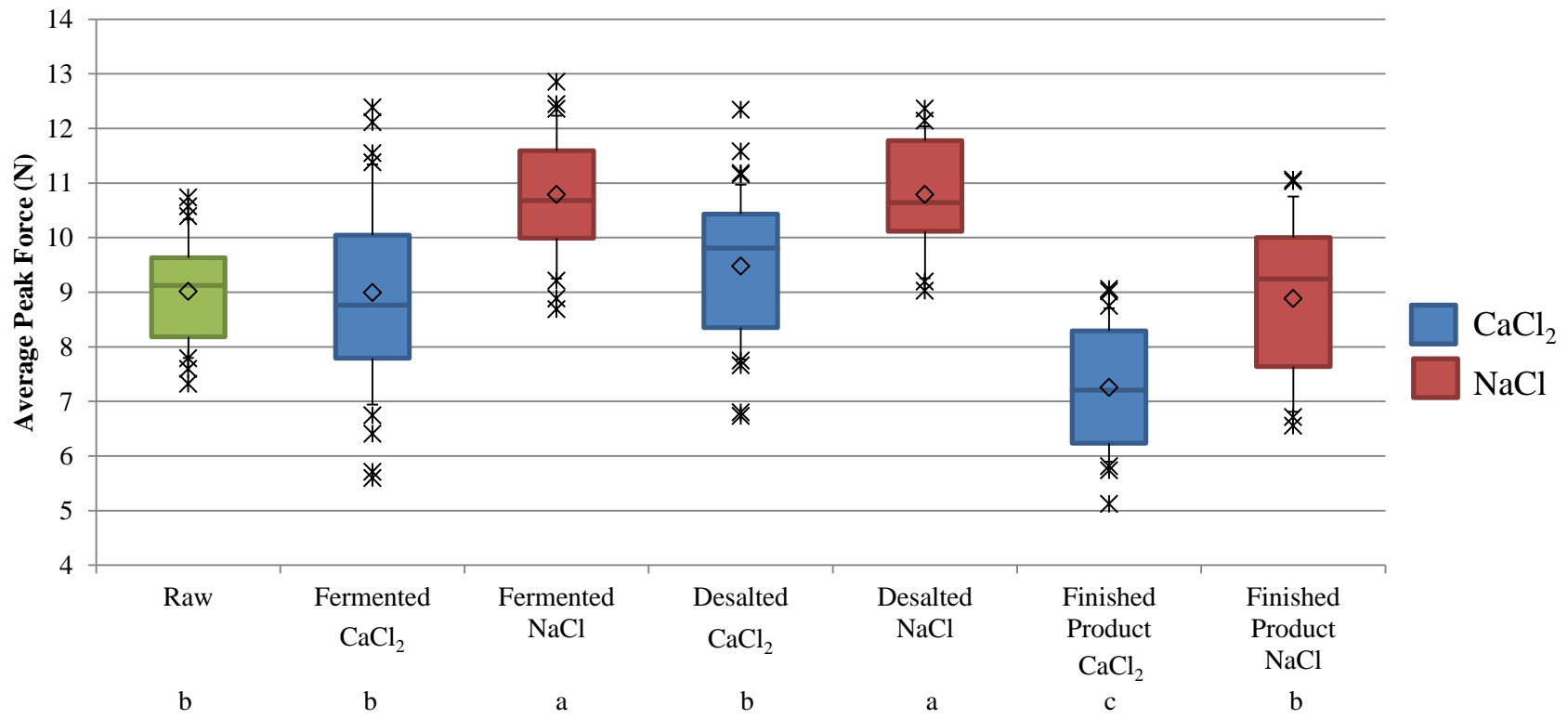
<sup>3</sup>NaCl concentrations are calculated from chloride concentrations with the chloride contributed by CaCl<sub>2</sub> subtracted from the chloride concentration.



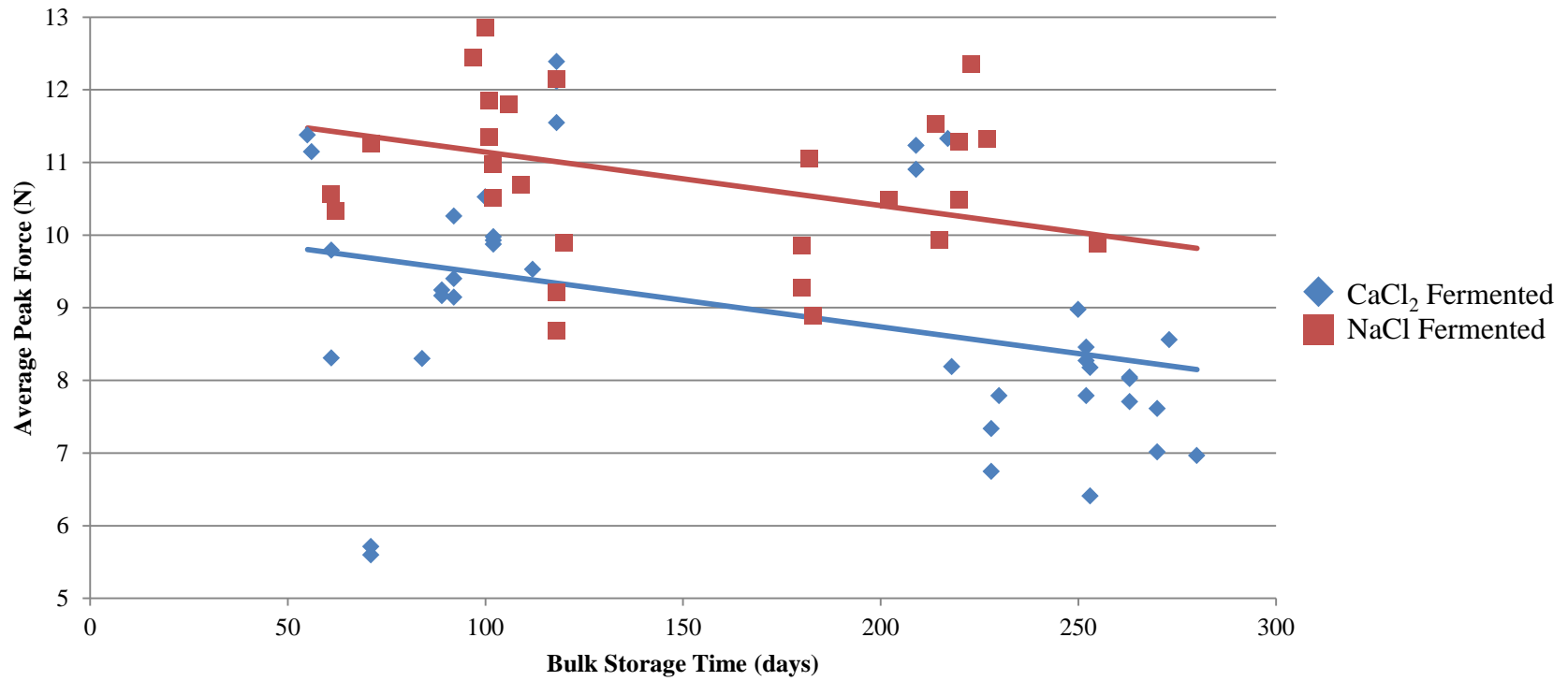
**Figure 2.1 - Total, Balloon, Lens, and Honeycomb Bloater Indices for Fermented and Desalted cucumbers by size and brining process. Error bars represent standard error.**



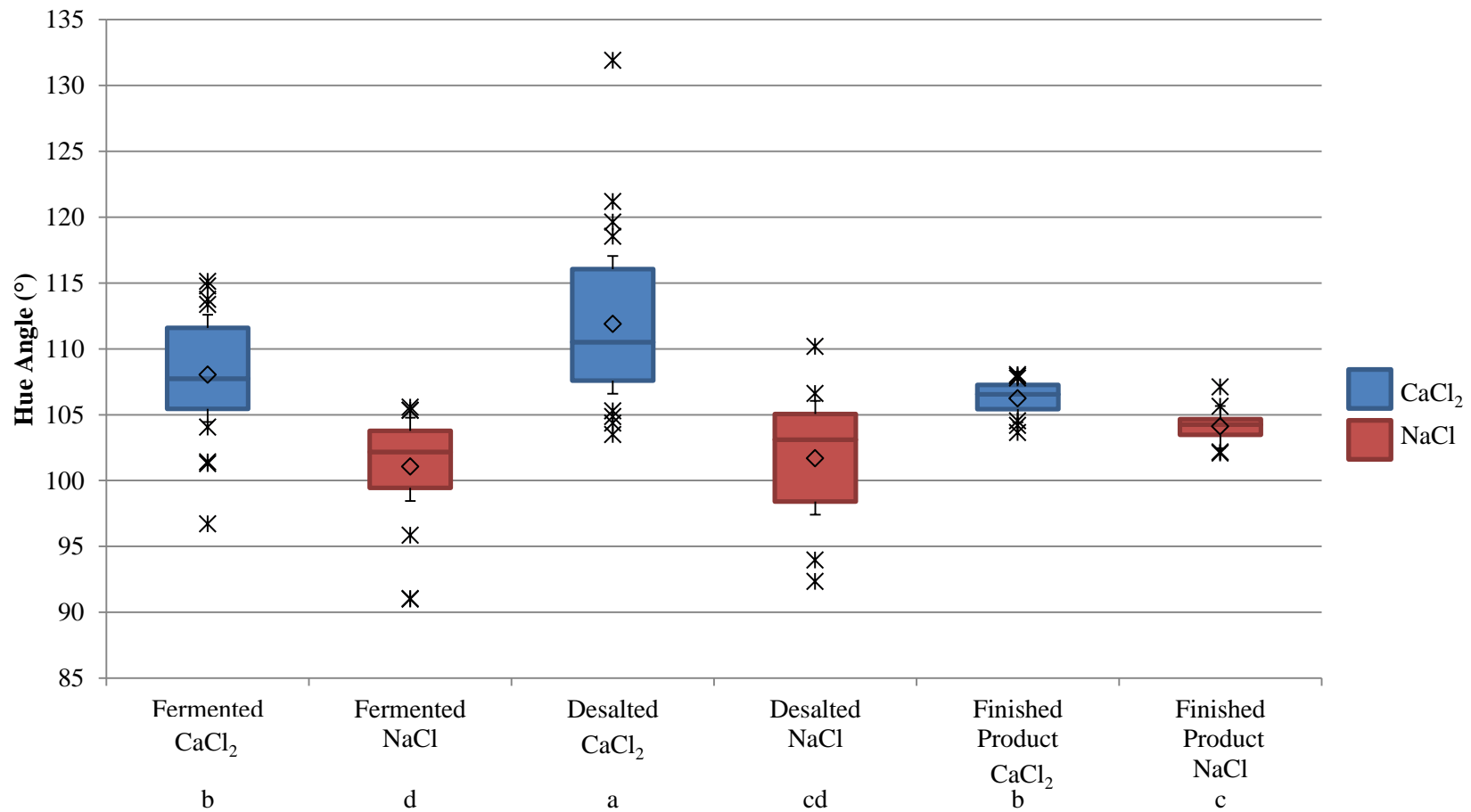
**Figure 2.2 – Bloater Indices for fermented cucumbers by brining process and purging procedure. The minimal purging routine of 2 hours, 2 times a day was only used in CaCl<sub>2</sub> brines. The 8hrs, 2xday treatment was used by processor 1 and the 20hrs, 1xday treatment was used by the remaining processors.**



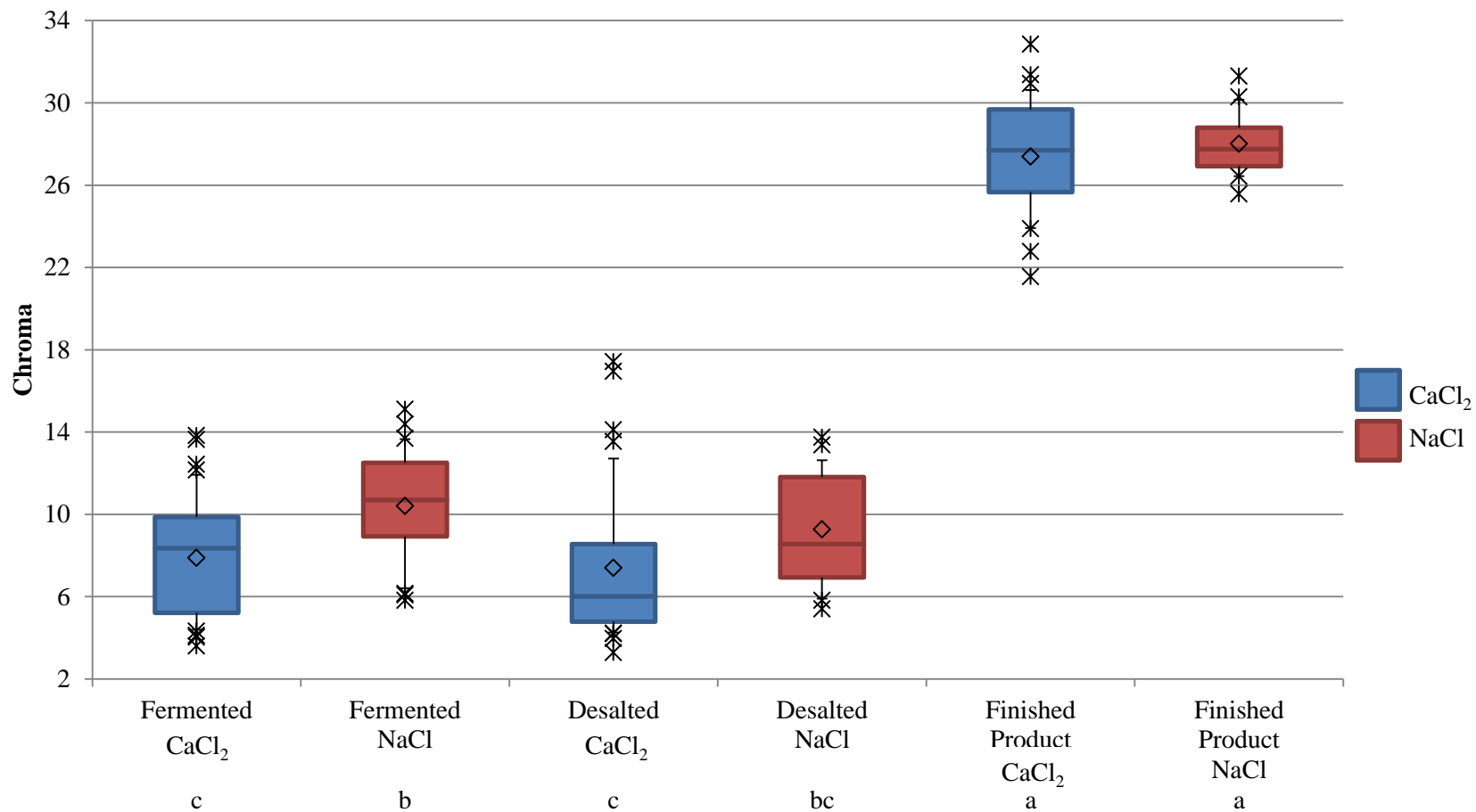
**Figure 2.3 - Mesocarp firmness (N) for cucumbers brined in CaCl<sub>2</sub> compared with NaCl at the various stages in processing. Peak force values were significantly different between brining processes at each stage but were not significantly different between Fermented and Desalted stages. Peak Forces are significantly different at  $P < 0.05$  for treatments with different letters below the axis.**



**Figure 2.4 - Changes in mesocarp firmness associated with increased time spent in bulk storage**



**Figure 2.5 - Mesocarp hue for cucumbers brined in CaCl<sub>2</sub> and cucumbers brined in NaCl at the various stages in processing. Hue is measured as the degrees from red on a polar coordinate system with 90° being in the yellow direction and 180° being in the green direction. Hue values were significantly different at  $P < 0.05$  for treatments with different letters below the axis.**



**Figure 2.6 - Mesocarp chroma for cucumbers brined in CaCl<sub>2</sub> and cucumbers brined in NaCl at the various stages in processing. Chroma is measured radially from the pole and is the intensity of the color. Chroma values were significantly different at  $P < 0.05$  for treatments with different letters below the x-axis**





Figure 2.7 – Fermented cucumbers representative of the average color of each fermentation process. The average hue value for the cucumbers in the CaCl<sub>2</sub> fermentation process is significantly larger than the average hue of the cucumbers in the NaCl fermentation process ( $P < 0.05$ ). The chroma values are significantly different ( $P = 0.0269$ ) but the L\* values are not significantly different from each other ( $P = 0.9432$ ).

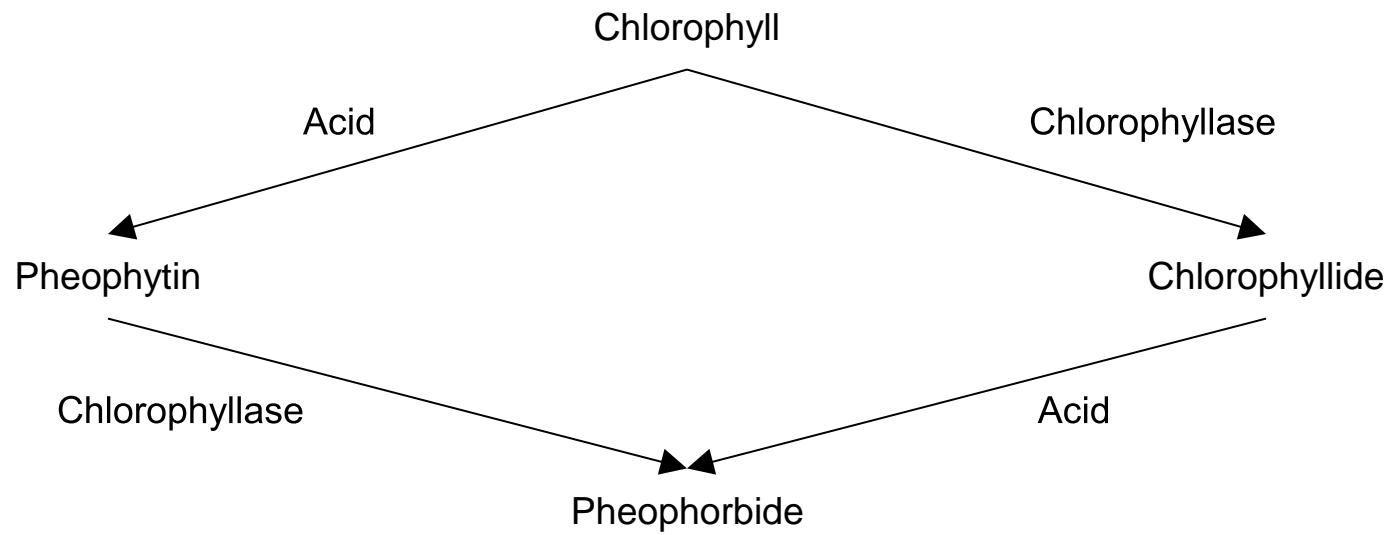


Figure 2.8 - Scheme for the degradation of Chlorophyll to Pheophorbide via Pheophytin or Chlorophyllide as intermediates. Adapted from Heaton and others 1996.

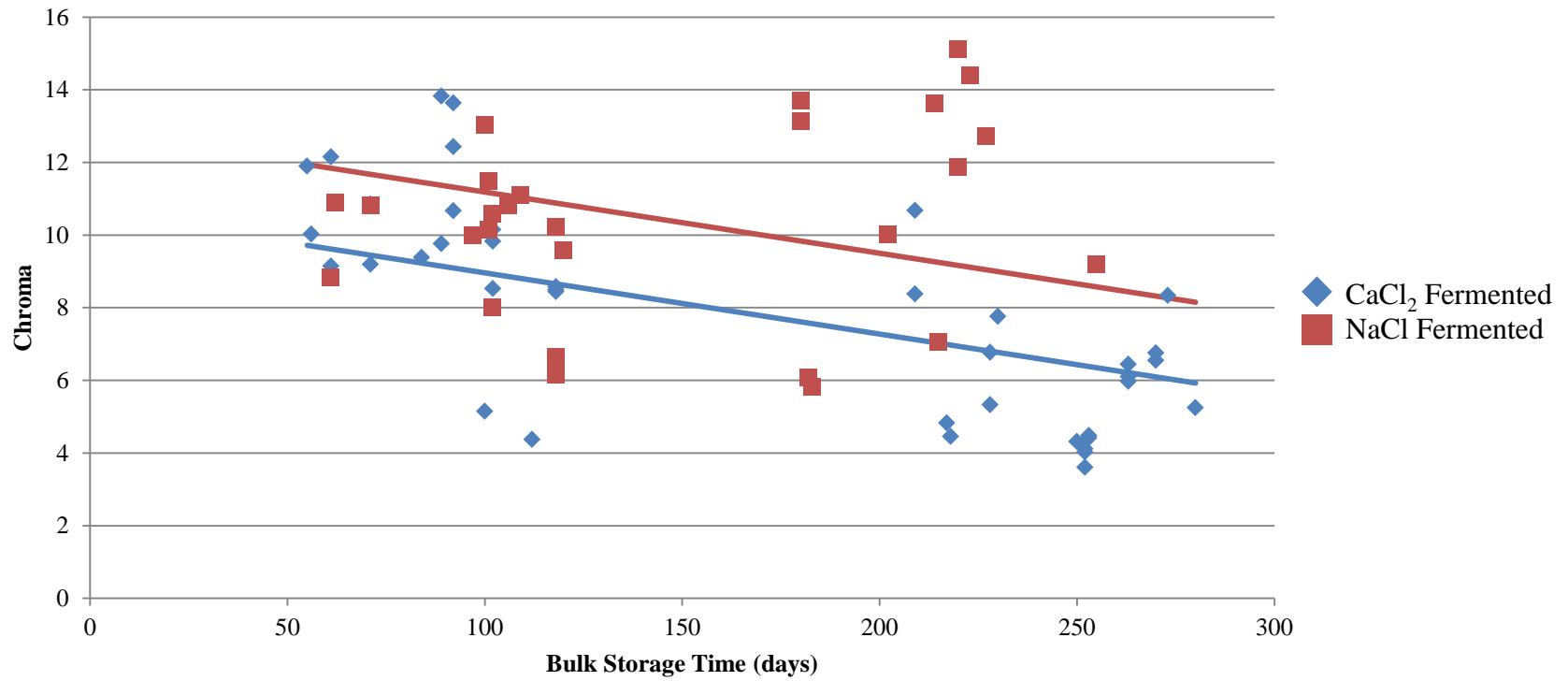


Figure 2.9 - Chroma changes with greater length of time spent in bulk storage for fermented cucumbers brined in CaCl<sub>2</sub> or NaCl.

## CHAPTER 3 – Effect of Brine Acidification on Fermentation Microbiota and Texture Quality of Cucumbers Fermented in a Calcium Chloride Brine

Target Journal: Journal of Food Science

### 3.1 Abstract

Commercial fermentation for bulk preservation of cucumbers relies on natural microbiota and 1 M sodium chloride (NaCl) brines, resulting in large volumes of high salt wastewater. An alternative fermentation process utilizing 0.1 M calcium chloride (CaCl<sub>2</sub>) as the only salt has been developed to eliminate NaCl from fermentation brines for reduced environmental impact. This process, initiated in brines of neutral pH, resulted in variable texture quality of fermented cucumbers at the commercial scale. Initial brine pH affects microbial ecology, activity of pectinolytic enzymes, non-enzymatic softening, and preservative efficacy. This study determined the effect of brine acidification on the fermentation microbiota and texture quality of cucumbers fermented in CaCl<sub>2</sub> brine. Cucumber fermentations were conducted in sealed, 946 mL, glass jars for 6 independent lots of cucumbers in a randomized complete block design with a full-factorial treatment structure for brine acidification (acetic acid, hydrochloric acid, or non-acidified) and brining salt (1 M NaCl or 0.1 M CaCl<sub>2</sub>). Fermentations were inoculated with 10<sup>6</sup> CFU/mL *Lactobacillus plantarum* and included 4 mM potassium sorbate, a common yeast and mold inhibitor. Fermentations were monitored for 21 days by quantification of acids and sugars using HPLC and enumeration of *Enterobacteriaceae*, yeasts and molds, and lactic acid bacteria. Mesocarp tissue firmness was measured after 7, 14, 21, and 100 days using a puncture test on a 6.7 mm slice from the center of 15 cucumbers/fermentation. *Enterobacteriaceae spp.* survived longer and were > 1 log CFU/mL higher in fermenting cucumbers than in brines.

Addition of 25 mM acetic acid to fermentation brines (but not the addition of HCl at the same pH) reduced *Enterobacteriaceae spp.* in brines and cucumbers ( $P < 0.002$ ) during the initiation of fermentation for both brining salts. However, acidification had no effect on texture quality of fermented cucumbers for either brining salt ( $P = 0.8235$ ). Interestingly, cucumber firmness was 2.9 N higher in CaCl<sub>2</sub> brined cucumbers compared to those brined in NaCl and stored for 100 days ( $P < 0.0001$ ). These results differ from previous commercial trials, indicating that there are production variables negatively affecting texture of CaCl<sub>2</sub> brined, fermented cucumbers that require further optimization.

### 3.2 Introduction

Current commercial practice for the bulk preservation of cucumbers as pickles utilizes the microbiota present on the cucumbers for a natural fermentation. As such, brines that equilibrate to at least 5% sodium chloride (0.86 M NaCl) are used to select for lactic acid bacteria (LAB) and inhibit salt sensitive spoilage and pathogenic bacteria on the cucumbers (McFeeters and Pérez-Díaz 2010). However, this concentration of salt puts the wastewater generated by processors in excess of the EPA's Secondary Drinking Water Standards for chloride concentrations (250 mg/L or 7 mM) by several orders of magnitude (U.S. Environmental Protection Agency 2009). Recycling of fermentation brines has successfully reduced the high NaCl wastewater generated and has been widely implemented (McFeeters and Pérez-Díaz 2010). However, concentrations of NaCl used for fermentation and bulk storage must be lowered to create a palatable finished product (2-3% NaCl). Fresh water is used to dilute the salt in the cucumbers, but this desalting water has too low of a salt concentration to be recycled and yet is still high enough to cause concern for the environment. In an attempt to eliminate NaCl from fermentation brines, an alternative fermentation process was developed by McFeeters and Pérez-Díaz (2010) using 100 mM calcium chloride ( $\text{CaCl}_2$ ) and 25 mM acetic acid in a 55:45 (wt/vol) cucumber: brine pack ratio inoculated with a *Lactobacillus plantarum* LA0445 starter culture. This fermentation brine contributes no sodium ions to wastewaters and a fifth of the concentration of chloride ions compared to the typical commercial fermentations. Commercialization of this alternative fermentation process was completed with several modifications to the lab scale process (Pérez-Díaz and others 2015). Removal of the acetic acid, addition of 6 mM potassium sorbate as a preservative, and introduction of a minimal purge routine were

determined to be commercially necessary. However, the texture quality was found to be more variable at the commercial scale using the new process. Several consumer sensory tests of cucumber pickles produced with the modified CaCl<sub>2</sub> fermentation process and salt fermentations found a lower texture quality in the pickles produced with the CaCl<sub>2</sub> brine (Wilson and others 2015, Pérez-Díaz and others 2015). This overall reduction in texture quality was shown to be directly related to the CaCl<sub>2</sub> fermentation process in a series of commercial fermentations from the 2013 and 2014 growing seasons (Chapter 2).

The changes made in the commercial scale up included the removal of acetic acid from the brine formulation, addition of the potassium sorbate, and reduction of the air purging routine (Pérez-Díaz and others 2015), all of which were considered necessary for a stable, complete fermentation. The acetic acid was removed because it can serve as a substrate for oxidative yeast and other spoilage microorganisms in the absence of sugar, which can be a problem in open-top commercial tanks (Franco and Pérez-Díaz 2012). They also determined that potassium sorbate was needed because it has preservative effects against these yeast and other spoilage microorganisms while being non-inhibitory towards the lactic acid bacteria leading the fermentation. However, they did not test fermentations using the calcium chloride fermentation process with both acetic acid and sufficient potassium sorbate to inhibit spoilage organisms. Excluding the acetic acid from the cover brine results in a fermentation process with an initial brine pH above 7 which may in part explain the higher *Enterobacteriaceae* counts found in the brines during the first week of fermentation in CaCl<sub>2</sub> brines (Pérez-Díaz and others 2015).

Based on the commercially significant reasons for modifying the method, it is of interest if the deviations from the original protocol (McFeeters and Pérez-Díaz 2010) are

causing the texture variability (Pérez-Díaz and others 2015, Wilson and others 2015, Chapter 2). The modified calcium chloride brine have initial pH values above neutral (unpublished data) and Day 1 values have been reported as  $5.9 \pm 0.5$  in  $\text{CaCl}_2$  brines compared to  $4.15 \pm 0.1$  in NaCl brines (Pérez-Díaz and others 2015). The pH is initially higher in the  $\text{CaCl}_2$  brines because of the elimination of the acetic acid and the rise in pH associated with the dissociation and subsequent protonation of the potassium sorbate. The use of a starter culture without the NaCl inhibition initiates a quick fermentation that rapidly decreases the pH to  $4.0 \pm 0.5$  by day 3 (Pérez-Díaz and others 2015). However, neutral pH values can affect the rate of softening through a number of direct and indirect mechanisms including non-enzymatic activity, enzymatic activity, microbial growth and survival, and preservative efficacy. Non-enzymatic softening rates of blanched cucumbers have been shown to be much higher at neutral pH values, presumably by  $\beta$ -elimination reactions (Krall and McFeeters 1998, McFeeters and Fleming 1991). Additionally, the neutral pH range of the initial brining conditions is a much more suitable environment for the survival of microorganisms that may exist on the surface of the cucumber such as bacteria in the *Enterobacteriaceae* family as well as yeasts and molds that have been found to produce softening enzymes (Bell and others 1950, Etchells and others 1958, Costilow and others 1980, Walter 1991, Collmer and Keen 1986). Etchells and others (1975) found that acidification of cover brine was sufficient for reduction of coliform bacteria within the *Enterobacteriaceae* family allowing for controlled fermentation. Additionally, McDonald and others (1991) found that brines acidified using acetic acid or a buffered system with equivalent protonated acetic acid reduced *Enterobacteriaceae* counts during the initiation of natural fermentation compared to non-acidified brines. The acetic acid treatment was more effective than the buffered treatment at



reducing *Enterobacteriaceae*, indicating the potential for a pH effect as well as the organic acid effect as the cause of the *Enterobacteriaceae* death. *Enterobacteriaceae* species also survived longer on the inside of cucumbers where the pH decreases more slowly during the initiation of fermentation because of the time required for the acid in the brine and the acid generated by fermentation to equilibrate with the interior of the cucumber. The acidified brines reduced the number of *Enterobacteriaceae* inside the cucumber much faster than in non-acidified brines. The preservative potassium sorbate was also deemed necessary for successful commercial fermentations by the inhibition of spoilage microorganisms, but it is much more effective at low pH values in its conjugate acid form, sorbic acid (Dharmadhikari 1992, Sauer 1977). Therefore, it is plausible that in the first few days of the fermentation, when the cucumbers in the calcium chloride brined tanks are at neutral pH values, the cucumbers are softening by non-enzymatic softening or due to pectin degrading enzymes produced by the increased metabolic activity of *Enterobacteriaceae* or other undesired organisms found on the cucumbers due to lessened inhibition by potassium sorbate.

The addition of acetic acid was excluded from the  $\text{CaCl}_2$  fermentation process because it could be used as a substrate for oxidative yeasts, however acetic acid has many other effects in commercial fermentations. At its simplest, it functions as an acid, lowering the pH to inhibit microorganisms. It also functions as an organic acid capable of crossing the cell membrane of bacteria to dissociate inside the cell lowering the internal pH of microorganisms making it more effective at killing microorganisms than inorganic acids like hydrochloric acid. Additionally, 28 mM (0.16%) equilibrated acetic acid has been shown to prevent enzymatic softening by the inhibition of molds in commercial air purged fermentations, resulting in significantly firmer cucumbers (Potts and Fleming 1982).

However, the use of acetic acid in the initial brine can be detrimental in some situations. Several organic acids have been shown to increase softening rates of cucumbers in fresh pack cucumbers (Bell and others 1972). Acetic acid was shown to cause the least softening compared with lactic, citric, malic and oxalic, but softening did occur over storage with 350 mM (2%) acetic acid. However, 350 mM is a much higher concentration than would be expected in a commercial fermentation and calcium chloride has been shown to protect against the softening caused by acetic acid in pickled peppers so softening by acetic acid would not be expected to occur in fermentation brines if used as an acidulant (Fleming and others 1993, Howard and others 1994).

It is hypothesized that acidification of the initial fermentation brines with an organic acid such as acetic acid in the presence of sufficient potassium sorbate preservative will eliminate the difference in firmness between cucumbers fermented in NaCl and CaCl<sub>2</sub> brines. Additionally, it is hypothesized that the acidification of the fermentation brine with an inorganic acid such as hydrochloric acid in the presence of sufficient potassium sorbate will lessen the softening difference between brining treatments because it will provide mild inhibition to undesired microorganisms by lowering the pH of the fermentation. However, an organic acid such as acetic acid will be more effective at inhibiting *Enterobacteriaceae*, yeast, and mold populations than HCl at the same pH, so the treatments acidified with organic acid are hypothesized to result in less softening of the cucumber pickles during storage than the treatments acidified with inorganic acid. The objective of this experiment was to determine the effect of brine acidification on texture quality of fermented cucumbers and fermentation microbiota.

### 3.3 Materials and Methods

#### 3.3.1 Fermentations and Sample Collection

Fresh, size 2B (3.5-3.8 cm diameter) pickling cucumbers were obtained from a local processor and packed into 32 oz. (946 mL) jars. A full-factorial treatment structure for brining salt (equilibrated concentrations of 1 M NaCl and 0.1 M CaCl<sub>2</sub>) and acidification (equilibrated 0.025 M acetic acid, hydrochloric acid at the same pH, and no acidification) was used to create six brining treatments. Before equilibration, the pH values of the brines were  $3.58 \pm 0.07$  in the acidified treatments while the non-acidified treatments were  $9.58 \pm 0.73$  and  $7.05 \pm 1.10$  for the CaCl<sub>2</sub> brine and NaCl brine respectively. Using each treatment, 16 jars were packed at a 55:45 (w/w) cucumber to brine ratio using 6 independent lots of cucumbers in a randomized complete block design. All treatments included 4 mM potassium sorbate and were inoculated at  $10^6$  CFU/mL *Lactobacillus plantarum* (LA0445, USDA-ARS, Food Science Research Unit, Raleigh, NC culture collection). *L. plantarum* starter culture was grown at 30°C in deMan, Rogosa and Sharpe (MRS, Becton, Dickinson and Co.) broth to a population of approximately  $10^9$  CFU/mL, centrifuged to pellet cells, and re-suspended in saline. After inoculation, the jars were sealed and incubated at 28°C. Fermentations were monitored for 21 days as well as at 100 days to simulate typical bulk storage time. At each time point (1, 2, 3, 5, 7, 14, 21, and 100 days) at least one jar was removed for sampling and was not included in the rest of the experiment. For each time point, the cucumbers were aseptically separated from the brines and blended using autoclaved Waring blenders to form a slurry. The slurries were stomached (model 400; Tekmar Co., Cincinnati, Ohio) and both the slurries and brine were used for microbiological analysis, pH, and HPLC analysis as discussed in the following sections.

### *3.3.2 Microbial enumeration of microorganisms*

Samples were serially diluted using 0.85% saline solution and spiral plated using an Autoplate 4000 (Spiral Biotech, Norwood, MA). Enumeration of *Enterobacteriaceae* was done by plating on Violet Red Bile agar (VRBG, Becton, Dickinson and Co.) containing 1% glucose (Sigma) incubated at 37°C for 24 hours. Lactic Acid Bacteria (LAB) were enumerated using Lactobacilli deMan, Rogosa, and Sharpe agar (MRS, Becton, Dickinson and Co.) supplemented with 0.001% cycloheximide (OXOID, New England) incubated at 30°C for 3 days. Yeasts and molds were enumerated using yeast and mold agar (YMA, Becton, Dickinson and Co.) supplemented with 0.01% chloramphenicol (Sigma) and 0.01% chlortetracycline (Sigma) at 30°C for 3 days.

### *3.3.3 Instrumental Texture Analysis*

Texture measurements were performed using a mesocarp puncture test on a 6.7 mm slice obtained from the center of 30 raw cucumbers to represent the initial cucumber mesocarp firmness on day 0 and on slices obtained aseptically from the center of 15 cucumbers on days 7, 14, 21, and 100 prior to the remaining cucumber being blended into a slurry. The mesocarp puncture test was conducted on a TA.XT Plus Texture Analyzer (Texture Technologies Corp, Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) using a 3 mm punch probe to puncture the mesocarp tissue (Thompson and others 1982). In turn, the mesocarp of one lobe of each slice was centered above a 3.1 mm hole in the base plate and the probe was lowered at a test speed of 2.5 mm/sec through the sample (Yoshioka and others 2009). The test was conducted and data analyzed using Texture Expert software (version 6.1.3.0, Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems,

Godalming, Surrey, UK). The peak force of 15 slices per fermentation were averaged and recorded in Newtons (N) as the average peak force.

#### *3.3.4 Detection of Fermentation Metabolites*

Concentrations of organic acids, residual sugars, and some spoilage metabolites were measured by High Performance Liquid Chromatography analysis using an Agilent 1260 Infinity HPLC system (Agilent Technologies Inc., Santa Clara, California, USA) equipped with a HPX-87H ion exchange column for organic acid analysis (Bio-Rad Laboratories, Hercules, California, USA). Chromatography was conducted with 0.03 N sulfuric acid as an eluent at a flow rate of 0.6 mL/minute and using a column temperature of 37°C. Organic acids were quantified using an Agilent 1260 DAD detector (G4212B, Agilent Technologies Inc., Santa Clara, California, USA) at 210 nm. Residual sugars and some spoilage compounds were quantified using an Agilent 1260 RI detector (G1362A, Agilent Technologies Inc., Santa Clara, California, USA) that connected in series after the DAD detector. HPLC analysis was conducted on both the brines and the cucumber slurries. Slurries were produced by aseptically blending the cucumbers in a Waring Blender until homogenous as described earlier. Slurries were frozen at -80°C and then thawed to lyse the cucumber cells for more accurate sugar measurements. The samples were centrifuged at 10,000 g for 7.5 min prior to HPLC analysis.

#### *3.3.5 pH and Analysis of Salt*

A Fisher Accumet (model AR25) pH meter calibrated with standard pH 2, 4, and 7 buffers (Fisher Scientific) was used for pH measurement. Calcium and chloride concentrations were determined according to Fleming and others (2001) and AOAC Method 968.31 (2000) for the first replication to determine how quickly the brine components

equilibrated with the cucumbers. The calcium method used 0.025 N disodium ethylenediaminetetraacetate dihydrate (EDTA) as a titrant and hydroxy naphthol blue as the indicator. Titration by Fajans Method using 0.171 N silver nitrate ( $\text{AgNO}_3$ ) as a titrant and dichlorofluorescein as the indicator determined chloride concentration.  $\text{CaCl}_2$  concentrations were estimated to be equal to calcium concentrations although raw cucumbers do have about  $3.0 \pm 0.8$  mM calcium in them (Chapter 2). NaCl concentrations were estimated to be equal to the chloride concentrations since no other sources of chloride should exist in the fermentations using NaCl as the only salt.

### *3.3.6 Statistical Analysis*

All results were analyzed using SAS statistical software (version 9.4, SAS Institute, Cary, NC, USA). A randomized complete block design with a full-factorial treatment structure was performed for the salt and acid levels. A general linear model two-way analysis of variance (ANOVA) was used with least square means. The day of analysis, salt used, and acidification treatment used were designated as fixed effects. Statistical significance was indicated at  $P < 0.05$ . Values are presented as means  $\pm$  standard deviation. Figures are represented with error bars using standard error.

## **3.4 Results and Discussion**

All cucumber fermentations for each lot of cucumbers were completed within 21 days of fermentation, indicated by the depletion of reducing sugars (Figure 3.1), equilibrated lactic acid concentrations (Figure 3.2), and stable pH values (Figure 3.3). All treatments were stable during 100 days of bulk storage as indicated by constant fermentation metabolite concentrations. The reducing sugars were utilized faster in the  $\text{CaCl}_2$  brines and significantly more reducing sugars were utilized in the  $\text{CaCl}_2$  brine treatments than in the NaCl brines

(Figure 3.1 and Table 3.1). This is likely because of the higher LAB counts and activity in the CaCl<sub>2</sub> brine treatments compared with the NaCl brine treatments (Figure 3.4) due to NaCl acting as a greater inhibitor of growth than CaCl<sub>2</sub> (Naewbanij and others 1986). The reduced inhibition allowed the *L. plantarum* inoculated into the brines as well as any natural microbiota brought in on the cucumbers to grow and utilize glucose and fructose resulting in lactic acid production and a decrease in the pH. The higher LAB populations that survived longer and utilized more reducing sugars (Table 3.1 and Figure 3.4) likely accounts for the significantly greater production of lactic acid and significantly lower pH values in the treatments with CaCl<sub>2</sub> brines (Table 3.1). Significantly greater acetic acid and ethanol concentrations were also produced in the fermentations with CaCl<sub>2</sub> brines. This could be because of greater utilization of substrate as each treatment produced acetic acid concentrations of about 4.5-5.5% of the concentration of lactic acid. The ethanol concentrations were less consistent between treatments when accounting for the increased LAB fermentation activity with ethanol concentrations being 15-18.5% of the concentration of lactic acid in the treatments without acetic acid. Treatments with acetic acid added to the fermentation significantly reduced the *Enterobacteriaceae* counts (Figure 3.5) and ethanol concentrations (Table 3.1), suggesting some production of ethanol in the fermentations without acetic acid added may have been produced by uninhibited *Enterobacteriaceae* (Etchells and Fabian 1945).

During the initiation of fermentation, the environments inside the cucumber and in the brine are vastly different for microbial survival and growth (McDonald and others 1991). Immediately after brining, the cucumbers have ample supplies of substrates for microorganisms with minimal inhibition as the brine components have not yet equilibrated

making the cucumbers a nutritious environment for the growth of the microorganisms present on the cucumbers. The brine is a very different environment containing 2.29 M (13.3%) NaCl or 0.22 M (2.4%) CaCl<sub>2</sub> with 8.9 mM potassium sorbate and depending on treatment may have a very low pH (3.6-3.7) by the use of HCl or 0.055 M acetic acid, deterring growth. The acidification of the brines resulted in day 0 brine pH values of 3.6-3.7 while the non-acidified brines started upwards of 7 compared to the cucumber environment which has a pH value around 6. The *L. plantarum* starter culture was inoculated into the brine at 10<sup>6</sup> CFU/mL, but this harsh brine environment reduced the lactic acid bacteria counts in the treatments with NaCl as the brining salt by 2 log CFU/mL within the first day (Figure 3.4c). As nutrients diffused from the cucumbers into the brine and the salt equilibrated into the cucumbers, the LAB populations in the NaCl brines nearly matched the LAB populations in the CaCl<sub>2</sub> brines by day 3 before dramatically decreasing by 2 log CFU/mL by the end of the first week and another 2 log CFU/mL by the end of the second week (Figures 3.4a and 3.4c). The LAB populations in the cucumbers (Figures 3.4b and 3.4d) showed a similar pattern but the high nutrient levels and relatively low diffused salt concentrations helped the LAB match the growth pattern of the CaCl<sub>2</sub> brined cucumbers for most of the first week. Around day 7 the LAB population in the cucumbers started steadily declining, as they had in the brine, likely because the salt had equilibrated and organic acids had started replacing the nutrients, lowering the pH; both the salt and pH would have provided mild inhibition.

Initial yeast and mold counts from the cucumbers were 10<sup>5</sup> CFU/mL, but by the day 1 time point had decreased below 10<sup>2</sup> CFU/mL in all treatments except the non-acidified CaCl<sub>2</sub> brined treatment which matched the other treatments by the second day. The potassium sorbate was added to help inhibit the growth of yeast and mold and seemed to sufficiently do



so in each treatment tested as the sorbate concentrations were nearly equilibrated by the first day (data not shown).

Of particular concern in this experiment was the effect of brine salt and acidification on the growth and survival of *Enterobacteriaceae* spp. naturally found on the raw cucumbers. Counts on the raw cucumber were  $4.8 \pm 0.2$  log CFU/mL and stayed more than 1 log CFU/mL higher in the cucumbers than in the brine for the first 3 days (Figure 3.5). By day 5, the *Enterobacteriaceae* had decreased below detection levels in all treatments for both cucumbers and brine. The treatments with acetic acid included in the fermentation brines significantly reduced *Enterobacteriaceae* spp. counts in brines and cucumbers ( $P < 0.002$ ). However, the addition of HCl at the same pH did not have an effect nor did the use of NaCl compared to CaCl<sub>2</sub>. In Pérez-Díaz and others (2015), *Enterobacteriaceae* counts were measured in non-acidified CaCl<sub>2</sub> brine and in a recycled brine. They found that it took a week for the non-acidified CaCl<sub>2</sub> treatment to decrease below detection limits and the non-acidified CaCl<sub>2</sub> treatment had much higher counts than the recycled NaCl brine treatment. Our data suggests that it was the acetic acid in the recycled brines that lowered the *Enterobacteriaceae* counts rather than the salt.

Fermented cucumber mesocarp firmness was unaffected by the different brining treatments during the fermentation period. However, after being stored for 100 days to simulate a typical bulk storage period, a large difference was observed between the salt treatments. The average peak force of raw cucumber mesocarp was increased by 1.5-2 N during the first week of brining in all treatments (Figure 3.6), but after 100 days of storage, the cucumbers fermented in NaCl brines had average peak force values lower than the raw cucumbers while those in CaCl<sub>2</sub> brines maintained the higher firmness values (Figure 3.7).

After storage, the treatments brined in NaCl had an average firmness of 7.5 N while their CaCl<sub>2</sub> brined counterparts were at 10.4 N. Cucumber firmness was significantly higher in CaCl<sub>2</sub> brined cucumbers compared to those brined in NaCl at 100 days after brining by 2.9 N ( $P < 0.0001$ ). Acidification using acetic acid or HCl had no effect on texture quality of fermented cucumbers for either brining salt ( $P = 0.8235$ ).

The finding that cucumbers fermented in CaCl<sub>2</sub> brines were significantly firmer than cucumbers fermented in NaCl brines is contrary to what was observed in commercial trials (Chapter 2, Pérez-Díaz and others 2015, Wilson and others 2015). Commercial trials have shown that a CaCl<sub>2</sub> fermentation process using 0.1 M CaCl<sub>2</sub>, 6 mM potassium sorbate, and a starter culture inoculated at 10<sup>6</sup> log CFU/mL resulted in product with an average peak force 1.7 N less firm than current commercial fermentations in 1 M NaCl and 0.03 M CaCl<sub>2</sub>. Since a clear opposing trend was found in this experiment, it is likely that there are commercial production variables that negatively affect the texture in the CaCl<sub>2</sub> process. There are many possible variables that we controlled for by using a sealed jar experiment that are uncontrolled in the open-top tanks used in commercial production. These production variables include oxygen that is introduced through air-purging operations and solubilization at the surface of open-top tanks, rainwater that freely falls into open tanks, introduction of new microbes from the air, and evaporation of water or organic acids from the brine causing varying concentrations of brine components. Other variables we controlled for that are less precise in commercial fermentation include exact pack out ratios of 55:45 cucumbers: brine (w/w) resulting in exact concentrations of brine components, constant incubation temperature of 28°C as opposed to temperatures varying with the environmental temperature, and the use of a starter culture instead of a natural fermentation in the NaCl brined treatments.

Commercial fermentation of cucumbers involves filling a fermentation tank with hundreds of bushels of cucumbers and then filling it with brine made to equilibrate to the desired concentrations of NaCl, CaCl<sub>2</sub>, acetic acid, and/or potassium sorbate. As the pack ratio is not strictly controlled, the brine may not equilibrate to the right concentrations and may not inhibit pectinolytic organisms as well as if controlled. The temperature in a fermentation prepared in June will usually have a different temperature profile than a fermentation started in October. The temperature is well stabilized because of the heat capacity of the water and size of the fermentation, but the temperature in the tank will still vary somewhat through the year and has been shown to increase the softening rate as the temperature was raised (McFeeters and others 1995) as well as affecting the fermentation microbiota. All of these factors can have direct or indirect effects on the firmness of a fermented and stored cucumber. It seems plausible that since minimal firmness changes were seen in the treatments with CaCl<sub>2</sub> brines, that the firmness differences seen in Chapter 2 for CaCl<sub>2</sub> brined tanks were due to a variable controlled for in this experiment and that another variable was introduced that reduced the firmness of the NaCl brined cucumbers. The use of closed jars as opposed to open-top fermentation tanks would prevent the free exchange of atmospheric oxygen coupled with the introduction of new microorganisms that could result in greater growth of pectinolytic organisms such as yeasts and molds. Softening was not observed with the presence of greater *Enterobacteriaceae spp.* in this study, but aerobic yeasts and molds that can produce pectinolytic enzymes could have been a factor in the commercial tanks that were not present in the closed jars. Commercial fermentations commonly have 0.03 M CaCl<sub>2</sub> added as a firming agent (McFeeters and others 1995). This was not included in this experiment to examine the difference between the brining salts individually, but the lack of

CaCl<sub>2</sub> as a firming agent in NaCl brines could explain the observed softening during extended storage (Buescher and Burgin 1988). The addition of acetic acid to fermentation brines was the only factor found to significantly affect the *Enterobacteriaceae* populations during the initiation of fermentation. Since the use of acetic acid did not affect the mesocarp firmness, it seems unlikely that these *Enterobacteriaceae* are causative agents in cucumber softening. However, since the CaCl<sub>2</sub> brined cucumbers were not found to be softer than the NaCl brined cucumbers in this study, an interaction effect between initial brine acidification and a production variable cannot be ruled out as explaining the softening observed in commercial fermentations.

### **3.5 Conclusion**

The use of acetic acid in cucumber fermentations reduced the *Enterobacteriaceae* population faster than acidification with HCl and non-acidified treatments in both NaCl and CaCl<sub>2</sub> brines. However, acidification did not affect the mesocarp firmness during fermentation or after 100 days storage, so the texture differences observed are not likely a result of *Enterobacteriaceae spp.* There was significantly different mesocarp firmness at 100 days storage between the two brining salts with CaCl<sub>2</sub> brined treatments found to be firmer than NaCl brined treatments. This result is different from commercial experiments and the cause could not be definitively identified in this study but may be due to production variables in open-top 40,000 L tanks not well represented in sealed 1 L jars.

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### Chapter 2

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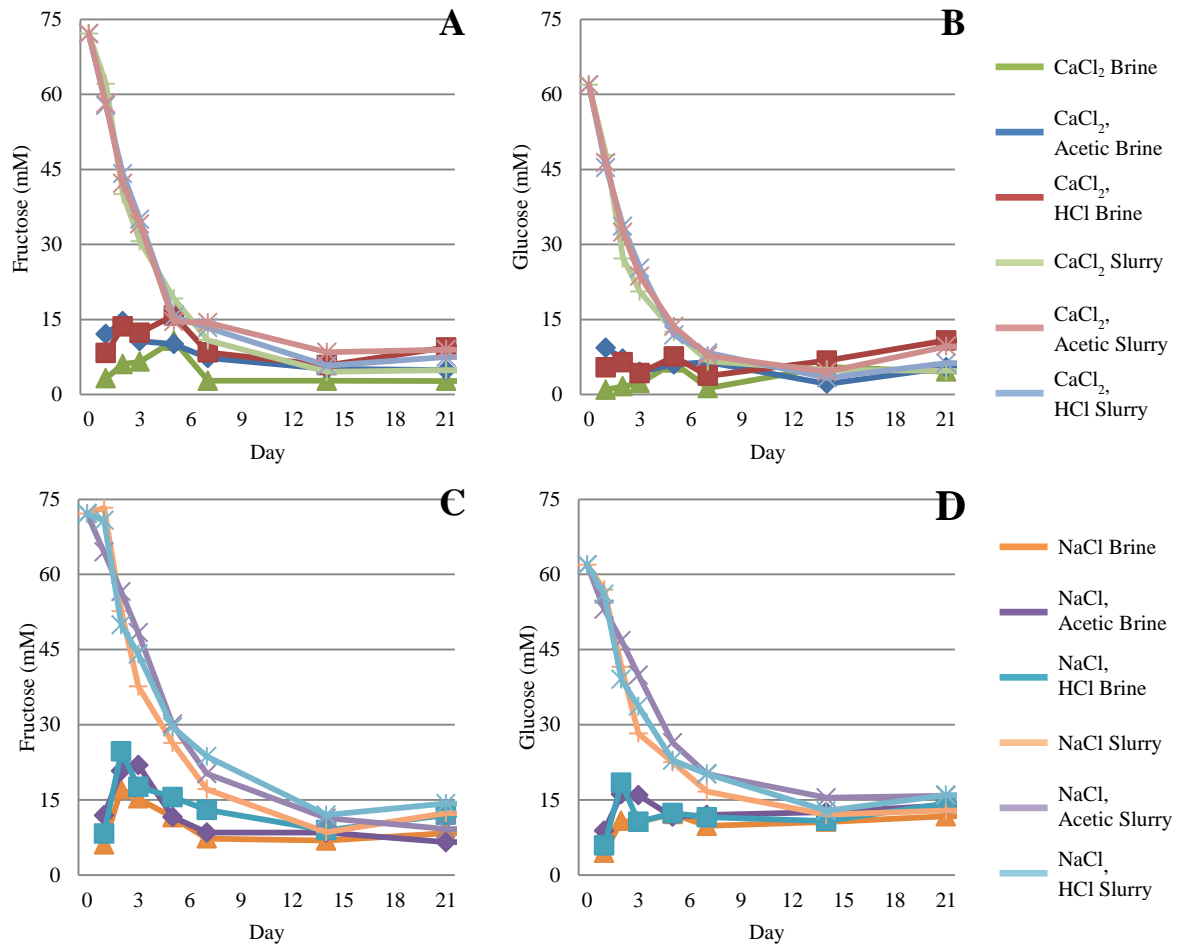
Yoshioka Y, Horie H, Sugiyama M, Sakata Y. 2009. Quantifying Cucumber Fruit Crispness By Mechanical Measurement. *J Breeding Sci.* 59 (2): 139-147.

**Table 3.1 – Effect of brine composition on fermentation chemistry (21 days)**

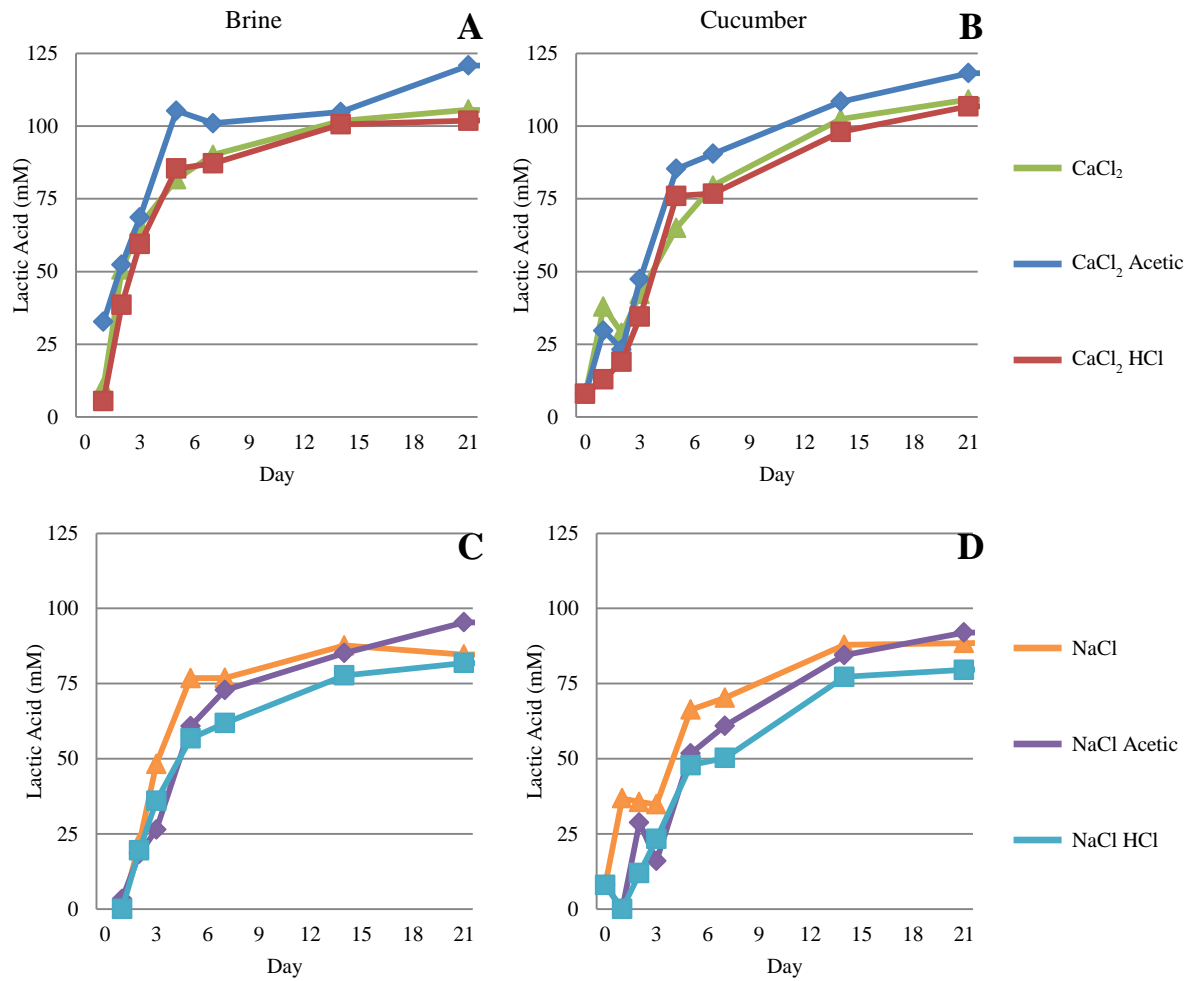
Salt	Acid	Fructose (mM) <sup>1</sup>	Glucose (mM) <sup>1</sup>	pH <sup>1</sup>	Lactic Acid (mM) <sup>1</sup>	Acetic Acid (mM) <sup>1</sup>	Ethanol (mM) <sup>1</sup>
CaCl <sub>2</sub>	No Acid	3.8 ± 3.8 <sup>b</sup>	1.8 ± 2.4 <sup>c</sup>	3.14 ± 0.10 <sup>b</sup>	107.4 ± 13.8 <sup>ab</sup>	5.9 ± 1.2 <sup>b</sup>	19.3 ± 4.7 <sup>a</sup>
	Acetic	4.6 ± 4.3 <sup>ab</sup>	2.2 ± 3.5 <sup>c</sup>	3.12 ± 0.07 <sup>b</sup>	119.5 ± 13.7 <sup>a</sup>	31.1 ± 1.4 <sup>a</sup>	11.5 ± 2.3 <sup>bc</sup>
	HCl	6.9 ± 7.2 <sup>ab</sup>	5.1 ± 6.1 <sup>bc</sup>	3.11 ± 0.09 <sup>b</sup>	104.3 ± 18.7 <sup>abc</sup>	5.4 ± 0.5 <sup>b</sup>	19.3 ± 4.3 <sup>a</sup>
NaCl	No Acid	8.8 ± 7.0 <sup>ab</sup>	12.3 ± 5.6 <sup>ab</sup>	3.26 ± 0.03 <sup>a</sup>	86.6 ± 20.1 <sup>bc</sup>	3.9 ± 0.5 <sup>c</sup>	12.9 ± 3.0 <sup>b</sup>
	Acetic	7.9 ± 4.7 <sup>ab</sup>	14.9 ± 6.6 <sup>a</sup>	3.25 ± 0.02 <sup>a</sup>	93.7 ± 14.7 <sup>abc</sup>	29.8 ± 0.9 <sup>a</sup>	6.7 ± 4.9 <sup>c</sup>
	HCl	13.2 ± 8.1 <sup>a</sup>	15.1 ± 5.5 <sup>a</sup>	3.29 ± 0.06 <sup>a</sup>	80.7 ± 21.8 <sup>c</sup>	3.6 ± 0.5 <sup>c</sup>	13.9 ± 2.7 <sup>ab</sup>

<sup>1</sup>Means for different treatments followed by different letters are significant at  $P < 0.05$ .





**Figure 3.1 - Depletion of the fermentable sugars fructose (a and c) and glucose (b and d) during the first 21 days of fermentation for treatments using the CaCl<sub>2</sub> brines (a and b) or NaCl brines (c and d). The concentrations in the brines are the bolder colors and the concentrations in the cucumbers are the fainter colors.**



**Figure 3.2 - Production of lactic acid during the first 21 days of fermentation in CaCl<sub>2</sub> brines (a and b) or NaCl brines (c and d).**

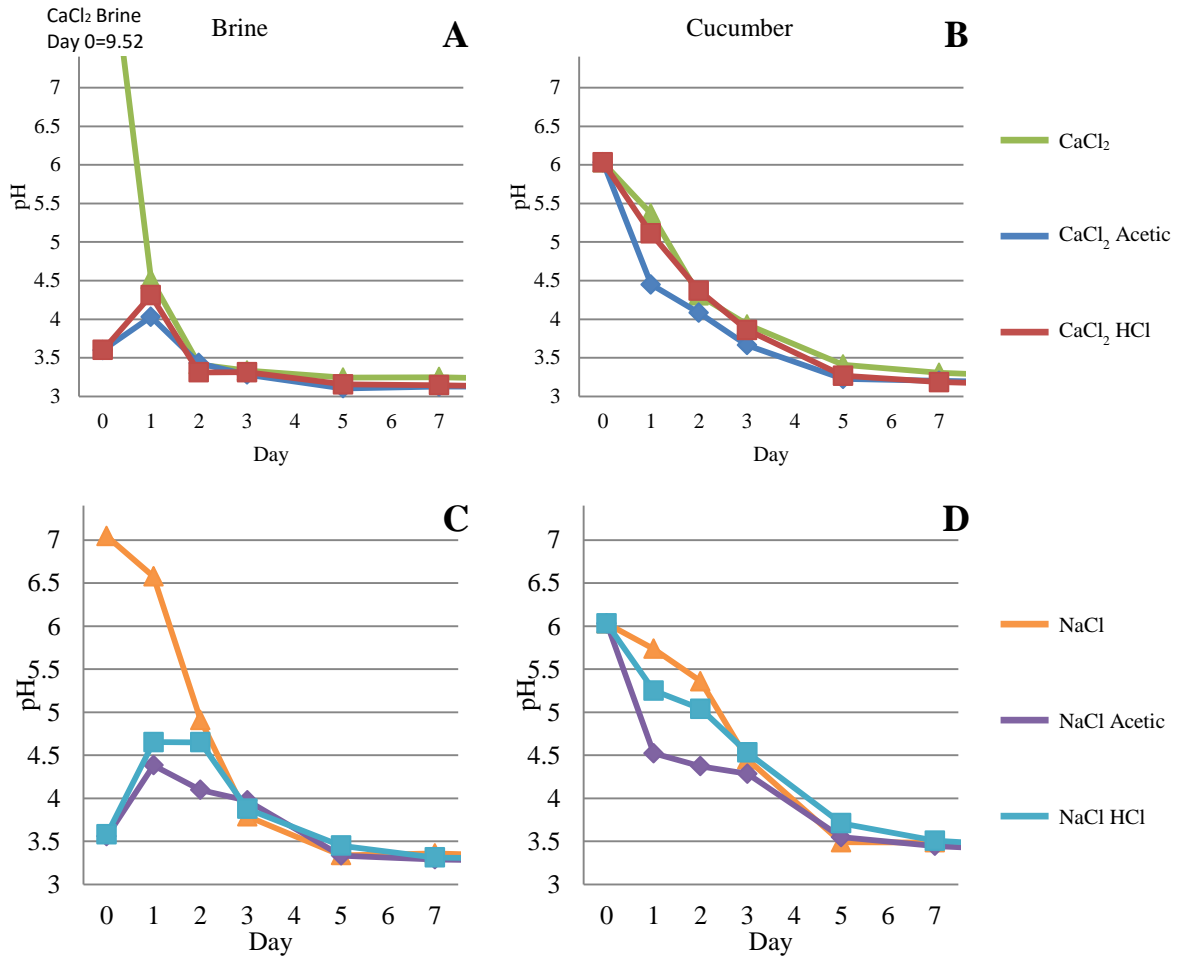


Figure 3.3 - pH change during the first 7 days of fermentation in CaCl<sub>2</sub> brines (a and b) or NaCl brines (c and d).

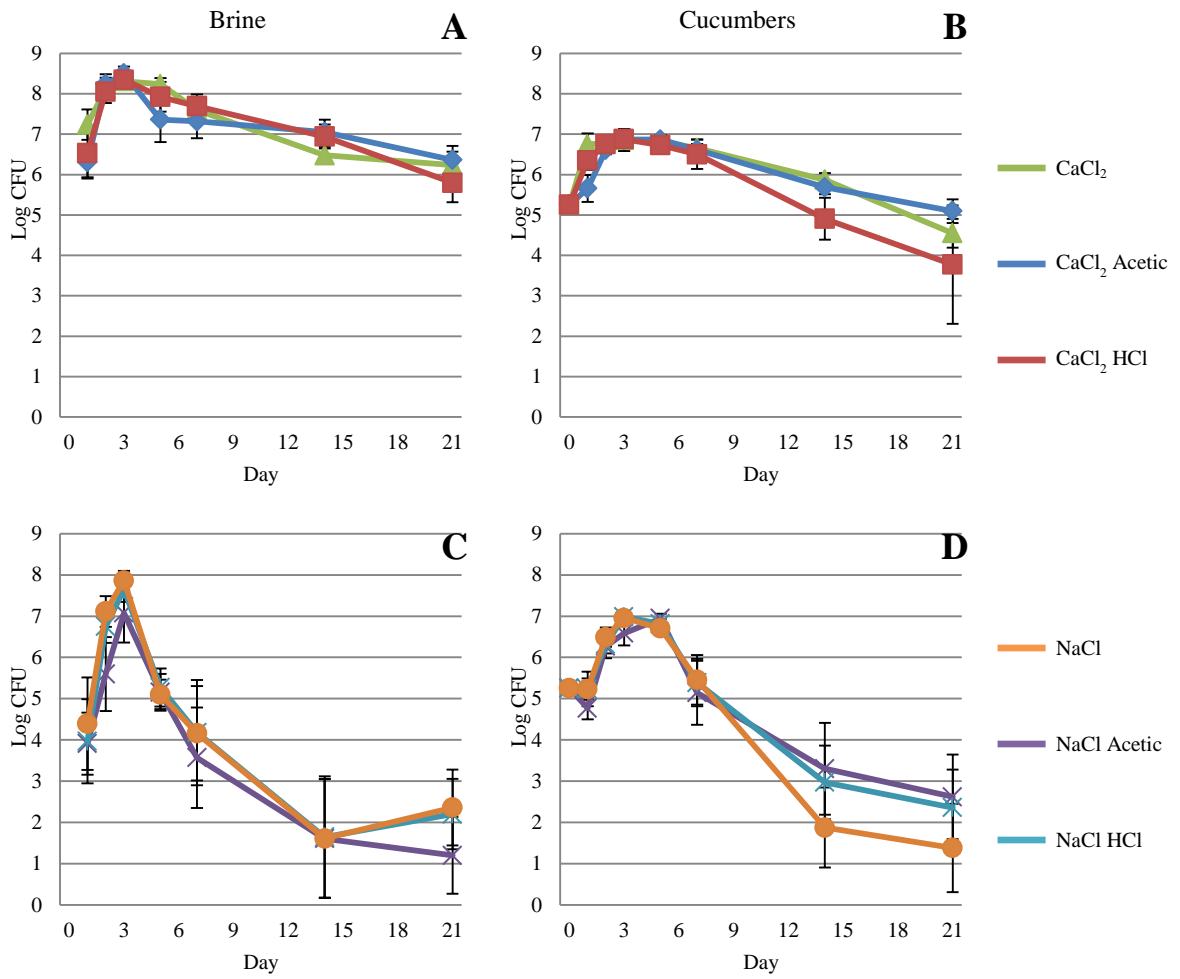
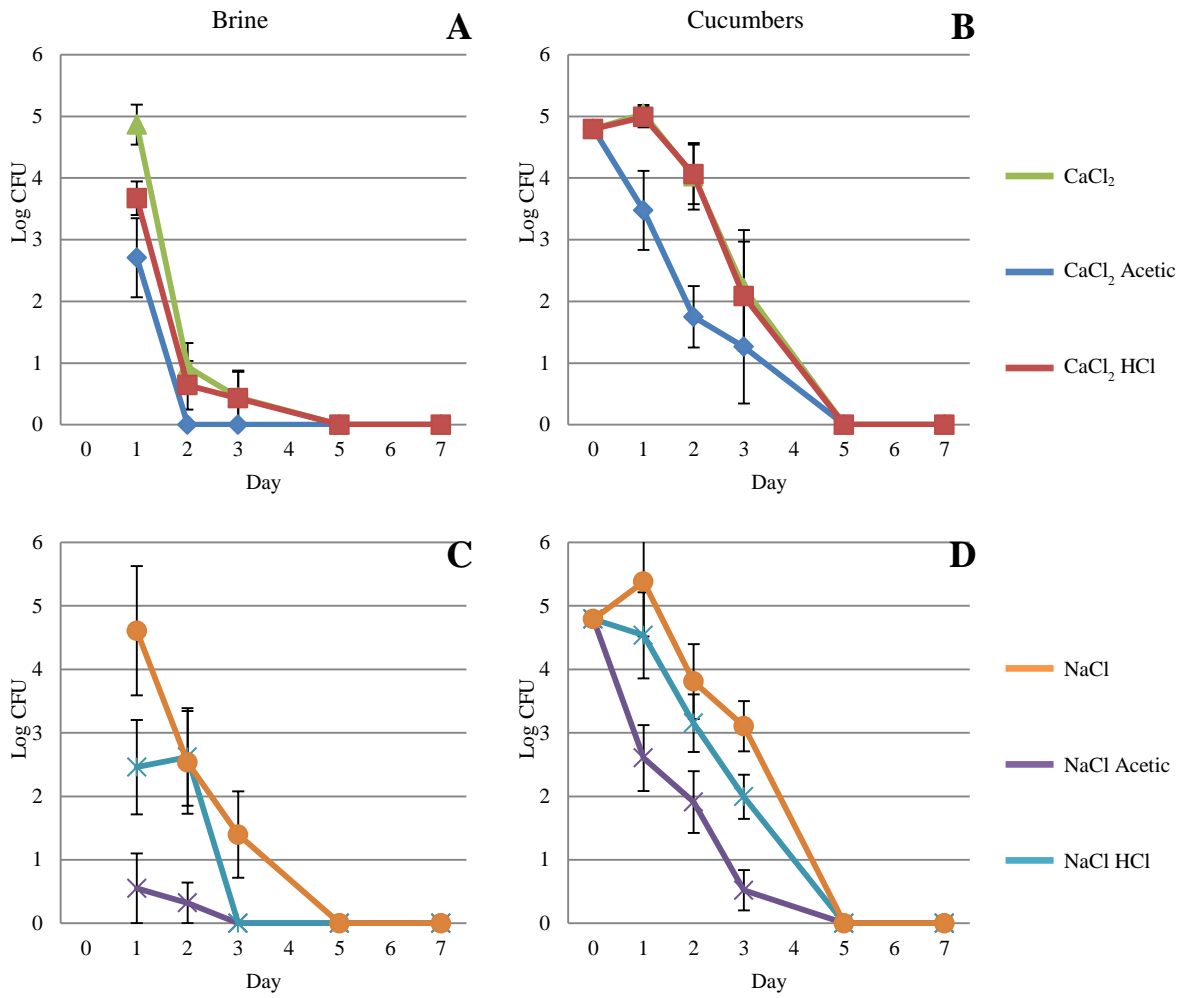
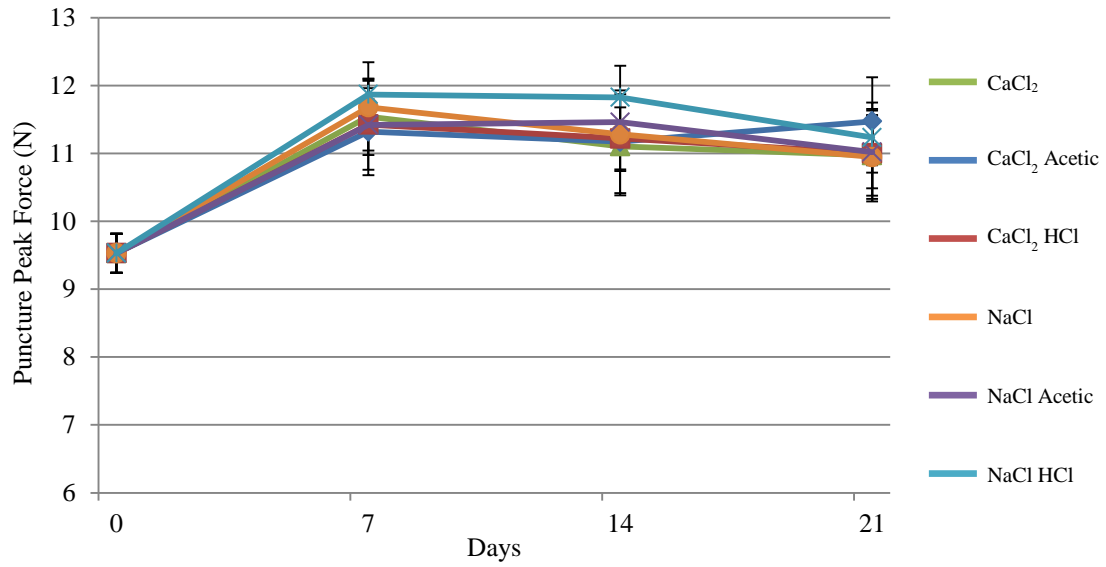


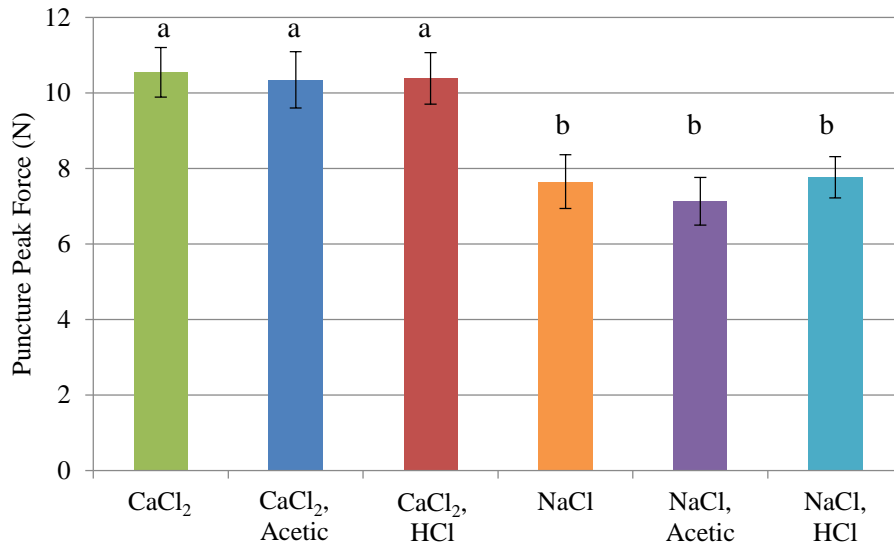
Figure 3.4 - LAB populations enumerated in the brine (a and c) and cucumbers (b and d) during the first 21 days of fermentation in CaCl<sub>2</sub> brines (a and b) and NaCl brines (c and d).



**Figure 3.5 - *Enterobacteriaceae* populations enumerated in the brine (a and c) and cucumbers (b and d) during the first week of fermentation in the CaCl<sub>2</sub> brines (a and b) and NaCl brines (c and d). All treatments were below detection levels by day 5.**



**Figure 3.6 - Cucumber mesocarp firmness (N) over the course of fermentation.**



**Figure 3.7 - Cucumber mesocarp firmness (N) after typical bulk storage of 100 days.**

## APPENDICES



## **Appendix A: Supplementary Statistical Information**

### **Supplemental Material to Chapter 2**

#### *Statistical Model*

This research project used an unbalanced design that had an unequal number of tanks in each treatment and not all combinations of cucumber size and fermentation process were used symmetrically. Additionally, raw, fermented, desalted, and finished product samples were not collected from each fermentation. To help accommodate for this unbalance design, a mixed model was used with the fermentation process, cucumber size, and stage in processing designated as fixed effects and the individual fermentations were designated as random effects. A least square means with Tukey adjustment was used to find significance. The statistical analyses for Bloater Index, Firmness, and Color follow.

#### *Statistical Analysis for Bloater Indices*

The statistical analysis of the total bloater index as well as Balloon, Lens, and Honeycomb type indices were completed using the following variables and code to determine whether significant differences existed between the cucumber size, fermentation process and stage in processing. The ANOVA, Least Squares Means, and Difference in Least Square Means produced by SAS follow the code. Statistical significance was indicated at  $P < 0.05$ .

#### *Variables*

**sampletype** = the stage in fermentation (raw, fermented, desalted, finished product)

**brinetype** = the fermentation process used

**csize** = the size of the cucumbers

**tank** = the fermentation

**BloaterIndex** = The bloater index approximates the damage to the cucumbers based on the severity and type of bloaters present.

*SAS Code:*

```
Proc mixed data=CommercialTrials method=type3;  
  
  class sampletype brinetype csize tank tankyear;  
  
  model BloaterIndex = brinetype|csize sampletype tankyear/solution;  
  
  random tank(sampletype*csize2*brinetype*tankyear);  
  
  lsmeans brinetype*csize /pdiff adjust=tukey;  
  
run;  
  
quit;
```

**Table A.1 – Analysis of Variance (ANOVA) table for the statistical analysis of Total Bloater Index**

Type 3 Analysis of Variance						
Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
CSize	3	718.610731	239.536910	111.93	5.53	0.0014
BrineType	1	27.115133	27.115133	112.05	0.63	0.4303
BrineType*CSize	3	302.676651	100.892217	111.95	2.33	0.0783
SampleType	1	340.133500	340.133500	111.3	7.81	0.1601
Tankyear	1	662.644878	662.644878	111.87	15.29	0.0002
Tan(Sam*Bri*CSi*Tan)	111	4848.212840	43.677593	2	2.69	0.3098
Residual	2	32.500000	16.250000	.	.	.

**Table A.2 – Least Squares Means table for the statistical analysis of Total Bloater Index**

Least Squares Means							
Effect	BrineType	Csize	Estimate	Std Error	DF	t Value	Pr >  t
BrineType*CSize	CaCl2	2A	5.1000	3.4825	112	1.46	0.1459
BrineType*CSize	CaCl2	2B	6.6818	1.4521	112	4.60	<.0001
BrineType*CSize	CaCl2	3A	9.7991	1.0787	112	9.08	<.0001
BrineType*CSize	CaCl2	3B	6.2709	3.1175	112	2.01	0.0467
BrineType*CSize	NaCl	2A	4.1667	2.8434	112	1.47	0.1456
BrineType*CSize	NaCl	2B	4.7955	1.5588	112	3.08	0.0026
BrineType*CSize	NaCl	3A	5.8743	1.6931	112	3.47	0.0007
BrineType*CSize	NaCl	3B	15.5773	3.4973	112	4.45	<.0001

**Table A.3 – Difference in Least Squares Means table for the statistical analysis of Total Bloater Index**

Effect	Differences of Least Squares Means					Estimate	Standard Error	DF	t Value	Pr >  t	Adjustment	Adj P
	BrineType	CSize2	_BrineType	_CSize2								
BrineType*CSize	CaCl2	2A	CaCl2	2B	-1.5818	3.7731	112	-0.42	0.6758	Tukey-Kramer	0.9999	
BrineType*CSize	CaCl2	2A	CaCl2	3A	-4.6991	3.6457	112	-1.29	0.2001	Tukey-Kramer	0.9012	
BrineType*CSize	CaCl2	2A	CaCl2	3B	-1.1709	4.6740	112	-0.25	0.8027	Tukey-Kramer	1.0000	
BrineType*CSize	CaCl2	2A	NaCl	2A	0.9333	4.4959	112	0.21	0.8359	Tukey-Kramer	1.0000	
BrineType*CSize	CaCl2	2A	NaCl	2B	0.3045	3.8154	112	0.08	0.9365	Tukey-Kramer	1.0000	
BrineType*CSize	CaCl2	2A	NaCl	3A	-0.7743	3.8722	112	-0.20	0.8419	Tukey-Kramer	1.0000	
BrineType*CSize	CaCl2	2A	NaCl	3B	-10.4773	4.9355	112	-2.12	0.0360	Tukey-Kramer	0.4069	
BrineType*CSize	CaCl2	2B	CaCl2	3A	-3.1173	1.8177	112	-1.71	0.0891	Tukey-Kramer	0.6776	
BrineType*CSize	CaCl2	2B	CaCl2	3B	0.4109	3.4441	112	0.12	0.9052	Tukey-Kramer	1.0000	
BrineType*CSize	CaCl2	2B	NaCl	2A	2.5151	3.1928	112	0.79	0.4325	Tukey-Kramer	0.9935	
BrineType*CSize	CaCl2	2B	NaCl	2B	1.8864	2.1344	112	0.88	0.3787	Tukey-Kramer	0.9870	
BrineType*CSize	CaCl2	2B	NaCl	3A	0.8075	2.2373	112	0.36	0.7188	Tukey-Kramer	1.0000	
BrineType*CSize	CaCl2	2B	NaCl	3B	-8.8954	3.7981	112	-2.34	0.0209	Tukey-Kramer	0.2807	
BrineType*CSize	CaCl2	3A	CaCl2	3B	3.5282	3.2942	112	1.07	0.2865	Tukey-Kramer	0.9615	
BrineType*CSize	CaCl2	3A	NaCl	2A	5.6324	3.0412	112	1.85	0.0667	Tukey-Kramer	0.5863	
BrineType*CSize	CaCl2	3A	NaCl	2B	5.0037	1.8916	112	2.65	0.0093	Tukey-Kramer	0.1511	
BrineType*CSize	CaCl2	3A	NaCl	3A	3.9248	2.0008	112	1.96	0.0523	Tukey-Kramer	0.5122	
BrineType*CSize	CaCl2	3A	NaCl	3B	-5.7781	3.6495	112	-1.58	0.1162	Tukey-Kramer	0.7592	
BrineType*CSize	CaCl2	3B	NaCl	2A	2.1042	4.2195	112	0.50	0.6190	Tukey-Kramer	0.9996	
BrineType*CSize	CaCl2	3B	NaCl	2B	1.4755	3.4831	112	0.42	0.6727	Tukey-Kramer	0.9999	
BrineType*CSize	CaCl2	3B	NaCl	3A	0.3966	3.5435	112	0.11	0.9111	Tukey-Kramer	1.0000	
BrineType*CSize	CaCl2	3B	NaCl	3B	-9.3064	4.6763	112	-1.99	0.0490	Tukey-Kramer	0.4932	
BrineType*CSize	NaCl	2A	NaCl	2B	-0.6288	3.2427	112	-0.19	0.8466	Tukey-Kramer	1.0000	
BrineType*CSize	NaCl	2A	NaCl	3A	-1.7077	3.3093	112	-0.52	0.6069	Tukey-Kramer	0.9996	
BrineType*CSize	NaCl	2A	NaCl	3B	-11.4106	4.5074	112	-2.53	0.0127	Tukey-Kramer	0.1933	
BrineType*CSize	NaCl	2B	NaCl	3A	-1.0789	2.2982	112	-0.47	0.6397	Tukey-Kramer	0.9998	
BrineType*CSize	NaCl	2B	NaCl	3B	-10.7818	3.8236	112	-2.82	0.0057	Tukey-Kramer	0.1005	
BrineType*CSize	NaCl	3A	NaCl	3B	-9.7029	3.8762	112	-2.50	0.0137	Tukey-Kramer	0.2049	

### *Statistical Analysis for Firmness*

The statistical analysis of the firmness as determined by the average peak force using a mesocarp puncture test was accomplished using the following variables and code to determine the significant differences shown in Figure 2.2. The ANOVA, Least Squares Means, and Difference in Least Square Means produced by SAS follow the code. Statistical significance was indicated at  $P < 0.05$ .

#### ***Variables***

**sampletype** = the stage in fermentation (raw, fermented, desalted, finished product)

**brinetype** = the fermentation process used

**csize** = the size of the cucumbers

**tank** = the fermentation

#### ***SAS code:***

```
Proc mixed data=CommercialTrials method=type3;  
  
    class sampletype brinetype csize tank;  
  
    model PeakForce = sampletype|brinetype csize/solution;  
  
    random tank(csize*brinetype);  
  
    lsmeans sampletype*brinetype /pdiff adjust=tukey;  
  
run;  
  
quit;
```

**Table A.4 – Analysis of Variance (ANOVA) table for the statistical analysis of Peak Puncture Force**

Type 3 Analysis of Variance						
Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
SampleType	3	98.112111	32.704037	216	60.36	<.0001
BrineType	1	4.349134	4.349134	216	5.26	0.0226
SampleType*BrineType	3	6.668458	2.222819	216	4.10	0.0074
CSize	3	50.144150	16.714717	102.04	8.68	<.0001
Tank(BrineType*CSize)	77	246.925756	3.206828	216	5.92	<.0001
Residual	216	117.032132	0.541815	.	.	.

**Table A.5 – Least Squares Means table for the statistical analysis of Peak Puncture Force**

Least Squares Means							
Effect	SampleType	BrineType	Estimate	Std Error	DF	t Value	Pr >  t
SampleType*BrineType	Desalted	CaCl2	9.7027	0.2224	216	43.63	<.0001
SampleType*BrineType	Desalted	NaCl	10.8223	0.2604	216	41.56	<.0001
SampleType*BrineType	Fermented	CaCl2	9.3849	0.2157	216	43.51	<.0001
SampleType*BrineType	Fermented	NaCl	10.7287	0.2401	216	44.68	<.0001
SampleType*BrineType	Finished	CaCl2	7.5687	0.2105	216	35.96	<.0001
SampleType*BrineType	Finished	NaCl	9.2285	0.2549	216	36.21	<.0001
SampleType*BrineType	Raw	.	9.3050	0.2217	216	41.97	<.0001

**Table A.6 – Difference in Least Squares Means table for the statistical analysis of Peak Puncture Force**

Effect	Differences of Least Squares Means				Estimate	Std Error	DF	t Value	Pr >  t	Adjustment	Adj P
	SampleType	BrineType	_SampleType	_BrineType							
SampleType*BrineType	Desalted	CaCl2	Desalted	NaCl	-1.1195	0.3002	216	-3.73	0.0002	Tukey-Kramer	0.0059
SampleType*BrineType	Desalted	CaCl2	Fermented	CaCl2	0.3179	0.1811	216	1.76	0.0807	Tukey-Kramer	0.6510
SampleType*BrineType	Desalted	CaCl2	Fermented	NaCl	-1.0260	0.2812	216	-3.65	0.0003	Tukey-Kramer	0.0078
SampleType*BrineType	Desalted	CaCl2	Finished	CaCl2	2.1340	0.1568	216	13.61	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	CaCl2	Finished	NaCl	0.4742	0.2891	216	1.64	0.1024	Tukey-Kramer	0.7253
SampleType*BrineType	Desalted	CaCl2	Raw	.	0.3977	0.1739	216	2.29	0.0232	Tukey-Kramer	0.3059
SampleType*BrineType	Desalted	NaCl	Fermented	CaCl2	1.4374	0.2959	216	4.86	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	NaCl	Fermented	NaCl	0.09352	0.2419	216	0.39	0.6994	Tukey-Kramer	0.9999
SampleType*BrineType	Desalted	NaCl	Finished	CaCl2	3.2536	0.2902	216	11.21	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	NaCl	Finished	NaCl	1.5937	0.2252	216	7.08	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	NaCl	Raw	.	1.5173	0.2985	216	5.08	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Fermented	CaCl2	Fermented	NaCl	-1.3439	0.2763	216	-4.86	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Fermented	CaCl2	Finished	CaCl2	1.8162	0.1603	216	11.33	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Fermented	CaCl2	Finished	NaCl	0.1564	0.2853	216	0.55	0.5842	Tukey-Kramer	0.9994
SampleType*BrineType	Fermented	CaCl2	Raw	.	0.07988	0.1749	216	0.46	0.6483	Tukey-Kramer	0.9998
SampleType*BrineType	Fermented	NaCl	Finished	CaCl2	3.1600	0.2706	216	11.68	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Fermented	NaCl	Finished	NaCl	1.5002	0.2327	216	6.45	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Fermented	NaCl	Raw	.	1.4237	0.2793	216	5.10	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Finished	CaCl2	Finished	NaCl	-1.6598	0.2777	216	-5.98	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Finished	CaCl2	Raw	.	-1.7363	0.1432	216	-12.13	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Finished	NaCl	Raw	.	-0.07648	0.2867	216	-0.27	0.7899	Tukey-Kramer	1.0000

### *Statistical Analysis for Color*

The statistical analysis of the hue, chroma, and L\* were accomplished using the following variables and code to determine the significant differences shown in Figures 2.4 and 2.5. The ANOVA, Least Squares Means, and Difference in Least Square Means produced by SAS follow the code. Statistical significance was indicated at  $P < 0.05$ .

#### ***Variables***

**sampletype** = the stage in fermentation (raw, fermented, desalted, finished product)

**brinetype** = the fermentation process used

**csize** = the size of the cucumbers

**tank** = the fermentation

**L** = L\*

#### ***SAS code:***

```
Proc mixed data=CommercialTrials method=type3;  
  
  class sampletype brinetype csize tank;  
  
  model L = sampletype|brinetype csize/solution;  
  
  random tank(csize*brinetype);  
  
  lsmeans sampletype*brinetype /pdiff adjust=tukey;  
  
run;  
  
quit;
```



```
Proc mixed data=CommercialTrials method=type3;  
  
    class sampletype brinetype csize tank;  
  
    model hue = sampletype|brinetype csize/solution;  
  
    random tank(csize*brinetype);  
  
    lsmeans sampletype*brinetype /pdiff adjust=tukey;  
  
run;  
  
quit;
```

```
Proc mixed data=CommercialTrials method=type3;  
  
    class sampletype brinetype csize tank;  
  
    model chroma = sampletype|brinetype csize/solution;  
  
    random tank(csize*brinetype);  
  
    lsmeans sampletype*brinetype /pdiff adjust=tukey;  
  
run;  
  
quit;
```

**Table A.7 – Analysis of Variance (ANOVA) table for the statistical analysis of L\***

Type 3 Analysis of Variance						
Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
SampleType	2	314.974644	157.487322	157.59	21.97	<.0001
BrineType	1	1.009942	1.009942	163.53	0.14	0.7045
SampleType*BrineType	2	4.670126	2.335063	154.48	0.32	0.7256
CSize	3	537.745679	179.248560	193.31	28.65	<.0001
Tank(Samp*Brin*CSize)	152	1115.908473	7.341503	81	2.31	<.0001
Residual	81	256.944470	3.172154	.	.	.

**Table A.8 – Least Squares Means table for the statistical analysis of L\***

Least Squares Means							
Effect	SampleType	BrineType	Estimate	Std Error	DF	t Value	Pr >  t
SampleType*BrineType	Desalted	CaCl2	44.5598	0.4648	152	95.87	<.0001
SampleType*BrineType	Desalted	NaCl	44.1285	0.5722	152	77.12	<.0001
SampleType*BrineType	Fermented	CaCl2	42.8817	0.4450	152	96.36	<.0001
SampleType*BrineType	Fermented	NaCl	43.1864	0.4952	152	87.20	<.0001
SampleType*BrineType	Finished	CaCl2	46.1165	0.4656	152	99.06	<.0001
SampleType*BrineType	Finished	NaCl	45.8041	0.6003	152	76.31	<.0001

**Table A.9 – Difference in Least Squares Means table for the statistical analysis of L\***

Effect	Differences of Least Squares Means					Estimate	Std Error	DF	t Value	Pr >  t	Adjustment	Adj P
	SampleType	BrineType	_SampleType	_BrineType								
SampleType*BrineType	Desalted	CaCl2	Desalted	NaCl	0.4313	0.6885	152	0.63	0.5320	Tukey-Kramer	0.9889	
SampleType*BrineType	Desalted	CaCl2	Fermented	CaCl2	1.6781	0.5652	152	2.97	0.0035	Tukey-Kramer	0.0399	
SampleType*BrineType	Desalted	CaCl2	Fermented	NaCl	1.3734	0.6276	152	2.19	0.0302	Tukey-Kramer	0.2494	
SampleType*BrineType	Desalted	CaCl2	Finished	CaCl2	-1.5567	0.5717	152	-2.72	0.0072	Tukey-Kramer	0.0766	
SampleType*BrineType	Desalted	CaCl2	Finished	NaCl	-1.2443	0.6841	152	-1.82	0.0709	Tukey-Kramer	0.4568	
SampleType*BrineType	Desalted	NaCl	Fermented	CaCl2	1.2468	0.6791	152	1.84	0.0683	Tukey-Kramer	0.4459	
SampleType*BrineType	Desalted	NaCl	Fermented	NaCl	0.9421	0.7224	152	1.30	0.1942	Tukey-Kramer	0.7825	
SampleType*BrineType	Desalted	NaCl	Finished	CaCl2	-1.9879	0.6836	152	-2.91	0.0042	Tukey-Kramer	0.0472	
SampleType*BrineType	Desalted	NaCl	Finished	NaCl	-1.6755	0.7762	152	-2.16	0.0324	Tukey-Kramer	0.2634	
SampleType*BrineType	Fermented	CaCl2	Fermented	NaCl	-0.3048	0.6151	152	-0.50	0.6210	Tukey-Kramer	0.9963	
SampleType*BrineType	Fermented	CaCl2	Finished	CaCl2	-3.2348	0.5593	152	-5.78	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Fermented	CaCl2	Finished	NaCl	-2.9224	0.6805	152	-4.29	<.0001	Tukey-Kramer	0.0004	
SampleType*BrineType	Fermented	NaCl	Finished	CaCl2	-2.9300	0.6203	152	-4.72	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Fermented	NaCl	Finished	NaCl	-2.6176	0.7234	152	-3.62	0.0004	Tukey-Kramer	0.0053	
SampleType*BrineType	Finished	CaCl2	Finished	NaCl	0.3124	0.6656	152	0.47	0.6395	Tukey-Kramer	0.9971	

**Table A.10 – Analysis of Variance (ANOVA) table for the statistical analysis of Hue**

Type 3 Analysis of Variance						
Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
SampleType	2	208.565471	104.282735	152.6	7.00	0.0012
BrineType	1	1553.817764	1553.817764	153.25	108.78	<.0001
SampleType*BrineType	2	489.998594	244.999297	152.26	16.09	<.0001
CSize	3	619.415916	206.471972	156.88	17.72	<.0001
Tank(Samp*Brin*CSize)	152	2356.020607	15.500136	81	21.73	<.0001
Residual	81	57.783768	0.713380	.	.	.

**Table A.11 – Least Squares Means table for the statistical analysis of Hue**

Least Squares Means							
Effect	SampleType	BrineType	Estimate	Std Error	DF	t Value	Pr >  t
SampleType*BrineType	Desalted	CaCl2	110.15	0.6238	152	176.58	<.0001
SampleType*BrineType	Desalted	NaCl	100.95	0.7650	152	131.97	<.0001
SampleType*BrineType	Fermented	CaCl2	106.08	0.5982	152	177.33	<.0001
SampleType*BrineType	Fermented	NaCl	99.9482	0.6616	152	151.06	<.0001
SampleType*BrineType	Finished	CaCl2	104.41	0.7185	152	145.32	<.0001
SampleType*BrineType	Finished	NaCl	102.60	0.9283	152	110.53	<.0001

**Table A.12 – Difference in Least Squares Means table for the statistical analysis of Hue**

Effect	Differences of Least Squares Means				Estimate	Std Error	DF	t Value	Pr >  t	Adjustment	Adj P
	SampleType	BrineType	_SampleType	_BrineType							
SampleType*BrineType	Desalted	CaCl2	Desalted	NaCl	9.1961	0.9200	152	10.00	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	CaCl2	Fermented	CaCl2	4.0644	0.7584	152	5.36	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	CaCl2	Fermented	NaCl	10.1998	0.8390	152	12.16	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	CaCl2	Finished	CaCl2	5.7337	0.8464	152	6.77	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	CaCl2	Finished	NaCl	7.5457	1.0304	152	7.32	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	NaCl	Fermented	CaCl2	-5.1317	0.9087	152	-5.65	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Desalted	NaCl	Fermented	NaCl	1.0038	0.9639	152	1.04	0.2994	Tukey-Kramer	0.9032
SampleType*BrineType	Desalted	NaCl	Finished	CaCl2	-3.4624	0.9815	152	-3.53	0.0006	Tukey-Kramer	0.0072
SampleType*BrineType	Desalted	NaCl	Finished	NaCl	-1.6504	1.1390	152	-1.45	0.1494	Tukey-Kramer	0.6970
SampleType*BrineType	Fermented	CaCl2	Fermented	NaCl	6.1354	0.8232	152	7.45	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Fermented	CaCl2	Finished	CaCl2	1.6693	0.8329	152	2.00	0.0468	Tukey-Kramer	0.3448
SampleType*BrineType	Fermented	CaCl2	Finished	NaCl	3.4813	1.0283	152	3.39	0.0009	Tukey-Kramer	0.0115
SampleType*BrineType	Fermented	NaCl	Finished	CaCl2	-4.4662	0.9031	152	-4.95	<.0001	Tukey-Kramer	<.0001
SampleType*BrineType	Fermented	NaCl	Finished	NaCl	-2.6542	1.0747	152	-2.47	0.0146	Tukey-Kramer	0.1398
SampleType*BrineType	Finished	CaCl2	Finished	NaCl	1.8120	1.0697	152	1.69	0.0923	Tukey-Kramer	0.5379

**Table A.13 – Analysis of Variance (ANOVA) table for the statistical analysis of Chroma**

Type 3 Analysis of Variance						
Source	DF	Sum of Squares	Mean Square	Error DF	F Value	Pr > F
SampleType	2	17045	8522.605624	156.63	761.62	<.0001
BrineType	1	69.271466	69.271466	161.57	6.36	0.0126
SampleType*BrineType	2	28.172069	14.086035	154.05	1.24	0.2922
CSize	3	194.526074	64.842025	187.04	6.77	0.0002
Tank(Samp*Brin*CSize)	152	1747.154498	11.494437	81	2.80	<.0001
Residual	81	332.632034	4.106568	.	.	.

**Table A.14 – Least Squares Means table for the statistical analysis of Chroma**

Least Squares Means							
Effect	SampleType	BrineType	Estimate	Std Error	DF	t Value	Pr >  t
SampleType*BrineType	Desalted	CaCl2	8.1971	0.5734	152	14.30	<.0001
SampleType*BrineType	Desalted	NaCl	9.6198	0.7053	152	13.64	<.0001
SampleType*BrineType	Fermented	CaCl2	8.8100	0.5492	152	16.04	<.0001
SampleType*BrineType	Fermented	NaCl	10.8228	0.6104	152	17.73	<.0001
SampleType*BrineType	Finished	CaCl2	28.0851	0.5898	152	47.62	<.0001
SampleType*BrineType	Finished	NaCl	28.4824	0.7612	152	37.42	<.0001

**Table A.15 – Difference in Least Squares Means table for the statistical analysis of Chroma**

Effect	Differences of Least Squares Means					Estimate	Std Error	DF	t Value	Pr >  t	Adjustment	Adj P
	SampleType	BrineType	_SampleType	_BrineType								
SampleType*BrineType	Desalted	CaCl2	Desalted	NaCl	-1.4227	0.8486	152	-1.68	0.0957	Tukey-Kramer	0.1807	
SampleType*BrineType	Desalted	CaCl2	Fermented	CaCl2	-0.6128	0.6972	152	-0.88	0.3808	Tukey-Kramer	0.9510	
SampleType*BrineType	Desalted	CaCl2	Fermented	NaCl	-2.6257	0.7736	152	-3.39	0.0009	Tukey-Kramer	0.0112	
SampleType*BrineType	Desalted	CaCl2	Finished	CaCl2	-19.8880	0.7182	152	-27.69	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Desalted	CaCl2	Finished	NaCl	-20.2853	0.8626	152	-23.52	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Desalted	NaCl	Fermented	CaCl2	0.8099	0.8372	152	0.97	0.3349	Tukey-Kramer	0.9275	
SampleType*BrineType	Desalted	NaCl	Fermented	NaCl	-1.2030	0.8901	152	-1.35	0.1786	Tukey-Kramer	0.7557	
SampleType*BrineType	Desalted	NaCl	Finished	CaCl2	-18.4653	0.8535	152	-21.63	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Desalted	NaCl	Finished	NaCl	-18.8626	0.9734	152	-19.38	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Fermented	CaCl2	Fermented	NaCl	-2.0128	0.7583	152	-2.65	0.0088	Tukey-Kramer	0.0269	
SampleType*BrineType	Fermented	CaCl2	Finished	CaCl2	-19.2751	0.7035	152	-27.40	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Fermented	CaCl2	Finished	NaCl	-19.6725	0.8588	152	-22.91	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Fermented	NaCl	Finished	CaCl2	-17.2623	0.7766	152	-22.23	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Fermented	NaCl	Finished	NaCl	-17.6596	0.9095	152	-19.42	<.0001	Tukey-Kramer	<.0001	
SampleType*BrineType	Finished	CaCl2	Finished	NaCl	-0.3973	0.8512	152	-0.47	0.6413	Tukey-Kramer	0.9972	