

**Efficacy of Ultraviolet Light in Combination with Chemical Preservatives for the
Reduction of *Escherichia coli* in Apple Cider**

By

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A Thesis submitted in partial fulfillment of the
Requirements for the degree of
Master of Science in Food Science and Technology
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June 3, 2005
Blacksburg, Virginia

Keywords: *Escherichia coli*, Apple Cider, Ultraviolet, Preservatives, Dimethyl
Dicarbonate, Hydrogen Peroxide, Potassium Sorbate, Sodium Benzoate

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ABSTRACT

Hazard Analysis Critical Control Point (HACCP) regulations for juice manufacture require the application of a process that will result in a 5-log reduction (99.999%) of the pertinent pathogen in the juice being processed. The use of ultraviolet (UV) light, as an alternative to traditional thermal processing, has been adopted by some juice processors as a means of meeting the HACCP 5-log performance standard. However, little research had been performed to determine the effect of UV when used in combination with antimicrobial agents that are commonly added to juice products. Therefore, the objectives of this work were (1) to determine if chemical preservatives and ultraviolet light have a combined effect on the reduction of *Escherichia coli* in apple cider, and (2) to determine the influence of adding chemical preservatives at different points in the processing of juice (i.e., either prior to or after ultraviolet light processing) on the reduction of *Escherichia coli* in apple cider. In this study, refrigerated (4°C) pasteurized apple cider that contained no added preservatives was inoculated with *E. coli* ATCC 25922, a surrogate strain for *E. coli* O157:H7, and exposed to UV (peak output: 254 nm). The following chemical preservatives were added to apple cider either prior to or after UV exposure: dimethyl dicarbonate (75 and 150 ppm), hydrogen peroxide (75 and 150 ppm), potassium sorbate (1000 and 2000 ppm), and sodium benzoate (1000 and

2000 ppm). Following UV exposure and chemical preservative application, inoculated juices were stored at 4°C for 72 hours. Samples were collected prior to and immediately after UV exposure and at 24, 48, and 72 hours of storage. At each sampling point, juice portions (0.1 ml) were serially diluted in peptone diluent (0.1%) and surface plated onto Tryptic Soy Agar (TSA). Counts of the bacterial colonies were made 48 hours after incubating plates at 35°C. Overall, reductions of *E. coli* were greater in cider treated with preservatives after UV processing than when preservatives were added prior to UV processing ($P < 0.05$). Furthermore, dimethyl dicarbonate and hydrogen peroxide were more effective than potassium sorbate and sodium benzoate in reducing *E. coli* populations in conjunction with UV ($P < 0.05$). When added prior to UV exposure, potassium sorbate was the least effective, allowing for the greatest survival ($P < 0.05$). This study describes the use of UV in conjunction with hydrogen peroxide and dimethyl dicarbonate as an effective method for producing a 5-log or greater reduction of *E. coli* O157:H7 in apple cider.

ACKNOWLEDGMENTS

I would like to thank my advisor and friend, Dr. Robert C. Williams, for his assistance in completing my research and thesis. He provided me with his knowledge, as well as, some comic relief and encouragement throughout the duration of my project. Thanks for being so patient with me and helping me recognize my potential as a graduate student. Secondly, I would like to thank my other committee members Dr. Joseph E. Marcy and Dr. Susan S. Sumner for their advice and support.

I am grateful for all the graduate students, faculty and staff that I have met in the Food Science Department at Virginia Tech. Thanks to Fletch Arritt, Michael Bazaco, and Angie Hartman for their friendship and advice. I thank Trina Pauley for our daily conversations during my many research breaks. Additionally, I would like to thank my lab assistant, Jackie Miles, for all her time and effort in the lab. Also, I want to thank Joe Boling and Wen Wan for helping me with my SAS statistics.

I extend my sincere thanks and love to my parents, Joselito and Nida Quicho, for their support and encouragement. They taught me the value of education and hard work. I thank my sisters, Joanne, Jela, and Jocelyn, for their love and encouragement. Finally, I would like to express my love and gratitude to Tiffany Lindfors. Without your support and encouragement I would never have completed this endeavor. You've all been there when times were tough, and for that I am truly grateful.

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LITERATURE REVIEW

Raw, unpasteurized cider has been recognized as a vehicle for *Escherichia coli* O157:H7 infections in recent years. The U. S. Food and Drug Administration's (FDA) HACCP program that includes a 5-log reduction of pertinent pathogens in fresh juices may be difficult on small cider operations. Treatment with UV is proven to produce a 5-log reduction of *E. coli* O157:H7 in apple cider, but there is always the possibility contamination can occur after initial treatment. Chemical preservatives in conjunction with UV can provide downstream protection from *E. coli* O157:H7 contamination and extend the shelf-life of the apple cider after bottling. Using low concentrations of chemical preservatives in conjunction can allow for reduced UV dosage compared to treatment with UV alone. This combination can be economical, while maintaining organoleptic properties and safety of fresh apple cider.

I. *Escherichia coli* O157:H7

E. coli O157:H7 was first recognized as a cause for human illness following an outbreak linked to undercooked ground beef in 1982 (Riley *et al.*, 1983). The O157:H7 strain an enterohemorrhagic *E. coli* (EHEC), produces a verotoxin that has cytotoxic effects on Vero (African green monkey kidney) cells (Brooks *et al.*, 1998). *E. coli* O157:H7 is known to cause an estimated 7,000 to 20,000 infections, 150 to 300 deaths and \$230-\$600 million in medical costs and lost productivity costs in the United States annually (Kaspar, 2000). Infants, young children (< 5 years old), the elderly and

immunocompromised individuals are more susceptible to *E. coli* O157:H7 infections than healthy, older children and adults (CDC, 2003).

A. Characteristics

Escherichia coli serotype O157:H7 is a Gram-negative, catalase-positive, oxidase-negative, rod-shaped facultatively anaerobic bacterium that produces Shiga-like toxin(s) (SLT's) (Buchanan and Doyle, 1997). The verotoxin has similar properties to the Shiga toxins that are produced by *Shigella dysenteriae* type 1, but are both antigenically and genetically different (Brooks *et al.*, 1998). *E. coli* O157:H7 has an optimal growth temperature at 37°C, but grows well between 30-42°C (Doyle and Schoeni, 1984). Furthermore, Doyle and Schoeni (1984) showed that *E. coli* O157:H7 can survive with little reduction in population for 9 months in frozen ground beef (-20°C). Growth rates of *E. coli* O157:H7 are similar between pH 5.5 and 7.5 with populations declining at lower pH levels (Buchanan and Klawitter, 1992). *E. coli* O157:H7 can grow at a minimum pH 4.0-4.5, so it has the ability to survive in a low-acid food such as apple cider (Buchanan and Bagi, 1994).

B. Illness

The incubation period of *E. coli* O157:H7 is typically 3 to 4 days following ingestion, but can incubate from 1 to 10 days after ingestion (FDA, 2001b). The most common symptom associated with *E. coli* O157:H7 infection is hemorrhagic colitis, bloody diarrhea, and other complications caused by affecting the large intestine (Buchanan and Doyle, 1997). Bloody and nonbloody diarrhea, vomiting, and abdominal

cramps are common symptoms of infection, with a typical duration of 7-10 days (FDA, 2002).

More serious, life-threatening infections can result from hemorrhagic colitis. The young and elderly are more susceptible to a more severe consequence of infection called hemolytic uremic syndrome (HUS), which is a disorder distinguished by acute hemolytic anemia, thrombocytopenia, and renal insufficiency (Garg *et al.*, 2003). Infection with *E. coli* O157:H7 has been recognized as the leading cause of HUS in the United States. Most patients diagnosed with HUS require dialysis and blood transfusions, but the condition may lead to death (Doyle, 1991).

C. Pathogenicity

The pathogenicity of *E. coli* O157:H7 is due to a number of virulence factors, including cytotoxins. These cytotoxins include verotoxins and SLT's that are present in more than 60 *E. coli* serotypes. Serotype O157:H7 is the one most associated with foodborne illnesses and it the most notable pathogen in the EHEC group (Feng, 1995). The pathogenicity of *E. coli* O157:H7 is due to the ability of the pathogen to adhere to the intestinal tract, where it can colonize and produce cytotoxins. The infectious dose is unknown, but may be very low, i.e. less than 10 cells (Anonymous, 1996).

D. Reservoirs and Disease Sources

The main mode of transmission of *Escherichia coli* O157:H7 infections is the fecal-oral route. *E. coli* O157:H7 is commonly shed by cattle, deer, and sheep, which may contaminate water supplies used for drinking, irrigation, and recreational purposes

(Buchanan and Doyle, 1997). Dropped and damaged fruit are a potential source of *E. coli* and other microorganisms. Intact tree-picked fruit has significantly lower bacterial contamination than dropped, damaged, and decayed fruit (Riordan *et al.*, 2001). Wright *et al.* (2000a) reported in their 1998 survey of Virginia cider producers that 32% used dropped apples.

1. Water

E. coli O157:H7 has been associated with outbreaks in drinking and recreational water (Akashi *et al.*, 1994 and Keene *et al.*, 1994). Waterborne-illness was caused most likely by ingesting fecally contaminated water. Wang and Doyle (1998) determined that *E. coli* O157:H7 can survive in municipal, reservoir, and recreational water at 8°C for 91 days and 48 days at 25°C. Application of Good Manufacturing Practices (GMP) is important to minimize the use of contaminated agricultural water, and processing water. Using antimicrobial chemicals and filtering recirculated water may improve water quality and reduce *E. coli* O157:H7 contamination (FDA, 1998a).

2. Manure and Feces

Human and animal fecal matter are major sources for foodborne pathogens. *Escherichia coli* O157:H7 may be in the intestinal tracts of asymptomatic animals such as cattle, deer, and sheep. Nonpathogenic *E. coli* is ubiquitous in the environment, and has been found in damp environments such as soil, vegetation, moist and wet areas in factories and inadequately treated water supplies (Anonymous, 1996). *E. coli* O157:H7 is most often associated with cattle, undercooked ground beef, raw milk, and other beef-

associated products. The most likely source of contamination beef occurs during the slaughter stage where intestinal contents (fecal material), and milk from milking, can come in contact with the carcass (Anonymous, 1996). Raw, fresh fruit and vegetables have been known to be contaminated with *E. coli* O157:H7 when untreated cow manure has been applied to crops. Using properly treated manure and making crops inaccessible to wild and domestic animals can reduce the prevalence of *E. coli* O157:H7 (FDA, 1998a).

E. Factors Affecting Growth of *Escherichia coli* O157:H7

1. Temperature and pH

E. coli O157:H7 grows poorly at 44 to 45.5°C, the temperatures in which most fecal coliforms and *E. coli* are detected in foods (Doyle and Schoeni, 1984). *E. coli* O157:H7 is heat-sensitive microorganism that becomes less resistant in low pH (< 4.0) at temperatures above 50°C (Splittstoesser *et al.*, 1995). Short-term storage (≤6 h at 21°C) of apple cider (pH 3.4) prior to pasteurization decreases the thermotolerance of *E. coli* O157:H7, thus increasing the efficacy of the thermal process (Ingham and Uljas, 1998). The thermal pasteurization process for Wisconsin apple cider producers has been shown to be effective at 68.1°C for 14 s, but New York State recommends 71.1°C for 6 s to achieve a 5-log reduction of *E. coli* O157:H7. (Mak *et al.*, 2001 and NYSDAM, 1998). In frozen ground beef patties (-18°C), holding patties at elevated temperatures (> 3 °C) prior to cooking increased heat sensitivity (Jackson *et al.*, 1995). The FDA recommends that ground beef be cooked to at least 68.3°C. The heat resistance of *E. coli* O157:H7 can be influenced by storage and holding temperatures of various food products.

F. Mechanisms of Acid Resistance

Acid tolerance of *E. coli* O157:H7 allows a small number to survive in the gastric liquids of humans. This factor is important to the virulence of *E. coli* O157:H7 since it is believed that very small numbers of cells (< 10) are required to cause illness (Leyer *et al.*, 1995). Gordon and Small (1993) determined that nonpathogenic *E. coli* is significantly less acid tolerant than enteroinvasive and enteropathogenic *E. coli* strains. There are several acid resistance mechanisms (e.g. oxidative-, arginine- and glutamate-resistance systems), which allows for survival as it enters the stomach (pH 1-3) and help *E. coli* O157:H7 colonize in the weak acid environment of the small intestine (pH 4.5 -7) (Lin *et al.*, 1996). Ryu *et al.* (1999) showed that unadapted *E. coli* O157:H7 are less tolerant to various organic acids (i.e. malic, citric, lactic or acetic) than acid-adapted cells at pH 3.4 - 3.9. *E. coli* O157:H7 can survive in extremely low pH environments, which can enhance acid tolerance in a low acid food such as apple cider.

G. Survival in Acidic Food Products

The acid resistance of *E. coli* O157:H7 gives it the ability to survive in various acidic food products. *E. coli* O157:H7 can survive during a meat fermentation process (pH 4.4 - 5.0), in shredded salami (Leyer *et al.*, 1995), in fermented dry sausage (Glass *et al.*, 1992), and in mayonnaise (Zhao and Doyle, 1994). Fresh apple cider has been implicated in a number of outbreaks of *E. coli* O157:H7 infections in recent decades. Unpasteurized, refrigerated apple cider that does not contain preservatives has shown survival of *E. coli* O157:H7 for up to four weeks (Luedtke and Powell, 2002). Zhao *et al.* (1993) have shown that *E. coli* O157:H7 can survive long periods of time in apple cider

with and without preservatives at 8°C. Leyer *et al.* (1995) previously demonstrated that acid-adapted *E. coli* O157:H7 can survive in apple cider (pH 3.46) for an extended period of time (81-100 h) compared to non-adapted cells. Refrigerating or freezing apple cider can enhance the *E. coli* O157:H7 survival in cold storage due to its mechanisms of acid resistance (Lin *et al.*, 1996).

H. *Escherichia coli* O157:H7 Outbreaks

The consumption of contaminated, undercooked ground-beef has been the main cause of *E. coli* O157:H7 associated foodborne illness (Feng, 1995). Outbreaks from ground beef and its associated products, and raw milk can be prevented by properly cooking or pasteurizing the product. Fresh cheese curds made from raw milk have been implicated in *E. coli* O157:H7 infections (CDC, 2000). The United States Department of Agriculture (USDA) regulations require cooking ground beef hamburgers to an internal endpoint temperature of 71.1°C (instantly) for consumers; 68.3°C (16 second holding) for food service operations. *E. coli* O157:H7 can survive in frozen ground beef for nine months with little reduction in numbers (Doyle and Schoeni, 1984).

On June 30, 2002, the ConAgra Beef Company recalled 18.6 million pounds of fresh and frozen ground beef and beef trimmings nationwide. Eighteen reported illnesses in Colorado were caused by *E. coli* O157:H7 infected meat, and 8 more cases related to the outbreak were identified in six other states (California, Iowa, Michigan, South Dakota, Washington, and Wyoming) (CDC, 2002). Undercooked ground beef was also implicated in an outbreak of *E. coli* O157:H7 infections linked to the consumption of hamburgers from a popular restaurant chain. The infections were traced back to

contaminated, undercooked burgers from a local fast food chain. There were 51 cases of HUS and 4 deaths in Washington, Idaho, California, and Nevada, which reported more than 1000 laboratory-confirmed infections (CDC, 1993).

The state health departments of Michigan and Virginia reported in June and July 1997 an increased number of *E. coli* O157:H7 infections based on the numbers from the previous year. The outbreaks were traced back to alfalfa sprouts that were grown from seeds contaminated with *E. coli* O157:H7 (Breuer *et al.*, 2001). A similar case in Japan involved contaminated radish sprouts (Itoh *et al.*, 1998).

I. Methods of Detection and Isolation from Foods

Isolation of low levels of *E. coli* O157:H7 in food is difficult due to the presence of other bacteria that can interfere with detection. Typically, *E. coli* O157:H7 is isolated in stool samples of infected patients. Successful detection and isolation from a stool sample is attributable to the increased numbers of *E. coli* O157:H7 in infected patients relative to the amount of natural background flora (Anonymous, 1996).

Isolation of *E. coli* O157:H7 from a food sample begins in a selective enrichment followed by plating on sorbitol-MacConkey agar (SMAC). *E. coli* O157:H7 is negative (colorless colonies) on SMAC (sorbitol is present instead of lactose), unlike other *E. coli*, which do not ferment sorbitol (March and Ratnum, 1986 and Brooks *et al.*, 1998). Fecal sampling with SMAC medium is more accurate than with food samples, due to larger percentage of non-sorbitol fermenters that are in foods (March and Ratnum, 1989). Since, foods may contain lower levels of *E. coli* O157:H7 than fecal samples further presumptive tests are performed. The latex agglutination test uses a rabbit antibody that

is reactive with O157 antigen and is observed for agglutination (March and Ratnum, 1989). The latex agglutination test in conjunction with SMAC cultures can help detect *E. coli* O157:H7 in foods.

Almost all strains of *E. coli* O157:H7 are negative in the 4-methylumbelliferyl-B-D-glucuronide (MUG) assay. Most *E. coli* O157:H7 does not contain the enzyme B-glucuronidase, which cleaves MUG to produce a fluorescent product that appears with long-wave UV (CDC, 1994). Doyle and Schoeni (1984) tested eight different strains of *E. coli* O157:H7 with one exception testing positive in the MUG assay.

Confirmed tests for *E. coli* O157:H7 require identification of the H7 flagellar antigen and tests for the production of SLT's. If tests result in a nonmotile or negative for the H7 flagellar antigen, they are further tested for the production of SLT's.

There have been several foodborne outbreaks in various food products in recent years. The ability to identify outbreaks has been improved by the use of pulse-field gel electrophoresis (PFGE) and comparison patterns by PulseNet (CDC, 2000). PulseNet is the National Molecular Subtyping Network for Foodborne Disease Surveillance.

II. Apple Cider

A. Apples

In the United States alone there are approximately 2,500 known varieties of apples grown, and more than 7,500 grown worldwide (Anonymous, 2003). There are 36 states that grow apples commercially, and the top six producing states include (in millions of 42-lb units): Washington (116.6), New York (24.3), Michigan (23.0),

California (12.1), Pennsylvania (10.5) and Virginia (7.0). Of the 39% of apples that were used for processed products, 18% were used in juice and cider (Anonymous, 2003).

The Virginia Apple Board reports that 70% of Virginia apples are sold for processing into products such as: applesauce, apple juice, apple butter, slices and cider. The Virginia apple industry provides a major contribution to the state's economy with an estimated \$235 million annually (Anonymous, 2004b). Virginia produces eleven major varieties: Red delicious, Golden Delicious, York, Rome, Stayman, Winesap, Granny Smith, Jonathan, Gala, Ginger Gold, and Fuji.

B. Processing

The Processed Apple Institute (PAI) has given guidelines for handling, receiving, and production to the processed apple industry since 1974. This organization updates its guidelines as new technologies are developed and standards change. Apples that are determined to be "mature" (based on amount of sugar, firmness, seed, and skin color) are picked by hand or by a mechanical method. They are placed in canvas bags or lined buckets for transporting to a central loading area. Bruised, cut, damaged, and diseased apples are removed before storage (Anonymous, 2004a).

Apples are typically stored in controlled atmosphere storage (CA), where temperature, oxygen, carbon dioxide and humidity are controlled to delay further ripening in airtight warehouses (Anonymous, 2004b and 2004c). Cider apples are traditionally brushed and washed in water that does not contain additives prior to milling to reduce the risk of cider contamination to improve shelf-life. Apples are usually treated with a sanitizing rinse since using untreated water has been shown to be an ineffective

way to reduce contamination (Senkel *et al.*, 1999 and CDC, 1997). However, a sanitizing rinse may not be completely effective due to internalization of *E. coli* in cider apples and the resistance of *Cryptosporidium* oocysts to chlorine and iodine (Senkel *et al.*, 1999). The fruit is flumed from receiving stations to processing lines before they transported by dry conveyors through water sprays and scrubbers before processing.

There is no clear definition for apple cider, but cider can be defined as “juice from freshly squeezed apples separated from the pomace with no further clarification” (Kozempel *et al.*, 1998). The caramel color and opaqueness comes from apple solids in the juice that turn color when exposed to the air (Anonymous, 2003). Cider pH and °Brix is dependent on the apple varieties and where they are grown (Chikthimmah *et al.*, 2003). Cider is traditionally thermally pasteurized but in recent years it has been UV treated to kill microorganisms that might be present. Pasteurization treatment can assure safety of the product either before or after bottling.

The PAI recommends that samples from the beginning, middle, and end of production lot be collected and stored for inspection and testing. After inspection and testing any product that does not meet quality standards is recognized and subjected to appropriate corrective actions (Anonymous, 2004a).

C. Outbreaks

An outbreak of *E. coli* O157:H7 due to the consumption of unpasteurized apple juice occurred in October 1996 in Connecticut (CDC, 1997). The cider was processed at a small mill and contamination was believed to be due to the use of dropped apples for cider production. Fourteen cases of *E. coli* O157:H7 infections were reported.

On October 30, 1996 *E. coli* O157:H7 was implicated in an outbreak in unpasteurized apple cider (CDC, 1996). According to the Seattle-King County Department of Public Health, Odwalla brand unpasteurized apple juice and juice mixtures containing apple juice were associated with the outbreak. Forty-five cases of *E. coli* O157:H7 infection in British Columbia, California, Colorado, and Washington State were reported. Odwalla Inc. issued a voluntary recall of Odwalla brand pure apple juices and 12 other blended juices containing apple juice at the request of the FDA (FDA, 1996).

Twenty-three cases of *E. coli* O157:H7 associated outbreaks were linked to the consumption of fresh-pressed apple cider, in the fall of 1991. The cider was unpasteurized, contained no preservatives, and had a pH between 3.7 - 3.9. The cider was made with unwashed “dropped” apples from a local farm. The source of *E. coli* O157:H7 contamination was not confirmed, but contamination by cow manure of the dropped apples was suspected (Besser *et al.*, 1993).

III. Processing

A. Thermal Pasteurization

Thermal pasteurization is recognized as the most effective method for reducing contamination in apple cider (Senkel *et al.*, 1999). The lethality of a thermal process is based on the D- and Z-value of the target microorganism in a food product. Small juice producers and larger companies typically pasteurize apple juice with batch and ultra-high temperature (UHT) pasteurization, respectively (Anonymous, 1997).

Batch or vat pasteurization requires juice to be heated to 145°F and held for 30 minutes, whereas, UHT is < 250°F for 0.1 seconds. These processes can cause more

unfavorable flavor qualities than high-temperature / short-time (HTST) pasteurization which is rarely used in the juice industry. Improved quality and safety of the juice is minimized because the juice is subjected to 161°F for 15 seconds (Anonymous, 1997). This method requires refrigeration of the product to retard the growth of microorganisms and to extend shelf-life.

Thermal pasteurization is proven to be an effective safety treatment for fresh juices, but can be economically unfeasible to implement in small juice production. Thermal pasteurization units in the past have ranged from \$163,000-185,000 depending on the type of unit purchased (Kozempel *et al.*, 1998). Thermal pasteurization is a costly solution with undesirable effects on the quality of the final product.

B. Alternatives Processing Technologies

Alternative processes are becoming more prevalent in improving the safety and quality of juice products. Ultimately, alternative processes are being explored in the hope of providing the safety of traditional thermal pasteurization techniques without compromising the organoleptic qualities of juices. An “alternative process” in the juice industry would be an alternative to thermal processing. Some alternative processes that are applicable to juice processing include: high pressure processing, pulsed electric field, pulsed x-ray, UV, ohmic heating, inductive heating, pulsed light, combined UV and low concentration hydrogen peroxide, ultrasound, filtration, oscillating magnetic fields, and antimicrobial treatments (FDA, 2000).

Under the current Juice HACCP regulations, apple cider processors are required to treat the juice to achieve at least a 5-log reduction of the pertinent pathogen. At the

time the final ruling was made the FDA was not aware of processing technology that could produce a 5-log reduction in apple juice products without a “kill step.” The “kill step” did not have to be pasteurization, allowing the potential use of alternative processing technologies to achieve a 5-log reduction (FDA, 2001a). Recently, there has been an effort to develop and research alternative processes that can achieve the 5-log reduction of pertinent pathogens required under Juice HACCP regulations. Alternative processing can provide smaller entities more options to meet the required Juice HACCP regulations.

IV. Ultraviolet Light (UV)

A. General

UV processing uses radiation from light with wavelengths shorter than the violet end of the visible spectrum. Wavelength for the UV spectrum ranges from 100 to 400 nm (Bolton, 1999). This UV range is divided into three subdivisions: UVA (315 to 400 nm) causes changes to skin that leads to tanning in humans, UVB (280 to 315 nm) causes burning of the skin and may lead to cancer, UVC (200 to 280 nm, germicidal range) is effective against inactivating bacteria and viruses, and the vacuum range (100 to 200 nm) is absorbed by almost all substances and can only be transmitted in a vacuum (Fraise *et al.*, 2004 and Bolton, 1999).

B. Susceptibility of Microorganism to UV

Populations of microorganisms undergo inactivation by UV in the shape of a sigmoidal curve. According to Sastry *et al.* (2000) microorganisms in response to an

injury phase produce the initial plateau. After the initial plateau, the maximum amount of injury is reached; so minimal UV exposure will produce increased lethality in microbial populations. Microbial resistance to UV and the presence of suspended solids block UV may produce the tail end of the inactivation curve.

Chemical disinfectants destroy or damage a microorganism's cellular structure, whereas UV inactivates the microbe by damaging its DNA. The germicidal properties of UV irradiation are caused by mutations of DNA molecules that have a maximum absorbance of UV at approximately 254 nm, the wavelength that most commercial UV lamps emit UV (Fraise *et al.*, 2004). Sterilizing UV (UVC) penetrates the outer structure of the cell and produces crosslinks between successive pyrimidines on cellular DNA strands forming dimers, which prevent cells from replicating, leading to cell death (Bolton, 1999 and Shechmeister, 1991). UVC is the most effective in disinfecting smooth surfaces because light is not scattered and the surface is in the direct path of the beam (Yaun *et al.*, 2003).

The most important factors that make bacteria susceptible to UV are (1) pH, (2) sensitivity to different stage of the bacterial growth phase (logarithmic phase cells are most susceptible), (3) and the ability to form spores (Shechmeister, 1991). Vegetative cells are more susceptible to UV radiation than bacterial spores, but the degree of sporulation can affect sensitivity (Fraise *et al.*, 2004). Yaun *et al.* (2000) demonstrated that *E. coli* O157:H7 is more susceptible to UV inactivation than *Salmonella* on smooth, agar surfaces. Viruses and cysts of waterborne protozoa (i.e. *Giardia lamblia* and *C. parvum*) are also inactivated by UV, and are more resistant than non-sporulating bacteria but less resistant compared to spore producing bacteria (Fraise *et al.*, 2004).

C. Factors that Affect UV Sterilization

There are many variables that influence the efficacy of UV processing. These variables include: the light intensity that is emitted by the lamps, the amount of time the microorganisms are exposed to the UV, and the ability of the UV to transmit its energy through the processing medium to the microorganism. In liquid mediums, as the absorptivity coefficient increases the required D-value needed to inactivate *E. coli* increases (Oteiza *et al.*, 2005).

UV processing technology is being used as an alternative to pasteurization for the reduction of bacteria in cider. One drawback of UV is its poor ability to penetrate food substances. Ngadi *et al.* (2003) determined that by reducing fluid thickness that vegetative pathogenic cells are more susceptible to UV in foods that have low UV transmission. It is more difficult to inactivate microorganisms in food substances that are more turbid or contain more suspended solids. The blockage of light due to suspended solids helps shield the microbes. Therefore, it is important to expose apple cider in a thin film to UV. The design of a UV sterilization unit depends on UV intensity and dose, penetration depth, and the pertinent microorganism to absorb UV (Ngadi *et al.*, 2003).

D. Applications

UV has been used for various processes for the inactivation of microorganisms. UV has been used as an antimicrobial application in water treatment, surface and air disinfection, and in prepared foods and food containers (Blatchley and Peel, 2001). UV is not used for sterilization through solids since there is little or no penetration, and in

glass and plastics that can readily absorb UV radiation (Russell *et al.*, 1999b). In order for UV radiation to be effective as sterilization agent on these surfaces impractical UV doses must be applied.

UV technology has become a more popular alternative to chlorination for the treatment of drinking water from municipal wells in parts of the United States. The UV process does not impart any tastes or odors to the water, and does not form any harmful byproducts that chlorine has been known to produce (Protasowicki, 2002). UV has yet to be approved by the U. S. Environmental Protection Agency (EPA) for drinking water disinfection under the Surface Water Treatment Rule (SWTR), but state regulatory agencies have approved UV treatment on a case-by-case basis (Protasowicki, 2002).

E. Treatment of Apple Cider

The conventional method for reducing microorganism populations in apple cider is thermal pasteurization. The advantage of UV processing is that it preserves the sensory qualities of fresh, non-thermally pasteurized apple cider. UV treated cider has no significant differences in taste compared to fresh unpasteurized ciders (Tandon *et al.*, 2003). In addition, UV processing has been tested and proven to be effective in reducing microbial populations in apple cider. Wright *et al.* (2000b) used a thin film UV disinfection unit (peak output at 254 nm) to get a mean reduction of 3.81 log CFU/ml *E. coli* O157:H7, for treated samples subjected to a dosage ranging from 9.402 to 61,005 $\mu\text{W}\cdot\text{s}/\text{cm}^2$. Their results determined that in order to produce a 5-log reduction, an additional reduction measure would be necessary. Worobo (1999) showed that in a

single pass through the CiderSure® 3500A UV disinfection unit, *E. coli* O157:H7 strains reduced 5.83 to 6.12 log CFU/ml.

Hanes *et al.* (2002) showed that exposure to 14.32 mJ/cm² of UV irradiation for ≤ 1.9 seconds can reduce *C. parvum* oocysts in apple cider by a 5-log oocysts/ml reduction. Their results determined that UV processing is an effective method for reducing *C. parvum* in fresh apple cider. UV has a limited effect on yeasts and molds in apple cider, yet produces a quality cider with a reduced shelf-life compared to thermally pasteurized cider (Tandon *et al.*, 2003). Differences in UV susceptibility of *E. coli* O157:H7 have been shown in multiple varieties of apple cultivars used to produce apple cider (Basaran *et al.*, 2004). Regardless of the cultivars used, Basaran *et al.* (2004) showed that UV (14 mJ/cm²) is capable of producing ≥ 5-log CFU/ml reduction of various strains of *E. coli* O157:H7. UV treatment is effective in reducing *E. coli* O157:H7 populations in multiple apple cider varieties.

V. Prevention and Control Measures

A. Contamination Sources

Targeting contamination sources may drastically improve the safety of apple supply before apple cider production. Contamination of a single apple may affect an entire batch of cider produced. The most likely source of contamination comes from fecal material from such animals as cattle, deer, and sheep that harbor target pathogens (FDA, 1999). Other possible sources of contamination can come from poor worker-hygiene, birds, rodents, and insects. Direct contamination of the apples can occur when apples are dropped on the ground and may come in contact with feces (FDA, 1999).

Contaminated crates, irrigation and spraying water and windfall can indirectly spread target microorganisms during the growing and harvesting phase of production (FDA, 1999).

B. Cleanliness

Contamination can occur in the washing stage if tubs and flumes do not contain water that is frequently changed, or when water is used from an unprotected and untested source (FDA, 1999). Apples can remain in flumes and baths for as little as one to two minutes or as long as 30-45 minutes. The flume water typically contains chlorine dioxide, hypochlorite or other chlorine compounds, since the water is recirculated and this aids in controlling microbial buildup (Anonymous, 2004a).

C. Niches

Apples typically undergo a washing step by immersion into a dump tank or low pressure spray. This step is effective in the removal of dirt, pesticide residue and some microbial contaminants, but is ineffective for the removal of well-adhered contaminants. *E. coli* has the ability to adhere to the calyx and stem portions of apples better than on the skin surface and can grow in punctures (Sapers *et al.*, 2000). Therefore, *E. coli* O157:H7 can internalize and survive surface disinfection that prepares apples from pressing.

VI. Regulations

A. Juice HACCP

Unpasteurized fruit and vegetable juice products have been associated with numerous foodborne outbreaks in recent years. The FDA proposed a new plan to improve the safety of fresh juice on August 26, 1997 (FDA, 1997). On January 19, 2001 the FDA passed a ruling that required all juice producers, regardless of size, to implement a HACCP program with a 5-log reduction performance criterion (66 FR 6138) (FDA, 2001a). This ruling followed the juice labeling rule, which required “all juice shipped in interstate commerce or made from ingredients shipped in interstate commerce, including that produced by small businesses, that has not been processed to achieve a 5-log reduction in pathogens must be labeled with a warning to consumers” (§ 101.17) (FDA, 2001a). The juice labeling rule allowed small producers to continue selling cider with the following statement: “WARNING: This product may contain harmful bacteria which can cause serious illness in children, elderly, and persons with weakened immune systems” (FDA, 1998b). The FDA estimates that 140 juice-related illnesses were prevented yearly, as a result of the juice labeling rule making more consumers aware of consuming untreated juice (FDA, 2001a).

The FDA reluctantly gave temporary alternatives to small entities to relieve the financial burden of developing a HACCP program. The first alternative provided an exemption to small entities “that [made] juice on their premises and whose total sales of juice and juice products [did] not exceed 40,000 gallons per year and [sold] directly to consumers and retailers” (FDA, 2001a). These businesses were required to label their packaged products sold according to the labeling rule. The second alternative gave small entities an extension of the HACCP compliance period, giving the smallest businesses more time to meet the terms of the final ruling. A one year extension was given to small

firms (< 1000 employees) and two years to the smallest (< 100 employees), saving each entity approximately \$1,000-31,000 and \$900-61,000 during the compliance period, respectively. The amount of savings was directly related to the time in which the process was expedited (FDA, 2001a). Effective January 22, 2002 the FDA required small and very small businesses to develop and implement HACCP systems for their processing operations. On January 21, 2003 small business regulations became effective, and very small businesses were required to comply as of January 20, 2004 (FDA, 2003). The only exemption to the regulations are those that are produced by a retail only establishment (§ 120.3). Retail establishments are defined as operations that only provide juice directly to consumers. “‘Provides’ includes storing, preparing, packaging, serving, and vending” as long as the establishment does not sell or distribute juice to other businesses (FDA, 2001a).

Implementation of a HACCP program showed significant reduction in bacterial contamination compared to previous seasons without a safety program (Senkel *et al.*, 1999). Juice HACCP helps processors improve sanitation, fruit treatment, and processing of the juice to reduce the potential for foodborne illnesses.

B. Definition of Juice

The FDA defines juice as “the aqueous liquid expressed or extracted from one or more fruits or vegetables, purees of the edible portions of one or more fruits or vegetables or any concentrations of such liquid or puree (§ 120.1)” (FDA, 1999). Juice HACCP principles apply to all processors that produce a “100 percent juice or a concentrate of that juice for subsequent beverage” (FDA, 2003). If a beverage consists of less than 100

percent juice, the juice ingredient is required to be produced under HACCP regulations (FDA, 2003).

VII. Chemical Preservatives in Foods

A. General

Chemical preservatives play a key role in food preservation. They extend product shelf-life, aid in retention of wholesomeness and improve the safety of the food supply through delaying or preventing microbial decomposition and by inhibiting or hindering growth of pathogens (Foegeding and Busta, 1991). Foodborne outbreaks caused by pathogens such as *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* spp. has concerned the food industry about the safety of their products. Foods that contain undesirable microbial growth can make foods unfit for human consumption and affect the food supply worldwide (Russell *et al.*, 1999a). Physical microbial control processes, such as thermal processing, can be limited due to the characteristics of certain food products. Chemical preservatives are commonly used in combination with physical processes to control microbial growth in foods. The efficacy of a chemical food preservative is based on the interaction of the food product with its chemical and physical properties (Foegeding and Busta, 1991).

B. Use and Efficacy for Control of *E. coli* O157:H7

Sodium benzoate and potassium sorbate are commonly used food preservatives, but have minimal effect on *E. coli* O157:H7 at acceptable concentrations. Sodium benzoate (0.1%) is capable of producing a 5-log reduction of *E. coli* O157:H7 with

unfavorable flavor qualities at increased concentrations ($> 0.0125\%$) (Zhao *et al.*, 1993 and Salunkhe, 1955). Sodium benzoate is eight times more effective in reducing the heat resistance of *E. coli* O157:H7 than potassium sorbate (Splittstoesser *et al.*, 1995).

Potassium sorbate has a reduced effect on *E. coli* O157:H7 at lower temperatures and pH (Tsai and Chou, 1996). Apple cider (pH 4.1) treated with 0.1% sorbic acid at various storage times then subjected to freeze-thawing (48 h at -20°C ; 4 h at 4°C) can produce a 5-log reduction of *E. coli* O157:H7 (Uljas and Ingham, 1999). Comes and Beelman (2002) showed that a combination of fumaric acid (0.15%, wt/vol) and sodium benzoate (0.05%, wt/vol) is capable of a 5-log reduction in apple cider followed by a holding period (25°C for 6 h). Increasing pH (3.2 to 4.7) and decreased temperature (5°C) storage decreases the rate of destruction using the fumaric acid/sodium benzoate (0.15/0.05%) combination (Chikthimmah *et al.*, 2003). Although, sodium benzoate and/or potassium sorbate is ineffective for controlling *E. coli* O157:H7 but can be useful in conjunction with a pasteurization process.

C. Sodium Benzoate

Benzoic acid was first identified as an antifungal agent in 1875 (Fraise *et al.*, 2004). Sodium benzoate is the more water-soluble salt form of benzoic acid ($\text{C}_6\text{H}_5\text{COOH}$), which has a sweet faint balsamic odor and sweet sour to astringent taste (Foegeding and Busta, 1991 and Burdock, 2002). The sodium benzoate in the form of white granules, crystalline powder or flakes dissolves in water (66 g/100 ml at 20°C) (Burdock, 2002 and Davidson *et al.*, 2002). It is generally recognized as safe (GRAS)

substance, and one of the most widely used preservatives in the U. S. and other countries, due in most part to its low cost.

Sodium benzoate mechanism for inactivation involves the disruption of the cell membranes. Nutrients are not as readily available to the cell due to the effect on the permeability to the cell membrane (Freese *et al.*, 1973). The undissociated, molecular form of benzoic acid is more effective at permeating cell membranes than the dissociated form (Foegeding and Busta, 1991). A microorganism's uptake and ability of the cells to transport the compound out of the cell affects its sensitivity to sodium benzoate (Russell *et al.*, 1999a). The optimum pH for sodium benzoate antimicrobial activity is between 2.5 and 4.0, and its effectiveness against microorganisms is lower when pH is greater than 4.5 (Russell *et al.*, 1999a).

Sodium benzoate is readily used as a preservative in acid or acidified food products like fruit juices, beverages and many other food products. It is a natural component in many foods (i.e. berries, cinnamon, etc.) but it is commonly used at concentrations of 0.03-0.30% and 0.00075-1.25% in the U.S. and other countries, respectively (Russell *et al.*, 1999a). The typical concentration of sodium benzoate in apple cider is 0.05-0.1% (Davidson *et al.*, 2002). Sodium benzoate is also effective against *E. coli* O157:H7 by reducing its heat resistance (Splittstoesser *et al.*, 1995). Processors that add sodium benzoate to apple cider can increase the thermal death time at higher temperatures (70°C) and may obtain less than a 5-log reduction (Dock *et al.*, 2000).

D. Potassium Sorbate

Potassium sorbate is a widely used preservative in the food industry. It was first derived from the mountain ash tree and discovered by the French in the 1850's, but is now manufactured by organic synthesis (Burdock, 2002). Potassium sorbate is the water-soluble salt form of sorbic acid ($\text{CH}_3\text{-CH=CH-CH=CH-COOH}$), a *trans-trans* unsaturated fatty acid (Foegeding and Busta, 1991). Potassium sorbate is a white, fluffy powder with a distinctive odor and sour taste (Foegeding and Busta, 1991). It is the most frequently used salt form of sorbic acid and is highly water-soluble (58.2 g/100ml at 20°C) and decomposes at about 270°C (Davidson *et al.*, 2002 and Burdock, 2002). Potassium sorbate is an inexpensive preservative, and is rather tasteless and odorless in food. It is a GRAS substance, but is commonly used between 0.1 to 0.2% (Foegeding and Busta, 1991).

The inhibitory action of potassium sorbate is caused by the inhibition of enzyme and nutrient transport (Foegeding and Busta, 1991). Many enzymes (e.g. enolase, lactate dehydrogenase, fumarase, etc.) are affected by sorbates at different levels (Foegeding and Busta, 1991). Potassium sorbate reduces the heat resistance of *E. coli* O157:H7 (Splittstoesser *et al.*, 1995). The effectiveness of potassium sorbate is the greatest when the pH < 6.5 (Davidson *et al.*, 2002). The pH of apple cider also aids in reducing the heat resistance of *E. coli* O157:H7. Sorbates are used in cheese products, baked goods, fruits, vegetables, wines, soft drinks and other food products to extend shelf life and for mold and yeast inhibition (Foegeding and Busta, 1991).

E. Hydrogen Peroxide

Hydrogen peroxide was first discovered in 1818 by French chemist Thénard, from a reaction of hydrochloric acid on barium dioxide that produced oxygen. Hydrogen peroxide (H_2O_2) is a colorless, water miscible liquid with a nitrous smell that obtained by hydrolysis of other peroxides such as peroxidisulfuric acid (Foegeding and Busta, 1991). It has been used as a disinfectant for milk preservation, water, and fruit juices in many countries, including the U. S., since 1913 (Fraise *et al.*, 2004).

Hydrogen peroxide is an effective antimicrobial agent against a variety of microorganisms (bacteria, fungi, yeast, spores, viruses), even those having defense mechanisms against oxidizing agents (Fraise *et al.*, 2004 and Naidu, 2000). At the proper concentration, hydrogen peroxide works quickly against microorganisms (Foegeding and Busta, 1991). The antimicrobial activity is a function of concentration and contact time, and is most effective against Gram-negative bacteria than Gram-positive bacteria (Fraise *et al.*, 2004). The local formation of highly active hydroxyl radicals, $\text{OH}\bullet$, are attributed to the efficacy of hydrogen peroxide (Fraise *et al.*, 2004). Hydroxyl radicals are among the strongest oxidants, and they are known to disrupt membrane lipids, DNA, and double bonds of significant cell components (Fraise *et al.*, 2004). Bayliss and Waites (1979) discovered that the efficacy hydrogen peroxide in low concentrations was enhanced in combination with UV radiation for the reduction of bacterial spores.

Hydrogen peroxide is GRAS in the U. S., and its typical uses in the food industry are with raw milk (0.05%), a variety of cheeses and addition to whey (0.04%) and starch (0.15%) (Russell *et al.*, 1999a). When the raw milk is treated with H_2O_2 it can be pasteurized at a lower temperature (Foegeding and Busta, 1991). It is also used to

decontaminate packaging materials for aseptic processing of juices and other foods, as an oxidizing and bleaching agent, and it inhibits microbial spoilage in fish marinades (Russell *et al.*, 1999a).

The use of hydrogen peroxide can be beneficial to the juice industry. Sapers *et al.* (2000) confirmed that washing apples in a 5% H₂O₂ solution reduced populations by < 3-log. Decontamination was less effective when followed by a rinse step, due to residual H₂O₂ concentration decreasing considerably (2000 mg/L to 20-50 mg/L). The FDA allows the use of hydrogen peroxide as a wash for fruits and vegetables that are not considered raw agricultural commodities when it is used in low concentrations in combination with acetic acid to form peroxyacetic acid (FDA, 2002a). The EPA approved the use of ≤ 1% H₂O₂ applied to all post-harvest agricultural commodities from the requirement of a tolerance (FDA, 2002b). Recent studies have shown that a 1% H₂O₂ treatment can be an effective alternative to chlorinated washes for inactivating human pathogens, i.e. *E. coli* O157:H7, attached to produce (Sapers *et al.*, 2003).

F. Dimethyl Dicarbonate

Dimethyl dicarbonate (DMDC) (CH₃-O-O-C-O-C-O-O-CH₃) is a dicarbonic ester that is colorless liquid with a fruity aroma (Ough, 1993). DMDC is unstable in aqueous solutions and hydrolyzes to methanol and CO₂ (Ough, 1993). It can be used as a microbial control agent in non-carbonated juice beverages containing up to and including 100 percent juice (FDA, 2004). DMDC may be used in foods as a food contact substance. According to the FDA, no more than 250 ppm of DMDC may be added to non-carbonated juice beverages containing up to and including 100 percent juice. DMDC

complies with the requirements listed in 21 CFR 172.133 (a) and (c). The beverages must be produced under good manufacturing conditions and their microbial load must first be reduced by current technologies such as heat treatment, filtration, etc., prior to the addition of DMDC (FDA, 2004).

The inhibition of microorganisms is due to the inactivation of intracellular enzymes (Ough, 1993). According to Foegeding and Busta (1991), dimethyl dicarbonate does not react with sugars or artificial sweeteners in sports drinks. Microorganisms must be inactivated prior to DMDC hydrolysis in order for the molecule to be effective (Ough, 1993). Hydrolysis occurs at a faster rate at higher temperatures (Ough, 1993). Fisher and Golden (1998) reported that DMDC was effective against *E. coli* O157:H7 in apple cider at 25°C. Uncarbonated fruit juices may require larger doses of DMDC and/or a more vigorous pretreatment to reduce microbial load prior to the addition of DMDC (Cirigliano *et al.*, 1998).

Bayer Corporation's Organic Chemicals Business Group originally marketed DMDC as Velcorin® cold sterilant for beverages, but LANXESS Corporation took over production in 2005 (Iams, 2000 and Anonymous, 2005). It is commonly used as yeast inhibitor in wine, ready-to-drink tea beverages, sport drinks and fruit or juice sparklers (Iams, 2000). Velcorin® is added directly to the juice immediately before it is filled in conventional packaging materials such as glass, metal or plastic (Bolz, 2003). It is used at 0.025% in these products (Davidson *et al.*, 2002). DMDC is also approved for use in single strength juices since it also exhibits inhibitory action in molds and bacteria (Fisher and Golden, 1998). According to Cirigliano *et al.* (1998) the microbial threshold in beverages must be lowered in order for DMDC to be more effective. DMDC is most

effective when used in conjunction with a preliminary treatment, e.g. pasteurization, to reduce microbial counts (Cirigliano *et al.*, 1998).

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**Efficacy of Ultraviolet Light in Combination with Chemical Preservatives
for the Reduction of *Escherichia coli* in Apple Cider**

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Keywords: *Escherichia coli*, Apple Cider, Ultraviolet, Preservatives, Dimethyl Dicarboxylate, Hydrogen Peroxide, Potassium Sorbate, Sodium Benzoate

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INTRODUCTION

Outbreaks of foodborne illnesses caused by *Escherichia coli* O157:H7 and other pathogens have been associated with consumption of unpasteurized apple cider and other juices. As a result, the FDA implemented hazard analysis critical control point (HACCP) regulations that require juice processors to utilize a process that will produce a 5-log reduction of the pertinent pathogen in the juice being processed (FDA, 2001). The final ruling for small businesses to implement a HACCP program was binding as of January 20, 2004; therefore, this rule is currently in effect for juice processors in the United States.

Cider producers are faced with the responsibility of applying an effective process to comply with current HACCP regulations. Thermal pasteurization has been the most commonly used process to improve the safety and shelf-life of apple cider. However, the costs of setting up and maintaining a pasteurization unit may be cost prohibitive for many small processors, and the number of cider processors that are considered small businesses is substantial. A 1998 survey performed by Wright *et al.* (2000a) revealed that 59 percent of apple cider producers in Virginia produce less than 5,000 gallons of apple cider annually. Furthermore, most cider processors generate a majority of their income in the peak four-week fall season to cover operating and production costs (McCandless, 1998).

The initial capital investment for an installed thermal pasteurization unit has been estimated to be upwards of \$185,000, which is unfeasible for many small cider producers (Kozempel *et al.*, 1998). In addition to the initial capital cost, annual operating costs can reach \$93,000. An investment in thermal pasteurization units may be unfeasible for

small cider producers, and low-cost, safe alternatives are needed. In addition to the cost of thermal pasteurization, some believe that heating causes negative organoleptic effects that make the pasteurized apple ciders less appealing than fresh, unheated cider.

Nonthermal processing alternatives for cider, such as ultraviolet (UV) light, have been a focal point of current research in food microbiology. Until relatively recent times, UV radiation had not been used as a processing method for commercially produced apple cider due to poor UV transmission in fluids that contain suspended solids. However, recent advances in UV technology have resulted in processes that are effective for reducing pathogens, and there has been growing interest among cider processors in the use of UV. Surveys have revealed that 80% of Virginia apple cider producers were interested in alternative processing methods (Wright *et al.*, 2000a).

Since the FDA approved UV processing in 2000, commercial use of UV for the 5-log reduction of pertinent pathogens has gained popularity. Equipment manufacturers have designed UV processing systems specifically for use in the juice industry. The CiderSure® 3500 was designed to produce a 5-log reduction or greater in fresh apple cider, by automatically adjusting to differences in total solids and color in apple cider varieties to ensure that a minimum effective UV dose is delivered to the product (Worobo, 1999).

Although research has shown that UV processing can produce a 5-log reduction of *E. coli* O157:H7 in apple cider, little work has been performed to determine the effect of UV when used in combination with chemical preservatives that are commonly used for juices or juice products. It is known that UV treatment alone is not effective against spoilage microorganisms, such as yeasts and molds in apple cider (Tandon *et al.*, 2003).

Chemical preservatives such as hydrogen peroxide, dimethyl dicarbonate, potassium sorbate and sodium benzoate have been used to control spoilage yeasts and molds in beverages (Fraise *et al.*, 2004, Cirigliano *et al.*, 1998 and Foegeding and Busta, 1991).

OBJECTIVES

The specific objectives of this research were:

1. To determine if chemical preservatives and ultraviolet light have a combined effect on the reduction of *Escherichia coli* in apple cider.
2. To determine the effect of chemical preservative addition prior to and after ultraviolet light processing on the reduction of *Escherichia coli* in apple cider.

MATERIALS AND METHODS

Culture Purification and Preparation –

A culture of *E. coli* ATCC 25922, a surrogate for *E. coli* O157:H7, from frozen medium was grown in Tryptic Soy Broth (TSB; Difco; Becton-Dickinson; Sparks, MD) at 35°C and consecutively transferred (10 µl) at 24 h intervals into fresh TSB. A portion of the culture (10 µl) was streaked onto sorbitol-MacConkey agar (SMAC; Difco; Becton-Dickinson; Sparks, MD) and incubated at 35°C for 24 h. A typical colony from the SMAC was streaked onto Eosin Methylene Blue Agar (EMB; Difco; Becton-Dickinson; Sparks, MD) and incubated at 35°C for 24 h to select for *E. coli*. A typical colony from the EMB plate was streaked onto a Tryptic Soy Agar (TSA; Difco; Becton-Dickinson; Sparks, MD) slant and grown at 35°C for 24 h. An API 20E identification strip (BioMérieux; Marcy l'Etoile, France) was used to further identify the culture as *E. coli*. To prepare stock cultures, *E. coli* ATCC 25922 was grown in TSB at 35°C for 24 h prior to cryogenic freezing. Purity of the culture was assessed periodically to ensure that no contamination of stock cultures occurred; the culture was plated on selective media (i.e. SMAC, EMB) for 24 at 35°C followed by testing on an API 20E identification strip. Additionally, an isolated colony was Gram stained to confirm the presence of small, rod-shaped, Gram-negative bacteria. All media for culture purification and preparation throughout these studies were prepared according to manufacturers specifications.

Apple Cider –

A commercial apple cider (Murray Cider Company, Inc. - Roanoke, VA 24019) was used in this project. This unfiltered apple cider (pH ~3.7) was thermally treated, shelf-stable with no colors or preservatives added. Since, studies were conducted at 4°C (initial temp.) the apple cider was stored under refrigeration prior to experimentation. The apple cider was analyzed for pH, °Brix, and background microflora prior to and during experimentation. In order to assess background microflora, uninoculated cider (0.1 ml) was surface plated in duplicate onto TSA and incubated at 35°C for 24 h.

Preparation of Inoculum and Inoculation of Apple Cider –

E. coli ATCC 25922 was grown in TSB (10 ml) at 35°C for 24 h. The culture was centrifuged (11,000 x g for 10 minutes) (Sorvall Superspeed Refrigerated Centrifuge w/ Sorvall SS34 rotor head, Dupont Industries; Wilmington, DE) and resuspended in sterile 0.1% peptone diluent (PW) (Becton, Dickinson, and Company; Sparks, MD). Inoculated cider was tested to determine initial inoculum level prior to UV exposure by serially diluting and surface plating (0.1 ml) on TSA. Plates were incubated at 35°C for 24 h.

Apple cider (1980 ml) was transferred to sterile (2L) media bottles. The juice was inoculated with the 24 h resuspended culture (20 ml) to achieve an initial population of approximately 7 log CFU /ml, and was mixed by the arc method (10 arcs) prior to UV treatment or addition of chemical preservatives. Stir bars were not used to avoid physical hazards entering the UV processor and damaging the pump.

UV Treatment System –

All studies were performed using the CiderSure® 3500A (FPE Inc., Rochester, NY) thin film UV system at 4°C. Inoculated apple cider in every treatment was processed at a constant flow rate in a processing tube containing eight germicidal lamps. The inoculated cider was pumped through the CiderSure® 3500A (peak output 254 nm; dosage determination based on proprietary algorithms) in a thin film (< 0.0035 in) using a positive displacement pump. Sensor values from the UV apparatus were manually recorded to calculate the approximate UV dose applied to the cider. Sensors were located on the outermost wall of the interior of the flow chamber (**Appendix I**). The flow rate through UV treatment chamber was 15.3 liters of cider/min.

Cleaning and Sanitation –

The CiderSure® 3500A was cleaned, sanitized, and rinsed, prior to and after each treatment. The apparatus was first cleaned with warm tap water on the wash cycle for 5 min. Then a diluted sodium hypochlorite solution was pumped through the unit for sanitation for 10 min. The UV lamps were turned on before the unit was rinsed with warm water that was run through for 1 min. An additional rinse step followed for 1 min with the UV lamps turned on before samples were processed. Samples were prepared to process following the final rinse step.

All collection vessels were cleaned with tap water and autoclaved prior to each experiment. Collection vessels were 10-liter Nalgene® (Nalge Nunc International; Rochester, NY), autoclavable (15 min, 121°C), graduated polypropylene buckets. Collection vessels were sealed with aluminum foil during sterilization and storage.

Chemical Preservatives –

Studies performed to determine the combined effect of UV and antimicrobials agents utilized potassium sorbate (KS; 99% purity; Avocado Research Chemicals; Heysham, Lancs., England), sodium benzoate (NaB; granular; Fisher Scientific; Fair Lawn, NJ), dimethyl dicarbonate (DMDC; Sigma-Aldrich; Steinheim, Germany), and hydrogen peroxide (HP; 30%; Fisher Scientific; Fair Lawn, NJ) added directly to the juices. Test levels of each antimicrobial were determined based upon minimum effective levels reported in literature and through preliminary experimentation. The effect of NaB (1000 and 2000 ppm), KS (1000 and 2000 ppm), HP (75 and 150 ppm) and DMDC (75 and 150 ppm) added prior to UV exposure at 4°C was determined. The effect of adding each antimicrobial agent after exposing inoculated cider to UV at 4°C was also determined. The juices treated with the antimicrobial were mixed by using the arc method (10 arcs) prior to introduction of UV.

Application of UV on Apple Cider Containing Preservatives –

Refrigerated (4°C) juice was kept in an ice-water bath prior to sampling to maintain the temperature. Two liters of inoculated apple cider with each chemical preservative (as described below) was processed for each repetition. Uninoculated juice, which was run through the unit without UV, served as a negative control. Samples were mixed using the arc method (10 arcs). Samples (~50 ml) were collected in sterile 50 ml disposable centrifuge tubes. Samples taken before and after processing were serially diluted in PW and surface plated onto TSA. Plates were incubated at 35°C for 48-h prior

to colony enumeration. Collected samples (stored at 4°C) were plated at 0, 24, 48, and 72 h.

Addition of Chemical Preservative to Juice Following UV Exposure –

Immediately after UV exposure, juice (~500 ml) was collected in sterile 500 ml media bottles. The UV- treated cider (250 ml) was then transferred using sterile 250 ml graduated cylinders to sterile 250 ml media bottles for addition of chemical preservatives and subsequent storage (4°C). UV-treated juices were mixed to disperse the added chemical preservatives using the arc method (10 arcs). Samples taken before and after processing were serially diluted in PW and surface plated onto TSA. Plates were incubated at 35°C for 48 h prior to colony enumeration. Collected samples (stored at 4°C) were plated at 0, 24, 48, and 72 h.

Additional Measurements –

Initial and final readings of pH and temperature were taken for each experimental unit. The pH (Accumet Model 15 pH Meter; Denver Instrument Company; USA) of the apple cider was measured before and after processing. The pH meter was calibrated with pH 4 and 7 buffer solution (Fisher Scientific; Fair Lawn, NJ) before each use. The thermometer was sanitized with a 70% ethanol (ETOH; CDA19 denatured; Fisher Scientific; Fair Lawn, NJ) solution before and after sampling. The temperature of the juice was measured before and after sampling. The representative samples of soluble solids (°Brix, Abbe Mark II Digital Refractometer; Cambridge Instruments, Inc.; Buffalo, NY) in the apple cider were measured.

Statistical Analysis –

The entire experiment was performed in triplicate. Recovery of *E. coli* O157:H7 (log CFU/ml) by direct plating was statistically analyzed using the mixed procedure (PROC MIXED) of SAS version 9.1 (SAS Institute, Cary, NC). Mean separation test was performed using the Tukey-Kramer procedure. The experimental design for the complete study was a 2 x 4 x 2 factorial experiment in randomized complete block design with repeated measures and sub-sampling at each time point.

RESULTS

Populations of background microflora in the apple cider used for this study were consistently undetectable (< 1.0 log CFU/ml). Initial populations of *E. coli* ATCC 25922 in inoculated cider were approximately 7.0 log CFU/ml and the limit of detection was 1.0 log CFU/ml based on the direct plating method used. Therefore, an approximate 6-log reduction was achieved if populations reached the detection limit. Initial populations of *E. coli* ATCC 25922 in untreated juice (i.e., treated with neither UV nor preservatives) decreased by 1.2 log CFU/ml after 72 hour storage at 4°C. Inoculated apple cider without any added preservatives was subjected to approximately 13.7 mJ/cm² of UV resulting in a reduction of 4.7 log CFU/ml immediately after UV treatment. There was little or no change in pH (~0.02) in treated cider following UV exposure. Apple cider (4°C) increased in temperature to $16 \pm 2^\circ\text{C}$ after processing through the UV apparatus.

Overall, addition of chemical preservatives after UV treatment was more effective in reducing *E. coli* ATCC 25922 populations than when chemical preservatives were added prior to UV treatment ($P < 0.05$). There was no significance difference between low and high concentrations of each chemical preservative whether added to apple cider prior to or after UV ($P > 0.05$). Inactivation of *E. coli* ATCC 25922 in apple cider treated with KS prior to UV treatment (4°C) was less than inactivation caused by UV alone ($P > 0.05$).

Pre-UV Application of Chemical Preservatives –

NaB (1000 and 2000 ppm) in combination with UV had a minimal initial reduction, but reduction rate increased during storage at 4°C. Initial reductions of *E. coli* ATCC 25922 in apple cider containing NaB 1000 and NaB 2000 pre-UV were 3.34 log CFU/ml and 2.26 log CFU/ml, respectively. Populations remained steady over 24 and 48 h. *E. coli* ATCC 25922 populations at 72 h were 4.38 and 5.39 log CFU/ml. Cider containing NaB 1000 and NaB 2000 were subjected to 12.7 and 11.4 mJ/cm² of UV, respectively, which was slightly lower compared to cider that did not contain preservatives.

Samples containing KS (1000 and 2000 ppm) in apple cider prior to exposure to UV proved to be ineffective for reducing *E. coli* ATCC 25922 populations compared to inoculated juice that received neither UV or preservatives. Initial reduction of *E. coli* ATCC 25922 was 0.1 and 0.2 log CFU/ml, respectively. Positive controls that contained inoculum then exposed to UV produced an initial 4.7 log CFU/ml reduction, in comparison to apple cider treated with a chemical preservative. Samples containing 1000 and 2000 ppm of KS received the lowest dose, 6.9 and 6.0 mJ/cm², respectively, of all treatments compared to cider without preservatives.

Treatment with HP (75 and 150 ppm) produced a substantial reduction in *E. coli* ATCC 25922 populations in apple cider prior to UV processing. HP 75 and HP 150 reduced *E. coli* ATCC 25922 populations by 4.30 and 4.17 log CFU/ml, respectively, after initial UV exposure. There was a lethal effect on *E. coli* ATCC 25922 populations, producing a 7.2 and 7.0 log CFU/ml reduction with HP 75 and HP 150 after 24 hours,

respectively. The addition of 75 and 150 ppm HP in apple cider prior to UV produced a similar UV dosage, 13.7 mJ/cm^2 , as cider without preservatives.

DMDC (75 and 150 ppm) was effective in reducing *E. coli* ATCC 25922 populations in apple cider in conjunction with UV. Both concentrations produced $> 6 \text{ log}$ reduction of *E. coli* ATCC 25922 populations. All three repetitions had similar results after initial plating following UV exposure. One repetition maintained a 2-3 log CFU/ml population during 24, 48, and 72 hour sampling, while the other two repetitions produced $> 7 \text{ log CFU/ml}$ reduction. Apple cider containing 75 and 150 ppm DMDC had a UV dose of 13.7 mJ/cm^2 , in both samples similar to cider without chemical preservatives.

Post-UV Application of Chemical Preservatives –

Initial populations of *E. coli* ATCC 25922 were approximately 7.2 log CFU/ml in apple cider before being subjected to UV. Initial populations of *E. coli* ATCC 25922 after UV exposure and before the addition of chemical preservatives were $\sim 3.2 \text{ log CFU/ml}$. The average UV dosage for cider prior to the addition of each chemical preservatives was approximately 13.7 mJ/cm^2 .

NaB (1000 and 2000 ppm) was effective in reducing *E. coli* ATCC 25922 populations over a 72 h period. NaB 1000 and NaB 2000 produced an initial reduction of ~ 3.9 and $\sim 4.0 \text{ log CFU/ml}$. Population declined steadily over 24 and 48 h in both concentrations. A total population decrease of $\sim 5.2 \text{ log CFU/ml}$ occurred in NaB 1000 treated apple cider, while NaB 2000 produced a $\sim 6.5 \text{ log CFU/ml}$ reduction.

KS (1000 and 2000 ppm) remained ineffective in reducing *E. coli* ATCC 25922 populations in UV treated cider. Initially, in UV treated apple cider *E. coli* ATCC 25922

populations decreased ~3.9 and ~4.0 log CFU/ml in KS 1000 and KS 2000, respectively. In both KS 1000 and KS 2000 populations remained steady over the total time period. In KS 1000 and KS 2000 there was a final reduction of ~3.6 and ~4.3 log CFU/ml, respectively.

Apple cider subjected to UV then treated with HP (75 and 150) was effective in inactivation of *E. coli* ATCC 25922. Initially, HP 75 and HP 150 reduced *E. coli* ATCC 25922 populations by ~4.2 and ~4.1 log CFU/ml, respectively. Both treatments produced > 7.0 log CFU/ml reductions after 24 h. No growth occurred at 24, 48, and 72 h.

DMDC (75 and 150 ppm) in combination with UV treated apple cider was effective in reducing *E. coli* ATCC 25922 populations. DMDC 75 and DMDC 150 produced a ~4.3 and ~4.8 log CFU/ml reduction of *E. coli* ATCC 25922, the largest initial reduction of any treatments. After a 24 h period DMDC 75 and DMDC 150 decreased the population by > 7 log CFU/ml.

DISCUSSION

All combinations of chemical preservatives (UV/NaB 1000 ppm, UV/NaB 2000 ppm, UV/HP 75 ppm, UV/HP 150, UV/DMDC 75 ppm, or UV/DMDC 150 ppm) added following UV resulted in ≥ 5 log reduction of *E. coli* ATCC 25922 after 72 h storage at 4°C. The only exceptions were UV/KS 1000 and UV/KS 2000 which reduced *E. coli* ATCC 25922 < 1 log CFU/ml after 72 h storage. *E. coli* ATCC 25922 is a surrogate for *E. coli* O157:H7, with similar UV sensitivity (Sastry *et al.* 2000). Initial populations of *E. coli* ATCC 25922 inoculum in the apple cider were approximately 7- to 8 log CFU/ml to allow determination of a 5-log CFU/ml or greater reduction. The results indicate that HP and DMDC have a substantial lethal effect on *E. coli* ATCC 25922 populations throughout storage for 72 h at 4°C, while KS produces a minimal reduction (< 1 log CFU/ml) compared to 24 h results.

Population reductions were overall more effective when chemical preservatives were added post-UV compared to apple cider treated with chemical preservatives prior to UV ($P < 0.05$). All post-UV combinations with the exceptions of post-UV/KS 1000 and post-UV/KS 2000 produced similar reductions compared to preservative addition prior to UV exposure. Reduction in inoculated cider without preservatives was ~ 4.3 log CFU/ml after 72 h storage, both post-UV/KS 1000 ppm and post-UV/KS 2000 produced < 1 log CFU/reduction of *E. coli* ATCC 25922. Overall, post-UV antimicrobial treatment was improved due to the initial *E. coli* ATCC 25922 population reductions. These results may indicate that UV may be inhibited by the addition of KS in apple cider. Samples containing 1000 and 2000 ppm KS prior to UV exposure received almost half the dose

(6.9 and 6.0 mJ/cm²) compared to cider without chemical preservatives (13.7 mJ/cm²). KS may affect the transmission of UV in apple cider, which can reduce the lethality of UV. Further studies need to be performed in order to determine the interaction between UV and KS in an acidic medium.

Both prior to and following UV, DMDC and HP were very effective during 24 h storage. Both treatments produced > 5 log CFU/ml reductions of *E. coli* ATCC 25922 after 24 h, with the exception of one repetition of UV/DMDC 75 and UV/DMDC 150 that did not completely inhibit *E. coli* ATCC 25922 growth. In cider treated with NaB 1000 and NaB 2000 ppm following UV exposure, produced ~0.9 log and ~1.2 log CFU/ml improved reduction of *E. coli* ATCC 25922 compared to treatment prior to UV, respectively. This may indicate that NaB is more effective against UV injured *E. coli* ATCC 25922.

Duffy *et al.* (2000) validated the use of a thin film disinfection unit (CiderSure®) for a 5 log or greater reduction of *E. coli* O157:H7 in raw, unprocessed apple cider. UV processing tubes (n = 70) were tested to produce a 5 log reduction of *E. coli* ATCC 25922. *E. coli* ATCC 25922 mean reductions of the various processing tubes ranged 5 to 8.5 logs. Worobo (1999) used the same UV disinfection unit to produce 5.9 - 6.1 log CFU/ml in various strains of *E. coli* O157:H7.

Wright *et al.* (2000) exposed cider inoculated with *E. coli* O157:H7 with UV in a thin film through a UV disinfection unit (peak 254 nm; max. 107,366 µW-s/cm²). They achieved a mean reduction of 3.8 CFU/ml of *E. coli* O157:H7 populations.

Unpasteurized cider was used so there was an increase in background microflora (i.e.

yeasts and molds). A > 5 log reduction was achieved at the highest UV dosage (61,005 $\mu\text{W}\cdot\text{s}/\text{cm}^2$) and minimal background microflora (1.0 log CFU/ml).

Leyer *et al.* (1995) used acid-adapted *E. coli* O157:H7 strains grown in broth acidified with HCl (pH 5.0) and compared their survival in apple cider (pH 3.46) to nonadapted cells. Nonadapted cell populations were completely inactivated after 28 h, but adapted cell populations were ~60 CFU/ml after 81 h. They showed that acid-adaptive response enhances the survival of *E. coli* O157:H7 in acidified food products such as apple cider.

Unpreserved apple cider has shown that *E. coli* O157:H7 can survive 31 days at 8°C and 18 days at 4, 10, and 25°C (Zhao *et al.*, 1993 and Fisher and Golden, 1998). Duffy *et al.* (2000) and Worobo (1999) reported that a 5-log reduction of *E. coli* O157:H7 can be achieved with UV treatment in a thin film. Fisher and Golden (1998) determined that DMDC was effective in inactivating *E. coli* O157:H7 in unpreserved apple cider at 4, 10, and 25°C. In their study, DMDC 250 ppm treated cider showed recovery of *E. coli* O157:H7 populations after 72 h at 4°C. However, in this study 75 ppm and 150 ppm DMDC both pre- and post-UV exposure produced < 1 log CFU/ml *E. coli* ATCC 25922, a surrogate strain for *E. coli* O157:H7, in apple cider after 24 h at 4°C.

This study demonstrates that the combination of UV and chemical preservatives (NaB, HP, and DMDC) may have an additive effect to produce a 5-log CFU/ml or greater reduction of *E. coli* O157:H7 in apple cider. KS is ineffective as chemical preservative treatment in apple cider with UV. Studies were performed at refrigeration temperature (4°C), which may have reduced the efficacy of NaB and KS. Both NaB and KS reduce the heat resistance of *E. coli* O157:H7 in acidic mediums (pH 3.6-4.0) at elevated

temperatures ($\geq 50^{\circ}\text{C}$) (Splittstoesser *et al.*, 1995). Future studies at room temperature (25°C) or greater may improve or reduce the efficacy of chemical preservatives used in this study.

CONCLUSION

The objectives of this study were (1) to determine if chemical preservatives and UV have a combined effect on the reduction of *Escherichia coli* in apple cider and (2) to determine the effect of chemical preservative addition prior to and after UV processing on the reduction of *Escherichia coli* in apple cider. The results of this study revealed that using chemical preservatives following UV exposure was more effective than addition of chemical preservatives prior to UV exposure for reduction of *E. coli* ATCC 25922 in apple cider ($P < 0.05$).

Of the chemical preservatives tested (NaB, KS, HP and DMDC), DMDC and HP were the most lethal in combination with UV. Cider containing either NaB or KS received lower doses of UV compared to cider that contained no added preservatives, indicating that these preservatives reduced the transmission of UV through the juice. In apple cider containing chemical preservatives prior to UV exposure inactivation was least effective in KS samples ($P > 0.05$). Lower concentrations of each preservative was as effective as the higher concentrations in almost all samples ($P < 0.05$).

Thin film UV alone has been proven to be effective in producing a 5-log decrease of *E. coli* O157:H7 in apple cider (Worobo, 1999 and Duffy *et al.*, 2000). UV in combination with chemical preservatives may have an additive effect to produce a ≥ 5 -log decrease of *E. coli* O157:H7 in apple cider. UV may be able to be used at lower doses ($< 14 \text{ mJ/cm}^2$) with low concentrations of certain chemical preservatives when combined before or after UV exposure. Adding low concentrations of chemical preservatives, i.e. HP and DMDC, may help reduce microbial load prior to UV pasteurization. Addition of

chemical preservatives following UV exposure may help provide downstream protection prior to bottling and during storage of apple cider to improve shelf-life.

Further studies need to be performed to determine UV and chemical preservative interaction in acidic mediums. KS was ineffective in combination with UV to produce a 5-log reduction of *E. coli* ATCC 25922 in apple cider. Apple cider treated with certain chemical preservatives (i.e. KS) may receive lower doses of UV by affecting transmission. Studies of UV in combination with chemical preservatives at room temperature (25°C) or greater are needed. Elevated temperatures may improve the lethality of chemical preservatives, such as sodium benzoate and potassium sorbate, which help lower the heat resistance of *E. coli* O157:H7 (Splittstoesser *et al.*, 1995). Furthermore, chemical preservatives in combination with UV should be tested on raw, unpasteurized apple cider. UV combined with chemical preservatives may be an effective, economically feasible alternative to thermal pasteurization for the reduction of *E. coli* O157:H7 in apple cider.

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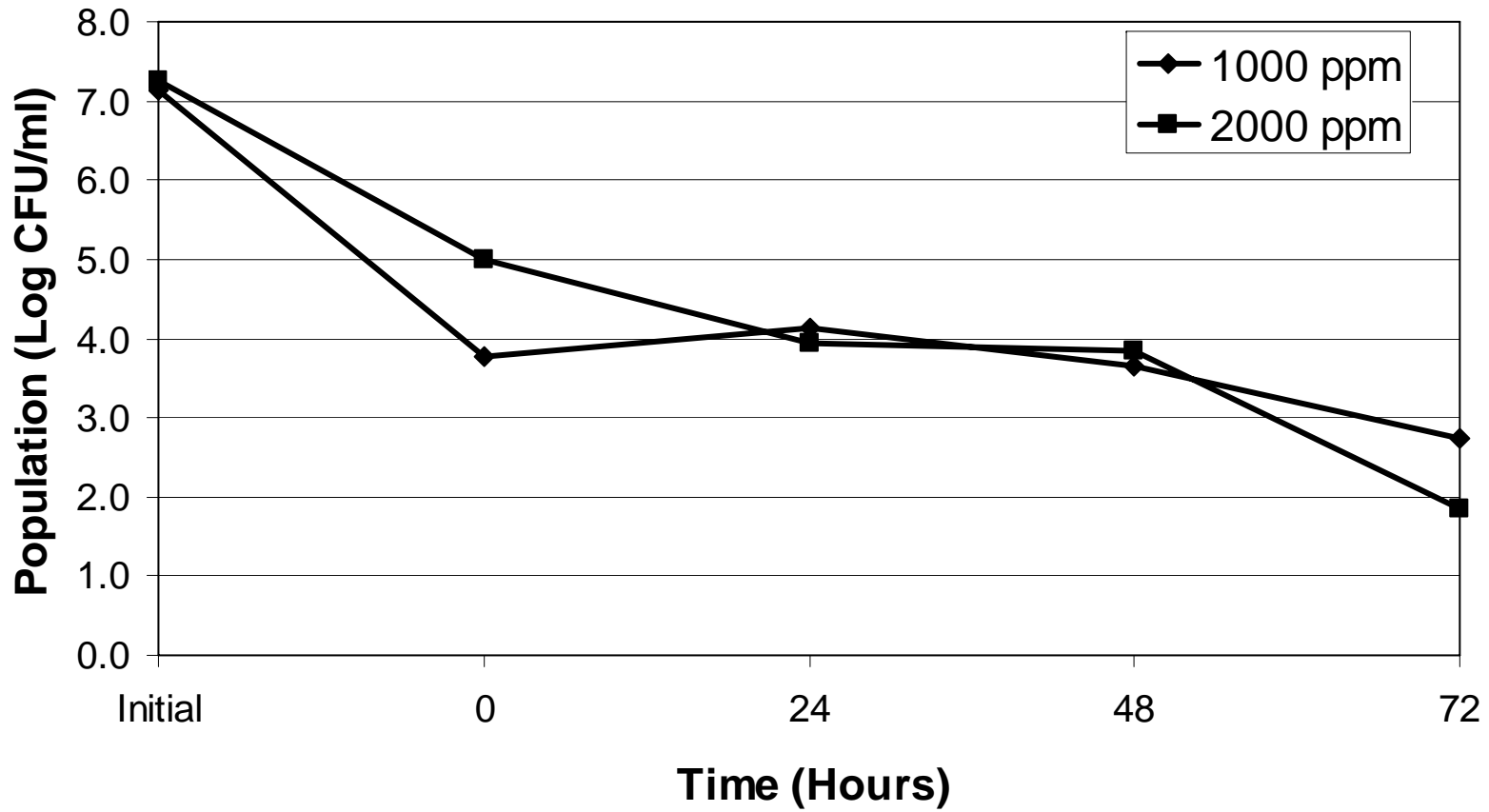


Figure 1: Fate of *Escherichia coli* ATCC 25922 during ultraviolet light (peak output 254 nm) processing and subsequent storage (4°C) in apple cider containing sodium benzoate (1000 or 2000 ppm) added prior to ultraviolet light exposure. n = 3.

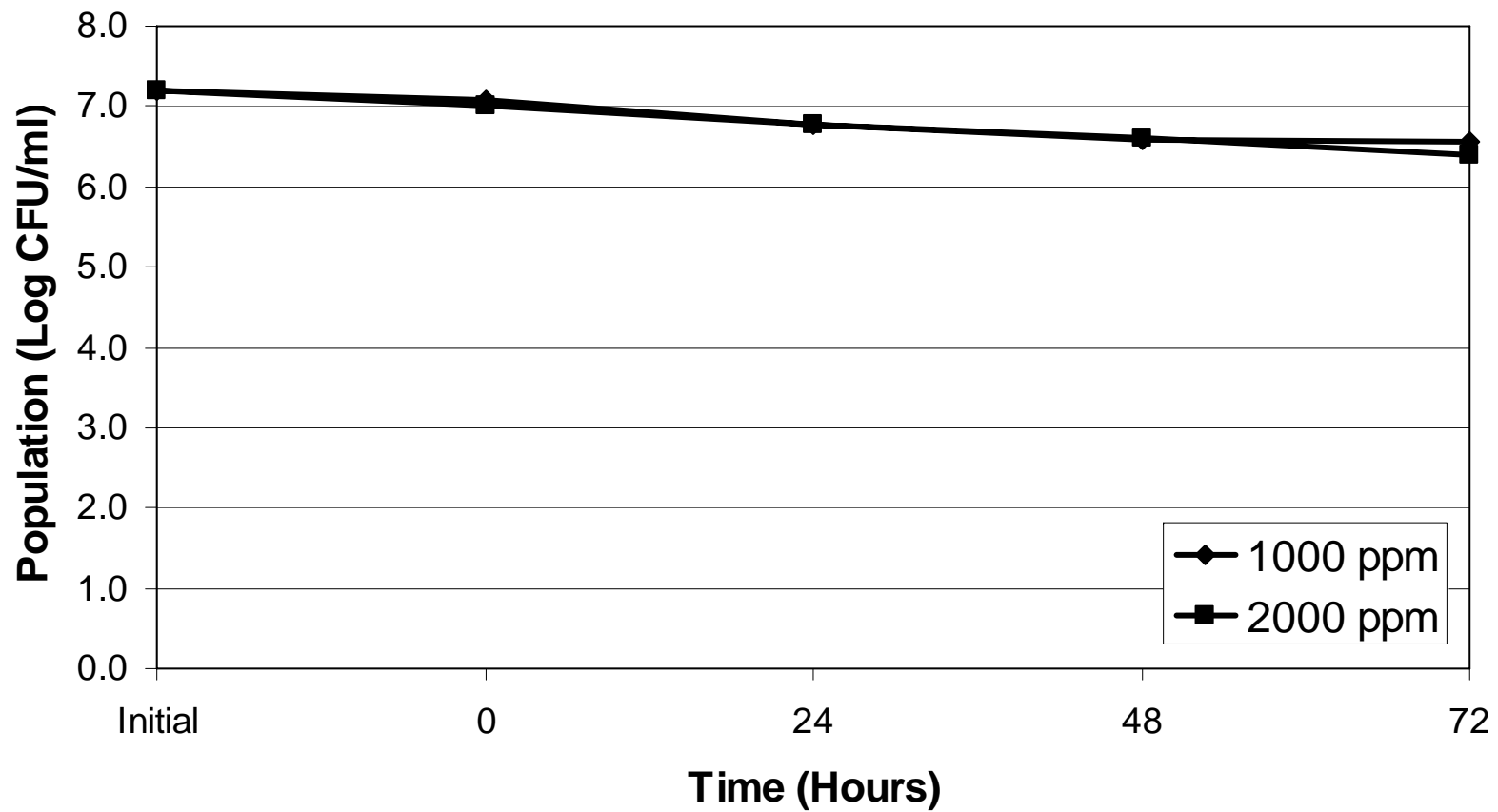


Figure 2: Fate of *Escherichia coli* ATCC 25922 during ultraviolet light (peak output 254 nm) processing and subsequent storage (4°C) in apple cider containing potassium sorbate (1000 or 2000 ppm) added prior to ultraviolet light exposure. n = 3.

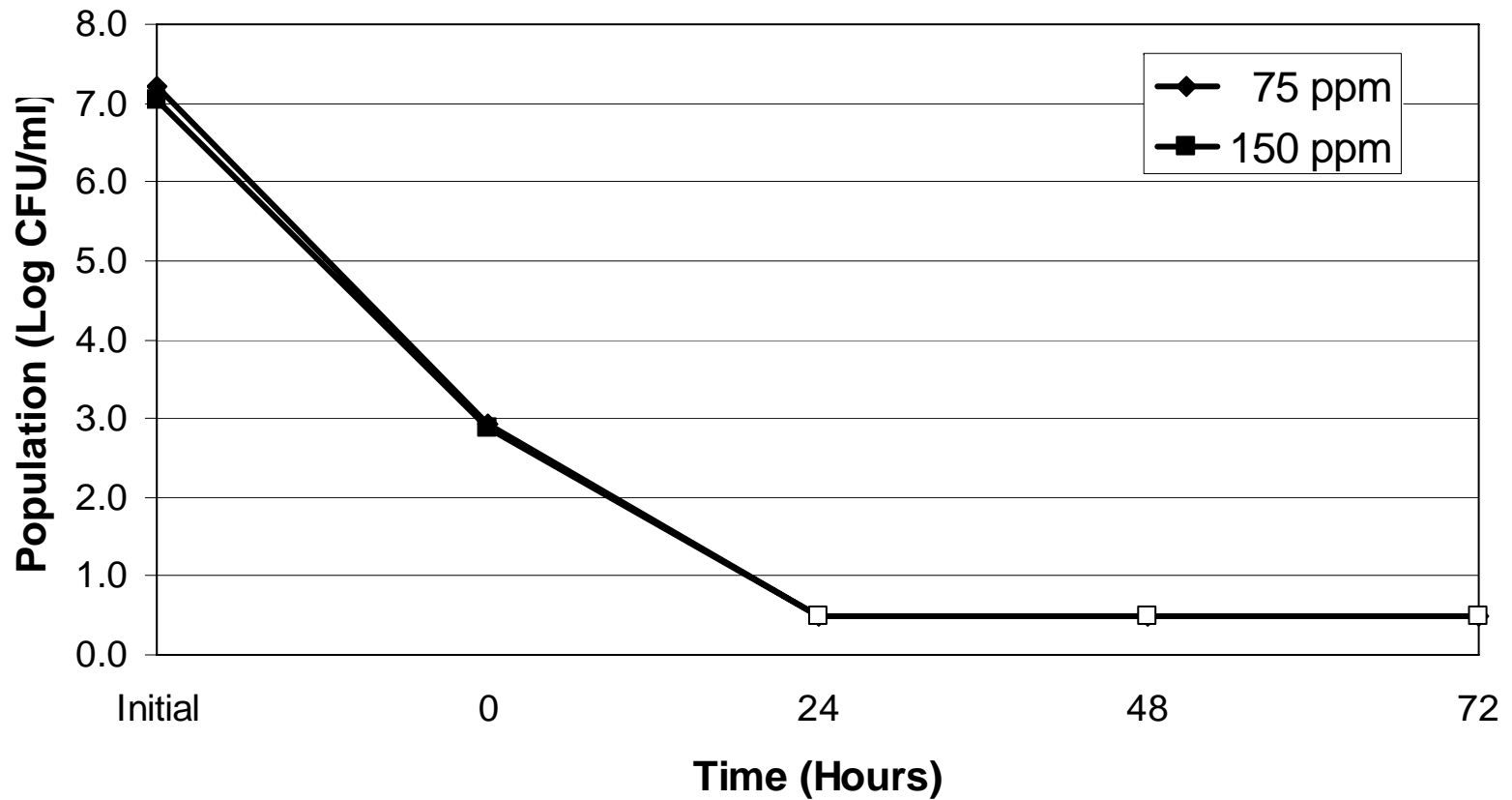


Figure 3: Fate of *Escherichia coli* ATCC 25922 during ultraviolet light (peak output 254 nm) processing and subsequent storage (4°C) in apple cider containing hydrogen peroxide (75 or 150 ppm) added prior to ultraviolet light exposure. n = 3. Open symbols indicate that populations were below the limit of detection (1 log CFU/ml).

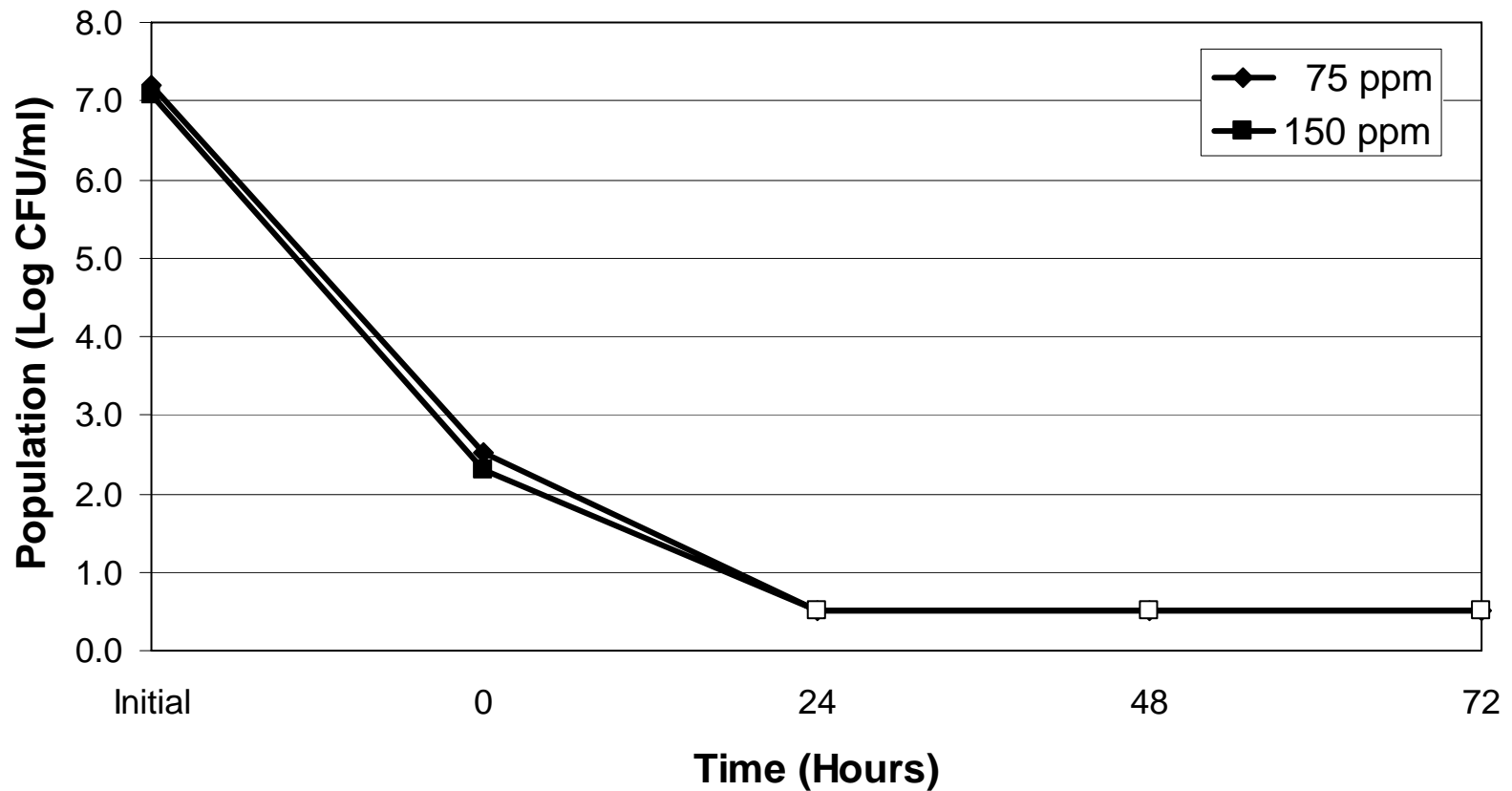


Figure 4: Fate of *Escherichia coli* ATCC 25922 during ultraviolet light (peak output 254 nm) processing and subsequent storage (4°C) in apple cider containing dimethyl dicarbonate (75 or 150 ppm) added prior to ultraviolet light exposure. n = 3. Open symbols indicate that populations were below the limit of detection (1 log CFU/ml).

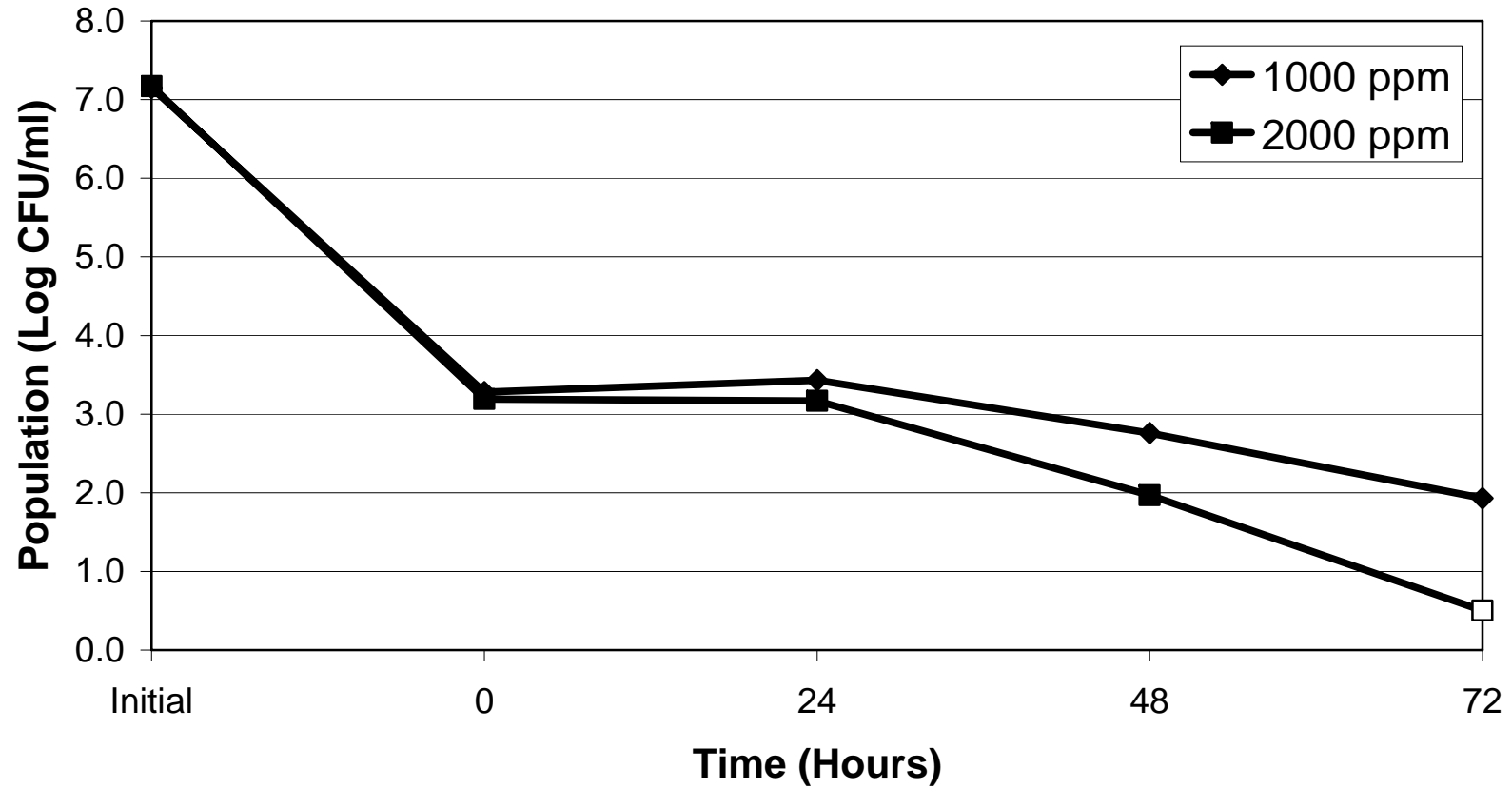


Figure 5: Fate of *Escherichia coli* ATCC 25922 during ultraviolet light (peak output 254 nm) processing and subsequent storage (4°C) in apple cider containing sodium benzoate (1000 or 2000 ppm) added after ultraviolet light exposure. n = 3. Open symbol indicates that populations were below the limit of detection (1 log CFU/ml).

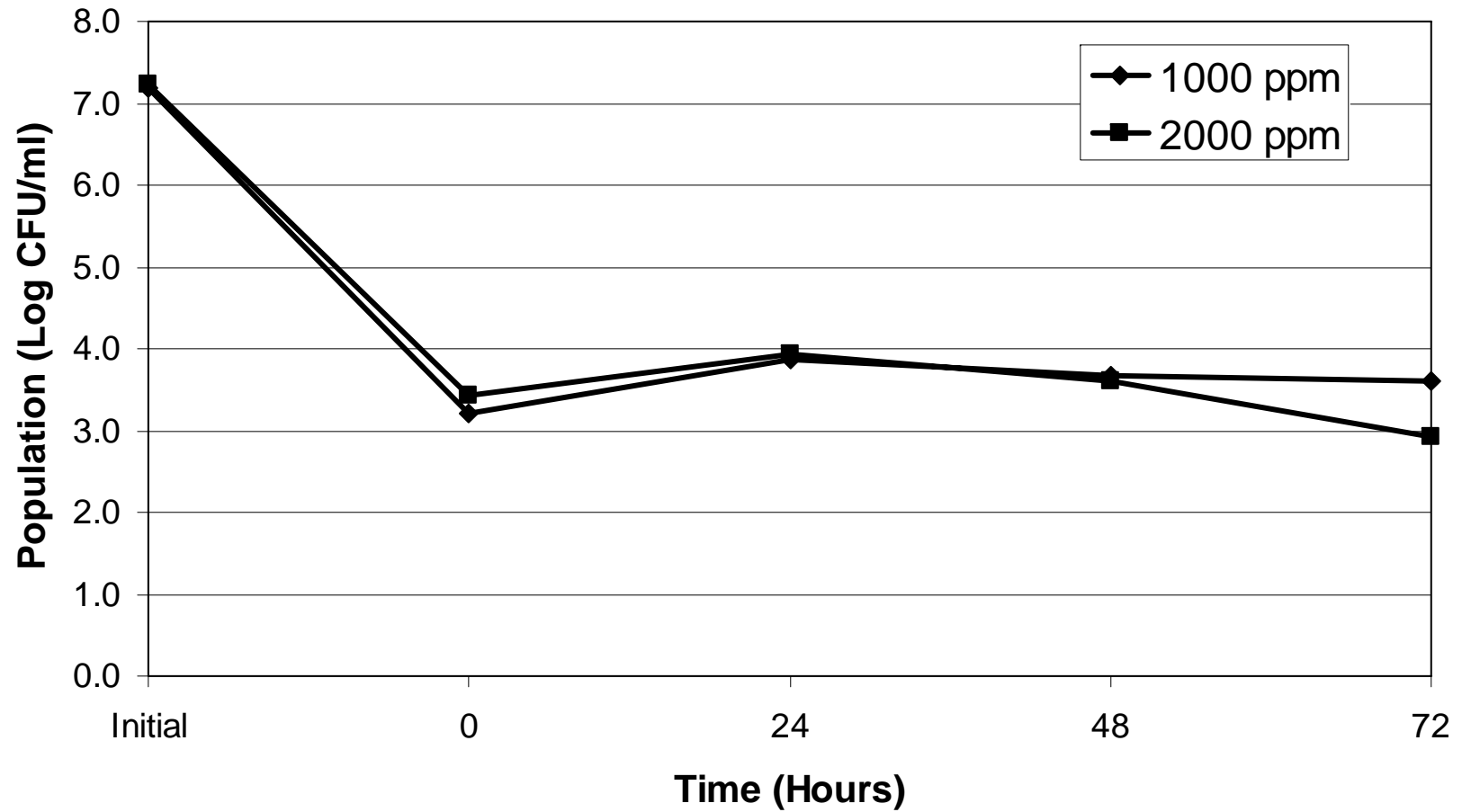


Figure 6 Fate of *Escherichia coli* ATCC 25922 during ultraviolet light (peak output 254 nm) processing and subsequent storage (4°C) in apple cider containing potassium sorbate (1000 or 2000 ppm) added after ultraviolet light exposure. n = 3 (2000 ppm, n = 2.)

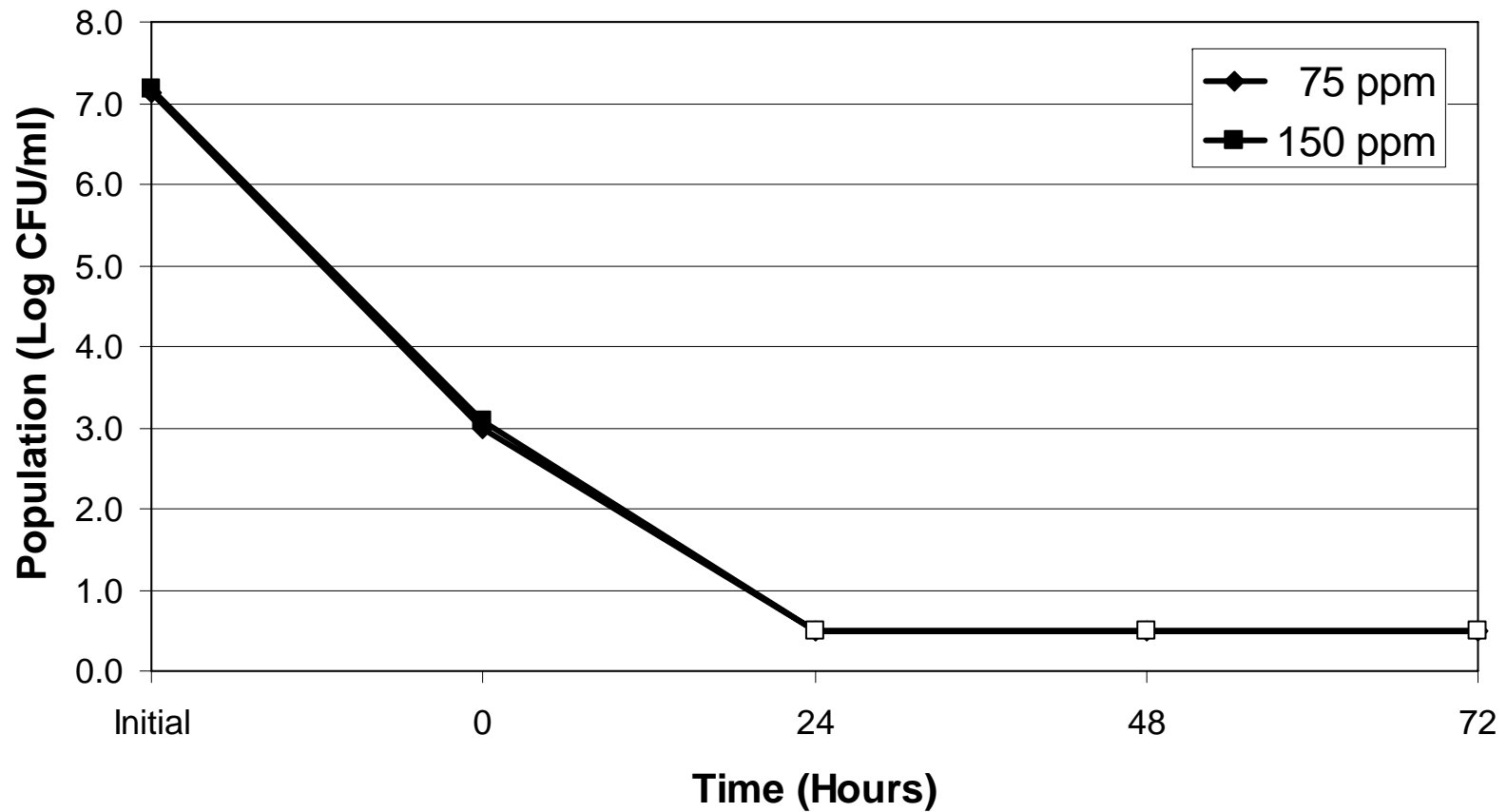


Figure 7: Fate of *Escherichia coli* ATCC 25922 during ultraviolet light (peak output 254 nm) processing and subsequent storage (4°C) in apple cider containing hydrogen peroxide (75 or 150 ppm) added after ultraviolet light exposure. n = 3. Open symbols indicate that populations were below the limit of detection (1 log CFU/ml).

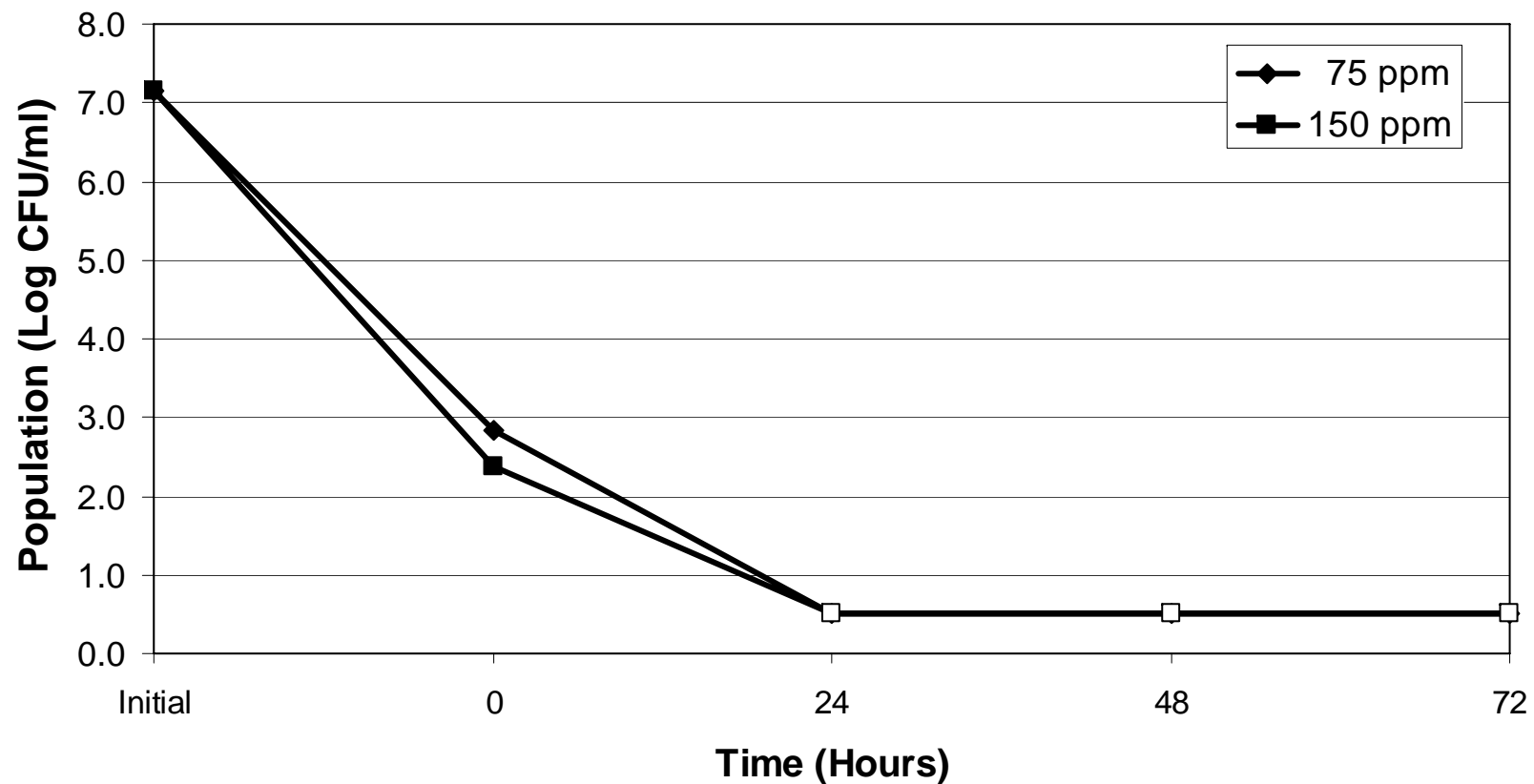
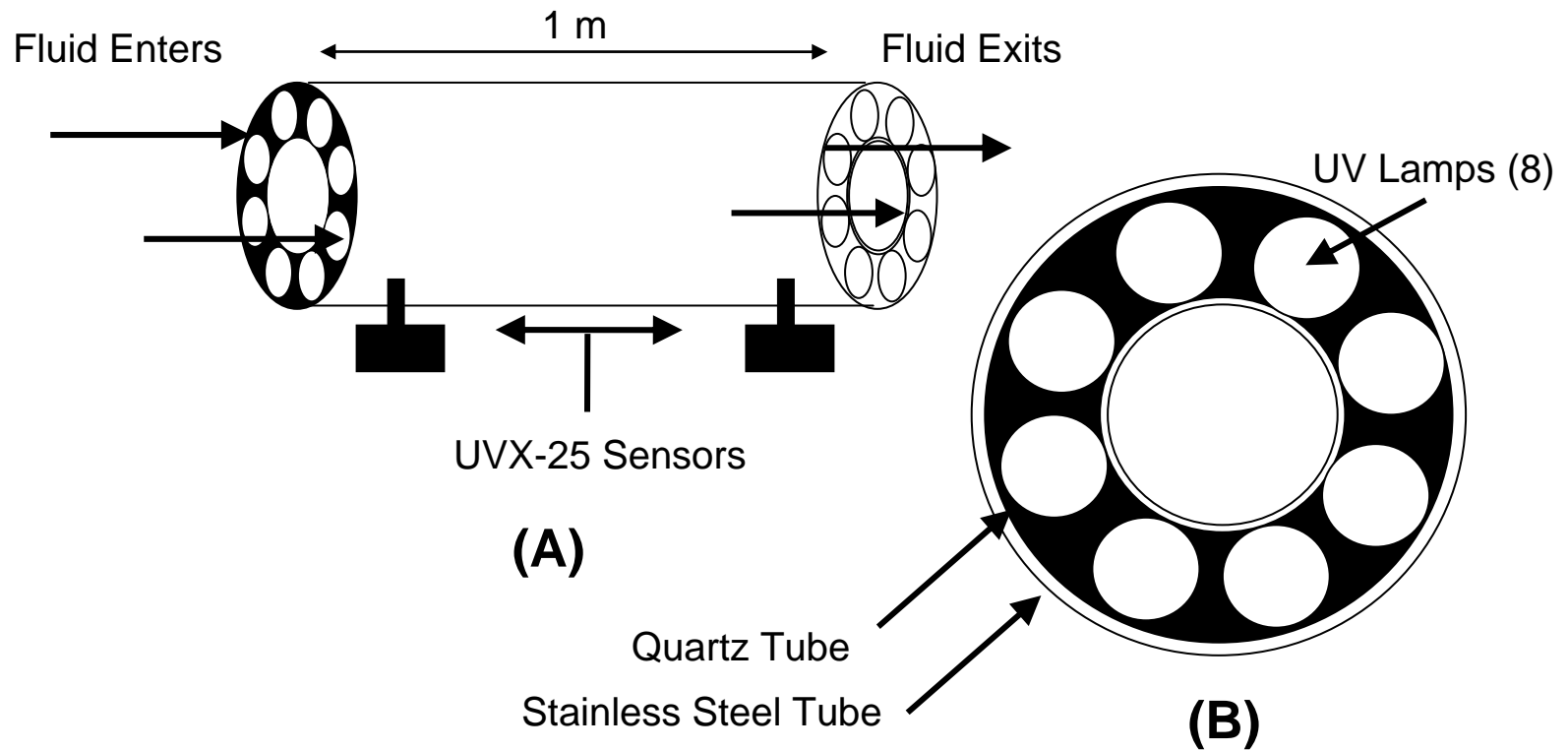


Figure 8: Fate of *Escherichia coli* ATCC 25922 during ultraviolet light (peak output 254 nm) processing and subsequent storage (4°C) in apple cider containing dimethyl dicarbonate (75 or 150 ppm) added after ultraviolet light exposure. n = 3. Open symbols indicate that populations were below the limit of detection (1 log CFU/ml).

ACKNOWLEDGEMENTS

This research was supported by a grant from the National Integrated Food Safety Initiative (Award # 2002-51110-01961). I would like to thank Dr. Randy Worobo, Cornell University, for loaning the CiderSure® 3500A for the duration of this study. Finally, to Phil Hartman, FPE Inc., for assisting me with technical issues pertaining to the CiderSure® 3500A.



Appendix I: Diagram of the UV chamber (A) and layout of UV lights (B) within the CiderSure® 3500A UV Processor (FPE Inc., Rochester, NY) (Matak *et al.*, 2004).

Appendix II: Experimental Design

Efficacy of Ultraviolet Light in Combination with Chemical Preservatives for the Reduction of *Escherichia coli* in Apple Cider

Micro	<i>Escherichia coli</i> ATCC 25922															
Juice	Apple cider (unfiltered, pasteurized, shelf-stable)															
Temp (°C)	4															
UV Treatment	Antimicrobial addition prior to UV treatment								Antimicrobial addition following UV treatment							
Antimicrobial	KS		NaB		HP		DMDC		KS		NaB		HP		DMDC	
Level (ppm)	2000	1000	2000	1000	150	75	150	75	2000	1000	2000	1000	150	75	150	75
Sampling Time (hours)	Init.	Init.	Init.	Init.	Init.	Init.	Init.	Init.	Init.	Init.	Init.	Init.	Init.	Init.	Init.	Init.
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48	48
	72	72	72	72	72	72	72	72	72	72	72	72	72	72	72	72
Media	TSA	TSA	TSA	TSA	TSA	TSA	TSA	TSA	TSA	TSA	TSA	TSA	TSA	TSA	TSA	TSA
Duplicates	x2	x2	x2	x2	x2	x2	x2	x2	x2	x2	x2	x2	x2	x2	x2	x2
Replications	3	3	3	3	3	3	3	3	2	3	3	3	3	3	3	3

VITAE

Joemel Mariano Quicho was born on July 2, 1979 in Orion, Bataan, Philippines. He moved to San Diego, CA at the age of 3 and has considered Virginia Beach, VA his hometown since the age of 5. He graduated from Salem High School (Virginia Beach, VA) in June 1997. He received a Bachelor of Science in Biology, with an emphasis in Microbiology, and Chemistry minor, from Virginia Polytechnic Institute and State University (Blacksburg, VA) in December 2001. In August 2002, Joemel enrolled in the Master's Program in Food Science and Technology at Virginia Polytechnic Institute and State University, with a concentration in Food Microbiology.