INTRODUCTION

Although the number of food borne illnesses associated with the consumption of fresh fruits and vegetables has increased over the past years, there is still a growing demand for these products by the consumer, resulting in a large number of these products in the market. In the past decade there have been far too many food borne disease outbreaks associated with the consumption of fresh produce and juices. Among the most recognized outbreaks are those involving *Escherichia coli* O157:H7 in unpasteurized apple cider, and *Salmonella* in unpasteurized orange juice. The low pH of these juices (apple cider and orange juice have pH's of 3.7 and 4.0 (respectively) on average) was once considered a safeguard against growth or survival of pathogens, but we now know that this is not true. Some strains of *E. coli* O157:H7 are able to survive in apple cider with 0.1% sodium benzoate added as a preservative for up to 21 days (45), and still more strains can survive refrigeration and freezing (to -20^{o}C), showing little or no decline in total cell count (20).

The FDA published its final rule regarding juice safety in 2001, requiring a 5 log reduction in the most resistant pathogen (for example *E. coli* O157:H7 in apple cider) in hopes of making fresh juice products safer. This regulation would require pasteurization or other preservation methods such as treatment with UV light or ozone or the addition of organic acids. Unfortunately preservation methods, such as pasteurization are too expensive for many small producers of juices. It is in their best interest that the method of contamination is revealed, be it in the field or in the processing facility. This would allow processors to concentrate on reduction of the pathogen by preventing its internalization and produce safer products.

Of particular interest is the ability of microbial pathogens to internalize and survive in fresh produce that are commonly used for juices. When bacteria are internalized they are not susceptible to traditional washing and sanitizing methods used on the surface of fruits because the fruit protects the bacteria from sanitizers and removal by scrubbing. If pathogens are internalized in the fruit there is an increased food safety risk for minimally processed juices. In order to reduce the likelihood of internalization of pathogens by fresh fruits a better understanding of the internalization process itself is needed. This includes research that examines specific areas on the fruit where

internalization occurs, and also the nature of the time during the growing season that internalization occurs, as well as investigating potential sources of contamination.

Chapter 1: Literature Review

Escherichia coli

Characteristics

Escherichia coli is commonly found in the intestines of warm blooded animals, such as humans and cattle. Most strains of the Gram negative non-sporeforming rod-shaped bacteria are not pathogenic to humans. Of several strains of *Escherichia coli*, it is a strain commonly found in cattle, *Escherichia coli* O157:H7, that is pathogenic to humans and is commonly involved in foodborne disease outbreaks. The organism ferments glucose to produce acid and gas, and is motile through use of peritrichous flagella. *E. coli* can commonly survive and grow over a wide pH range, from 4.4 to 9.0 (13, 31). However, there are certain strains of *E. coli* O157:H7 that are able to survive in apple cider at pH 3.7 for 31 days at a temperature of 8^{o}C (45, 53).

Escherichia coli O157:H7-Illness, reservoirs, and pathogenicity

Escherichia coli O157:H7 infection is one of the more than 200 known illnesses that can be transmitted through food. This serotype is the predominant pathogen in the Enterohemmorrhagic *Escherichia coli* (EHEC) group, and is "associated most frequently with human infections worldwide"(24). It is estimated that in 1999 there were over 73,000 total cases of infection caused by *E. coli* O157:H7, of which only 3,674 active cases were reported, with 85% being linked to food borne transmission, of these food borne cases, 52 resulted in deaths (38). It has also been estimated that the pathogen causes as many as 20,000 cases of infection and 250 deaths per year in the United States (1). *E. coli* O157:H7 produces a toxin which causes bloody and non-bloody diarrhea (hemorrhagic colitis). It is also the leading cause of hemolytic uremic syndrome, which is a side effect of hemorrhagic colitis (29). Hemolytic uremic syndrome is the leading cause of acute kidney failure in children. Another characteristic of the pathogen include its inability to ferment sorbitol, although there are now strains that have shown the ability to ferment sorbitol. It also displays slow, or a lack of, growth above 44.5^{o}C (40). *Escherichia coli* O157:H7 was first recognized as a human pathogen in 1982, and in a

time span of 21 years there have been numerous food-borne outbreaks in many different foods, ranging from fresh fruits and vegetables to ground beef.

The majority of these outbreaks have been associated with foods derived from cattle, such as ground beef and milk (9). *Escherichia coli* O157:H7 is naturally occurring in the bovine intestine, and also has reservoirs in many other healthy animals (30). In a large number of outbreaks the source of *E. coli* O157:H7 has been confirmed "to be from cattle that asymptomatically carry and shed the pathogen in their feces" (43). *Eschericia coli* O157:H7 can remain viable in bovine feces for up to 70 days, dependent on conditions (6). It can be found in the feces of domestic animals, birds, and feral animals such as deer (30). Wild birds apparently pick up the pathogen while feeding on garbage, sewage, fish, or lands grazed by cattle.

Contamination of Produce by *Escherichia coli* O157:H7

Improperly washed fruits and vegetables can be contaminated with human or animal feces, and pathogens can then contaminate the interior of the produce during cutting. What is even more important is that pathogens can be internalized by produce, protecting the pathogens from traditional washing methods that only clean the surface of the produce (1). *Escherichia coli* O157:H7 has been shown to survive in a viable but non-culturable state in water, especially cold water, for prolonged periods of time. In this state it cannot be isolated by traditional microbiological methods, making it nearly impossible to detect in water supplies (41).

Apples

Morphology-Wax and Cutin

The cuticle of the apple is made up of cutin, a biological polyester, and is made by the outermost layer of cells, called epidermal cells. Cutin is not responsible for the "water-proofing" effect of the skin on the apple itself. It is when particles of cutin are embedded in wax that there is a highly impermeable barrier. The cuticle is attached to the epidermal cell walls by pectin. The natural wax that the cutin is embedded in plays two major functions; it provides water repellency to the fruits surface; and it reduces water permeability through the skin (34). Therefore damage to this layer will cause a decrease in its functionality. The wax layer prevents damage by wind and acid rain, and

reduces risk of infection by microorganisms (28). As an apple grows the amount of natural wax increases so that total wax amount does not really change per unit of surface area on the fruit. After harvest and during storage the quantity as well as the quality of natural waxes can change. This is often demonstrated during storage of fruit. It is important to note that changes in the wax quantity and quality are dependant on the cultivar of apple, with some cultivars showing little change, and some showing considerable change. The wax layer also plays an important role in preventing moisture loss during storage, the better quality and quantity of wax, the less moisture loss during storage (34).

Condition and Damage

The condition and type of fruit plays a major role in internalization of any pathogen. Damage to the fruit may encourage uptake of bacteria, and damaged areas may provide conditions necessary for growth or survival of pathogens. There are many opportunities for apple damage to occur from tree to table. Injuries such as bruises and punctures can be caused while picking, washing, during storage or shipment, etc. In 1978 Dr. George Mattus conducted extensive studies on the condition of apples in distribution centers and retail stores in Virginia. He found that a mere six inch drop in a foam or fiber tray resulted in bruises in over 50% of the apples, and if apples were dropped while packed in bag-master cartons they could sustain bruising and cuts or punctures (8).

Environmental conditions can also alter apples so that they may more readily internalize pathogens. Golden Delicious apples may contain russet sports (red tissue) which significantly decrease the cuticle thickness during apple growth through harvest. Severe cases of russet also effect epidermal cells of fruit, causing them to be less elastic so that they may rupture more frequently (46). Russeting is often attributed to cold, frosty, rainy, or humid seasons, as well as mechanical injury during the early life of the fruit (49). Apples contain stomata, much like those of the leaf. These stomata lose function as the cuticle grows and the stomata cells become rigid. Lenticels come from scars and breaks in the cuticle. Cuticles vary in structure, composition, thickness and permeability by cultivar, the fruits stage of development, and environmental conditions.

As the apple grows and cuticle cells "tear", the epidermal cells prepare a seal around the tear before any further damage can occur (19).

Apples naturally have native microflora that include bacteria and yeasts. The native microflora that are present in any type of produce are thought to play an important role in maintaining the quality and safety of that food (36), but the relationship of those found in apples and how they may prevent pathogen contamination have not been investigated.

Confocal Scanning Laser Microscopy

Confocal microscopy is a powerful research tool as it permits the generation of three-dimensional images. It is suitable for the analysis of structural details of thick specimens. When compared to conventional light microscopy, in which illumination of the specimen is uniform, confocal microscopy illumination is sequential. Illumination from the laser source is focused on one spot of the specimen as an element of the volume of the sample. A plane is then imaged. This allows the researcher to image each section in a sample, and to create images through a sample using a Confocal Scanning Laser Microscopy (CSLM). This also allows for topographic measurements. The use of CSLM combined with fluorescence microscopy is extremely valuable. Different wavelengths of light can be selected by using optical filtration, including ultraviolet and infrared excitations. This produces high-resolution images in a single fast scan, but, in order to use highly reflective or fluorescent sources smaller, stable samples must be used (47).

When green fluorescent protein (GFP) is expressed in prokaryotes, such as *Escherichia coli*, it produces a fluorescent product; however, the entire intact protein is needed for fluorescence. Bacterial cells are in this way visible through the use of confocal microscopy. Purified GFP absorbs blue light (from 395 nm to 470 nm) and emits green light (peak emission at 509 nm) and the observed fluorescence is stable with virtually no photobleaching. When bacterial colonies of GFP producing *E. coli,* isolated on plates containing the inducer isopropyl-beta-D-thiogalactoside (IPTG), are excited by blue light, a strong green fluorescence is observed (16).

When samples are examined by traditional microscopy methods there is often a problem of samples becoming dehydrated. This is especially true with fruit and vegetable samples that have to be cut into very small pieces, when they must be fixed and dehydrated for observation. When using CSLM it is possible to observe larger samples that are intact, therefore there is no distortion in the morphology of cell structure (44).

Regulatory Implications

In January of 2001 the FDA published a final rule requiring Hazard Analysis Critical Control Point (HACCP) plans to be put into place in all juice processing facilities and that juices must be processed to obtain a 5-log reduction in microbial population (26, 27). The FDA's department of Health and Human Services stated that "implementation of a HACCP system will increase the protection of consumers from illness-causing microbes and other hazards in juices" (27). HACCP systems are a scientifically based analysis of "potential hazards, determination of where the hazards can occur in processing, implementing control measures at points where hazards can occur to prevent problems, and rapid corrective actions if a problem occurs" (27). All juice processors must now reevaluate their process to determine any hazards (microbiological, chemical, or physical), and will be required to use a process that can achieve a 5-log reduction in the most resistant pathogen (26, 27).

Simple pasteurization of fresh juices, such as apple juice, will provide this required 5-log reduction in the target pathogen population. Unfortunately this is a high cost process and is therefore not desirable to small producers of apple cider (25). Juices that are sold through retail operations that are not processed to obtain this 5-log reduction must contain a warning label (27). HACCP principles include a hazard analysis, and for each significant hazard there be a one or more critical control points along with critical limits. The prerequisite of an appropriate HACCP plan is that good manufacturing practices (GMPs) are established. GMPs include sanitation standard operating procedures (SSOPs) and cleaning for the entire facility, as well as the equipment and raw materials. At the absolute minimum, processors of apple cider must follow GMP regulations (32). Unfortunately the HACCP plans do not extend to the growing and harvesting of raw agricultural products (27). The contamination of apples with

Escherichia coli O157:H7 likely occurs in the orchard, and so the HACCP program does not apply. It is therefore important to determine the source of contamination and the population dynamics of *E. coli* O157:H7 in apples.

Post-harvest contamination may be lessened through proper sanitation. The most commonly used sanitizer in wash, spray, and flume waters in industry is chlorine; however, the antimicrobial activity of chlorine depends on the amount of free available chlorine in the wash water that comes into contact with microorganisms. Chlorine is the most "widely used sanitizing agent available for fresh produce…the most that can be expected at permitted concentrations is a 1- to 2- log population reduction" (52). Sanitizers that can be used to wash fresh produce are regulated by the FDA, in accordance with the Federal Food, Drug and Cosmetic Act (CFR, Title 21, Ch. 1, Section 173.315). Unfortunately, there is no effective chlorine substitute for sanitizing fruits and vegetables (6).

Food Borne Disease Outbreaks due to *Escherichia coli* O157:H7

Fresh Produce and Juices

Since its emergence, *Escherichia coli* O157:H7, has been linked to an increasing number outbreaks in fresh produce, and fresh produce products, such as apple cider, unpasteurized apple juice, and lettuce. The pathogen *Salmonella* has been linked to many outbreaks in fresh, unpasteurized orange juice (15). Since 1974 there have been at least ten outbreaks of human illness due to *E. coli* O157:H7, *Salmonella*, and *Cryptosporidium* in juices in the United States and Canada, resulting in at least 2 deaths (10). *Escherichia coli* O157:H7 has also been isolated from cilantro and coriander, which originated in Mexico. There was an outbreak in 1994 linked to the consumption of food from a salad bar that was possibly cross-contaminated with raw ground beef (5). The total estimated annual cost resulting from outbreaks of food borne *E. coli* is $659,100,000 (29). The first outbreak of *E. coli* O157:H7 in fresh-pressed apple cider occurred in 1991 in Massachusetts with 23 reported cases. Before this, outbreaks in cider were thought unlikely because it was thought that cider could not support the growth or survival of food borne pathogens. In this particular outbreak drop apples that were contaminated with animal manure were implicated (4, 29).

Outbreaks like this increase the interest in determining the efficacy of decontamination treatments (10) as well as the source of contamination. It is also likely that an increased awareness about *E. coli* O157:H7 infection and improved characterization methods such as pulsed field gel electrophoresis sub typing have increased the reported number of infections with *E. coli* O157:H7 (9). Another reason for an increased number of outbreaks from fresh produce is the increase in fresh fruit and vegetable consumption of nearly 50% in the past 30 years. This change in food consumption has brought forth more microbial hazards associated with fresh produce. There is an increase in the number of outbreaks associated with fresh produce in the summertime, and while not fully understood, this phenomenon is likely explained by the fact that fresh produce is more attainable at this time and therefore more highly consumed (5). There is also an increasing popularity in retailing of freshly cut fruits and vegetables, and these foods are thought generally safe to eat by the public. However the Center for Disease Control and Prevention (CDC) found that from 1973 to 1987 and 1988 to 1991 that 5 to 6% of total food borne outbreaks could be attributed to the fruits and vegetables category (37).

In 1996 there were four outbreaks associated with cider contaminated with *E. coli* O157:H7, resulting in the death of one child, a similar outbreak occurred in 1993 (30). Two of these cases implicated cider that was locally produced at a small cider mill, where the apples were crushed whole in presses, and the cider was typically not pasteurized before sale (14). Another case in 1996 implicated a widely know commercial brand of unpasteurized apple juice or juice mixtures that contained apple juice. DNA "fingerprinting" allowed officials to link these cases to a previously unopened container of the same brand of apple juice (12). In 1997, Tauxe reported that in 1996 there were 71 reported cases in three states, and that the production, harvesting, growing, picking and bundling events could lead to contamination by irrigation water or manure (48).

In June and July of 1997, the state health department of Virginia received an increased number of reports of *E. coli* O157:H7 infections than in previous years. Thirty-two cases were reported in 1997, compared with only 11 cases being reported during the same time period in 1996. Many of these cases were linked to consumption of alfalfa sprouts grown from contaminated seeds, with a confirmed case described as "diarrheal

illness... with onset of symptoms from June 1 to September 5, 1997"(9). There was also a related outbreak in Michigan at the same time. This was the first time that an outbreak of *E. coli* O157:H7 infections was linked to consumption of alfalfa sprouts, and it resulted in the removal of 6,000 pounds of remaining seeds from the marketplace in order to prevent more illnesses (9).

Consumption of raw sprouts has been previously linked to outbreaks of various serotypes of *Salmonella*, which can survive for months under the dry storage conditions used for seed storage, and it is thought that *E. coli* O157:H7 follows a similar survival pattern. Research has also shown that *E. coli* O157:H7 can proliferate during alfalfa seed germination and can exist on the outer surface as well as in the sprout vessels. Identification by PFGE suggests the contamination of a lake commonly used for swimming by the feces of a patient ill from consumption of sprouts caused more infections (9).

Water

There have been reported cases of outbreaks of *E. coli* O157:H7 infections linked with swimming water (lakes and pools) as well as drinking water. Because of the low infectious dose, water is an efficient vector for the pathogen. Watersheds that are vulnerable to infiltration by animals, such as lakes, streams, and ponds run a risk of contamination. The first reported outbreak in drinking water occurred in 1989 in rural Missouri. In 1998 there were a reported 157 illnesses that were significantly associated with drinking unchlorinated water from a municipal supply in Wyoming, of these cases hemolytic uremic syndrome developed in three children and one adult (41). A.M. Emmerson recommends that stored water be protected from contamination from birds and other vermin, should be free from bacteria, and that it should be treated at the source by filtration. Mr. Emmerson also states that water should be distributed in a system that is under pressure and in a chlorinated form (23).

Control of the pre-harvest contamination risk due to water would be quite difficult. Contact with wildlife and surface water runoff was implicated in the contamination of the spring fed water supply, and water analysis performed by the EPA

revealed high coliform counts (41). As reported in the World Health Organizations Disease Outbreaks Reports, on May 30, 2000, there was an outbreak in the water supply of Walkerton, Ontario. As of the date of the report there were five reported deaths and 27 reported hospitalizations (51). Contamination of water supplies is relevant to produce safety in that contaminated water may in turn contaminate the produce pre- or post-harvest.

Current Research

Pathogen Control in Juices

Miller and Kaspar, (40) examined the survival properties of *E. coli* O157:H7 in cider containing potassium sorbate or sodium benzoate or no preservative, as well as survival in Tryptic Soy Broth (TSB) adjusted to pH 2, 3, 4, 11, and 12. The study found that compared to the control, *E. coli* O157:H7 were resistant to low pH, and strain number 43895 remained unchanged after 24 hours of incubation at pH 3, 4, and 11, and decreased by 0.5 log at pH 2. Of the *E. coli* O157:H7 inoculated into the cider, 84 to 91% were viable after 21 days at 4^{o}C in the cider containing 0.1% potassium sorbate, and 93% of the cells were viable in the cider containing 0.5%. In cider containing sodium benzoate decreases in numbers ranged from 2 to 57% after 21 days, however the cider that had the 57% decrease still contained 10^{4}CFU/ml. Ciders with no preservative decreased very little with 98% and 91% of cells recovered after 21 days (40).

Conner, *et* al. (18), studied the effect of pH reduction on growth and survival of *E. coli* O157:H7 in tryptic soy broth plus yeast extract (TSB+YE). They found that when held at 25^{o}C populations decreased in acetic, citric, lactic, malic, and tartaric acids. Higher bacterial numbers were recovered at 4^{o}C than 10^{o}C. They concluded that at the pH values tested, *E. coli* O157:H7 has the ability to survive in acidic conditions for up to 56 days (18).

Fresh Produce

Janisiewicz, *et al.* (30), conducted a study to determine population dynamics of *E. coli* O157:H7 in wounded (damaged) apple tissue. This is important because it is a common practice to use drop apples, which can be damaged (30). Wounding and contamination of apples can occur during harvesting or transportation through mis-

handling. In a majority of apple orchards inexperienced manual labor is used for harvesting, which can possibly be a source of contamination as proper harvest methods may not be used (35). Janisiewicz, *et al.* (30), found that populations of *E. coli* were able to survive and grow exponentially after inoculation into wounded apple tissue. The largest increase in numbers was shown in populations with the smallest amount of inoculum, with all inocula eventually reaching a density of 10^6 to 10^7 CFU per wound. It was thought that the ability of *E. coli* to grow in the tissue was due to modification of the adjacent microenvironment by the pathogen (30). Similarly, D.W. Dingman found that when the bruised tissue of Golden Delicious, Red Delicious, Macoun, and Melrose apples was inoculated with *E. coli* O157:H7 the growth of the pathogen was promoted. This was independent of the date of harvest or the source of the apple (21).

Time is an important factor in the survival and growth of *E. coli* O157:H7. In a previous study similar to this one, conducted by B.K. Seeman at Virginia Polytechnic Institute and State University in 2001, it was shown that early in the growth and development of the apple (June through August) there was no internalization after inoculation on day 0 (in June) in the Redfree cultivar of apple. During the two week period before harvest of apples it was found that *E. coli* O157:H7 was readily internalized and was able to survive. It is important to note that research suggests from bacterial sampling of whole fruits that the bacterial counts of native bacterial flora (of uninjured apples) are highest in June and show a steady decline through October. The majority of these bacteria are acidophilic species (acetic and lactic acid bacteria), which are better suited to survive the acidic environment of the apple. The most common of the microflora native to apples are weakly fermentative nonsporing yeasts, not bacteria and mold, and yeast show a peak in growth in September, at a count of 10^2 to 10^6 organisms per apple. Sound (uninjured) apples typically carry a mold load of 10^3 to 10^5 organisms per apple (22).

In a 2001 study of U.S. orchards, Riordan, *et al*. (42), found no *E. coli* O157:H7 in the core areas of collected apples (and pears), or in any environmental samples. However, *E. coli* was found in 6.3% of the fruit that was collected, this includes intact fruit on the tree, wounded fruit on the tree and drop apples. They also found that the calyx and stem ends of the core contained higher total counts than the inner core of the

apples, and there were overall higher total counts in dropped apples. Fruit that was intact on the tree had no difference in counts between fruits that were oriented either calyx up or calyx down (42).

In 2001, Fleischman, *et al.* (25), found that a one log reduction in *E. coli* O157:H7 could be achieved after dipping apples in 40^{o}C water for 90 seconds when apples were inoculated by submersion in a liquid culture. They concluded that since this temperature was sub-leathel to the pathogen that the reduction was due to rinsing of the surface of the apple itself. The greatest reduction in the target pathogen was achieved when apples were dipped in 95^{0}C water, with a reduction of 2 logs when apples were inoculated by submersion in a liquid culture. Results at 80^{0}C were similar, and when apples were dipped in 60^{0}C water the effect was once again attributed to rinsing. When apples were spot inoculated the reduction in the target pathogen was greatest when dipping into water at 95^{0}C and 80^{0}C, showing a 7 to 6 log drop, respectively (25).

Janes, *et al.* (39) found that apples inoculated with different strains of *Escherichia coli* O157:H7, temperature abused (37^{o}C) for 24 hours, and then stored at cold temperatures produce higher viable counts than apples kept at the cold temperature for the entire storage time. Five different strains of *E. coli* O157:H7, and three common cultivars of apple obtained from local grocery stores were used in this study. Apples were surface sterilized by soaking in sodium hypochlorite for 5 minutes and allowed to air dry. Apples were inoculated by stabbing with a pipette tip and expelling culture directly into the apple flesh, the apples were then stored for 28 days at 25 and 4^{o}C. After 28 days apples were sampled and it was concluded that at higher temperatures the pathogen was much more persistent and higher counts were found. Red Delicious apples were treated in the same way but temperature abused at 37^{o}C for 24 hours before cold storage at 4^{o}C, these apples were found to have a higher bacterial load than apples that were kept at a consistent colder temperature (29). In 2001, McKellar, *et al.*, found that at a lowered pH (5.0-5.5) and increased acetic acid content, *Escherichia coli* O157:H7 was more likely to grow at 25^{o}C than at 4^{o}C, being a mesophilic microbe it is not possible for *E. coli* O157:H7 to grow at 4^{o}C in any media, but it can survive at this temperature (39).

There are many potential sources of contamination by *E. coli* O157:H7 in an apple orchard, and many more are presented during harvesting and transportation. While

the apples remain in the orchard the source of pathogen contamination is most likely from contaminated irrigation water or pesticide sprays in which unfiltered or untreated water is used. Drop apples may come into contact with contaminated ground water or animal feces (11). There is also a possibility that apples are contaminated with unclean wash waters or wash water with a low concentration of chemical sanitizer. Many researchers have demonstrated that the use of wash waters that are at a cooler temperature than the temperature of the fruit increases the chances of bacterial internalization. This can be explained by the general gas law. When the apples are submerged in the cooler wash water the gases in their tissues exert reduced pressure, in order for atmospheric and hydrostatic forces to equilibrate with the produce water is taken into the apple (11).

Burnett, *et al.* (10) found that when apples were inoculated under a negative temperature differential (wash water cooler than apple temperature) a "higher number of cells infiltrated the intact skin" as opposed to a lower number found in apples inoculated under a positive temperature differential. A greater number of cells were found not in the loosely packed cells of the lenticels and russet areas, but in cuticular cracks and the narrow crevices that radiate from them. Preferential attachment to damaged areas of the apple by *E. coli* was also found, and infiltration could be related to temperature differential of the inoculum to the apple. In all the samples, regardless of temperature differential there was infiltration into the core of the apple and microscopy showed that the infiltration was through the blossom end of the calyx, progressing up the floral tube and into the core region. All of this was observed using confocal scanning laser microscopy (CSLM) (11).

Kenney, *et al.* (33) used scanning confocal microscopy to visualize *E. coli* O157:H7 GFP in unwaxed organic Red Delicious apples. Warm apples were immersed in chilled peptone which contained the cell suspension in sterile bags. Some apples were bruised in order to observe attachment of the pathogen to bruised tissue. Samples of apple tissue were visualized with a 40x oil immersion objective, and bacteria were detected using an excitation wavelength of 488 nm. Kenney concluded that approximately 5% of the lenticels on a mature Red Delicious cultivar apple remain open, and bruising of the skin may be sufficient to disrupt the cutin that has formed in the lenticels and force microbes deeper within. This results in protection of the pathogen

from traditional washing. Even apples that were dropped from 0.48 m onto padding designed to reduce or eliminate bruising had a 100% chance of being bruised. In this study microscopy showed that the lenticels were colonized by *E. coli* O157:H7 more often than wax platelets, but overall, the bacterial cells tended to penetrate more deeply into the wax platelets. Colonization was also observed in and on wax platelets and lenticels and crevices of apples that were rubbed to simulate washing (33).

In 1999, the USDA-CFSAN studied the infiltration of *E. coli* O157:H7 in oranges. The pathogen was applied to the stem scar of the fruit that were then subjected to a temperature decrease. The fruit were then juiced and the resulting juice was analyzed for numbers of *E. coli* O157:H7. They also performed survival and growth studies by applying pathogens to simulated puncture wounds. They found that oranges had the potential to internalize the pathogen and support its survival (50).

Rutgers University researchers sterilized lettuce leaves with bleach, and still found *E. coli* within the internal tissue of the leaves. They suggested that "small gaps in growing roots are a known port of entry for plant pathogens, and may allow *E. coli*" to enter (17). Similar uptake into tomatoes has also been shown to occur through research. Bartz, *et al*., found that tomatoes with fresh stem scars were more susceptible to infiltration of bacteria, as were fruit that were green (i.e. younger) (3).

In 2001, Beuchat, *et al* (7), explored the possibility of standardizing the protocol for inoculation of fresh produce with pathogenic microorganisms in order to develop standard methods that could be used for a wide array of produce and pathogens. They recommended that several criteria, such as the type of produce, the pathogen of interest, the procedure for inoculation, and evaluation of the test condition, as well as retrieval of the pathogens and reporting of results. These considerations would then shape the method of experimentation. This is because of the standardization results from different researchers and laboratories could be theoretically compared (7).

Research involving *Escherichia coli* O157:H7

In a survey of Wisconsin cider mills Ulijas, *et al.*(21) found that most mills only used hand picked apples, and they inspected apples before washing. They also brushed apples as well as washed them. Very few mills sanitized apples before pressing; however most of the cider mills did sanitize cider making equipment between pressings. Of all of

the cider studied, 43% was pasteurized, 4% was UV light treated, 30% contained preservatives. For all cider, however, 31% of respondents reported that they relied solely on refrigeration and/or freezing for preservation and to prevent pathogen growth. From August 1997 to March 1998, D.W. Dingman, collected as many as 314 samples of apple cider from 11 cider mills in Connecticut. Of these 4% contained *Escherichia coli*. However, all isolates were shown to ferment sorbitol, suggesting that they were not *E. coli* O157:H7 (21).

In 2003, Avery, *et al.*(2), found that of shiga toxin producing *E. coli* O157 isolates originating from meats, bovine and ovine feces, bovine hides, and human isolates an average of 22.9% could survive after drying on concrete. Those that were isolated from cases of human disease, were on average more sensitive to drying. It was concluded that the ability to resist drying would produce a natural selection for these isolates of *E. coli* O157 in the meat chain, thus making it more likely that they would reach consumers. It is entirely possible that this could occur in other foods, as contamination with these drying resistant isolates of *E. coli* O157 from livestock and livestock products could occur (2).

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Chapter 2: Internalization of *Escherichia coli* under Field Conditions in Four Varieties of Apple

Megan L. Hereford*[1], Susan S. Sumner[1], Merle D. Pierson[1], Richard P. Marini[2], Robert C. Williams[1], Randy Worobo[3], D. Kang[4]

[1]Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061

[2]Department of Horticulture, Virginia Tech, Blacksburg, VA 24061

[3]Department of Food Science and Technology, Cornell University, Geneva, NY 14456

[4]Department of Food Science and Human Nutrition, Washington State University, Pullman, WA 99614

KEYWORDS: *Escherichia coli*, Apples, Internalization

***Corresponding author**: Mailing Address: Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061-0418
Phone: 540-231-8679. Fax: 54-231-9293. Electronic mail address: mherefor@vt.edu

ABSTRACT

Fresh, unpasteurized, fruit juices have been implicated as vehicles of transmission of foodborne disease. Of particular interest are outbreaks of *Escherichia coli* O157:H7 associated with apple cider. In this research internalization of *E. coli* into apples during growth on the tree was investigated. Redfree cultivar apples were spray inoculated on the tree (10^6 CFU/apple) with *E. coli* ATCC 25922, which was used as a surrogate strain for *E. coli* O157:H7, at the beginning of the apple growth stage and 30 days into their growth and were sampled every 72 hours. Apples were divided into skin, flesh, inner core, and outer core and sampled for *E. coli* ATCC 25922. For both inoculations there was no recovery of the inoculated bacteria ($< 10^1$ CFU/apple) after 15 days of sampling. Red Delicious, Golden Delicious, and York Imperial apples were spray inoculated on the tree two weeks before expected harvest date with *E. coli* ATCC 25922 and harvested and sampled every other day for two weeks. Apples were divided into skin, flesh, inner core, outer core-stem end, and outer core-blossom end. Results were similar to those found after the early sprayings of the Redfree apples, after 6 days (3 sampling days) there were no *E. coli* ATCC 25922 found in any section of all apple cultivars ($< 10^1$ CFU/apple). It appears that internalization of *E. coli* in apples does not occur while the fruit is on the tree if apples are sprayed two weeks before harvest or during early growth stages, and post harvest internalization should be the focus of concern.

INTRODUCTION

Over the past two decades there have been several outbreaks of foodborne illness associated with the consumption of fresh produce and fresh fruit juices. Among these outbreaks, those in apple cider involving *E. coli* O157:H7 are of particular concern. Increasing popularity of fresh juices and produce may be linked to the increasing trend in foodborne illness associated with the consumption of these products (3, 12). Before the first recognized outbreak of *E. coli* O157:H7 associated with apple cider it was assumed that the low pH of the juice was successful in preventing survival or growth of pathogens (2, 8).

The first reported outbreak of the *E. coli* O157:H7 illness associated with apple cider occurred with fresh-pressed apple cider in 1991 in Massachusetts, with 23 cases reported (2,8). In this particular case, drop apples that were contaminated with animal manure were used to make the cider. In 1993 and 1996 there were two similar outbreaks of *E. coli* O157:H7 illness in which fresh apple cider pressed at a small local cider mill was implicated. The outbreak in 1996 resulted in the death of one child (5, 9). There have also been numerous outbreaks of *E. coli* O157:H7 in which water was the implicated vehicle of transmission. When water comes into contact with fecal material it is possible that it could become contaminated with *E. coli* O157:H7 and watershed runoffs may contaminate municipal supplies that are not properly protected, or lakes, ponds, and swimming pools (13). In January of 2001 the FDA published a final rule requiring Hazard Analysis Critical Control Point (HACCP) plans be put into place in all juice processing facilities. This rule requires that all juices be processed to obtain a 5-log reduction in the most resistant pathogen, for example, *E. coli* O157:H7 in apple cider (6, 7). It is hoped that "implementation of a HACCP system will increase the protection of consumers from illness-causing microbes and other hazards in juices" (7).

It is possible that produce can be contaminated by pathogens either pre- or post-harvest. Pathogens may be internalized into produce before typical washing and sanitizing steps are performed (1). Contamination of the fruit on the tree with *E. coli* O157:H7 could possibly occur from spray water used to apply pre-harvest pesticides. Subsequently the microorganism on the apple could internalize into the fruit.

The morphology of the apple skin allows bacteria to attach to the skin and be protected from washing and brushing. Approximately 5% of lenticels in mature Red Delicious apples remain open at harvest time, providing a place of bacterial attachment. Also damage to the skin of the apple may disrupt the cutin formed in the lenticels of the apples, providing more areas of possible bacterial protection (10). Cutin is a biological polyester that is embedded in wax, this highly water impermeable barrier is attached to epidermal cell walls of the flesh of the apple by pectin (11).

This research explores the possibility of apples being contaminated with *Escherichia coli* pre-harvest in four cultivars of apple, Redfree, Red Delicious, Golden Delicious, and York Imperial. Apples were sprayed while still on the tree with an *E. coli* O157:H7 surrogate strain, *E. coli* ATCC 25922 and sampled to determine if the microorganism was internalized by the apple, and in which part of the apple it was most readily internalized.

MATERIALS AND METHODS

Preparation of Inoculum:

Escherichia coli ATCC 25922 (American Type Culture Collection, Rockville, MD) was used as a surrogate strain for *E. coli* O157:H7. This strain is commonly used in research as a surrogate for *E. coli* O157:H7. Stock cultures were maintained at -76^{o}C in a 50/50 solution of Brain Heart Infusion (BHI) Broth (Difco, Detroit, MI) and glycerol (Acros, New Jersey). Cultures were grown at 37^{o}C in BHI broth and isolated and purified on MacConkey Agar (Difco, Detroit, MI). Typical colonies were confirmed using an API 20 E strip (bioMerieux, Hazelwood, MI). *E. coli* ATCC 25922 was then grown for approximately 24 hours at 37^{o}C in 40 ml of BHI broth. This solution (5 ml) was added to sterilized commercial spring water to achieve a solution of approximately 10^7 cfu/ml. When apples were sprayed evenly with this solution it resulted in an inoculation level of approximately 10^6 cfu/apple.

Inoculation of Apples:

Four cultivars of apple were used in this study, Redfree, Red Delicious, Golden Delicious, and York. The apple orchards are located at Virginia Tech's College of Agriculture and Life Sciences Kentland Farm (Montgomery County, VA). Redfree is an early harvest cultivar, with an anticipated harvest date of late July to mid-August. Red Delicious and Golden Delicious have an anticipated harvest date of mid-September, while York is harvested in mid-October. Redfree apples were sprayed with a handheld sprayer on June 2, 2002, and July 10, 2002 and sampled on days 3, 6, 9, 12, and 15. Red Delicious, Golden Delicious, and York apples were sprayed approximately two weeks before their anticipated harvest dates, on September 4, September 10, and October 1, 2002, respectively and sampled on days 0, 1, 3, 5, and 7. Different trees in different rows were sprayed for each spray date. For each spray date two Redfree trees were sprayed, and three trees were sprayed for other cultivars, approximately 150 apples were randomly selected to be sprayed, except in the case of the Golden Delicious when 200 apples were sprayed. Hand-held sprayers were used to spray each apple individually and then each apple was tagged for identification purposes. The hand held sprayer was a home use two-liter polyethylene compression sprayer. One apple was tested after each spraying to assure proper inoculation. This apple was sprayed and allowed to dry on the tree before being sampled. Two uninoculated apples were randomly sampled as negative controls.

Apple Analysis and Bacterial Enumeration:

Long Term Study

On each sample day, 5 sprayed apples and 2 control apples were picked and sectioned into skin, flesh, inner core, and outer core using a sterilized apple peeler-corer-slicer. Each section was stomached in filtered stomacher bags for two minutes at 230 rpm in a stomacher (Seward, London, England) with 9 ml of sterile peptone water (Difco, Detroit, MI). Samples were plated in duplicate at a 10^{-1} dilution on a weight/weight basis onto MacConkey agar using an Autoplate 4000 Spiral Plater (Spiral Biotech, Norwood, MA). Approximately 1 ml of each sample was added to EC-MUG (4-Methylumbelliferyl-beta-D-Glucuronide) (Difco, Detroit, MI) as a positive/negative confirmatory test. A positive result was identified as fluorescence under long wave UV light and the presence of gas bubbles. *E. coli* was identified on MacConkey agar

primarily by color of colonies and confirmation tests were performed using API 20 E strips.

Short Term Study

During the short term study inoculum and spraying methods were identical to those mentioned above. However, control apples were only picked on the spray date (day 0). For Red Delicious and York, 8 apples were picked on every sampling day, and for Golden Delicious, 10 apples were picked every sampling day. For this cultivar a larger sample size was desired in order to determine if there was an effect of position on the tree where the apple was growing (East versus West). Apples were then sectioned into skin, flesh, inner core, outer core-blossom end, and outer core-stem end, and were prepared for spiral plating as in the long term study. Each sample was then saved and 9 ml of LST broth (Difco, Detroit, MI) was added to each stomacher bag and incubated at room temperature for 24 hours. If, when spiral plates were counted, there was no detectable *E. coli* the corresponding enriched sample was streaked onto MacConkey agar as a positive/negative confirmatory test.

Statistical Analysis:

For the long term study Analysis of Variances (ANOVA) was performed with the Statistical Analysis System (SAS Institute, Cary, NC), and the main effect means were compared with Tukeys Test. For the short term study ANOVA was performed on the 3x4x5 factorial experiment (3 varieties, 4 dates, and 5 samples). The 3-way interaction was significant, so ANOVA was performed using SAS (SAS Institute, Cary, NC) for each cultivar to test the main effects of sample and day. Sample and day did not interact, so means were compared with Tukeys Test.

RESULTS

On the first day of sampling in June and July there were no recoverable amounts of *E. coli* ATCC 25922 on spiral plates. The percentage of positive confirmation tests was compared using ANOVA and Tukeys Test (SAS Institute, Cary, NC). There was no significant effect ($P \geq 0.05$) of apple section (skin, flesh, inner core, and outer core), and there was no significant interaction ($P \geq 0.05$) between day and section. There was a

significant effect ($P \leq 0.0001$) of day for apples sprayed in June. The percentage of positive confirmation tests for days 3, 6, and 9 were significantly different from days 12 and 15 (Table 1). When apples were sprayed in July there were no detected amounts of *E. coli* ATCC 25922 on spiral plates and no positive confirmation tests for any sampling day beginning on day three.

All apples that were analyzed for the short term study had a wide array of background growth that was identified using an API 20 E strip. Red Delicious and Golden Delicious apples commonly had *Klebsiella oxytoca* as well as a unidentified yeast. York apples were found to have *Enterobacter agglomerans*, and possibly *Enterobacter aerogenes, K. oxytoca,* and *Serratia odorifera*, as well as a unidentified yeast. When the percentage of positive confirmation tests was compared using ANOVA and Tukeys test (SAS Institute, Cary, NC) for Golden Delicious apples there was no significant effect of day ($P = 0.7447$), and there was no interaction between day and section of apple ($P = 0.3030$). There was a significant effect of section of the apple ($P \leq 0.0001$), skin sections had a significantly higher percentage positive confirmation tests than flesh, inner core, outer core-stem end, and outer core-blossom end samples (Table 2). For Red Delicious apples there was no significant effect of day ($P = 0.0767$), and there was no significant interaction between day and section of apple ($P = 0.7766$). There was a significant effect of section of the apple ($P \leq 0.0001$), skin, flesh, and outer core-stem end sections had a significantly higher percentage of positive confirmation tests than outer core-blossom end, and inner core sections (Table 2). For York apples there was a significant effect of day ($P \leq 0.0001$), and there was a significant interaction between day and section of apple ($P = 0.0131$). There was no significant effect of section of apple ($P = 0.0549$). Days 1 and 3 had a significantly different percentage of positive confirmation tests than days 5 and 7 (Table 3).

DISCUSSION

When apples were inoculated under field conditions in the summer and fall of 2002 there was no significant survival (recovery) of *E. coli* ATCC 25922 after the day of inoculation. Apples were sprayed and harvested in the morning before 10:00 a.m. and it

is possible that cell death from UV damage prevented internalization of the pathogen. The general weather conditions for that particular year were very dry, and apples ripened and were harvested earlier than their anticipated harvest date. The temperature differential between inoculum water and the apples may not have been sufficient for internalization to occur. If the water that contains the pathogen is colder than the tissue of the apple then gasses in the apple tissues exert a reduced pressure, and in order for atmospheric and hydrostatic forces to equilibrate with the produce, water (which contains the pathogen) is taken into the apple (4).

Results of the long term study indicate that *E. coli* did not survive on immature apples (those sprayed in June), or apples sprayed approximately one month before harvest. This may be due to the lack of intracellular spaces, as cells are constantly dividing during apple growth. All apple cultivars supported the survival of native microflora, with the most common in all apple cultivars being *Klebsiella oxytoca*. Results of the short term study show that all three cultivars of apple, Red Delicious, Golden Delicious, and York had no significant amounts of *E. coli* ($P \geq 0.05$) recovered after one week of sampling. Golden Delicious had the highest amount of internalization of all three cultivars, with the skin supporting a low amount of survival compared to other areas of the apple. For Red Delicious this occurred in the outer core-stem end as well as the skin and flesh, and in York all parts of the core showed a higher amount of internalization relative to other parts of the apple. When Burnett, *et al.*, examined apples using confocal scanning laser microscopy they found, regardless of temperature differential between the inoculum and the apple, that infiltration into the core of the apple occurred (4). Initial internalization occurred through the blossom end of the apple and progressed up the floral tube and into the core region. This research did not use whole apples on the tree, instead whole apples were immersed in the inoculum. Conflicting data was presented by Riordan, *et al.* (14), in a survey of U.S. orchards the core areas of collected apples and pears there was no *E. coli* O157:H7.

Even though this data supports the argument that internalization occurs at low levels pre-harvest, there is still a possibility that it could occur and at high enough levels to provide a risk to consumers of fresh, unpasteurized apple cider. Kenney, *et al.* (2001) found that *E. coli* was able to colonize lenticels of Red Delicious apple, which protects

the pathogen from washing and scrubbing. The pathogen also penetrated deeply into wax platelets (10). It is possible that this also prevents recovery of the pathogen by traditional microbiological methods such as stomaching. *Esherichia. coli* O157:H7 also preferentially attached to damaged areas of apple (4). Janisiewicz, *et al.*, (9), found that *E. coli* populations were able to survive and grow exponentially in wounded apple tissues (9). Wounding of apples is less likely to happen on the tree, and therefore wounded areas due to bruising and puncturing would not be available to pathogens in the event of internalization occurring in the orchard. Damage to the apples from russet sports or fungal growth is much more likely and could provide these damaged areas that would support the survival and growth of the pathogen before the apples are harvested.

There was no significant amount of the surrogate species, *E. coli* ATCC 25922, found in the four cultivars of apple after day zero. Redfree apples did not support the growth or survival of *E. coli* when they were sprayed during early growth stages. Red Delicious, Golden Delicious, and York apples that were sprayed with the surrogate species two weeks before harvest also did not support the growth or survival of *E. coli* past one week after inoculation. It is possible that if apples are harvested and consumed immediately after a possible spraying with a pathogen such as *E. coli* O157:H7 then infection could occur.

ACKNOWLEDGEMENTS

Funding for this research was supported by a grant from the Cooperative State Research, Education and Extension Service/United States Department of Agriculture's Integrated Research Education and Extension Grant Program (Project 208-11-110A-333-1) entitled Internalization of *E. coli* in Apples Under Field and Laboratory Conditions.

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Percentage of positive confirmation tests	Day
70a	3
65a	6
50a	9
0b	12
0b	15

Table 1. Percentage of positive confirmation tests for Redfree cultivar of apples sampled in June. N = 20 for each day. Means followed by the same letter are not significantly different at an alpha level of 0.05.

Percentage of positive confirmation tests- Golden Delicious	Percentage of positive confirmation tests- Red Delicious	Section
5a	6a	Skin
3b	5a	Flesh
2b	1b	Inner Core
2b	5a	Outer Core-Stem End
1b	1b	Outer Core-Blossom End

Table 2. Percentage of positive confirmation tests for Golden and Red Delicious cultivar of apples in five sections of the apple. N = 50 for each Golden Delicious section, N = 40 for Red Delicious. Means followed by the same letter are not significantly different at an alpha level of 0.05.

Percentage of positive confirmation tests	Day
4b	1
4b	3
8a	5
1c	7

Table 3. Percentage of positive confirmation tests for York cultivar of apples over 4 successive sampling days. N = 40 for each day. Means followed by the same letter are not significantly different at an alpha level of 0.05.

Chapter 3: Post Harvest Internalization of *Escherichia coli* O157:H7 in Apples as Examined by Confocal Scanning Laser Microscopy

Megan L. Hereford*[1], Susan S. Sumner[1], Merle D. Pierson[1], Richard P. Marini[2], Robert C. Williams[1], Randy Worobo[3], D. Kang[4]

[1]Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061

[2]Department of Horticulture, Virginia Tech, Blacksburg, VA 24061

[3]Department of Food Science and Technology, Cornell University, Geneva, NY 14456

[4]Department of Food Science and Human Nutrition, Washington State University, Pullman, WA 99614

KEYWORDS: *Escherichia coli* O157:H7, Apples, Internalization, Confocal Microscopy

***Corresponding author**: Mailing Address: Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061-0418
Phone: 540-231-8679. Fax: 54-231-9293. Electronic mail address: mherefor@vt.edu

ABSTRACT

Internalization of pathogens in fresh produce may be responsible for outbreaks of illness associated with consumption of fresh fruits and vegetables, and fruit juices. The objective of this project was to examine internalization of *Escherichia coli* O157:H7 in four cultivars of apple, Red Delicious, Golden Delicious, Rome, and York, and to determine which cultivar more readily internalized the pathogen, and if bruising or puncturing had any significant effect on internalization rate. Apples were left unblemished or were manually bruised or punctured. They were then immersed in cold spring water (4^{o}C) containing *E. coli* O157:H7 and incubated for three days at room temperature (23^{o}C) before sampling and microbiological enumeration, as well as examination by confocal scanning laser microscopy. In all cultivars except Golden Delicious the punctured apples had the highest rate of internalization. Unblemished Golden Delicious apples had the highest rate of internalization when compared to bruised and punctured apples of this cultivar. Red Delicious apples had the highest counts in the outer core-stem end samples, while the other three cultivars had the highest counts in the skin samples. Damage to apples can slightly increase the rate of internalization of *E. coli* O157:H7. Washing of apples, unblemished or damaged, in water containing the pathogen, could promote the internalization of *E. coli* O157:H7 in fresh juices produced from apples.

INTRODUCTION

Escherichia coli O157:H7 is associated with over 70,000 estimated cases of illness each year (8). When the microbe is ingested by humans it produces a toxin that causes bloody and non-bloody diarrhea (hemorrhagic colitis). *Escherichia coli* O157:H7 is also the leading cause of hemolytic uremic syndrome, a rare and potentially fatal disease that is the leading cause of acute kidney failure in children (5, 9). This pathogen was first recognized in 1982, and has been associated with food borne outbreaks in beef, apple cider, and water, as well as many other foods. The majority of these outbreaks are associated with ground beef (2), which may be associated with the fact that *E. coli* O157:H7 is naturally occurring in the bovine intestine, in which it is asymptomatically carried and shed (11).

It is possible for produce to become contaminated with *E. coli* O157:H7 of bovine origin as well as from birds, deer, and other sources (6). Washing may not remove the pathogen from the surface of produce, and internalization of the pathogen by the produce protects the microbe from the effects of washing and sanitization, although thorough washing still may not remove the pathogen from the fruits surface (1). The condition of fruit (apples) plays a major role in the internalization of *E. coli*. Damage to the skin of the apple makes the fruit more vulnerable to bacterial invasion. Injury of the fruit can possibly occur anytime from picking to storage, and possibly on the tree. *Escherichia coli* O157:H7 can also survive in low pH juices that were traditionally thought to prevent the survival of pathogenic microorganisms. Apple cider has an average pH of 3.7, and research has shown that *E. coli* O157:H7 can survive in cider at this pH for up to 31 days at refrigeration temperatures of approximately 8^{o}C (12, 14). The pathogen is viable but non-culturable state in water for prolonged periods of time (10).

Confocal Scanning Laser Microscopy (CSLM) is a powerful tool, permitting the generation of three-dimensional images, and can be used to analyze structural details of thick slices of sample. Combining this microscopy technique with fluorescence microscopy is extremely valuable, producing high-resolution images in a single fast scan (13). *E. coli* O157:H7 GFP (green fluorescent protein) has the ability to express a fluorescent product. This gene is located on a plasmid that carries ampicillin resistance to ease in the identification of the microbe on agar plates containing the inducer

isopropyl-beta-D-thiogalactoside (IPTG). Purified GFP absorbs blue light from 395 nm to 470 nm and emits green light at approximately 509 nm (4).

When apples are inoculated by immersion under a negative temperature differential, bacterial cells infiltrate the skin of the apple, and infiltration can be related to the temperature differential (3). Kenney, *et al.* (7), have shown attachment of *E. coli* O157:H7 GFP to bruised tissue of Red Delicious apple, visualized by confocal scanning laser microscopy (CSLM). They also found that green fluorescent protein (GFP) *E. coli* O157:H7 colonized apple skin lenticels of Red Delicious apples. Burnett, *et al.* (3), found that *E. coli* O157:H7 GFP preferentially attaches to damaged areas of apples, as well as through the blossom end of the calyx and up the floral tube to the inner core. This research project examines the ability of the *Escherichia coli* O157:H7 to be internalized in whole apples. The rate of internalization was compared among cultivars, as well as the location of internalization.

MATERIALS AND METHODS

Preparation of Inoculum

Escherichia coli O157:H7 ATCC 43889 (American Type Culture Collection, Rockville, MD) was transformed to express fluorescent proteins through insertion of the Clontech plasmid (Clontech, Palo Alto, CA) pEGFP for green fluorescent protein expression (Worboro, R. Cornell University, Geneva, NY). In addition to GFP expression the plasmid also contains an ampicillin resistance gene. When in the presence of isopropyl-beta-D-thiogalactoside (IPTG), the lac promoter is "turned on", inducing expression of the GFP, which fluoresces green under long wave UV light.

Stock cultures were maintained at -76^{o}C in a 50/50 solution of Brain Heart Infusion (BHI) Broth (Difco, Detroit, MI) and glycerol (Acros, New Jersey). Cultures were grown at 37^{o}C in BHI broth supplemented with 100 µg/ml of ampicillin (Sigma, St. Louis, MO) and 10 µg/ml of IPTG (Sigma, St. Louis, MO) for 24 hours and were isolated and purified on MacConkey Agar (Difco, Detroit, MI) containing 100 µg/ml of ampicillin and 10 µg/ml of IPTG. Typical colonies were confirmed by fluorescence under long wave UV light. *Escherichia coli* O157:H7 was then grown for approximately 24 hours at 37^{o}C in 40 ml of BHI broth supplemented with ampicillin and IPTG.

Preparation and Inoculation of Apples

Apples were obtained from the Virginia Tech College of Agriculture and Life Sciences Kentland Farm research orchard near Blacksburg, VA. Four cultivars of apple were used, Red Delicious, Golden Delicious, Rome, and York. Unblemished mature apples were harvested in September and October of 2002. They were stored for approximately 3 to 4 months at 4°C. Apples were held at room temperature (23°C) for 24 to 48 hours before inoculation. Apples were mechanically bruised by dropping them from a 15 cm height onto a sanitized metal surface, or they were punctured four times with a sterile metal inoculating needle to a depth of 2.5 cm. For each cultivar: six apples were left unblemished, of these, four were inoculated and two were used as negative controls; six apples were bruised, of these, four were inoculated and two were used as negative controls; six apples were punctured, of these, four were inoculated and two were used as negative controls. Twenty ml of a 24-hour-old culture of GFP *E. coli* O157:H7 was added to 1980 ml of sterilized commercial spring water at 4°C in a sterile 4L beaker. Apples at room temperature (approximately 27°C) were immersed in the spring water solution for 10 minutes. Inoculated apples were allowed to air dry in a biohazard hood on sterile baking racks for approximately 30 minutes. All apples, treated and control, were then placed into separate Whirl-pak bags (Nasco, Fort Atkinson, WI) and incubated at room temperature (approximately 27°C) for three days.

Apple Analysis and Bacterial Enumeration

After incubation, three of the six apples (one control and two inoculated apples) for each treatment (i.e. unblemished, bruised, punctured) from each cultivar (i.e. Red Delicious, Golden Delicious, Rome, and York) were divided into sections (i.e. skin, flesh, inner core, outer core-blossom end, and outer core-stem end) using an at home use apple-peeler-corer-slicer. This equipment was sanitized with chlorine (200 ppm) and 20% ethanol before and after each apple was sectioned. Untreated controls were sampled first. Each section was homogenized in filtered stomacher bags for two minutes at 230 rpm in a stomacher (Seward, London, England). Samples were plated in duplicate at a 10^{-1} dilution (w/w) onto MacConkey agar supplemented with ampicillin and IPTG using a Autoplate 4000, Spiral Plater (Spiral Biotech, Norwood, MA). All remaining homogenized sample was enriched by addition of 10 ml of LST broth (Difco, Detroit,

MI) and incubated at room temperature. Spiral plated MacConkey plates were incubated at 37°C for 24 hours. If there were no *E. coli* O157:H7 present on spiral plates then the enrichment samples were streaked onto MacConkey agar supplemented with ampicillin and IPTG and incubated at 37°C for 24 hours. If bacteria were observed after 24 hours of incubation the result was recorded as positive, if there was no growth then negative was recorded.

Confocal Scanning Laser Microscopy

Two inoculated and one control apple for each treatment of each variety was examined using a Zeiss LSM 510 Laser Scanning Microscope on an Axiovert 100M using a 40X water immersion lens with a 1.2 numerical aperature. An argon laser provided excitation at 480 nm, and a 505-550 nm emission filter was used to prevent detection of autofluorescence of the parenchyma cells of the apple. Apples were sliced by hand using a sterilized knife. Thin slices of the apple flesh and core were then examined and imaged. Presence of *E. coli* O157:H7 GFP was recorded, as well as location of the microbe in relation to cellular structure of the apple.

Experimental Design and Statistical Analysis

Apples were randomly picked from the orchard and stored. When required for laboratory sampling only unblemished apples of each cultivar were chosen. For each cultivar the total number of samples plated on MacConkey agar was 15. Of these 15 samples, 5 were from one control apple (i.e. skin, flesh, inner core, outer core-stem end, outer core-blossom end). Microbial counts were averaged for duplicate samples (Log cfu/ml of sample= Log cfu/g of sample) so there was one count reported for each sample. The experiment was a 4x3x5 factorial in a completely randomized design. There were four cultivars, three treatments (unblemished, bruised, and punctured), and five fruit samples (skin, flesh, inner core, outer core-stem end, outer core-blossom end). Analysis of Variance was used to analyze each cultivar separately using the General Linear models procedure of the Statistical Analysis System (SAS Institute, Cary, NC) because the 3-way interaction was significant ($P<0.05$). Tukey's HSD Test was used to compare mean counts (Log cfu/ml of sample=Log cfu/g sample)

RESULTS

When each cultivar of apple was analyzed (plate counts) separately there was a significant ($P < 0.001$) effect of section (i.e. skin, flesh, inner core, outer core-blossom end, and outer core-stem end), but no significant ($P \geq 0.05$) effect of treatment (i.e. untreated, bruised, and punctured) for number of *E. coli* O157:H7. However, when each cultivar within treatments was analyzed there was a significant difference among the cultivars of apple ($P \leq 0.05$). York had the lowest counts for each treatment (Figure 1), and punctured York apples had higher counts than bruised or untreated York apples. When apples were punctured or bruised, the Red Delicious had the highest overall counts of all cultivars. When apples were left uninjured and inoculated, Golden Delicious had the greatest amount of internalization.

When sections were compared among cultivars, Red Delicious apples had the greatest amount of internalization in the outer core-stem end of the apple (Figure 2), as well as the outer core-blossom end and the inner core. Golden Delicious apples had the highest rate of internalization in the skin and the flesh among all cultivars of apple. York apple sections were consistently lowest among all cultivars of apples for all sections ($P \leq 0.05$).

Red Delicious inner core samples had a significantly ($P=0.027$) lower *E. coli* O157:H7 count than the stem end of the outer core (Figure 2). In Golden Delicious apples internalization was significantly higher in the skin and flesh than the inner core ($P=0.0021$) different lower counts than skin and flesh of the apple (Figure 2). The outer core-blossom end and skin sections of Rome apples had significantly ($P=0.0073$) higher numbers of *E. coli* O157:H7 than the inner core section of the apple (Figure 2). *E. coli* O157:H7 counts for inner core samples were significantly less ($P=0.0182$) than skin samples for York apples (Figure 2).

Examination of flesh and core sections of all cultivars of treated apples revealed the presence of *E. coli* O157:H7 GFP in the extracellular spaces of the apple parenchyma cells. The bacteria were highly clustered in areas of flesh that had been bruised and

punctured, and were more evenly distributed throughout the flesh of uninjured apples. Untreated York apples was most difficult to image the microbe in after examination of numerous samples.

DISCUSSION

Kenney, *et al.* (7), found that when apples were inoculated under a negative temperature differential bacterial cells preferentially attached to bruised (damaged) sections of the apple. Bacterial cells also colonized the lenticels, of which 5% on mature Red Delicious apples remain open. In this study internalization occurs more readily in certain cultivars than others, and the area that microbes are most likely to be internalized is dependent on the cultivar of apple. Damaging the apple has an effect on the rate of internalization, but not the area of internalization for each cultivar. If apples are washed in water containing pathogenic microbes it is possible that the microbes could be internalized, presenting a hazard to consumers of fresh apple products such as minimally processed cider. Lack of chlorination, or insufficient chlorination of wash water means that pathogens that are washed off of apples or debris could contaminate the water and then other apples. *Escherichia coli* O157:H7 may survive in a viable but non-culturable state in water, especially cold water (10).

In this study damage to apples increased the rate of internalization into all sections of the apple, except in the case of Golden Delicious apples. Use of damaged (non table quality) apples is a common practice for cider mill operations. Apples can be damaged during harvest or transportation and storage, as well as during washing steps. Damage could consist of bruising and puncturing. Damaged apples show a higher rate of internalization than undamaged apples, when the damage occurs immediately before contact with the pathogen. If pathogens have been internalized into the flesh and core of the apple, it is possible for them to be present in the resulting juice after pressing. Washing of this damaged produce may remove microbes from the surface, but will have no effect in removing those that are protected inside the apple.

Confocal scanning laser microscopy (CSLM) showed the presence of *E. coli* O157:H7 GFP in all cultivars of apple that were unblemished, punctured, or bruised, in the core and flesh of the apples when examined with a Zeiss LSM 510 Laser Scanning

Microscope using a 40x water immersion lens. The bacteria were not within cells of the apple, but were present in intracellular spaces between cells. Bruised and punctured flesh of the apples had large, clumped groups of bacteria present, and damage to cellular structure of the apple was apparent, especially in the Golden Delicious apples. York apples had the lowest amount of recoverable *E. coli* O157:H7 GFP and this was also true when sections of the flesh and core of unblemished, bruised, or punctured apples were examined by CSLM. The visual presence of *E. coli* O157:H7 GFP as observed by CSLM reinforces microbiological findings that internalization does occur in apples. Infiltration occurs through the skin and core of undamaged apples, with the presence of bacteria detected in the inner most sections of the apple (i.e. inner core). Damage to the apple allows a greater amount of bacteria to be internalized for most cultivars.

ACKNOWLEDGEMENTS

Funding for this research was provided by a grant from the Cooperative State Research, Education and Extension Service/United States Department of Agriculture's Integrated Research Education and Extension Grant Program (Project 208-11-110A-333-1) entitled Internalization of *E. coli* in Apples Under Field and Laboratory Conditions.

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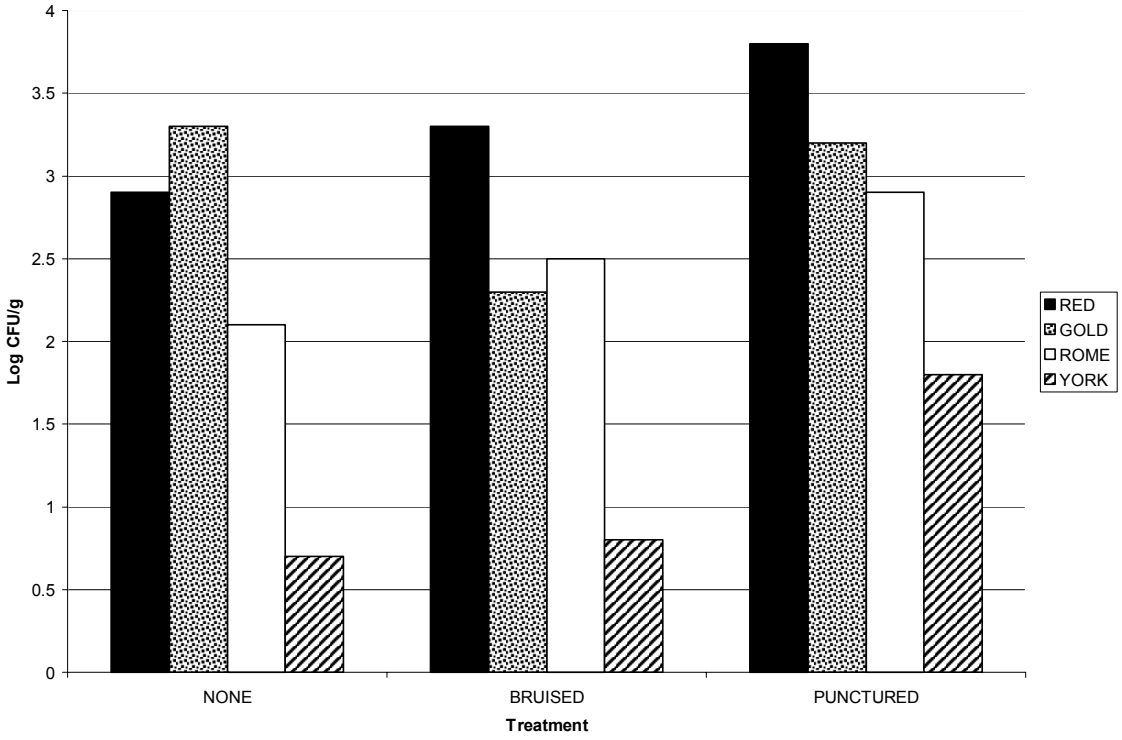

Figure 1: Count of *Escherichia coli* O157:H7 present in apples (Log cfu *E. coli* O157:H7/g apple) for each treatment (none, bruised, punctured) by cultivar (Red, Gold, Rome, York).

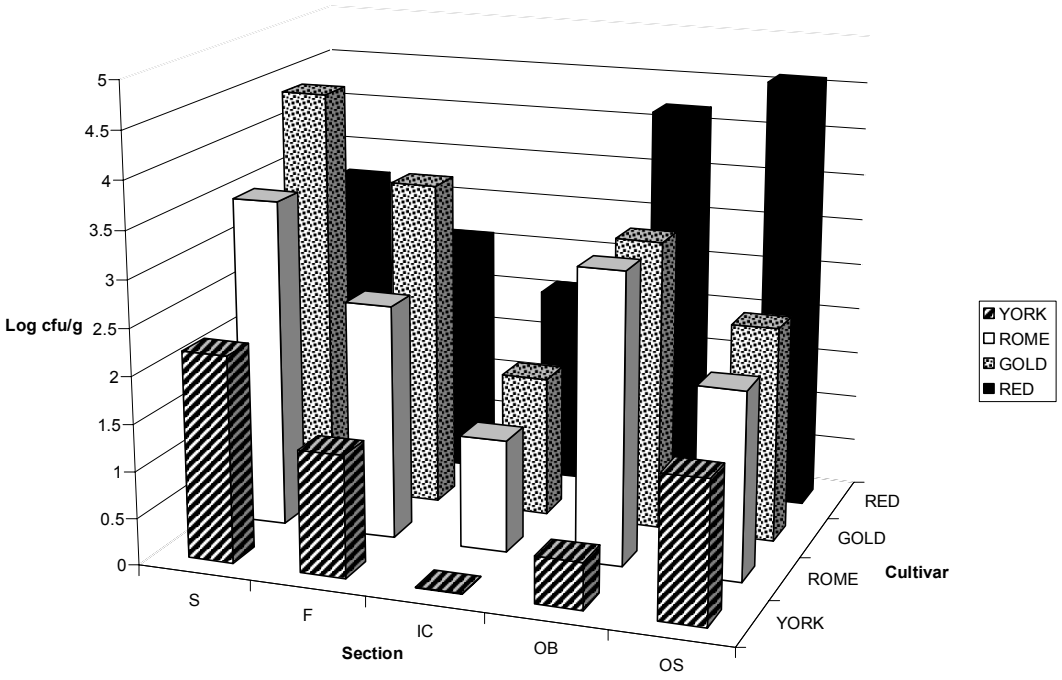

Figure 2: Count of *Escherichia coli* O157:H7 in apple sections (log cfu *E. coli* O157:H7/g apple) for each cultivar (Red, Gold, Rome, York) by section (S=skin, F=flesh, IC=inner core, OB=outer core-blossom end, OS=outer core-stem end).

VITAE

Megan L. Hereford

Megan Hereford began her college education at Virginia Tech in the fall of 1997, and received her bachelor's degree in biology in the spring of 2001. She started her masters program at Virginia Tech in the Department of Food Science and Technology in the fall of 2001 under Dr. Susan Sumner. During her two years as a graduate student she has been active in the Institute of Food Technologists Student Association chapter (Food Science Club), and was Communications Officer for one year. She has also been very active in the student professional development group for the International Association of Food Protectionists (IAFP-SPDG), holding the office of treasurer for the IAFP-SPDG in the fall of 2002 and spring of 2003. She is looking forward to continuing her education when she starts her PhD work in the fall of 2003 at Virginia Tech.