INTRODUCTION

Many consumers are drawn to fresh, unpasteurized juices and ciders over heat treated products because of their distinct flavors and aromas as well as enhanced nutritional values. In the past, regulations on juice products have been limited since these products are naturally acidic thus limiting microbial growth. However, recently the consumption of fresh apple juice and apple cider has been linked to outbreaks associated with pathogens such as *Escherichia coli O157:*H7, *Salmonella* species and *Cryptosporidium parvum*. Most of the attention has been drawn to *E. coli* O157:H7 due to the severity of infection, especially in younger children and the elderly. Two major outbreaks, 1991 and 1996, linked *E. coli* O157:H7 to be the cause of 23 and 66 persons to become ill, respectively. One child died in the 1996 outbreak (22). As a result of the escalating numbers of juice-associated outbreaks, the Food and Drug Administration (FDA) announced plans for a program to address food borne illnesses linked to juices in order to improve the safety of fresh and minimally processed fruits and fruit products (24).

The FDA issued a statement on the importance of the specific issue of bacterial internalization, relative to the production of fresh fruits and juices in August of 1997. A better understanding of internalization is paramount for improving the safety of fresh and minimally processed fruits and fruit products. After several comment periods, the final rule was published on January 19, 2001. In this document, the FDA addressed a series of comments regarding alternatives to HACCP programs. The agency concluded that although there are many likely alternatives to controlling food borne illness associated with juices, a HACCP plan allows for the most flexibility among processors as well as the most effective means of assuring food safety (26). The final rule is intended to force processors to determine likely sources of microbial, chemical and physical hazards that could contaminate their products. Processors are now required to implement strategies to control these hazards, as well as implementing a five-log reduction of the most resistant pathogen in the finished juice product (26).

This project addresses the issue of food borne pathogen internalization in apples through laboratory studies using *E. coli* O157:H7 and outside the laboratory under field

conditions using a surrogate, *E. coli* ATCC 25922. The purpose of this project is to provide a scientific basis for determining if, how, and when there is internalization of apples by human pathogens such as *E. coli* O157:H7. Such information can be used to develop control measures for preventing fruit infection that can be adopted by table apple and apple cider producers. Literature supports the concept that microorganisms can internalize, survive, and under the right conditions, grow within fruits and vegetables. Currently, the entry and movement of microorganisms in the interior of the fruit is not well understood. Research needs to be done to improve the assurance and awareness of microbiological safety relative to apples and apple products where internalization of pathogenic microorganisms could be a concern.

LITERATURE REVIEW

A. *Escherichia coli* Species

1. General Characteristics

Escherichia coli is a Gram negative, nonsporeforming rod-shaped organism. The organism ferments glucose with the production of acid and gas and is motile through peritrichous flagella. The bacteria grow at pH levels between 4.4 and 9.0 (37). *E. coli* is a member of the Family *Enterobacteriacaea.* The organism is found in the intestines of most warm-blooded animals, such as cows, deer, and humans. The vast majority of strains are harmless to humans; however, one strain, which is commonly found in cows, O157:H7, is pathogenic to humans (49).

Overall, there are more than 170 different serogroups known. There are over 200 types of O antigens, 100 K antigens and 50 H antigens (32). The O antigen is the polysaccharide that projects from the core polysaccharide while the K antigen is the polysaccharide capsular antigen. The H antigen is the flagellar protein. There are considerably less H antigens because the carbohydrate side chains are less heterogeneous than that of the O antigen (32). The different O, H and K antigen formula gives each strain its name (32).

Further classification of the bacteria categorizes them into several groups: enterohemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EaggEC), enteroinvasive *E. coli* (EIEC), and enteropathogenic *E. coli* (EPEC). EHEC or STEC organisms cause hemorrhaging and loss of blood due to the production of one or more toxins that are identical to that produced by *Shigella dysenteriae* (15). ETEC are often associated with outbreaks in childcare centers and with traveler's diarrhea. This disease requires colonization of the organisms before the toxins will be produced. The toxin produced by ETEC is similar to the cholera toxin, producing diarrhea without a fever. EPEC organisms do not contain the heat labile or heat stable toxin that the ETEC organisms contain, nor do they require the colonization factors. However, EPEC is similar to ETEC by its production of watery diarrhea. EaggEC are noninvasive, cause no inflammation and are identified by the cell's tendency to clump together. EaggEC causes persistent non-bloody, watery diarrhea in young children. Unlike the ETEC, EPEC and EaggEC,

the EIEC is an invasive organism that grows within the cytoplasm of the intestinal epithelial cells. EIEC resembles *Shigella* due to the pathogenic mechanism. There is no toxin associated with EIEC; however, infection produces dysentery-like symptoms including diarrhea and fever.

2. *Escherichia coli* O157:H7 Characteristics

Escherichia coli O157:H7 is set apart from other *E. coli* species by its inability to ferment sorbitol within 24 hours, the absence of β-glucuronidase, and its slower growth at temperatures higher than 44.5°C. *Escherichia coli* O157:H7 can grow between pH 4.0 to 9.0 (37). It was first identified by the Centers for Disease Control and Prevention in 1975, but it was not until a 1982 outbreak associated with undercooked hamburger meat that the organism was identified to be the cause of enteric disease (15).

Escherichia coli O157:H7 is the most common member of the EHEC group. Other members include O103:H21, O111:MN, and O145:H25. The virulence genes associated with EHEC organisms are located on a plasmid and on a pathogenicity island located on a chromosome. The plasmid, which is about 60 mega-Daltons, encodes for the enterohemolyin as well as for fimbriae to help the organism adhere to the surface of the intestine (32). The pathogenicity island allows the organism not only to adhere to the intestinal walls but also to create lesions in the mucosa (15). In addition to the plasmid and the chromosomal pathogenicity island, there are two shiga-like toxins, SLT-I and SLT II, which are encoded on a bacteriophage. New terminology has renamed these toxins Stx1 for SLT-I and Stx2 for SLT-II (32). The verotoxins are referred to as shiga-like due to their similarity to the toxins produced by *Shigella dysenteriae* (32).

3. *E. coli* O157:H7 Reservoirs and Transmission

The main reservoir for pathogenic strains of *E. coli* is the intestinal tract of warm-blooded animals (48). Cattle are specifically the most common reservoir of this organism. It is unknown how the cattle are infected with this organism; however, the organism does not cause illness in the cattle (48). Smith (2001) found that the organism could be introduced to cattle through drinking contaminated water (45). Here, the route

of infection would be oral, not fecal (45). Irrigation water and surface run-off water can be a reservoir of pathogenic microorganisms (9,50).

Most commonly, *E. coli* O157:H7 is spread from host cattle to other livestock as well as to food products through cattle feces (45). Smith (45) showed the prevalence of the organism in feedlot cattle. The results showed that 23% of the tested animals shed the organism in their feces (45). *E. coli* O157:H7 can remain viable for up to 70 days in cattle feces (9).

Research has also shown that wild birds, mostly gulls, can be a source of transmission. In this case, the birds were thought to have been picking up the organisms by feeding on sewage or lands where cattle graze. Fecal samples obtained at urban landfills were positive for vero cytotoxin-producing *E. coli* O157:H7 (2). Other routes of transmission include recreational (27) and drinking water (16), and person-to-person transmission, as in child day-care facilities (4). However, the primary sources of transmission are through improperly prepared meat products (27) and contaminated produce.

4. Illness and Pathogenicity

In the United States, *E. coli* O157:H7 is the most common cause of Hemolytic Uremic Syndrome, HUS, and hemorrhagic colitis (6). Bacterial infections by *E. coli* O157:H7 are more severe among children under the age of 5, the elderly, and the immunocompromised. The infective dose is unknown and is thought to be as low as 1000 cells. The ease of person-to-person transmission supports the theory of a low infective dose (26). However, the more bacteria that are consumed, the sooner symptoms will appear (49).

Once ingested, the bacteria travel through the stomach, pass through the small intestine and attach to the lining of the large intestine causing inflammation of the intestinal wall. The inflammatory response is the body's reaction to the toxin produced by the bacteria (15). Symptoms of infection can begin to appear within hours or within several days; however, the typical incubation period is between three and nine days (32). Hemorrhagic colitis begins with intense abdominal pains and severe cramping, followed by diarrhea and vomiting. The diarrhea will become watery and bloody. Other

symptoms include lowered red blood cell counts, also known as hemolytic anemia, and lower platelet counts (26). The infection is usually self-limiting and lasts approximately eight days (32). Antibiotics do not help to clear the infection; the usual treatment is supportive care (15).

Most illnesses occur without any further problems, however secondary problems may take place. Between 2 and 7% of the patients develop HUS, which causes kidney failure in children (15). This occurs when the bacteria travel through the inflamed intestinal wall and release a shiga-like toxin. This toxin attaches to the inside of blood cells and is then carried throughout the body. Most damage occurs in the kidney, pancreas, and brain where the blood supply is the highest. Once HUS begins, there is no way to stop the progression of the disease. The majority of patients with HUS require blood transfusions; however, there is a 3- 5% mortality rate associated with HUS (26). HUS is the foremost cause of acute kidney failure in children (15). The CDC estimates approximately 73,000 cases, 2,100 hospitalizations and 61 deaths annually in the United States alone (15).

5. Acid Resistance

Escherichia coli O157:H7 is able to withstand lower pH levels than other *E. coli* species. This is due, in part, to its ability to adapt to the environment (28). Exposure to sub-lethal pH levels causes microorganisms to adjust and subsequently to survive at even lower pH levels (28). Tolerance to lower pH levels is also dependent on the growth phase of the organism. Acid tolerance peaks at the late stationary phase and is followed by a secondary peak at the early stationary phase (5). Similar results have been shown to occur with other microorganisms such as *Shigella flexneri* (5). In addition to stationary phase induced acid tolerance, Arnold and Kaspar (1) showed acid tolerance to be induced in log-phase cells through starvation (1). This is due to starvation-induced proteins that are produced by the cell. The proteins protect the cell from further chemical and physical challenges (1).

The ability of organisms to persist in acidic pH environments may account for a low infective dose. Research has shown that the oral infective dose is proportional to the relative level of acid resistance (35). For example, the infective dose for *V. cholerae* is

approximately 10^9. This organism is not as resistant to higher levels of acid as *Shigella flexneri*, which has an infective dose of 10^2 (35). Acid resistance may help them pass through the stomach, where the pH may be as low as 1, into the intestines. Here the organisms will encounter a higher pH, ranging from 4 to 6 (35). Having already passed through the low pH, they will be able to flourish and cause disease in the intestines. Oral antacids and similar medications reduced the infective dose of acid-sensitive organisms by 50% (41). Organisms have a higher chance of reaching the intestines if they are able to withstand the extreme pH levels of the stomach (35).

6. Foodborne Outbreaks Associated with *E. coli* O157:H7

Escherichia coli O157:H7 has been associated in many foodborne outbreaks. The most widely known outbreak linked the pathogen to undercooked hamburger meat. This outbreak occurred in 1993 at a Jack-In-The-Box fast food restaurant. The outbreak caused 600 people to become ill and three people to die. Aside from ground beef, *E. coli* O157:H7 outbreaks have occurred in many other types of meat products. For example, a 1994 outbreak associated the organism with dry-cured salami (12). Twenty-three patients, twenty from Washington and three from northern California, became ill after consuming dry-cured salami at a local grocery chain. The manufacturer of the product voluntarily recalled 10,000 pounds of infected meat product in order to prevent further disease (12). Other outbreaks have associated *E. coli* O157:H7 poisonings with water, unpasteurized juices and produce (48).

The first and, by far largest, waterborne outbreak occurred in 1989. Over 240 Missouri residents were affected by *E. coli* O157:H7 resulting in 32 hospitalizations and four deaths. The original source of contamination was never identified; however, a leak in the main flow is thought to be the cause (27). Another similar outbreak occurred in 1999 when contaminated drinking water was found to be responsible for a large outbreak in New York (16)

There have been numerous outbreaks associated with fresh, unpasteurized apple juices and ciders (48). One outbreak, which occurred in Massachusetts, linked unpasteurized apple cider to 23 illnesses (6). A similar outbreak occurred in 1996, when Odwalla brand unpasteurized apple juice was linked to the illness of 28 people. The

agent of contamination was identified through the use of DNA fingerprinting techniques. It was shown that all patients were infected with the same strain of *E. coli* O157:H7 (13).

Minimally processed fruits and vegetables have also been implicated as a source of infection. For example, in the summer of 1997, 60 people from 16 counties throughout central and southern Michigan were diagnosed with *E. coli* O157:H7 infections (14). At the same time, 48 cases of the infection were diagnosed in Virginia. After investigation, it was found that contaminated alfalfa seeds were distributed to two farms, one in Michigan and one in Virginia (14). Romaine lettuce was also found to be a vehicle for transmission of the organism in 1999. Here the lettuce caused a two community, mulitstate outbreak (16).

7. Factors Affecting Growth in Apples and Apple Products

Under the right conditions it is possible that once inside the fruit, bacteria cannot only survive, but can grow (31). One study showed that *E. coli* O157:H7 survived inside ground apples at both refrigeration and room temperatures with survival increasing when stored at refrigerated temperatures (31). The bacteria were found to be present in all cultivars after18 days, at which point mold spoilage occurred (24). However, refrigeration temperatures reduce survival of the pathogen in areas of injury on the skin of the apples (43).

Due to high acid tolerance, not only can this pathogen survive in apples, *E. coli* O157:H7 can also survive in apple juice and apple cider. Miller et al. (37) found that at cold storage temperatures (4˚C), *E. coli* O157:H7 can survive after 14- 21 days in apple cider (37). Miller examined the difference of survival in refrigerated cider products with and without preservatives. Two types of preservatives were examined: potassium sorbate and sodium benzoate. Potassium sorbate at 0.1% and 0.5% showed little effect on the organism, leaving a minimum of 84% viable cells. Sodium benzoate at 0.1% caused a greater decrease in the number of viable cells, however 10^4 cells remained after the initial count dropped 57 percent. Refrigerated cider that contained no preservatives showed the highest decrease with no detection of viable *E. coli* O157:H7 cells after 11 days.

In addition to Miller's studies, Besser et al found the organism to survive up to seven days in non-refrigerated, unpreserved apple cider (6, 40). Zhao et al. (56) also

found *E. coli* O157:H7 to survive up to 31 days in apple cider held at 8° C at a pH of 3.7. The length of survival in cider allows enough time for the pathogen to transmit disease. With this in mind, the efficiency of surface treatments in order to insure the safety of untreated juices and ciders is now in question.

Escherichia coli O157:H7, unlike other *E. coli* serotypes, is able to withstand higher levels of acid (37, 43). The organism can withstand pH levels below 4.0 for up to 56 days (19). Conner and Kotrola (19) performed experiments to test the survival of *E. coli* O157:H7 under different acidic conditions. The studies showed that mandelic acid was the most effective in inhibiting the growth of *E. coli* O157:H7. In this study, mandelic acid was compared against acetic, malic, citric, lactic and tartaric acids. However, even though mandelic acid was the best control agent against the bacteria, the acid is not considered to be "generally regarded as safe" or GRAS. Therefore, mandelic acid cannot be used as a food additive to prevent contamination by bacteria (19).

Most juices and ciders are considered to be high-acid food and therefore regulations have not previously required heat treatment, or pasteurization processes. Due to the increasing number of outbreaks, it has become necessary to reexamine the protocol. Using pasteurization as the final step in the processing of apple juice or cider can eliminate pathogenic organisms; however, in doing so, the flavor and appearance of the product can be adversely affected. In addition to adverse effects on the final product, pasteurization is costly and many smaller producers cannot afford the necessary equipment (52). However, the current practices that these producers are using, surface treatments, addition of potassium sorbate or sodium benzoate and refrigeration, may be allowing contaminated apple juice and cider to be marketed.

B. Apples

1. General

Apple trees are classified as members of the rose family due to the similarities between the apple and rose blossoms. Many other fruit bearing trees such as the pear, cherry, plum and peach, are also classified in the rose family (55). They are considered to be pome fruits (46). A pome is a fleshy fruit with seed cavities and a fleshy outer part (WorldNet, Princeton University Dictionary). Further classification places apples into

the genus Pyrus, which is the Latin term for "pear-tree". When distinguishing the apple tree from the pear tree, it is important to look at the trunk of the apple tree. The apple tree is much studier and shorter than that of the pear. The two can also be distinguished by looking at the color of the flowers. The flowers of the apple tree are pink to red while flowers of the pear tree are white (29).

Every tree produces around 50,000 to 100,000 apple blossoms, which are clumped in groups of either five or six. Usually, these clusters form at the end of fruiting spurs made of short, woody stems; however, single blossoms can also form along the branch itself where the bark is thinner. Although the number of blossoms is high, usually only between two and five percent actually develop into apples (55).

2. Development of the Apple from the Flower

i. Seed Development

For fruit development to begin, pollination and fertilization are usually required. However, there are some species of apples that do not require fertilization, such as the Baldwin variety. Pollination occurs when the pollen, which is originally made in the anther, is transferred to the stigma. Better fruit set occurs when the pollen from one cultivar is transferred to the stigma of another variety. This is termed "cross-pollination". Most species have a chemical mechanism that disallows self-pollination, however, some cultivars can fertilize the egg with its own pollen. Pollen can be transported from flower to flower by means of wind or insects such as the honeybee.

Once the pollen has reached the stigma, it germinates down the style. This sends a pollen tube into the carpel cavity of the flower, which encloses the ovule and the egg (46). The egg itself only carries half the number of chromosomes needed for the fruit, while the pollen carries with it the other half. The pollen tube is responsible for carrying the sperm to the ovule. Fertilization occurs when the sperm and the egg come together and the chromosomes from the egg and the pollen unite (55). The emerging egg begins its development within the ovule using the endosperm as a food source. Eventually, the egg moves into the next stage of development, the embryo. Next, the seed, which is the embryo and the endosperm, is formed. The seed is thought to have some hormone-like effect, which induces the fruit growth and development.

ii. Carpel Development

Once the seed is formed, further development of the carpels begins. The interior layers of the carpel, which also make up the ovary wall, start to become coarser and eventually make up the inedible portion of the apple core. These layers grow rapidly between the second and fourth weeks post bloom and reach maximum size transversely earlier than any other of the fruit tissues. The exterior layers of the carpel, however, begin to form a ring of vascular bundles, which develop into the edible flesh of the fruit. This section grows from the second week until the sixth week post bloom.

iii. Flesh Development

The epidermis is the outer layer of the developing apple fruit. During full bloom, these cells are thin walled, elongated, full of protoplasm and are dividing continuously. After a couple weeks of development, cell division has ceases, and the cells maintain a broader radial size than tangential while the cytoplasm begins to show vacuole formation. Although cell division has stopped, it is believed that the cells are still alive at this point. The epidermal cells begin to change shape by broadening and flattening so that the tangential size exceeds that of the radial. Here, as the apple grows in size, so does the cell. Next, the cells begin to separate and the space left is filled with cuticle. The final epidermal cell is elongated and flat with cellular contents being completely disintegrated. It is believed that the actual cell is dead (55).

During development, epidermal hairs can be found on the surface of the apple. The epidermal cells form these hairs. Only a few weeks full bloom, the hairs begin to die off and their roots are filled by cuticle. Hairs around the stem and the calyx remain on the apple; however, these too are eventually shed and filled by cuticle.

Also noticeable on the skin are the lenticels. It is believed that these cells come from stomata that stopped functioning sometime during the developmental stages of the apple or from breaks in the epidermis. These breaks can be due to the removal of the entire epidermal hairs or to a quicker expansion of the apple and a slower development of epidermal cells (46). Lenticels either remain open and permit the passage of gases through the apple or close and prevent the passage of gases (55). The number of lenticels

on each apple variety varies tremendously. For example, there can be anywhere from 450 – 800 lenticels on a Winesap apple where there could be anywhere from 1500 – 2500 lenticels on a Spitzenberg apple.

Just below the epidermis is the hypodermis. At full bloom, the hypodermis is only approximately four layers deep. The precise arrangement of the cells is eventually lost as the apple develops and the cells become filled with a deposit that is heavy and granular. By harvest time, however, the cells return to a normal arrangement of densely packed, thick-walled cells. Similar to the epidermis, the cells are tangentially longer and appear to be flat (46).

The main flesh of the apple is made of the "parenchyma of the fused bases of the calyx, corolla, and stamens" (46). These tissues can be found below the hypodermis stretching as far into the apple as the carpels. Cell division in the fleshy part of the fruit is complete approximately three weeks post bloom. Therefore, any enlargement in size is due to growth of the cells themselves and of the spaces between the cells. As with the cells of the main flesh, a period of cellular division is followed by a period of cellular growth and increases of intracellular spaces.

iv. The Mature Apple

A large section of the edible portion of the mature apple is comprised of the parenchyma of the floral tube. Because the size of the cells varies depending on apple variety, the respiration rate varies between cultivars. The respiration rate of the apple is inversely proportional to the parenchyma cell size. It has also been shown that approximately 25% of the parenchyma is made of intracellular space (46). This space allows for the diffusion and exchange of gases throughout the apple.

The middle and outer ovary wall make up only a fraction of the edible portion of the apple, while the inner carpel remains rough and leathery in texture. This is called the "core lining of the seed cavities" (55). With five carpels, there are usually five individual seed cavities, which developed after fertilization from the ovules of the original flower. With each carpel containing two seeds, there can be ten seeds per fruit.

The sepals at the top of the apple are also known as the calyx. The calyx can close completely and cover the cavity or can remain open depending on the apple variety.

3. Chemical and Physiological Changes in the Apple throughout Development

Throughout the growing season, many changes take place within each individual apple. For example, the total amount of water in the apples changes tremendously. As the fruit grows in size, the amount of water within the apple increases proportionally. This is due to the nature of the fruit being composed mainly of water. Like water, the absolute amount of nitrogen in the apple increases during development. The larger the apple, the more absolute nitrogen is present. Carbohydrates, such as sugars, acids, and polysaccharides are affected. Changes in the levels of vitamin and mineral, fat and enzyme can also be noted throughout the development of the apple.

i. Minerals

The content of most minerals in the apple increases during fruit development. However, the overall percentage of the minerals in the apple decreases. Potassium, which makes up the majority of the mineral content of the apple flesh, follows this trend with the content increasing as the apple develops while the total percentage on a dry weigh basis of potassium in the fruit decreases. Phosphorus and calcium also follow this trend, while iron does not. Iron content increases during development while the total percentage remains constant.

ii. Fatty Materials

As previously mentioned, at the beginning of the growing season, the epidermal layer of the apple is much thinner than it is at the end. Since the layer of cells is considered to be a form of a fat, it is understandable that the fat increases as the apple continues to develop through the season. Beyond the epidermis and the seeds, the levels of fat in the apple are minimal.

iii. Enzymes

An enzyme known as diastase maintains the hydrolysis of starch into maltose. The level of diastase increases as the fruit matures and ripens. Diastase causes an

increase in the level of sugars present in the apple. This increase in sugars causes the overall pH of the apple to increase. The concentration of acid also declines.

The level of oxidase decreases throughout the growing season. Oxidase is the enzyme, which causes browning of the apple once the flesh has been exposed to oxygen. It is thought that the specific cellular components that are affected are the tannins.

4. Microbiology of Apples

Previous knowledge of intact, sound apples has presumed that microorganisms are found only on the skin of the apple, and that the internal tissues of the fruit remain sterile with the exception of a few species (23). The natural microflora of apples can be broken down into two main sources: primary and secondary microflora. The primary microflora is considered the resident microflora and consists of those microorganisms that are found to naturally adhere the surface of the fruit. The resident microflora of the apple is limited to acidophilic and lactic acid organisms due to the pH and composition of the fruit. The secondary microflora consists of all other microorganisms that come into contact with the fruit due to the external environment, such as from water, soil, birds, insects and wind (23). It has been shown that bacterial counts peak in June and steadily decline from then on. Both primary and secondary microflora depend on the cultivar of apple, the geography, climate and processing conditions (23).

The predominant organisms that are found in apples are molds and weakly fermentative nonsporeforming yeast, such as *Candida*, *Saccharomyces* and *Kloeckera* and (23). The average load of molds ranges from 10^3 to 10^5 organisms per apple. Total counts of yeasts range from 10^2 to 10^6 organisms per apple or as much as 5.0×10^3 organisms per square centimeter. In contrast to the bacterial load, yeast counts tend to peak in September (23). Both yeasts and molds can cause significant damage to the fruit if present in extremely high numbers (23).

5. Culitvars of Apples

Apples cultivars are distinguished from each other on the basis of tree and fruit descriptions. The tree can be categorized by size, shape, branch and trunk structure, leafs and lenticels and flowers. Fruit is also grouped by size but also by date of ripening, stem

and calyx, skin and flesh composition and color and seeds are among the few ways to categorize the cultivars (29). In general apples are consumed one of three main ways, as juice, as applesauce or as fresh fruit. Apple trees that are used for the production of fresh fruit require constant care throughout the growing season. During the winter, fertilizer is spread beneath the trees so that the necessary nutrients are present for proper growth. The majority of the care begins at bloom. After the initial bloom, the trees must be thinned in order to maintain the proper amount of fruit on the tree. This is usually done with chemicals. Next, the trees are sprayed with a variety of fungicides and pesticides to insure that the crop will be suitable to be used as fresh fruit (33). Apples that are not used as fresh fruit but are processed into sauce and juice often do not get the constant care that the fresh fruit trees get. Processed fruit orchards attempt for greater tonnage rather than the general appeal and look of the fruit. Therefore, the work and money put into the growth of the processed fruit is decreased to the minimum (36).

Emphasis will be placed on the following cultivars in this overview: Delicious, Golden Delicious, Rome Beauty and York Imperial. Red and Golden Delicious and Rome Beauty are the three leading cultivars grown in Virginia but can be found throughout the United States. York, however, is only grown in Pennsylvania, West Virginia, Maryland and Virginia.

i. Red Delicious

The Red Delicious apple variety ripens between 140 to 150 days after the full bloom with harvest usually taking place in September. The fruit is a medium red color. It is considerably large and is oblong with tapering toward the end of the blossom. The texture of the flesh is juicy, fine-grained and crisp; however, when overripe, the fruit becomes mealy. The fruit has a low degree of acidity. The apples produced are mainly consumed as fresh fruit (36).

The Red Delicious tree is a large, upright spreading tree that is considered to be very vigorous. These trees take an average of five to eight years to bear its first fruit. Red Delicious trees are very susceptible to scab, fire blight and cedar rot; however, bitter rot and quince rust do not affect the trees. In general, the Red Delicious tree variety is fairly resistant to colder weather conditions (55,46).

ii. Golden Delicious

The Golden Delicious apple variety ripens later than the Red Delicious, with harvest most often occurring in late September. Maturity occurs between 155 and 160 days after full bloom. Golden Delicious apples are tapered like the Red Delicious, but are much longer in shape. The apple is medium to large in size with the skin, like the name depicts, being a bright golden yellow color. However, sometimes a "rosy cheek" may show up (55). The texture of the flesh is also crisp, tender and firm. This fruit has a medium degree of acidity. Golden Delicious apples are generally used as fresh fruit and for applesauce production (Personal conversation with 36).

The Golden Delicious tree is a medium sized spreading tree. These trees take a shorter time period to their first bearing than does Red Delicious. Golden Delicious will bear fruit within four to six years. The trees are much more susceptible to cedar-apple rust, fire blight, bitter rot, and spray injury. Golden Delicious trees are a moderately vigorous variety.

iii. Rome Beauty

Rome Beauty is also known simply as Rome. This apple matures later in the season with harvest occurring 160 to 165 days after full bloom. Rome apples are round to oblate in shape with medium to intense red coloring. The flesh is white in color and is firm and crisp. However, if overripe, the flesh becomes mealy. The fruit has a medium to below medium acidity level. Rome Beauty apples are a desirable variety for commercial sales. These apples are generally used in the production of apple juice and sauce (36).

The Rome tree is a late blossomer, which helps it to avoid damage due to spring frost. It is a medium sized spreading tree. The age to the first bearing of fruit is similar to that of the Golden Delicious with the range between four and six years. The Rome variety is very susceptible to scab, cedar-apple rust, powdery mildew, and somewhat susceptible to fire blight. However, the tree is resistant to bitter rot and spray injury. Rome Beauty trees are moderately vigorous.

iv. York Imperial

York Imperial ripens between 155 and 165 days after full bloom with harvest occurring in mid to late October. These apples are medium to large in size and are described as oblate and oblique, meaning that the apple is lopsided. The flesh of York is si slightly yellowish, juicy, hard and crisp. The skin of the apple is a mix of reds and greens. The degree of the acidity of the flesh is medium. As with Rome Beauty, York apples are primarily used in juice slices and sauce production.

York Imperial is a large, upright spreading tree. The age to the first bearing is between six and eight years, which is closest to Red Delicious. The tree is moderately vigorous. York is resistant to scab; however, it is susceptible to fire blight, cedar-apple rot and spray injury.

C. Electron Microscopy

1. Transmission Electron Microscopy

There are three types of electron microscopes: transmission electron microscopes (TEM), scanning electron microscopes (SEM), and scanning transmission electron microscopes (STEM) (30). The first to be developed was the TEM. TEM are known for having the highest resolving power (30). TEM uses scattered electrons and transmission electrons, while reflecting other rays and electrons, to produce an image (30):

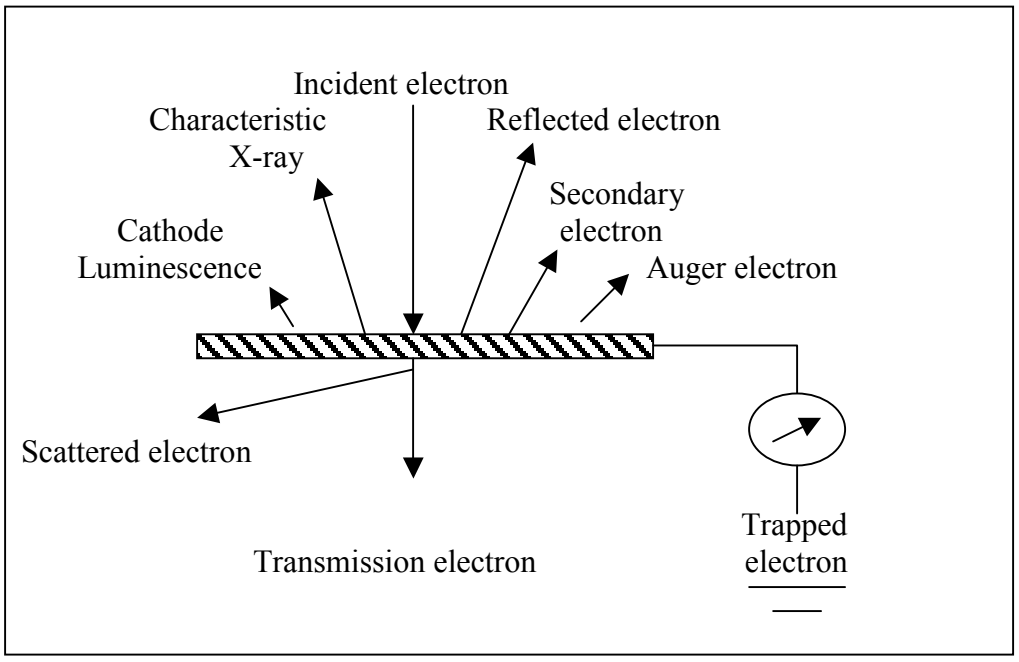

Figure 1. Light Scattering Pattern Produced by Transmission Electron Microscopes

The typical TEM consists of six electromagnetic lenses, three projector lenses, two condenser lenses, and an objective lens (47). The specimen chamber is located between the condenser and the objective lenses and beneath the projector is the viewing chamber (47). 'O' ring vacuum seals, that provide a vacuum tight column, separate each component. The components can easily be moved in relation to each other by a controls system (47). TEM can be used to visualize many types of materials and tissues, including metals, animal and plant tissues (47).

2. Laser Scanning Confocal Microscopy

Marvin Minsky first introduced the principle behind confocal microscopy in 1995. The theory of image collection differs greatly from that of the conventional wide field microscope. In conventional light microscopy, a two-dimensional image is formed through illuminating the entire sample along a certain plane (39). The image formed by the conventional scope can be viewed directly by the human eye (39). However, due to the blurring of the image above and below the focal line, the resolution and the contrast are drastically reduced. The light field that the confocal microscope uses sequentially

illuminates a single defined section of the sample. These light sources are normally from a laser. This focused beam allows the image to have increased clarity and contrast, as well as the ability to form a three-dimensional image (39). Three-dimensional imaging is obtained by stacking a number of the single vertical sections from successive focal planes called Z-series. Confocal microscopy allows the user to separate the sample with light, rather than by physical means (39).

The laser scanning confocal microscope (LSCM) is built on the underlying principles of the conventional scope, however, this scope also utilizes photomultiplier tube detectors as well as a computer, which controls the scanning mirrors and facilitates the compilation and display of images. The image is obtained through a single point illumination, which can be successively scanned through the sample (39). These light points then are detected by the photomultiplier located behind a pinhole. The computer takes the output and builds an image. Adjusting the size of the pinhole allows for larger areas of sampling (39).

LSCM can be used for a wide variety of applications. Microscopy has been used to image sea urchin fertilization, immunolabeled *Drosophila* embryos (39) as well as different types of fruits and vegetables (11). Burnett et al. (11) used LSCM to image *E. coli* on apples. Wachtel et al (50) and Seo (44) also used CSLM to image *E. coli*, however, this research focused on lettuce seedling and seed coats.

3. Using Green Fluorescent Protein as a Marker

Fluorescent labeling improves the ease of locating specimens while using the laser scanning confocal microscope. For example, when using green fluorescent protein (GFP), the sample will fluoresce under ultraviolet or blue light (39). GFP is a small protein, around 27 kDa (39), which is purified from the bioluminescent jellyfish *Aequorea victoria* (17). It can easily enter the nucleus of the cell and cross from the cytoplasm to the cytoplasmic extensions (39). The protein absorbs blue light at around 490 nm and emits green light around 517 nm (17). The excitation and emission spectra of GFP are similar to that of fluorescein isothiocyatenate, FITC, which is available on most conventional confocal scopes (39).

The protein can easily be transformed into bacterial cells using electroporation methods (53). Once the GFP protein has been inserted into the living cells, expression can be promoted on plates containing isopropyl-β-D-thiogalactoside. Under ultraviolet illumination, green fluorescence can be seen in transformed cells (17). The plasmid also encodes for ampicillin resistance. This resistance can be used as a method to maintain the plasmid in the cells (18). The T7 promoter regulates the expression of GFP In *Escherichia coli* (17).

When using fluorescent markers, autofluorescence issues become important. Many tissues fluorescence naturally when they are struck with light of certain wavelengths. For example, many yeasts and plant cells autofluoresce in the red spectrum (39). Autofluorescence is also seen with apple tissues in the red spectrum due to parenchyma cells (11). This problem can be overcome by using fluorescent markers outside the wavelength that causes the autofluorescence (39).

D. Contamination Issues in Apples and Apple Products

1. Regulation of Juice Products

Recently, the consumption of fresh apple juice and apple cider has been linked to outbreaks associated with *Escherichia coli O157:*H7, *Salmonella* species and *Cryptosporidium parvum*. However, most of the attention has been drawn to *E. coli* O157:H7 due to the severity of infection, especially in younger children and the elderly. Two major outbreaks, 1991 and 1996, linked *E. coli* O157:H7 to be the cause of 23 and 66 persons to become ill, respectively. One child died in the 1996 outbreak (22). As a result of the escalating numbers of juice-associated outbreaks, the Food and Drug Administration (FDA) announced plans for a program to address food borne illnesses linked to juices in order to improve the safety of fresh and minimally processed fruits and fruit products (24).

The FDA published a notice of intent on August 28, 1997 to address problems associated with the consumption of raw juices. The agency asked for comments regarding three issues: (a) the initiation of mandatory Hazard Analysis and Critical Control Point (HACCP) programs, (b) the usages of labels to inform consumers of the dangers of food borne illness that are associated with the consumption of untreated

products (24), and (c) the initiation of education programs for juice processors in order to minimize the number of food borne illnesses linked to the consumption of fresh juices (26). The initial program allowed a fifteen-day period for comments.

The proposed rule was published in the Federal Register of April 14, 1998. Again, the FDA allowed a comment period, which extended from the date of the publishing until August 7, 1998. This proposal required all unpasteurized juice and cider producers who do not process their juice products to obtain a five-log reduction of harmful bacteria to place a warning label on the product. Also under this rule, the FDA considered requiring the implementation of HACCP plans in unpasteurized juice production. Since the issuance of the proposal, the FDA continued to accept comments on the safety of juices.

The proposed HACCP plan is based on the assumption that pathogens are located only on the surface of fruits and vegetables and not in the interior. However, this assumption is not necessarily correct. The safety of fresh fruits and vegetables has been questioned mainly due to the possibility of bacterial internalization.

The final rule was published on January 19, 2001. In this document, the FDA addressed a series of comments regarding alternatives to HACCP programs. The FDA concluded that although there are many likely alternatives to controlling food borne illness associated with juices, a HACCP plan allows for the most flexibility among processors as well as the most effective means of assuring food safety (26). The final rule is intended to force processors to determine likely sources of microbial, chemical and physical hazards that could contaminate their products. Processors are now required to implement strategies to control these hazards, as well as implementing a five-log reduction of the most resistant pathogen in the finished juice product (26). Although the plan does not call for mandatory pasteurization, the HACCP plan presented by each processor must allow for the five-log reduction of the target pathogen in some manner. For example, the FDA cited ultra-violet light irradiation as an effective alternative technique to obtain the required reduction.

The HACCP regulation will affect those processors who do not pasteurize their juice product as well as those who do. The agency stated that the HACCP plan does not require the inclusion of harvesting, picking, and transportation of the fruits, but begins

with the incoming produce. Processors must keep detailed records of the implementation and the verification of the HACCP plans (26). The final rule does not include the use of warning labels as a preventative measure, however labels must be used until individualized HACCP plans are implemented. The FDA commented that the warning labels were only considered to be an intermediate step, not a solution to the hazards presented by fresh juice products (26).

2. Sources of Apple Contamination

In the fall of 1996 alone, there were four outbreaks of *E. coli* O157:H7 associated with unpasteurized apple cider. The source of the pathogen was not determined, but various possible points of contamination were noted. *Escherichia coli* O157:H7 is associated with many healthy animals and can be found in the feces of deer (42), cows, birds, and of domestic animals (31). Although manure has been suspected to be the primary source of some outbreaks, the definite route of contamination is unknown. Other suspected environmental contamination sources are infestation by insects, run-off from nearby pastures, manure and contaminated irrigation systems (31). Flume or wash waters (38), harvesting equipment and poor worker hygiene are also possible contamination points (11, 43). Although flume and wash waters are chlorinated, the amount of chlorine in these conditions is unknown; however, some pathogenic microorganisms are resistant to chlorine and related decontaminants (10).

As previously mentioned, one possible route of contamination is internalization, or infiltration, of pathogens. Internalization is the uptake of bacteria into the fruit. Due to the possibility of internalization and the extreme hazards associated with it, the FDA reopened a comment period in late November 1999. The agency used this period to allow for further research to gain more information on the internalization and survival of pathogens in fruits that are used in juice production.

Common practices used in apple plants may not only be allowing infiltration to occur, but may in fact be promoting the uptake of bacteria into apples. In juice processing, apples are routinely submerged in water while being transported by flume systems or while being washed in dump tanks (51, 46). Both of these practices provide conditions for vacuum formation within the apple (51, 46).

Apples can become contaminated even before they reach the processing center. In order to prevent damage to apple crops, producers spray the trees with insecticides throughout the growing season. This could be another possible route of contamination. The base water that is added to powder mixes could come from unsanitary sources, for example run-off water near pastures, well water, or stock pond water. In this case, the contaminated water would be directly sprayed on to the apples themselves allowing contact between the apples and the bacteria. In addition, windfall apples are picked and later used for cider production. These apples are subjected to bacteria that may be present on the ground and are therefore at a higher risk for contamination by internalization (31, 46). In Virginia, 5% of the producers allow grazing animals in the orchard, 8% fertilize orchards with manure, and 32% use drop apples (54). Apples that are picked under these conditions have increased possibility of contamination due to exposure to *E. coli* O157:H7 from animals. Drop apples may also have injuries in the skin that allow fungal decay which causes physiological changes in the structure of the apple which (43). These changes can also promote the growth potential of *E. coli* O157:H7 by causing the pH of the injured part of the apple to increase (43). Although the FDA encourages juice producers to use fruit of the highest quality, the Federal Register allows for the use of drops in most juice products (25).

Other studies have shown that insects and birds can carry *E. coli* O157:H7 and can therefore contaminate apples through damaged areas in the skin. Janisiewicz et al. (31) showed that flies inoculated with the pathogen resulted in a high incidence of contamination of surface wounds on the apple (31). Once the bacteria are inoculated onto the surface of the apple, it is possible that they can then spread further into the apple's flesh. In this case, topical sanitizers will not be affective.

3. Mechanism of Internalization

Bacteria can enter the fruit in two ways: one, through the leaves of the plant, and two through the fruit itself. Bacteria can enter the leaf through the stoma, which are tiny pores in the epidermis of a leaf or a stem that allows the passage of gases and water vapor. When entering directly into the fruit, bacteria can enter the morphological

structure in the fruit itself through the calyx, stem or stem scar. Bacteria can enter the fruit through injuries in the skin, such as areas of decay (51, 24).

In 1981, Bartz and Showalter demonstrated the internalization of bacteria in tomatoes due to negative temperature differentials (3). This research used the general gas law to predict the infiltration of the bacteria from the environment into the fruit. The general gas law states that any change in pressure of an ideal gas in a closed container of constant volume is directly proportional to a change in the temperature of the gas. In this case, the tomato itself is the closed container, however, any fruit could be substituted as a "closed container" (10). Fruit has a relatively constant volume and its morphological structure allows for many pockets of gas. Therefore, a decrease in temperature within the fruit causes a decrease in the internal gas pressure. This results in partial vacuum within the fruit. Equilibrium in the fruit is obtained through the uptake of gases from the external environment. This vacuum and consequential uptake of gas can also allow the influx of bacteria in the fruit. Vacuum formation can also occur when immersing the fruit in water, which causes an increase in external pressure. The amount of material that is drawn into the fruit is proportional to the pull of the vacuum. The extent of the pull depends on the length of exposure, the amount of cooling and the depth of the immersion of the fruit (10).

Internalization has not only been found to occur in apples, but also in oranges, tomatoes, and melons (10). Walderhaug et al. (51) showed internalization in oranges through the use of dye. In this study, oranges were allowed to equilibrate at 37˚C overnight. Next, a solution of Brilliant Blue dye and *E. coli* O157:H7 was applied to the stem scar of the orange. The oranges were then held at 4˚C. During the equilibration period, the oranges appeared to draw the solution through the stem scar into the center of the orange. Here the temperature differential is thought to have allowed for the infiltration into the orange. This study showed that the oranges can uptake human pathogens at a rate of 3% (51).

Buchanan et al. (10) used apples and obtained comparable results. This research showed that a vacuum occurs between the stem and the calyx of the apple under temperature differentials causing the uptake of cold dye into a warm apple. The dye accumulated in the inner core of the apple (10). Dye was also shown to enter the skin

through areas of damage, such as puncture holes and bruises. Buchanan et al. (10) showed that under laboratory conditions, the uptake, or internalization, of *E. coli* O157:H7 was also possible. Bacteria were found in the inner and outer core, skin and flesh of the exposed apples.

Untreated apple juice and cider might have the potential of causing an outbreak due to internalization if the product is not further treated (34). Research has shown that once bacteria are internalized into the fruit, surface treatments are unsuccessful in decreasing microbial populations (51). Once inside the fruit, the bacteria are protected from any type of topical cleansing, such as chemical surface treatments or removal by physical methods, like brushing. Liao and Sapers (34) showed that after attachment to the stem and calyx of the apple, *Salmonella* Chester was found to be either resistant to or protected from sanitizing treatments (34).

4. Preventing Contamination in Apples

Current practices for decontamination include water rinsing, chlorine treatments, organic acids, and irradiation (Nyguyen-the1994). However, as previously mentioned, some of these techniques might be increasing the chances for internalization. For example, although chlorinated wash waters can decrease the external load, not all bacteria will be killed (7,1).

Chemical sanitizers, such as quaternary ammonium compounds, peroxy compounds and organic acids, can also be employed in the flume systems (20). Organic acids, such as acetic, lactic, citric and propionic acids (38), act on microbes by lowering the overall pH of the environment (21). This causes a disruption in the membrane function as well as the enzymatic systems of the cell (21). The effectiveness depends on the type of acid being used, the concentration of the solution, and the amount of undissociated acid. The higher the amount of undissociated acid, the more effective it will be (21). However, it should be noted that not all organic acids are approved for all food products (Nguyen–the).

Hazard analysis critical control point programs can be implemented to decrease risks of illness. These programs can include critical control points from the farm to the table, including the actual picking and processing of the apple (8). Learning and

following these new procedures on a daily basis can help workers better understand the principles behind foodborne illnesses (8). This could reduce the risks of illness (8).

Alternative methods to juice or cider pasteurization can also be implemented. For example, ozone and irradiation methods can replace pasteurization and provide a product that does not have the undesirable sensory characteristics of heat treatment. Irradiation at low doses (2kGy) is more efficient than chemical sanitizers (38). However, these treatments are not always allowable for use with fresh produce. Irradiation is not approved in all cases (7). Also, when dealing with fresh produce, ozone penetration can sometimes cause damage to the produce (7).

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SECTION II: INTERNALIZATION OF *ESCHERICHIA COLI* IN APPLES UNDER NATURAL CONDITIONS

Title: Internalization of *Escherichia coli* in Apples Under Natural Conditions

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ABSTRACT

Foodborne illnesses in unpasteurized apple cider have been attributed to the pathogenic bacteria *Escherichia coli* O157:H7. Contamination is likely to occur during the fruit growing and harvesting phases. In apple cider production where the entire apple is pressed, pathogens found within the apple core and surrounding tissue are a potential problem. Internalization of *E. coli* in apples under natural environmental conditions was addressed in this study using a controlled outdoor setting. In this study a surrogate *E. coli* species (ATCC 25922) was used as an alternative to the pathogenic species. The bacterial culture was applied to topsoil and spread evenly on a 6x6-foot area. Red Delicious, Golden Delicious, and Rome Beauty apples were placed randomly on the soil much like a drop or windfall apple. The position was noted as to whether the apple fell calyx up, down or on its side. Apples sampled 1, 3, 8, and 10 days after placement were examined for the presence of *E. coli*. Skin, flesh, inner, and outer core samples were plated on MacConkey agar supplemented with cycloheximide and MUG to ease in identification. *Escherichia coli* was found in the inner core and flesh samples of all apple cultivars, indicating the potential for infiltration by the organism outside laboratory conditions.

INTRODUCTION

Over the past 10 years, the consumption of unpasteurized apple juice has been linked to increasing numbers of food borne outbreaks associated with *Escherichia coli* O157:H7 (18, 4, 8, 9), *Salmonella* (10) and *Cryptosporidium parvum* (14,9). These outbreaks have raised questions as to the safety of unpasteurized juice as well as to the use of surface treatments to decontaminate fruits. These issues are addressed in the U.S. Food and Drug Administration final rule and Hazard Analysis and Critical Control Point (HACCP) guide for juice processing (1). The direct causes of these outbreaks are difficult to determine; however, several possible routes of transmission exist, including: irrigation water, manure, sewage, poor worker hygiene, harvesting equipment and containers, insects, birds, and processing equipment. Microbial contamination can occur through the use of damaged, scald, or windfall fruit used in the production of apple cider that may harbor disease-causing microorganisms (19, 20). Common practices of apple cider producers could support bacterial infiltration. Historically, apple cider is produced from "cider apples" or windfalls. These apples may be at a greater risk of infiltration by bacteria on the ground; still, tree apples are at risk from dust, insects, birds, and handling practices. In general, cider producers are knowledgeable about good agricultural practices that should be used to prevent contamination. In one survey from 1993, 100% of New Englanders reported using drop apples (4). In part due to Good Agricultural Practices and increased awareness the use of drop apples has generally decreased; however, about 5% of the producers in Virginia allow grazing animals in their orchards, 8% fertilize with manure, 32% use drop apples, and 37% do not use a sanitizer after washing the apples (20). These practices may still be fairly common among apple cider producers across the country, as similar practices were observed in Wisconsin (19). According to recent surveys (19, 20) the use of drop apples in cider is decreasing. This most likely indicates that juice producers and orchard managers are aware of microbial contamination on some level.

Even when drop apples are not used, the potential exists that apples may contain microorganisms in bruised or wounded areas (6). Most bacterial diseases of fruit require an opening in the plant tissue to cause infection. For example, fire blight (*Erwinia*

amylovora) can infect apple trees during bloom by entering through openings in the flower tissues or it can infect trees later in the season if the succulent tissues are wounded by hail or strong winds. Therefore, to control fire blight, commercial fruit producers spray trees with an antibiotic at times when the trees are susceptible to infection. The knowledge that *E. coli* can infiltrate non-injured tissue suggests that there may be several opportunities in the production/harvest/handling system for bacterial infiltration.

The route of entry for microorganisms into the apple may occur in two ways: through the leaves of the plant, or through the fruit itself (7,12,6). Microorganisms may enter the leaf through the stoma, tiny pores in the epidermis of a leaf or stem that allows the exchange of gases and water vapor. Similarly, microorganisms may infiltrate the flesh of the fruit directly through the calyx, stem scar, or natural micropores in the lenticels of the skin. The infiltration of microorganism may be enhanced by fruit surface damage pre- or post-harvest from hail, dust, insects, birds, strong winds, or wash water.

Infiltration of fruit tissues, based on the general gas law, was first demonstrated by Bartz and Showalter (3) using tomatoes. This law states that any change in pressure in a closed container of constant volume is directly proportional to a change in the temperature of the gas. Fruit acts as a "closed container" (3). The morphological structures of the fruit contain pockets of gas at relatively constant volume. A decrease in temperature within the fruit reduces the pressure within the fruit, which results in a decrease in the internal gas pressure. The concern exists that as this vacuum forms, uptake of gas and liquid may allow the influx of bacteria into the fruit. Researchers have demonstrated the formation of a vacuum capable of pulling a bacterial or dye suspension into the fruit core as a result of an increase in external pressure that results from immersing fruit below the water surface (3,5,13). This phenomenon may be enhanced by differences in water and fruit temperature (5).

A recent study identified the potential for infiltration of bacteria under laboratory conditions into specific structures of apples, including lenticels and the floral tube (6). Other studies have shown that bacteria can enter fruit tissues through puncture wounds (15), while we show here that bacteria can enter fully intact apples. This study describes the potential for bacterial infiltration outside a laboratory through lenticels, the stem scar, and the calyx/floral tube. The potential for bacterial infiltration under natural conditions

was analyzed using three apple cultivars: Red Delicious, Golden Delicious, and Rome Beauty. Apple cultivars were compared for ease of bacterial infiltration and electron microscopy was used to gain a better understanding of the role of cellular morphology. *Escherichia coli* ATCC 25922 was used as a surrogate organism since this study was conducted in the field.

METHODS AND MATERIALS

Apples

Red Delicious, Golden Delicious, and Rome apples were harvested by hand from the Virginia Tech College of Agriculture and Life Sciences Kentland Farm (Montgomery County, VA) and stored at 2°C in an apple cooler at the Department of Food Science and Technology until used. These apple cultivars are used in the preparation of apple cider on the East Coast of the United States. Red Delicious and Golden Delicious apples are used in the production of apple cider in many parts of the country.

Outdoor Apple Pen

A six-foot pen was constructed from wire mesh to encase the apples. The entire floor of the pen was lined with a plastic tarp, which formed a barrier between the ground and a 3-inch layer of topsoil applied at the start of the experimental period (200 pounds spread evenly). A thin wire mesh supported at four corners and at a center pole was laid over the pen to prevent birds and other animals from interacting with the apples. Apples were sterilized in 2,000 mg/L sodium hypochlorite (5.25%) prior to being dropped on the soil. A total of seventy intact apples were dropped gently from a height of three feet at random around the floor of the pen in two duplicate trials. Upon sampling, apples were still intact.

Inoculum

Escherichia coli ATCC 25922 (American Type Culture Collection, Rockville, MD) was incubated for approximately 24 hours at 37°C in Brain Heart Infusion Broth (Difco, Detroit, MI). Three hundred ml of inoculum containing 1.0×10^8 cfu/ml was then gently spread over the soil in the apple pen. One apple of each cultivar was selected at

random and tested as a control. Soil samples were tested for bacterial contamination before and after inoculation. The remaining apples were placed on the soil within thirty minutes after soil was inoculated.

Apple Sample Preparation

Each day six apples, two per cultivar, were randomly chosen and aseptically removed from the pen. The position of the apple on the soil was noted: calyx up, calyx down or on its side. Each apple was then cored and cut into sections: outer core, inner core, flesh and skin (5). Each section was placed into a filtered stomacher bag and stomached at 230 rpm for 30 sec. (Seward, London, England). Twenty ml of sterile peptone solution (Difco, Detroit, MI) was added to the core and skin sections (flesh samples stomached with natural juices). Petri plates contained MacConkey agar (Difco, Detroit, MI) supplemented with cycloheximide (Sigma, St. Louis, MO) to control mold contamination and MUB (4-Methlyumbelliferyl-β-D-Glucuronide, Sigma, St. Louis, MO) to ease in identification of *E. coli*. Few to no bacterial colonies other than *E. coli* were observed from the plated apple samples. Dilutions were prepared individually based on the weight of the apple skin, inner core, outer core, and flesh samples. In each case a 10^{-1} dilution was prepared on a wt/wt basis and duplicate plates were incubated at 37°C for 24 hr and then counted according to Spiral Plater System directions. Plate counts (cfu/ml) were compared as log cfu/ml counts using ANOVA analysis and Tukeys test to compare means (p value<0.05, SAS Software, SAS Institute, Cary, NC). The effects of apple variety, apple positions (calyx up, calyx down, or side) and day of trial were analyzed and compared.

Two identical trials were conducted. The first was conducted in the fall and the second in the early spring of the following year. Temperatures ranged from 50-70° F during the days of both trials, falling into the 30s at night; however, apples did not show signs of ice crystals at any time. During the second trial, precipitation in the form of rain occurred (<2 inches), but did not viably disrupt the topsoil or apples within the pen.

Electron Microscopy

Transmission Electron Microscopy (TEM) was performed on a JEOL 100 CX-II Scanning Transmission Electron Microscopy (STEM) with a magnification range of 360 to 320,000x in TEM mode. Apple slices were taken from clean intact apples of all three cultivars and fixed in 2.5% gluteraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 and processed for TEM.

RESULTS

Microbial Analysis

Apples were obtained from an outside area as described above. There was no visible difference in the soil level at the start of each study (uniformly 3.0 inches deep) compared to that at the end of each study (2.8-3.0 inches deep). Tables 2.1 and 2.2 show mean values in log cfu/ml ± standard deviation from two trials where two apples were taken each day. All three apple cultivars were sampled during each trial at 1, 3, 8, and 10 days. Data are separated according to values from inner and outer core (Table 1) and from skin and flesh (Table 2). Cultivar means differed significantly for flesh and skin ($p<0.05$ by ANOVA). This indicates that the skin or cuticle thickness and natural lenticel openings may influence bacterial entry to skin or flesh between apple cultivars (Figures 2.1 and 2.2). Bacterial counts were significantly affected by both day and position for outer core values only ($p<0.05$ by ANOVA). This indicates that apples with calyx facing down toward the soil had a greater chance for internalization to occur, compared to those with calyx facing up or those on their sides. Position was only significant in data obtained from outer core samples. There was no significant difference in values for inner cores. Importantly, *E. co*li was not obtained from control apples sampled prior to the start of each study or form control apples placed on the ground prior to inoculation. MUG in the plates ensured proper identification of *E. coli*, and while *E. coli* was not found in control apples, any *E. coli* found in the apples after day zero was attributed to that placed on the soil. Variation in sample means are shown in the calculated standard deviation and may be attributed in part to the rain in trial two or slight variation in temperature during the two trials.

Electron Microscopy

Transmission Electron Microscopy was used to analyze possible differences in apple skin and tissue structure of the three cultivars. There are differences in lenticel size and number among apple cultivars (Figure 1). As lenticels are no longer needed for gas exchange in mature apples, like those used in this study, they are often filled with waxy cells, as shown in the first two panels, Rome Beauty (RB) and Red Delicious (RD). In Golden Delicious (GD) lenticels may be filled with "cork" cells as shown by the upper arrow or may be unfilled and remain open as shown by the lower arrow. In Golden Delicious apples the lenticels are a possible source for internalization of bacteria. The cultivars also have different wax platelet arrangements.

There are also differences in cell wall structure (Figure 2). The thickness of the cell wall is a characteristic often used to identify and differentiate apple cultivars. The cellulose and hemicellulose fibers within the cell wall of a young fruit are often quite intact and dense. As the fruit ripens vacuoles begin to grow and changes within the cell wall may occur. On average, the cells of a Golden Delicious apple have thinner cell walls compared to the cells of Rome Beauty and Red Delicious apples. The cells in Figure 2.3 are believed to be at similar stages of ripening. A thinner cell wall is more prone to internalization of bacteria. As an apple ripens, the parenchyma cells move about, cell walls change, and internalization may occur more readily in all apple cultivars.

DISCUSSION

Bacteria were found in all four parts (skin, flesh, inner core, outer core) of the intact "drop" apples used in this study. It appears that bacteria can infiltrate apple tissue and the inner core under natural environmental conditions. Bacteria may enter these areas of the apple through stem and calyx openings or through openings that exist within the skin. Some bacteria may actually have the ability to preferentially bind to the fruit surface through the formation of biofilms, especially near the stem and calyx areas (16, 2) this could ease the infiltration of these microorganisms to the apple core.

The entrance of bacteria was different among the three apple cultivars studied, Rome Beauty, Red Delicious, and Golden Delicious. This may be due to innate

differences in the apple cultivars, which may in turn influence how and if an apple becomes contaminated during development. Apples have a myriad of potential entry routes for bacteria. Studies are currently underway to evaluate routes of entry during development. In brief, as an apple develops lenticels (Figure 1) form as minute openings on the apple surface. These may or may not be visible to the naked eye depending on the apple variety. Lenticels can range in number from 450 to 800 or from 1500 to 2500 per fruit (17). Lenticels form through the following ways. Stomata may develop into lenticels early in fruit development. Lenticels may also arise from breaks in the epidermis caused by a complete removal of epidermal hairs in that area, or from breaks brought by the inability of the epidermis to keep pace with the expanding inner tissues of hypodermis and parenchyma during growth. During fruit development, lenticels usually become closed through cutinization or suberization processes, which prevent the free passage of gasses from the inner tissues to the outside air.

Dingham (11) reported that susceptibility to bacterial contamination or bacterial growth varies with apple cultivars. Differences in apple cultivars that may influence infiltration may be a function of ripening, perhaps by association with pH, which was not studied here, or along with natural anatomical differences. Lenticels, cuticle structure, cuticle area, and the width of stem and calyx areas are all used in differentiating apple cultivars (17). In the Golden Delicious apple variety, lenticels may not close or may only partially close after the formation of "cork" cells (17). Additionally, cork cells may form in response to tissue damage and therefore may not be as efficient as waxy cells from inhibiting infiltration of microorganisms through lenticels.

Golden Delicious apples have a cracked and discontinuous cuticle unlike other cultivars that have a fairly oily cuticle. Environmental factors can also influence cuticle formation. For example parts of the fruit that develop in the shade, such as the skin located directly around the stem, have a thinner cuticle. While the fruit ripens, air spaces within fruit cells may grow or expand, allowing increased infiltration of microorganisms. The process of ripening may lead to changes in apple tissue. The further influence of environmental factors and fruit growth are currently being evaluated in field studies for their potential role in microbial infiltration.

This study indicates that bacteria can infiltrate apple tissues outside laboratory conditions. It appears that environmental conditions may be most influential on infiltration of bacteria into the core, where subsequent studies (data not shown) have indicated the possible need for the inclusion of a final processing step such as UV light, high pressure, or heat pasteurization to assure a proper kill of reduction of microorganisms (5-log reduction of the target microorganism). Theses findings also suggest that careful culling of apples may not be enough to ward off potential microbial contamination, since bacteria may be inside the tissue of intact apples. To our knowledge the infiltration of bacteria in dropped apples outside a laboratory has not be demonstrated before.

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Table 1. Location of E. coli ATCC 25922 infiltration in the outer and inner cores of Rome Beauty, Red Delicious and Golden Delicious.

Apple Variety	Day	Outer Core Log cfu/g ± sd n=4 per day	Inner Core Log cfu/g ± sd n=4 per day
Rome Beauty	0	nd	nd
	1	2.5±1.2	1.98±0.37
	3	4.4±1.5	2.72±0.88
	8	2.8±0.1	2.06±0.37
	10	3.9±0.84	2.39±0.42
Red Delicious	0	nd	nd
	1	1.3±0.43	1.00±0.10
	3	4.8±0.43	1.46±0.65
	8	2.7±1.4	1.73±1.03
	10	4.1±0.57	2.79±0.60
Golden Delicious	0	Nd	nd
	1	3.8±0.53	2.15±1.2
	3	4.9±0.75	1.46±0.65
	8	2.5±1.1	3.02±0.38
	10	4.4±0.32	2.22±1.7

[a] nd (not detected)

Table 2. Location of E. coli ATCC 25922 infiltration in the skin and flesh of Rome Beauty, Red Delicious and Golden Delicious.

Apple Variety	Day	Skin Log cfu/g ± sd n=4 per day	Flesh Log cfu/g ± sd n=4 per day
Rome Beauty	0	nd	nd
	1	2.7±1.4	1.91±0.28
	3	3.2±1.3	1.00±0.10
	8	2.0±0.07	3.10±0.71
	10	2.8±0.67	1.68±0.96
Red Delicious	0	Nd	nd
	1	3.0±0.05	1.79±0.11
	3	2.12±0.28	1.93±0.38
	8	1.6±0.86	1.32±0.10
	10	1.5±0.21	1.70±0.98
Golden Delicious	0	Nd	nd
	1	4.4±0.20	2.73±0.10
	3	3.1±0.20	1.31±0.43
	8	3.0±0.06	1.92±0.29
	10	3.1±1.1	1.00±0.10

[a] nd (not detected)

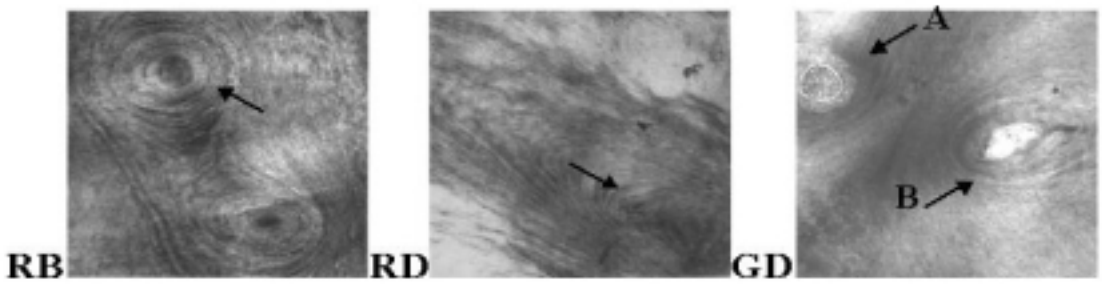

Figure 1. Lenticels from Rome Beauty (RB), Red Delicious (RD), and Golden Delicious (GD) skin samples are indicated by the black arrows.

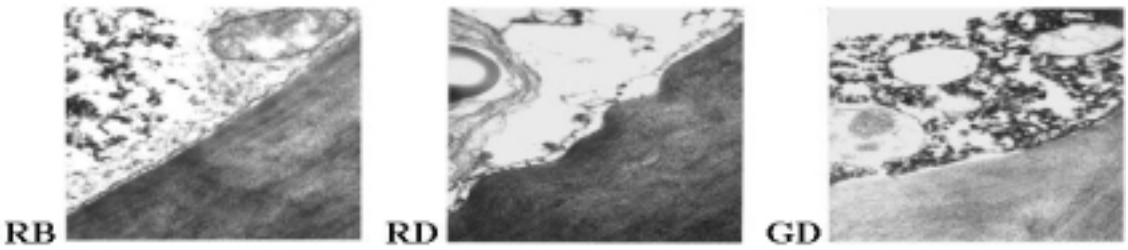

Figure 2. Cell walls of Rome Beauty (RB), Red Delicious (RD) and Golden Delicious (GD).

SECTION III: INTERNALIZATION OF *ESCHERICHIA COLI* IN APPLES IN FIELD AND LABORATORY CONDITIONS

Title: Internalization of *Escherichia coli* in Field and Laboratory Conditions

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ABSTRACT

The purpose of this project was to study bacterial internalization in apples during growing conditions using *Escherichia coli* ATCC 25922 as a surrogate for *E. coli* O157:H7. Five cultivars of apples were studied: Redfree, Rome, York, Golden, and Red Delicious. Redfree was used in a long-term study in which individual apples were spray inoculated at the beginning of the growing season with *E. coli* ATCC 25922 at 10^4 cfu/apple. The apples were picked 1, 30 and 60 days after treatment and sectioned into skin, flesh, inner and outer cores. The remaining four cultivars were used in a two-week study. Apples were inoculated two weeks prior to harvest and picked every other day until harvest. In the long-term study, the surrogate *E. coli* was not found after day 1. Other coliforms, such as *E. vulneris, Klebsiella pneumoniae* and *Kl. ozaenae* were present in each pick. The two-week study showed higher rates of internalization in Red and Golden Delicious than in Rome and York, with the *E. coli* present in all four sections of the fruit. Red Delicious showed a trend of increasing counts of bacteria over the two-week period with initial counts ranging from less than one cfu/ml to final counts as high as 2.89±1.90 log cfu/ml. Again *Klebsiella* species and *E. vulneris* were found in the apples. Microscopy techniques were used to compare differences of internalization rates in confined laboratory conditions. Internalization occurred to a limited extent in mature Red and Golden Delicious tree apples.

INTRODUCTION

Many consumers prefer fresh, unpasteurized juices and ciders over heat treated products because of their distinct flavors and aromas as well as enhanced nutritional values. In the past, regulations on juice products have been relaxed; these products are naturally acidic thus limiting microbial growth (7). However, recently the consumption of fresh apple juice and apple cider has been linked to outbreaks associated with pathogens such as *Escherichia coli O157:*H7, *Salmonella* species and *Cryptosporidium parvum*. Most of the attention has been drawn to *E. coli* O157:H7 due to the severity of infection, especially in younger children and the elderly. Two major outbreaks, 1991 and 1996, linked *E. coli* O157:H7 to be the cause of 23 and 66 persons to become ill, respectively. One child died in the 1996 outbreak (4). As a result of the escalating numbers of juice-associated outbreaks, the Food and Drug Administration announced plans for a program to address food borne illnesses linked to juices in order to improve the safety of fresh and minimally processed fruits and fruit products (7).

The cause of these outbreaks is difficult to determine; however, there are many possible sources. *Escherichia coli* O157:H7 is associated with many healthy animals and can be found in the feces of deer (13), cows, birds, and of domestic animals (9). Although manure has been suspected to be the primary source of some outbreaks, the definite route of contamination is unknown. Other suspected environmental contamination sources are infestation by insects, run-off from nearby pastures, manure and contaminated irrigation systems (9). Flume or wash waters (11), harvesting equipment and poor worker hygiene are also possible contamination points (3, 13). Although flume and wash waters are chlorinated, the amount of chlorine in these conditions is unknown; however, some pathogenic microorganisms are resistant to chlorine and related decontaminants (2).

Research has shown that contamination is no longer restricted to the surface of the produce, but that bacteria can be taken up, or internalized, into the core and flesh. Bartz and Showalter (1) demonstrated internalization of bacteria in tomatoes due to negative temperature differentials. When cold tomatoes were submerged in warm water, a vacuum occurred. The tomato then allows an influx of water into the fruit in order for the

pressure to equilibrate. The amount of material that is drawn into the fruit is proportional to the pull of the vacuum (1). The extent of the pull depends on the length of exposure, the amount of cooling and the depth of the immersion of the fruit (2).

Buchanan et al. (2) used apples and obtained comparable results. This research showed a vacuum occurs between the stem and the calyx of the apple under temperature differentials again causing the uptake of cold dye into a warm apple. The dye accumulated in the inner core of the apple (2). Dye also shown entered the skin through areas of damage, such as puncture holes and bruises. Buchanan et al. (2) showed that under laboratory conditions, the uptake, or internalization, of *E. coli* O157:H7 was also possible.

Both fluming and dump tanks provide conditions for vacuum formation within the apple (8,17). Common practices used in apple plants may not only be allowing infiltration to occur, but may in fact be promoting the uptake of bacteria into apples. During processing, apples are routinely submerged in water while being transported by flume systems or while being washed in dump tanks (8,17). Both fluming and dump tanks provide conditions for vacuum formation within the apple (8,17).

The possibility of internalization prior to reaching the processing plant presents a concern. Irrigation water or spray water could be another possible source of contamination. In order to prevent damage to apple crops, producers spray trees with insecticides and fungicides throughout the growing season. In these cases, dehydrated powders must be solubalized in water. The base water that is added to powder mixes could come from unsanitary sources, for example run-off water near pastures, well water, or stock pond water. In this case, the contaminated water would be directly sprayed on to the apples themselves allowing contact between the apples and the bacteria. If bacteria are present within the flesh and inner core of the apple, simple surface washes will not affect internalized bacteria.

Transmission electron microscopy (TEM) and laser scanning confocal microscopy (LSCM) have been used to show differences in tissue structure as well as uptake of bacteria into produce (3,18,16). Transmission electron microscopy shows morphological differences in structure, including cell wall thickness, presence of lenticels and thickness of the cellulose fibers within the tissue. However, samples must be fixed

and dehydrated to observe using TEM. Laser scanning confocal microscopy (LSCM) allows observation under fully hydrated conditions (16). Burnett et al. (2000) used LSCM to image *E. coli* on surface of apples. Wachtel et al (2002) also used LSCM to image *E. coli*, however, this research focused on lettuce seedlings and seed coats (18).

This project addresses the issue of food borne pathogen internalization in apples through laboratory studies using *E. coli* O157:H7 and outside the laboratory under field conditions using a surrogate, *E. coli* ATCC 25922. The purpose of this project is to provide a scientific basis for determining the extent and location of internalization in apples by human pathogens such as *E. coli* O157:H7. *E. coli* O157:H7 strains that express green fluorescent proteins were used to visualize bacteria in apple tissue using CSLM. Transmission electron microscopy was also used to determine morphological differences between apple cultivars.

METHODS AND MATERIALS

FIELD STUDIES

Preparation of Inoculum

Escherichia coli ATCC 25922 (American Type Culture Collections, Rockville, MD) was used as a surrogate species for *E. coli* O157:H7. Stock cultures were maintained at -80˚C in a 50/50 solution of Brain Heart Infusion broth (Difco, Detroit, MI) and glycerol (Acros, New Jersey). Cultures were grown at 37˚C on Brain Heart Infusion broth and isolated and purified on MacConkey Agar (Difco, Detroit, MI) and confirmed using an API strip (bioMérieux, Hazelwood, MI). *Escherichia coli* ATCC 25922 was grown in 100 ml of Brain Heart Infusion Broth for approximately 24 h at 37˚C. One milliliter of this suspension was added to sterile well water to achieve a solution of approximately 10^6 cfu/ml. Preliminary studies indicated showed that over a 72 h period, the cell suspension increased approximately one log when held at room temperature (22˚C) in sterile well water (Fig. 1). This suspension functioned as the inoculum for spraying the test apples.

Inoculation of Apples

Five cultivars of apple were used in this study: Redfree, Red Delicious, Golden Delicious, Rome Beauty and York. The apple trees were located at Virginia Tech's College of Agriculture and Life Sciences Kentland Farm in the Virginia Tech Research Orchard (Montgomery County, VA). Redfree is an early harvest cultivar. The remaining four apple cultivars are typically harvested in the fall and are used in the manufacturing of apple juices and ciders throughout the United States. Long-term study apples were spray inoculated on the trees to obtain 10^5 cfu/apple. Short-term study apples were spray inoculated on the trees to obtain 10^4 cfu/apple. The inoculum was evenly distributed across the entire surface of the apple using a home use handheld two-liter polyethylene compression sprayer. One apple was tested to assure accurate inoculation and one unsprayed apple was tested as a negative control.

Analysis and Enumeration

Spray inoculations were preformed twice during the growing season, at the beginning of the growing season and two weeks prior to harvest. Redfree was used in the long-term study and the remaining four apples cultivars were used in an intensive, two-week study. The inoculation process was the same for the long-term and the short-term study.

During the long-term study, apples were picked on days 1, 30 and 60. Five sample apples and two uninoculated control apples were picked per day and sectioned into skin, flesh, inner and outer cores using a home use apple corer-peeler-slicer (2). Each section was placed into separate filtered stomacher bags and stomached at 230 rpm for 2 min (Seward, London, England). Nine ml of sterile peptone water (Difco, Detroit MI) was added to the skin, inner and outer core sections while the flesh was stomached with natural juices. Samples were plated using the Spiral Platter System (Spiral Biotech, Norwood, MA) onto MacConkey agar supplemented with cycloheximide (Sigma, St. Louis, MO) to prohibit yeast and mold growth. Each sample was plated in duplicate at a 10^{-1} dilution on a weight/weight basis. All plates were incubated at 37˚C for 24 h and counted using the Spiral Biotech directions. Identification of *E. coli* was primarily based on the color of the colonies; however, confirmation tests were performed using API

strips. Samples were also placed into EC broth (Difco, Detroit, MI) supplemented with MUG (4-Methylumbelliferyl-ß-D-Glucuronide, Sigma, St. Louis, MO) to ensure lower counts of bacteria were detected. Little to no growth was noted in these tubes.

In the short-term study, apples were spray inoculated two weeks prior to harvest. Five sample apples and two control apples were picked every other day until harvest. Samples were prepared in the same manner as described for the long-term study.

Experimental Design and Statistical Analysis

Five treated and two unsprayed apples were picked on each sampling day using a randomized design in both the long- and short-term studies. The total number of samples plated on MacConkey agar per day was 20 and 8, respectively. Microbial counts (log cfu/mL) were determined in duplicate for each sample. Counts were analyzed using single and multiple linear regressions, multiple contrast and analysis of variance (ANOVA) tests using the Statistical Analysis System (SAS Institute, Cary, NC) to determine significant differences ($P<0.05$ unless otherwise noted). Simple and multiple linear regressions were used to determine significant differences in samples within the same cultivar. Backward selection was performed to leave only the significant variables. In the cases in which only one variable was significant, simple linear regression was performed; two or more significant variables required the use of multiple linear regression. Multiple contrast was used to determine significant differences between the cultivars.

MICROSCOPY

Laser Scanning Confocal Microscopy

Red Delicious flesh and core samples were imaged with a Zeiss LSM 510 laser scanning confocal microscope on an Axiovert 100M using a 40X water immersion lens with a 1.2 Numerical Aperture. An argon laser provided excitation at 488 nm. A 505-550 nm emission filter was also used to prevent detection of autofluorescence due to the parenchyma cells naturally present in the apple tissue.

Preparation of Inoculum

Escherichia coli ATCC 25922 (American Type Culture Collections, Rockville, MD) was used as a surrogate species for the field studies. In order to validate findings, both the surrogate species and the pathogenic species, ATCC 43889 were used in the microscopy studies. Both cultures were transformed to express fluorescent proteins. Transformation was possible through the insertion of the Clontech plasmid (Clontech, Palo Alto, CA) pEGFP for green expression. In addition to fluorescence expression, the plasmid also contained an ampicillin resistance gene to further aid identification of the transformed bacteria. When in the presence of isopropyl-ß-D-thiogalactoside (IPTG), the bacteria will turn on a lac promoter and will therefore fluoresce under UV light. Stock cultures were maintained at -80˚C in a 50/50 solution of Brain Heart Infusion broth and glycerol and grown initially at 37˚C on Brain Heart Infusion broth supplemented with 100 μg/ml of ampicillin (Sigma, St. Louis, MO). Both cultures were isolated and purified on Luria-Bertani Agar (Difco, Detroit, MI) supplemented with ampicillin and IPTG (Sigma, St. Louis, MO) and confirmed by fluorescence under UV light.

Escherichia coli species were grown in 40 ml of Brain Heart Infusion Broth supplemented with ampicillin and IPTG for approximately 24 h at 37˚C. Twenty milliliters of this suspension was added to 1980 ml of well water (5˚C) to achieve a solution of approximately 10^7 cfu/ml. This suspension was used as the inoculum for the dipping of the test apples.

Inoculation and Examination of Apples

Twenty ml of an overnight culture of *E. coli* ATCC 25922 transformed to express a fluorescent protein and 1980 ml of cold well water (5˚C) were mixed together in a four-liter beaker. Four whole, intact Red Delicious apples (30˚C) were fully submerged into the inoculum for 10 min. They were then individually washed in 1 liter of fresh water for another 10 min and allowed to dry. Each apple was placed in separate Whirl-pak (Nasco, Fort Atkinson, WI) bags and incubated at room temperature for three days. On the third day, two apples were analyzed using LSCM. Each apple was examined using the LSCM to determine how deep the bacteria penetrated into the apple. The apple was cored and sliced every 0.5 cm down from the stem and up from the calyx. The remaining two

apples were analyzed following the field study protocol. Uninoculated control apples were used in both studies. The study was replicated using *E. coli* O157:H7 ATCC 43889.

A second study was preformed to analyze bruised apples. The same procedure was used to inoculate the apple; however, in this study, a 0.5 cm deep puncture wound was positioned in the side of each apple using a thick needle.

Experimental Design and Statistical Analysis

Four Red Delicious apples were examined per treatment, intact and bruised. In addition two uninoculated apples were analyzed each day as a control. Two inoculated apples and two uninoculated apples were examined using the microbiological techniques as described in the field study. The total number of samples plated on MacConkey agar per day was 8, for both inoculated and control apples. Microbial counts (log CFU per mL) were determined in duplicate for each sample. Statistical analysis followed the procedure for the field studies. The remaining two inoculated apples were examined under CLSM.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was preformed using a JEOL 100 CX-II Scanning Transmission Electron Microscope (STEM) with magnification of 25,000X in TEM mode. Apples slices were taken from Golden Delicious, Rome Beauty and York, fixed in 2.5% gluteraldehyde in 0.1 M sodium phosphate butter (Difco, Detroit, MI), adjusted to pH 7.4 and processed for TEM. Transmission electron microscopy was used to analyze the morphological differences in skin samples.

General Microscopy

Thick sections of Red Delicious, Golden Delicious, and Rome Beauty apple skins were imaged using an Olympus BX60 Fluorescent Optical Microscope with magnification of 300X. Sections were prepared in the same manner as the TEM, fixed in 2.5% gluteraldehyde in 0.1 M sodium phosphate butter (Difco, Detroit, MI), and adjusted to a pH of 7.4.

RESULTS

FIELD STUDIES

Long Term Study

The number of culturable *E. coli* ATCC 25922 are shown in Table 1 and are presented as mean log cfu/g ± standard deviation for Redfree apples 1, 30 and 60 days after treatment. On day 1, *E. coli* was found in the outer core sample, but not in the skin, flesh or inner core where culturable cells had decreased to undetectable numbers (<1 cfu/g). On days 30 and 60, *E. coli* was not detected in any of the apple sections. However, on days 30 and 60, *Klebsiella pneumonia*, *Kl. ozaenae* and *E. vulneris* were detected in the flesh and outer core of the samples. All control apples were negative for *E. coli*.

There was no precipitation on the day of or the day after inoculation and little rain accumulation was recorded throughout the study. Temperature ranged from 16°C to 25°C on the day of inoculation. Temperature throughout the long-term study ranged from 8°C to 29°C.

Escherichia coli did not survive on immature apples past day 1. During the first months of development, apple cells are constantly dividing; however, growth of the apple cells does not take place until cell division halts. Therefore, there is little intracellular space for the bacteria to grow until the fruit matures (17). Also, pH levels, sugars and nutrients are very low until two weeks prior to harvest (17). These conditions prevent microbial growth.

Short Term Study

Escherichia coli was found in all sections of the Red Delicious apple (Table 2). Maximum *E. coli* counts were reported on day 13 for the skin, flesh, inner and outer cores. High *E. coli* counts in the outer core corresponded with higher counts in the skin, flesh, and inner core samples ($p<0.05$, Figure 2). Figure 3 shows *E. coli* counts in the outer core increased significantly over the two-week period ($p<0.05$). This same response was noted when comparing the proportion of apples with *E. coli* present in the inner core ($p<0.05$). Finally, *E. coli* log counts were highest in the skin when indigenous

bacteria were present in high numbers. On inoculation day, temperature ranged from 15°C to 29°C, and no rainfall was observed. During harvest, the temperature was consistent every day, ranging from 15°C to 29°C. 2.5 cm of rainfall was noted four days after inoculation.

Escherichia coli was also detected in the treated Golden Delicious (Table 2). Maximum skin and inner core *E. coli* counts were noted on day 3; however, maximum outer core and flesh counts were noted on day 9 and 13, respectively. When the initial proportion of skin samples with *E. coli* present was high, the proportion of inner core samples with bacteria present was also high throughout the two-week period ($p<0.05$, Figure 4). *Escherichia coli* log counts were highest in the skin and the inner core when indigenous bacteria were present in high numbers. Figure 5 shows higher counts of *E. coli* in the outer core are related to increasing counts in the flesh ($p<0.05$). On treatment day, temperature ranged from 6°C to 27°C with no rainfall. During harvest, the temperature was consistent every day, ranging from 15°C to 29°C.

Treated York Imperial also had *E. coli* present in all sections (Table 3). Maximum counts were obtained on day 1 for skin and outer core and day 7 for flesh and inner core. Samples with high numbers of indigenous bacteria in the flesh corresponded with high numbers of *E. coli* in the flesh ($p<0.05$). *Escherichia coli* counts in the outer core were related to higher counts of indigenous bacteria as well as to higher counts in the skin ($p<0.05$). Outer core counts decreased over the 14-day period ($p<0.05$). On treatment day, the temperature ranged from 8°C to 12°C, with no rainfall observed. During harvest, the temperature was inconsistent, ranging from -1°C to 18°C. No rainfall was observed.

E. coli was found in the outer core of Rome Beauty on days 3 and 9. On inoculation day, temperature ranged from 2°C to 20°C, with no rainfall. During harvest, the temperature was inconsistent, ranging from -5°C to 25°C. No rain was detected.

Internalization in the cultivars differed significantly. When comparing the skin and flesh samples, Red Delicious differed from all other cultivars ($p<0.05$). For inner core samples, Red Delicious differed from Rome Beauty, Golden Delicious and York ($p<0.05$). Red Delicious and Rome Beauty differed for the outer core samples ($p<0.05$).

Due to the inconsistency of the uptake of *E. coli* ATCC 25922, the standard deviation of each sample day is high.

Internalization was much greater in all areas of the apples in the indoor studies (Table 4). An initial higher count in the flesh was related to initial higher counts in the skin and inner core ($p<0.05$); however, over the fourteen-day period, all three sections decreased over one log (Figure 7). Initially high counts in the outer core were also related to high counts in the inner core (Figure 6, $p<0.05$); however, where the inner core decreases over 2 logs, the outer core remained stable.

Internalization was significantly higher in all four sections for the indoor compared to the outdoor study. Temperature was held constant at 23°C with no simulated rainfall in the indoor study. Environmental conditions, such as fluctuating temperature, play an important role in the amount of bacterial internalized.

MICROSCOPY

Laser Scanning Confocal Microscopy

Escherichia coli ATCC 25922 and *E. coli* O157:H7 were both imaged in the tissue of Red Delicious apples using the laser scanning confocal microscope (LSCM) after dip inoculation. The organism was found 0.5 cm into the stem and calyx end of intact apples and 0.5 cm into the flesh of a bruised apple.

The microbiological analysis, which followed the procedure for the field studies, is shown in Table 5. Statistical analysis by ANOVA found no significant difference between the surrogate species and the pathogen ($p<0.38$). There was also no difference between the amount of bacteria internalized in the intact and bruised apples. The amount of bacteria observed using standard microbiological techniques showed a significant amount of bacteria internalized in all four sections of both the intact and bruised apple. However, results differed for LSCM. When viewing the apple through the microscope, single layers are scanned for fluorescence emitted by the transformed *E. coli.* The small viewing area makes it difficult to find the bacteria in a large sample, such as an apple.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to analyze morphological differences in apple skin and flesh of Red Delicious, Golden Delicious and York Imperial. Figure 7 shows lenticels found in the skin samples of the three cultivars. Lenticels can be filled with waxy cells, as in Red Delicious, or by corky cells as in Golden Delicious. Lenticels may also be left open, as shown in Figure 7C (17). Figure 8 shows cell wall differences in the flesh samples. Cell structure also varies among apple cultivars. Apple cultivars are often characterized based on differences in the cellulose and hemicellulose of the cell wall and the cell membrane (17). As seen in Figure 9, the Golden Delicious and York Imperial cell walls are thinner than the Red Delicious.

General Microscopy

The cuticle on the skin of mature apples also varies in thickness and chemical composition depending on the variety. Some cultivars are known to have cracks or breaks in the cuticle (17). As shown in Figure 10A, these cracks are most common in the Golden Delicious cuticle.

DISCUSSION

The results of the long-term study show that *E. coli* was unable to survive on immature apples past day 1. During the first 50 days of development, apple cells are constantly dividing; however, cell growth does not occur until cell division halts. Therefore, there is little intracellular space for the bacteria to grow until the fruit matures. Also, pH levels, sugars and nutrients are very low until two weeks prior to harvest. These conditions prevent microbial growth. Internalization is a function of apple maturity.

Red Delicious showed the highest amount of internalization in the short-term study. The remaining three cultivars showed considerably less the uptake of *E. coli* ATCC 25922. One possible explanation is the trend of the indigenous bacteria. The natural microflora in an apple is most abundant in June and steadily decreases thereafter (6). Therefore, apples that are picked in September, Red and Golden Delicious, are likely to have more bacteria present then those harvested in October, York Imperial and Rome Beauty. Dingman (5) stated that susceptibility to contamination by bacteria varies

depending on the apple variety. As previously mentioned, morphological structure, pH, sugar and nutrient levels are linked to microbial growth. Environmental conditions during this growing season did not allow the fruit to reach full maturity in terms of sugar levels (12). Due to the dry season, apples did not have the usual amount of available sugar. Drought stress inhibits photosynthesis and causes a decrease in the starch and sugar content of the fruit (12). Lower temperatures could explain the drop of *E. coli* found in the York Imperial and the Rome Beauty.

Indoor studies showed high initial uptake into all four sections of the apple. Over the two-week period, all sections decreased. Indoor study apples were held at room temperature and spoilage was detected after day 11. The decrease in counts may be due to fruit spoilage. Spoilage organisms could have played a competitive role causing the growth of *E. coli* to be suppressed.

Contaminated spray or irrigation water is a potential source of contamination. Earlier harvest cultivars, such as the Delicious cultivars, are more likely to uptake the organism. When comparing the indoor and the outdoor study, it is evident that environmental conditions affect the uptake of the organism into the fruit.

While the field study did not differentiate between the stem and the calyx, the CSLM found no difference of uptake between the two. However, as previously mentioned, sample size made it difficult to obtain more accurate results.

Comparing York Imperial, Red and Golden Delicious images under the TEM showed differences in the skin samples though the presence of lenticels. Lenticel size, shape and number vary among apple cultivars. Some cultivars such as Winesap average 450 – 800 lenticels per apple, while other cultivars have a higher range of 1500 – 2500 per apple. Lenticels are openings in the skin, which may arise from nonfunctional stoma, or from breaks in the cuticle due to the removal of the entire epidermal hairs (17). Lenticels provide bacteria a possible route of entry into the flesh of an apple. Depending on the type of material the variety uses to fill the lenticel, the gap becomes more or less susceptible to internalization. For example, when Golden Delicious does not fill the lenticels, the inner tissue of the apple is left exposed.

When imaging the cells walls, major differences were seen in the primary cell walls, which are composed mainly of cellulose, hemicellulose and pectins. These early

stage cell walls must be easily expandable in order for the fruit to grow. However, once growth ceases, secondary cell walls begin to form. These cell walls contain cellulose, other complex sugar-based molecules and lignins to ensure rigidity (15). As the cell walls change from the primary to the secondary wall, the fibers within the flesh expand and vacuoles appear (17). This expansion allows easier diffusion of gas and other particles throughout the tissue (17). Due to thinner cell walls, internalization and movement within the tissue may occur easily when bacteria are present in higher numbers.

The discontinuity of the cuticle leaves exposed tissue and may provide a route of entry. Exposed apple tissues are more susceptible to contamination by bacteria. Kenney et al. (10) found cells introduced to areas of the skin with lenticels and cracks in the cuticle were protected by the platelets present in the apple skin. Therefore, apples in which these cracks are common can harbor bacteria and potentially show higher rates of internalization.

Although there have been numerous outbreaks associating *Escherichia coli* O157:H7 with meat products, only recently has the organism been associated with apple products. Many practices used in orchards and processing facilities may be contributing to the internalization of pathogens into apples, specifically irrigation and spray water, and flume systems. This study indicates that internalization can occur to a limited extent in field conditions and to a more severe extent when held in laboratory conditions. Flume systems used for the washing or transportation of fruit are potential sources of contamination or cross contamination. Stricter guidelines dictating temperature control and the amount of free sanitizing agents needed could lower the possibility of cross contamination. However, the most effective treatment involves the inclusion of a five-log reduction of the target organism, *Escherichia coli* O157:H7. The five-log reduction can be obtained through one step or the combination of two or more steps.

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Table 1. Location of *E. coli* ATCC 25922 infiltration in the skin, flesh, inner and outer cores of immature Redfree apples, treated June 5 and harvested through August.

Cultivar	Days after Treatment	Skin Mean Log cfu/ml ± sd n = 5 per day	Flesh Mean Log cfu/ml ± sd n = 5 per day	Inner Core Mean Log cfu/ml ± sd n = 5 per day	Outer Core Mean Log cfu/ml ± sd n = 5 per day
Redfree	1	<1	<1	<1	3.1±0.62
	30	<1	<1	<1	<1
	60	<1	<1	<1	<1

[a] Samples that were below the lower limit of detection (log (CFU/g) = 1.00) are noted as <1.

Table 2. *E. coli* ATCC 25922 infiltration in the skin, flesh, inner and outer cores of mature Red Delicious and Golden Delicious apples, spray inoculated outdoors August 28 and September 6 respectively.

Cultivar	Days after Treatment	Skin Mean Log cfu/g (+/Total) n = 5 per day	Flesh Mean Log fu/g (+/Total) n = 5 per day	Inner Core Mean Log cfu/g± (+/Total) n = 5 per day	Outer Core Mean Log cfu/g (+/Total) n = 5 per day
Red	0	<1	<1	<1	<1
Delicious	1	1.9 (3/5)	1.7 (2/5)	<1	0.56 (1/5)
	3	<1	<1	0.81 (1/5)	<1
	5	1.2 (2/5)	<1	0.40 (1/5)	0.74 (1/5)
	7	<1	1.1 (2/5)	0.70 (1/5)	0.95 (1/5)
	9	1.2 (2/5)	1.1 (2/5)	0.64 (1/5)	0.97 (1/5)
	11	2.0 (3/5)	1.5 (2/5)	0.46 (1/5)	2.4 (3/5)
	13	2.6 (4/5)	2.2 (4/5)	1.7 (3/5)	2.9 (5/5)
Golden	0	<1	<1	<1	<1
Delicious	1	0.68 (1/5)	<1	<1	1.1 (2/5)
	3	0.96 (2/5)	<1	0.66 (1/5)	<1
	5	0.60 (1/5)	<1	0.52 (1/5)	0.40 (1/5)
	7	<1	<1	<1	1.2 (2/5)
	9	0.55 (1/5)	0.20 (1/5)	0.34 (1/5)	1.2 (4/5)
	11	<1	<1	<1	<1
	13	<1	0.70 (1/5)	<1	0.79 (1.5)

[a] Samples that were below the lower limit of detection (log (CFU/g) = 1.00) are noted as <1.

Table 3. Location of *E. coli* ATCC 25922 infiltration in the skin, flesh, inner and outers core of York Imperial and Rome Beauty apples, spray inoculated outdoors September 25 and October 1, respectively.

Cultivar	Days after Treatment	Skin Mean Log cfu/g (+/Total) n = 5 per day	Flesh Mean Log cfu/g (+/Total) n = 5 per day	Inner Core Mean Log cfu/g (+/Total) n = 5 per day	Outer Core Mean Log cfu/g (+/Total) n = 5 per day
York Imperial	0	<1	<1	<1	<1
	1	0.50 (1/5)	<1	<1	2.13 (4/5)
	3	<1	<1	<1	0.46 (1/5)
	5	<1	<1	<1	<1
	7	<1	0.34 (1/5)	0.56 (1/5)	<1
	9	<1	<1	<1	<1
	11	<1	<1	<1	<1
Rome Beauty	0	<1	<1	<1	<1
	1	<1	<1	<1	<1
	3	<1	<1	<1	0.40 (1/5)
	5	<1	<1	<1	<1
	7	<1	<1	<1	<1
	9	<1	<1	<1	0.70 (1/5)
	11	<1	<1	<1	<1
	13	<1	<1	<1	<1

[a] York Imperial apples were not available for sampling on day 13.
[b] Samples that were below the lower limit of detection (log (CFU/g) = 1.00) are noted as <1.

Table 4. Location of *E. coli* ATCC 25922 infiltration in the skin, flesh, inner and outer cores of Red Delicious apples in a controlled indoor setting.

Cultivar	Days after Treatment	Skin Mean Log cfu/g (+/Total) n = 5 per day	Flesh Mean Log cfu/g (+/Total) n = 5 per day	Inner Core Mean Log cfu/g (+/Total) n = 5 per day	Outer Core Mean Log cfu/g (+/Total) n = 5 per day
Indoor:	0	<1	<1	<1	<1
Red	1	3.9 (5/5)	4.9 (5/5)	3.7 (5/5)	4.4 (5/5)
Delicious	3	3.8 (5/5)	2.8 (5/5)	1.5 (3/5)	4.0 (5/5)
	5	4.3 (5/5)	3.1 (4/5)	2.1 (3/5)	5.1 (5/5)
	7	2.8 (4/5)	2.4 (5/5)	1.0 (2/5)	4.6 (5/5)
	9	3.8 (5/5)	3.3 (5/5)	1.3 (3/5)	3.1 (4/5)
	11	2.3 (5/5)	1.3 (2/5)	0.90 (2/5)	4.4 (5/5)

[a]Samples that were below the lower limit of detection (log (CFU/g) = 1.00) are noted as <1.

Table 5. Location of *E. coli* ATCC 25922 and infiltration in the skin, flesh, inner and outer cores of mature Red Delicious apples in a controlled indoor setting using dip inoculation after a three day incubation.

Intact	Skin	Flesh	Inner Core	Outer Core
Escherichia coli ATCC 25922	4.0±0.38	4.3±2.7	2.2±0.11	6.1±0.28
Escherichia coli O157:H7	4.2±1.5	4.2±2.3	3.1±4.4	6.1±0.34

Bruised	Skin	Flesh	Inner Core	Outer Core
Escherichia coli ATCC 25922	5.8±0.38	5.8±0.27	5.9±0.11	6.1±0.28
Escherichia coli O157:H7	4.3±0.89	4.7±0.43	2.5±3.5	6.4±0.90

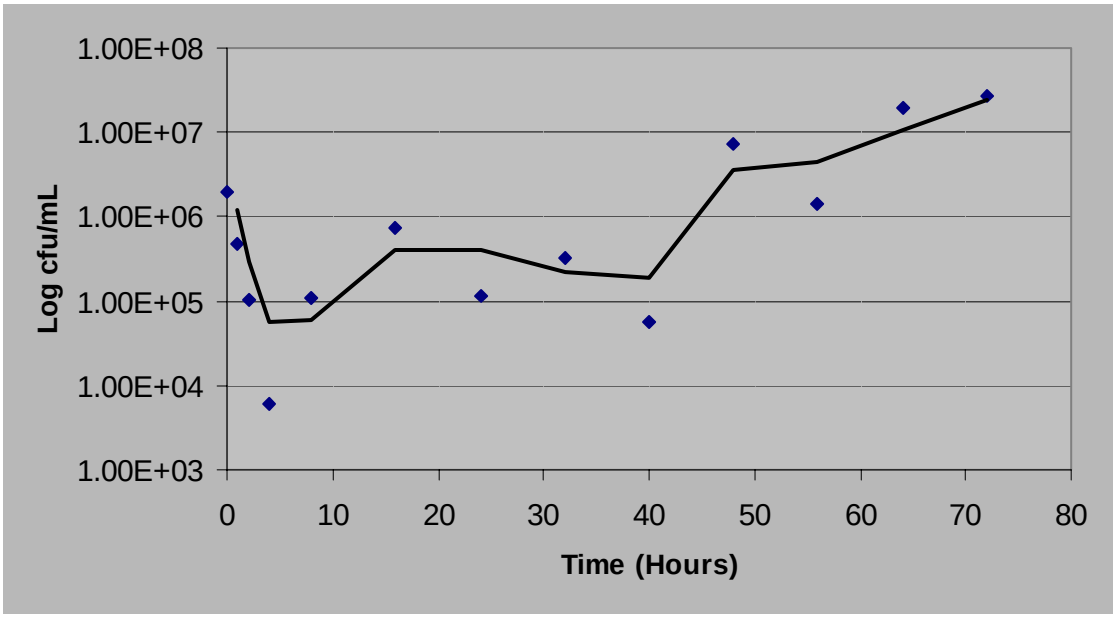


Figure 1. Preliminary Growth Studies of Escherichia coli ATCC 25922 held at room temperature in sterile spring water for a three-day period.

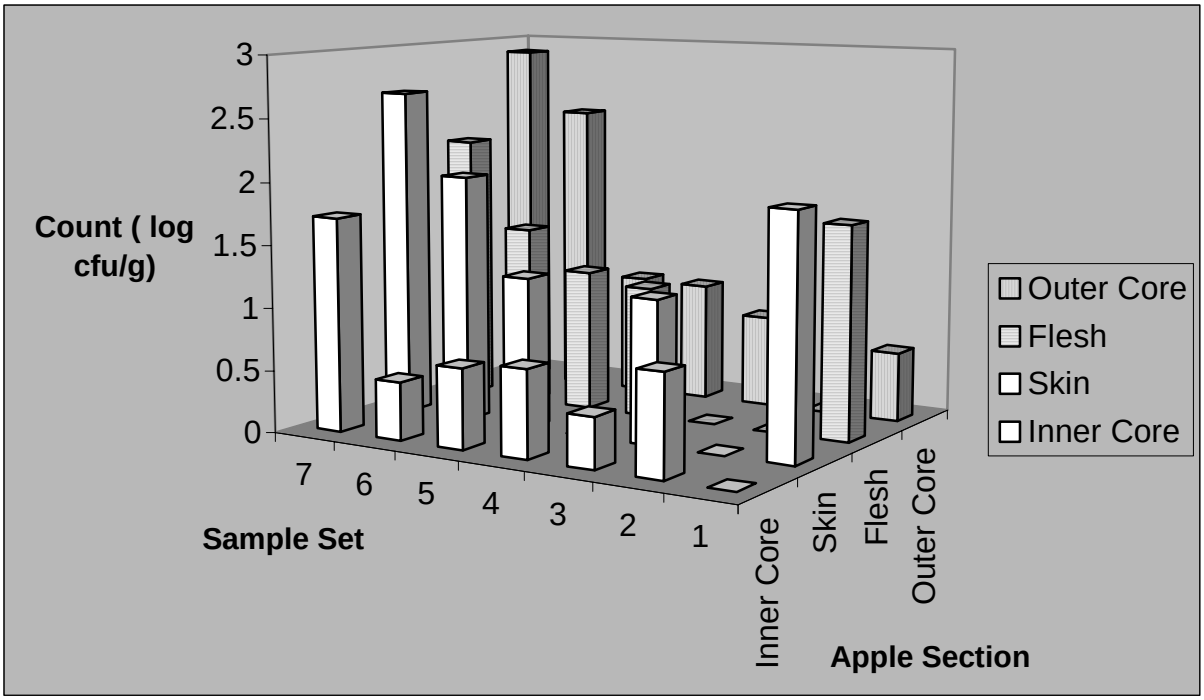


Figure 2. *E. coli* counts in the skin, flesh and inner core of the Red Delicious over the two-week period of the short-term study. The r^2 value for the outer core, skin, flesh and inner core are 0.56, 0.61, 0.59 and 0.60.

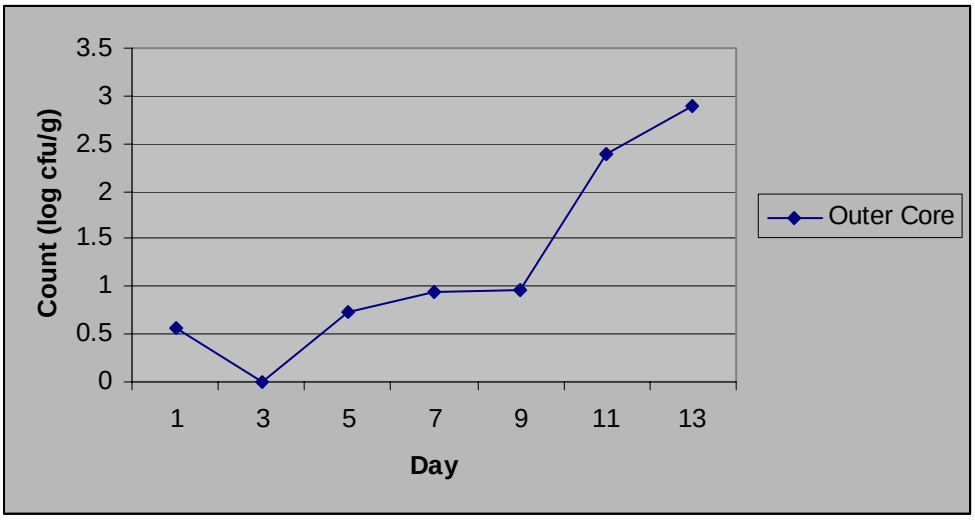


Figure 3. The rate of internalization in the outer core of the short-term Red Delicious apple is related to the day ($r^2 = 0.55$).

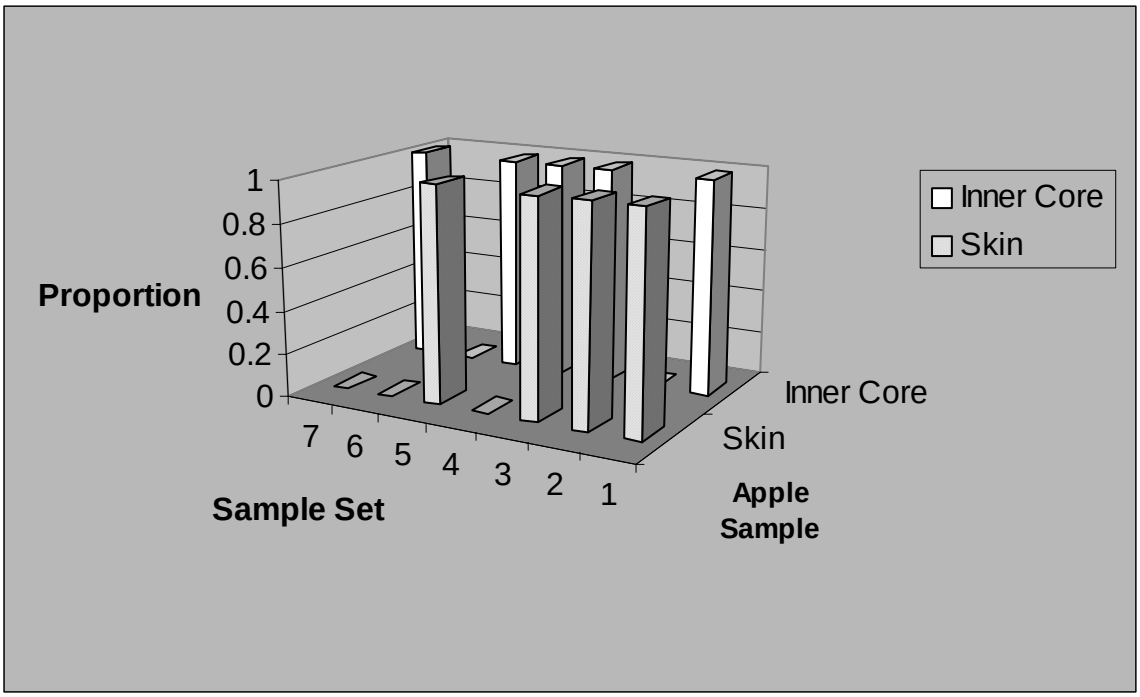


Figure 4. The proportion of Golden Delicious apples with *E. coli* present in the skin and in the inner core over the two-week period of the short-term study. The r^2 value is 0.59.

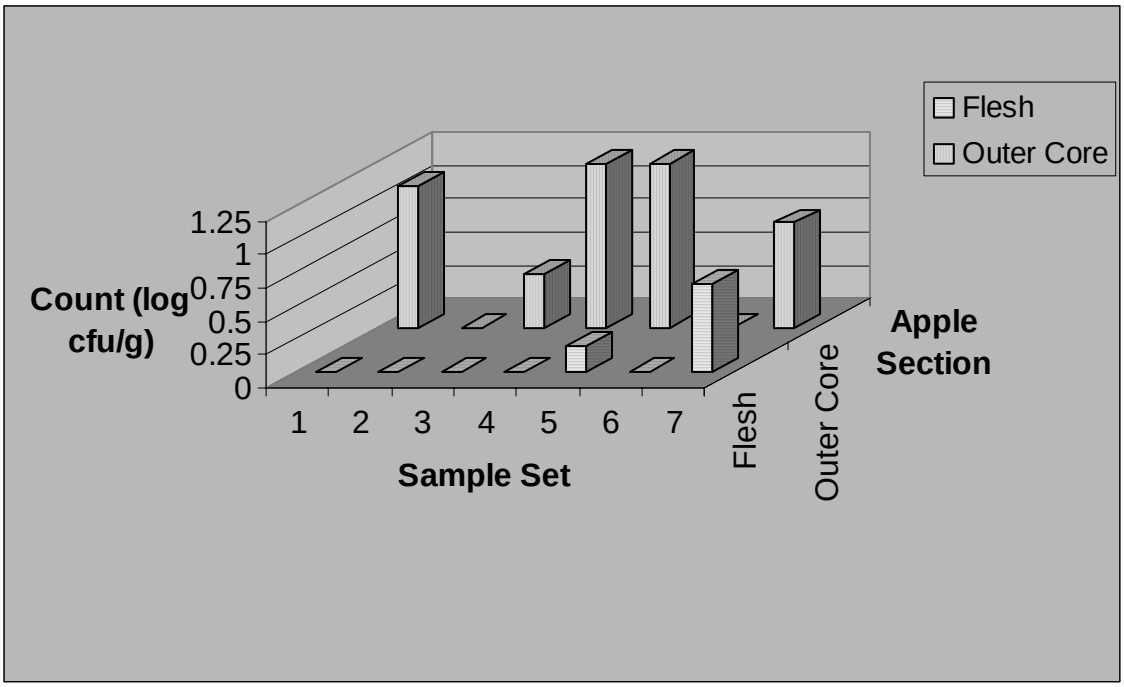


Figure 5. Counts in the flesh, skin and inner core of the indoor Red Delicious apple over the two-week period of the short-term study. The r^2 value is 0.65.

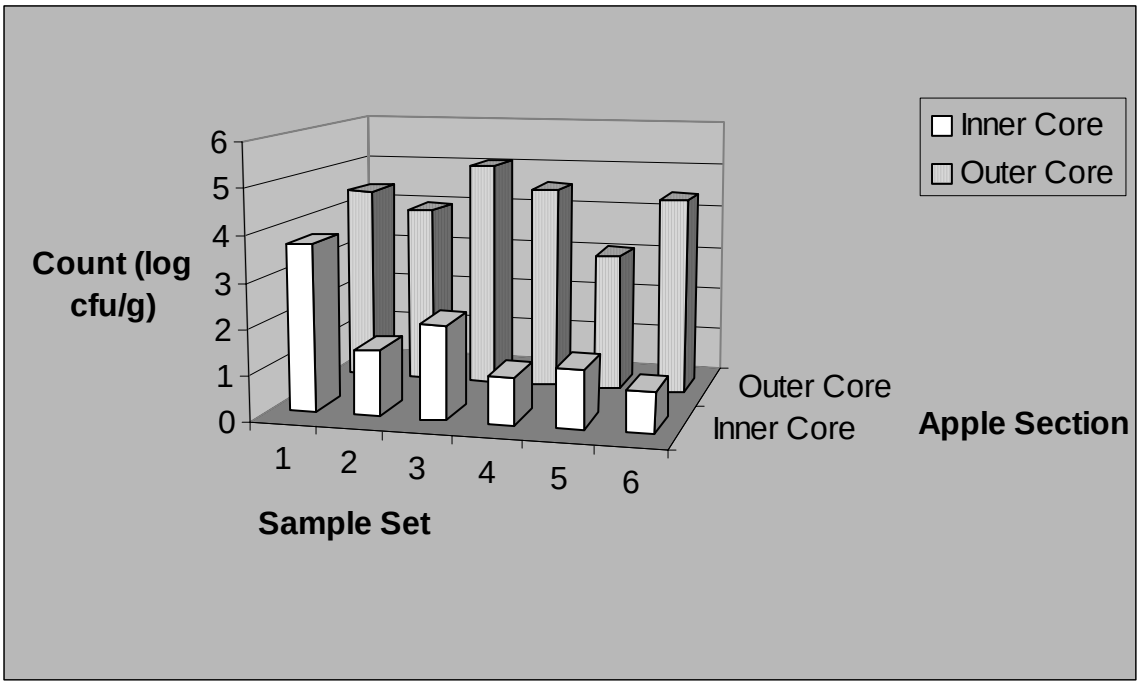


Figure 6. Counts in the outer core and the inner core of the indoor Red Delicious apple. The r^2 value is 0.64.

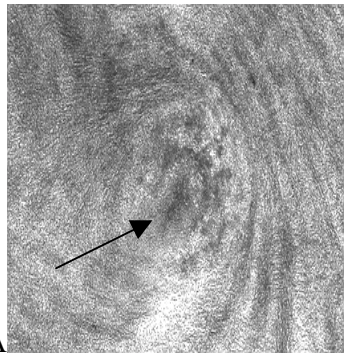
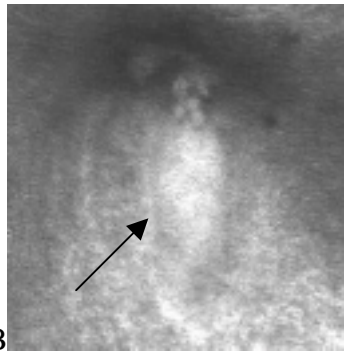
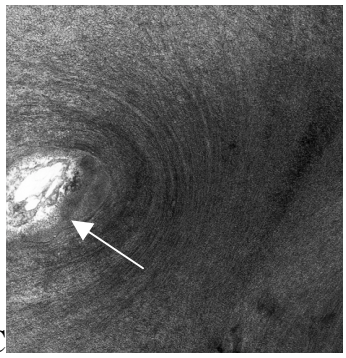
A  B  C 

Figure 7. Lenticels on mature skin samples, Golden Delicious (A), York Imperial (B) and Red Delicious (C) at a magnification of 25,000X, are indicated by arrows.

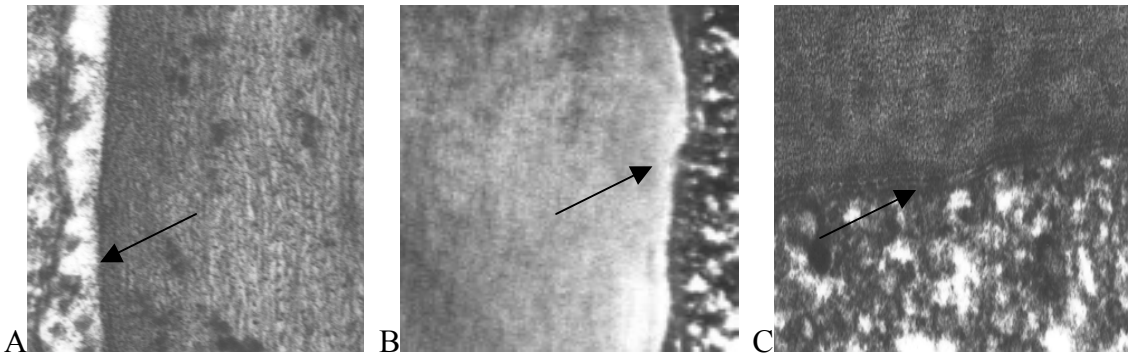


Figure 8. Cell wall and hemicellulose of mature Golden Delicious (A), York Imperial (B) and Red Delicious(C) at 25,000X magnification. Arrows point to the separation of the cell wall and the hemicellulose.

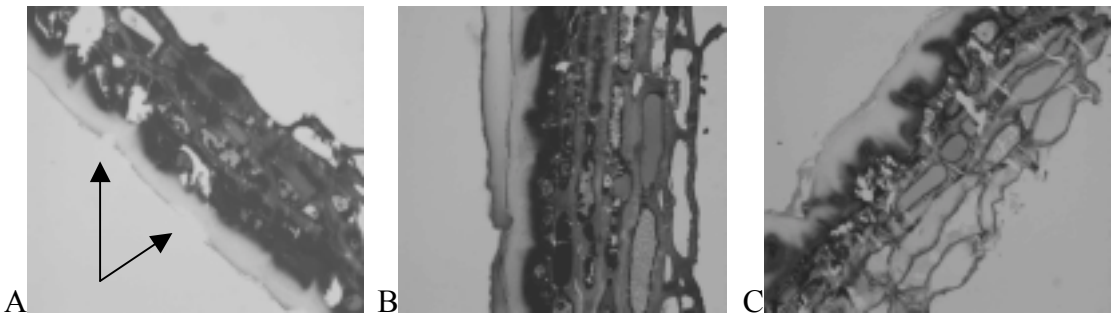


Figure 9. Cuticle of mature Golden Delicious (A), York (B) and Red Delicious (C) at 300X magnification. Arrows show cracks in the cuticle.

VITAE

Brooke Seeman was born and raised in Richmond, Virginia. She began her career at Virginia Tech in the fall of 1996 and completed her Bachelor's degree in Biology in the spring of 2000. During the summer of 1998, Brooke worked at the Medical College of Virginia in a Microbiology/Immunology lab as a summer intern. The following summer, she began undergraduate research under Dr. Susan Sumner in the Food Science and Technology Department at Virginia Tech. It was during this time that Brooke became interested in food science and decided to enroll as a graduate student. She began her Master's program in Food Science and Technology at Virginia Tech in the fall of 2000.

While at Virginia Tech, Brooke was a member of the International Association for Food Protection and the Institute of Food Technologists. Her undergraduate research was presented at the International Association for Food Protection meeting in August of 2000 and was then accepted for publication in the Journal of Dairy, Food and Environmental Sanitation in May of 2002. During her second year of graduate school, Brooke served as the Communications Officer for Virginia Tech's chapter of the Institute of Food Technologists Student Association. Also while at Virginia Tech, Brooke was a member of the Virginia Tech Field Hockey team and of Alpha Chi Omega Fraternity.