Novel wash water formulation from sustainable resources to improve safety and shelf-life of leafy greens

by

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Abstract

In the past two decades, multiple outbreaks occurred due to consumption of fresh leafy-greens contaminated with Shiga toxin-producing *Escherichia coli* (STECs). There is, therefore, a large need to find effective intervention strategies to control microbial contamination in fresh produce. Current practices include the use of potable water or at best with a wash-water sanitizer, commonly peracetic acid, to clean debris, reduce microbial load and extend shelf-life. Surface characteristics of produce as well as other factors (e.g., organic load) limit the efficacy of existing technologies. Recent studies have analyzed the combination of essential oils with surface-active ingredients to improve antimicrobial effectiveness. The amphiphilic structure of surface-active antimicrobials allows them to penetrate the phospholipid membrane, altering the membrane structure and fluidity. This increases cell permeability and can disrupt metabolic processes. Oxidizers are effective antimicrobials due to the oxidizing effect on enzymes, cell membrane, structural proteins and other cellular functions depending on the type of oxidizer. Combining a surface-active antimicrobial (e.g., lauric arginate) and an oxidizer (e.g., organic acids), can create synergistic effects to offer a solution as sustainable ingredients with enhanced antimicrobial effectiveness at reduced concentrations.

The objectives of this study were to: 1) evaluate the *in vitro* effectiveness and stability of individual and combined treatments of surface-active (lauric arginate (LAE) and LAE and cinnamaldehyde (CA) emulsions) and oxidizing sanitizers (lactic acid (LA) and peracetic acid (PAA) against pathogenic *E. coli* as a function of time and concentrations; 2) assess the antimicrobial effectiveness of treatments (lactic acid, peracetic acid, lauric arginate and lauric arginate emulsion) applied individually or sequentially to *E. coli*-inoculated spinach in a pilot-washing system as a function of time and organic load (1% and 10% v/v); 3) implement effective

treatments from pilot-wash system for use in field washing trials to compare the shelf-life (yeast and mold growth, aerobic plate counts and visual quality) as a function of leafy green type (lettuce, spinach and green mix) and treatment type (LAE (1%) + CA (1%) individually and LAE (1%) + CA (1%) then PAA and SaniDate 5.0 control).

To evaluate the *in vitro* effectiveness and stability of individual and combined treatments of surface-active and oxidizing sanitizers food grade emulsions were created using LAE (1%) + CA (1 and 2%) with or without coconut oil (CO) (8%) as a carrier. Emulsions were characterized for particle size, surface charge and hydrophobicity. Treatments (1mL) also included lactic acid (LA) solution (1%), LAE solution (1%) and their combinations (0.5mL LA with 0.5mL LAE) and peracetic acid (PAA) (100ppm). Treatments were applied to a STEC *E. coli* cocktail (O157:H7, O26 and O121) (10^8 CFU/mL) that was grown overnight. Solutions were neutralized and enumerated at 0.08, 0.16, 0.5, 1, 3, 5 and 24 hours. The most effective single treatment was LAE (1%) + CA (1%) with a 3.78 CFU/mL log reduction after 5 minutes contact. Positive (PAA) and negative (water) controls had a 0.22 CFU/mL log and 0.08 CFU/mL log reduction, respectively. LAE (1%) + CA (1%) had a positive surface charge and an average particle size of 192.7nm. Therefore, LAE (1%) + CA (1%) emulsions are proven effective against *E. coli* and show potential for use in the food industry.

To determine the antimicrobial effectiveness of treatments (lactic acid, peracetic acid, lauric arginate and lauric arginate emulsion) applied individually or sequentially, spinach (10 g) was inoculated with an overnight STEC cocktail (final concentration of 10^6 CFU/g). Spinach was then washed in a pilot setup (1 L treatment) with the following antimicrobial treatments: LA solution (1%), LAE solution (1%), and LAE (1%) + CA (1%) emulsions. PAA (100 ppm) and water were used as positive and negative controls, respectively. Treatments were applied

individually (5 or 10 min) or sequentially (2.5+2.5 or 5+5 min) with or without organic load (OL) (1% and 10%). The samples were then neutralized, and *E. coli* population enumerated. Spinach color was measured before and after washing in the treatments. The most effective individual treatment was LAE (1%) + CA (1%) no OL with 2.23 CFU/g log reduction after 10 minutes contact time. Positive (PAA) and negative (water) control had a 1.33 CFU/g and 0.22 CFU/g log reduction, respectively. The most effective sequential treatment was LA (1%) followed by LAE (1%) + CA (1%) with no OL. However, at 10% OL, sequential treatment of LAE (1%) + CA (1%) and PAA was the most effective treatment with a 1.83 CFU/g log reduction. Overall, combinations of surface-active antimicrobials (LAE), essential oils (CA) and organic acids can provide sustainable wash-water solutions to effectively control and reduce STECs contamination on leafy greens.

To determine the effectiveness of new treatments on shelf-life in field washing trials, fresh lettuce, leafy green mix and spinach were washed at a commercial produce farm in northeast Kansas using the best performing treatments at 10% OL (LAE (1%) + CA (1%) emulsion individually and sequentially followed by PAA) to compare to their existing washing treatment (SaniDate 5.0). Shelf-life of the products were predicted by leafy green color, yeast and mold counts (CFU/g) and total aerobic plate counts (CFU/g) at varying days after harvest and washing (0, 2, 4, 7 and 10 days). After 10 days, for spinach, mixed greens and lettuce, respectively, control (no treatment) showed yeast and mold counts of 6.05 CFU/g, 4.37 CFU/g and 4.02 CFU/g and aerobic plate counts of 6.92 CFU/g, 6.90 CFU/g and 7.40 CFU/g. LAE (1%) + CA (1%) had yeast and mold counts of 3.22 CFU/g, 1.52 CFU/g and 3.48 CFU/g and aerobic plate counts of 4.06 CFU/g, 6.51 CFU/g and 7.12 CFU/g. LAE (1%) + CA (1%) followed by PAA had yeast and mold counts of 2.79 CFU/g, 2.98 CFU/g and 3.59 CFU/g and aerobic plate

counts of 3.70 CFU/g, 6.47 CFU/g and 5.41 CFU/g. However, sequential application showed the greatest change in green color (*a) after 10 days. Spinach washed in LAE (1%) + CA (1%) retained green color (*a) the best. LAE (1%) + CA (1%) individually and sequentially with PAA improved the shelf-life of leafy greens by having the lowest yeast and mold and aerobic plate counts. In some cases, sequential application of improved visual quality. A survey was developed using field washing trial results and given to produce growers at farmers' markets to determine their willingness to implement and pay for the new wash-water treatments. Most farmers (79%) believed that spinach washed in the new treatment showed a visual difference after 7 days compared to control. Most (83%) also said they would be willing to pay more for this treatment and 26% would pay up to $0.30 more per unit to use this new treatment.

These studies conclude that when combined with or used sequentially, lauric arginate and cinnamaldehyde emulsions are effective at inactivating *E. coli* and prolonging shelf-life by reducing yeast, mold and aerobic plate counts on leafy greens. This technology can also be used for other types of produce and has been found effective in other food matrixes. Lauric arginate and essential oil emulsions can provide the food industry with effective antimicrobials that are both sustainable and natural. These treatments can be used by the produce industry to improve the efficacy of their current antimicrobial interventions for fresh produce and reduce the potential for future foodborne outbreak events.

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Dedication

I would like to dedicate this thesis to my family who have supported me my entire life; my friends who are always there for me; and my husband Aaron who has always encouraged me and believed in me every step of the way.

Chapter 1 - Literature Review

1.1 History of leafy greens and related outbreaks

From 1970 to 2019 in the United States (US), consumer demand for produce increased. The consumption of fresh vegetables increased from 94.9 to 153.42 pounds per capita per year which is an additional 379 million pounds per year (USDA, 2019). This is related to a positive correlation between eating produce and health. For example, earlier research showed that eating cruciferous vegetables like arugula and kale can lower the risk of lung and breast cancer (National Cancer Institute, 2012). Due to this information, the US Department of Agriculture (USDA) has increased the number of recommended vegetables from four servings in the 1960s to seven servings today (Davis & Saltos, 1999; ChooseMyPlate, 2020). As society becomes more health conscientious, consumer demand for produce will continue to grow. However, when produce has to be recalled due to microbial contamination, it causes consumers to question the safety of their food. More than 50% of Americans consider foodborne illness a top food safety concern (International Food Information Council, 2021). Furthermore, food safety outbreaks have a significant economic impact, costing the US around $7 billion a year (Hussain & Dawson, 2013). In 2020, there were ten multistate foodborne outbreaks, seven of them were caused by produce (CDC, 2020).

Leafy greens were responsible for 30% of all produce- related outbreaks from 1996-2017 (CDC, 2018). Several foodborne pathogens have been linked to leafy green outbreaks. Thirty-nine percent of all leafy green outbreaks from 1997-2017 were linked to *Escherichia coli* (*E. coli*) (CDC, 2018). One of the deadliest leafy green outbreaks of *E. coli* occurred in 2006 (CDC, 2006). This was a multistate outbreak of O157:H7 associated with fresh bagged spinach originating in California. The outbreak strain was isolated from cattle fields near the growing

site. This outbreak caused 199 illnesses across 26 states, and 31 people developed hemolytic-uremic syndrome (HUS) which led to the death of four people (CDC, 2006). Another notable *E. coli* outbreak occurred in fall 2019, where leafy greens sickened 167 people across 27 states (CDC, 2020a). This outbreak was traced back to romaine lettuce grown in Salinas Valley of California that was contaminated from cattle fields in the area (CDC, 2020a). The most recent *E. coli* outbreak started in August 2020; the map of cases within this outbreak is shown in Figure 1.1. This outbreak was caused by leafy greens but has still not been traced back to an original source (CDC, 2020b). As shown in multiple outbreaks since 2006, the common cause is normally animals, specifically cattle near the growing region. *E. coli* is commonly found in the gastrointestinal tract of cattle and studies (Smith et al., 2001; Matthews et al., 2006) have found that *E. coli* O157:H7 is shed from approximately 20% of cattle. When animals are raised near produce fields, it is important to properly manage all waste coming from the animals to ensure runoff is not contaminating the produce fields.

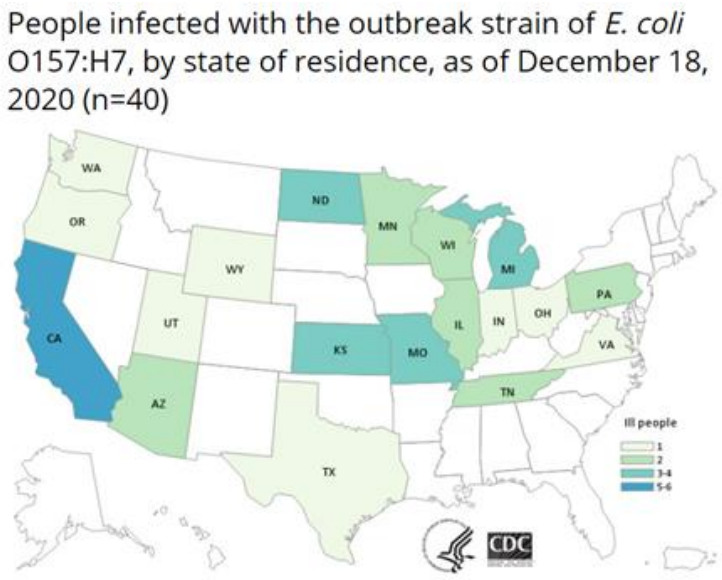

Figure 1.1. Map of reported *E. coli* cases by state as of December 18, 2020, for the multistate *E. coli* outbreak linked to leafy greens (CDC, 2020b).

1.2 *Escherichia coli* in the leafy green industry

Most stains of *E. coli* are not pathogenic and are part of the healthy intestinal microflora (CDC, 2014). Six major *E. coli* pathogenic groups have been identified: Shiga toxin-producing (STEC) previously called Enterohemorrhagic (EHEC), Enterotoxigenic (ETEC), Enteropathogenic (EPEC), Enteroaggregative (EAEC), Enteroinvasive (EIEC) and Diffusely adherent (DAEC). STEC represents the group that is most associated with foodborne human illnesses. A symptom of a life-threatening complication of STEC infection is the Hemolytic Uremic Syndrome (HUS), which is related to two Shiga-like cytotoxins, Stx1 and Stx2 (Delaquis, Bach, & Dinu, 2007). HUS can cause acute renal failure leading to hospitalization and possible death with approximately 63,000 cases annually (Scallan et al., 2011). STEC pathogenesis requires ingestion of the bacteria. Once ingested, bacteria colonize the intestine through attachment and proliferation. After attachment, the Stx(s) are released and attack the endothelia cells causing apoptosis or altering of gene/protein expression of the cells (Melton-Celsa, 2014). STECs were reportedly responsible for most leafy green outbreaks from 1998-2017 (CDC, 2018). As shown in Figure 1.2., out of the different STEC serotypes, O157:H7 is the most prevalent causing 81% of leafy green outbreaks (CDC, 2018). The other leafy green outbreaks have been associated with other *E. coli* non-O157 STEC, commonly called “big six,” which includes: O26, O45, O103, O111, O121 and O145 (CDC, 2014). Non-O157 strains have a similar gene, stx2, which is found to be highly associated with HUS (Johnson, Thorpe, & Sears, 2006). However, non-O157 STECs do not always cause the same symptoms as O157. The O26 serogroup has been found to cause the most similar symptoms as the O157 serogroup (CDC, 2014).

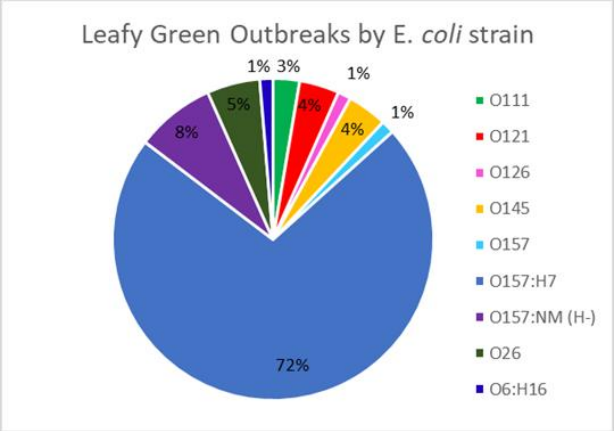

Figure 1.2. Leafy green outbreaks associated with different *E. coli* strains (CDC, 2018).

1.3 *Escherichia coli* contamination mechanisms

Leafy greens can become contaminated with *E. coli* at various times throughout the production chain. There are many vehicles that can spread *E. coli* to leafy greens. Common sources are improper sanitary procedures, contaminated water and feces from animals or insects in the growing region (Jay et al., 2005). *E. coli* follows the fecal-oral route of transmission as shown in Figure 1.3. Furthermore, *E. coli* can be internalized by leafy greens through their vascular system or via portals of entry such as the stomata or hydathodes on stems and leaves. Various issues during processing can cause the internalization of pathogens such as product damage, and water temperature. Leafy greens are very delicate and can be easily damaged during processing; common types of damage are wounds and cuts. These types of product defects can lead to internalization of pathogens and product quality losses. When lettuce goes through additional processing like chopping or shredding, cellular fluids are released, these fluids provide nutrients for organisms' growth (Wulfkuehler et al., 2013). Differences in temperature between the wash water and product can cause a pressure differential, which causes the water to be absorbed into the leafy greens through the stomata (Solomon, Yaron & Matthews, 2002). If the

wash water is at a lower temperature than the produce, it might cause the water to be internalized along with any pathogens present (Fan et al., 2009). Internalized pathogens can lead to difficulties in ensuring a safe product as they may become more resistant to chemical application during washing.

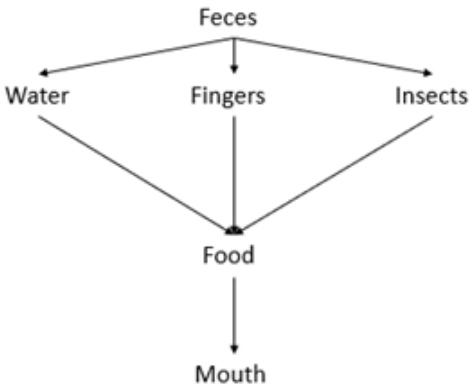

Figure 1.3. Fecal-oral route of transmission in food.

1.4 Current industry practices to prevent microbial contamination on leafy greens

The leafy green industry utilizes a variety of postharvest strategies to reduce microbial contamination, improve shelf-life and product quality. Multiple washing steps and sequential chemical application can all help reduce microbial load on leafy greens. Processor's techniques might vary depending on their size, facilities available and target consumer. Depending on the size and target consumer of the producer, there also might be different regulatory requirements about product handling and processing. The Food Safety Modernization Act (FSMA) Produce Safety Rule establishes the food safety and growing standards for any fruits and vegetable grown for human consumption (FDA, 2021). Under the FSMA Produce Safety Rule, unprocessed whole fruits and vegetables sold directly to consumers, for example, farmers markets, are

generally exempt (FDA, 2021). Commercial farms that sell to wholesale, processors or distributors are governed by the FSMA Produce Safety Rule (Laborde, 2018).

1.4.1 Washing steps

Washing leafy greens is a common step to remove any dirt and debris that is on the product. Larger facilities often apply multiple stages of washing using large equipment to improve washing efficiency and technique. Figure 1.4. outlines the steps in leafy green processing (Gehringer et al., 2017). Smaller farms often have different methods to produce and market their products as they may not have the infrastructure in place to implement multiple washing steps. The main customers of small farms are often farmers' markets, food hubs or other local food movements. The small farm classification is an annual gross cash income of $350,000 or less (Economic Research Service, 2021). These farmers often sell whole leafy greens without a chopping or bagging step. Without large processing equipment to clean the leafy greens, small farms will often forego any product washing. However, small-scale washing can be done by farmers, often in smaller facilitates on the farm. This type of washing can be done with or without sanitizing step. Washing in just water can help remove large debris and reduce produce water loss. However, water quality is a major factor in the effectiveness of washing; if the water is contaminated it can potentially introduce pathogens to the food. Water used for washing should be potable and from an approved source. Small farms, like those who sell at farmers' markets, may choose to not wash their produce. The addition of a washing step requires the proper equipment and resources available to properly ensure they are not introducing pathogens or allowing pathogens to spread between items (Burrows, 2019). After washing, the produce are then packed into boxes for distribution. Leafy greens can also be placed in water before transport, this prevents the leafy greens from drying out and losing moisture (FDA, 2006).

Larger production facilities often rely on large machines that harvest the product or can help workers harvest and then transport the product out of the field. After harvesting, the products are transferred to the processing facility. Leafy greens are often chilled during transportation to better control temperature prior to washing and further processing. Once received at the facility, produce is inspected to ensure quality, sorting off any significantly damaged produce. After the product has been received and passed inspection it is then allowed to move along to the other processing steps. The first washing step at the facility can be used to remove large debris. In many large-scale productions, a flume system is used to wash the product. Flumes inject air into the water creating turbulence which washes the product as shown in Figure 1.5A. A large centrifuge can also be used during washing; this type of system can help separate the product from large debris and gently wash the product. The first washing step can be water only or can contain antimicrobials. By applying multiple antimicrobials and utilizing hurdle method technology, processors can increase the overall reduction in pathogens.

Hurdle method technology is a method used in the food industry to reduce or control the growth of pathogens through the application of multiple approaches and technologies. Hurdle technology aims to improve the total quality of foods while reducing the high treatment intensities of individually- applied treatments (Leistner, 1995). By applying multiple interventions, hurdle method technology has been proven to have a synergistic effect by using various mechanisms to target microorganisms (Rahman et al., 2016). Microorganisms react homeostatically when exposed to stress factors in order to try and maintain balance. Microorganisms have different homeostatic reactions, depending on the type of stressor. Using a combination of stressors can disrupt multiple homeostasis mechanisms and thus prevent the microorganisms from multiplying which inactivates them or causes cell death (Lee, 2004).

Examples of hurdle technology for fresh produce include applying electrolyzed water combinations (Rahman et al., 2010; Hao et al., 2013), ozone + thermal processing (Achen & Yousef, 2001; Koseki & Isobe, 2006) and application of lower levels of antimicrobial chemicals (McMeekin & Ross, 2002; Raijkowski & Baldwin, 2003). Using different chemical applications has been determined as one of the best hurdle methods for fresh produce, as it does not significantly impact quality and is a cheaper process to implement (McMeekin & Ross, 2002). Often, different types of chemicals are used as they have different modes of action against pathogens. It is common for chlorine to be used first followed by an organic acid. If applicable, the leafy greens can go into a cutting or chopping step. This is commonly done for bagged pre-cut lettuce sold at supermarkets.

There are two main techniques to apply antimicrobials to leafy greens: a spray technique as show in Figure 1.5B. or submersion as show in Figure 1.5C. The spray technique can help reduce the amount of water and antimicrobial used. Therefore, it is more cost effective and environmentally friendly. However, it can have reduced effectiveness as there is less contact surface area. The submersion technique ensures that all product surfaces are sanitized. However, the submersion technique increases water and antimicrobial use, which increases cost. The cutting or chopping stage is a critical point in the process. If there is still contamination on the outside of the product, cutting or chopping will spread the contamination to other parts of the product and to other products along the line. The second washing, after cutting, is commonly used to apply antimicrobials. This is to ensure that any microorganisms that was potentially spread during cutting, or chopping is removed.

Wash water quality is also critical to reducing pathogens on leafy greens and maintaining a quality product. The efficacy of the antimicrobial agent may be affected by the water

temperature, pH, water hardness, organic material in the water and other factors. If in the processing facility the produce wash water is recycled and not properly treated, it can spread microorganisms onto the product. If recycled wash water is used, it is filtered to remove any large debris. It is then treated with an antimicrobial, often chlorine to prevent cross contamination when applied to the next batch of product. The recycled water is then tested for microbial load and pH before being recirculated into the washing system. If the wash water is recycled into the antimicrobial application step, the concentration of the chemical will also be checked. This is to ensure that the chemical being used doesn't exceed allowed limits. After rinsing and draining, approximately 0.5-1.0% water can remain on the product (Baur et al., 2004). Removing of this excess water is a critical step to avoid growth of microorganisms and improve quality and shelf life of leafy greens. Therefore, after washing, the product is often dried using a centrifuge to remove any excess water (Baur et al., 2004). During and after processing, leafy greens need to be cooled quickly and stored at refrigerator temperatures (0 to 2°C). Maintaining a high humidity during storage can also prevent the leafy greens from wilting (Burrows, 2019).

Figure 1.4. Commercial leafy green processing steps

*step applies to chopped lettuce only

Figure 1.5A. Leafy greens going through flume washing. Retrieved from Sormac (2020).

Figure 1.5B. Antimicrobial application using spray technique on leafy greens. Retrieved from AMBiT Projects Limited (2020).

Figure 1.5C. Antimicrobial application using submersion technique. Retrieved from AMBiT Projects Limited (2020).

1.4.2 Chemical treatments

There are many different chemical treatments that are approved to be used in produce wash water with varying concentrations. Industry practice is either to use a chlorine-based sanitizer or organic acids like lactic acid or peroxyacetic acid (PAA). Chlorine is commonly used as a produce wash-water because it has been proven effective and is economical for processors. It is commonly used at a range of 75 to 200ppm (Suslow, 2000). Chlorine is electronegative; therefore, it oxidizes the peptide links in the cell membrane of microorganisms which results in denaturing of structure proteins which can lead to cell lysis and death (Maris, 1995). This oxidizing agent can also alter cellular metabolism and decreases the uptake of nutrients and oxygen (Shaw, 2013). When used in wash water for lettuce, the efficiency of chlorine is limited to 1-to-2 log reductions at ranges of 50- 200mg/liter (Fan et al., 2009; Haute et al., 2013). Chlorine efficacy can be reduced however, based on the amount of organic matter in the wash water. High concentrations of organic matter in wash water deactivates chlorine. Chlorine is also ineffective below 7°C and is highly corrosive to metals (Shaw, 2013). Limiting contact times to one to two minutes and keeping pH values between 6 and 7.5 can minimize corrosion of equipment. The use of high chlorine concentrations can generate chlorine gas in the facility which poses a safety risk (Haute et al., 2013).

Lactic acid is a common organic acid used to wash fruit and vegetables, it is generally recognized as safe (GRAS) and has no limitations for use in food. Lactic acid's mode of action is in its ability to penetrate the cytoplasmic membrane, which results in reduced pH and structure degradation. Processors may choose to use lactic acid instead of other chemicals because it is noncorrosive to stainless steel and is a natural product. It is stable in the presence of organic matter and does not have a strong odor (Fan et al., 2009). However, lactic acid also works best in acidic conditions so pH will have to be constantly monitored without impacting produce quality (Shaw, 2013). This constant monitoring and adjustments can lead to relatively high cost for the producer (Fan et al., 2009). During a study done by (Ho et al., 2011) lactic acid at 4,500 and 6,000 ppm was found to decrease *E. coli* K-12, a known pathogenic *E. coli*, attached to spinach leaves by 0.43 log and 0.83 log, respectively. Another study done by (Wang et al., 2019) found a 1.22 log reduction in *E. coli* O157:H7 on lettuce when 1% lactic acid was applied.

Peracetic acid (PAA) is a common sanitizer used in the produce industry; it is generally used at concentrations of 100-200ppm. However, it is approved for use up to 2,000ppm for use on food products (Clark Burton & Gibbins, 2017). PAA (CH_3CO_3H) is a combination of acetic acid (CH_3COOH) and hydrogen peroxide (H_2O_2). Its primary mode of action is oxidation and PAA has a stronger oxidizing potential than chlorine. It disrupts the bonds in proteins and enzymes which denatures them and causes rupturing of the cell wall (Maris, 1995; Fan et al., 2009). PAA is a broad-spectrum antimicrobial because it can be used in a wide pH range and in warm or cool water. It is also effective against bacterial endospores, yeast and mold (CDC, 2008). Another main advantage PAA has over other oxidizing agents is the higher tolerance for organic matter in the water (Hirneisen et al., 2009). PAA is an approved antimicrobial washing additive for fresh and fresh-cut produce at a maximum concentration of 80 mgL-1 (Fan et a.,

2009; Zoellner et al., 2018). PAA is a clear and colorless liquid that has a strong and pungent odor. Previous studies have analyzed PAAs' use as a wash water treatment for lettuce; PAA (50ppm) had an average of 1.35 log CFU/g reduction of *E. coli* O157:H7 after 90 seconds (Davidson, Kaminski-Davidson, & Ryser, 2017). PAA (75 mg/L) had a 5 log CFU/g reduction of *E. coli* after contact treatment for 90 minutes (Banach et al., 2020).

1.5 Encapsulation approaches

Lauric arginate (LAE) is a cationic surfactant derived from lauric acid, L-arginate and ethanol. It was first developed and patented in Spain in 1983. LAE's antimicrobial properties are attributed to its positive charge which can disrupt the cell membranes of bacteria and target other intracellular components (Ma, Davidson, & Zhong, 2019). Complex food matrixes can potentially require higher concentrations of LAE due to its interaction with negatively charged foods and microbial binding (Ma, Davidson, & Zhong, 2013). However, with LAE's amphiphilic structure, it can be emulsified with other surfactants to create a physically stable antimicrobial (Ma, Davidson, & Zhong, 2019). LAE was granted its GRAS status by the FDA in 2005 for use on food products up to 200ppm. Many studies have evaluated the antimicrobial activity of LAE against various microorganisms, used alone or in combination with essential oils compounds. It was found to have a minimum inhibitory concentration of 11.8ppm against *E. coli* O157:H7 (Ma, Davidson, & Zhong, 2019). When LAE was tested against lettuce leaves inoculated with *E. coli* O157:H7, it was found to have a 2.5 log reduction at a concentration of 100mg/L (Nubling et al., 2016).

There is a growing concern among consumers about where food comes from and how it is processed. A recent trend shows consumers wanting to ensure that the food they purchase for their family is the highest quality and free of chemicals or any potentially harmful additives.

Over 50% of consumers consider chemicals found in food as the top food safety concern (International Food Information Council, 2021). There is also a rising market for organic and all-natural products, especially produce. In order to be USDA certified organic, foods must be grown and processed according to federal guidelines (USDA, 2019). The term all-natural is generally regarded as meaning not artificial or synthetic, however, this term is not regulated by the FDA or USDA (FDA, 2016). Consumer demand for organic goods has shown double-digit growth with the fruit and vegetable sector accounting for 46% of total organic sales (Greene et al., 2017). The increase in demand for organic goods will continue to impact produce industry practices. Certain types of fertilizers, pesticides, antimicrobials, etc. are not considered certified organic and therefore, not allowed for use on organic produce. Consumers are also focused on the environmental impact of the products they purchase. Four in ten consumers believe their food purchase choices have a significant impact on the environment. Furthermore, out of those consumers, 67% say that environmental sustainability is their primary purchase motivation (International Food Information Council, 2021). Sustainability is generally defined as the avoidance of the depletion of natural resources in order to maintain and ecological balance (Oxford University Press, n.d.).

Overall, consumers are demanding environmentally sustainable, and all natural ingredients in their food, while still maintain the strictest food safety guidelines. Therefore, recent studies focused on essential oils, which are natural plant derivatives, that create a synergistic effect when combined with lower levels of traditional antimicrobials. This synergistic effect reduces the overall amount of chemicals used while still maintaining effectiveness.

1.5.1 Essential oils

Essential oils are natural compounds that have been used in many different applications such as food, cosmetics, and pharmaceuticals. Essential oils have both non-volatile and volatile compounds, which are extracted using different methods including solvent and steam (Chen & Huang, 2016). Non-volatile compounds do not easily evaporate into a gas, compared to volatile compounds that evaporate at room temperature or below. Essential oils can be classified into three types: terpenes, phenols and aldehydes (Chang, McLandsborough, & McClements, 2015). Terpenes are the main constituent of essential oils and are derived from the isoprenoid pathway; their antimicrobial activity is related to the presence of hydroxyl groups (Guimarães et al., 2019). Phenols are a secondary metabolite from plants; though most essential oils are terpenes, some are phenolic compounds like thymol (Aldred, Buck, & Vall, 2009). Phenols can denature proteins and disrupt cellular functions (Dydek, 2014). Aldehydes are another plant constituent with antimicrobial properties due to its ability to alter the function of membrane proteins (Walsh et al., 2001).

The use of essential oils in foods has been increasing due to consumers preferring natural over synthetic additives (International Food Information Council, 2021). Different types of essential oil derivatives are known for their antimicrobial properties include thymol, eugenol and cinnamaldehyde. These compounds are hydrophobic in nature which makes them difficult to combine in aqueous solutions. To overcome this problem researchers encapsulated essential oils by emulsifying them with lipids or surfactants into droplets called emulsions (Chang, McLandsborough, & McClements, 2015). The addition of lipids, like triglycerides, corn oil, coconut oil, etc., to emulsions can help improve stability and prevent phase separation. These water-insoluble, non-polar oils are generally referred to as ripening inhibitors due to their high

molecular weights (McClements et al., 2011). These lipids are also referred to as carrier oils and can prevent the change in particle shape during storage that is responsible for particle aggregation (Qian et al., 2012).

1.5.2 Emulsion stability

Encapsulation in emulsions can improve physical stability by providing larger surface area allowing for greater absorption and more interaction with the solvent, which allows ingredients to be homogenously dispersed in aqueous environments. Colloidal dispersions, commonly referred to as emulsions, generally are defined as a suspension of small particles that is distributed within a liquid (McClements, 2012). Colloidal dispersions have many uses; however, in the food industry a colloidal dispersion with smaller particles is preferred. Smaller particle size means they are more stable and are easier to incorporate into food as they are turbid or clear in color. Being relatively small compared to the wavelength of light means nanoemulsions can be combined in clear food. The small size of nanoemulsions also increases stability compared to conventional emulsions >100nm (McClements, 2011).

There are two types of emulsions with small particles (radius < 100), nanoemulsions and microemulsions. Microemulsions are thermodynamically stable and are formed by mixing oil, water and surfactants. Microemulsions, also referred to as micellar emulsions, are organized so that the non-polar tails face each other to form a hydrophobic core. This helps improve stability as it reduces contact area between water and the non-polar groups. Nanoemulsions are classified as being thermodynamically unstable dispersions that contain two immiscible liquids. Nanoemulsions also helps protect the compound from faster degradation as they allow for a slower release of essential oils (Sari et al., 2014). Oil-in-water nanoemulsions are considered thermodynamically unstable consisting of two immiscible liquids. Nanoemulsions will disrupt

over time; the rate of breakdown depends on the kinetic stability of the emulsion due to the energy barrier between states. The speed at which nanoemulsions breakdown can also be determined by the mechanisms in which oil droplets collide. These mechanisms include creaming, flocculation, coalescence and Ostwald ripening.

The addition of emulsifiers to nanoemulsions can improve stability of emulsions (McClements, 2012). Emulsifiers are surface-active substances that adsorb to oil–water interfaces and develop a protective layer around droplets. This additional layer around the essential oil prevents droplet aggregation (McClements, 2011). Some examples of emulsifiers include, small molecule surfactants, like tween or LAE, phospholipids, amphiphilic proteins and amphiphilic polysaccharides (McClements, 2011).

There are a few measurement techniques to measure the stability of emulsions. Particle size is one of the key indicators of emulsion stability by measuring the diameter of the droplet (Iyer et al., 2015). The size of the droplet will determine its classification, conventional or nanoemulsion as well as what type of instability effects will act upon the system. For example, nanoemulsions are more resistant to gravitational separation, flocculation and coalescence but are susceptible to Ostwald ripening (McClements, 2011). Particle size can also give an indication of how long the emulsions are stable for. Emulsions below 150nm can maintain stability at various storage temperatures for over a month, whereas emulsions with 250nm particle size show separation after one month storage (Iyer et al., 2015). Zeta potential is another common measurement technique to evaluate stability of emulsions. Zeta potential is the electrical potential at the slipping plane which is exhibited by any particle in a suspension. Magnitude of zeta potential gives an indication about the potential stability of colloidal system. Emulsions with a higher zeta potential, positive or negative, are considered stable due to the repulsive forces

exceeding the attractive forces, thus preventing coagulation or flocculation (Wei Lu & Gao, 2010).

1.5.3 Combined applications

Essential oils can be used in combination with many different traditional antimicrobials to create a synergistic effect. For example, LAE and essential oils have both been proven effective antimicrobials (Ma, Davidson, & Zhong, 2019). However, using lauric arginate or essential oils alone can impact product quality. LAE gives the product a bitter taste at greater than 50 mg/L and essential oils can affect the sensory palatability of food (Ma, Davidson, & Zhong, 2016b). Researchers have studied combining LAE and essential oils to increase antimicrobial effectiveness and decrease quality issues. Another complication with using essential oil nanoemulsions is stability. These nanoemulsions are susceptible to Ostwald ripening, the growth of large oil droplets that reduce smaller oil droplets through diffusion. Ostwald ripening, the development of larger particles in a solution as smaller particles dissolve and attach to them, can be reduced by adding a high concentration of water-insoluble oils (Chang, McLandsborough, & McClements, 2015). Recent studies have found that surfactants like LAE can enhance antimicrobial effectiveness of essential oils by increasing their solubility in aqueous solutions (Ma, Davidson, & Zhong, 2013). A study done by (Chang, McLandsborough, & McClements, 2015) found that the combined presence of LAE and 4% Thyme oil was able to reduce the spoilage yeast *Zygosaccharomyces bailii* below detectable limits approximately 36 hours sooner after inoculation when compared to 4% Thyme oil with no LAE. Another study found that when combining LAE with cinnamon oil and then cinnamon oil and EDTA there was a 0.42 and 4.7 log reduction respectively in *E. coli* O157:H7 in tryptic soy broth (Ma et al., 2016a). In addition, the incorporation of LAE increases loading capacity and

stability of nanoemulsions, reducing the effect of Ostwald ripening (Chang, McLandsborough, & McClements, 2015). Overall, using a combination of LAE and essential oils can help create a synergistic effect that increases antimicrobial effectiveness, palatability and stability.

1.6 Conclusion

There have been many studies focusing on the methodology behind using essential oils as an antimicrobial in foods, as well as using nanoemulsions as a delivery method for these essential oils. However, these techniques have yet to be thoroughly studied using a leafy green wash water system. Contact time needed for the nanoemulsions to be effective in a complex matrix such as leafy greens also need to be more thoroughly evaluated. Sustainability and environmental impact are a growing concern among the food industry. One way for companies to become more environmentally friendly is to reduce water usage. When developing a new wash water application there needs to be a focus on reducing water usage. By developing a more effective wash water system, contact time can potentially be reduced, thus saving the amount of water used. Many studies previously done have focused on the effect of essential oils as an antimicrobial and ensuring the nanoemulsion delivery system worked. However, the impact of essential oils on different food products and their quality has not been studied as extensively. This study will focus on the quality issues that may arise from applying essential oils and organic acids to the leafy greens, as well as evaluate the total microorganism being shed into the wash water.

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Chapter 2 - Knowledge Gap and Research Importance

Multiple outbreaks of *E. coli*- contaminated leafy greens in recent years shows that there is a need for more effective interventions to eliminate pathogens on produce surfaces. Current chemical interventions include organic acids like lactic acid and peracetic acid (PAA). However, with the growing concern among consumers about chemical usage in the food industry, a novel treatment that uses natural ingredients is needed. Developing a novel produce washing treatment that needs to be both effective and sustainable can be challenging; however, recent studies have proven that essential oils are effective antimicrobials for use in the food industry when combined with surface-active ingredients. Cinnamaldehyde (CA) is one such essential oil and when combined with the cationic surfactant lauric arginate (LAE) it has proven effective against pathogenic *E. coli* as well as other food related spoilage organisms (Chen et al., 2015; Hilbig et al., 2016; Ma et al., 2016). Researching the antimicrobial effectiveness of cinnamaldehyde and lauric arginate emulsions in combination or in sequence with conventional treatments might provide the industry with improved effectiveness of interventions and reduce chemical usage. For these reasons, the present research aims to:

- To demonstrate that cinnamaldehyde- based emulsions can provide an effective and natural solution against *E. coli* strains *in vitro*. By evaluating the effectiveness and stability of individual and combined treatments of surface-active (lauric arginate and lauric arginate and cinnamaldehyde emulsions) and oxidizing sanitizers (lactic acid and peracetic acid) against pathogenic *E. coli*. Treatments were analyzed as a function of time and concentrations over a 24-hour period and emulsions characterized for stability.
- To determine which treatment (lactic acid, peracetic acid, lauric arginate and lauric arginate emulsion) is the most effective against *E. coli* contaminated spinach without

having a negative effect on fresh spinach color. By assessing the antimicrobial effectiveness of treatments applied individually or sequentially to *E. coli* inoculated spinach in a pilot-washing system, as a function of time and organic load (1% and 10%) while measuring for changes in spinach color.

- To analyze the impact of effective treatments (LAE (1%) + CA (1%) individually and LAE (1%) + CA (1%) then PAA and SaniDate 5.0 control) from the pilot-wash system for use in field washing trials to determine which treatment shows greater improvement on the shelf-life of leafy greens. By comparing the yeast and mold growth, aerobic plate counts and visual quality as a function of leafy green type (lettuce, spinach and green mix) and treatment type.

Chapter 3 - Interaction of Surface-Active Ingredients and Oxidizers on *in vitro* Performance of Shiga toxin-producing *Escherichia coli*

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Abstract

In the last two decades, multiple outbreaks linked to produce contaminated with Shiga toxin-producing *Escherichia coli (STEC)* have occurred. Surface characteristics of produce limit the effectiveness of current technologies. Studies have begun to examine the combination of surface-active ingredients, such as lauric arginate, with essential oils, to improve antimicrobial efficacy and offer more sustainable produce washing solutions. The objective of this study was to evaluate the *in vitro* effectiveness and stability of individual and combined treatments of surface-active ingredients (lauric arginate) and organic acids (lactic acid and peracetic acid) against STEC *E. coli*. Emulsions were created using lauric arginate (1%) and cinnamaldehyde (1 and 2%) with or without coconut oil (8%) as a carrier. Treatments also included lactic acid (1%), lauric arginate (1%) and their combination. Peracetic acid and water were used as positive and negative controls. Solutions were neutralized and enumerated up to 24 hours. Combination of lauric arginate and lactic acid as well as lauric arginate + cinnamaldehyde (1%) emulsion showed significant ($p < 0.05$) reduction after 5 minutes with 3.98 CFU/mL and 3.79 CFU/mL log reduction, respectively. Lauric arginate (1%) + cinnamaldehyde (2%) + coconut oil (8%) emulsion showed significant ($p < 0.05$) impact after 60 minutes with the greatest log reduction of 6.83 CFU/mL. Positive and negative control showed no significant reduction in *E. coli*. Overall, the combination of lauric arginate with organic acids or cinnamaldehyde are effective treatments that can provide a suitable alternative wash-water treatment to reduce *E. coli* contamination on produce.

3.1 Introduction

There have been multiple outbreaks related to fresh produce in the past two decades (CDC, 2018). Leafy greens were responsible for 30% of all produce related outbreaks from 1996-2017 (CDC, 2018). These outbreaks are often caused by Shiga-toxin producing (STEC) *Escherichia coli* linked to cattle near the produce fields. The route of contamination for these outbreaks was suspected to be because of improper storage and handling of animal waste (CDC, 2006; CDC, 2020a). The most recent *E. coli* O157:H7 outbreak related to leafy greens started in August 2020, sickening 40 people, and hospitalizing 20 (CDC, 2020b). Current food safety interventions are proving ineffective at preventing *E. coli* outbreaks in leafy greens. These current industry practices include washing leafy greens in potable water or organic acids, like peracetic acid and lactic acid (Shaw, 2013; Burrows, 2019). Antimicrobials can be applied to produce in two different ways, using either a spray or a submersion technique (Baur et al., 2004; Wulfkuehler et al., 2013). However, surface characteristics of leafy greens can limit the effectiveness of traditional chemical applications (Doan et al., 2020). Furthermore, pathogens can be internalized into the plants vascular system through wounds or cuts created while processing (Van Haute et al., 2013). These types of product damages are common due to the delicate nature of leafy greens (Baur et al., 2004). The internalization of pathogens greatly reduces external antimicrobial application effectiveness (Ryser et al., 2009). Seeking for alternatives to the current cleaning and processing steps of leafy greens, numerous studies (Chen et al., 2015, Hilbig et al., 2016, Ma et al., 2016) have examined the antimicrobial effectiveness of essential oils combined with surfactants like lauric arginate. Essential oils are gaining popularity for use as antimicrobials against various foodborne pathogens since they are GRAS (Generally Recognized as Safe) and broadly accepted by consumers due to being naturally derived instead

of synthetic. Essential oils are hydrophobic, this makes them difficult to combine in aqueous solutions. To improve application for aqueous solutions essential oils can be encapsulated with surfactants to act as an emulsion delivery system (Chang et al., 2012, Chang et al., 2015). LAE is a cationic surfactant derived from lauric acid, L-arginine and ethanol, it was patented in Spain in 1983 (García Domíngues et al., 1983). Also this compound is GRAS and FDA recommends its use on food products up to 200ppm. Surfactants have an amphiphilic structure, which allows them to penetrate the membrane of bacteria, altering the membrane structure which disrupts metabolic processes (Shen et al., 2021). The aim of this study was to: (1) evaluate the *in vitro* effectiveness of cinnamaldehyde and lauric arginate emulsions on STEC *E. coli* compared to and in combination with organic acids, like lactic acid and peracetic acid; and (2) analyze the effect of cinnamaldehyde concentrations and the addition of a carrier oil, coconut oil, on the efficacy and stability of emulsions.

3.2 Materials and Methods

3.2.1 Emulsion preparation and characterization

Emulsions were prepared by mixing cinnamaldehyde (CA) (1% and 2%) (Sigma-Aldrich Company, Milwaukee, WI., U.S.A.) with LAE (1%) (VEDEQSA, Barcelona, Spain) with or without coconut oil (CO) (8%) (refined coconut oil composed of 1:1 caprylic and capric acids, Better Body Foods, Lindon, UT., U.S.A.). Emulsions with sodium-caseinate (SC) powder (1%) (Alfa Aesar, Ward Hill, MA, U.S.A.) were also prepared with CA (2%) and CO (8%) at pH 6.5 and 3.5). Using the method previously described by McDaniel et al. (2019) the emulsions were combined using a high-shear mixer (Brinkman Polytron, Brinkman Instruments Inc., Westbury, NY., U.S.A.) for one minute and thirty seconds. The coarse emulsion was then homogenized using a 2-stage valve homogenizer (Panda Plus 2000, GEA) at 500 bars for 3 passes. The particle

size and zeta potential of the emulsions was characterized using DelsaMax Pro (Beckman Coulter, Indianapolis, IN., U.S.A.). Surface characteristics of LAE were evaluated at five different concentrations (1, 0.5, 0.25, 0.1 and 0.05%) using the Attension Theta Lite Drop Tensiometer (Attension Theta Lite C311, Nanoscience Instruments, Phoenix, AZ., U.S.A). The tensiometer was calibrated and measurements taken using the Oneattension user manual protocol.

3.2.2 Bacterial strains

Three strains of *Escherichia coli* were used for this study: CDC 97-3068 (serotype O121:H19), H30 isolated infant with diarrhea (Konowalchuk et al., 1977) (serotype O26:H11), ATCC 3550 (serotype 0157 Stx 1 and Stx 2) isolated from human feces. Strains were obtained from Dr. Luchansky (United States Department of Agriculture, Wyndmoor, PA., U.S.A). Overnight cultures were grown in TSB (Tryptic Soy Broth, Difco Laboratories, Spark, MD., U.S.A.) at 37° for 24 hours with 70rpm shaking. The cultures were chosen based upon their prevalence in produce postharvest outbreaks (CDC, 2018).

3.2.3 Antimicrobial activity

Overnight *E. coli* cultures were combined to create a cocktail (10^8 CFU/mL) and then treated with the following treatments up to 24 hours: LAE (1%), lactic acid solution (LA) (1%) (Purac® Corbion, Blair, NE., U.S.A.) and their combinations (0.5mL+0.5mL) and CA emulsions. Peracetic acid solution (PAA) (100ppm) (Synergex™, EcoLab, St. Paul, MN., U.S.A.) and water were used as positive and negative controls, respectively. Solutions were neutralized using double strength Dey/Engley (D/E) Neutralizing broth (Difco Laboratories, Spark, MD., U.S.A.). Remaining population was enumerated at 0.08, 0.16, 0.5, 1, 3, 5 and 24

hours using serial dilutions in peptone water (Difco Laboratories, Spark, MD., U.S.A.) and plating on Tryptic Soy Agar (TSA) (Difco Laboratories, Spark, MD., U.S.A.) overnight at 37°C.

3.2.4 Experimental design

The effect of treatments on the reduction of *E. coli* cocktail was evaluated. All experiments were performed in triplicate and the results were presented as mean values. Significant differences and comparison among means were determined using a Tukey multiple comparison test (Minitab 20.2, State College, PA., U.S.A.). A level of significance of $P < 0.05$ was considered for the analysis of variance (ANOVA) tests.

3.3 Results and Discussion

3.3.1 Emulsion particle size

Particle size is one of the key indicators of emulsion stability (Iyer et al., 2015). A study done by Iyer et al. (2015) analyzed the effect of particle size of oil-water emulsion on stability over a month. This study noted that emulsions below 150nm maintained stability at different storage temperatures over a month-long period compared to 250nm emulsions, which showed phase separation at the end of the storage period. All emulsions used in our study were between 146nm and 180nm, indicating that some are considered stable, but others could eventually phase separate. LAE (1%) + CA (1 and 2%) had similar particle sizes (d_{32}≈166 nm and d_{32}≈165 nm). The addition of CO (8%) decreased particle size (d_{32}≈150 nm) and would be considered stable, which is explained by Qian et al. (2012) who notes that the addition of carrier oil can greatly improve the stability of systems prone to aggregation. That study also further explains that incorporating carrier oils into emulsions can prevent the change in particle shape during storage that is responsible for particle aggregation. SC (1%) emulsions with CA (2%) and CO (8%) had the lowest particle size (d_{32}≈146 nm) at original pH (6.5). When pH of SC emulsions was

adjusted to 3, particle size increased (d_{32}≈177 nm) (Figure 3.1.). All samples showed monodisperse behavior, the particles were of a uniform size in the dispersed phase after homogenization. McDaniel et al. (2019) showed similar results for SC (2%) emulsions with CO (8%) and CA (2%) (d_{32}≈180 nm).

3.3.2 Zeta potential

LAE (1%) + CA (1 and 2%) had similar ζ-potentials (≈7.68mV) and (≈9.74mV). The addition of CO (8%) to the emulsions increased the ζ-potential (≈35.50mV). SC (1%) emulsions with CA (2%) and CO (8%) had the lowest ζ-potential (≈0.38mV) at original pH (6.5). When pH of SC emulsions was adjusted to 3, ζ-potential increased (≈2.12mV) (Figure 3.2.). The impact of pH on zeta potential was analyzed were found by Hu et al. (2020) who analyzed the effect of pH on stability of CA (1%) and chitosan (1%) emulsions, pH 6.5 had a lower zeta potential (≈4.0mV) compared to pH 4.0 (≈28.0mV). Magnitude of zeta potential gives an indication about the potential stability of colloidal system. Emulsions with higher zeta potentials, positive or negative, are considered stable due to the repulsive forces exceeding the attractive forces, thus preventing coagulation or flocculation (Wei Lu & Gao, 2010). Having a magnitude of >30mV indicates that LAE (1%) + CA (2%) + CO (8%) is generally considered a stable suspension. However, as noted by Roland et al. (2003) any differences in zeta potential should be at least 10 mV in order to conclude variations in stability.

3.3.3 Surface tension

Five different concentrations of LAE were measured (1, 0.5, 0.25, 0.1 and 0.05%). As the concentration of LAE decreased, the surface tension (ST) increased. LAE (1%) had the ST at 11.9 mN/m (Figure 3.3). The highest ST was 0.05% LAE at 24.51 mN/m. Huang & Nitin, 2017, evaluated the surface tension of different surfactants, including LAE. This study found that at

0.1% concentration LAE had a surface tension of approximately 36 mN/m and concluded that the addition of surfactants to produce wash water is likely to improve the antibacterial effectiveness. As a surfactant LAE can self-assemble as micelles until a critical concentration is reached called critical micelle concentration (CMC) (Ma et al., 2020). As evaluated in Chi & Catchmark, 2017, the surface tension gradually decreased with the increase in LAE concentration until the CMC was reached. The concept of measuring interfacial tensions is useful for many applications, especially where fluids are being selected for (micro) fluidic handling applications (Berry et al., 2015).

3.3.4 Antimicrobial activity

The antimicrobial effectiveness of individual sanitizers was first analyzed over a 24-hour period (Figure 3.4.). LAE (1%) and its combination LAE (1%) + LA (1%) showed a significant ($p < 0.05$) reduction in *E. coli* after 1 hour with 5.66 CFU/mL and 5.34 CFU/mL log reduction. These two treatments continued to show significant ($p < 0.05$) antimicrobial effectiveness after 24 hours with 5.58 CFU/mL and 6.13 CFU/mL log reduction. Hilbig et al. (2016) noted a log reduction of approximately 2 log CFU/mL reduction in *E. coli* O157:H7 after 8-hour exposure to LAE at 6ppm. Positive control PAA showed a significant ($p < 0.05$) reduction at hour 5 with 5.09 CFU/mL log reduction and 5.71 CFU/mL log reduction after 24 hours. Similar results were found by Ghostlaw et al. (2020) who after a starting *E. coli* O157:H7 concentration of 6.77 log CFU/mL reported below detectable limit after being exposed to PAA (30ppm) for 4 hours. Negative control (water) showed no significant ($p > 0.05$) reduction over the 24-hour period and in fact had a negative reduction, which indicates *E. coli* cell growth.

To better examine the antimicrobial effectiveness of individual sanitizers, the *E. coli* reduction was then analyzed over a 60-minutes period. Figure 3.5. demonstrates the log

reduction of individual sanitizers within 60 minutes. After 5 minutes combination of LA (1%) and LAE (1%) had significant ($p < 0.05$) reductions with a 3.98 CFU/mL log reduction. Individual application of AE (1%) also had a significant ($p < 0.05$) effect with 3.08 CFU/mL log reduction after 5 minutes. Similar findings were found by Becerril et al. (2013) who noted a log reduction of 3.5 CFU/mL of *E. coli* ATCC 29213 after 4 minutes exposure to LAE at 25 mg/L. Lactic acid showed very little activity against *E. coli* after 5 minutes with only a 0.1 log CFU/mL reduction. However, this is similar to what Lopez-Galvez et al. (2009) found when treating produce wash water with 2500 mg/L Purac FCC 80 (PURAC Bioquímica, Montmeló, Spain), which is 80% lactic, and showed a 0.2 reduction [log (N/N_0)] immediately after applying to *E. coli* cocktail. In this study, peracetic acid was found to have no significant ($p > 0.05$) effect on the *E. coli* cocktail compared to negative control, with a reduction of 0.22 log CFU/mL after 5 minutes. Two similar studies (Lopez-Galvez et al., 2009; Zhang et al., 2020) also analyzed the effect of peracetic acid on *E. coli*. With different concentrations of PAA, these studies were able to find a greater log reduction in *E. coli* within 3 minutes. Lopez-Galvez et al. (2009) used Tsunami 100 (Ecolab®, Barcelona, Spain), containing 15% peroxyacetic acid. They applied Tsunami at 125 mg/L, and it showed a 1.5 reduction [log (N/N_0)] immediately after application. Zhang et al. (2020) applied PAA (96 μM) and reproted a *E. coli* ATCC 10798 reduction of 2.1 log CFU/mL after 3 minutes. After 60 minutes both individual LAE and in combination with LA showed significant ($p < 0.05$) log reductions of 5.38 CFU/mL and 5.74 CFU/mL, respectively. However, other treatments were not significantly different ($p > 0.05$) after 60 minutes, with water control having a negative log reduction, indicating *E. coli* cell growth.

After the application of individual sanitizers, CA emulsions were analyzed over 60 minutes for *E. coli* cocktail reduction (Figure 3.6.). After 5 minutes, LAE (1%) and CA (1 and

2%) had the most significant ($p < 0.05$) effect with 3.79 CFU/mL and 3.65 CFU/mL log reduction, respectively. The addition of a carrier oil to LAE (1%) + CA (2%) showed a delay in antimicrobial effectiveness with 1.23 CFU/mL log reduction at 5 minutes. However, after 60 minutes LAE (1%) + CA (2%) + CO (8%) was the most effective treatment with a 6.83 CFU/mL log reduction significantly ($p < 0.05$) higher than any other treatment, sanitizer, or emulsion. Similar studies conducted by Hilbig et al. (2016) and Park et al. (2017) analyzed the effect of cinnamon bark oil emulsions on *E. coli* O157:H7. Hilbig et al. (2016) analyzed the effect of LAE (6ppm) and cinnamon bark oil (16ppm) emulsions which resulted in approximately 2 log CFU/mL reduction after 3 hours. Park et al. (2017) evaluated the effect of cinnamon bark oil at 0.25% and cetylpyridinium chloride, as a surfactant at 0.05% and found a reduction of 4.21 log CFU/mL after 2 minutes. This indicates that the addition of surfactants to cinnamon derived essential oils can enhance antimicrobial effectiveness. Adjusting the pH of SC (1%) + CA (1%) emulsion from 6.5 to 3 showed a slight but not significant ($p > 0.05$) increase in antimicrobial activity indicating that the change in pH had no effect on antimicrobial effectiveness.

3.4 Conclusion

Based on the results observed, this study concluded that surface-active ingredients like LAE are effective antimicrobials against STEC *E. coli in vitro*. Antimicrobial efficacy might be improved when LAE is applied in combination with organic acids like lactic acid and emulsified to act as a delivery system for cinnamaldehyde. Overall, more research is needed to further understand how to effectively utilize these technologies on food matrixes to maximize antimicrobial efficacy.

3.5 Acknowledgement

This project was funded by the Kansas Department of Agriculture Specialty Crop Block Grant KDA 2019-3.

3.6 Tables and Figures

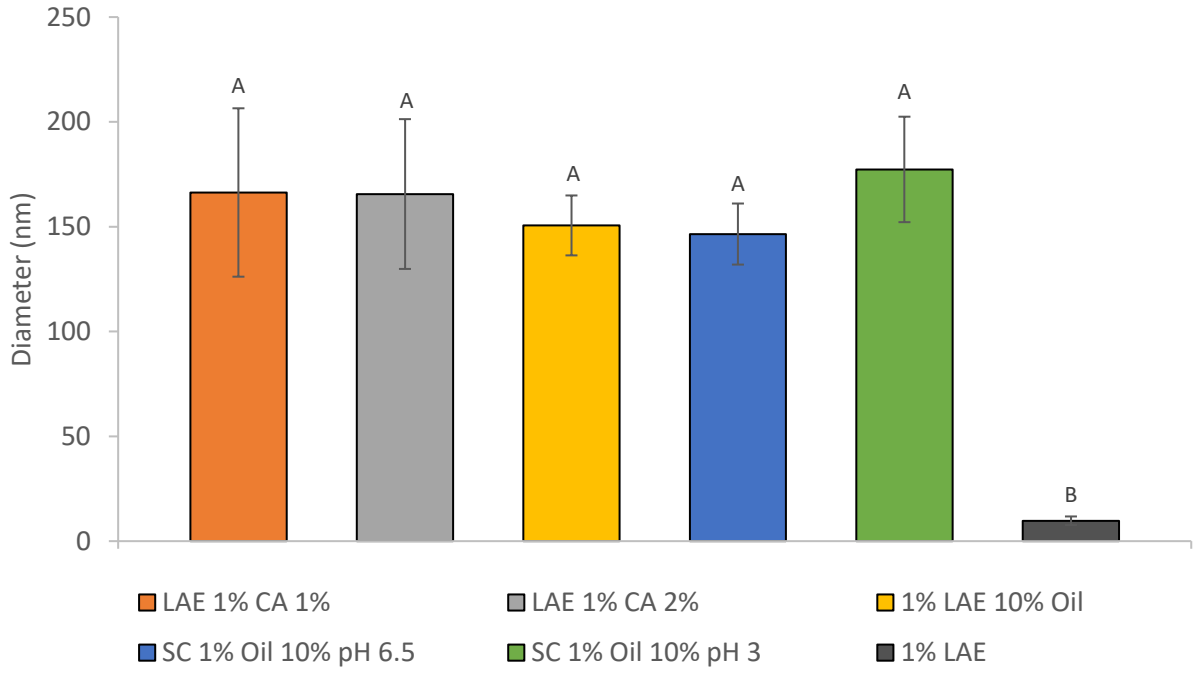

Figure 3.1. Particle Size results. The results show the average values. Error bars indicate standard deviation. Values that do not share letters are significantly different ($P < 0.05$).

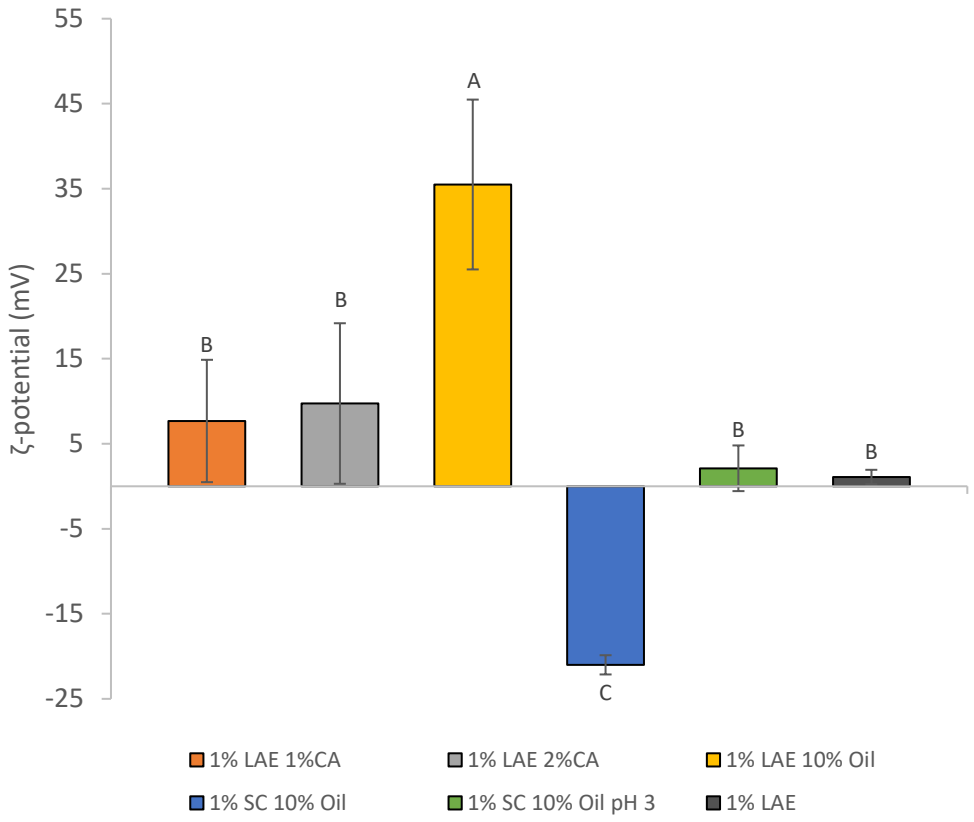

Figure 3.2. Zeta potential results. The results show the average values. Error bars indicate standard deviation. Values that do not share letters are significantly different ($P < 0.05$).

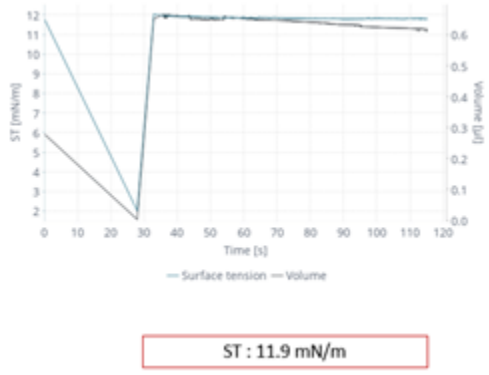

Figure 3.3. Surface tension (mN/m) results of 1% over time.

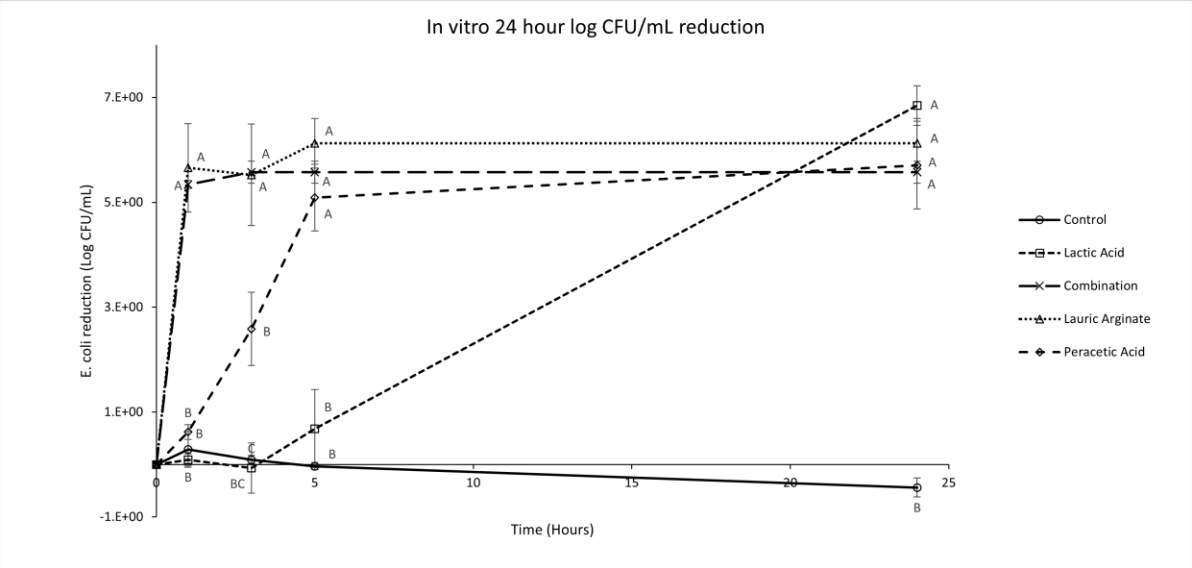

Figure 3.4. In vitro log CFU/mL reduction of *E. coli* over 24 hours after treatment with control (o), lactic acid (□), combination (x), lauric arginate (Δ) and peracetic acid (◊). Data is presented as mean values of replicates. Error bars indicate standard deviation. Lines do not represent a model but are used to aid comprehensibility. Values that do not share letters at the same time are significantly different ($P < 0.05$).

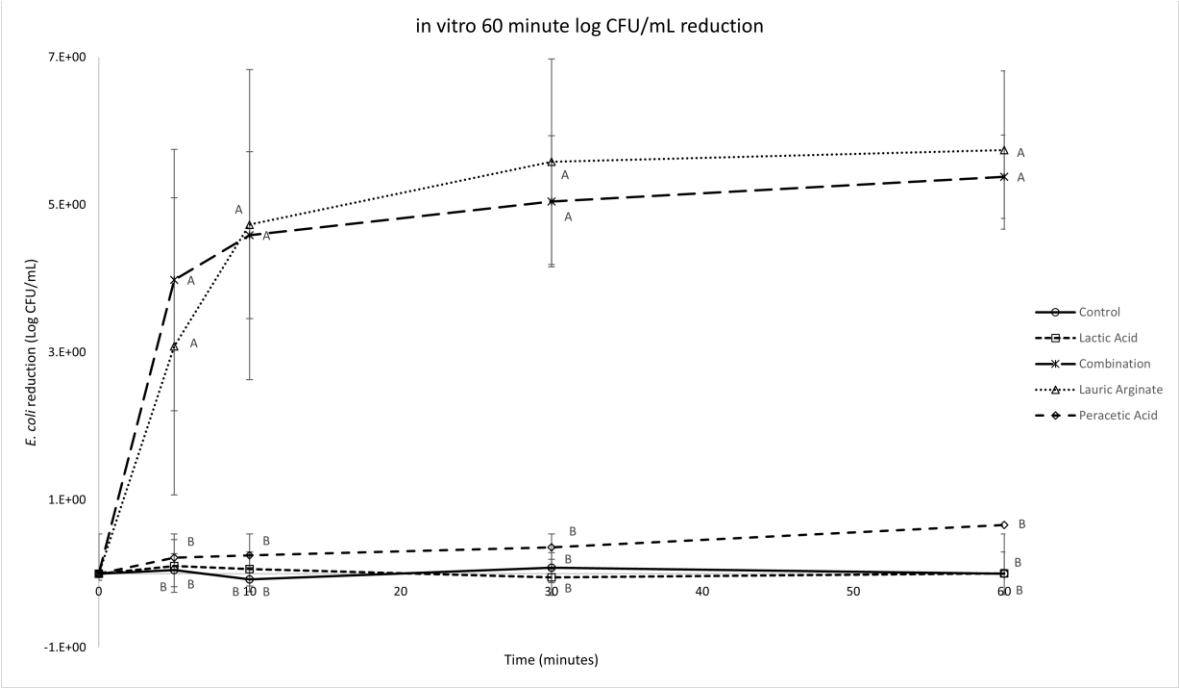

Figure 3.5. In vitro log CFU/mL reduction of *E. coli* over 60 minutes after treatment with control (o), lactic acid (□), combination (x), lauric arginate (Δ) and peracetic acid (◊). Data are presented of mean values of replicates. Error bars indicate standard deviation. Lines do not

represent a model but are used to aid comprehensibility. Values that do not share letters at the same time are significantly different ($P < 0.05$).

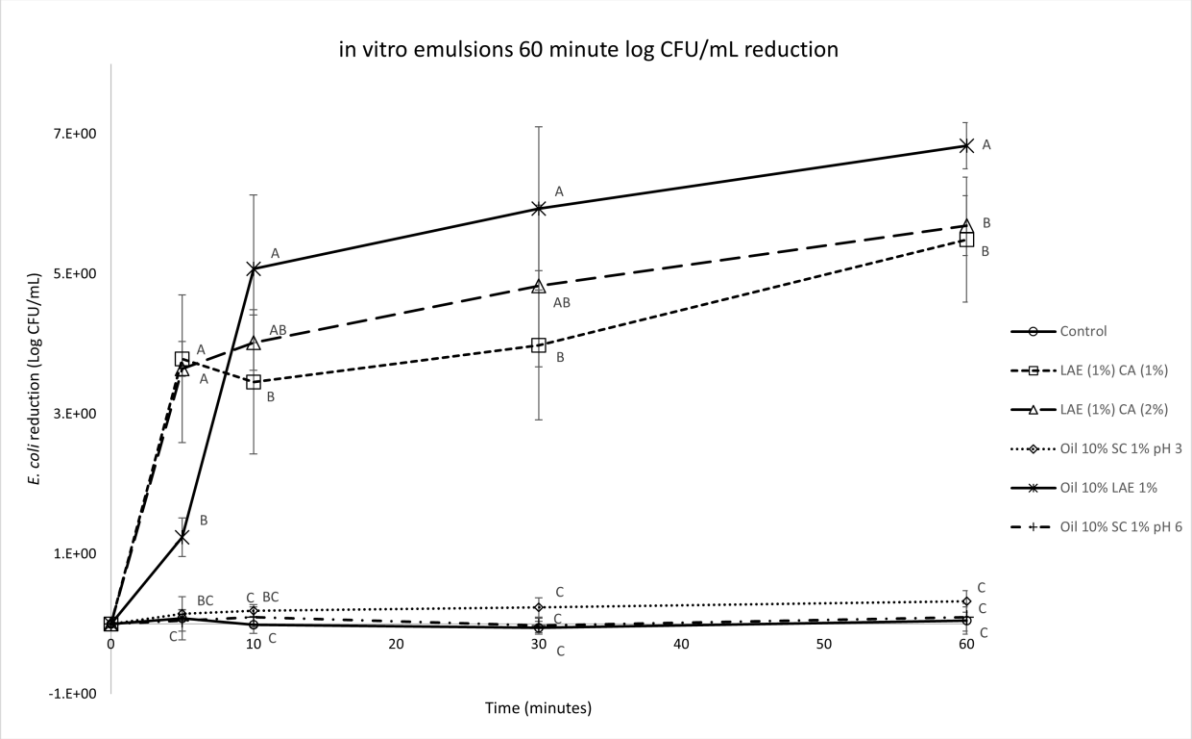

Figure 3.6. In vitro emulsions log CFU/mL reduction of *E. coli* over 60 minutes after treatment with control (o), LAE (1%) + CA (1%) (□), Oil 10% LAE 1% (x), LAE (1%) + CA (2%) (Δ), Oil 10% SC 1% pH 3 (◊) and Oil 10% SC 1% pH 6.5 (+). Data are presented of mean values of replicates. Error bars indicate standard deviation. Lines do not represent a model but are used to aid comprehensibility. Values that do not share letters at the same time are significantly different ($P < 0.05$).

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Chapter 4 - Pilot-test of Lauric Arginate Emulsions along with Organic Acids as a function of Application Sequence against Shiga toxin-producing *Escherichia coli* on Fresh Spinach

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Abstract

Multiple leafy green outbreaks due to Shiga toxin-producing *Escherichia coli* (STEC) have occurred in the past two decades. Furthermore, current approved industry antimicrobial treatments are mainly based on chemical use and consumers are increasingly concerned with chemical usage. The objective of this study was to assess the effectiveness of different antimicrobial treatments, lactic acid (LA), peracetic acid (PAA), lauric arginate (LAE) and cinnamaldehyde (CA) LAE emulsions, applied individually or sequentially over time on *E. coli*-inoculated spinach in a pilot-washing system with or without organic load. Spinaches were inoculated overnight with an STECs cocktail and then washed in a washing pilot setup. The most effective individual treatment was LAE (1%) + CA (1%) showing a significant ($p < 0.05$) reduction of 2.30 ± 0.19 log CFU/g after 10 minutes. The most significantly ($p < 0.05$) effective sequential treatment was LA followed by LAE (1%) + CA (1%) with a 2.30 ± 0.06 CFU/g log reduction. When 10% organic load was added, the most effective treatments were PAA followed by LAE (1%) + CA (1%) and LAE (1%) + CA (1%) followed by PAA. These two treatments were significantly ($p < 0.05$) more effective with a 1.86 ± 0.10 CFU/g and 1.83 ± 0.17 CFU/g log reduction, respectively, after 10 minutes. Overall, combinations of surface-active antimicrobials, essential oils and organic acids can provide sustainable wash-water solutions to reduce STECs contamination on leafy greens.

4.1 Introduction

Since 2006 multiple outbreaks have occurred due to fresh leafy-greens contaminated with Shiga toxin-producing *Escherichia coli* (STECs) (CDC, 2018). The most recent outbreak occurring in August 2020, from *E. coli* O157:H7 causing 40 people to become infected and 20 hospitalized (CDC, 2020). Another notable outbreak occurred in 2012 and was linked to *E. coli* O157:H7 contaminated organic spinach, causing 33 people to fall ill and 46% of them to be hospitalized (CDC, 2012). Currently, the leafy green industry uses a variety of techniques, such as washing, to reduce microbial contamination, improve shelf-life and enhance product quality. Washing leafy greens is one of the best methods to remove any dirt and debris on the product and by applying multiple antimicrobials during the washing steps, processors can increase the overall reduction in pathogens (FDA, 2006). Current wash-water treatments for leafy greens include potable water and organic acids, like lactic acid and peracetic acid (FDA, 2006; Shaw, 2013; Burrows, 2019). Because of the surface characteristics of leafy greens as well as other factors, like organic load, the antimicrobial efficacy of conventional sanitizers, like peracetic acid, can be inhibited (Van Haute et al., 2013; Doan et al., 2020). Leafy greens are very delicate and can be easily damaged during processing, causing wounds, cuts and other quality defects, like loss of green color. When leafy greens are wounded or cut it allows the release of cellular fluids, a valuable nutrient source for mciroorganism growth (Wulfkuehler et al., 2013). Leafy green color is one of the primary quality and freshness indicators that consumers use to innfluence their purchasing decions (Leon et al., 2006; Pathare et al., 2013). Produce that appears dull or off-colored is seen as not fresh, spoiled and undesirable (Nisha et al., 2011). There is also growing concern among consumers about chemicals and additives in food. Over 50% of consumers consider chemicals used in food processing to be the top food safety concern in 2021

(International Food Information Council, 2021). There is, therefore, a need for more effective antimicrobial treatments that are also naturally derived and sustainable while maintaining product quality.

Essential oils have been studied extensively for their antimicrobial effectiveness (Hammer et al., 2001; Elgayyar et al., 2001; Teles Andrade et al., 2014) and are recently gaining popularity for their use against various foodborne pathogens. Essential oils have obtained Generally Recognized as Safe (GRAS) status (FDA, 2021) and are broadly accepted by consumers due to being naturally derived instead of synthetic. Cinnamaldehyde, a derrivative from cinnamon bark oil, is one of the prevelant essential oils being studied for its antimcirobial activity (Hilbig et al., 2016; Ma et al., 2016; Hu et al., 2020). However, essential oils are hydrophobic, this makes them difficult to combine in aqueous solutions (Chang et al., 2012; Chang et al., 2015). To improve application for aqueous solutions numerous studies (Chen et al., 2015; Hilbig et al., 2016; Ma et al., 2016) have evaluated the ability of essential oils encapsulated with cationic surfactants, like lauric arginate (LAE) to act as an emulsion delivery system. LAE is a cationic surfactant derived from lauric acid, L-arginine and ethanol, it was patented in Spain in 1983 (García Domíngues et al., 1983) and is GRAS for use on food products up to 200ppm (FDA, 2021). Surfactants have an amphiphilic structure, which allows them to penetrate the membrane of bacteria, altering the membrane structure which disrupts metabolic processes (Shen et al., 2021). LAE individually was found to have a minimum inhibitory concentration (MIC) of 11.8ppm against *E. coli* O157:H7 (Ma et al., 2020). When LAE (100mg/L) was tested against lettuce leaves enoculated with *E. coli* O157:H7 it was found to have a 2.5 CFU/g log reduction (Nubling et al., 2016). When combined with cinnamaldehyde, LAE was found to have a MIC of 7ppm against *E. coli* O157:H7 (Hilbig et al., 2016). This study

aims to assess the antimicrobial effectiveness of lauric arginate and cinnamaldehyde emulsion, lactic acid and peracetic acid applied individually or sequentially to *E. coli* inoculated spinach in a pilot-washing system as a function of time and organic load (1% and 10%).

4.2 Materials and Methods

4.2.1 Nanoemulsion preparation and characterization

Emulsions were prepared by mixing cinnamaldehyde (CA) (1%) (Sigma-Aldrich Company, Milwaukee, WI., U.S.A.) with lauric arginate (LAE) (1%) (VEDEQSA, Barcelona, Spain) with or without coconut oil (CO) (8%) (refined coconut oil composed of 1:1 caprylic and capric acids, Better Body Foods, Lindon, UT., U.S.A.). Using the method previously described by McDaniel et al. (2019) the emulsions were combined using a high-shear mixer (Brinkman Polytron, Brinkman Instruments Inc., Westbury, NY., U.S.A.) for one minute and thirty seconds. The coarse emulsion was then homogenized using a 2-stage valve homogenizer (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) at 500 bars for 3 passes.

4.2.2 Bacterial strains and spinach inoculation

Three strains of *Escherichia coli* were used for this study: CDC 97-3068 (serotype O121:H19), H30 isolated infant with diarrhea (Konowalchuk et al., 1977) (serotype O26:H11), ATCC 3550 (serotype 0157 Stx 1 and Stx 2) isolated from human feces. Strains were obtained from Dr. Luchansky (United States Department of Agriculture, Wyndmoor, PA., U.S.A.). Cultures were grown in TSB (Tryptic Soy Broth, Difco Laboratories, Spark, MD., U.S.A.) at 37° for 24 hours with 70rpm shaking. The cultures were chosen based upon their prevalence in produce postharvest outbreaks (CDC, 2018). *E. coli* cocktail (10^8 CFU/mL) was prepared by centrifuging overnight individual strains at 1,800rpm for 10 minutes at 22°C using the Allegra X-I4R centrifuge (Beckman Coulter, Indianapolis, IN., U.S.A.). The supernatant was discarded,

and pellet was resuspended in 5mL of phosphate buffer solution (PBS) (Difco Laboratories, Spark, MD., U.S.A.) and strains were combined in equal ratio. Pre-washed baby spinach was obtained from the local grocery store (Dillons, Manhattan, KS., U.S.A.). Fifty grams of spinach and 1mL of *E. coli* cocktail was added to a Stomacher® bag (Seward Ltd, West Sussex, United Kingdom) and shaken for five minutes. Inoculated spinach (10^6 CFU/mL) was then laid flat to dry in Class II A2 Biological Safety Cabinet (ESCO, Singapore) for 30 minutes.

4.2.3 Pilot-washing

Spinach (subsample of 10g for each condition) was washed individually or sequentially with or without organic load (OL) (1 or 10%) in the following treatments (1L): LAE (1%), lactic acid solution (LA) (1%) (Purac® Corbion, Blair, NE., U.S.A.) and CA emulsions. Peracetic acid solution (PAA) (100ppm) (Synergex^{TM}, EcoLab, St. Paul, MN., U.S.A.) and water were used as positive and negative controls, respectively. OL was created by blending 100mL deionized water with 100g of spinach then was added at either 1% or 10% concentration. Spinach was washed individually at 5 or 10 minutes. Sequential application spinach was washed in two different treatments, described above, the first treatment for 2.5 minutes or 5 minutes and then transferred to the second treatment for an additional 2.5 or 5 minutes. After washing all spinach was spun-dry for one minute. Spinach was then transferred to 90mL double strength Dey/Engley (D/E) Neutralizing broth (Difco Laboratories, Spark, MD., U.S.A.). Spinach bags were hand stomached for 1 minute then solution was transferred to peptone water (Difco Laboratories, Spark, MD., U.S.A.) for serial dilutions. Wash water samples (1mL) were taken and evaluated for bacterial counts by neutralizing in double strength D/E broth (9mL) and serial diluting. *E. coli* populations were plated on MacConkey Agar (Difco Laboratories, Spark, MD., U.S.A.) overnight at 37°C.

4.2.4 Color measurements

The color of spinach was evaluated before and after treatment using Hunter MiniScan EZ spectrophotometer (HunterLab, Reston, VA., U.S.A.). Five spinach leaves were sampled per treatment and averages were reported for L*, a*, and b*. Total color difference (ΔE) was calculated using Equation 1:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2} \quad \text{Equation (1)}$$

4.2.5 Cellular contents leakage

An individual colony of each *E. coli* strain was placed in 50mL TSB overnight at 37°C. Cells were centrifuged for 10 min at 4,000 rpm, supernatant was discarded, and the pellet resuspended in 10mL PBS twice. *E. coli* strains were combined to form a cocktail (10^{10} CFU/mL), 9mL of the cocktail was added to 1mL of the following treatments: LA (1%), PAA (100ppm) and LAE (1%) + CA (1%). Samples were incubated for 1 hour at 37°C at 70 rpm on the GeneMate Orbital Shaker (VWR, Solon, OH., U.S.A.). Samples were centrifuged for 15 min at 4,300 rpm, supernatant was collected and filtered using MicroLiter nylon 0.20µm PF for LA and PAA. LAE (1%) + CA (1%) was filtered using Microsep Advance Centrifugal Device with Omega Membrane 100K (Pall Corporation, Port Washington, NY., U.S.A.) for 10 minutes at 4,300 rpm. Two mL of supernatant was used to measure UV absorption at 260 nm using Genesys 10S Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA., U.S.A.).

4.2.6 Experimental design

The effect of treatments on the reduction of *E. coli* inoculated spinach was evaluated. All experiments were performed in triplicate and the results were presented as mean values. Significant differences and comparison among means were determined using a Tukey multiple

comparison test (Minitab 20.2, State College, PA., U.S.A.). A level of significance of $P < 0.05$ was considered for the analysis of variance (ANOVA) tests.

4.3 Results and Discussion

4.3.1 Pilot-wash antimicrobial activity

This study focused on cinnamaldehyde combined with a cationic surfactant, LAE on fresh spinach. The CFU/g log reduction of *E. coli* cocktail inoculated spinach after individual (Figure 4.1.) and sequential (Figure 4.2.) washing of treatments was observed. The most effective individual treatment was LAE (1%) + CA (1%) showing a significant ($p < 0.05$) reduction of 2.30 ± 0.19 log CFU/g after 10 minutes. LAE and LA showed similar results with reductions of 1.48 ± 0.11 CFU/g and 1.54 ± 0.13 CFU/g, respectively. In this study, sequential application of treatments overall, improved the reduction of *E. coli* cocktail after 10 minutes. The most siginficantly ($p < 0.05$) effective treatment was LA for 5 minutes follwed by LAE (1%) + CA (1%) for 5 minutes with a 2.30 ± 0.06 CFU/g log reduction. This indicates a synergist effect between lactic acid, an oxidizer, which oxidizes the enzymes, cell membrane and structural proteins of the cell and the already effective individual treatment of LAE (1%) + CA (1%) as proven earlier. The least effective treatment was LAE for 5 minutes followed by LA for 5 minutes with a 1.36 ± 0.07 CFU/g log reduction. This indicates then when LAE is applied individaully without the addition of CA it is less effective and when it is applied first followed by LA, there is a signficiantly less ($p < 0.05$) synergistic effect between the two antimicrobials. Previous studies have evaluated the effect of various essential oil emulsions as washing treatments for leafy greens to reduce *E. coli* O157:H7 (Bhargava et al., 2015; Ruengvisesh et al., 2015; Nubling et al., 2016; Huang & Nitin, 2017). A treatment of 0.1% oregano oil emulsified with Tween 80 showed a 2.8 CFU/g reduction after 3 hours (Bhargava et al., 2015). While this

study used a different essential oil and emulsifying agent, Tween 80, it still showed when combined to form emulsions essential oils do show antimicrobial activity. However, in this study both a lower concentration of essential oils 0.1% compared to the 1% used in our study and the addition of Tween 80 which can impact antimicrobial effectiveness (Nielsen et al., 2016) showed an overall a decrease *in E. coli* reduction over time compared to the present study. Ruengvisesh et al. (2015) evaluated the effect of CytoGaurd LA 20 (0.125%) and eugenol (0.003125%) emulsion on fresh spinach and it was found to have a 2.65 CFU/cm^2 log reduction after immersion for 5 minutes. In this current study, LAE (1%) and CA (1%) emulsions showed a 1.63 CFU/g log reduction after washing for 5 minutes. The differences in results could be attributed to the use of different essential oils, washing techniques as well as difference in quantification. Park et al (2017) washed basil leaves in cinnamon bark oil (0.25%) and cetylpyridinium chloride (0.05%) emulsions and showed a 4.10 CFU/g reduction in *E. coli* O157:H7 after washing for 3 minutes. The results of all these emulsions studies conclude that type and concentration of essential oil as well as the type of emulsifying or surfactant ingredient added can dramatically alter antimicrobial effectiveness of emulsions. A study done by Huang & Nitin (2017) showed approximately 2.25 CFU/g log reduction in *E. coli* O157:H7 after washing lettuce for 20 minutes in 0.1% LAE. Compared to the current study, which washed for a maximum of 10 minutes and saw 1.48 CFU/g log reduction of *E. coli* cocktail, it is possible that by washing longer, we would see a similar or greater reduction as shown by Huang & Nitin (2017). Nubling et al. (2016) washed lettuce in LAE (100 mg/L) individually and saw a 2.6 CFU/g reduction of *E. coli* O157:H7 after washing for 120s.

4.3.2 Organic load

Organic load, which is characterized as product tissue, cellular fluids, soil, insects and microbes (Herdt & Feng, 2009) can reduce antimicrobial effectiveness of wash water treatments when allowed to accumulate (Zhang et al., 2009; Davidson et al., 2017). Multiple studies have been conducted on the effect of organic load on organic acids like peracetic acid (Lopez-Galvez et al., 2009; Davidson et al., 2013; Davidson et al., 2017). However, no previous studies have evaluated the effect of organic load on LAE and CA emulsions. In this study the effect of two different concentrations of organic load (1% and 10%) was evaluated. All individual wash-water treatments (Table 4.1.) showed a decrease in effectiveness when organic load was increased from 1% to 10%. However, there is a significant difference ($p < 0.05$) between LAE (1%) + CA (1%) and other treatments at both 5 and 10 minutes and 1% and 10% OL. This shows that the LAE and CA emulsion is not affected by organic load and showed the least amount of reduction in antimicrobial efficacy compared to other treatments. Studies evaluating the effect of OL on other types of essential oils have shown similar results. Harness III (2015) applied 0.5% thyme oil emulsion to *E. coli* O157:H7 on spinach, and at 1% organic matter there was no significant loss in antimicrobial effectiveness. Zhang et al. (2016) also evalauted the effect of organic load on thyme oil (1%). This study found that the addition of organic load up to 5% had no signficant effect on antimcirobail effectiveness agaisnt *E. coli* O157:H7. Positive control, peracetic acid, showed the second highest *E. coli* reduction at both OL concentrations after 10 minutes. A similar study (Davidson et al., 2017) evaluating the effect of organic load on peracetic acid (50ppm) saw no significant ($p < 0.05$) difference in *E. coli* O157:H7 log CFU/g reduction between four different organic load concentrations. The reduction on inoculated lettuce ranged from 0.97 CFU/g to 1.74 log CFU/g at 10% and 0% OL, respectively after being washed for 90s.

Table 2. shows the effect of organic load on sequential application of treatments. The most effective treatment was LA for 2.5 minutes followed by PAA for 2.5 minutes with a 2.56 ± 0.03 CFU/g log reduction at 1% organic load. However, when increasing the organic load to 10% the most effective treatments were PAA followed by LAE (1%) + CA (1%) emulsions and LAE (1%) + CA (1%) emulsions + PAA. Considering the individual treatments of PAA and LAE (1%) + CA (1%) emulsions were least effected by high concentrations of organic load, it can be concluded that when applied sequentially these treatments would remain unaffected. Which was proven when PAA + LAE (1%) + CA (1%) emulsions and LAE (1%) + CA (1%) emulsions + PAA were significantly ($p < 0.05$) more effective with a 1.86 ± 0.10 CFU/g and 1.83 ± 0.17 CFU/g log reduction, respectively, after 10 minutes. Based on the individual treatments of being the most effective at 10% OL at 10 minutes, applying them sequentially would only further improve antimicrobial efficacy. Wash-water samples were also taken to evaluate the amount of *E. coli* that detached from spinach during washing and remained in the water. Negative control showed 5.19 ± 0.08 log CFU/mL and 4.76 ± 0.10 log CFU/mL at 10% and 1% OL, respectively, after spinach was washed for 10 minutes. The only treatment to have detectable colony counts from wash-water samples was individual treatment of lactic acid with 1.71 ± 0.26 log CFU/mL and 1.92 ± 0.36 log CFU/mL at 1% and 10% respectively, after 10 minutes. All other treatments were below detectable limit, which indicates that wash-water treatments are still effective at eliminating *E. coli* cells at 1% and 10% OL. Similar results were found by Nubling et al. (2016), which detected no remaining colonies after LAE wash-water treatment was applied.

4.3.3 Color measurements

Fresh produce color is one of the most important factors on consumer purchasing decisions (Leon et al., 2006; Pathare et al., 2013). Produce that appears dull or off-colored is

seen as not fresh, spoiled and undesirable (Nisha et al., 2011). In this study we evaluated the effect of wash-water treatments on the color of fresh spinach. The results of ΔE and a* are show in Table 4.3., ΔE represents the difference in total color. None of the treatments showed a significant difference in total color ($p > 0.05$) which indicates that there is a less likelihood of visual quality defects on spinach after being washed in these treatments. *a was used as an indicator for green color, a greater negative number of *a shows a darker green color in fresh produce (Pathare et al., 2013). There were no significant differences in spinach color after being washed for 5 minutes. Spinach washed in LAE (1%) + CA (1%) individually showed a significantly ($p < 0.05$) lower *a after 10 minutes. A similar study (Park et al., 2011) evaluated the effect on *a after washing lettuce in organic acid treatments. It was found that after washing lettuce for 10 minutes with 1% LA there was no significant difference between and of the organic acid treatments ($p > 0.05$). When the effect of LAE (0.1%) was analyzed by Huang & Nitin (2017) it was noted that only a marignal change in the color of lettuce leaves was obsereved compared to control samples, however, the total change in color (ΔE) did not exceed 3, which falls into acceptable range of total color change (Chen & Mujumdar, 2008). Previous studies (Bhargava et al., 2015; Ruengvisesh et al., 2015) involving the use of essential oil emulsions as wash water treatments for leafy greens have not evaluated color changes. However as noted by Ruengvisesh et al. (2015) there was not a visual difference in color after washing spinach. Overall, these results indicate that the wash-water treatments do not significantly impact fresh spinach color.

4.3.4 Cellular contents leakage

Three different treatments, PAA, LA and LAE (1%) + CA (1%) were analyzed for their effect on the cell and the release of cellular contents as shown in Figure 4.3., PAA and LAE

(1%) + CA (1%) had statistically similar results at A_{260}≈0.726 and A_{260}≈0.752 ($p<0.05$). LA showed significantly ($p<0.05$) less cellular content release into the supernatant (A_{260}≈0.422) than other treatments. A similar experiment by Ma et al. (2016) showed absorbance at 260nm of approximately 0.600 against *E. coli* O157:H7 when treated with a combination of 10mg/mL LAE and 400 mg/L cinnamon oil for 3 hours. Moghimi et al. (2016) compared the effects of nanoemulsions to pure oil and control treatmetns. This study found that there was a signficant increase in cell leakage when *E. coli* was treated with the thyme oil nanoemulsion for 60 minutes compared to control treatment. These results show that both PAA and LAE (1%) + CA (1%) cause significant cellular content release, which indicates effective antimicrobial activity.

4.4 Conclusion

Based on the results observed, this study concluded that surface-active ingredients like LAE and cinnamaldehyde are effective antimicrobials against STEC *E. coli* when applied in spinach pilot-washing experiment. Furthermore, antimicrobial efficacy might be improved when LAE and CA emulsion are applied sequentially with organic acids like peracetic acid, and the addition of organic load does not impact the effectiveness of the treatments. Overall, more research is needed to further understand how to effectively utilize these technologies in field settings to maximize antimicrobial efficacy.

4.5 Acknowledgements

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4.6 Tables and Figures

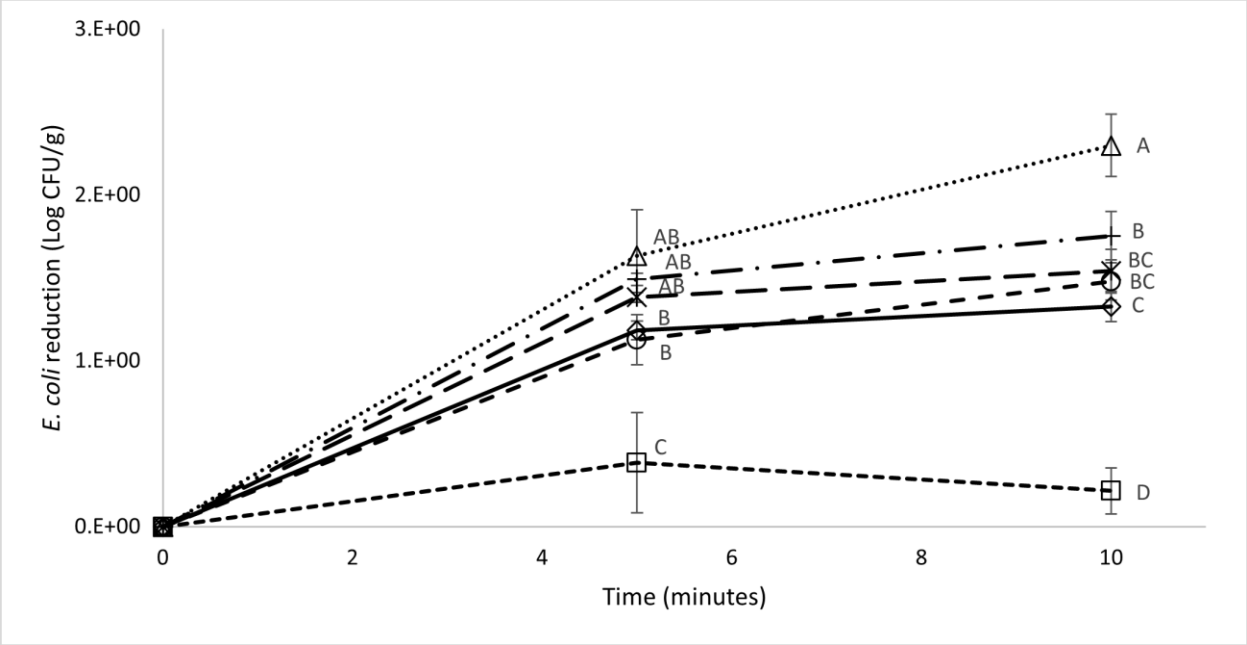

Figure 4.1. Individual application of treatments to spinach.
Values shown are log CFU/mL reduction of *E. coli* over time (min) after treatment with control (□), LAE (1%) + CA (1%) (Δ), LAE (o), LA (x), PAA (◊) and LAE 1% Oil 10% (+). Data are presented of mean values of replicates. Error bars indicate standard deviation. Lines do not represent a model but are used to aid comprehensibility. Values that do not share letters at the same times are significantly different ($P < 0.05$).

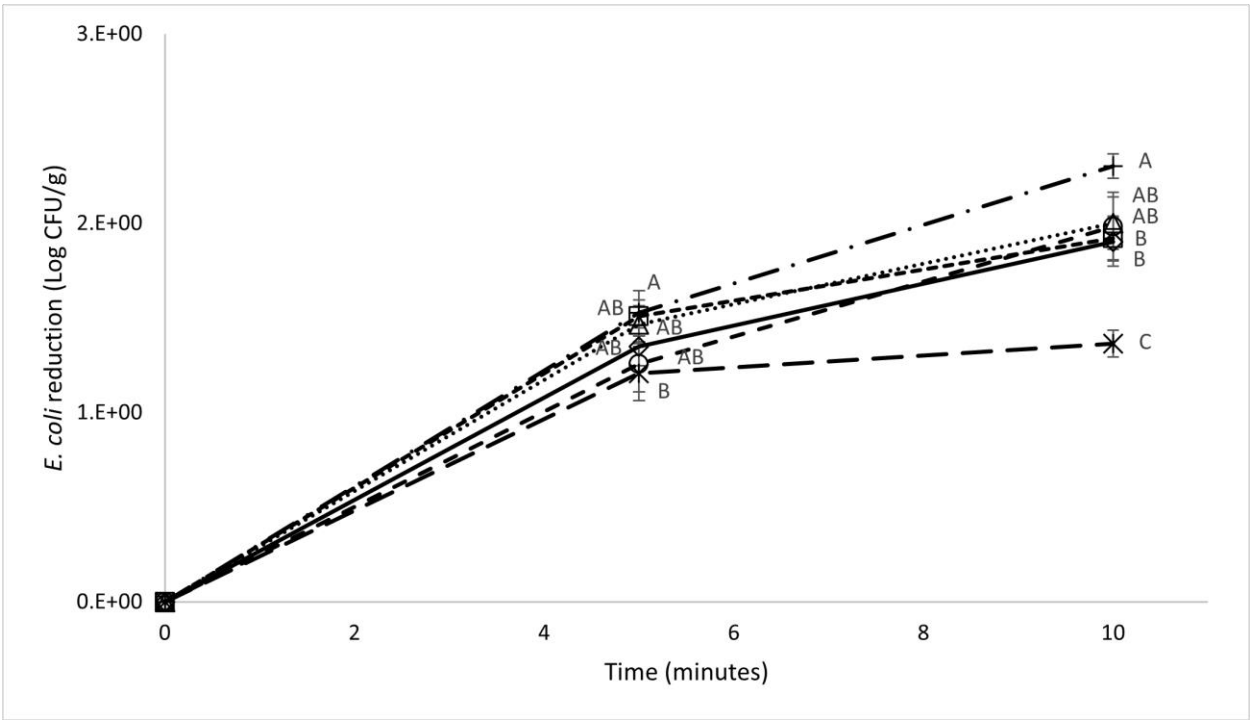

Figure 4.2. Sequential application of treatments to spinach.
Values shown are log CFU/mL reduction of *E. coli* over time (min) after treatment with LAE + PAA (□), LAE (1%) + CA (1%) + PAA (Δ), PAA + LAE (1%) + CA (1%) (o), LAE + LA (x), LAE (1%) + CA (1%) + LA (◊) and LA + LAE (1%) + CA (1%) (+). Data are presented of mean values of replicates (n=3). Error bars indicate standard deviation. Lines do not represent a model but are used to aid comprehensibility. Values that do not share letters at the same times are significantly different ($P < 0.05$).

Table 4.1. Individual treatments with organic load (1% and 10%) at 5 and 10 minutes.
Data is presented as mean values of replicates (log CFU/g reduction) (n=3) ± standard deviation. Values that do not share letters at the same times are significantly different ($P < 0.05$).

	1% organic load		10% organic load	
	5 min	10 min	5 min	10 min
Control	0.52 ± 0.15^{c}	0.59 ± 0.16^{d}	0.33 ± 0.07^{c}	0.23 ± 0.20^{d}
Lactic Acid	1.03 ± 0.08^{b}	1.26 ± 0.20^{c}	0.51 ± 0.01^{bc}	0.52 ± 0.01^{cd}
Peracetic Acid	0.96 ± 0.08^{b}	1.72 ± 0.04^{b}	0.70 ± 0.16^{b}	1.19 ± 0.04^{b}
Lauric Arginate	0.86 ± 0.05^{b}	0.98 ± 0.04^{c}	0.40 ± 0.07^{c}	0.52 ± 0.10^{c}
LAE 1% CA 1%	2.04 ± 0.21^{a}	2.37 ± 0.17^{a}	1.52 ± 0.07^{a}	1.94 ± 0.03^{a}

Table 4.2. Sequential application of treatments with organic load (1% and 10%) at 2.5+2.5 and 5+5 minutes.
Data is presented as mean values of replicates (log CFU/g reduction) (n=3) ± standard deviation. Values that do not share letters at the same times are significantly different ($P < 0.05$).

	1% organic load		10% organic load	
	2.5 + 2.5 min	5 + 5 min	2.5 + 2.5 min	5 + 5 min
LAE + LA	1.48 ± 0.10^{bc}	2.15 ± 0.26^{b}	0.57 ± 0.11^{b}	1.38 ± 0.10^{b}
LA + LAE	1.31 ± 0.09^{c}	2.16 ± 0.13^{b}	0.64 ± 0.05^{b}	1.10 ± 0.06^{c}
LA + PAA	2.02 ± 0.30^{a}	2.56 ± 0.03^{a}	1.07 ± 0.10^{a}	1.40 ± 0.03^{b}
PAA + LA	1.81 ± 0.22^{ab}	2.28 ± 0.02^{ab}	1.04 ± 0.03^{a}	1.22 ± 0.08^{bc}
PAA + LAE 1% + CA 1%	2.15 ± 0.04^{a}	2.52 ± 0.05^{ab}	1.28 ± 0.13^{a}	1.86 ± 0.10^{a}
LAE 1% + CA 1% + PAA	2.17 ± 0.10^{a}	2.43 ± 0.15^{ab}	1.29 ± 0.10^{a}	1.83 ± 0.17^{a}

Table 4.3. Color measurements (a* and ΔE) of spinach washed individually (5 and 10 min) and sequentially (2.5+2.5 and 5+5 min).
Data is presented as the mean values of replicates ± standard deviation. Values that do not share letters at the same times are significantly different ($P < 0.05$).

	a*		Δ*E*	
	5 min	10 min	5 min	10 min
Control	-10.72 ± 1.94^{a}	-11.20 ± 1.80^{ab}	1.04 ± 0.37^{a}	3.34 ± 1.65^{a}
Lactic Acid	-9.03 ± 6.29^{a}	-11.24 ± 1.55^{ab}	6.81 ± 4.15^{a}	6.48 ± 4.27^{a}
Peracetic Acid	-10.96 ± 1.92^{a}	-10.74 ± 2.06^{a}	6.18 ± 4.51^{a}	6.34 ± 5.18^{a}
Lauric Arginate	-11.56 ± 1.65^{a}	-11.03 ± 1.89^{ab}	3.71 ± 2.32^{a}	3.18 ± 1.94^{a}
LAE 1% + CA 1%	-11.15 ± 1.48^{a}	-13.63 ± 2.49^{b}	5.24 ± 3.37^{a}	7.72 ± 4.14^{a}
	2.5 + 2.5 min	5 + 5 min	2.5 + 2.5 min	5 + 5 min
PAA + LAE 1% + CA 1%	-10.82 ± 1.95^{a}	-12.07 ± 1.55^{ab}	3.81 ± 1.51^{a}	3.09 ± 2.16^{a}
LAE 1% + CA 1% + PAA	-10.48 ± 2.10^{a}	-10.58 ± 3.23^{a}	7.39 ± 5.22^{a}	6.79 ± 4.83^{a}
LA + LAE 1% + CA 1%	-11.39 ± 0.76^{a}	-11.58 ± 1.66^{ab}	10.62 ± 1.61^{a}	4.15 ± 2.61^{a}
LAE 1% + CA 1% + LA	-11.33 ± 1.35^{a}	-10.93 ± 1.75^{a}	5.7 ± 4.97^{a}	5.26 ± 4.55^{a}

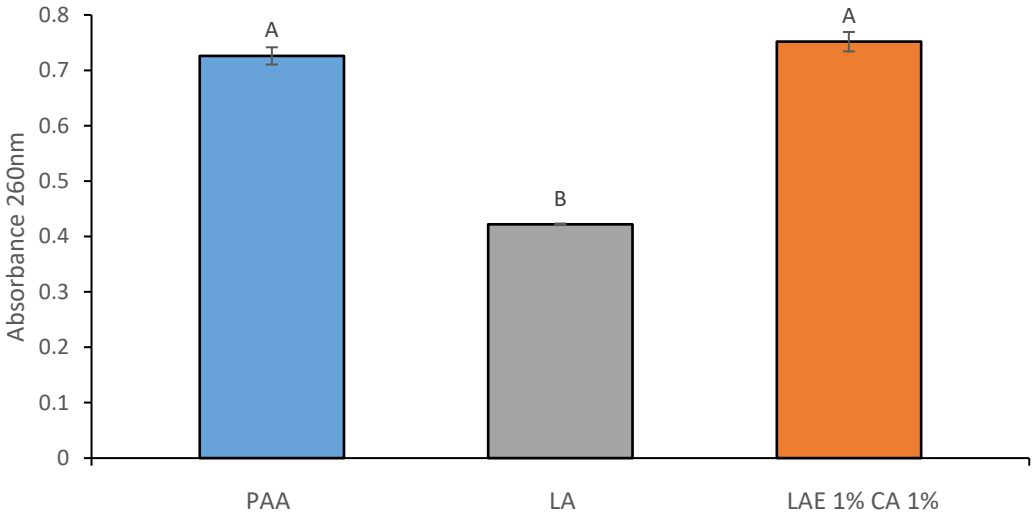

Figure 4.3. Cell content release results. PAA (100ppm), LA (1%) and LAE (1%) + CA (1%). The results show the average of values (n=3). Error bars indicate standard deviation. Values that do not share letters are significantly different ($P < 0.05$).

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Chapter 5 - Field Washing Test of Lauric Arginate Emulsions and Organic Acids Against Spoilage Organisms to Evaluate the Effect on Shelf-Life of Leafy Greens

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Abstract

Farmers' markets are increasing in popularity, indicating consumer preference for local and all-natural food. About 76% of surveyed Kansas growers who sell at farmers' markets currently have a produce rinsing step. A novel wash-water formulation is needed to provide farmers with an all-natural treatment. Leafy greens were washed using lauric arginate and cinnamaldehyde emulsions individually and sequentially followed by peracetic acid to compare to existing washing treatment. Shelf-life was evaluated by leafy green color, yeast and mold counts and aerobic plate counts over a 10-day period. LAE (1%) + CA (1%) emulsion followed by peracetic acid was the most effective treatment with mean yeast and mold counts of 2.79 CFU/g, 2.98 CFU/g and 3.59 CFU/g and aerobic plate counts of 3.70 CFU/g, 6.47 CFU/g and 5.41 CFU/g for spinach, mixed greens and lettuce, respectively. LAE (1%) + CA (1%) emulsion, individually and followed by peracetic acid, improved the shelf-life and visual quality of leafy greens. A survey was developed using field-washing results and given to produce growers to determine willingness to implement new treatments. Most surveyed farmers (79%) believed that there was a visual difference in the new treatment after 7 days compared to the control. Most (83%) would be willing to pay more and 26% would pay up to $0.30 more for this treatment.

5.1 Introduction

In 1994 there were only 1,755 recorded farmers' markets in the United States. That number has almost tripled by 2015 to 8,476 farmers' markets (Economic Research Service, 2021). In Kansas there are currently 89 registered farmers' markets (From the Land of Kansas, 2021) which is an increase from 55 markets in 1990 (Hughes & Mattson, 1992). In 2015, 45% of adults living in Kansas said that they have purchased fruits and vegetables from a farmers' market. There is also a significantly higher percentage of people in urban counties who purchase produce from farmers' markets compared to rural counties (Kansas Bureau of Health and Promotion, 2019). Produce is the most common product purchased at farmers' markets, accounting for more than 82% of total sales (Economic Research Service, 2015), and 45% of vendors at farmers' market sell produce (Agricultural Marketing Service, 2006). This growing number of markets can indicate a changing consumer preference for locally sourced, more natural produce instead of conventional retail produce. Consumers have also shown an increased desire for organic goods, which has shown double-digit growth with the fruit and vegetable sector accounting for 46 percent of total organic sales (Greene et al., 2017).

The US Food Safety Modernization Act (FSMA) Produce Safety Rule establishes the regulations for any fruits and vegetable grown for human consumption. Under the FSMA Produce Safety Rule, unprocessed whole fruits and vegetables sold by smaller farmers are exempt (Kansas State University Research and Extension , 2021). Most of the produce sold at farmers' markets falls under the exemption, so the oversight of maintaining produce safety best practices relies on the farmers. There are no regulations requiring produce growers to rinse any of their produce. Any equipment used to transport and display produce needs to be clean (Agricultural Marketing Service, 2019). Kansas State Research and Extension currently

recommends farmers understand the FSMA Produce Safety Rule and apply it even if they are exempt. They also recommend that farmers sell only clean produce and wash the produce, if necessary, in portable water (Kansas State University Research and Extension , 2021). Most practices at farmers' markets are currently being driven by consumer demand. Consumers will not purchase produce covered in dirt and debris therefore, the farmers rinse any visible contamination off. Most of the rinsing steps are to just remove any visible contamination, not to sanitize the product. Rinsing to remove visible debris is often done with potable water. Of the few farmers that have an additional produce sanitizing step, organic acids like peracetic acid are common (Shaw, 2013). The addition of a produce rinsing step increases costs and can introduce new contamination to the product if not done properly.

There is a need for a novel wash-water treatment that is effective, all-natural and that can be utilized by small farmers who are interested in selling directly to consumers. This study aims to analyze the effect of cinnamaldehyde and lauric arginate- based wash water treatments for use in field washing trials in order to compare the shelf life of leafy greens washed in novel treatments and current antimicrobial treatment. In addition, we conducted a survey with an aim to better understand farmers' current opinions about the addition of rinsing steps in conjunction with their willingness to implement a new washing solution that improves product shelf-life.

5.2 Materials and Methods

5.2.1 Treatment preparation

Three solutions were used for field washing: a positive control, cinnamaldehyde (CA) and lauric arginate (LAE) emulsion individually and sequentially with peracetic acid (PAA). The emulsion was prepared by mixing CA (10%) (Sigma-Aldrich Company, Milwaukee, WI., U.S.A.) with LAE (10%) (VEDEQSA, Barcelona, Spain). Using the method previously

described by McDaniel et al. (2019), the emulsions were combined using a high-shear mixer (Brinkman Polytron, Brinkman Instruments Inc., Westbury, NY., U.S.A.) for one minute and thirty seconds. The coarse emulsion was then homogenized using a 2-stage valve homogenizer (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) at 500 bars for 3 passes. The emulsion was added to potable wash water, so the final concentration was LAE (1%) and CA (1%). The PAA solution (Synergex^{TM}, EcoLab, St. Paul, MN., U.S.A.) was made by adding it to potable wash water to a concentration of 100ppm PAA. The control treatment, SaniDate 5.0 (BioSafe, Omaha, NE., U.S.A.), was the current antimicrobial wash water treatment used by the produce grower at the concentration of 1 oz of SaniDate 5.0 per 15 gallons of potable water. SaniDate 5.0 has hydrogen peroxide and peroxyacetic acid as its active ingredients.

5.2.2 Field-washing

Trials were conducted at a commercial produce farm in northeast Kansas. Three leafy green types: spinach, lettuce and green mix were used in this study. Five pounds of each leafy green type was washed in each treatment for approximately 3 minutes, then greens were transferred to a water rinsing sink. Leafy greens washed in the control treatment and the individual treatment of LAE (1%) + CA (1%) were rinsed twice. Leafy greens washed in sequential application of LAE (1%) + CA (1%) followed by PAA received one rinse. Leafy greens were spun dry for 5 minutes after washing. Leafy green samples (25g) were taken before washing, after sanitizing and after drying. At the sampling points, leafy greens were transferred to 225mL double strength Dey/Engley (D/E) Neutralizing broth (Difco Laboratories, Spark, MD., U.S.A.) in a Stomacher® bag (Seward Ltd, West Sussex, United Kingdom). Leafy green bags were hand stomached for 30 seconds and then the solution was transferred to 0.1 % peptone water (PW) (Difco Laboratories, Spark, MD., U.S.A.) for serial dilutions. Wash water samples

(10mL) were taken out of the sanitizer and rinsing tanks after the leafy greens were washed. Samples were evaluated for bacterial counts by neutralizing in double strength D/E broth (90mL) and serial diluting. Samples of each leafy green type for each treatment were taken after being washed and spun dry and used for microbial shelf-life experiments. Leafy greens were stored at refrigerated temperatures and packed in plastic leafy green boxes. At day 2, 4, 7 and 10 after harvest, 10 g of each leafy green type was transferred into 90mL of 0.1% PW in a Stomacher bag and then placed into the Stomacher® 80 Biomaster (Seward Ltd, West Sussex, United Kingdom) for 1 minute 30 seconds. Microbial populations were plated onto Rapid Yeast and Mold (RYM) petrifilms and Aerobic Plate Count (APC) petrifilms (3M, St. Paul, MN., U.S.A.). RYM and APC petrifilms were incubated for 48 hours at 25°C and at 37°C, respectively.

5.2.3 Color measurements

The color of spinach was evaluated at day 0, 2, 4, 7 and 10 of the shelf-life studies using a Hunter MiniScan EZ spectrophotometer (HunterLab, Reston, VA., U.S.A.). Spinach was chosen as a representative leafy green type. Five spinach leaves were sampled per treatment and averages were reported for L*, a*, and b*. Total color difference (ΔE) was calculated using Equation 1:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2} \quad \text{Equation (1)}$$

5.2.4 Survey questions development and implementation

Survey questions were formulated to measure produce growers' food safety opinions and willingness to implement a new wash water formulation. The survey was reviewed for ease of understanding the questions, appropriateness of questions, and survey flow. Revisions to the survey were made based on feedback from an expert in food safety education and outreach as well as a produce safety extension associate. The survey was also pilot tested by a produce

grower and subsequently revised. The survey was approved by the KSU IRB-10627. The survey included 11 multiple choice questions with some referring to pictures or tables. A sample of the survey questions is provided in Table 5.1. In July and August 2021, 40 survey responses were collected. Participant criteria required that respondents sold produce at farmers' markets or farm stand. Surveys were given to produce vendors at Kansas farmers' markets and were also distributed during Kansas State University Olathe Commercial Fruit and Vegetable Research Field Day.

5.2.5 Farmers' markets and produce growers

The farmers' markets selected to administer the surveys were based on the suggestion of the Extension Food Safety Specialist. The selection criteria included the number of produce vendors attending and proximity to Manhattan, Kansas . The four farmers' markets selected were Manhattan, Lawrence, Lenexa and Overland Park. Survey participants were selected based on crops grown, farmers' market or field day attendance and their willingness to participate in the survey. Farmers who grew a variety of specialty crops and crops that typically undergo a washing step were the main focus of this survey. The study was reviewed by the Kansas State University Institutional Review Board which determined it to be exempt. The survey contained a statement with a description of the research objectives. The survey did not collect personal identifying information such as farm name, participant name, address, or phone number. Participants could voluntarily provide their email address to be entered into a drawing. Questions were grouped based on the following topics: personal and farm demographics, food safety opinions and willingness to adopt this new rinsing formulation.

5.2.7 Statistical Analysis

The effect of treatments, as well as days of storage on spinach color and the growth of yeast and molds and aerobic plate count was evaluated. The results of each trial were averaged and presented as mean values. Significant differences and comparison among treatment and leafy green type were determined using a Tukey multiple comparison test (Minitab 20.2, State College, PA., U.S.A.). A level of significance of $P < 0.05$ was considered for the analysis of variance (ANOVA) tests.

5.3 Results and Discussion

5.3.1 Field-washing results

Tables 5.2 and 5.3 demonstrate the results of microbial plating before washing, after sanitizing and after spinning dry. Initial APC and yeast and mold (YM) counts varied depending on leafy green type. However, a mean of 6.50 CFU/g and 3.79 CFU/g, respectively, were observed. Previous studies have found similar results with APC counts around 7 CFU/g on fresh spinach and lettuce samples (Valentin-Bon et al., 2008; Poimenidou et al., 2016). After sanitizing, there was a significant difference ($p < 0.05$) in YM counts between treatments. LAE (1%) + CA (1%) + PAA was the most effective treatment in reducing YM across all leafy green types, with 1.63 CFU/g, 2.00 CFU/g and 1.09 CFU/g for spinach, green mix and lettuce, respectively. However, there was not a significant difference ($p > 0.05$) between the APC results of the leafy green mix and lettuce samples washed in different treatments after sanitizing. The most effective treatment after rinsing was LAE (1%) + CA (1%) + PAA, with a 3.73 log CFU/g APC counts on lettuce. After drying, there were significant differences in both the APC and YM results between different treatments. This could indicate that there is still residual antimicrobial activity occurring even after rinsing. Similar results were found by Poimenidou et al. (2016) who

used an oregano oil-based treatment to wash fresh cut spinach and lettuce samples and saw approximately a 1.5 CFU/g and 3.0 CFU/g reduction in APC, respectively, after sanitizing and rinsing. Samples of the sanitizer and rinse water were also taken to analyze for APC and YM in the water after washing. There were no significant differences in any of the wash water samples, indicating that treatments are equally effective at removing microbial populations from water.

5.3.2 Shelf-life results

Tables 5.4 and 5.5 demonstrate the APC and YM shelf-life results of all leafy green types. After 10 days, LAE (1%) + CA (1%) individually and sequentially with PAA shows the most significant ($p < 0.05$) impact on APC of fresh spinach. Sequential application of LAE (1%) + CA (1%) and PAA showed the lowest APCs on all green types after 10 days. However, the individual treatment of LAE (1%) + CA (1%) showed significantly ($p < 0.05$) lower counts of YM in two of the leafy green types compared to control treatment. Overall, the control (Sanidate 5.0) treatment shows the highest APC and YM counts for all green types after 10 days. Yossa et al. (2012) analyzed the effect of cinnamaldehyde (800ppm) individually and combined with either Tween 20 or acetic acid as wash water treatments for spinach. The shelf-life of spinach was analyzed over 7 days and YM and APC were measured. This study found after 7 days storage an increase in APCs by approximately 1.30 log CFU/g and YM increase of approximately 2.20 log CFU/g growth for each treatment with cinnamaldehyde. A similar study, (Poimenidou et al., 2016), used an oregano oil wash water treatment on fresh cut spinach and lettuce samples analyzed shelf-life for 7 days. The results in this study indicated that oregano oil had significant ($p < 0.05$) antimicrobial effect on APC compared to other treatments like citric acid and NaOCl. However, when oregano oil was applied sequentially after citric acid, these

treatments showed significant ($p < 0.05$) effects on APC during shelf-life studies with 2.2 log CFU/g after 7 days.

5.3.3 Color measurements and pictures

The color of fresh produce is one of the critical elements on consumer buying decisions (Leon et al., 2006; Pathare et al., 2013). Any dullness or off-colors seen in produce results in it appearing not fresh, spoiled and unsatisfactory (Nisha et al., 2011). The color of all leafy greens was evaluated and *a was used as an indicator for green color, a greater negative number of *a shows a darker green color in fresh produce (Pathare et al., 2013). ΔE represents the difference in total color. There were no significant differences ($p > 0.05$) in *a and ΔE for all treatments for each day (Figure 5.1.). These results indicate that washing treatments used have similar impact on leafy green color over the 10-day period. When spinach leaves were washed in oregano oil by Poimenidou et al. (2016) the oregano significantly ($p < 0.05$) affected the appearance resulting in a dark green color as exhibited by a decrease in *a value. Sequential application of citric acid and oregano oil had a negative effect on color and showed an increase in *a value which indicates a loss in fresh green color. However, application of cinnamaeldehyde individually and with citric acid or tween 20 did not signficantly ($p > 0.05$) impact spinach color when measured by Yossa et al. (2012). This indicates that cinnamaldehyde treatments do not signficaintly impact fresh spinach color.

5.3.4 Survey results

Previous Kansas farmers' market surveys have been focused on market demographics (Hughes & Mattson, 1992; Otto & Varner, 2005) current farmer practices (Lewis Ivey et al., 2012) and consumer preferences (Steele, 2014; Bureau of Health and Promotion, 2019). A few

have focused on new food safety techniques such as refrigeration practices and willingness to pay for antifungal packaging (McDaniel, 2019).

In this current study there was a broad age demographic of the farmers surveyed. The largest group was 60+ (35%) and the next largest age classification was 30-39 (23%). Similar findings were found from a 2018 survey of Kansas and Missouri specialty crop producers with 60% of respondents being below the age of 50 and this survey with 55% of the respondents being below 50 (McDaniel, 2019). This shows a change compared to a survey of Iowa farmers' market vendors with an average of 51-65 years in 2005 (Otto & Varner, 2005). This difference could indicate that the farms surveyed are multi-generational or there are more young people starting to farm. There was a comparable number of male (47%) and female (50%) respondents with 3% choosing not to provide. A similar survey reported 56% female respondents in 2018 (McDaniel, 2019). Produce growers surveyed represented small (92%) and midsize (8%) farms. This classification is measured by annual gross cash farm income (GCFI)- a measure of the farm's revenue that includes sales of crop and livestock (From the Land of Kansas, 2021). The small farm classification is an annual GCFI of $350,000 or less. Annual revenue from produce sales for Kansas produce growers surveyed in this study are shown in Figure 5.2. A national farmers' market survey (Agricultural Marketing Service, 2006) found that the North Central region, to which Kansas belongs, had an average of $155,000 in annual sales of produce. Most of the producers responding to this survey currently grow specialty crops (67%) with some planning to in the future (8%). Specialty crops are defined as "fruits and vegetables, tree nuts, dried fruits and horticulture and nursery crops, including floriculture" (Agricultural Marketing Service, 2014).

5.3.5 Growing techniques

Certified organic farms made up 8% of the farms surveyed with 82% farms being conventional and 8% planning to become certified organic in the future. This differed from (McDaniel, 2019) who reported 25% organic farms surveyed in Kansas and Missouri and an earlier survey (Agricultural Marketing Service, 2006) which reported 39.8% farmers' market vendors in North Central region sell organic certified products. Farmers were asked if they currently have a rinsing step for at least one of their produce products. Most (76%) of the farms responded that they currently implement a produce rinsing/washing step in their process. Similar numbers were reported by a farmer's survey in another Midwest state which found that 60% of farmers surveyed wash produce on the farm (Lewis Ivey et al., 2012). Many farmers commented that they wash anything that is grown in the ground like potatoes and carrots or has an extensive root system. However, most of the farmers specified that they do not wash their leafy greens.

5.3.6 Food safety opinions

Almost all farmers surveyed believe that rinsing is important in some cases with only 5% responding that a rinsing step is not important. Out of the farmers who believe rinsing is important in some cases, a further 59% believe it is important in all cases (Figure 5.3.). A survey of farmers in another Midwestern state found that the average response was farmers somewhat agree that adding a treatment to the wash water is important, with approximately 38% strongly agreeing (Lewis Ivey et al., 2012). A few farmers that we surveyed believed that if the products have dirt on them, then they should be washed. Others believed that you only needed to wash produce that have a root system. Some farmers commented that washing leafy greens would damage them and cause the shelf life to decrease just by washing them. Some believe that washing is important however, they do not currently implement a washing step because of how

they sell their products. Many also do not currently have a washing step as it would increase their cost of production and consumers are not currently demanding it from them. A previous study analyzed produce farmers awareness of foodborne pathogens on fresh produce. Farmers were asked if specific bacteria can contaminate produce; 83%, 90% and 73% for *E. coli*, *Salmonella* and *Listeria*, respectively, said that yes produce can be contaminated with those bacteria (Ki et al., 2018).

5.3.7 Implementation of new wash water formulation

Farmers were shown pictures and graphs of the new washing formulation. They were asked if they could see a difference, the degree of difference and finally if they would be willing to pay more for this new formulation. Almost all farmers saw a visible difference in spinach that had not been washed and spinach after being rinsed, only 5% of farmers did not see a difference (Figure 5.4.). Few (21%) farmers surveyed saw no difference in pictures of spinach washed in the control treatment and the new wash water formulation after seven days of storage at refrigerated temperatures. However, 38% saw a large difference and 41% a slight difference in spinach quality. When survey respondents were shown Figure 5.5., the results of yeast and mold growth over seven days after treatment with control and new wash water formulation, most (79%) responded that the difference in growth between control and the new wash water formulation was significant. The remaining farmers were either not sure about the figure's findings (18%) or saw no difference (3%.) (Figure 5.6). When asked what the maximum amount they would be willing to pay per clamshell (170g) extra to implement this new formulation, most (83%) farmers agreed they would pay more. Out of the farmers who said they would pay more for the new formulation, 37% would pay $0.10 more, 31% would pay $0.20 more and 32% would pay $0.30 more (Figure 5.7.). There were similar findings when Kansas and Missouri

producers were asked if they would be willing to pay more to implement a new antifungal packaging film. Farmers said they would be willing to pay upwards of $0.39 more for this new technology that improves shelf-life of strawberries (McDaniel, 2019). These results conclude that generally, farmers are willing to accept and pay more for new technology if it improves shelf-life.

5.4 Conclusion

This study found that when used individually or when combined with organic acids such as PAA, LAE (1%) + CA (1%) is an effective wash water treatment. It shows an improvement on shelf-life of leafy greens compared to control treatment. This treatment can provide an effective and all-natural leafy green wash water alternative to farmers. The survey was used as an indicator of Kansas farmers' current practices and their willingness to accept to new rinsing treatments. It showed the improvements in quality and safety that a rinsing step could have. Most farmers agreed that there were differences between washed and unwashed spinach and spinach treated with a control treatment and the new wash water formulation. Almost all farmers said that they would be willing to pay more for this new formulation that extends shelf-life of leafy greens. However, current recommendations to farmers are that they do not wash the produce if possible. If they chose to wash their produce because they see an improvement due to washing, it should be done with a sanitizer at the proper concentration. A challenge with this methodology is that farmers need to be first educated about proper rinsing techniques as well as new rinsing formulations that meet consumer demand and reduce microbial levels.

5.5 Acknowledgments

Thank you to Juniper Hill Farms for allowing us the use of their facilities, leafy greens and other equipment. This project was funded by the Kansas Department of Agriculture Specialty Crop Block Grant KDA 2019-3

5.6 Tables and Figures

Table 5.1. Questions asked to survey respondents.

Topic areas	**Questions**
Personal demographics	What is your age?
	What is your gender?
Farm demographics	What is your average annual revenue from produce sales?
	Do you currently grow specialty crop?
	Are you currently USDA organic certified?
	Do you currently have a produce washing/rinsing step after harvesting for any of your produce?
Food safety opinions	Do you think having a produce washing/rinsing step after harvesting is important?
Implementation of new wash water formulation	Do you see a visible difference in the spinach before being washed/rinsed and after being washed/rinsed?
	Do you see a visible difference in spinach washed/rinsed using a control formulation compared to the new wash/rinse water formulation stored for seven days after washing/rinsing?
	Do you think there is a significant difference in the results of yeast and mold growth over seven days when comparing the formulations to the control?
	What is the maximum amount you would be willing to pay per unit of product more for this new formulation?

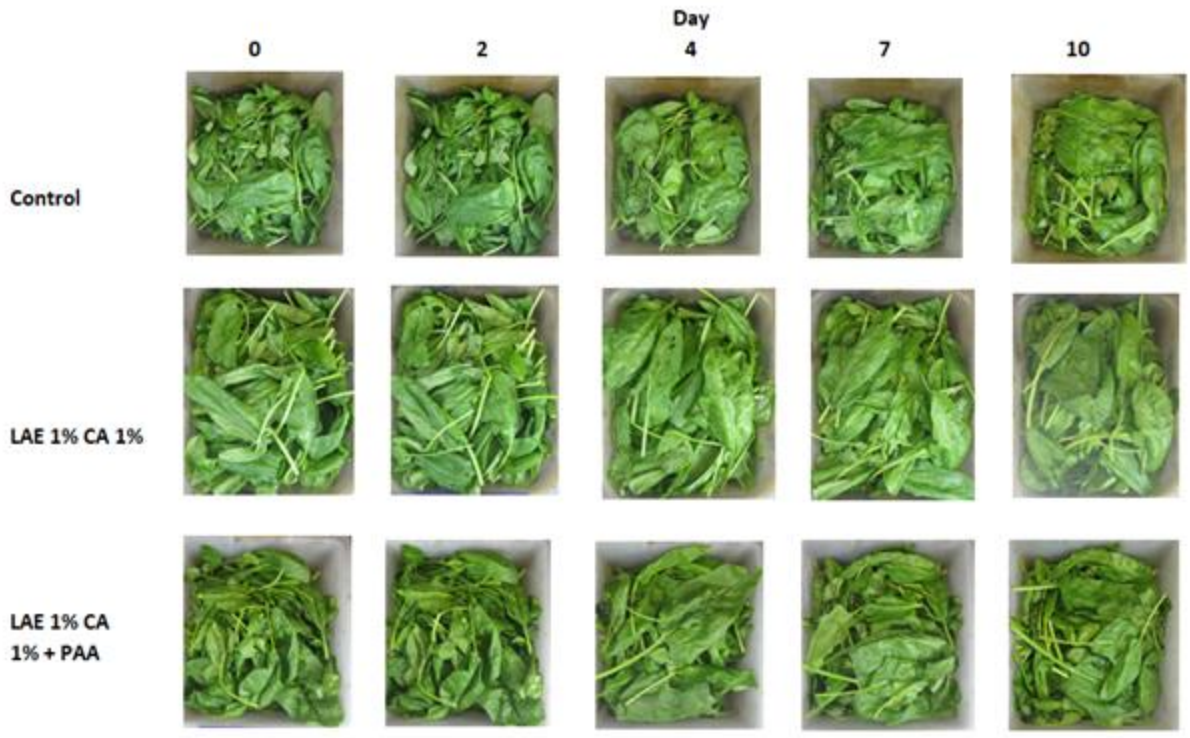

Figure 5.1. Visual quality of **s**pinach undergoing different treatments stored for 10 days at 3°C.

Table 5.2. Application of treatments to spinach, green mix and lettuce.
Values shown are aerobic plate counts log CFU/g with standard deviation. Treatments include control, LAE (1%) + CA (1%) and LAE (1%) + CA (1%) + PAA. Data is presented as mean values of replicates. Values that do not share letters for the same leafy green type at the same sampling point are significantly different ($P < 0.05$).

APC	**Spinach**		
Day	Prewash	After Sanitizer	After Centrifuge
Control	6.69 ± 0.12 [a]	4.97 ± 0.06 [ab]	5.02 ± 0.05 [a]
LAE (1%) CA (1%)	6.29 ± 0.69 [a]	4.63 ± 0.17 [b]	4.23 ± 0.19 [a]
LAE (1%) CA (1%) + PAA	6.82 ± 1.10 [a]	5.22 ± 0.12 [a]	4.83 ± 0.72 [a]
	Green Mix		
Day	Prewash	After Sanitizer	After Centrifuge
Control	5.92 ± 0.24 [b]	4.87 ± 0.59 [a]	4.78 ± 0.54 [a]
LAE (1%) CA (1%)	6.54 ± 0.37 [a]	4.68 ± 0.56 [a]	5.01 ± 0.25 [a]
LAE (1%) CA (1%) + PAA	6.74 ± 0.29 [a]	4.41 ± 1.08 [a]	4.24 ± 0.35 [b]
	Lettuce		
Day	Prewash	After Sanitizer	After Centrifuge
Control	6.36 ± 0.07 [a]	4.36 ± 0.01 [a]	4.30 ± 0.01 [a]
LAE (1%) CA (1%)	6.65 ± 0.31 [a]	4.31 ± 0.07 [a]	4.33 ± 0.18 [a]
LAE (1%) CA (1%) + PAA	6.77 ± 0.02 [a]	3.94 ± 0.53 [a]	3.73 ± 0.28 [a]

Table 5.3. Application of treatments to spinach, green mix and lettuce.
Values shown are yeast and mold log CFU/g with standard deviation. Treatments include control, LAE (1%) + CA (1%) and LAE (1%) + CA (1%) + PAA. Data is presented as mean values of replicates. Values that do not share letters for the same leafy green type at the same sampling point are significantly different ($P < 0.05$).

RYM	**Spinach**		
Day	Prewash	After Sanitizer	After Centrifuge
Control	3.28 ± 0.00[a]	2.46 ± 0.24 [a]	3.76 ± 0.13 [a]
LAE (1%) CA (1%)	3.62 ± 0.00 [a]	1.88 ± 0.68 [a]	1.24 ± 0.34 [b]
LAE (1%) CA (1%) + PAA	3.78 ± 0.31[a]	1.63 ± 0.04 [a]	0.85 ± 0.21 [b]
	Green Mix		
Day	Prewash	After Sanitizer	After Centrifuge
Control	3.97 ± 0.34 [a]	3.90 ± 0.30 [a]	3.75 ± 0.29 [a]
LAE (1%) CA (1%)	3.20 ± 0.67 [a]	1.84 ± 1.01 [b]	2.18 ± 0.54 [b]
LAE (1%) CA (1%) + PAA	3.64 ± 0.20 [a]	2.00 ± 0.89 [b]	1.65 ± 0.96 [b]
	Lettuce		
Day	Prewash	After Sanitizer	After Centrifuge
Control	4.44 ± 0.05 [a]	2.94 ± 0.00 [a]	3.10 ± 0.42 [a]
LAE (1%) CA (1%)	4.45 ± 0.12 [a]	2.08 ± 0.24 [ab]	1.24 ± 0.09 [b]
LAE (1%) CA (1%) + PAA	3.99 ± 0.09 [b]	1.09 ± 0.55 [b]	2.78 ± 0.12 [a]

Table 5.4. Shelf-life analysis of spinach, green mix and lettuce.
Values shown are aerobic plate counts log CFU/g with standard deviation. Washing treatments include control, LAE (1%) + CA (1%) and LAE (1%) + CA (1%) + PAA. Data is presented as mean values of replicates. Values that do not share letters for the same leafy green type on the same day are significantly different ($P < 0.05$).

APC	**Spinach**				
Day	0	2	4	7	10
Control	5.02 ± 0.05 [a]	6.07± 0.23 [a]	6.81 ± 0.56 [a]	6.92± 0.03 [a]	6.92± 0.08 [a]
LAE (1%) CA (1%)	4.23 ± 0.19 [a]	4.17 ± 0.13 [a]	4.21± 0.14 [b]	3.80± 0.15 [b]	4.06 ± 0.22 [b]
LAE (1%) CA (1%) + PAA	4.83 ± 0.72 [a]	3.54 ± 0.05 [a]	3.42 ± 0.06 [b]	4.42± 0.95 [b]	3.70 ± 0.21 [b]
	Green Mix				
Day	0	2	4	7	10
Control	4.78 ± 0.54 [a]	5.79 ± 0.26 [a]	6.51 ± 0.18 [a]	6.51 ± 0.47 [a]	6.90 ± 0.66 [a]
LAE (1%) CA (1%)	5.05 ± 0.25 [a]	5.42 ± 0.42 [a]	5.51 ± 0.71 [a]	5.70 ± 1.37 [a]	6.51 ± 0.75[a]
LAE (1%) CA (1%) + PAA	4.24 ± 0.35 [a]	5.34 ± 0.48 [a]	5.47 ± 0.93 [a]	5.56 ± 1.17 [a]	6.47 ± 0.67 [a]
	Lettuce				
Day	0	2	4	7	10
Control	4.29 ± 0.01 [a]	5.40 ± 021 [a]	5.96 ± 0.08 [a]	6.18 ± 0.00 [a]	7.40 ± 0.50 [a]
LAE (1%) CA (1%)	4.33 ± 0.18 [a]	5.62 ± 0.12 [a]	6.15 ± 0.27 [a]	6.58 ± 0.00 [a]	7.12 ± 0.08 [a]
LAE (1%) CA (1%) + PAA	3.73 ± 0.28 [a]	4.84 ± 0.80 [a]	6.28 ± 0.06 [a]	6.12 ± 0.15 [a]	5.42 ± 0.0 [a]

Table 5.5. Shelf-life analysis of spinach, green mix and lettuce.
Values shown are yeast and mold log CFU/g with standard deviation. Washing treatments include control, LAE (1%) + CA (1%) and LAE (1%) + CA (1%) + PAA. Data is presented as mean values of replicates. Values that do not share letters for the same leafy green type on the same day are significantly different ($P < 0.05$).

RYM	**Spinach**				
Day	0	2	4	7	10
Control	3.76 ± 0.13 [a]	3.75 ± 0.054 [a]	4.62 ± 0.06 [a]	6.92 ± 0.03 [a]	6.05 ± 0.07 [a]
LAE (1%) CA (1%)	1.24 ± 0.34 [b]	3.48 ± 0.28 [a]	3.41 ± 0.02 [b]	3.80 ± 0.15 [b]	3.22 ± 0.08 [b]
LAE (1%) CA (1%) + PAA	0.85 ± 0.21 [b]	3.29 ± 0.05 [a]	3.20 ± 0.06 [c]	4.43 ± 0.95 [b]	2.79 ± 0.13 [c]
	Green Mix				
Day	0	2	4	7	10
Control	3.75 ± 0.29 [a]	4.29 ± 1.05 [a]	3.44 ± 1.24 [a]	4.03 ± 0.94 [a]	4.37 ± 1.11 [a]
LAE (1%) CA (1%)	2.18 ± 0.54 [b]	2.57 ± 0.99 [a]	1.71 ± 0.33 [b]	3.76 ± 2.08 [a]	1.52 ± 0.85 [b]
LAE (1%) CA (1%) + PAA	1.65 ± 0.94 [b]	2.54 ± 0.30 [a]	2.46 ± 0.67 [ab]	4.39 ± 0.84 [a]	2.98 ± 1.20 [ab]
	Lettuce				
Day	0	2	4	7	10
Control	3.10 ± 0.42 [a]	2.75 ± 0.09 [a]	3.63 ± 0.19 [a]	4.26 ± 0.01 [a]	4.02 ± 0.17 [a]
LAE (1%) CA (1%)	1.24 ± 0.09 [b]	1.30 ± 0.43 [a]	1.77 ± 0.53 [b]	3.18 ± 0.24 [b]	3.48 ± 0.19 [a]
LAE (1%) CA (1%) + PAA	2.78 ± 0.12 [a]	2.34 ± 0.66 [a]	2.83 ± 0.08 [ab]	3.36 ± 0.23 [b]	3.59 ± 0.00 [a]

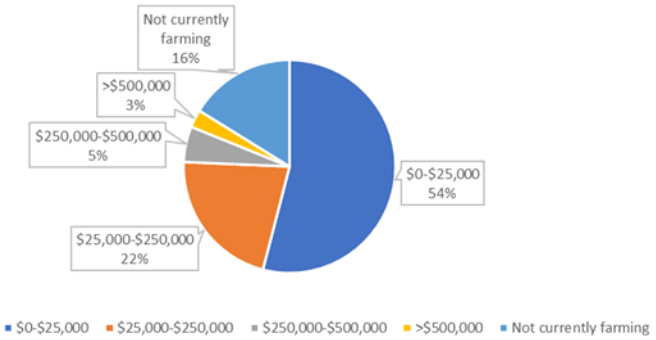

Figure 5.2. Kansas produce farmers average annual revenue ($) from produce sales.

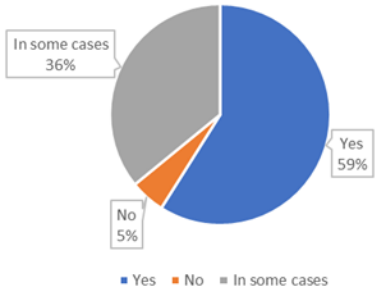

Figure 5.3. Kansas produce farmers' opinions on whether they believe a produce rinsing step is important.

Figure 5.4. Pictures shown to produce farmers before (left) and after (right) spinach is rinsed with new wash-water formulation.

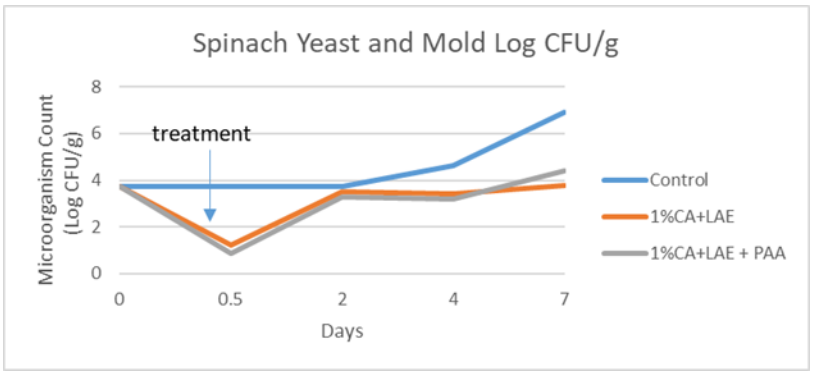

Figure 5.5. Table shown to produce farmers that depicts the yeast and mold counts (log CFU/g) after storage for 7 days at 3°C.

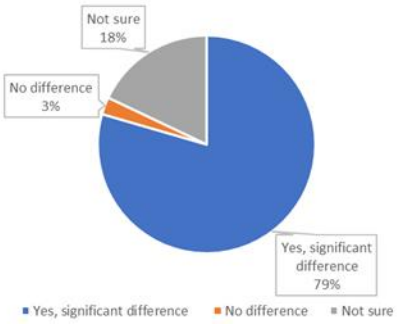

Figure 5.6. Produce farmers' opinions on whether they think there is a difference in the yeast and mold growth over seven days.

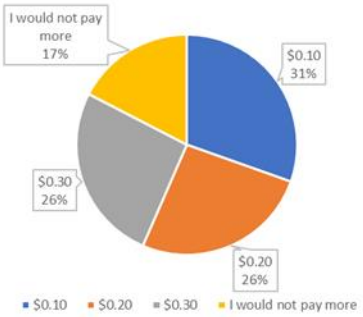

Figure 5.7. Produce farmers' opinions on whether they would be willing to pay more per clamshell (170g) extra for new wash-water treatment.

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Appendix A - Supplementary Information

Table A.1 Field washing shelf-life color results at day 0, 2, 4, 7 and 10.
Values shown are *a and ΔE with standard deviation. Washing treatments include control, LAE (1%) + CA (1%) and LAE (1%) + CA (1%) + PAA. Data is presented as mean values of replicates. Values with different letters on the same day are significantly different ($p<0.05$).

	***a**				
Day	0	2	4	7	10
Control	-10.26 ± 0.74 [a]	-9.10 ± 1.54 [a]	-10.42 ± 0.72 [a]	-10.24 ± 0.58 [a]	-9.61 ± 0.36 [a]
LAE (1%) + CA (1%)	-10.06 ± 0.68 [a]	-10.32 ± 0.52 [a]	-10.29 ± 1.16 [a]	-10.26 ± 0.33 [a]	-9.75 ± 0.35 [a]
LAE (1%) + CA (1%) + PAA	-8.71 ± 1.71 [a]	-9.10 ± 1.54 [a]	-10.44 ± 0.45 [a]	-9.64 ± 0.83 [a]	-9.71 ± 0.57 [a]
	ΔE				
Day	0	2	4	7	10
Control	5.86 ± 2.81 [a]	7.41 ± 5.47 [a]	7.76 ± 3.15 [a]	4.92 ± 2.42 [a]	6.80 ± 2.81 [a]
LAE (1%) + CA (1%)	4.66 ± 3.45 [a]	4.30 ± 3.55 [a]	5.72 ± 2.64 [a]	4.69 ± 3.84 [a]	5.94 ± 2.28 [a]
LAE (1%) + CA (1%) + PAA	8.17 ± 2.00 [a]	5.67 ± 3.96 [a]	5.59 ± 2.23 [a]	6.44 ± 3.59 [a]	4.57 ± 3.03 [a]