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Destruction of Escherichia coli O157:H7 on whole apples via single and sequential application of chemical sanitizers

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Destruction of *Escherichia coli* O157:H7 on whole apples via single and sequential application of chemical sanitizers

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Unpasteurized apple cider is one of several fruit and vegetable products that has been involved in the categorization of *Escherichia coli* O157:H7 as a food-related pathogen. First implicated as a hazard in ground beef, this gram-negative bacterium can be found in raw fruits, vegetables, milk, and water [Archer, 2000]. Humans are also known carriers [Jay, 2000].

Feces from grazing animals such as cattle, sheep, and deer are targeted as the source of contamination for apples used in cider production [Alzamora, 2000]. Outbreaks involving raw apple cider have caused government agencies, such as the Food and Drug Administration to question the safety of fruit and vegetable juices [CDC, 1996; Breur, 2001]. Mandatory sanitation and labeling regulations have been issued by the FDA for the juice industry in hopes of eradicating potential microbial hazards in fruit and vegetable juice processing [FDA, 2001;1998].

Heat pasteurization is the most effective method for destroying pathogens in juice [Anonymous, 1998; Jay, 2000]. However, the high cost incurred by purchasing and operating a pasteurization facility can prove to be too great for small-scale apple cider producers [Cummins, 2002; Kozempel, 1998]. Even so, heat pasteurization is known to alter the organoleptic properties native to fresh apple cider, rendering the product undesirable [Fisher,1998; Wisniewsky, 2000]. Techniques other than pasteurization are allowable if they can achieve a 100,000-fold reduction in pathogen number for the specific raw product [Anonymous, 1998]. The process of sanitizing apples shows the greatest potential at removing unwanted bacteria during post harvest processing. The success of a chemical

sanitizer would potentially allow apple cider processors, who cannot afford pasteurization equipment or do not desire it for aesthetic reasons, to produce a product considered to be safe by governmental standards.

The research outlined in this thesis was conducted to determine if selected chemical sanitizers could reduce the amount of pathogens on apple surfaces by 5 log (100,000-fold). Selected chemical sanitizers were used singly and in sequential combinations on apples inoculated with *E. coli* O157:H7. The efficacy of the same chemical sanitizers was evaluated during mild heat application for removal of this microorganism.

Thesis organization

This thesis consists of two papers to be submitted to the Food Protection Trends. Each paper constitutes a chapter and will contain the following sections: an abstract, introduction, materials and methods, results, discussions, and references cited. Chapter 4 will be a comprehensive conclusion that will encompass the findings from both papers. References are located at the end of each chapter and will follow the work-cited format for the Journal of Food Protection.

Literature review

Escherichia spp. are gram negative bacilli, belonging to the family of bacteria know as *Enterbacteriaceae.* Genera of this family are environmentally ubiquitous; found in water, soil, and vegetation and can be pathogenic or non-pathogenic. Specific antigens, protein markers found on the outer membrane (O), capsule (K), and/or flagellum (H) of *Escherichia coli* cells are used to classify this species for epidemiological purposes. The tendency of an *Escherichia coli* species to cause disease is indicative of the antigenic diversity found within this genus [Murray,1998].

With optimal growth temperatures of 35-37°C, *E. coli* have the ability to maintain growth in human systems [Campbell, 1987]. This may account for the variety of diseases associated with pathogenic *E. coli* including meningitis, inflammation of the brain; urinary tract infections; sepsis, blood poisoning; and gastroenteritis, inflammation of the intestinal tract [Murray, 1998].

These microorganisms are especially common to the intestinal microflora of most animals including humans [Murray, 1998]. *E. coli*'s commensal relationship with the human gastrointestinal tract can sometimes become hazardous if bacterial populations reach and/or exceed infective levels. Gastroenteritis can occur when as few as 100 cells of *E. coli* O157:H7 are allowed to infect the human body [Doyle, 1997]. Serotypes causing gastroenteritis are divided into six groups, enterotoxigenic, enteroinvasive, enteropathogenic, enteroaggregative, diffuse-adhering and enterohemorrhagic. These grouping are based on virulence properties, mechanisms of pathogenicity, clinical syndromes, and distinct O:H serogroups.

Enterotoxigenic *E. coli* (ETEC) are responsible for traveler's diarrhea and are linked to episodes of infant diarrhea in developing countries [Doyle, 1997]. There are fourteen ETEC serogroups identified to be responsible for these human illnesses. Adhesins, or fimbrial colonization factors, enable ETEC to inhabit the proximal small intestine. Heat labile (LT-I) and heat stable (LT-II) enterotoxins of ETEC cause sodium chloride, potassium bicarbonate and water to expel from cells into the intestinal lumen [Murray, 1998]. This reaction induces cramping, nausea, vomiting, and watery diarrhea [Doyle, 1997].

Enteroinvasive *E. coli* (EIEC) initiates diarrhea and dysentery, much like that of *Shigella spp.* [Doyle, 1997; Wang, 2002]. Blood and leukocytes have been found to pass

through the feces of EIEC patients as well [Murray, 1998]. Eleven serogroups are found to reside in humans, with serotype O124 being most frequently encountered. This group of *E. coli* invades the human colon and begins to proliferate rapidly; thereafter, EIEC destroys the epithelium of the colon. EIEC is equipped with a large plasmid (p1NV) encoding for outer membrane proteins (OMPs) that execute the invasion of the colon epithelium [Murray, 1998].

Enteropathogenic *E.coli* (EPEC) are known to cause pediatric diarrhea without the use of LT-I, LT-II, or invasive OMPs [Doyle,1997; Murray,1998]. The organism destroys the microvilli of the small intestines by affixing itself to the adjacent enterocytes. The membrane proteins, Bfp and intimin assist with the attachment and destruction of the small intestine's cellular architecture. Infected cells lose the absorptive properties necessary to prevent diarrhea. Diarrhea in this case is persistent and profuse. Other symptoms include, fever, vomiting, and abdominal pain. In adults, diarrhea can contain mucous but is without blood. [Bell, 1998].

Enteraggregative *E. coli* (EAggEC) are a newly discovered cause of infantile diarrhea in underdeveloped countries. Infants afflicted with EAggEC, experience watery diarrhea, vomiting, dehydration, and low grade fever [Murray, 1998]. This group acts much like EPEC by adhering to HEp-2 cells of the intestinal mucosa [Doyle, 1997]. Further studies are being conducted to fully understand this group's pathogenic mechanisms.

Diffuse-adhering *E. coli* (DAEC) do not possess any of the toxins normally associated with *E.coli spp.* such as shiga, heat labile, or heat stable toxins. Diffuse aggregative attachment to HEp-2 or HeLa cell lines enables these bacteria to inaugurate mild

diarrhea without fecal expulsion of blood or leukocytes. The occurrence of DAEC has been limited to young children that are older than infants for reasons.

Enterhemorrhagic *E. coli* (EHEC) are identified as human pathogens that cause disease, ranging from mild, uncomplicated diarrhea to more severe diseases such as hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic pupura (TTP) [Jay, 2000]. EHEC which is commonly found in developed countries accounts for an estimated 73,000 cases and 61 deaths occurring each year in the United States alone [CDC, 2001]. On average, the onset of illness occurs four days after initial infection and ranges between three and nine days. Duration of illness is usually two to nine days if not severe [Bell, 1998].

The presence of the *eae* chromosomal gene, a virulence factor, affords the entrance of the bacterium into eukaryotic cells [Buchanan, 1997; Doyle 1997]. After intimin mediated attachment of EHEC to the epithelium of the terminal ileum, cecum, and colon, non-bloody diarrhea develops. Thereafter, the cytotoxic verotoxins, shiga-like toxin I (SLT-I) and shiga-like toxin II (SLT-II), (now termed Stx 1 and Stx 2 respectively) bind to the glycolipid, globotriaosylcermide (Gb3) on the host cell. Gb3, a toxin receptor is Stx sensitive [Jay, 2000]. Once the toxin is internalized and granted transport to the trans-Golgi network, all present and future protein synthesis is disrupted [Jay, 2000; Murray, 1998]. EHEC infections are usually sudden, tragic and sometimes deadly.

Escherichia coli O157:H7: An Overview

Escherichia O157:H7 is the principal serotype of EHEC and much is known about this serotype's genome and pathogenesis. *E. coli* O157:H7 is a facultative anaerobe, (capable of surviving with or without the presence of air) that grows rapidly at 30 to 42°C [Jay, 2000].

Dissection of the serotype O157:H7 indicates that the species contains markers on its outer membrane or lipopolysaccharide (LPS) layer of the membrane and the flagellum [Murray, 1998]. The serotypes H7 and O157 were discovered and named separately in 1944 and 1972 respectively. The O157:H7 was rediscovered in 1975 by isolation from human feces [Jay, 2000]. Unlike most *E.coli* strains, O157:H7 grows poorly at $\geq$ 44.5°C, is negative for sorbitol fermentation, and does not produce β-glucuronidase necessary for the hydolysis of 4-methly-umbelliferyl--glucuronide (MUG) [Doyle, 1997].

The principal reservoir of *E.coli* O157:H7 is believed to be the bovine gastrointestinal tract [Alzamora, 2000]. Studies of *E. coli* O157:H7 infected cattle by Brown et al. (1997) show initial localization of the pathogen to be in the forestomachs (rumen, omasum, and reticulum). Large volumes of *E.coli O157:H7* are characteristically shed in cow feces. To hamper this, it is suggested that cattle and dairy farmers eliminate corn from a cow's diet a few weeks prior to slaughter, thus cutting down the amount of shedding [Jay, 2000].

It should be noted that bovine species supply the human food chain with such foods as meat and milk products and in practice provides a direct route for the pathogen to enter the human food supply. Via direct and cross contamination, *E. coli* O157:H7 enters foods, food processing equipment, food contact equipment and food handlers, just to name a few. It is important to maintain proper hygiene and sanitation of food processing facilities and equipment to prevent the spread of *E. coli* O157:H7 and the occurrence of the deadly diseases associated with this pathogen.

Associated Diseases

Epidemic and endemic disease caused by serotype O157:H7 have resulted from consumption of undercooked ground beef or other beef products, water, raw fruits and

vegetables, and unpasteurized milk and fruit juices [CDC, 2002a; 2002b; 2000b; 1996; Jay, 2000]. About one third of persons who become infected with *E. coli* O157:H7 are hospitalized [Murray, 1998]. In some instances an *E. coli* O157:H7 infection can be fatal especially in the very young and the elderly [Doyle; 1997]. The major syndromes caused by *E. coli* O157:H7 infections are hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic pupura.

Hemorrhagic colitis, results in abdominal cramps, bloody and non-bloody diarrhea, vomiting, and occasionally fever [Jay, 2000]. The disease usually progresses through a successive series of these symptoms. At the start of infection, abdominal cramps occur. One to two days later, non-bloody diarrhea ensues. The non-bloody diarrhea progresses into bloody diarrhea one to two days later and lasts four to ten days. The diarrhea can persist for several days to weeks. [Doyle, 1997]. The disease was first identified as a foodborne disease in 1982. Undercooked, ground beef sandwiches eaten at a fast food restaurant in Oregon and Michigan caused all victims to suffer bloody diarrhea and severe abdominal cramps [Riley, 1983; Jay, 2000].

Hemolytic uremic syndrome (HUS) encompasses a triad of features: acute renal insufficiency, kidney dysfunction; microangiopathic hemolytic anemia, intravascular coagulation of erythrocytes which can block blood vessels; and thrombocytopenia, low platelet circulation due to blood clotting in the brain [Doyle, 1997]. HUS has been preferentially associated with the production of Stx2, which is shown to destroy renal endothelial cells selectively [Murray, 1998; Jay, 2000]. Children are highly affected; ten percent of children fewer than ten years of age suffer from HUS [Murray, 1998]. Most cases of kidney failure in children are a result of HUS [Doyle, 1997].

Thrombotic thrombocytopenic pupura (TTP) occurs mostly in adults. Histologically similar to HUS, TTP initiates potentially reversible platelet aggregation in the brain. Blood clot formation leads to neurological alterations and deficiencies [Doyle, 1997].

Foodborne Illness Outbreaks Associated with *Escherichia coli* O157:H7

Foods such as those mentioned in the previous section are modes of *E. coli* O157:H7 transmission to humans. Person to person contact is also cited as a form of transmission [Doyle, 1997]. In 2001, 61% of reported cases of *E. coli* O157:H7 infections were attributed to food, 18% to animal contact or environmental contamination in an animal setting, 14% person-to-person contact and 7% to swimming exposures. Locations reported were fairs, petting zoos, restaurants, daycare centers, prisons/correctional facilities, and elementary and middle schools [CDC, 2001]. Associated diseases outbreaks are common in warm months, typically May to October. Age specificity for *E. coli* O157:H7 infections is high for children younger than five and the elderly [Doyle, 1997; Murray 1998] due to an often times immature or weaken immune system.

Alfalfa sprouts were implicated in *E. coli* O157:H7 illness outbreaks during June and July 1997. Cases were identified in Michigan and Virginia. Microbiological analysis of alfalfa seeds confirmed its role in the outbreaks [Breur, 2001]. Also in June 1998, The Center for Disease Control (CDC) was notified of 55 laboratory confirmed cases of *E. coli* O157:H7 infections stemming from fresh cheese curds processed at a dairy plant in Wisconsin. Twenty-five of the 55 ill persons were hospitalized. Patients suffered from bloody diarrhea, cramps, fatigue, and nausea [CDC, 2000]. More recently in Colorado, five persons developed HUS after consuming recalled beef products manufactured by ConAgra Beef Company [CDC, 2002b]. After in-plant inspections by the United States Department of

Agriculture (USDA), the original recall of 354,000 lbs was expanded to 18.6 million lbs of fresh and frozen ground beef and beef trimmings [CDC, 2002b].

E. coli O157:H7 disease outbreaks attributed to fruit and vegetable juices were rare until 1991. Contaminated, raw apple cider devastated 13 families with illness and 4 children with HUS in Massachusetts [Doyle, 1997]. Again in 1996, unpasteurized apple cider was implicated in the center of a trans-American foodborne illness outbreak with reports made in Canada, Colorado, and Washington [CDC, 1996]. The company involved, Odwalla, Inc. apparently used fallen (drop) apples for 90% of cider produced [Doyle, 1997]. These apples very likely contacted animal feces directly or by an insect carrier. For some cider processors, the appearance of an apple is often not a juice quality indicator.

Acid Tolerance of *Escherichia coli* O157:H7

Acidic food products like fruit and vegetable juices were once thought to inhibit bacterial growth and survival. Weagant et al. (1994) working with salads dressed with mayonnaise acidified with different acids, acetic, citric, and lactic acid found that temperature and pH played a vital role in *E. coli* O157:H7's mortality. Salads inoculated with the pathogen were stored at 5, 21, or 30°C for 72 hours. At 5°C, bacterial populations were significantly reduced during the first 4 hours. The same researchers also demonstrated survival of *E. coli* O157:H7 for 35 days at the same temperature.

Apple cider is noted for its high acidity (pH < 4). Studies have shown survival of *E. coli* O157:H7 in unpasteurized apple cider for 10 to 31 days at 8°C [Alzamora, 2000]. Even longer survival was seen at 5 and 25°C for up to 42 days by Ryu et al. (1998). Apple cultivars with pH's ranging from 3.47-5.11 were able to sustain the pathogen for 5 days at 25°C [Fisher, 1998].

Evidence shows that *E. coli* O157:H7's predominant ability to withstand very low pH environments stems from preexistent mechanisms inside the cell. These mechanisms allow the cell to resist the toxic effects of acids. The pathogen is known to produce mucoid colonies with layers of exopolysaccharides [Alzamora, 2000; Erickson, 1995].

Current Regulations and Sanitation Practices

After the 1996 outbreak associated with raw apple cider, the FDA developed a strategic plan for fruit and vegetable juice safety with input from public, industry, and the National Advisory Committee on Microbiological Criteria for Foods (NACMCF). The NACMCF is an advisory panel of independent experts who provide guidance to FDA and USDA on matters concerning the safety and regulation of foods [Annonymous, 1998]. The group evaluated the science, technology and manufacturing practices related to the safe production of juices [Annonymous, 1998]. The overall conclusion was that major safety concerns are directly linked to unpasteurized juice processing and distribution. The initial action taken by the FDA was the requirement of processors to implement Hazard Analysis Critical Control Point (HACCP) plans.

HACCP is defined as a management system focused on prevention of problems in order to assure the production of food products that are safe for consumers [Stevenson, 1999]. The program relies on common sense, technical and scientific elements relating to processing in order to formulate good manufacturing procedures for food products. The underlying HACCP theme is viewed as preventive control from field to table [Annonymous, 1998; Stevenson, 1999]. This regulation would be in effect for domestic and foreign vegetable and fruit juices. Size would not exclude a plant from the new regulations. Larger

processing plants would be expected to implement HACCP within a year of the issuance of the new regulations. Smaller facilities would have more time to meet standards.

Unpasteurized juices do not undergo a treatment for the control of harmful microorganism. Thus the survival of *E. coli* O157:H7 in unpasteurized juice is the likely reason for the previously mentioned foodborne disease outbreaks. The second part of the FDA regulations requires processors of unpasteurized juices to adjust their processes to achieve a 100,000-fold or 99.999% reduction (5 log) in the numbers of harmful microbes in their finished product [Anonymous, 1998].

Pasteurization is the most effective way to achieve a 99.999% reduction in harmful bacteria. The high temperature process causes the destruction of the most heat resistant, non-sporeforming pathogenic organisms and reduction in the number of spoilage organisms native to a given product [Jay, 2000]. In 1998, it was estimated that 98% of juices sold in the United States were pasteurized [Anonymous, 1998]. The 2% that is not, could very well spark future foodborne disease outbreaks.

Under the new regulations, processors are not limited to the use of pasteurization. This is helpful when considering the cost of purchasing and operating a pasteurization facility. Based on a medium sized plant, processing 56 million L (14.85 million gal) of apple cider/year with a design capacity of 170 L/min (45 gal/min), the estimated installation cost for pasteurization equipment is $185,000. This estimate is separate from yearly unit operating costs, which are estimated to be $93,000 [Kozempel, 1998]. This increases the total estimate to $278,000. There are currently no plans for the government to provide financial assistance to smaller processors that may not be able to afford the necessary equipment.

For juices distributed that have not been exposed to some type of pathogen reduction step, a special label must be affixed alerting consumers of the potential dangers associated with consuming the product. The label states: "WARNING: This product has not been pasteurized and, therefore, may contain harmful bacteria which can cause serious illness in children, the elderly, and persons with weakened immune systems". All untreated, packaged juices are required to have this label [Anonymous, 1998].

Researchers are currently evaluating other methods suitable for the juice industry that will effectively reduce pathogens without the need for pasteurization. Options other than pasteurization include; washing, scrubbing, antimicrobial solutions, alternative technologies or a combination of techniques. The washing step and antimicrobial solutions have been areas with the most promise of helping juice processors produce products that are free of hazardous microorganisms. For cider, apples are normally selected, washed, and ground into a pulp [Jay, 2000]. For consumers, it is recommended that apples be rinsed under cool running water just prior to consumption, and when possible, that scrubbing with a clean brush be utilized [Parnell, 2003]. Unfortunately, water has only been found to eliminate pathogens by ≤ 2 logs. Water can mechanically dislodge bacterial cells from the apple surface but has no killing effect on bacteria. In fact, plain wash water may spread contamination to other apples. Stronger chemicals with antibacterial properties, such as sanitizers can be combined with washing to impact pathogen growth and survival during processing.

Chemical Sanitizers

Chemical sanitizers are useful antimicrobial chemicals because they are able to destroy the vegetative cells of microorganisms [FDA, 1998]. Several antimicrobial

biological compounds are currently used in commercial sanitizers and others are being evaluated for their ability to kill microbes. These compounds are noted to break down cellular membranes and disrupt biosynthetic pathways of microorganisms [Cherry, 2000].

Sanitizers are indirect food additives used to control the growth of microorganisms on food processing equipment and utensils and other food contact articles [Alzamora, 2000]. Indirect food additives come in contact with foods but are not a part of the finished product's composition. Regulations for proper use of indirect food additives are found in CFR, Title 21, Ch. 1, Section 178.1010, 4/1/96 edition [Alzamora, 2000]. Declaration of sanitizers is not required on fruit and vegetable labels. Chemical sanitizers are regulated by the FDA, under the Federal Food, Drug and Cosmetic Act (FFDCA) and by the U.S. Environmental Protection Agency (EPA) under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA).

The FDA *Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables* states that antimicrobial chemicals in processing water are useful in reducing the microbial build-up in water and may reduce microbial loads on the surface of produce. When used properly, less cross contamination of produce and a high degree of sanitation can be expected. Scientists of the United States Department of Agriculture, Agriculture Research Station (ARS), Eastern Regional Research Center (ERRC) have tested different sanitation methodologies in its commercial-size pilot plant in Wyndmoor, Pennsylvania [Core, 2002]. The tests involved produce artificially contaminated with harmless bacteria similar in behavior to disease-causing organisms. Several studies have also demonstrated that under optimal conditions, this process can achieve effective pathogen attenuation [Annous, 2001; Lin, 2002; FDA, 2001].

Other studies have shown the efficacy of different sanitation methodologies specifically on apples. Chemical sanitizers, such as chlorine and hydrogen peroxide have been studied for their effectiveness in removing *E. coli* from the flesh of Golden Delicious apples. After a 1 min exposure at room temperature, sanitizers were able to achieve reductions up to 3 $\log_{10}$ CFU/apple [Sapers, 1998]. The efficiency of aqueous commercial cleaners in removing the same pathogen from the surface of Red Delicious apples has been evaluated by Kenney et al. (2002). Populations of *E. coli O157:H7* were reduced 2.27-3.11 $\log_{10}$ CFU/apple by the commercial cleaner, Shield-Brite Field Clean®. The selected chemicals chlorine, hydrogen peroxide, lactic acid, and sodium bicarbonate and the commercial sanitizers, Tsunami™100 and Pro-San™ are the focus of this research.

Chlorine

Chlorine in various forms is widely used as a chemical sanitizer of fresh and fresh-cut fruits and vegetables [Alzamora, 1998; Beuchat, 1997; Cords and Dychdala, 1993]. It is advantageous for several reasons: 1) it can kill microbes rapidly; 2) it is safe and FDA approved for use; 3) it has no adverse effects on food; 4) it is economical; 5) it is readily soluble in water; and 6) it can be tested for solution concentration [Schlimme, 1997]. Chlorine exerts its antimicrobial effect by forming N-chloro compounds with cell membrane proteins. Chlorine is then able to impair the transport of nutrients into the cell and decrease membrane permeability. RNA and DNA are released from the cell due to chlorine's ability to alter membrane permeability [Alzamora, 2000].

For post-harvest treatment of produce, chlorine is often added to water (chlorination). The chemistry of chlorine in solution is as follows:

$NaOCl + H_2O \leftrightarrow HOCl + NaOH$ (elemental chlorine - Cl_2)

$Ca(OCl)_2 + 2H_2O \leftrightarrow Ca(OH)_2 + 2HOCl$ (hypochlorous acid – HOCl)

$Cl_2 + H_2O \leftrightarrow HOCl + H^+ + Cl^-$ (hypochlorite ion – OCl^-)

Figure 1.1. Reactions and compound(s) formed by chlorine in solution [Cords and Dychdala, 1993].

Chlorine is typically utilized between 50-200 parts per million (ppm) at a pH of 4.0 to 7.5 (at which it is in the HOCl form) and with a contact time of 1-2 minutes [Cords and Dychdala, 1993; FDA, 1998].

There are some limitations to chlorine's antimicrobial capacity. Its antimicrobial activity depends on the amount of available free chlorine as hypochlorous acid (HOCl), elemental chlorine (Cl_2), or hypochlorite ion (OCl^-) [Cords and Dychdala, 1993], which is influenced by temperature, and pH of water that comes in contact with microbial cells [Alzamora, 2000; Schlimme, 1997]. The waxy cuticle found on the surface of some fruits and vegetables is hydrophobic which can prevent chlorine from reaching microbes. The amount and kinds of organic matter found in wash water greatly affect the dissociation of chlorine [Alzamora, 2000; FDA, 1998]. Chlorine also has no residual effect, so after use, the antimicrobial power of chlorine is reduced [Schlimme, 1997].

E.coli O157:H7 is sensitive to chlorine. Chlorine was demonstrated to be most effective in reducing numbers of *E. coli* O157:H7, aerobic microorganisms, yeasts, and molds from cantaloupe surfaces at 2,000 ppm [Park and Beuchat, 1999]. Lisle et al. (1998) determined that chlorine inactivated *E. coli* O157:H7 by damaging the respiratory and transport processes of the cell membrane. In water, Zhao et al. (2001) observed the sensitivity of *E. coli O157:H7* at 1.1 ppm free chlorine with inactivation of 4 $\log_{10}$CFU/ml

within 1 min. Chlorine, as an already commonly used sanitizer was used in this research to compare current practices in apple sanitation to potential future practices.

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a peroxy compound that acts as a microbicide when mixed with water. H_2O_2 is an oxidizing agent and is highly toxic because it forms an intermediate in oxygen reduction, superoxide oxygen ($O_2^{\bullet}$). This property initiates the production of hydroxyl radicals (OH-) during the breakdown of O_2. These radicals cause damage to nucleic acids, proteins, and lipids [Alzamora, 2000].The reactions are listed below:

$$
\begin{array}{lcl}
O_2 + e^- & \rightarrow & O_2^- \\
O_2 + e^- + 2H^+ & \rightarrow & H_2O_2 \\
H_2O_2 + e^- + H^+ & \rightarrow & H_2O_2 + OH \\
OH + e^- + H^+ & \rightarrow & H_2O
\end{array}
$$

Figure 1.2. Reactions and compounds formed during the reduction series of O_2 by a single electron [Cords and Dychdala, 1993]

H_2O_2 is able to diffuse and pass through membranes rapidly during oxidation [Sapers, 1998]. It maintains a generally recognized as safe (GRAS) classification for use as a bleaching, oxidizing agent, and antimicrobial chemical in food (21CFR184.1366). Use of hydrogen peroxide as an antimicrobial agent is already approved by the FDA for treating milk in cheese production, preparation of modified whey, and thermophile-free starch production [Cherry, 2000].

Studies have demonstrated the potential of hydrogen peroxide as a produce sanitizer. In a study by Saper et al. (1999), unwaxed Golden Delicious apples were inoculated with a non-pathogenic strain of *E.coli* O157:H7 and dipped for 1 min in different sanitizer

treatments such as 5% hydrogen peroxide, chlorine, selected commercial sanitizers, and tap water at ambient temperature, 50°C, or 60°C. Hydrogen peroxide treatments at ambient temperature demonstrated a 3.4 log reduction. Treatments at 50°C were not much different from reductions achieved at ambient temperature and remained in the 3-4 logs range. In most cases, population reductions were slightly greater at the higher treatment temperature [Sapers, 1999].

Microorganisms like *Listeria monocytogenes* and *Escherichia* spp., can be found in soil particles and dust which come in contact with fresh fruits and vegetables. H_2O_2 can serve as an added defense against microbes located in dirt and dust particles. Complete removal of debris on the surface of mushrooms is enhanced by hydrogen peroxide's capacity to react with catalase on the mushroom surface [Sapers, 1998]. Some fruits and vegetables contain natural catalase. Catalase is an enzyme that serves to protect cells from the toxic effects of hydrogen peroxide. This enzyme decomposes hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) as bubbles [Cords and Dychdala, 1993]. The oxygen bubble formation loosens the attachment of microorganisms and dissolves soil particles [Sapers, 1998].

Hydrogen peroxide may demonstrate increased effectiveness when used in combination with certain chemicals or in the absence of debris. A study by Peters et al. (1995) showed that the antimicrobial activity of hydrogen peroxide can be enhanced when used in combination with certain acids. Hydrogen peroxide acidified with acetic acid was responsible for a 4 log CFU/g reduction of *Shigella spp* on lettuce, although physical defects occurred. On whole apples, a 4.1 log reduction of this pathogen was observed when inoculated whole apples were treated with 5% hydrogen peroxide and acidic surfactants at 50°C [Peters, 1995].

For this work, 1.5% hydrogen peroxide was combined with a 1.5% concentration of lactic acid to evaluate the efficacy of this combination for the elimination of *E.coli O157:H7* from apple surfaces. This exact combination was able to reduce *Salmonella enteritidis* by 6.0 $\log_{10}$CFU and *E. coli* O157:H7 and *Listeria monocytogenes* to undetectable levels when used on apples, oranges, and tomatoes at 40°C for 15 min [Venkitanarayanan, 2002].

Lactic acid

Lactic acid is one of the most active organic acids. It has been successfully used in washes and sprays for the decontamination of beef, lamb, pork, and poultry carcasses [Alzamora, 2000]. There is the potential for lactic acid to be applied to the surface of vegetables and fruits for eliminating and/or reducing pathogens of concern. Lactic acid's antimicrobial activity is highly pH dependent. The undissociated form of the acid usually possesses the most antimicrobial activity [Doyle, 1997]. Bacteria maintain a normal internal pH at neutrality in order to prevent sudden changes to structural proteins, enzymes, nucleic acids, and phospholipids involved with its life systems [Doyle, 1997]. In the undissociated form, lactic acid has ability to cross the cytoplasmic membrane. Once inside the cytoplasm, organic acids will dissociate and acidify the cytoplasm. This would in turn cause the denaturation of proteins, enzyme inactivation and damage to nucleic acids.

Other than on meat and contact surfaces, lactic acid has been experimentally used on vegetables, fruits, and vegetable/fruit products. Chemical combinations with lactic acid were effective at eliminating *Salmonella spp.* and *E. coli* by 4 log from fresh-cut lettuce leaves [Lin, 2002]. Particularly, the use of lactic acid in combination with H_2O_2 on apples will be reported in chapters 2 and 3 for destroying *E. coli* O157:H7.

Sodium bicarbonate

If one were to open the refrigerator in any home across the United States, a box of sodium bicarbonate would most likely be found sitting in a back corner. Sodium bicarbonate, a multiple-purpose, generally recognized as safe (GRAS) food compound is well known for its use as a leavening agent and a pH, taste and texture control in foods [Davidson, 1993]. Its antibacterial effects were established relatively recently in 1980. The first studies on sodium bicarbonate were performed using oral bacteria [Davidson, 1993].

Sodium bicarbonate is a salt derived from reacting sodium hydroxide (strong base) with carbonic acid (weak acid). The compound is prepared from sodium carbonate, water, and carbon dioxide [Davidson, 1993]. Its uses and GRAS status are listed in 21 CFR 582.1613 and 582.1721. Sodium bicarbonate is non-toxic and used in foods up to a 2% concentration [Davidson, 1993].

Sodium bicarbonate imposes its effect by elevating the pH of foods. Increases in pH after bicarbonate use have been cited for changes in the microbial ecology of acid foods. These changes brought about spoilage by atypical bacteria. Buffering the solution in some way has been found to alleviate this problem. Studies have been conducted on sodium bicarbonate's antimicrobial and antifungal properties and have found it to be effective under certain parameters. Sodium bicarbonate has been shown to decrease green and blue mold on the surface of citrus fruits. When applied at room temperature, sodium bicarbonate at 2 to 4% reduced blue mold by more than 50% [Palou, 2001]. Both blue and green molds were reduced by 40-60% on mandarins dipped for 60 to 150 sec in 2 or 3% sodium bicarbonate [Palou, 2002]. On beef carcass, 1% sodium bicarbonate was tested as a wash for the removal of *E. coli, L. innocua* and *Salmonella wentworth* [Bell, 1997]. A three-step rinse process

using sodium bicarbonate and hydrogen peroxide was tested and patented for use on poultry carcasses processed into food [Fletcher, 1993].

As related to foodborne and food-related bacteria, sodium bicarbonate has mostly been observed for its efficacy in model systems. Corral et al. (1988) observed sodium bicarbonate's inhibition of the bacteria *E. coli*, *Lactobacillus plantarum*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* and the yeast *Saccharomyces cerevisiae* and *Hansenula wingei*. Aerobic plate counts for bacteria were reduced 10,000-fold and yeasts 100,000-fold.

Model systems are good preliminary indicators as to how particular sanitizers may perform; however, due to the different physical and chemical properties of foods, direct application to foods is best for evaluating a sanitizer's performance. Sodium bicarbonate is an inexpensive chemical that can easily be incorporated into washing systems. If its ability to reduce bacterial populations on apples is greater than water alone, sodium bicarbonate could serve as a powerful sanitation aid in apple cider processing. Its evaluation is discussed in chapters 2 and 3.

Tsunami™100

Tsunami™100 is a commercially available sanitizer manufactured by EcoLabs, St.Paul, MN. The sanitizer is recommended for use in the waters of processed fruits and vegetables in both batch and continuous operations [Anonymous, 1997]. The sanitizer is a peroxyacetic acid solution and is a strong oxidizing agent used on food surfaces. FDA classifies peroxyacetic acid as a no-rinse food contact surface sanitizer [Alzamora, 2000].

Peroxyacetic acid maintains antimicrobial activity pH up to 7.5; in the pH range of 7-8 activity begins to decrease [Alzamora, 2000; Cords and Dychadala, 1993]. Greatest

potency is at colder temperatures and lower concentrations and organic matter does not affect its stability [Davidson, 1993]. The sanitizer leaves no residues and readily breaks down after use into water, oxygen, and acetic acid. Tsunami™100 is approved for use in dipping fruits and vegetables to control microbial growth on the surface [Anonymous, 1997]. Some studies have tested the sanitizer's efficacy on apples for the removal of *E. coli* O157:H7 [Wisniewsky 1999; Wright, 2000]. Effectiveness varies for yeast and molds. The antimicrobial efficacy of Tsumani™ 100 against *E. coli* O157:H7 on whole apples is compared to that of other selected chemical sanitizers used in chapter 2.

Pro-San™

Pro-San™ is a fruit and vegetable wash specially formulated using food grade sequestrants and an anionic surfactant. The powder concentrate contains citric acid, sodium acid pyrophosphate, and sodium dodecyl benzene sulfonate. Combining 10g Pro-San™ with 1 L of water makes an instant cleaning solution. The solution manufactured by Microcide, Inc., Detroit, MI, is odorless, colorless, biodegradable and free of preservatives. The solution washes away dirt, chemical residues, and other surface contaminants. Its use on pathogens has not been extensively studied. The acidic and surfactant properties of the chemical ingredients may prove to be effective at destroying vegetative cells on produce. In the present study, Pro-San™ was examined for destroying *E. coli* O157:H7 via single and sequential usage on apple surfaces. Appropriate information on Pro-San's effectiveness against this pathogen is stated in chapters 2 and 3.

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CHAPTER 2. EFFICACY OF SELECTED CHEMCIAL SANITIZERS FOR KILLING *ESCHERICHIA COLI* O157:H7 ON WHOLE APPLES AT 25°C AND 55°C

A paper to be submitted to Food Protection Trends

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ABSTRACT

The viability of GFP-transformed *E. coli* O157:H7 (strain B6-914) on whole apples was evaluated in response to a two-minute exposure to distilled water (control) and six different sanitizers – sodium hypochlorite (CHLOR; 200 ppm chlorine), 5% hydrogen peroxide (HP), sodium bicarbonate buffer (SB, pH 11.5), 1.5% H_2O_2 + 1.5% lactic acid (HPLA), Tsunami100™ (TSU; 80 ppm) and ProSan™ (PROS; 1%). The stem area of unwaxed Red Delicious apples, spot-inoculated with *E. coli* O157:H7, was immersed in distilled water or sanitizers at 25°C or 55°C for 2 min, and rinsed for 5 seconds in fresh distilled water. Survivors were enumerated by vigorously washing apples in buffered peptone water (BPW), surface plating samples of BPW onto Tryptic Soy agar (TSA) and Sorbitol MacConkey agar (SMA), and counting bacterial colonies after incubation (37°C, 24 h). Numbers of *E. coli* on apples were ~ 5.64 $\log_{10}$ CFU/apple before immersing in water or sanitizers. Log_{10} reductions in numbers of *E. coli* on apples following immersion in water, CHLOR, TSU, SB, HP, PRO and HPLA at 25°C were 1.35, 1.56, 1.77, 1.99, 2.44, 3.07 and 3.01 $\log_{10}$CFU/apple, respectively, based on TSA counts. Log reductions based on SMA counts for the same treatments ranged from 1.82, 2.51, 2.95, 3.21, 3.17, 3.05 and 3.77 respectively. Increasing the temperature to 55°C enhanced the antimicrobial effectiveness of the sanitizers irrespective of plating medium used; $\log_{10}$ reductions at 55 ^{O}C ranged from 1.46 to 3.72

(TSA) and 2.31 to 3.90 (SMA). At 55°C, PROS and HPLA consistently reduced numbers of *E. coli* O157:H7 by more than 3 log cycles based on SMA counts. The use of PROS or HPLA at 55°C may be successful at achieving reductions ≥5 log for *E. coli* O157:H7 populations on whole apples when used in combination with other intervention methods.

INTRODUCTION

Escherichia coli O157:H7, a deadly human pathogen shed in the feces of cattle, sheep, and deer, has been linked to foodborne illness outbreaks associated with unpasteurized apple cider and juice [2, 12]. In 1991, 23 illnesses occurred in Massachusetts as a result of apple cider contaminated with *E. coli* O157:H7 [15]. In 1996, Odwalla, Inc was required to recall its unpasteurized apple juice and cider products after they had initiated a multi-state outbreak of *E. coli* O157:H7. The outbreak involved 66 illnesses and the death of a young child [6, 14]. These *E. coli* O157:H7-associated outbreaks have prompted the Food and Drug Administration (FDA) to examine the potential hazards to consumer safety linked to fresh fruit and vegetable juice. To improve the microbial safety of juice processing, the FDA now requires that all processors implement a Hazard Analysis Critical Control Point (HACCP) plan and utilize a sanitation regime that eliminates the most resistant pathogen relevant to the final product by 5 log cycles [12].

For apple cider production, the destruction of *E. coli* O157:H7 can easily be achieved by heat pasteurization [9, 14, 15]; however, many small-scale producers cannot afford this equipment. Based on a study of small cider plants in Iowa, processing about 20,000 liters of apple cider/per year, a fully installed pasteurization facility plus operation can cost as much

as $35,000/year [10]. Therefore, the incorporation of adequate, low cost interventions into current processing procedures would be ideal to prevent drastically increased production costs for small-scale processors.

Apple cider producers can use alternatives to pasteurization to meet the FDA 5-log standard. For example, fresh produce usually undergoes a washing and/or surface sanitizing step, to remove debris, pesticides, and other waste materials before being processed [9, 12]. This post-harvest practice could provide an effective means for eliminating *E. coli* O157:H7 and other pathogens from fresh fruits and vegetables well before juice processing occurs. Several studies have demonstrated that surface treatment of produce with sanitizers can achieve effective pathogen attenuation [9, 14]. Reductions in the range of 1 to 6 log have resulted from the use of chemical sanitizers at levels that far exceed the manufacturer's recommendations [18, 23] or for prolonged contact times that are not practical for current production practices [19, 22, 27].

Contact time and temperature are two parameters that have been shown to influence the effectiveness of sanitizers. Infiltration of microorganisms into cuts, abrasions, and natural openings on fruits and vegetables is likely to occur when warm produce are submerged into cold, contaminated water [5, 13, 21]. Current research has shown that ≥3 log reductions in pathogen numbers can occur when sanitizers are used at temperatures greater than ambient temperature [16, 25]. Reductions of ≥ 5 log in *E. coli* O157:H7 numbers on fresh produce via use of chemical sanitizers have mostly been observed for contact times exceeding 5 minutes [19, 23]. After microbiological analysis, *E. coli* O157:H7 was undetectable on apples immersed in 1.5% lactic acid + 1.5% hydrogen peroxide at 40°C for 15 min [25]. Prior to a 10-min Tsunami™ exposure, viable counts on whole apples were

decreased by ≥ 5 log [23]. Contact times longer than 5 minutes would extend the length of processing and potentially decrease the amount of cider produced by a plant per day.

The use of selected sanitizers at high temperatures with a sufficient exposure time acceptable for current industry practices may be an alternative to pasteurization for small-scale apple cider producers. Accordingly, the purpose of this study was to examine the efficacy of selected chemical sanitizers (chlorine (200 ppm), 5% hydrogen peroxide, sodium bicarbonate buffer, Tsunami™ 100, Pro-San™ and 1.5% hydrogen peroxide + 1.5% lactic acid) for reducing *E. coli* O157:H7 whole apples, from a 2-minute exposure to sanitizers at 25°C and 55°C.

MATERIAL AND METHODS

Bacterial strain and preparation of inoculum. *Escherichia coli* O15:H7 strain B6-914 90 ec was utilized as the test organism for all experiments. The strain is non-pathogenic and produces a bright green fluorescent protein for ease of identification under ultraviolet light. The culture was maintained at -70°in Tryptic Soy broth (TSB; Difco, Detroit MI) supplemented with 10% glycerol. Prior to each experiment, the stock culture was transferred three times (twice in 10 ml and once in 100 ml of TSB) and incubated at 37°C for 18 h. Cells were harvested by centrifugation (10,000 x g, 10 min, 4°C) and suspended in 30 ml of 0.1% peptone water (Difco). The cell suspension contained approximately 5.0 x 10^7 cells/ml and was used to inoculate the apples.

Apple preparation and inoculation. Organically grown, 'Red Delicious' apples were obtained from a local grocery store in Ames, Iowa. The apples were stored at 4°C in

the original container until needed. Prior to each experiment, unblemished apples were selected and allowed to reach ambient temperature (approximately 30 min). Apples were washed and mildly brushed with a soft bristle brush in soapy, lukewarm water to remove dirt, debris, and other surface contaminants, then thoroughly rinsed in distilled water and dried in a laminar flow hood (Fisher Scientific, Pittsburgh, PA) for 1 hr. Using a micropipeter (Fisher), 100 µl of the inoculum was dispensed in droplets (10-12) around the stem area of each apple; each 100 µl aliquot contained 5.0×10^6 cells of GFP-transformed *Escherichia coli O157:H7*. Apples were then held in a laminar flow hood for 24 h at 23 ± 1°C.

Chemical sanitizers and preparation. Chemical sanitizers used were 200 ppm chlorine (CHLOR; Clorox Co., Oakland, CA), a sodium hypochlorite solution; 5% hydrogen peroxide (HP; Fisher); sodium bicarbonate buffer (SB; Fisher); Tsunami™100 (TSU; Ecolabs, St. Paul, MN); 1.5% hydrogen peroxide + 1.5% lactic acid (HPLA; Fisher and Sigma Aldrich, Inc. Milwaukee, WI) and 1% Pro-San™ (PROS; Microcide, Inc., Detroit, MI). All chemical treatments were combined with sterile distilled water. Distilled water at 25°C or 55°C, was used as the control treatment for the experiment. The 200 ppm solution of chlorine was acidified to pH 6.4 ± 0.1 with 5% citric acid (Fisher). A 30% hydrogen peroxide solution (Fisher) was used to prepare all chemical sanitizers containing hydrogen peroxide. Sodium hydroxide (Fisher) was used to buffer the sodium bicarbonate to pH 11.5 ± 0.1. An 85% lactic acid solution (Sigma Aldrich) was combined with hydrogen peroxide to prepare the 1.5% hydrogen peroxide + 1.5% lactic acid sanitizer. Tsunami™100 was applied at a concentration of 80 ppm. Following manufacturer instructions, 10 g of Pro-San™ (powder) was added to 1 L of distilled water.

Chemical sanitation of apples. Apples were immersed individually, with stem end down in a sterile stomacher bag (17.7 x 30.4 cm, Seward, London, UK) containing 200 ml of sanitizer for 2 min at either 25°C or 55°C without agitation. For the high temperature treatment (55°C), bags were submerged in a thermostatically controlled heated water bath (Neslab, Portsmouth, NH) set to maintain the sanitizer temperature inside the bag at 55°C. The temperature of the sanitizers was measured before usage with a thermometer (Fisher). After the 2-min treatment, each apple was rinsed in fresh, distilled water for 5 sec then placed in a sterile stomacher bag for microbiological analysis.

Microbiological analysis. Fifty milliliters of buffered peptone water (BPW; Fisher) was added to each bag containing an apple. The stem end of the apple was vigorously rubbed by hand for 2 min. The whole apple was discarded thereafter. The remaining solution was serially diluted (1:10) in sterile 0.1% peptone water (Difco). Aliquots (0.1 ml) of appropriate serial dilutions were surface-plated on tryptic soy agar (TSA; Fisher) and Sorbitol MacConkey agar (SMA; Difco) in duplicate. All inoculated agar plates were incubated at 37°C for 24 h before green fluorescent colonies were counted. Colony identity of *E. coli* O157:H7 B6-914 was confirmed using an ultraviolet lamp (Optica Engineering, Santa Rosa, CA).

Statistical analysis. Four replicate experiments were conducted. During each experiment, three apples were analyzed per sanitizer treatment for each of the temperatures (25°C and 55°C) in a randomized complete block design. Mean values of numbers of *E. coli* O157:H7 survivors ($\log_{10}$CFU/apple) were subjected to Analysis of Variance (ANOVA) using the general linear models procedure of the Statistical Analysis Software program (SAS

Institute, Inc, Cary, NC) [18]. Differences were deemed statistically significant at $P < 0.05$ unless otherwise noted.

RESULTS

A listing of each sanitizer, its abbreviation, concentration used and pH is shown in Table 2.1. Populations of *E. coli* O157:H7 recovered from apples that were inoculated but not chemically sanitized (NTC) were 5.64 $\log_{10}$CFU/apple. CHLOR reduced microbial loads greater than distilled water but was less effective than all other sanitizers. For all sanitizer treatments, numbers of survivors were consistently lower at 55°C than at 25°C but mean differences were not statistically different ($P>0.05$).

Numbers of *E. coli* O157:H7 survivors and log reductions of this pathogen on whole apples, following treatment with sanitizers at 25°C are shown in Table 2.2. Based on TSA counts, log reductions of *E. coli* O157:H7 from sanitizing apples with TSU, CHLOR, SB, HP, PROS and HPLA at 25°C, as compared to *E. coli* populations from NTC, were 1.77, 1.57, 1.99, 2.44, 3.07 and 3.01 respectively. Log reductions from treatments with TSU, CHLOR, SB, HP, PROS and HPLA at 25°C were 2.95, 2.51, 3.25, 3.17, 3.38 and 3.77, respectively based on SMA counts.

Table 2.3 shows numbers of *E. coli* O157:H7 survivors and log reduction in pathogen numbers following treatment with sanitizers at 55°C. At 55°C, all chemical sanitizers achieved reductions > 3 log cycles with the exception of CHLOR which gave a 2.69 log reduction when SMA was used as the plating medium. Chemical sanitizers demonstrating the highest reductions at 55°C (SMA) were PROS, SB, and HPLA which gave 3.82, 3.69, and 3.90 log reductions, respectively. The antimicrobial efficacy of the sanitizers at 55°C

was reduced when TSA was employed as the plating medium (Table 2.3). Log reductions of the pathogen on whole apples were 2.42, 2.10, 2.66, 2.76, 3.72 and 2.81 following treatment with TSU, CHLOR, SB, HP, PROS, and HPLA, respectively. Survival of the organism was also consistently lower at 55°C than at 25°C for all treatments on SMA; however, differences in sanitizer effectiveness due to temperature were not statistically significant ($P>0.05$).

DISCUSSION

Apples were inoculated with *E. coli* O157:H7 by use of a spot inoculation method. This method closely simulates the way in which apples in the natural environment could be contaminated by soil, feces, or by human hands [4]. Also, a spot inoculation method has proven to be a consistent and reproducible method for applying a known amount of bacteria to the surface of apples [25] and tomatoes [4].

Determining the recovery level of *E. coli* O157:H7 from apples not receiving a water or sanitizer wash was necessary for analysis of sanitizer efficacy in relation to a 5-log reduction. Bacterial counts taken from untreated, inoculated apples indicated populations of *E. coli* O157:H7 were reduced by approximately 1.25 log before chemical sanitation. This may have resulted from death of a part of the cell population during drying of the inoculum on the apples [18]. Wright et al. (24) reported a 3-log reduction in numbers of *E. coli* on artificially inoculated Red Delicious apples following drying for 30 min.

Selective culture media can be inhibitory to *E. coli* O157:H7 that have been stressed by the environment, chemicals or heat [1, 7]. In the present study, TSA was used as a non-selective medium and SMA as a selective medium for enumerating *E. coli* O157:H7. For all

chemical sanitizers tested, numbers of *E. coli* O157:H7 recovered from treated apples were lower on SMA than on TSA. These results suggest that some *E. coli* O157:H7 cells were injured by the sanitizers and could not be recovered on SMA.

Treatment of apples with distilled water (25°C) resulted in a 1.34 $\log_{10}$ reduction in *E. coli* O157:H7 based on TSA counts. These results are similar to previous findings. For example, Wright et al. (24) reported a 1.1 $\log_{10}$ reduction in *E. coli* O157:H7 on apples following dipping in water only. In a more recent study, Venkitanaranayan et al. (25) demonstrated 0.78 log reductions in *E. coli* O157:H7 on apples dipped for 15 min in water (40°C). Since water at 25°C does not have antimicrobial activity, reductions observed in the present study are likely to be linked to physical removal of cell from the apple surface during immersion. Slight decreases in *E. coli* O157:H7 noted on apples dipped in water at 55°C were not statistically significant ($P>0.05$). This might be due to the short exposure time (2 min) at this temperature used in the present study.

Chlorine is widely used as a post-harvest cleaning agent for produce at a contact time of 1 to 2 min. [2, 3, 8, 10]. Chlorine is generally used in the 50 to 200 ppm concentration range [14] and its bactericidal activity is highest between pH 6 and 7.5, at which chlorine is in the hypochlorous form (HOCl) [2, 12]. In this experiment, the efficacy of a 2-min exposure to chlorine (200 ppm) acidified to pH 6.4 was tested. At 25°C, reductions achieved were 1.57 and 2.51 (TSA and SMA) and 2.10 and 2.69 at 55°C. Generally, as temperature increases, chlorine's bactericidal action is increased [26]. In the present study, an increase in effectiveness with respect to temperature was not statistically significant ($P>0.05$).

Use of chlorine at concentrations higher than 200 ppm has been tried on various produce. Park et al. (18) utilized a 2,000 ppm chlorine (25°C) dip for oranges, apples, and

asparagus inoculated with *E. coli* O157:H7 and *Salmonella*. Reductions of both pathogens ranged from 2.6-3.8 $\log_{10}$CFU after a 3-min exposure. Application of chlorine at such high concentrations has been shown to be effective but is not approved by the FDA for surface sanitation of fresh produce.

Hydrogen peroxide (HP) possesses bactericidal and inhibitory activity due to its properties as an oxidant and ability to generate cytotoxic oxidizing species such as hydroxyl radicals [12]. As a bactericide, H_2O_2 damages nucleic acids, proteins, and lipids [2]. The efficacy of H_2O_2 has been tested on a variety of whole and fresh-cut produce. Concentrations greater than 1% have shown effectiveness at removing *Salmonella* and *E. coli* O157:H7 on produce surfaces [18, 22]. Populations of *Salmonella* on alfalfa sprouts were reduced by 2 $\log_{10}$CFU/g after 2 min using 2% hydrogen peroxide [12]. Hydrogen peroxide (5%) eliminated *E. coli* O157:H7 by 3.4-3.8 log cycles on Golden Delicious apples dipped for 1 min at 25°C and 50°C [19]. In the present study, the use of 5% hydrogen peroxide at 25°C and 55°C reduced survival of *E. coli* O157:H7 on Red Delicious apples by 3.17 and 3.31 log cycles, respectively based on SMA. Lower reductions seen in the present study may be attributed to natural variations in *E. coli* O157:H7 survival among different apple cultivars. Red Delicious apples have been found to support the survival of *E. coli* O157:H7 better than Golden Delicious and Rome apples [11].

Sodium bicarbonate buffer reduced bacterial populations of *E. coli* O157:H7 greater than 5% H_2O_2 to give 3.25 and 3.69 log reductions at 25°C and 55°C, respectively based on SMA counts. The effectiveness of SB for reducing *E. coli* O157:H7 is related to pH. When applied at pH 11.5, SB creates an alkaline environment that results in bacterial cell destruction. Highly alkaline solutions solubilize membrane proteins and/or saponify lipids in

the bacterial cytoplasmic membrane to result in leakage of cytoplasmic constituents [17]. Mendonca et al (17) demonstrated that sodium bicarbonate buffer (pH 11) disrupted the cytoplasmic membrane on *E. coli* O157:H7 and *Salmonella typhimurium*. Measurement of cellular leakage by spectrophotometry after exposure of the pathogens to $NaHCO_3$ buffer (pH 9, 10, 11, and 12) at 25°C for 3 min, revealed that leakage of cellular contents increased as pH increased. For *E. coli* O157:H7, a 4 log reduction was seen in 5 min after exposure to NaHCO3 buffer (pH 11) at 37°C [15].

Tsunami™100 (TSU) and Pro-San™ (PROS) are commercially available chemical sanitizers used on fruit and vegetable surfaces. Tsunami™100, a peroxyacetic acid solution, is approved by FDA for dipping fruits and vegetables to control of microbial growth [23]. For the present study, reductions based on TSU exposure for 2 min at 25°C and 55°C ranged from 1.77 to 2.42 and 2.95 to 3.09 (SMA) $\log_{10}$CFU/apple, respectively. The current results are comparable to those observed in recent studies. In a study based on 2-min contact times at ambient temperature, the reduction range for TSU on bacterial colonies of *E. coli* was 2.57-2.76 log cycles [24]. Longer exposure and higher concentrations of TSU reduced in *E. coli* O157:H7 $\geq$ 5 log cyles. Viable *E. coli* counts on whole apples were decreased by 3.0 to--5.5 log when TSU (25°C) was used at 1 to 16 times manufacturer recommended concentration for 5 min [23].

In the present study, PROS performed better than TSU at both 25°C and 55°C. As noted in Table 2.1, PROS is highly acidic (pH 2.4). The sanitizer contains citric acid, sodium acid pyrophosphate and sodium dodecyl benzene sulfonate. Reductions of 3.07 (25°C, TSA) and 3.72 (55°C, TSA) log cycles were most likely due to the acidity of the solution and the surfactant action of sodium dodeyl benzene sulfonate. *E. coli* O157:H7 maintains minimum

growth at pH 5 [15]; below pH 5, stresses on a bacterial cell's enzyme function and nutrient transport could occur and lead to growth inhibition. Impairment of cell function could be further exacerbated by surfactant induced damage to the cytoplasmic membrane.

The sanitizer, 1.5% hydrogen peroxide + 1.5% lactic acid (HPLA) was the most effective treatment, having the highest log reduction (3.90 log cycles) at 55°C (SMA). The effectiveness of hydrogen peroxide at concentrations > 1% has been previously discussed. Lactic acid is often used as an antimicrobial spray on beef, lamb, pork and poultry carcasses [2]. The undissociated portion of organic acids, such as lactic acid, is responsible for antimicrobial activity. In a study by Lin et al. (2002), a larger reduction of *Listeria monocytogenes, E. coli* O157:H7 and *Salmonella enteritidis* was observed for lettuce treated with a lactic acid/ H_2O_2 combination than with H_2O_2 alone.

When evaluated by Venkitanarayanan et al. (25), 1.5% hydrogen peroxide + 1.5% lactic acid was most effective at reducing *E. coli* O157:H7 on Red Delicious apples to undetectable levels at 40°C for 15 min. Under the same conditions, populations of *Salmonella enteritidis* were reduced by 6 log cycles. A 2-min immersion in the same solution was able to reduce *E. coli* O157:H7 by 3.90 at 55°C in this study. Longer exposures times for *E. coli* O157:H7 to HPLA would most likely give greater reductions in pathogen numbers; however, the feasibility of a long exposure time for sanitizer application in cider production must be evaluated. Currently, a 1-2-min exposure is typically used in industry for some chemical sanitizers [12].

This study demonstrated that selected chemical sanitizers (SB, HPLA and PROS) can remove *Escherichia coli* O157:H7 from the surface of apples by as much as 3.48 to 3.90 log cycles after 2 min of exposure time at 55 ^{o}C. An increase in sanitizer temperature

increased sanitizer effectiveness. No physical alterations in apple appearance were observed after the selected chemical sanitizers were used at 25°C or 55°C. A contact time of 2-min was found to be acceptable for processors and sufficient for pathogen elimination.

There is the potential for these sanitizers to be used singly or in combinations at 25°C and 55°C. Combinations of sanitizers and/or other intervention methods, such as heat could have additive, synergistic or antagonistic interactions [14]. For example, hydrogen peroxide was found to produce higher population reductions when used in combination with commercial sanitizing agents at 50-60°C [18]. Chemical sanitizers, CHLOR, HP, HPLA, PRO and $NaHCO_3$ should be tested in sequential combinations at 25°C and 55°C to determine if a 5 log reduction in bacterial populations of *E. coli* O157:H7 on whole apples can be achieved.

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Table 2.1. List of Chemical Sanitizers and Abbreviations

Sanitizer	Abbreviation	Concentration	pH
Control	H2O	-	7.0
Chlorine	CHLOR	200 ppm	6.4
Hydrogen peroxide	HP	5%	4.1
Sodium bicarbonate buffer	SB	5 M	11.4
Tsunami™100	TSU	80 ppm	3.3
Hydrogen peroxide + lactic acid	HPLA	1.5%	2.4
ProSan™	PROS	1%	2.4

Table 2.2. Numbers of *E. coli* O157:H7 * survivors and log_{10}reductions for whole apples immersed in chemical sanitizers at 25°C

	SMA		TSA	
Treatment	**Log_{10}CFU/apple[1]**	**Log_{10}Reduction[2]**	**Log_{10}CFU/apple[1]**	**Log_{10}Reduction[2]**
H2O	3.82 [a]	1.82 [b]	4.29 [a]	1.35 [b]
CHLOR	3.13 [ab]	2.51 [b]	4.07 [a]	1.57 [b]
HP	2.47 [ab]	3.17 [b]	3.20 [a]	2.44 [b]
SB	2.39 [ab]	3.25 [b]	3.65 [a]	1.99 [b]
TSU	2.69 [ab]	2.95 [b]	3.87 [a]	1.77 [b]
HPLA	1.87 [b]	3.77 [b]	2.63 [a]	3.01 [b]
PROS	2.26 [ab]	3.38 [b]	2.57 [a]	3.07 [b]

*Mean populations of *E. coli* O157:H7 recovered after 4 replicate experiments.

[1] *E. coli* O157:H7 populations recovered from apples immersed in each chemical sanitizers for 2 min; colony counts based on SMA or TSA media.

[2] Reductions in *E. coli* populations as compared to an initial population of 5.64 log_{10}CFU/apple.

Means with the same letter within the same column are not significantly (P>0.05) different

Table 2.3. Numbers of *E. coli* O157:H7 * survivors and log_{10}reductions for whole apples immersed in chemical sanitizers at 55°C

	SMA		TSA	
Treatment[a]	**Log_{10}CFU/apple[1]**	**Log_{10}Reduction[2]**	**Log_{10}CFU/apple[1]**	**Log_{10}Reduction[2]**
H2O	3.33 [a]	2.31 [b]	4.18 [a]	1.46 [b]
CHLOR	2.95 [ab]	2.69 [b]	3.54 [a]	2.10 [b]
HP	2.33 [ab]	3.31 [b]	2.88 [a]	2.76 [b]
SB	1.95 [ab]	3.69 [b]	2.98 [a]	2.66 [b]
TSU	2.55 [ab]	3.09 [b]	3.22 [a]	2.42 [b]
HPLA	1.74 [b]	3.90 [b]	2.83 [a]	2.81 [b]
PROS	1.82 [ab]	3.82 [b]	1.92 [a]	3.72 [b]

*Mean populations of *E. coli* O157:H7 recovered after 4 replicate experiments.

[1] *E. coli* O157:H7 populations recovered from apples immersed in each chemical sanitizers for 2 min; colony counts based on SMA or TSA media.

[2] Reductions in *E. coli* populations as compared to an initial population of 5.64 log_{10}CFU/apple.

Means with the same letter within the same column are not significantly (P>0.05) different

CHAPTER 3. SEQUENTIAL APPLICATION OF CHEMICAL SANITIZERS FOR INACTIVATION OF *ESCHERICHIA COLI* O157:H7 ON WHOLE APPLES

A paper to be submitted to Food Protection Trends

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ABSTRACT

This study was conducted to determine if selected chemical sanitizers applied sequentially to the surface of whole apples would give a 5-log reduction in numbers of *Escherichia coli* O157:H7. Chemical sanitizer combinations of chlorine (CHLOR, 200 ppm); 5% hydrogen peroxide (HP); 1.5% hydrogen peroxide + 1.5% lactic acid (HPLA); sodium bicarbonate buffer (SB) and Pro-San™ (PROS) were chosen after preliminary trials involving individual application of each chemical sanitizer. After spot inoculation with GFP-transformed *E. coli* O157:H7 (strain B6-914), apples were immersed in distilled water or sequentially in two selected chemical sanitizers, stem end down for 2 min at 25°C or 55°C; each 2 min immersion was followed by a 5-sec distilled water rinse. *E. coli* O157:H7 survivors were recovered by massaging apples by hand in Buffered Peptone Water (BPW) and enumerated by surface-plating on Tryptic Soy agar (TSA) and Sorbitol MacConkey agar (SMA). Bacterial colonies were counted after 48-hr incubation at 35°C. Sequential treatments at 25°C, eliminated *E. coli* O157:H7 by 2.10 to 3.00 $\log_{10}$CFU/apple and 1.47 to 3.01 $\log_{10}$CFU/apple based on TSA and SMA counts, respectively. At 55°C, CHLOR and HP; SB and HPLA; HP and HPLA; CHLOR and HPLA; and PROS and HPLA reduced E. *coli* O157:H7 by 2.98, 3.03, 3.54, 2.55 and 2.87 $\log_{10}$CFU/apple and on TSA and by 3.02, 3.17, 3.24, 3.27 and 2.51 $\log_{10}$CFU/apple on SMA. These results suggest that sequential

application of chemical sanitizers was not effective in achieving a 5-log reduction of *E. coli* O157:H7 on whole apples based on conditions used in the present study.

INTRODUCTION

Foodborne illness outbreaks originating from the consumption of contaminated fruit and vegetable products have ignited major concern for consumer safety in recent years [4, 5, 6]. Contamination of fresh vegetables and fruits can occur during harvesting, post harvest treatment, processing, shipping, marketing, and in the home [2, 14]. Produce that comes in direct contact with water, soil, and/or animal feces are very likely to become contaminated with spoilage and/or pathogenic microorganisms. To reduce the incidence of foodborne disease outbreaks, the Food and Drug Administration (FDA) has mandated methods that will assure consumers that only safe fruit and vegetable products reach markets across the United States. The implementation of Hazard Analysis Critical Control Point (HACCP) plans and a 5-log reduction standard for pathogens are regulations formulated in an effort to improve the safety of fresh fruits, vegetables, and fruit/vegetable products [7, 8].

The cleanliness of produce is conventionally achieved by washing produce before processing and consumption [2, 14]. Washing produce with plain water does not achieve reductions >2-log [3, 14, 19]. Reductions less than 2-log are insignificant when compared to the microbial load of most fruits and vegetables. Microorganisms have been observed to occur on raw or minimally processed produce at populations ranging from 10^3 to 10^9 CFU/g [9, 11]. These microbial numbers must be reduced before produce are processed or distributed.

Treatments using chemical sanitizers have been shown to reduce pathogen populations on fresh fruits and vegetables. Sanitizers for use on produce surfaces are regulated by FDA in accordance with the Federal Food, Drug and Cosmetic Act (FFDCA) as outline in CFR, Title 21 [1]. Theses chemicals are often used during post harvest processing to clean and reduce the microbial load of wash waters [1]. Sanitizing agents destroy vegetative cells of microorganisms but are not capable of eradicating microbes completely [9].

A variety of chemical sanitizers, such as chlorine, peroxide, and organic acid have been studied as interventions for the removal of *E. coli* O157:H7 from apple surfaces [13, 20, 21, 22]. Washing inoculated whole apples with 200 ppm chlorine, reduced microbial populations by <1 log [15]. Wright et al. (24) demonstrated the efficacy of a phosphoric acid-based fruit wash in reducing *E. coli* from the surface of Red Delicious apples by 2.9 log. Hydrogen peroxide has shown reductions of the same pathogen in the range of 3 to 4 logs on apple halves [15].

Only usage of sanitizers at high concentrations and/or prolonged contact times has been effective at eliminating pathogens by ≥ 5 logs. Chlorine (2000 ppm) and acidified sodium chlorite (850 and 1200 ppm) were more effective than hydrogen peroxide at killing *Salmonella spp* by 5 to 7 log on asparagus [9]. Large reductions such as this are often not without some physical alteration or deterioration in produce quality [16, 20].

Published research provides evidence on the effectiveness of chemical sanitizers after single application. Reductions up to 4 logs have been attained for certain chemical sanitizers after one application [2, 19, 20, 22]. There is potential for greater reductions to be achieved

after an application with a second sanitizer if the procedure has an additive or synergistic effect on pathogen attenuation.

This study was conducted to determine if selected chemical sanitizers used in a sequential regime could reduce *E. coli* O157:H7 on whole apples by ≥5 log. It is expected that the individual action of each sanitizer will provide successive hurdles to pathogen survival. Per preliminary trials [18], the results observed for chemical sanitizers are projected to be additive when used one after the other, e.g. 5% hydrogen peroxide or sodium bicarbonate capable of individually reducing *E. coli O157:H7* by 3 log, theoretically should provide a 6 log reduction when applied sequentially.

MATERIAL AND METHODS

Preparation of inoculum. *Escherichia coli* O157:H7 strain B6-914 gfp 90ec, which fluoresces under ultraviolet light, was used as the target pathogen for this study. A stock culture of the microorganism was kept on reserve at -70°C in tryptic soy broth (TSB; Difco, Detroit, MI) supplemented with 10% glycerol. Prior to experimentation, the stock culture underwent 3 transfers in TSB (twice in 10 ml and a third time in 100 ml) with incubation at 35°C for 18-hr. Cells were harvested by centrifugation (10,000 x g, 10 min, 4°C) and suspended in 0.1% peptone water (30 ml; Difco). The cell suspension was used to inoculate the apples and contained approximately 4.6×10^7 cell/ml.

Preparation of apples and inoculation. Unwaxed, organic Red Delicious apples were acquired from a local supermarket in Ames, IA. Selected, unblemished apples were washed and mildy brushed in soapy, lukewarm water. After a thorough rinse in deionized water, apples were placed in a laminar flow hood (Fisher Scientific, Pittsburgh, PA) and

dried for 15-20 min. A micropipet was used to spot-inoculate apples with a 150-µl aliquot of the cell suspension around the stem end (12-15 droplets). Approximately 7.0 x 10^6 CFU was placed on the apple surface in each 150- µl aliquot. The apples were placed in the same laminar flow hood for 45-60 min to dry.

Preparation of chemical sanitizers. The following chemical sanitizers were prepared by mixing the respective solutes with deionized water; chlorine-200 ppm (CHLOR; Clorox Co., Oakland, CA), a sodium hypochlorite solution; 5% hydrogen peroxide (HP; Fisher); sodium bicarbonate buffer (SB; Fisher); Pro-San™ (PROS; Microcide, Inc., Detroit, MI), a commercial fruit and vegetable wash; and 1.5% hydrogen peroxide + 1.5% lactic acid (HPLA). The 200 ppm chlorine solution was acidified with 5% citric acid (Fisher) to pH 6.4±1. Sodium hydroxide (Fisher) was added to sodium bicarbonate forming a buffer solution of pH 11.5±1. Pro-San™ (1%) was prepared according to the manufacturer instructions by adding 10 g of Pro-San™ powder to 1 L deionized water. A 30% hydrogen peroxide solution (Fisher) was used to prepare 5% and 1.5% hydrogen peroxide. Lactic acid (Sigma Aldrich, Milwaukee, WI) at an 85% concentration was used for the 1.5% hydrogen peroxide + 1.5% lactic acid sanitizer.

Sequential treatment with chemical sanitizers. Sterile stomacher bags (17.7 x 30.4 cm, Seward, London, UK) containing 200 ml of deionzied water (used as the control) or chemical sanitizers were secured onto bag holders (Scienceware®, Pequannock, NJ) and allowed to temper to 25°C or 55°C. For applications at 55°C, stomacher bags were submerged in a constant temperature water bath (Neslab, Portsmouth, NH) and thermostatically maintained at the desired temperature. Using sterile tongs, apples were placed in chemical 1, stem end down for 2 min and rinsed for 5 sec in deionized water. The

same procedure was followed for chemical 2. Selected chemical sanitizer combinations were as follows: CHLOR and HP; CHLOR and HPLA; SB and HPLA; PROS and HPLA; HP and HPLA (See Table 3.1).

Microbiological analysis. After the second rinse with deionized water, apples were placed in sterile stomacher bags. Recovery of *E. coli* O157:H7 was performed by vigorously rubbing bagged apples by hand for 2 min in 50-ml of buffered peptone water (BPW, Difco). The recovered cells were diluted (1:10) in 9-ml dilution blanks containing 0.1% peptone water (Difco). Bacterial colonies of *E. coli* O157:H7 were plated in duplicate on tryptic soy agar (TSA; Fisher) and Sorbital MacConkey agar (SMA; Difco) and enumerated after 48 h incubation at 35°C.

Statistical analysis. Three replicate trials were conducted using a randomized complete block design. At 25°C and 55°C, two apples were analyzed per sanitizer treatment, for a totoal of 24 observations. Statistical Analysis Software (SAS, Institute, Cary, NC) was used to perform Analysis of Variance (ANOVA) using a general linear models procedure for mean values of number of *E. coli* O157:H7 survivors ($\log_{10}$CFU/apple). Differences were considered statistically significant at $P<0.05$ unless otherwise stated.

RESULTS

Table 3.1 shows the chemical combinations, codes, and estimated reductions for the present study. *Escherichia coli* O157:H7 populations were approximately 5.36 $\log_{10}$CFU/apple on apples inoculated but not treated with a chemical sanitizer combination (NTC). Table 3.2 shows the recovery of bacterial colonies of *E. coli* O157:H7 on TSA and SMA and the efficacy of each sanitizer combination at 25°C, as compared to NTC values.

Populations of *E. coli* O157:H7 recovered on TSA ranged from 2.34 to 3.41 $\log_{10}$CFU/apple and 2.20 to 3.74 $\log_{10}$CFU/apple on SMA. At 25°C, there were no significant differences ($P>0.05$) found among chemical treatments. Reductions of *E. coli* O157:H7 ranged from 2.24 to 3.17 log cycles on TSA and 1.47 to 3.01 log cycles on SMA. Among 25°C chemical sanitizers, the combination of C/HL was most effective and inactivated initial populations of *E. coli O157:H7* on apples by 3.01 log cycles (SMA).

E. coli O157:H7 survival and sanitizer efficacy for chemical sanitizer combinations applied at 55°C are shown in Table 3.3. Bacterial colonies of *E. coli* O157:H7 recovered after treatment (55°C) with water, C/HP, S/HL, H/HL, C/HL, and P/HL on TSA were 2.92, 2.53, 2.48, 1.97, 2.96 and 2.64 $\log_{10}$CFU/apple, respectively. On SMA, colony counts on apples subjected to those same treatments were 3.25, 2.20, 2.05, 1.98, 1.94 and 2.71 $\log_{10}$CFU/apple, respectively. At 55°C, there were no significant differences ($P>0.05$) found among chemical treatments. The treatment, C/HL (55°C) had the highest log reduction, at 3.27 $\log_{10}$CFU/apple (SMA). Irrespective of plating media used, reductions in *E. coli* populations were greater at 55°C when compared to 25°C for all treatments with the exception of C/HL; however, differences with respect to temperature were not statistically significant ($P>0.05$).

DISCUSSION

Mean values for numbers of *E. coli* O157:H7 on NTC apples were compared with values recovered from treated apples in order to determine sanitizer efficacy (Table 3.2 and 3.3). The spot-inoculation method used in this study allowed for a known level of *E. coli* O157:H7 to be applied to each apple surface (~ 6.84 $\log_{10}$CFU/apple). As a result, sanitizer

efficacies up to 5 logs could be measured. The average amount inoculated onto apple surfaces fell within the estimated range (10^3 to 10^9 CFU/g) of microorganisms observed to occur on raw or minimally processed produce [11, 12]. The level of inoculum applied to apples also allowed a testable amount of cells to remain on the apple surface after variable cell death occurred due to the apple drying process; approximately 1.50 $\log_{10}$CFU/apple were lost before sanitizer treatments were applied.

Two media, TSA (non-selective) and SMA (selective), were used in this study to enumerate injured and non-injured cells. Some degree of injury was expected as a result of environmental stress caused by the chemical sanitizers, heat, pH and drying [1]. Enumeration of bacterial cells on a selective medium, such as SMA would inhibit the growth of injured cells. In a recent study using GRAS chemicals to inactivate *E. coli* O157:H7 on apples, oranges and tomatoes, colony counts on TSA were consistently higher than those on SMA [23]. This was not observed for the majority of chemical sanitizer combinations used in this study. This may indicate that the selected sanitizer combinations potentially caused little or not injury in the surviving population of *E. coli* O157:H7 on apples.

A combination of sanitizers and good manufacturing practices are expected to achieve effective bacterial elimination [10, 13]. A study by Sapers et al. (16) evaluated the residual effect of sanitizers applied in sequential (2-stage) fashion for the removal of pathogenic and spoilage microorganisms on produce surfaces [15]. Apples were washed with an acidic surfactant or trisodium phosphate (TSP), rinsed in distilled water and sanitized with H_2O_2 (25°C). Reductions in *E. coli* O157:H7 populations were ≤ 2.85 $\log_{10}$CFU/g for 2-stage treatments. These reduction levels were comparable to the individual use of the same sanitizers [15].

At the beginning of this study, of the present study, it was expected that exposing the surface of inoculated apples to two different chemical sanitizers with and without mild heat would have an effect additive or synergistic to the individual use of each chemical sanitizer. The majority of sequential treatments (25 and 55°C) achieved log reductions approximately equal to the mean log reductions observed for the chemical sanitizers when used individually. These results are consistent with those reported by Sapers et al. (16).

Log reductions for the C/HL treatment were expected to be between 2.40-2.76 log cycles, based on reductions of 1.56-3.31 $\log_{10}$CFU/apple after a 2-min single immersion at 25 and 55°C [18]. In the present study, C/HP achieved reductions of 1.97 and 3.01 log cycles based on SMA counts and 2.24. and 2.98 log cycles on TSA counts at 25 and 55°C, respectively. At both temperatures, these results were not significantly different from the deionized water control.

The mean effect may have been the result of bacterial populations initially being reduced by chemical 1 and only a small population of *E. coli* cells being reduced by the antimicrobial action of chemical 2, thereafter. Some cells may have been able to resist the antimicrobial action of the chemicals sanitizers. Cells may have stringently adhered to the apple surface and been protected from chemical action through the formation of biofilms [23].

None of the sequential treatments were able achieve reductions ≥ 5-log. This occurred due to the mean effect on *E. coli* O157:H7 elimination discussed previously. The most effective sequential treatments were C/HL, H/HL. These treatment consistently achieved reductions > 2.70 $\log_{10}$CFU/apple. H/HL achieved the highest reduction of 3.78 log cycles at 55°C based on TSA counts. At 25°C, C/HL was able to reduce *E. coli* O157:H7

populations by 3.01 log cycles (TSA). The success of these chemical combinations may relate to the oxidizing properties of the sanitizers used.

At pH values up to 7.5, chlorine forms hypochlorous acid, the most germicidal form of aqueous chlorine solutions [1, 8]. Hypochlorous acid alters cellular membranes, disrupts protein synthesis, inhibits oxygen uptake and damages nucleic acids [8]. The 200 ppm chlorine used in this study was acidified to pH 6.4, therefore, this solution was applied at an optimal level for bacterial elimination. Trials on chlorine efficacy have rendered mixed results. The maximum reduction of *L. monocytogenes* on shredded lettuce and cabbage treated with 200 ppm chlorine as reported by Zhang et al. (24) was 1.3-1.7 $\log_{10}$CFU/g. Apples immersed in the same concentration (25°C) for 2 min achieved reductions of < 2.5 log cyles [23].

Hydrogen peroxide is noted for its lethal effect on microorganisms, depending on pH, temperature, and other environmental factors [1]. Reductions in populations of *Salmonell*a on alfalfa sprouts were approximately 2.0 $\log_{10}$CFU/g after treatment with 2% H_2O_2 for 2 min [3]. A 90-sec treatment at 50°C of lettuce with 2% H_2O_2 reduced *S. enteritidis, E. coli O157:H7,* and *L. monocytogenes* by 4.5, 4.7, and 2.7 $\log_{10}$CFU/leaf, respectively [2].

Hydrogen peroxide (1.5%) + 1.5% lactic acid, used as a sanitizer has been successful at reducing pathogen populations on fruits and vegetables [22]. Initial evaluations of the combination as a sanitizer were performed on *E. coli* O157:H7 suspended in peptone water. Reductions of > 7 logs in 10-20 min at 40 or 22°C were observed [21]. H_2O_2 + 1.5% lactic acid applied at 40°C for 15 min on apples, resulted in the removal of *E. coli* O157:H7 and *L. monocytogenes* to undetectable levels [22]. For this study, the sanitizer was applied at pH

2.4. At this pH level, 1.5% H_2O_2 + 1.5% lactic acid will denature cellular enzymes and proteins and adversely affect the transport of nutrients into the cell.

The use of two chemical sanitizers is not foreseen to pose an excessive expense to apple processors. None of the treatments combinations caused visible, physical deterioration to the apples. Chlorine, hydrogen peroxide and lactic acid are readily available, low cost interventions. Furthermore, a typical wash time for apples in commercial cider operations has been estimated to be 5 min [19]. This process will cut the current processing time by one minute per lot of apples processed.

Heat is also cited as an intervention method that could be used with combinations of sanitizers [9]. All 55°C treatments containing HPLA produced log reductions > 3 log cycles. Sanitizer temperatures must be at least 10°C higher than apples to prevent internalization of bacteria into areas such as punctures, bruises, and the apple core [3, 9, 17]. Internalized pathogens are protected from the antibacterial action of chemical sanitizers. Temperatures above the optimal growth range for *E. coli* O157:H7 (35-37°C) will naturally inhibit bacterial growth and cause cellular proteins to be denatured.

Outcomes of this study may have been different if certain parameters of the study were altered. Different modes of application, such as pressurized spraying may increase the effectiveness of chemical sanitizers when used sequentially. Pressurized spraying will help to mechanically dislodge cells from apples surfaces. After being sprayed with chlorine and allowed to stand for 1 to 10 min, *Salmonella* populations were significantly decreased on apple surfaces [3]. Under the same conditions, 200 and 2,000 ppm was equally effective on *E. coli* O157:H7.

To potentially increase the elimination of *Escherichia coli* O157:H7 from whole apple surfaces, sequential trials could be performed coupling sanitizers at different temperatures, e.g. one treatment at 25°C, and the consequent treatment at 55°C, or vice versa. It is suggested that this technique be tested further with different organisms implicated in foodborne illness outbreaks associated with fruits, vegetables, and fruit/vegetable products. It is important for sanitizers to have antimicrobial capabilities against *Salmonella* spp. and *L. monocytogenes* as well as *E. coli* O157:H7 [22]. The chemical sanitizers selected should be utilized under individualized optimal conditions for assured effectiveness.

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Table 3.1. Chemical combinations[1], codes, and estimated reductions for chemical sanitizers

Code	CHEMICAL 1	CHEMICAL 2	Predicted Reduction[2]
C/HP	chlorine 200 ppm	5% hydrogen peroxide	5.70-6.07
C/HL	chlorine 200 ppm	1.5% hydrogen peroxide + 1.5% lactic acid	6.28-6.67
H/HL	5% hydrogen peroxide	1.5% hydrogen peroxide + 1.5% lactic acid	6.98-7.2
S/HL	sodium bicarbonate buffer	1.5% hydrogen peroxide + 1.5% lactic acid	7.00-7.42
P/HL	ProSan™	1.5% hydrogen peroxide + 1.5% lactic acid	5.46-5.73

[1] All chemical combinations were applied for 2 min at 25 and 55°C.
[2] Estimate based on $\log_{10}$reduction results after single use of each sanitizer for 2 minutes.

Table 3.2. *E. coli* O157:H7 recovery* and chemical sanitizer efficacy after sequential application of sanitizers (2 min per sanitizer) at 25°C

	SMA		TSA	
Treatment	**Log_{10}CFU/apple[1]**	**Log_{10}Reduction[2]**	**Log_{10}CFU/apple[1]**	**Log_{10}Reduction[2]**
H_2O	3.74[a]	1.47[b]	3.41[a]	2.10[b]
C/HP	3.24[a]	1.97[b]	2.98[a]	3.17[b]
C/HL	2.20[a]	3.01[b]	2.34[a]	2.87[b]
H/HL	2.54[a]	2.67[b]	2.51[a]	3.00[b]
S/HL	2.38[a]	2.83[b]	2.92[a]	2.59[b]
P/HL	2.42[a]	2.79[b]	2.79[a]	2.72[b]

*Mean populations of *E. coli* O157:H7 recovered after 3 replicate trials.

[1] *E. coli* O157:H7 populations recovered from apples immersed in each chemical sanitizers for 2 min; colony counts based on SMA or TSA media.

[2] Reductions in *E. coli* populations as compared to an initial population of 5.51 log_{10}CFU/apple (TSA) and 5.21 log_{10}CFU/apple (SMA).

Means with the same letter within the same column are not significantly (P>0.05) different

Table 3.3. *E. coli* O157:H7 recovery* and chemical sanitizer efficacy after sequential application of sanitizers (2 min per sanitizer) at 55°C

	SMA		TSA	
Treatment	**Log_{10}CFU/apple[1]**	**Log_{10}Reduction[2]**	**Log_{10}CFU/apple[1]**	**Log_{10}Reduction[2]**
H_2O	3.25 [a]	1.96 [b]	2.92 [a]	2.59 [b]
C/HP	2.20 [a]	3.01 [b]	2.53 [a]	2.98 [b]
C/HL	1.94 [a]	3.27 [b]	2.96 [a]	2.55 [b]
H/HL	1.98 [a]	3.23 [b]	1.97 [a]	3.54 [b]
S/HL	2.05 [a]	3.16 [b]	2.48 [a]	3.03 [b]
P/HL	2.71 [a]	2.50 [b]	2.64 [a]	2.87 [b]

*Mean populations of *E. coli* O157:H7 recovered after 3 replicate trials.

[1] *E. coli* O157:H7 populations recovered from apples immersed in each chemical sanitizers for 2 min; colony counts based on SMA or TSA media.

[2] Reductions in *E. coli* populations as compared to an initial population of 5.51 log_{10}CFU/apple (TSA) and 5.21 log_{10}CFU/apple (SMA).

Means with the same letter within the same column are not significantly (P>0.05) different

CHAPTER 4. GENERAL CONCLUSIONS

The ultimate objective of this research was to evaluate the effectiveness of selected chemical sanitizers for the removal of *E. coli* O157:H7 on apples surfaces. The work performed in this thesis met the objective in its entirety.

All chemical sanitizers used performed better than plain water and chlorine (200 ppm) for reducing numbers of *E. coli* O157:H7 on whole apples. It can be concluded that chemical sanitizers used in the present study are more effective than water and chlorine, which are both currently used in industry. Statistically, increased temperature (55°C) did not significantly ($P>0.05$) affect chemical sanitizer performance. Differences attributed to temperature were seen when comparisons were made based on $\log_{10}$reductions.

Hydrogen peroxide (5%), sodium bicarbonate buffer, 1.5% hydrogen peroxide + 1.5% lactic acid and Pro-San™ were the most effective sanitizers tested. Hydrogen peroxide (1.5%) + 1.5% lactic acid effected the greatest reduction in numbers of *E. coli* O157:H7 (3.90 $\log_{10}$CFU/apple). The same chemical sanitizer was most effective at both 25°C and 55°C. The results from this study led to the selection of 5% hydrogen peroxide, sodium bicarbonate buffer, 1.5% hydrogen peroxide + 1.5% lactic acid Pro-San™ for sequential application.

Sequential usage of chlorine and 1.5% hydrogen peroxide + 1.5% lactic acid at 25°C and 55°C reduced bacterial populations of *E. coli* O157:H7 greater than any other combination utilized (3.01 and 3.27 $\log_{10}$CFU/apple). The chemical sanitizer, 1.5% hydrogen peroxide + 1.5% lactic acid could be used by processors currently using chlorine, as an effective sanitizer combination for reducing *E. coli* O157:H7 by greater than 3 log.

Reductions of 3 log cycles do not meet FDA standards but will provide cider producers with an effective intervention that can be combined with other interceptive steps during processing.

Results from the two studies showed that none of the chemical sanitizers were able to achieve the FDA mandated log reduction requirement. In order to fully examine the potentialities of chemical sanitizer efficacy on apple surfaces, there is a need to understand the relative amount of *E. coli* O157:H7 populations on apples based on cultivars, time of harvest, location in the canopy, and post-harvest storage. If populations are ≤ 3 log, the chemical sanitizers used in this research are able to destroy *E. coli* O157:H7 on whole apples, thus making the requirement of a 5 log reduction unnecessary.

For future studies it is recommended that different chemical sanitizer combinations be developed; for example, using some sanitizers at 25°C followed by a chemical sanitizer at 55°C and vice versa. Evaluation of the chemical sanitizers in a commercial washing system would determine their potency with large volumes of apples. The efficacy of the most effective chemical sanitizers from this study should be tested on pathogens known to contaminate fruits and vegetables.

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