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Influence of Infiltration and Attachment Site on Viability of *Escherichia coli* O157:H7 Cells in and on Apples Following Chlorine Sanitization
(Under the Direction of LARRY R. BEUCHAT)

Outbreaks of disease caused by *Escherichia coli* O157:H7 associated with the consumption of unpasteurized apple cider in recent years have raised interest to improve the efficacy of surface decontamination treatments applied to apples for the purpose of reducing populations of pathogens. The effectiveness of surface treatments may be limited due to the inaccessibility of bacterial cells sequestered in fruit tissues. Confocal scanning laser microscopy (CSLM) was used to characterize sites of attachment and infiltration of *E. coli* O157:H7 cells on and in raw apples as well as to differentiate viable and non-viable cells of the pathogen on apple tissues following treatment with water and 200 or 2000 ppm active chlorine solution. Results showed preferential cellular attachment to discontinuities in the waxy cuticle on the surface and to damaged tissue surrounding puncture wounds, where the pathogen was observed at depths up to 70 μm below the skin surface. Attachment to lenticels was sporadic, but cells were occasionally observed at depths of up to 40 μm. Infiltration through the floral tube and attachment to seeds, cartilaginous pericarp, and internal trichomes were observed in all apples examined. The mean percentage of viable cells located at each site after treatment with water or chlorinated water was in the following order, which also reflects the order of protection against inactivation: floral tube wall (20.5%) > lenticels (15.0%) > damaged cuticle surrounding puncture wounds (13.0%) > intact cuticle (8.1%). The location of viable cells within tissues was dependent upon the structure. With the exception of lenticels, the percentage of viable cells increased as depth into CSLM stacks increased, indicating that cells attached to subsurface structures were better protected against inactivation with chlorine than were cells located on exposed surfaces. Results suggest that *E. coli* O157:H7 cells attached to internal core structures or within subsurface tissues may evade treatment of up to 2000 ppm active chlorine. Further

research is warranted to investigate the efficacy of other chemical santizers as well as the influence of surfacants and solvents in combination with sanitizers for effectiveness in removing or killing *E. coli* O157:H7 lodged in protective structures on the surface and within apple tissues.

INDEX WORDS: *Escherichia coli* O157:H7, Apples, Confocal Scanning Laser Microscopy, Viability, Chlorine, Disinfection

INFLUENCE OF INFILTRATION AND ATTACHMENT SITE ON VIABILITY OF *ESCHERICHIA COLI* O157:H7 CELLS IN AND ON APPLES FOLLOWING CHLORINE SANITIZATION

by

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DEDICATION

To my best friend, Andrea.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW[1]

[1]Burnett, S.L., and L.R. Beuchat. Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. Submitted to J. Ind. Microbiol. Biotechnol.

Introduction

The frequency of documented outbreaks of human illness associated with consumption of raw fruits and vegetables, as well as unpasteurized juices, has increased in the United States in recent years (NACMCF, 1999). Salmonellosis has been linked to tomatoes, seed sprouts, cantaloupe, watermelon, apple juice, and orange juice. *Escherichia coli* O157:H7 infection has been associated with lettuce, alfalfa sprouts, and apple juice, and enterotoxigenic *E. coli* has been linked to carrots. Associations of shigellosis with lettuce, scallions, and parsley, cholera with strawberries, hepatitis A virus with lettuce, raspberries, and frozen strawberries, and Norwalk/Norwalk-like virus with melon, salad, and celery have also been documented. Most recently, *Cryptosporidium* infection linked to apple cider and *Cyclospora* infection linked to raspberries, lettuce, and basil have broadened awareness that produce-associated illnesses are not confined to bacteria and viruses as causative agents.

The epidemiology of foodborne diseases has undoubtedly contributed to an increased frequency of outbreaks of infections linked to raw produce. Changes in dietary habits, methods of produce production and processing, sources of produce, and the emergence of pathogens previously not recognized for their association with raw fruits and vegetables have been reported to contribute to increasing incidences of infection (NACMCF, 1999; Hedberg et al., 1994).

While much is known about the ecology of pathogens in foods of animal origin, the behavior of pathogens in association with naturally occurring microflora on fruits and vegetables is less defined. Differences in surface morphology and metabolic functions of leaves, stems, florets, roots, and tubers provide a wide range of diverse ecological niches selective for specific species or groups of microorganisms. Bruised and cut surface tissues exude fluids containing nutrients and numerous phytoalexins and other antimicrobials such as organic acids that may enhance or retard the growth of naturally occurring microflora and pathogens. Colonization and biofilm development

ensue, resulting in spoilage and growth of bacterial pathogens. Thus, viability of pathogens, parasites, and other infectious agents as affected by extrinsic and intrinsic factors unique to fruits and vegetables is largely unknown.

To date, chemical treatments administered to whole and cut produce for the purpose of killing or removing pathogens have not been demonstrated to reduce populations by more than about 3 $\log_{10}$ CFU/g (Beuchat, 1998). Reasons for their ineffectiveness stem largely from an inability of the potentially lethal chemical components to access microbial cells lodged in discontinuities and biofilms on the surface of produce. Protection against contact of cells with sanitizers results in an increased likelihood of the presence of pathogens on fruits and vegetables at the time of consumption and, therefore, an increased risk of illness.

Increased Incidence of Disease

Increases in numbers of produce-related outbreaks on an international scale have been attributed to several social and economic changes. Consumption of fresh or minimally processed fruits and vegetables has increased in the past two decades (NACMCF, 1999; Francis et al., 1999). Data from the National Agricultural Statistics Service of the U. S. Department of Agriculture reveal a rise in the per capita consumption of fresh fruits and vegetables in the United States by almost 20 pounds from 1988 to 1996 (USDA, 1996). This increase can be attributed, in part, to the consumer's desire to maintain diet that promotes better health (Alketruse and Swerdlow, 1996; Hedberg et al, 1994). Also, agronomic and harvesting practices, processing, packaging, distribution, and marketing have enabled importation of high-quality raw produce from Central and South America, as well as from countries in other parts of the world, to the United States, year-round, creating large and complex international networks of distribution. Brackett (1999) stated that improper

refrigeration is probably the single greatest hazard associated with the safety of chilled foods. Because of the complex nature of distribution channels that supply fresh produce to wholesale and retail markets, especially in the case of imported fruits and vegetables that may require longer delivery times, temperature abuse may result in an increased risk of microbiological spoilage and/or growth of pathogenic bacteria. At least two major produce-associated outbreaks can be attributed to the globalization of the food supply. Outbreaks of shigellosis in Norway, Sweden, and the United Kingdom in 1994 were linked to contaminated iceberg lettuce imported from Spain (Kapperud et al., 1995) and an outbreak of cyclosporiasis in the United States was linked to the consumption of raspberries imported from Guatemala (Herwaldt and Ackers, 1997).

Other factors contributing to the increase in incidence of disease associated with consumption of fresh fruits and vegetables include increases in the surveillance and reporting of foodborne diseases, international travel, and immigration (Alketruse and Swerdlow, 1996; Mead et al., 1999).

Sources of Contamination

The microbial ecosystem on the surface of raw fruits and vegetables is diverse and complex. The presence and numbers of microorganisms differ, depending on the type of produce, agronomic practices, geographical area of production, and weather conditions prior to harvest (Carmichael et al., 1994; Lund, 1992; Nguyen-the and Carlin, 1994). Numerous factors influence the range and populations of microorganisms associated with fruits and vegetables at any given point throughout their production and post-harvest handling, thus influencing the rate and type of spoilage. The environment in which plants are grown impose extrinsic factors that affect associated surface microflora, whereas intrinsic parameters such as the nature of the epithelium and protective cuticle, tissue pH, and the presence of antimicrobials

dictate which groups of produce may be more likely to harbor certain types of microorganisms in damaged tissues. The types of microorganisms recovered from raw fruits and vegetables at harvest most often reflect the microflora present in the field, orchard, grove, or vineyard at the time of harvest (NACMCF, 1999; Lund, 1992; Zagory, 1999). Climatic and agricultural determinants affecting the microbial ecosystem at harvest include geographical location, history of precipitation, wind, irrigation practices, pre-harvest, harvest, and post-harvest practices, and presence of insects, animals, and birds (Brackett, 1999). Microbial ecosystems on produce after harvesting can be greatly influenced by handling and storage conditions as well as conditions of processing, packaging, distribution, and marketing. Gram-negative bacteria dominate the microflora associated with most vegetables, while mold and weakly fermentative yeasts often comprise the dominant microflora on raw fruits, largely due to the acidic pH of fruit tissue, which is generally less than 4.0 (Splittstoesser, 1987).

Although spoilage bacteria, yeasts, and molds dominate the microflora of fruits and vegetables, the occasional presence of foodborne pathogens associated with these foods has been recognized for many years (Beuchat, 1998; Francis et al., 1999). Any type of produce has the potential to harbor pathogens (Brackett, 1999), but *Shigella* spp., *Salmonella*, enterotoxigenic and enterohemorrhagic *Escherichia coli*, *Campylobacter* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, *Yersenia enterocolitica*, *Bacillus cereus*, *Clostridium botulinum*, viruses, and parasites such as *Giardia lamblia, Cyclospora cayetanensis*, and *Cryptosporidium parvum* are of the greatest public health concern (Beuchat, 1996; Beuchat, 1998; NACMCF, 1999; Ortega et al., 1997; Taormina et al., 1999). Table 1.1 lists examples of raw fruits and vegetables from which pathogenic bacteria have been isolated.

Table 1.1. Examples of raw produce from which bacterial pathogens have been isolated[a]

Pathogen	Produce
Bacillus cereus	Cress sprouts, mustard sprouts, soybean sprouts
Camplyobacter jejuni	Mushrooms
Clostridium botulinum	Cabbage, mushrooms
Escherichia coli O157:H7	Alfalfa sprouts, cabbage, celery, cilantro, coriander
Listeria monocytogenes	Beans sprouts, cabbage, chicory, cucumbers, leafy salad greens
Salmonella	Alfalfa sprouts, artichoke, beet greens, cabbage, cauliflower, celery, eggplant, endive, fennel, lettuce, mungbeam sprouts, mustard cress, parsley, peppers, salad greens, spinach
Shigella	Lettuce, parsley, salad vegetables, scallions
Staphylococcus	Lettuce, parsely, salad vegetables, seed sprouts
Yersinia enterocolitica	Carrots, cucumbers, lettuce, tomatoes
Vibrio cholerae	Cabbage

[a]From Beuchat (1998) and NACMCF (1999)

Pathogens, along with spoilage microorganisms, may contaminate fruits and vegetables via several different routes and at several points throughout the pre-harvest and post-harvest system. Sources of contamination have been described (Beuchat, 1996; Beuchat and Ryu, 1997). Potential pre-harvest sources of microorganisms include soil, feces, irrigation water, water used to apply fungicides and insecticides, dust, insects, inadequately composted manure, wild and domestic animals, and human handling. Potential post-harvest sources include feces, human handling, harvesting equipment, transport containers, wild and domestic animals, insects, dust, rinse water, ice, transport vehicles, and processing equipment (Beuchat, 1996; Beuchat, 1998;

Beuchat and Ryu, 1997; Janisiewicz, 1999; NACMCF, 1999). Janisiewicz et al. (1999) demonstrated that fruit flies contaminated with a fluorescent-tagged nonpathogenic strain of *E. coli* served as vectors in colonizing apple wounds. These researchers isolated fluorescent *E. coli* from apple wounds within 48 h of exposure of apples to the flies.

Though the presence of pathogens on fruits and vegetables may be transient and secondary to spoilage microorganisms, produce has long been known to serve as a vehicle for infectious agents (Table 1.2). More recently, however, an increase in the number and frequency of outbreaks associated with produce has been documented. According to statistics compiled by the Centers for Disease Control and Prevention (CDC), the number of reported produce-related outbreaks per year doubled between the period 1973-1987 and 1988-1992 in the United States (Bean et al., 1997).

In response to the increase in produce-related outbreaks, the National Advisory Committee on the Microbiological Criteria for Foods identified research needs related to controlling the introduction and transmission of pathogens associated with fresh produce (NACMCF, 1999). An insufficiency in information concerning the nature of attachment of microorganisms to produce surfaces in addition to information about the efficacy of sanitizers in eliminating or reducing pathogens on fresh fruits and vegetables have been identified.

Surface Morphology Influences Ease of Removing Pathogens

Washing and rising some types of fruits and vegetables prolongs shelf life by reducing the number of microorganisms on the surface. However, only a portion of the microflora is removed with this simple treatment, thus only delaying the growth of spoilage and pathogenic microorganisms. With the addition of a disinfectant to wash water, the efficacy of decontamination can be enhanced by up to 100-fold (Beuchat,

Table 2.1. Examples of pathogens implicated in causing outbreaks of diseases associated with raw produce and produce products[a]

Produce	Pathogen
Whole and puree	
Soy, cress, mustard sprouts	*Bacillus cereus*
Raspberries, mesclun lettuce, basil	*Cyclospora cayetanensis*
Lettuce, alfalfa sprouts, radish sprouts	*Escherichia coli* O157:H7
Carrots	*E. coli* (enterotoxigenic)
Salad vegetables	*Giardia lamblia*
Lettuce, raspberries, frozen strawberries	Hepatitis A virus
Cabbage	*Listeria monocytogenes*
Sliced melon, green salad, celery	Norwalk/Norwalk-like virus
Tomatoes, watermelon, cantaloupe, sprouts (alfalfa, clover, mungbean) mamey, mango	*Salmonella*
Lettuce, scallions, parsley	*Shigella*
Cabbage	*Vibrio cholerae*
Juice	
Apple	*Cryptosporidium parvum*
Apple	*E. coli* O157:H7
Apple, orange	*Salmonella*
Coconut milk	*Vibrio cholerae*

[a]NACMCF (1999), Nguyen-The and Carlin (1994), and Taormina et al. (1999)

1998). Sanitizers vary greatly in their ability to disinfect raw produce. The mechanism of bactericidal action, the nature and the location of the microorganisms, and the type of produce all influence the efficacy of decontamination treatments. The inability of sanitizers to remove all microorganisms on the surface of raw produce suggests that they are ineffective in removing cells more intimately associated with morphological structures. Microorganisms, including pathogens, may reside in protected sites on the epidermis of fruits and vegetables (Beuchat, 1998; Frank, 1998; Seo and Frank, 1998). Although the protective mechanism of these sites is not well understood, the concept that the hyrophobic nature of plant structures is responsible for retarding the penetration of treatment solutions and thus affording microbial cells protection has been proposed.

The epidermis of fruits and vegetables is covered with a multi-layered hydrophobic cuticle (1-15 μm thick) which provides the primary barrier against fungal invasion, insect and physical damage, and desiccation. The cuticle is composed of cutin, which is composed of high molecular weight lipid polyesters of long chain substituted aliphatic acids (Pratt, 1988). Imbedded within the cutin are crystalline and amorphous wax molecules which are responsible for the highly repellant nature of plant surfaces. The cuticle of apples, for example, gradually increases in thickness concomitant with a gradual increase in fruit volume (Meyer, 1944). As the fruit develops, the physiochemical composition of the cuticle changes and tends to become more hydrophobic, with an accumulation of larger amounts of lipoidal components and the accretion of transcuticular and epicuticular waxes (Tukey and Young, 1942). As the cuticular membrane thickens in development, the microtopography becomes pronouncedly complicated in appearance. The formation of cracks in the cuticle of apples is attributed primarily to a tendency toward marked restriction of growth in the epidermal layer late in the growing season while the fleshy portions of the fruit enlarge rapidly (Verner, 1938). Cracks tend to occur at weak areas on the surface, e.g., around lenticels and trichomes on apples. Among the surface features are pits, clefts, craters,

pores, variable granulations, wrinkles, and occasionally a 'melted' appearance of the cuticle (Miller, 1981). Compromises in surfaces may also occur as a result of insect damage or mechanical injury during postharvest handling (Quitana and Paull, 1993), or may form naturally (Meyer, 1944; Verner, 1938).

Gas exchange takes places through pores in the epidermis called stomata. Stomata are protected by guard cells that open and close in response to changes in internal turgor pressure caused by environmental stimuli (Blanke, 1986). Lenticels are formed from stomata in maturing pome fruits and first appear to the unaided eye as small white or cream areas on the surface of the intact fruit. Glenn et al. (1985) observed that as apples mature, stomata open and become distorted. Following this transformation, 1000-1200 lenticels are formed on each apple. The development of lenticels on mature apples is a result of the formation of cutin within broken trichomes or stomata, whose guard cells remain permanently opened due to stretching which accompanies growth. As lenticels form and loosely packed wax platelets develop, the structure usually closes and remains impermeable to the passage of liquids and gases even under pressure. Approximately 5% of lenticels on Red Delicious cultivar apples, however, remain open (Clements, 1935), which may facilitate infiltration of pathogens. The invasion of fungi through lenticels on apples was studied as early as 1925 (Kidd and Beaumont).

Surfaces of several vegetables and fruits are covered with protuberances called trichomes that aid in inhibiting the invasion of insects and molds. As is the case with lenticels, the number of trichomes does not change with age, but simply are more widely dispersed over the surface of the vegetable or fruit as they increase in size. As the volume of pome fruits increases during development, trichomes break off at their base. Broken trichome bases may then fill with cutin, resulting in the formation of lenticels (Shaheen et al., 1981).

Infiltration and Attachment

Bacterial attachment on the surface of sound produce is limited in contrast to attachment on processed meat tissues. However, attachment and infiltration of microbial cells does occur and is facilitated by the stomata, lenticels, broken trichomes, and bruises and cracks in the skin surface of fruits and vegetables (Dingman, 2000; Wells and Butterfield, 1997). The occurrence of infiltration of microorganisms into plant tissues is a well-studied phenomenon, especially as it pertains to interactions between plant pathogens and their hosts. Kidd and Beaumont (1925) demonstrated that lenticels and cracks on the cuticle of apples served as sites of fungal invasion, which subsequently resulted in disease of the stored fruit. Species of *Xanthomonas, Pseudomonas,* Enterobacteriaceae*, Aerobacter,* and *Bacillus* have been isolated from the internal tissues of healthy tomatoes (Samish and Etinger-Tulczynska, 1962). The stem scar was shown to harbor more microorganisms than the underlying pulp. Investigators surmised that bacterial entry into developing tomatoes may occur in the vicinity of the sepals. Wilson et al. (1999) demonstrated and compared the internalization of pathogenic and nonpathogenic strains of *Pseudomonas syringae* into the leaves of bean plants. Their study determined that although the extent of colonization of the internal leaf tissues was greater for pathogenic than nonpathogenic strains, *P. syringae* was internalized within substomatal chambers and the interiors of broken trichomes of plant leaves. Fruit wounds have also been shown to harbor microorganisms. Mercier and Wilson (1994) inoculated the surfaces of apple wounds with a yeast, *Candida oleophila,* and subsequently isolated the organism from the internal tissues in the vicinity of wounds where populations had increased by ca. 2 $\log_{10}$ cfu/wound when stored for two days at 18°C.

The temperature differential between produce and its aqueous surrounding has been shown to influence uptake of water into plant tissues. Gases in the intercellular

spaces of fruits and vegetables exert reduced pressure during cooling, which allows the combined atmospheric and hydrostatic pressure on the immersed produce to force some of the external environment, for example, contaminated water, into its apertures (Bartz, 1982). Bartz and Showalter (1981) demonstrated that tomatoes submerged in a suspension of *Serratia marcensens* exposed to a negative temperature differential, i.e., the temperature of the fruit is higher than the temperature of the suspension in which it is immersed, not only contained the bacterium more frequently, but also gained more mass than tomatoes exposed to a positive temperature differential. The incidence of bacterial soft rot in tomatoes was demonstrated to be higher when fruit were inoculated with suspensions of *Erwinia carotovora* subsp. *carotovora* under a negative temperature differential than a positive temperature differential (Bartz, 1991).

The influence of a negative temperature differential on infiltration of bacteria of concern to public health into tissues of produce has been studied. Attachment of *E. coli* O157:H7 cells on apples is enhanced by a negative temperature differential (Buchanan et al., 1999). Higher numbers of the pathogen were isolated from the cores of apples inoculated under a negative temperature differential than a positive temperature differential. The U.S. Food and Drug Administration has recommended that packers consider the effects of water temperature when attempting to remove field heat from produce (FSIS, 1999). Other researchers have demonstrated that washing tomatoes in water containing *Salmonella* at a temperature cooler than that of the tomatoes results in infiltration of the pathogen into the stem scar tissue (Zhuang et al., 1995).

Researchers have incorporated dyes into bacterial suspensions to better visualize infiltration of aqueous milieu into citrus fruits (Merker et al, 1999; Walderhaug et al., 1999). Merker et al. (1999) observed that 3.6% of oranges internalized *E. coli* O157:H7 cells after applying the pathogen onto the stem scars of fruits. The uptake and internalization of dye occurred in approximately 3% of oranges analyzed. The authors suggested that dye uptake may serve as a useful surrogate for microbial pathogens, and

may be a useful tool in pathogen challenge studies where use of a pathogen would be unsuitable, e.g., in a processing facility (Merker et al., 1999). Pao et al., (2001) using stereomicroscopy, however, showed that solutions of dye and *Salmonella* cells in suspension behave differently in terms of infiltration and attachment to orange surfaces. Dye solutions penetrated deeper into tissues than did bacterial cells, and diffused laterally though orange tissues. Cells of *Salmonella* located within the xylem tissue of stem scars accumulated at the walls of reticulated vessels within vascular bundles. The pathogen was observed over 200 μm below the surface of the stem scar.

Infiltration and attachment of foodborne pathogens into lettuce leaf structures has been well characterized. Seo and Frank (1999) inoculated lettuce leaves with a suspension of *E. coli* O157:H7. They observed that *E. coli* O157:H7 cells preferentially attached to cut edges as opposed to the intact leaf surface. Cells penetrated the interior of the cut tissue and into substomatal chambers of leaves. Infiltration of *E. coli* O157:H7 into cut tissue of lettuce is enhanced at 4°C compared to 37°C (Takeuchi and Frank, 2000). Takeuchi et al. (2000) compared the attachment of *E. coli* O157:H7, *Listeria monocytogenes, Salmonella* Typhimurium, and *Pseudomonas fluorescens* to lettuce leaves. Their investigation demonstrated that each organism examined attached in a different manner to the intact lettuce cuticle and to cut edges. *E. coli* O157:H7 and *L. monocytogenes* attached preferentially to cut edges, whereas *P. fluorescens* was located to a higher extent on the intact cuticle. No preference of attachment site was found with *S.* Typhimurium. Differences in attachment were attributed to differences in hydrophobicities of bacterial surfaces as well as mechanisms of motility among the tested pathogens.

The mechanisms of microbial attachment to the tissues of fruits and vegetables are not well understood. It has been hypothesized, however, that hydrophobic interactions between the epidermal layer and microbial cells play a major role in facilitating attachment to cuticular cracks, stomata, lenticels, and trichomes (Frank,

1998). The nature of the initial attachment of foodborne pathogens to plant surfaces is nonspecific and reversible, and does not consist of complex physiochemical interactions. Planktonic bacteria move to or are attracted to a material surface through and by the effects of physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, the effect of surface electrostatic charge, and hydrophobic interactions (An and Friedman, 1997). The short-range interactions between bacterial cells and plant material surfaces are comprised of hydrogen bonding, ionic and dipole interactions, and hydrophobic interactions (Ong et al., 1999). Once bacteria are attracted to the surface, firm attachment (i.e., adhesion) requires several hours while presumably extracellular polymers are produced (Hermansson, 1999). Biofilms of pathogenic bacteria have been observed on produce surfaces (Carmichael et al., 1999; Itoh et al., 1998). Surface roughness influences bacterial attachment to plant surfaces as well as to other biomaterial. McAllister et al. (1993) reported that irregularities of polymeric surfaces promote bacterial adhesion, biofilm deposition, and accumulation of biliary sludge, whereas the smooth surface does not allow bacterial adhesion and biofilm deposition. Preferential attachment of *Salmonella* and *E. coli* O157:H7 cells to irregular and rough surfaces over smooth surfaces of tomatoes has been demonstrated (Lukasik et al., 2001)

Efficacy of Sanitizers

Although prevention of contamination is the most efficient way to ensure the safety of fresh fruits and vegetables, this is not always possible due to the intimate association of produce with soil, water, and manure, which may result in the introduction of foodborne pathogens. Several surface decontamination treatments have been employed to reduce populations of microorganisms on produce surfaces. The simple practice of washing raw fruits and vegetables in water removes a portion of the

pathogenic and spoilage microorganisms that may be present (Beuchat, 1998). Studies have shown, however, that the efficacy of such treatments are limited (Beuchat et al, 1998; Sapers et al., 2000). Additional 10- to 100- fold reductions can sometimes be achieved by treatment with disinfectants. It has been proposed (Adams et al., 1989; Beuchat et al., 1998) that limited efficacies of treatments in reducing populations of pathogens is a result of the inaccessibility of bacterial cells lodged within produce tissues at locations where they are afforded protection from physical removal by washing and disinfection by chemical sanitizers. Several types of treatments are known to be partially effective in removing disease-causing organisms from the surface of whole and cut raw fruits and vegetables. None of these treatments, however, can be relied upon to totally disinfect raw produce, at least when administered at levels which will not cause deterioration in sensory quality (Beuchat, 1998; Brackett, 1992).

Chlorine is widely used in the produce industry to extend the shelf life of fruits and vegetables. Pathogens vary in their sensitivity to chlorine. For example, *L. monocytogenes* is generally more resistant than *Salmonella* and *E. coli* O157:H7 (Beuchat, 1998). Although chlorine reduces populations of microbial cells exposed on the surface of produce by up to 2 or 3 $\log_{10}$ units, little is known about its efficacy in killing cells located in protected sites on the epidermis and within tissues. Adams et al. (1989) observed that treatment of salad greens with 100 ppm active chlorine resulted in limited improvement in disinfection. This small improvement was attributed to an increased concentration of chlorine gas formed within hydrophobic structures. The addition of a surfactant, Tween 80, to hypochlorite reduced microbial numbers by 99.6% but caused unacceptable changes in sensory quality. Significant populations of *Salmonella* Montevideo on and in raw tomatoes have been shown to remain after treatment with 320 ppm active chlorine (Zhuang et al., 1995). Cells of *Salmonella* Montevideo were surmised to have infiltrated into tomato tissues where they were protected from the treatment. Beuchat et al., (1998) proposed that the limited efficacy

they observed of 200 ppm active chlorine in reducing populations of *Salmonella, L. monocytogenes,* and *E. coli* O157:H7 on raw apples, tomatoes, and lettuce was a result of the elimination of active chlorine due to contact with organic material, i.e., produce tissue surfaces. Bacterial cells lodged within protective structures were thought to have survived the treatment. Bartz (1988) suggested that a negative temperature differential might be used to deliver active chlorine deeper within tissues of tomatoes than would a positive temperature differential. However, he recommended against the use of a negative temperature differential after an increase risk of contamination and ultimately diseased fruit was correlated with the practice. Several investigations seeking to determine efficacy of chlorine treatment of raw produce in reducing populations of pathogens have suggested similar reasons for ineffectiveness (Sapers et al, 1999; Liao et al., 2000; Annous et al, 2001). Wisniewsky et al. (2000) and Wright et al. (2000b) concluded that *E. coli* O157:H7 cells within lenticels, crevices, and cracks on the surface of apples were likely sheltered from the action of the sanitizers they employed.

Several studies have been reported in which confocal scanning laser microscopy (CSLM) was used to detect viable and dead *E. coli* O157:H7 cells attached to lettuce after treatment with chlorine. Seo and Frank (1999) demonstrated that *E. coli* O157:H7 cells located within substomatal chambers remained viable after treatment with 20 ppm active chlorine. Protection from the sanitizer was also afforded to cells internalized with in the cut lettuce tissue. Similar results were observed by Takeuchi and Frank (2000, 2001).

The use of 5 ppm chlorine dioxide (ClO_2) to wash whole fresh fruits and vegetables is allowed by the U.S. Food and Drug Administration. Its efficacy is less affected than hypochlorite by pH and organic matter (Beuchat, 1998). With regard to the ability of ClO_2 to penetrate into hydrophobic sites on produce surfaces, Reina et al. (1995) observed that although 2.8 ppm ClO_2 was effective in killing planktonic bacteria in cooling water used to treat pickling cucumbers, the disinfectant had little effect on

microorganisms on or in the fruit, suggesting that ClO_2 penetrated protective sites on the cucumber epidermis poorly. Han et al. (2000) compared the efficacy of 0.15 - 1.2 ppm ClO_2 gas in killing *E. coli* O157:H7 cells attached to damaged or intact surfaces of green peppers. Viable cells attached on and in damaged pepper cuticles were observed by CSLM after application of 0.6 ppm ClO_2 gas, whereas cell attached to the intact cuticle were not afforded protection from the sanitizer. Log_{10} cfu/g reductions of 7.3 and 3.0 were observed on injured and uninjured surfaces, respectively, confirming the observations made with CSLM.

Ozone has been applied in the food and bottled water industry in Europe for decades (Graham, 1997) and its use in food processing has recently been approved in the United States. Ozone has been shown to be a more powerful sanitizer than chlorine (Arana et al., 1999), and can be applied to foods without concern of potentially hazardous residual compounds remaining after treatment. However, Spotts and Cervantes (1992) reported that ozonated water did not control decay in wound-inoculated pears, nor did ozonated water effectively reduce fungal infection in inoculated wounds of apples. The authors suggested that ozone reacted with plant tissue and extracellular biochemicals at wound sites and thus failed to inactivate microorganisms attached to or embedded in plant tissue.

The used of hydrogen peroxide (H_2O_2) to disinfect minimally processed fruits and vegetables has been reviewed (Sapers and Simmons, 1998). Its use may be limited to produce which contain endogenous catalase activity to remove residual hydrogen peroxide, as mandated by the U.S. Food and Drug Administration. The antimicrobial behavior of hydrogen peroxide, applied either as a vapor or in solution, within specific hydrophobic locations on the surface of fresh produce has not been described. Sapers et al. (2000) investigated the used of H_2O_2 to reduce population of *E. coli* on the surfaces of apples. The treatment was ineffective in removing or killing *E. coli* cells located

near the stem and calyx end of apples, indicating that microorganisms located in cores of apples were afforded protection from the action of the sanitizer.

Little is known about the survival of pathogens located at protective sites on the surface of produce after the application of wax or oil coatings. However, protection against sanitizers is likely to be enhanced by the additional hydrophobic layer. In the fresh produce industry, waxes such as carnauba and shellac are sprayed onto tumbling fruits to prevent migration of water and to slow respiration rates (Kester and Fennema, 1986). Produce undergoes partial decontamination treatment before waxing. Wash and rinse treatments performed by the consumer may not be sufficient to remove pathogens lodged beneath and within the wax layer.

E. coli *O157:H7 and Unpasteurized Apple Cider*

The first confirmed isolation of *E. coli* O157:H7 in the United States was in 1975 from a California woman with grossly bloody diarrhea (Riley, 1983). The bacterium was first identified as a human pathogen in 1982, when it was associated with two foodborne outbreaks of hemorrhagic colitis (Karmali et al., 1983; Riley et al., 1983). The characteristics of *E. coli* O157:H7 uncommon to most other *E. coli* have been described (Doyle et al, 1997). These include: inability to grow well, if at all, at temperatures of $44.5°C, inability to ferment sorbitol within 24 h, inability to produce $-glucoronidase, possession of an attaching and effacing (*eae*) gene, carriage of a 60-Mda plasmid, and expression of an uncommon 5,000 to 8,000-molecular weight outer membrane protein. *E. coli* O157:H7 has also been demonstrated to be uniquely acid tolerant, surviving in food systems with a pH as low as 3.6 (Doyle et al., 1997).

Symptoms of disease caused by *E. coli* O157:H7 range from mild diarrhea to severe, acute abdominal pain and grossly bloody diarrhea (hemorrhagic colitis), hemolytic uremic syndrome, thrombotic thrombocyopenic purpura, and death (Adachi

et al., 1999). The originally recognized hemorrhagic colitis begins with the sudden onset of severe abdominal cramps and watery, non-bloody diarrhea, followed by the appearance of blood in the stool on the second or third day of the illness (Riley, 1987). Approximately half of patients experience vomiting, and fever occurs in less than one third. The illness lasts about a week, with most patients recovering without obvious sequelae (Parry and Palmer, 2000).

Cattle have been identified as the primary reservoir for *E. coli* O157:H7, although the pathogen has been detected in the feces of a wide range of domestic and wild mammals. Outbreaks of foodborne illness associated with *E. coli* O157:H7 have occurred from consumption of contaminated ground beef, cured and fermented meat products, raw milk, milk products (e.g., cheese, yogurt), apple juice, and raw vegetables and salads (Stinger et al., 2000; McClure and Hall, 2000). Person-to-person contact, environmental exposure, and drinking or swimming in contaminated water are also important sources of infection.

E. coli O157:H7 infections associated in recent years with the consumption of unpasteurized apple juice have raised interest in developing efficacious methods to kill human pathogens that may be present on raw apples and other produce (Steele et al., 1983; CDC, 1996; CDC, 1997; CDC, 1999; Buchanan et al., 1999, Sapers et al, 2000). Unpasteurized apple cider is the product of milled, pressed apples, and is traditionally consumed during harvest in autumn months in apple-growing regions. Since 1974, ten outbreaks of human infections caused by *E. coli* O157:H7, *Salmonella*, or *Cryptosporidium* have been associated with the consumption of fresh apple cider, resulting in 683 documented cases and two deaths (Luetdke and Powell, 2000b).

Researchers conducting an investigation to determine the cause of a 1996 outbreak of *E. coli* O157:H7 infection associated with apple juice concluded that apples delivered to a juice-production plant harbored the pathogen (Cody et al., 1999). Of the three lots suspected to be associated with the outbreak, two were shipped directly from

the same orchard. The juice company involved had issued written statements advising suppliers that it would accept only handpicked apples, but no mechanism was provided to ensure compliance. Furthermore, seasonal workers on the farm, who are paid by the number of bins harvested, were instructed not to harvest apples from the ground, but no system was in place to enforce this policy. Fresh deer feces collected from a wildlife refuge 0.25 miles from the farm was shown to contain *E. coli* O157:H7, although not with the same pulsed-field gel electrophoresis pattern as the isolates from patients and juice involved in the outbreak. However, it is plausible that deer, like cattle, may carry and excrete more than one strain (Cody et al., 1999). Thus, the probable use of drops in the production of juice implicated in this outbreak must be considered a possible way in which contamination occurred.

Recent surveys of cider manufacturing practices in three states (Senkel et al., 2000; Uljas and Ingham, 2000; Wright et al., 2000a) and one Canadian province (Luedtke and Powell, 2000a) indicate that less than half of the producers sanitize apples prior to milling. Chlorine solutions ranging in concentrations of 50 to over 300 ppm active chlorine are used as treatments to reduce numbers of microorganisms on apple surfaces. Research has shown, however, that significant populations of viable *E. coli* O157:H7 cells remain attached to apple tissues after the application of these treatments (Beuchat et al., 1998). In an investigation of the efficacy of a commercial flatbed brush washer in combination with several chemical treatments in reducing *E. coli* from the surface of Golden Delicious apples, Annous et al. (2001) observed that none of the washing treatments tested significantly reduced the population. The authors suggested that this may have been due to the failure of washing agents to reach inaccessible regions of apples near the calyx and stem, where bacteria have been demonstrated to reside (Buchanan et al., 1999). Thus, there is a need to develop methods to microscopically examine attachment sites of pathogenic microorganisms on produce,

and to differentiate viable and dead cells following disinfection treatments, in order to assess more accurately the effectiveness of produce sanitizers.

Methods for Microscopical Examination of Bacterial Cells in Produce Tissues

CSLM is a vital tool for examining plant structures including plant cuticles (Gilroy, 1997; Li et al., 1997; Fernandez, 1999), fruit parenchyma cells (Gray, 1999) and cut tissues (Han et al., 2000; Takeuchi and Frank, 2000, 2001a, 2001b). Because of its ability to markedly reduce out-of-focus light, compared to conventional wide-field microscopy, CSLM provides substantial improvement in resolution along the *z* axis and permits the optical sectioning of specimens. In addition, fully hydrated specimens can be examined, making possible a more accurate determination of viability of microbial cells attached to plant tissues.

Conceptualized in 1953, CSLM has only in recent years become a practical research technique. CSLM detects structures by collecting light from a single focal plane of the sample, excluding light that is out of focus. In CSLM, the microscope lenses focus laser light on one point in the specimen at a time (the focal point). The laser moves rapidly from point to point, resulting in a scanned image. Both fluorescent and reflected light from the sample passes back through the objective. The microscope and the optics of the scanner module focus the light emitted from the focal point to a second point, called the confocal point. The pinhole aperture, located at the confocal point, allows light from the focal point to pass through the detector. Light emitted from outside the focal point is rejected by the aperture (Hepler and Gunning, 1998). The confocal principle is illustrated schematically in Fig. 1.1.

As in conventional epifluorescent microscopes, one lens functions as both a condenser and an objective. The advantage is that the need for exact matching and co-orientation of two lenses is eliminated. A polarized laser beam from an aperture is

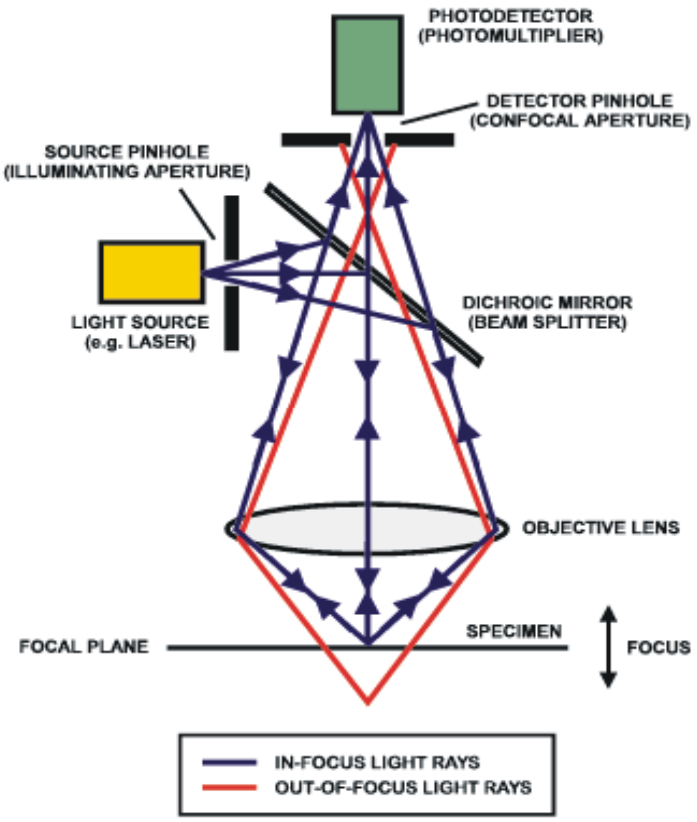

Figure 1.1. *Simplified optics of CSLM. Adapted from L. Ladic (1995)*

reflected by a beam splitter (dichroic mirror) into the rear of the objective lens and is focused on the specimen. The reflected light returning from the specimen passes back through the same lens. The light beam is focused into a small pinhole (i.e., the confocal aperture) to eliminate all the out-of-focus light, i.e., all light coming from regions of the specimen above or below the plane of focus (White et al., 1987). The achieved optical section thickness depends on several parameters such as the variable pinhole diameter and the wavelength. The in-focus information of each specimen point is recorded by a light-sensitive detector positioned behind the confocal aperture, and the analog output is digitized and fed into a computer.

The detector is a point detector and only receives light from one point in the specimen. Thus, the microscope sees only one point of the specimen at a time as

opposed to the conventional microscope where an extended field of the specimen is visible at one moment. To obtain an image, it is necessary to move the illuminated point and to move the specimen. CSLM makes it possible to image a single, in-focus plane as well as a series of planes. The advantage of having a stack of serial optical sections through the specimen in digital form is that either a composite projection image can be computed, or a volume-rendered 3-D representation of the specimen can be generated on a graphics computer.

Green fluorescent protein (GFP) has been demonstrated to be a useful fluorescent tag for studying the localization of bacterial cells in biological systems (Valdivia et al., 1996; Margolin, 2000; Prachaiyo and McLandsborough, 2000; Ling et al., 2000) and has been expressed in several Gram positive and Gram negative bacterial species (Valdivia et al., 1998). Originally derived from *Aeuquorea victoria*, a naturally fluorescent jellyfish, GFP is an 11-stranded $-barrel threaded by an "-helix running up the axis of the cylinder (Fig. 1.2). The chromophore is attached to the "-helix and is buried almost perfectly in the center of the cylinder, which has been called a $-can (Tsien, 1998). The chromophore is a *p*-hydroxybenzylideneimidazolionone, formed from residues 65-67, which are Ser-Tyr-Gly in the native protein (Valdivia et al, 1998). Correct conformational folding of the GFP chromophore requires atmospheric oxygen. The wild-type GFP has a major excitation peak at 395 nm, giving a peak emission at 503 nm. The fluorescent properties of wild-type GFP are stable up to pH 10-11 (Tsien, 1998). Several variants of the wild-type GFP are commercially available, including a red-shifted enhanced GFP (EGFP) containing mutations in the chromophore which shifts the maximal excitation peak to approximately 490 nm, resulting in more efficient detection using an argon laser, compared to wild-type GFP. EGFP offers some other advantages over staining to fluorescently label cells. It requires no additional substrate to fluoresce, facilitating sample preparation while providing ease in detection. Limitations in its use include instability of the EGFP plasmid following bacterial

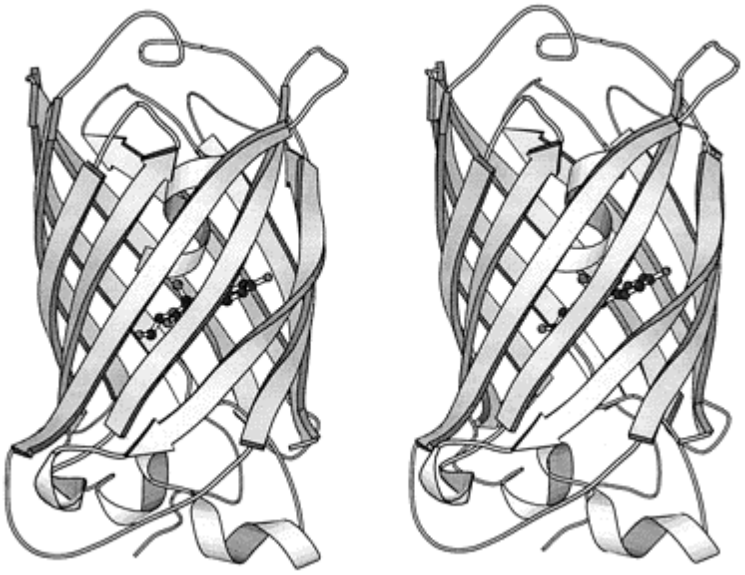

Figure 1.2. *Stereoview of the three-dimensional structure of GFP (30), showing 11 ß-strands forming a hollow cylinder through which is threaded a helix bearing the chromophore, shown in ball-and-stick representation. The drawing was prepared by the program MOLSCRIPT and is intended for viewing with a stereoviewer. Adapted from Tsien (1998).*

growth and possibly denaturation of protein upon exposure to sanitizers, resulting in loss of fluorescence. EGFP has been used to detect *E. coli* O157:H7 cells attached to surfaces of lettuce, tomatoes, and cauliflower (Takeuchi and Frank, 2001b).

Several staining techniques have been employed in studies using CSLM to differentiate viable and dead *Escherichia coli* O157:H7 cells attached to produce. To detect dead cells on lettuce tissues after treatment with 20 ppm active chlorine, Seo and Frank (1999) stained samples with propidium iodide, a membrane impermeant generally excluded from viable cells. The stain binds to nucleic acids within cells of compromised membranes, emitting red fluorescence (617 nm) when excited with green light (535 nm). Propidium iodide has been used in conjunction with purified antibodies to *E. coli* O157:H7 labeled with fluorescein isothiocyanate (Seo and Frank, 1999; Han et al., 2000) and Alexa Fluor 488 (Takeuchi and Frank, 2000), a fluorescent dye

manufactured by Molecular Probes, Eugene, OR, to detect viable and dead cells. SYTOX Orange nucleic acid stain (Molecular Probes) is also a cell impermeant nucleic acid stain with an excitation/emission profile similar to that of propidium iodide, but shown to have higher fluorescence intensity than propidium iodide when bound to DNA (Yan et al., 2000). The use of SYTOX Orange in studies investigating the efficacy of surface treatments of produce with sanitizers has not been reported. Takeuchi and Frank (2001a) stained lettuce samples inoculated with *E. coli* O157:H7 with SYTOX Green nucleic acid stain (Molecular Probes) to detect dead cells and affinity-purified antibodies labeled with Alexa Fluor 594 (Molecular Probes) to detect viable and dead cells. SYTOX Green behaves similarly to SYTOX Orange and propidium iodide, but emits green fluorescence (523 nm) when excited with the argon laser (488 nm) of a confocal scanning laser microscope.

Dissertation Objectives

The objectives of this work are to:

1. Characterize the attachment and infiltration of *E. coli* O157:H7 cells on and in the surfaces and tissues of raw apples.
2. Develop a method to detect and differentiate viable, dead, and total cells of *E. coli* O157:H7 on apples.
3. Microscopically and quantitatively assess the efficacy of treatment of apples with chlorine in killing *E. coli* O157:H7 cells attached to the surfaces and within tissues of apples.

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CHAPTER 2

ATTACHMENT OF *ESCHERICHIA COLI* O157:H7 TO THE SURFACES AND INTERNAL STRUCTURES OF APPLES AS DETECTED BY CONFOCAL SCANNING LASER MICROSCOPY[1]

[1]Burnett, Scott L., Jinru Chen, and Larry R. Beuchat. Submitted to Appl. Environ. Microbiol.

ABSTRACT

Confocal scanning laser microscopy (CSLM) was used to demonstrate the attachment of *Escherichia coli* O157:H7 transformed with a plasmid encoding for green fluorescent protein (GFP) to the surface and within the internal structures of nonwaxed Red Delicious cv. apples. Apples at 2° or 25°C were inoculated with an *E. coli* O157:H7 cell suspension at 2° or 25°C. The effect of a negative temperature differential (cold inoculum, warm apple), a positive differential (warm inoculum, cold apple), and no differential (warm inoculum, warm apple), in combination with a pressure differential (atmospheric vs. 10,130 Pa), on attachment and infiltration of cells was determined. CSLM stereo images of external surfaces of apples subjected to all combinations of test parameters showed preferential cellular attachment to discontinuities in the waxy cuticle on the surface and to damaged tissue surrounding puncture wounds, where the pathogen was observed at depths up to 70 μm below the skin surface. Attachment to lenticels was sporadic, but occasionally observed at depths of up to 40 μm. Infiltration through the floral tube and attachment to seeds, cartilaginous pericarp, and internal trichomes were observed in all apples examined, regardless of temperature differential during inoculation. Pressure differential had no effect on infiltration or attachment of *E. coli* O157:H7. Image analysis to count cells at various depths within tissues was used to quantitatively compare the extent of infiltration into various apple structures as well as the effects of temperature differential. Puncture wounds harbored greater numbers of the pathogen at greater depths than did other sites examined. Attachment or infiltration of cells was greater on the intact skin and in lenticels, russet areas, and the floral tube of apples inoculated under a negative temperature differential compared to those inoculated under no temperature differential. Results suggest that *E. coli* O157:H7 attached to internal core structures or within tissues of apples may evade decontamination treatments.

Interventions designed to deliver disinfectants to these locations or to remove viable cells of *E. coli* O157:H7 and other pathogens from apples by other means need to be developed.

INTRODUCTION

Escherichia coli O157:H7 infections associated in recent years with the consumption of nonpasteurized apple juice have raised interest in developing efficacious methods to kill human pathogens that may be present on raw apples and other produce (8, 9, 10, 18, 23). Among the obstacles in achieving this goal is the probability that pathogens infiltrate tissues within produce, giving them protection against chemical sanitizers, physical methods of removal such as brushing or high-pressure spraying, or other commonly used interventions for cleaning and sanitizing (1, 5, 19, 21).

Infiltration of internal structures and tissues of fruits and vegetables by pathogenic bacteria is thought to occur when produce surfaces come in contact with cells suspended in water. In the field, this may occur when rain, dew, or irrigation water collects on the surface of produce or, in the event that fruit falls from trees, as a result of contact with ground water. After harvest, wash and flume waters used to clean fruits and vegetables may provide a vehicle to facilitate infiltration of microbial cells (2, 3, 27). The potential for infiltration of viable cells is highest if the water is contaminated and antimicrobial agents such as chlorine are ineffective due to low concentration or pH (15).

The problem of bacterial ingress is exacerbated by differences in water and produce temperatures (2, 6). The United States Food and Drug Administration has recommended that packers consider the effects of water temperature when attempting to remove field heat, which is a primary consideration in maintaining the quality of many

types of produce (15). Several researchers have demonstrated that using wash water at a temperature cooler than that of produce (i.e., a negative temperature differential) will result in the absorption of water into tissues (2, 3, 6, 17, 27). This phenomenon is predicted from the general gas law. As the temperature of fruits and vegetables decreases, gases in their tissues exert a reduced pressure, which causes the combined atmospheric and hydrostatic forces on the immersed produce to equilibrate with the internal pressure, thus facilitating ingress of water (2). Bartz and Showalter (3) demonstrated that tomatoes submerged in a suspension of *Serratia marcesens* under a negative temperature differential not only contained the organism more frequently, but also gained more mass than tomatoes exposed to a positive temperature differential. A negative temperature differential enhances uptake of *Salmonella* into the stem scar tissues of tomatoes (27). Buchanan et al. (6) showed that apples immersed in an *E. coli* O157:H7 suspension had high populations of the pathogen in the outer core region, which afforded protection of cells against chlorine treatment. They concluded that the potential for aspirating the pathogen into the internal structures of the fruit was increased by a negative temperature differential.

To date, no research has been published investigating the potential for specific structures of apples such as lenticels, the intact epidermis, and the floral tube to harbor human pathogens. In the study described here, confocal scanning laser microscopy was used to determine and quantify the degree of infiltration and attachment of *E. coli* O157:H7 to specific tissues and locations on the surface and in the internal structures of intact Red Delicious cv. apples as affected by temperature and pressure differentials.

MATERIALS AND METHODS

Apples. Red Delicious cv. apples were harvested by hand at maturity from the orchards at the University of Georgia Mountain Experiment Station (Blairsville, Ga.)

and transported to our laboratory within 8 h. Apples were stored up to 110 days at 2°C until used.

Microorganism. *Escherichia coli* O157:H7 E318, isolated from ground beef, was transformed by Dr. Jinru Chen in Dr. Mansel W. Griffiths' laboratory, University of Guelph, Ontario, Canada using a pGFPuv plasmid (Clontech Labs, Inc., Palo Alto, Calif.). This plasmid encodes for the cycle 3 variant of green fluorescent protein (GFP), which shares a common excitation and emission profile with the wild-type GFP, but has been optimized for maximal excitation with UV wavelengths in the range of 360 – 400 nm. The GFPuv variant can be excited at 488 nm. A stock culture was maintained at -79°C in a water:glycerol (70:30, vol:vol) mixture. The organism was activated monthly and transferred to tryptic soy agar (TSA; Difco; Detroit, Mich.) slants containing 100 µg/ml ampicillin (Sigma; St. Louis, Mo.), incubated for 24 h at 37°C, and stored at 4°C.

Inoculum. For each apple to be inoculated, one Petri plate containing TSA supplemented with 100 µg/ml ampicillin (TSAA) was streaked to give confluent growth and incubated for 24±1 h at 37°C. Previous experiments in our laboratory demonstrated that fluorescence intensity of GFPuv-tagged cells is higher in cultures grown on agar media compared to liquid media as detected by epifluorescent microscopy. Bacterial suspensions were prepared by flooding each TSAA plate with 5 ml of 0.01 M sterile potassium phosphate buffer containing 0.85% saline (PBS, pH 7.2) and disrupting colonies with a sterile bent glass rod. The suspension was removed from the surface of the agar using a pipette and transferred to a sterile 50-ml centrifuge tube. Flooding, suspending cells, and pipetting was repeated twice to produce 15 ml of cell suspension, which was centrifuged at 2,000 x *g* for 15 min. Because two apples were inoculated at each time of analysis, pellets from cells harvested from two plates were combined after washing in 10 ml of PBS to make 20 ml of suspension. Ten milliliters of this suspension was added to 90 ml of Minimal Salts Medium (MSM) supplemented with

0.04% glucose (13) and 100 µg/ml ampicillin in a sterile polyethylene bag. MSM, which consisted of K_2HPO_4 (7.0 g/L), KH_2PO_4 (3.0 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.1 g/L), and yeast extract (1 mg/L), was supplemented with 0.04% glucose and autoclaved prior to use. Solutions of 1 mg/ml were filter sterilized (0.2-µm pore size) and added to MSM supplemented with 0.04% glucose appropriately to achieve a concentration of 100 µg/ml ampicillin. After vigorous mixing, bacterial cell suspensions were analyzed spectrophotometrically for absorbency at 590 nm and diluted with MSM as necessary to achieve the desired value of 0.88. The inoculum was then analyzed for population of *E. coli* O157:H7 by serially diluting in PBS and surface plating (0.1 ml in duplicate) on TSA and TSAA. Plates were incubated for 24 h at 37°C and presumptive *E. coli* O157:H7 colonies were counted. *E. coli* O157:H7 was confirmed by visualizing fluorescent colonies under a long-wave ultraviolet light source. Percent expression of the GFPuv plasmid was calculated by comparing counts obtained on the two media.

Inoculation of apples. Red Delicious apples selected for inoculation were of similar size and free of obvious bruises, cuts, wounds, or other assaults. Apple cortex pH was measured with a flat surface pH electrode (Fisher Scientific, Pittsburgh, Penn.) and pH meter (Denver Instruments, Arvado, Colo.). Immediately before inoculating, apple surfaces were punctured (1 cm deep x 1 mm wide) at five locations between the apex and the base with a sterile blunt nail to represent mechanical injury that may occur during handling. Sets of two apples per analysis were exposed to one of three temperature differentials during inoculation. For apples subjected to a negative temperature differential, two polyethylene bags (18 cm x 31 cm), each containing 100 ml of bacterial suspension were placed in an ice bath (2°C) for 15 min. To each bag, an apple tempered at 25°C was added. One apple from the set was subjected to a vacuum by placing one polyethylene bag in a commercial vacuum packager (Koch Model CE-95, Kansas City, Mo.) and removing 90% of the gaseous phase in the unit cavity. This

resulted in a pressure of 10,130 Pa within the bag. The other apple was maintained at atmospheric pressure. Apples subjected to both treatments were maintained in the ice bath for 30 min. For apples subjected to a positive temperature differential, 100 ml of the MSM cell suspension was tempered at 25°C before using to inoculate apples. Two apples at 2°C were separately placed in polyethylene bags containing the bacterial suspension at 25°C. Both bags (one under vacuum and one at atmospheric pressure) were maintained at 21°C for 30 min. For apples not subjected to a temperature differential, two apples tempered at 25°C were separately added to 100 ml of the MSM cell suspension at 25°C, and bags were maintained under vacuum or atmospheric pressure at 21°C for 30 min. Following the 30-min incubation period, vacuum within bags containing the apples exposed to a pressure differential was released by cutting the bag with a scissors. All apples were then incubated for 18 h at 25°C before prepared for examination using CSLM. All experiments were repeated three times.

Sample preparation. Bacterial attachment to the intact skin, russet area surrounding the stem, lenticels, and puncture wounds of apples was examined. Apples were removed from inoculum with sterile metal tongs and placed in a sterile plastic basket under a laminar flow hood (class II, type A/B3) for 30 min to dry. Apples were then placed on a sanitized cutting board. Ten 1 cm x 1 cm sections (5 containing puncture wounds) were removed from the surface of each apple with a sterile stainless steel scalpel and rinsed by placing in 10 ml of filtered (0.45 μm), autoclaved, distilled water by gentle agitation (90 rpm) on a rotary shaker for 2 min. Surface sections were then removed with a sterile forceps, placed in a sterile Petri plate under the laminar flow hood, and dried for 10 min.

Bacterial attachment to four internal structures, i.e., the floral tube, ventral cavity, seed locule, and seed integument, was examined (Fig. 2.1). Samples were prepared by removing the core of apples with a sterile stainless steel corer and slicing

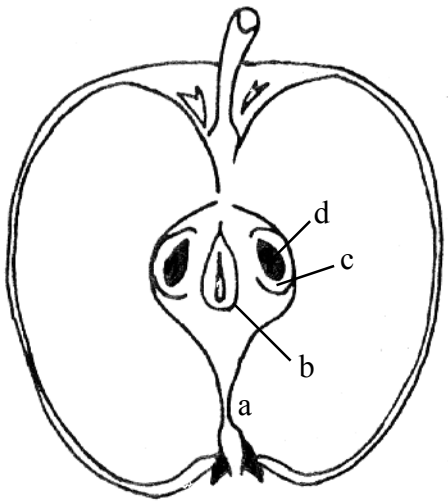

Figure 2.1. Longitudinal cross section of a Red Delicious apple showing the floral tube (a), ventral cavity (b), seed locules (c), and seeds (d).

the core cylinder transversely above and below seed locules with a sterile knife. The middle section containing the seeds was then cut longitudinally and sections of cartilaginous pericarp of locules and the ventral core cavity and seeds were removed using a sterile stainless steel blade. Internal structures were rinsed and allowed to dry for 10 min as described above.

Mounts for microscopic analysis were prepared by placing 0.3 ml of silica gel (Dow Corning, Midland, Mich.) on clean glass microscope slides. Inoculated apple sections were placed on silica gel followed by a drop of filtered (0.45 μm) glycerol. A coverslip was then placed on the apple specimen with gentle downward pressure to facilitate adherence to the silica gel.

Visualization with CSLM. Samples were analyzed using a BioRad MRC-600 confocal scanning laser microscope (BioRad, Inc., Hemel Hempstead, UK) equipped with a 50-W Argon/Krypton laser. The scanning head was mounted on a Nikon Optiphot microscope (Nikon, Tokyo, Japan) fitted with a 40X (numerical aperture = 1.30) oil immersion objective (Nikon, Tokyo, Japan). The system was operated by the Confocal Microscope Operating Software (COMOS) Vers. 7.1 supplied by BioRad. Green fluorescence of GFPuv-labeled *E. coli* O157:H7 cells was detected using an excitation wavelength of 488 nm. Emitted light was collected through a 480-nm dichroic mirror, a 520-nm long-pass filter, and a 680-nm short pass filter. Samples from random locations on each apple were examined for bacterial attachment to the eight external and internal areas and structures described above. Selected CSLM optical thin sections were stacked using COMOS to construct stereo projections which were formatted using CorelDRAW 8.0. Readers may attain three-dimensional views of these images by positioning a stereo viewer in front of the micrographs so that the left and right lenses are aligned above the left and right images, respectively. Alternatively, a three-dimensional view can be achieved without a viewer by separately observing the left and right figure with the left and right eyes, respectively.

Image analysis. Digital image analysis was performed using Scion Image, a software package based on NIH image for Macintosh created by the Scion Corporation (Fredrick, Md.). The number of cells at various depths within tissues was determined to quantitatively compare attachment to the eight structures examined as well as the effect of a negative or positive temperature differential during inoculation. Since qualitative observations revealed no influence on infiltration or attachment due to a pressure differential during inoculation of apples, quantitative analysis to determine numbers and position of cells on and in structures was not done using these apples. Representative CSLM stacks of samples prepared from apples that had not been subjected to a pressure differential were selected for quantitative examination by visualization of three-dimensional reconstructions. Cells were counted in optical slices positioned more than 3 μm apart to avoid counting the same cell more than once. Image thresholding and particle analysis were calibrated to program the software to count pixel clusters of an appropriate intensity and size. Each optical slice measured 326 x 218 μm with a depth of resolution along the z-axis of 3 μm, giving a volume of each region examined equal to approximately 2.13×10^5 μm^3. Data are expressed as number of cells per region examined, which are plotted on the y axis against depth (μm) into tissues on the x axis.

RESULTS

Attachment and infiltration of *E. coli* O157:H7 to external surface structures. Populations of *E. coli* O157:H7 in the MSM used to inoculate apples ranged from 8.23 – 8.31 $\log_{10}$ CFU/ml throughout this study. Attachment of *E. coli* O157:H7 cells to intact apple skin occurred primarily at discontinuities in the waxy cuticle, including clefts and against crests located between epidermal cells (Fig. 2.2A). The depth of clefts ranged from 10 to 16 μm below the surrounding cuticle and cells

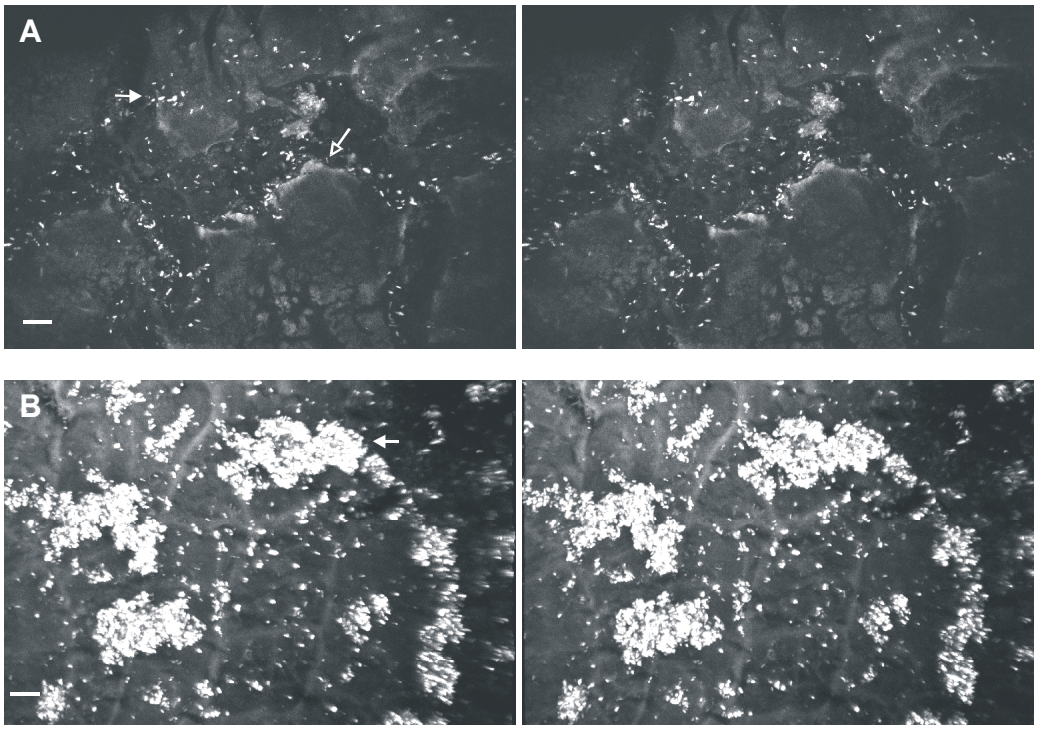

Figure. 2.2. CSLM stereo images showing attachment of *Escherichia coli* O157:H7 on intact apple surface. (A) Cleft (16-µm depth) in the waxy cuticle (open arrow); most cells are attached within the cleft (closed arrow). (B) Clusters of cells (arrow) on intact cuticle 34 µm in height. Cells were inoculated under a negative temperature differential. Bar = 10 µm.

were attached at various depths. In general, single cells attached to the waxy cuticle. However, clumping was observed on the apple shown in Fig. 2.2B that was inoculated under a negative temperature differential. Although qualitative differences in number of *E. coli* O157:H7 cells attached to or infiltrating external structures were difficult to discern, image analysis (Fig. 2.3) illustrates that a higher number of cells infiltrated intact skin, russet areas, and lenticels of apples inoculated under a negative temperature differential compared to the same structures of apples inoculated under a positive temperature differential.

Apple lenticels and russet areas, composed of loosely packed wax platelets, typically did not attract a high number of attached *E. coli* O157:H7 cells. However, cuticular cracks and narrow crevices radiating from these structures were heavily colonized (Fig. 2.4A). Colonization of lenticels was sporadic. For example, on a given apple, most lenticels contained a few cells just inside their openings, while an occasional lenticel would be heavily colonized with both attached and unattached cells (Fig. 2.4B). Cells in colonized lenticels were detected at depths of 40 – 50 μm below the surface with little overall influence of temperature (Fig. 2.3) or pressure differentials. Raised russet areas provided for moderate attachment; in general, cells were attached to russet walls and to waxy clefts within groves (Fig. 2.4C). Image analysis of russet areas inoculated under negative and positive temperature differentials indicate that representative samples do not vary significantly in cell numbers at depths greater than ca. 5 μm (Fig. 2.3).*E. coli* O157:H7 cells attached preferentially to damaged tissues surrounding puncture wounds in the skin. The pathogen heavily colonized these sites in samples subjected to all treatments and was detected at depths up to 70 μm below the tissue surface. Deep, narrow crevices radiating from torn skin tissues, in particular, held dense biofilm-like matrices of cells (Fig. 2.5A,B). Infiltration of tissues within puncture wounds was influenced by temperature differential. Fig. 2.3

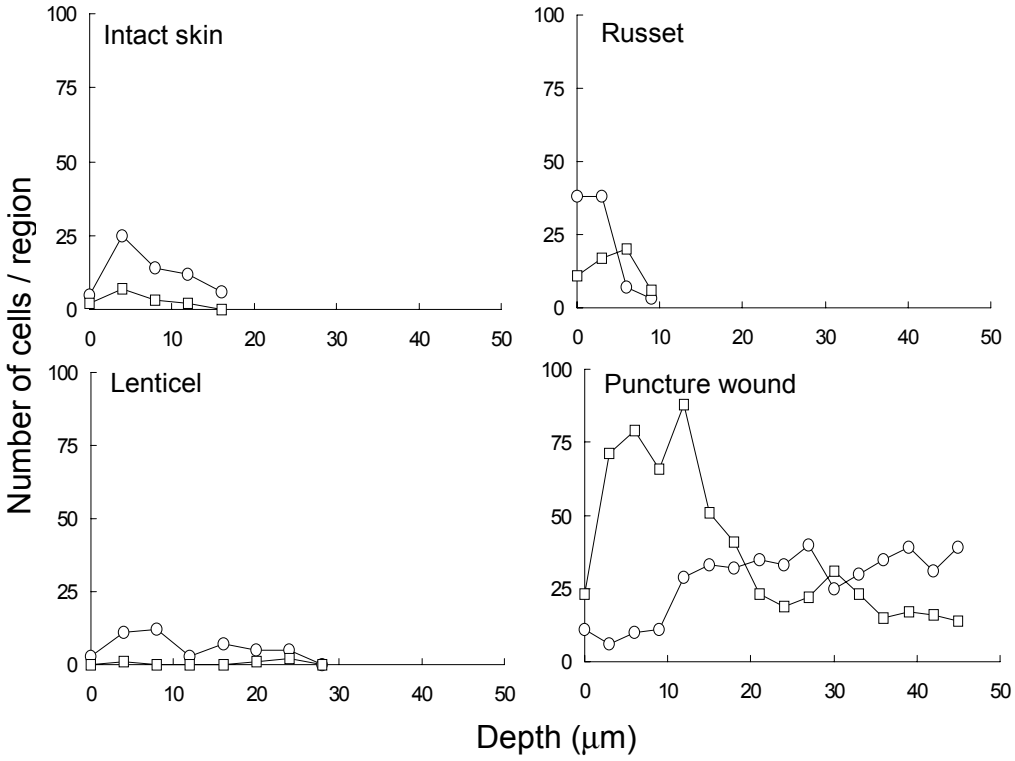

Figure 2.3. Infiltration of fluorescent *Escherichia coli* O157:H7 into external surface structures of apples as affected by negative (circles) or positive (squares) temperature differentials. The number of cells at various depths below the surface was determined by image thresholding and particle analysis in selected regions (213,000 μm^3) of CSLM stacks.

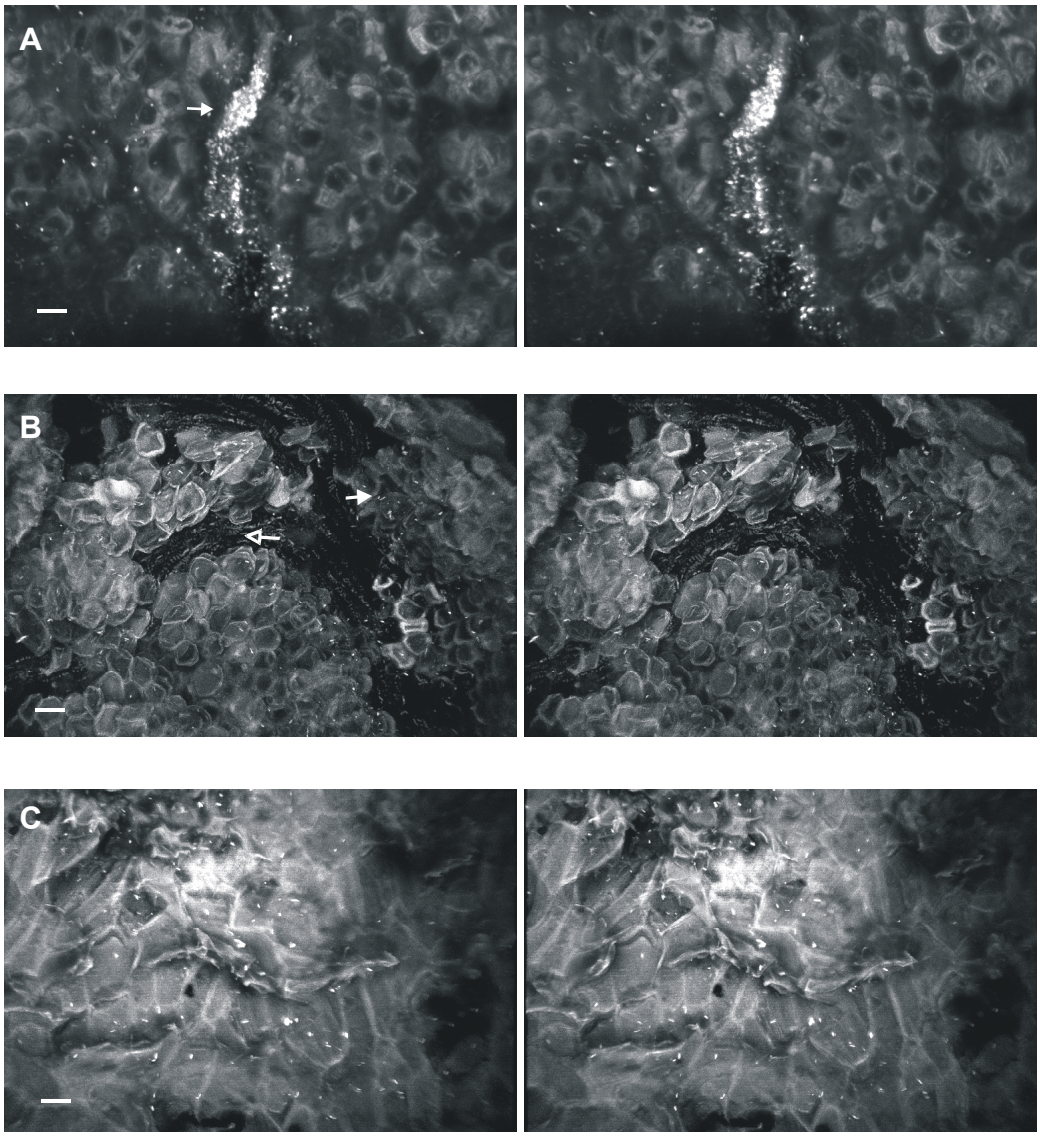

Figure 2.4. CSLM stereo images showing attachment of *Escherichia coli* O157:H7 to apple lenticels and russet areas. (A) Narrow fissure (42-µm depth) radiating from a lenticel heavily colonized with cells (arrow); (B) lenticel harboring attached (closed arrow) and unattached (open arrow) cells; (C) unattached and attached cells on wax platelets of russet. Bar = 10 µm.

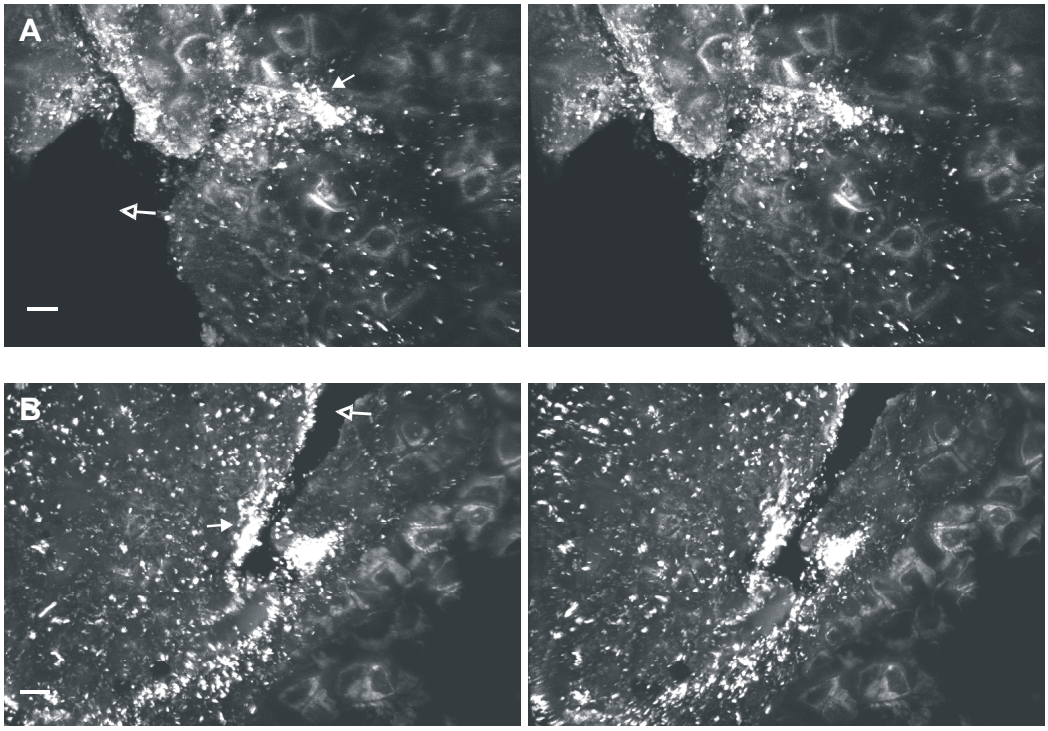

Figure 2.5. CSLM stereo images showing attachment of *Escherichia coli* O157:H7 to tissue surrounding skin puncture wounds (open arrows). Heavily colonization (closed arrows) of damaged tissue is shown to depths of a) 48 µm and b) 70 µm below the surface. Bar = 10 µm.

illustrates the consistent presence of cells at depths up to 45 µm within the tissue of representative samples inoculated under a negative and positive temperature differential. Higher numbers of cells at depths less than 18 µm in tissues of apples inoculated under positive pressure were observed, while cell numbers in tissues from apples inoculated under a negative pressure differential increased at 18 µm and remained higher to depths of up to 45 µm.

Attachment and infiltration of *E. coli* O157:H7 to internal structures. Infiltration of *E. coli* O157:H7 into the core of intact apples was observed in all samples, regardless of treatment. The floral tube of mature Red Delicious cv. apples remains open from the blossom to the cartilaginous percarp of the ventral core cavity. The wall of the floral tube, composed of waxy cuticle similar to that on the skin, did not harbor high numbers of attached cells. However, the pathogen attached readily to the apple flower remnants and internal trichomes just within the floral tube (Fig. 2.6A). Internal trichomes formed dense mats, commonly entrapping cells within. Yeast cells were often observed within the floral tube, with no relation perceived between their presence and the attachment of *E. coli* O157:H7. Image analysis of samples representing floral tube tissues from apples inoculated under negative or positive temperature differentials revealed higher numbers of cells at depths up to 20 µm in samples inoculated under a negative temperature differential (Fig. 2.7).

After passing through the floral tube, cells enter into the apple core, which consists of a ventral cavity and seed locules comprised of cartilaginous pericarp and seeds. The ventral core cavity of each inoculated apple contained fluid, indicating that several milliliters of the inoculum were imbibed. CSLM examination of the ventral cavity pericarp, locule pericarp, and seed integument revealed that dispersal and attachment of *E. coli* O157:H7 occurred throughout apple core. Moreover, discoloration of the cortex surrounding the core suggests that some degree of infiltration occurred into the intercellular air spaces of parenchyma cells that make up the cortex.

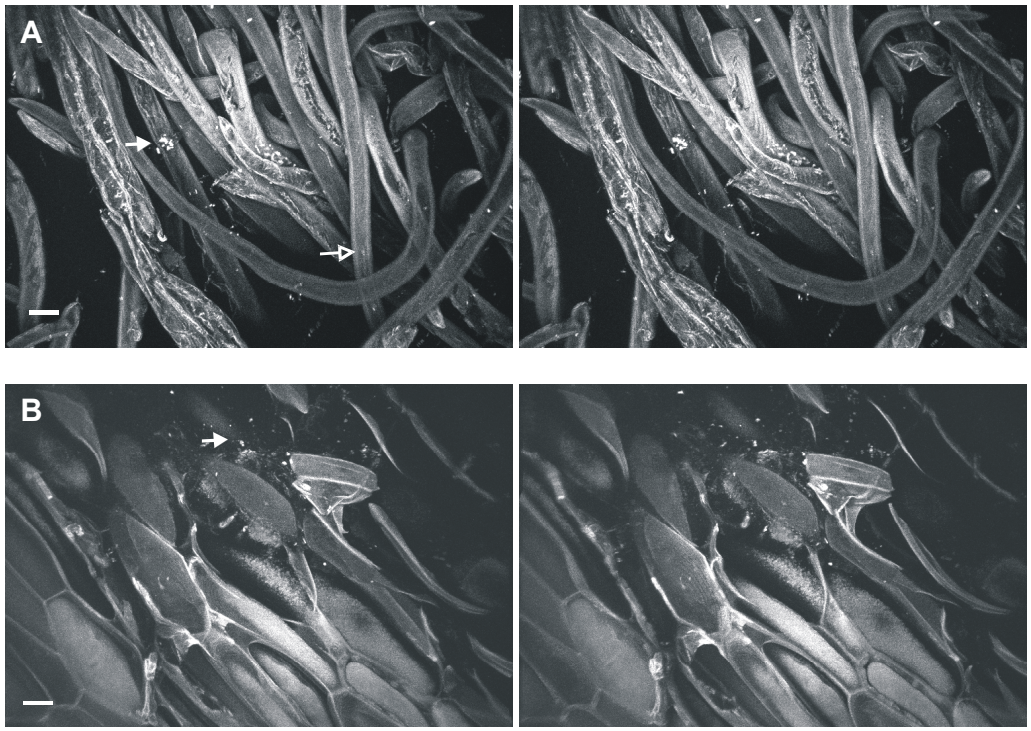

Figure 2.6. CSLM stereo images showing attachment of *Escherichia coli* O157:H7 to (A) internal trichomes (open arrow) of the floral tube at a depth of 52 µm where cells (closed arrow) are attached to or entrapped within the trichome network; (B) seed integument (18-µm depth), which harbored few attached cells (closed arrow). Bar = 10 µm.

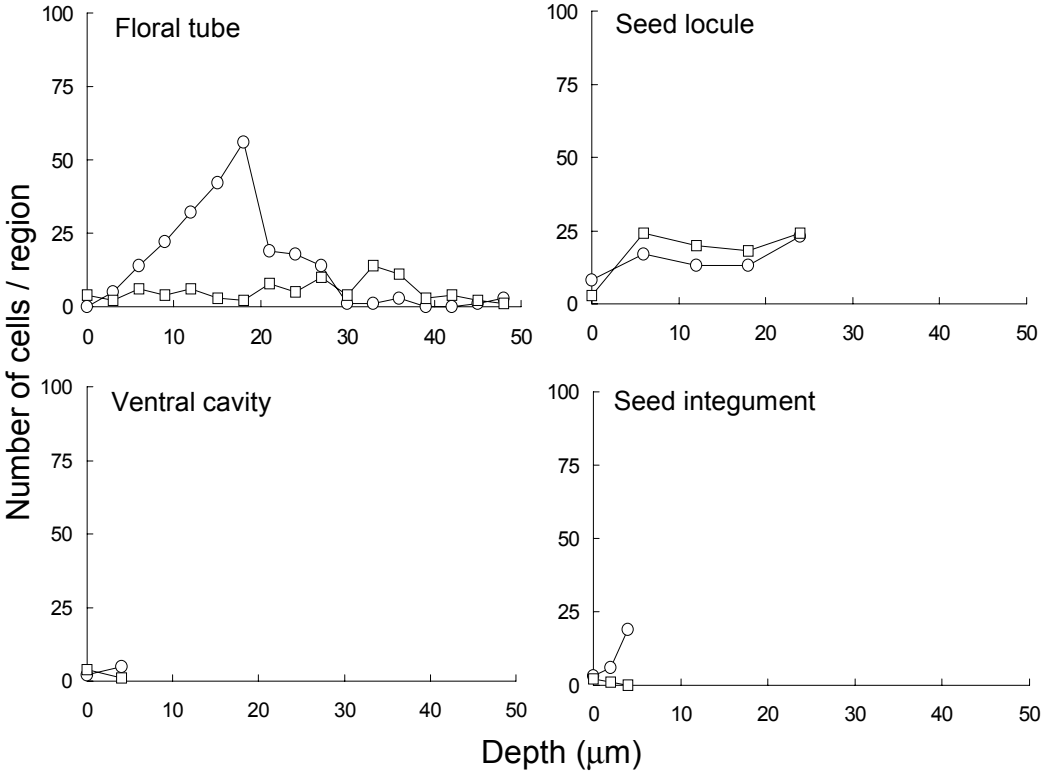

Figure 2.7. Infiltration of fluorescent *Escherichia coli* O157:H7 into the internal structure of apples as affected by negative (circles) or positive (squares) temperature differentials. The number of cells at various depths below the surface was determined by image thesholding and particle analysis in selected regions (213,000 μm^3) of CSLM stacks.

CSLM visualization of this tissue, however, is limited due to the extreme autofluorescent characteristics of the parenchyma cells. The cartilaginous pericarp of the ventral cavity and seed locules of mature apples consists of an irregular waxy tissue occasionally containing trichomes and deep crevices and ridges. Infiltration of cells into smooth regions of the cartilaginous pericarp of the ventral cavity and seed locules was minimal (Fig. 2.8A), whereas cells heavily colonized trichomes, crevices, and ridges (Figs. 2.8A,B) associated with these structures. Image analysis of representative ventral cavity and seed locule samples revealed no influence of temperature differential during inoculation on penetration of these tissues by *E. coli* O157:H7 cells (Fig. 2.7). However, a peak in cell numbers at a depth of 5 μm was observed in the seed integument sample of apples inoculated under a negative temperature differential compared with no penetration of cells into the integument of a seed from apples inoculated under a positive temperature differential.

DISCUSSION

Researchers conducting an investigation to determine the cause of a 1996 outbreak of *E. coli* O157:H7 infection associated with apple juice concluded that apples delivered to a juice-production plant harbored the pathogen (12). Of the three lots suspected to be associated with the outbreak, two were shipped directly from the same orchard. The juice company involved had issued written statements advising suppliers that it would accept only handpicked apples, but no mechanism was provided to ensure compliance. Furthermore, seasonal workers on the farm, who are paid by the number of bins harvested, were instructed not to harvest apples from the ground, but no system was in place to enforce this policy. Fresh deer feces collected from a wildlife refuge 400 m from the farm was shown to contain *E. coli* O157:H7, although not with the same pulsed-field gel electrophoresis pattern as the isolates from patients and juice involved

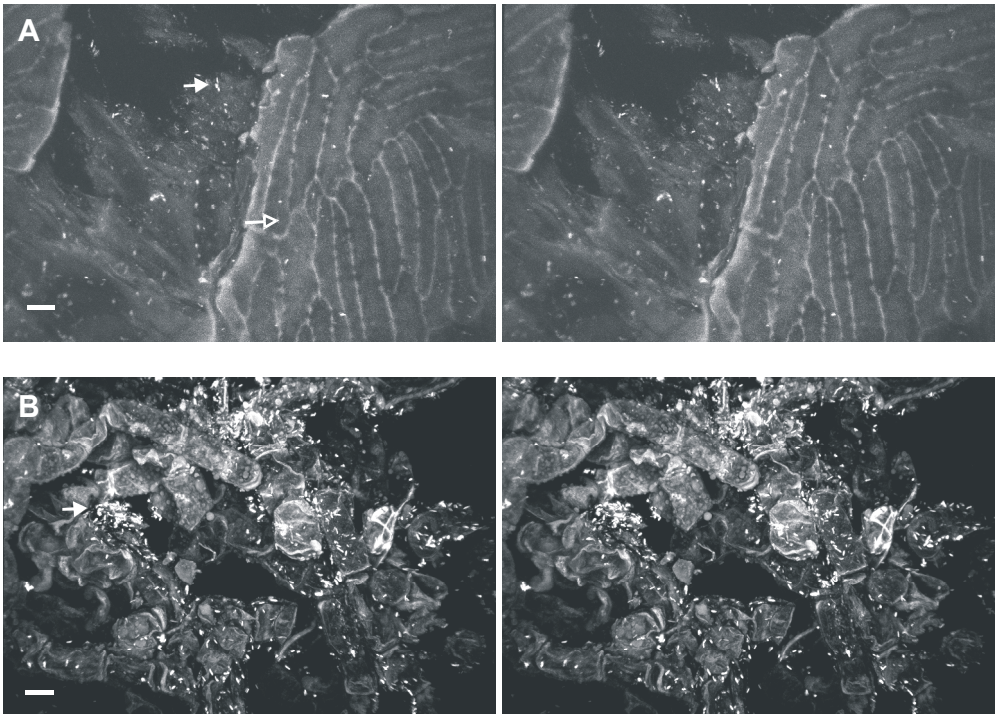

Figure 2.8. CSLM stereo images showing attachment of *Escherichia coli* O157:H7 to the ventral cavity. (A) More attached cells (closed arrow) were observed in crevices (38-µm depth) than on the smooth regions of cartilaginous pericarp (open arrow); (B) irregular tissue (42-µm depth) on the ventral cavity harboring many cells (closed arrow). Bar = 10 µm.

in the outbreak. However, it is plausible that deer, like cattle (12), may carry and excrete more than one strain. Thus, the probable use of drops in the production of juice implicated in this outbreak must be considered a possible way in which contamination occurred.

Contamination of apples may also occur at several points during pre- and post-harvest handling and processing of apple juice and cider. Dingman (14) found no correlation between the frequency of isolation of *E. coli* and the use of drops in the production of nonpasteurized apple juice after analyzing samples from 11 cider mills in Connecticut. Fruit flies (16), dust, harvesting equipment, and irrigation water (4), however, have been noted as serving as sources of pathogenic microorganisms on produce.

Our study has shown that *E. coli* O157:H7 can pervade the inner core of sound Red Delicious apples, dispersing and attaching to the cartilaginous pericarp of the ventral cavity and seed locules, and to seed integument. Infiltration occurs through the blossom end of the calyx and progresses up the floral tube into the core region. The internal trichomes within the floral tube entrapped the pathogen, which may contribute to observations made by other researchers (6) that higher numbers of *E. coli* O157:H7 inoculated onto intact apples were recovered from the outer core regions. Our study supports the evidence presented by others that the calyx end of the apple is an area of great concern with regard to infiltration of bacteria and the resulting inaccessibility to contact with sanitizers. High numbers of attached cells were observed not only in this region, but also throughout the core structures indicating that the pathogen may evade mechanical scrubbing, treatment with chemicals, and other interventions applied to reduce or eliminate pathogens on apples. Suffusion of the liquid cell suspension within the floral tube, ventral cavity, and seed locules further decreases the effectiveness of surface treatment solutions because of their dilution in and inability to replace the moisture present on the surface of these structures. Several researchers have noted that

contaminated flume and wash waters are likely vehicles of bacteria for ingress within the tissues of apples (5, 6, 15, 17). However, infiltration may be just as likely to occur on the field or in transport, as irrigation and rain water may serve as navigation systems to facilitate contact of pathogens with fruit. If in contact with the apple for sufficient time, the contaminated water may progress up the floral tube or into compromised areas on the surface by capillary action. Thus, contaminated apples entering into the processing facility would not be effectively sanitized in wash or flume waters.

E. coli O157:H7 cells attached preferentially to damaged tissues surrounding puncture wounds in the apple skin. The dense colonization of puncture wounds consistently observed throughout this study supports research by Janisiewicz et al. (16) who found that *E. coli* O157:H7 grew exponentially in Golden Delicious apple tissue when inoculated onto puncture wounds. Sapers et al. (20) observed higher counts of non-pathogenic *E. coli* associated with inoculated apple halves compared with intact apples. These workers surmised that more cells were associated with damaged surfaces than intact surfaces, which is in agreement with observations made in our study. Biofilm-like matrices (Figs. 2.2A, 2.5A, and 2.5B) were visualized in narrow fissures emanating from torn skin of wounds. While the acidic (pH 4.1) environment in the wound tissue may impose a stress on *E. coli* O157:H7, the incubation time (18 h) and temperature (25°C) were such to enable the growth of the pathogen in compromised tissue. Thus, the formation of a biofilm may have occurred. Biofilm formation on fresh produce has been observed with confocal microscopy (7) and has ramifications in terms of surface disinfection treatments applied to fruits and vegetables because of diffusion or penetration of active disinfectants into biofilm matrices (24). However, without the use of an appropriate fluorochrome to stain bacterial exopolysaccharide, which is produced during biofilm formation, we cannot conclude that the matrices visualized in this study were indeed a biofilm.

Although attachment to the waxy cuticle layer on the surface of intact skin occurred, fewer cells were observed compared to those in puncture wounds. Other workers (22, 25) have noted similar behavior of *E. coli* O157:H7 on lettuce leaves, where the pathogen attached preferentially to cut edges of leaves rather than to intact surfaces. In our study, MSM with 0.04% glucose was used as the suspension medium for inoculation, which may represent conditions approximating contaminated irrigation or surface water. Cells of *E. coli* O157:H7 cells grown in this medium were more hydrophobic than cells grown in tryptic soy broth (13), probably due to changes in bacterial membrane fatty acid composition that are brought about by the nutrient-limited environment. Thus, attachment to hydrophobic structures such as the waxy cuticle, lenticels, and russet areas would be maximized. Lenticels, however, only sporadically harbored *E. coli* O157:H7 cells. The development of lenticels on mature apples is a result of the formation of cutin within broken trichomes or stomata, whose guard cells remain permanently opened due to stretching which accompanies growth. In the young fruit, stomata serve as a vehicle for gas exchange. As lenticels form and loosely packed wax platelets develop, the structure usually closes and remains impermeable to the passage of liquids and gases even under pressure (11). Approximately 5% of lenticels on the Red Delicious cultivar, however, remain open (11), which is likely to account for the infrequent and sporadic observation of cells within lenticels. The bacterial suspension suffused into the open lenticel, whereas closed lenticels remained largely void of cells as a result of the pressure differential created.

Image analysis of CSLM stacks made possible the quantification of infiltration and attachment of *E. coli* O157:H7 to specific structures along the z axis of samples as influenced by temperature differential. A marked effect of negative or positive temperature differential was not readily discernible, due in part to a slow diffusion of the bacterial suspension within tissues over the 18 h incubation period following

inoculation. Inoculation under a negative temperature differential enhanced infiltration of the intact skin, lenticels, russet area, floral tube, and seed integument. The effect of temperature differential on infiltration of cells into the ventral cavity was inconclusive. However, a positive temperature differential appeared to enhance infiltration into the seed locule and clearly resulted in greater infiltration at depths up to 18 μm into the tissue surrounding puncture wounds. At depths of 20 – 45 μm, inoculation under a negative temperature differential enabled higher numbers of *E. coli* O157:H7 cells to infiltrate wound tissue compared to inoculation under a positive temperature differential. At the end of the 18-h incubation period at 25°C following inoculation, apples were removed from the inoculum and placed under the laminar flow hood at 21°C to dry for 30 min. Cooling of superficial air spaces in porous puncture wound tissue of the fruit during the drying process could have resulted in cell ingress, which may account for the high number of cells present within a depth of 2 – 18 μm below the surface of wound tissue of apples inoculated under positive pressure. We hypothesize that cells of *E. coli* O157:H7 on surfaces of apples inoculated under a positive temperature differential were less intimately associated with the surface than were cells inoculated using a negative temperature differential. It is likely that the drying step involved in the sampling process created a slight negative temperature differential, resulting in some degree of infiltration.

Infiltration of the pathogen into and throughout the core was observed in all apples analyzed, regardless of treatment. This observation may be, in part, a result of capillary action, which would draw the inoculum into the core area over the 18-h incubation period. Several researchers working with produce have demonstrated the influence of temperature differentials with the uptake of dye solutions (6, 17) and bacterial suspensions (2, 6). When exposed to a dye solution for 30 min under a negative temperature differential, 18 out of 113 (15.9%) nonwaxed Golden Delicious apples imbibed the dye within core structures, whereas no apples exposed to a positive

temperature differential were impregnated with the dye (6). Similar results showing dye uptake in oranges and grapefruits have been recorded (17, 26). Thus, we surmise that apples inoculated under a negative temperature differential experienced greater infiltration of *E. coli* O157:H7 cells into the core and surface structures within the first 30 min after exposure, compared to apples inoculated under a positive temperature differential, while subsequent incubation for 18 h at 25°C under no or a positive temperature differential allowed cell ingress of apples into puncture wound and core tissues. Such phenomena would not be unlikely to occur in the field, during transport, or in the packing house.

This study clearly demonstrates that *E. coli* O157:H7 can infiltrate the core and subsurface structures on the skin of apples, which may reduce the efficacy of chemical sanitizers, physical treatments such as brushing, and other methods applied to remove, reduce, or eliminate pathogenic microorganisms that may be present. Good agricultural and manufacturing practices are important to reduce the risk of contamination of apples by pathogens. Interventions designed to deliver disinfectants to locations within apple tissues or to remove viable *E. coli* O157:H7 and other pathogens from these tissues need to be developed.

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CHAPTER 3

COMPARISON OF METHODS FOR FLUORESCENT DETECTION OF VIABLE, DEAD, AND TOTAL *ESCHERICHIA COLI* O157:H7 CELLS IN SUSPENSION AND ON APPLES USING CONFOCAL SCANNING LASER MICROSCOPY[1]

[1]Burnett, Scott L., and Larry R. Beuchat. Submitted to Int. J. Food Microbiol.

ABSTRACT

The influence of treating *Escherichia coli* O157:H7 cells labeled with an enhanced green fluorescent protein (EGFP) plasmid with 20 µg/ml active chlorine, 10% hydrogen peroxide, and 8% acetic acid on fluorescence intensity was determined. In addition, fluorescent staining methods to differentiate viable and dead *E. coli* O157:H7 cells on the cuticle of Red Delicious cv. apples following treatment with water or 200 µg/ml active chlorine were evaluated. Suspensions of *E. coli* O157:H7 $EGFP^+$ cells were exposed to chemical treatment solutions for 0, 30, 60, 120, or 300 sec before populations (log_{10} cfu/ml) were determined by surface plating, and fluorescence intensities of suspensions and individual cells were measured using spectrofluorometry and confocal scanning laser microscopy (CSLM), respectively. The relative fluorescence intensity of suspensions and individual cells changed upon exposure to various treatments. Results indicate that the use of EGFP to tag *E. coli* O157:H7 may not be appropriate for investigations seeking to microscopically differentiate viable and dead cells on produce following surface treatment with sanitizers. SYTOX Orange and SYTOX Green nucleic acid stains fluorescently labeled dead *E. coli* O157:H7 cells attached to apple cuticles more intensely than did propidium iodide. A cross-signal occurred between CSLM photomultipliers when examining tissues treated with SYTOX Orange to detect dead cells and antibody labeled with Alexa Fluor 488 to detect total (dead and viable) cells. Because of the possibility of cross-signal resulting in an over-estimation of the number of dead cells on apples and, perhaps, other produce treated with these stains, SYTOX Green is preferred to detect dead cells and antibody labeled with Alexa Fluor 594 is preferred to detect the total number of cells on apple surfaces following treatment with santizers. The performance of SYTOX Green in combination with Alexa Fluor 594 to detect dead and total cells of *E. coli* O157:H7 on other produce remains to be determined.

INTRODUCTION

Fruits and vegetables have been implicated as vehicles of human pathogens with increasing frequency over the past two decades (NACMCF, 1999). In determining research needs related to controlling the introduction and transmission of pathogens associated with fresh produce, the National Advisory Committee on the Microbiological Criteria for Foods has recognized an insufficiency in information concerning efficacy of sanitizers in eliminating or reducing pathogen populations. Investigations have shown that the addition of sanitizers to water for treating fresh produce often results in only 10- to 100-fold additional reductions in microorganisms compared with water alone (Beuchat, 1998), indicating that pathogens may be located within tissues where they are afforded protection against physical removal by wash treatments and contact with sanitizers. Thus, there is a need to develop methods to microscopically examine attachment sites of pathogenic microorganisms on produce, and to differentiate viable and dead cells following disinfection treatments, in order to more accurately assess the effectiveness of produce sanitizers.

Confocal scanning laser microscopy (CSLM) is a vital tool for examining plant structures including plant cuticles (Fernandez, 1999) and fruit parenchyma cells (Gray, 1999). Because of its ability to markedly reduce out-of-focus light, compared to conventional wide-field microscopy, CSLM provides substantial improvement in resolution along the *z* axis and permits the optical sectioning of specimens (Hepler, 1998). In addition, fully hydrated specimens can be examined, making possible a more accurate determination of viability of microbial cells attached to plant tissues.

Several staining techniques have been used in CSLM studies to differentiate viable and non-viable *Escherichia coli* O157:H7 cells attached to produce. To detect dead cells on lettuce tissues after treatment with 20 µg/ml active chlorine, Seo and Frank (1999) stained samples with propidium iodide, a membrane impermeant

generally excluded from viable cells. The stain binds to nucleic acids within cells of compromised membranes, emitting red fluorescence (617 nm) when excited with green light (535 nm). Propidium iodide has been used in conjunction with purified antibodies to *E. coli* O157:H7 labeled with fluorescein isothiocyanate (Seo and Frank, 1999; Han et al., 2000) and Alexa Fluor 488 (Takeuchi and Frank, 2000), a fluorescent dye manufactured by Molecular Probes, Eugene, OR, to detect viable and dead cells. SYTOX Orange nucleic acid stain (Molecular Probes) is also a cell impermeant nucleic acid stain with an excitation/emission profile similar to that of propidium iodide, but shown to have higher fluorescence intensity than propidium iodide when bound to DNA (Yan et al., 2000). The use of SYTOX Orange in studies investigating the efficacy of surface treatments of produce with sanitizers has not been reported. Takeuchi and Frank (2001a) stained lettuce samples inoculated with *E. coli* O157:H7 with SYTOX Green nucleic acid stain (Molecular Probes) to detect dead cells and affinity-purified antibodies labeled with Alexa Fluor 594 (Molecular Probes) to detect viable and dead cells. SYTOX Green behaves similarly to SYTOX Orange and propidium iodide, but emits green fluorescence (523 nm) when excited with the argon laser (488 nm) of a confocal scanning laser microscope.

Green fluorescent protein (GFP) has been demonstrated to be a useful fluorescent tag for studying the localization of bacterial cells in biological systems (Burnett et al., 2000; Margolin, 2000; Prachaiyo and McLandsborough, 2000; Ling et al., 2000) and has been expressed in several Gram-positive and Gram-negative bacterial species (Valdivia et al., 1998). Several variants of the wild-type GFP are commercially available, including a red-shifted enhanced GFP (EGFP) containing mutations in the chromophore which shifts the maximal excitation peak to approximately 490 nm, resulting in more efficient detection using an argon laser, compared to wild-type GFP. EGFP offers some other advantages over staining to fluorescently label cells. It requires no additional substrate to fluoresce, facilitating sample preparation while

providing ease in detection. Limitations in its use include instability of the EGFP plasmid following cell division and possibly denaturation of protein upon exposure to sanitizers, resulting in loss of fluorescence. EGFP has been used to detect *E. coli* O157:H7 cells attached to surfaces of lettuce, tomatoes, and cauliflower (Takeuchi and Frank, 2001b). However the use of EGFP to label bacterial cells attached to produce surfaces for the purpose of determining efficacy of sanitizers has not been described.

In the study reported here, the influence of sanitizer treatment of *E. coli* O157:H7 cells labeled with EGFP on fluorescence intensity of was investigated. In addition, staining methods used to differentiate viable and dead *E. coli* O157:H7 cells attached to the cuticle of apples were evaluated.

MATERIALS AND METHODS

2.1. Microorganism, plasmid, and construction of EGFP-expressing E. coli *O157:H7*

E. coli O157:H7 SEA13B88, isolated from a patient whose infection was linked to unpasteurized, unfermented apple cider, was supplied by Dr. R. L. Buchanan at the U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition, Washington, D.C. The strain was maintained at 4°C on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI). The pEGFP plasmid (CLONTECH Laboratories, Palo Alto, CA), possessing an ampicillin resistance gene, was introduced to competent cells using a GenePulser electroporation apparatus (BioRad Laboratories, Palo Alto, CA) (Anonymous, 1988). Transformants (*E. coli* O157:H7 $EGFP^+$) were selected on TSA containing amplicillin (100 μg/ml) (TSAA). Expression of EGFP was confirmed by illumination of plates with UV light (Spectroline, Westbury, NY). Fluorescent colonies were selected and subcultured for further research.

2.2. Influence of chemical sanitizers on fluorescence of E. coli *O157:H7* EGFP$^+$

2.2.1. Preparation of cell suspension

E. coli O157:H7 EGFP$^+$ and *E. coli* O157:H7 EGFP$^-$ (the non-transformed parent strain) were streaked to give confluent growth on four TSAA plates and two TSA plates, respectively, and incubated at 37°C for 24 h. Previous experiments in our laboratory showed that fluorescence intensity of EGFP-expressing cells is higher in cultures grown on agar media than in liquid media as detected by epifluorescence microscopy. We have also determined by epifluorescence microscopy that incubation of TSAA inoculated with *E. coli* O157:H7 EGFP$^+$ at 37°C for 24 h results in higher numbers of fluorescent cells compared to incubation at 30 or 25°C for 24 h. Cell suspensions were prepared by flooding each TSA or TSAA plate with 5 ml of 0.01 M sterile potassium phosphate buffer containing 0.85% NaCl (PBS, pH 7.2) and disrupting the colonies with a sterile bent glass rod. Suspensions were transferred to sterile centrifuge tubes. Flooding, suspending cells, and transferring suspensions were repeated twice to produce 15 ml of cell suspension, which was centrifuged at 2,000 x *g* for 15 min. Pellets were washed in PBS. Suspensions of *E. coli* O157:H7 EGFP$^+$ or *E. coli* O157:H7 EGFP$^-$ were combined and resuspended in sterile distilled water (ca. 60 ml and 30 ml, respectively) to achieve an A_{590} value of 0.11. Suspensions were maintained at 4°C in the dark until used as inocula.

2.2.2. Preparation of chemical sanitizers

Chlorine solutions were prepared by diluting a stock solution of 40 mg/ml sodium hypochlorite (Sigma-Aldrich, St. Louis, MO) in 0.05 M potassium phosphate buffer (pH 6.8, 21°C). A working solution containing 222 µg/ml active chlorine was prepared by titrating with 0.0451 phenylarsine oxide using an amperometric titrator (Hach, Loveland, CO) and diluted 10-fold in 0.05 M potassium phosphate buffer to

prepare 9.0 ml of a solution containing 22.2 μg/ml active chlorine in sterile test tubes (25 x 150 mm). Hydrogen peroxide (Sigma) and acetic acid (Fisher Scientific Co., Pittsburgh, PA) were diluted in sterile distilled water to give concentrations of 111 and 89 mg/ml, respectively, and 9.0-ml aliquots were dispensed into sterile test tubes. Upon the addition of 1.0 ml of cell suspension to 9.0 ml of chemical solutions, concentrations of active chlorine, hydrogen peroxide, and acetic acid were 20 μg/ml, 100 mg/ml, and 80 mg/ml, respectively.

2.2.3. Measurement of fluorescence intensity

One milliliter of the *E. coli* O157:H7 $EGFP^+$ suspension was dispensed into tubes containing 9.0 ml of distilled water (positive control), 22.2 μg/ml active chlorine, 111 mg/ml hydrogen peroxide, or 89 mg/ml acetic acid. As a negative control, 1.0 ml of the *E. coli* O157:H7 $EGFP^-$ suspension was diluted in 9.0 ml of sterile distilled water. Cells were treated in each solution for 0, 30, 60, 120, or 300 sec before the active components were neutralized by transferring 4.0 ml of each suspension into tubes containing 4.0 ml of 950 μg/ml sodium thiosulfate (to neutralize chlorine and hydrogen peroxide) or 0.8 M potassium phosphate buffer (pH 6.8) (to neutralize acetic acid). Two milliliters of each suspension were then transferred to quartz cells. The relative fluorescence intensity of cell suspensions was determined using a spectrofluorometer (model RF-5301PC, Shimadzu, Columbia, MD) at excitation and emission wavelengths of 488 nm and 511 nm, respectively.

To determine the relative fluorescence intensity of individual cells as influenced by test sanitizers, a dilution of each treated cell suspension was filtered onto a black polycarbonate membrane (0.2 μm pore size, 25 mm diameter, Millipore, Bedford, MA), which was mounted on a clean glass microscope slide. Membrane filters were scanned using a Bio-Rad MRC-600 confocal scanning laser microscope (Bio-Rad, Inc., Hemel Hempstead, UK) equipped with a 50-mW argon-krypton laser. EGFP-expressing *E.*

coli O157:H7 cells were detected using an excitation wavelength of 488 nm. Emitted light was collected through a 480-nm dichroic mirror, a 520-nm long pass filter, and a 680-nm short pass filter. Fields containing at least 20 cells were scanned and images were recorded. Digital image analysis was performed using Scion Image (Scion Corp., Frederick, MD). Density profiling was used to measure fluorescence intensity of cells on the bases of gray level from 0 to 255, with the latter as complete saturation of the white level. Density profiles of 20 cells for each combination of chemical treatment and exposure time were measured and peak intensities were recorded and averaged.

2.2.4. Determination of populations of E. coli *O157:H7*

Treated suspensions were analyzed for number of viable *E. coli* O157:H7 cells by serially diluting (9:1) in sterile PBS and surface plating (0.1 ml) in duplicate on TSA (to enumerate *E. coli* O157:H7 $EGFP^-$) or TSAA (to enumerate *E. coli* O157:H7 $EGFP^+$). Presumptive colonies formed on TSA and TSAA were counted after incubating plates for 24 h at 37°C and three colonies from each sample were selected for confirmation by latex agglutination reaction (Oxoid, Basingstoke, Hampshire, UK).

2.3. Comparison of staining methods to detect viable and dead E. coli *O157:H7 cells on surfaces of apples*

2.3.1. Preparation of inoculum and inoculation of apples

E. coli O157:H7 $EGFP^-$ was cultured at 37°C in 10 ml of tryptic soy broth (TSB, pH 7.3) (Difco). Two successive 24-h loop transfers to 10 ml of TSB were made prior to a final loop transfer into 100 ml of TSB. Following static incubation at 37°C for 24 h, cells were harvested by centrifugation (2,000 x *g*, 15 min, 21°C), rinsed with 10 ml of sterile 0.1% (wt/vol) peptone water, recollected by centrifugation, and resuspended in 10 ml of 0.1% peptone water. This suspension served as an inoculum.

Mature Red Delicious cv. apples were harvested by hand from orchards at the University of Georgia Mountain Experiment Station (Blairsville, GA), transported to our laboratory within 8 h, and stored at 4°C and used within 90 days. Apples of similar shape and size, free of visible bruises, cuts, and other assaults, were selected for inoculation. Each apple, tempered at 25°C, was placed in a sterile polyethylene bag containing 10 ml of the inoculum and 290 ml of 0.1% peptone water at 4°C. Air was removed from the bags, which were then heat sealed to ensure complete immersion of apples in the inoculum. Each bag containing an apple immersed in diluted inoculum was placed in an ice bath (2°C) for 20 min. Apples were then removed from the inoculum and placed under a laminar flow hood (class II, type A/B3) for 18 h to enhance attachment of *E. coli* O157:H7 to apple surfaces.

2.3.2. Disinfection of apples

Inoculated apples were individually placed in resealable polytethylene bags (17.8 cm x 23.3 cm) to which 300 ml of 0 (sterile, filtered [0.20 μm pore size] distilled water) or 200 μg/ml active chlorine was added. Bags were sealed and shaken moderately by hand (12 cm arch) for 2 min, treatment solutions were decanted, and 300 ml of 0.2% (wt/vol) sodium thiosulfate (Sigma) was added to neutralize remaining active chlorine. Apples were submerged without agitation for 30 sec before the solution was decanted. The apples were removed from bags and placed in sterile plastic baskets under a laminar flow hood.

2.3.3. Preparation of stains

Three nucleic acid stains, propidium iodide (Sigma), SYTOX Orange (Molecular Probes, Eugene, OR), and SYTOX Green (Molecular Probes), were evaluated for effectiveness in labeling dead *E. coli* O157:H7 cells attached to surfaces of apples. These stains penetrate bacterial cells with compromised membranes but will

not cross the membranes of live cells. A stock solution of propidium iodide (1 mg/ml) was prepared in sterile, filtered (0.20 µm pore size) distilled water and maintained at 4°C in the dark. A working concentration of 1.0 µM propidium iodide was prepared immediately before application to inoculated apples. Stock solutions of SYTOX Orange and SYTOX Green, maintained at -30°C, were diluted in 10 mM Tris 1 mM EDTA (pH 7.1) (Sigma) at working concentrations of 0.1 and 2.5 µM, respectively. The two fluorescent dyes, Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes), were evaluated for their effectiveness in labeling viable and dead *E. coli* O157:H7 cells in conjunction with propidium iodide or SYTOX Orange stains and SYTOX Green stain, respectively, to stain dead cells. Affinity purified antibody to *E. coli* O157:H7 (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was conjugated with the fluorescent dyes using a protein labeling kit supplied by the manufacturer. The labeled antibody solutions were stored at 4°C in the dark until used. Working solutions were made by diluting stock solutions 1:100 in 1% (wt/vol) bovine serum albumin (Sigma) in PBS.

2.3.4. Preparation of specimens for CSLM analysis

E. coli O157:H7 cells on and within the intact cuticle following treatment with water or chlorine were examined. Apples were placed on a sanitized cutting board in a laminar flow hood. Three 1 cm x 1 cm x 1 mm sections were removed from the surface of each apple with a sterile stainless steel scalpel and placed in a sterile petri plate. To stain dead cells, samples were placed in sterile glass vials containing 2.0 ml of propidium iodide, SYTOX Orange, or SYTOX Green and incubated at 21°C on a rotary shaker (45 rpm) for 10 min. After rinsing (rotary shaker, 45 rpm) in sterile, filtered (0.2 µm pore size) distilled water (21°C) in sterile glass vials for 3 min, specimens stained with propidium iodide or SYTOX Orange were transferred to 1.0 ml of Alexa Fluor 488-Ab solution and incubated on a rotary shaker (45 rpm) for 30 min. Specimens

stained with SYTOX Green were transferred to 1.0 ml of Alexa Fluor 594-Ab solution and incubated as described. All samples were rinsed with distilled water, placed in sterile petri plates, and dried in a laminar flow hood for 10 min.

Mounts of apple specimens for microscopic analysis were prepared by placing 0.3 ml of silica gel (Dow Corning, Midland, MI) inside a plastic collar (2.8 mm high x 1.9 cm internal diameter) attached to a clean microscope slide with a rapid curing, general purpose epoxy adhesive (Devcon, Riviera Beach, FL). Stained apple samples were placed on the silica gel, followed by applying a drop of filtered (0.45 μm pore size) glycerol. A coverslip was then placed on the apple specimen with gentle downward pressure to facilitate adherence to the silica gel.

2.2.5. Visualization and image analysis with CSLM

Samples were analyzed using a Leica TCS SP II confocal scanning laser microscope (Leica Lasertechnik, Heidelberg, Germany), which is equipped with adjustable bandwidths for detecting fluorescent wavelengths. Fluorescence of cells treated with SYTOX Green and Alexa Fluor 488 were detected using a 50 mW argon/krypton laser at 488 nm. Fluorescence of cells treated with propidium iodide, SYTOX Orange, and Alexa Fluor 594 were detected using a 1 mW germanium/neon laser at 543 nm. Samples were scanned using a Leica DMR upright microscope (Leica) equipped with a 100x oil immersion objective (N.A. = 1.4). Bandwidths were adjusted appropriately for each stain and dye combination, and to compensate for autofluorescence of apple tissue. Both photomultipliers were adjusted to the same sensitivity. Each combination of stain and dye was evaluated qualitatively on the basis of its effectiveness in labeling cells such that fluorescent intensity of stained *E. coli* O157:H7 cells was greater than the intensity of autofluorescence of the surrounding tissue. Selected CSLM series of slices were stacked using Leica software and formatted using CorelDRAW 9.0.

2.3. Statistical analysis

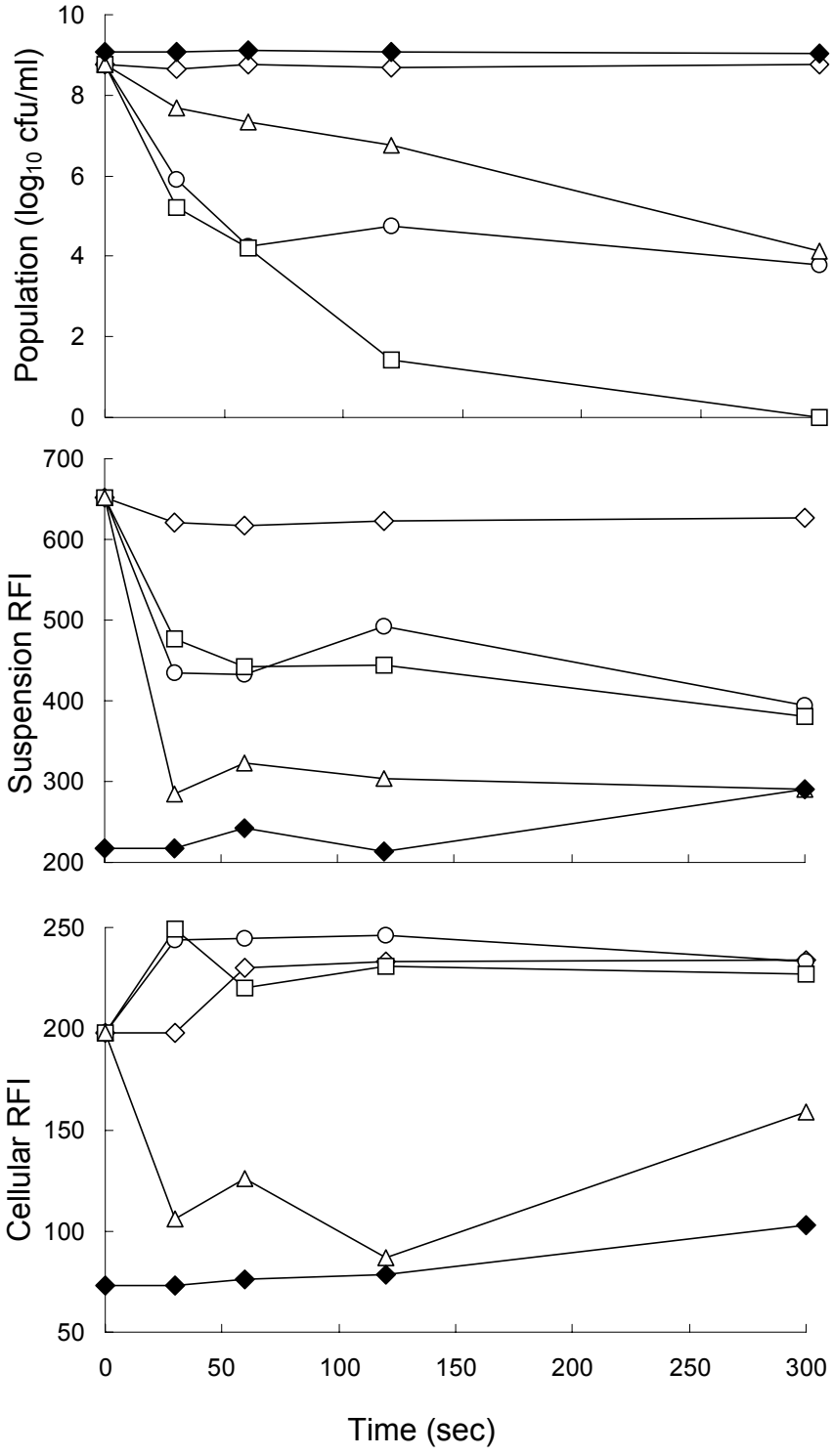
Experiments using sanitizers and EGFP-expressing cells were performed in triplicate. Data were subjected to the Statistical Analysis System (SAS; SAS Institute, Cary, NC) for analysis of variance with Duncan's multiple range tests to determine if significant differences ($P \leq 0.05$) in populations or relative fluorescence intensities of *E. coli* O157:H7 suspensions existed between mean values. Experiments comparing stains and dyes to fluorescently label *E. coli* O157:H7 cells attached to the surfaces of apples were performed in duplicate and qualitatively assessed based on ease of detection of viable, dead, and total cells.

RESULTS AND DISCUSSION

3.1. Influence of treatment with sanitizers on EGFP-expressing E. coli *O157:H7 in suspension*

The populations of *E. coli* O157:H7 $EGFP^+$ (8.8 $\log_{10}$ cfu/ml) reduced to 4.3 $\log_{10}$ cfu/ml upon exposure to 20 µg/ml active chlorine for 60 sec (Fig. 3.1). Within this exposure period, the relative fluorescence intensity (RFI) of the suspension declined from 650 to 430, significantly lower (P # 0.05) than the RFI of $EGFP^+$ suspensions treated with water for 60 sec. Thus, a reduction of 4.5 $\log_{10}$ cfu/ml *E. coli* O157:H7 $EGFP^+$ was concurrent with a significant decline in fluorescence intensity of the suspension. Relative fluorescence intensity of individual cells in suspensions treated with chlorine remained strong, increasing slightly during a 60-sec exposure and remaining stable after 300 sec of exposure. These observations suggest that EGFP

Figure 3.1. Influence of treatment with distilled water (◇), 20 μg/ml active chlorine (○), 80 mg/ml acetic acid (△), and 100 mg/ml hydrogen peroxide (□) on populations, relative fluorescence intensity (RFI) of suspensions, and RFI of cells of *E. coli* O157:H7 $EGFP^+$ (open symbols) and *E. coli* O157:H7 $EGFP^-$ (closed symbols).


Population (log10 cfu/ml)
10
8
6
4
2
0
Suspension RFI
700
600
500
400
300
200
Cellular RFI
250
200
150
100
50
0
50
100
150
200
250
300
Time (sec)

remained intact and retained fluorescence in cells surviving treatment with 20 μg/ml chlorine.

Populations of *E. coli* O157:H7 EGFP$^+$ were reduced to undetectable numbers (<1 cfu/ml) after treatment with 100 mg/ml hydrogen peroxide for 300 sec (Fig. 3.1). However, fluorescence intensity of suspensions remained as high as the fluorescence intensity of suspensions treated for 300 sec with chlorine (P # 0.05), indicating that fluorescent EGFP remained in suspensions of dead cells. Individual cellular fluorescence intensity of cells treated for 300 sec with 100 mg/ml hydrogen peroxide was not significantly different (P > 0.05) than the positive (water) control. This observation suggests that cells treated with hydrogen peroxide may retain fluorescent GFP. However, because the fluorescence intensity of the suspension was not as high as that of the postive control, GFP cannot be used as a fluorescent label to estimate the number of total cells in suspensions treated with 100 mg/ml hydrogen peroxide.

A steady reduction in populations of *E. coli* O157:H7 EGFP$^+$ was observed upon treatment with 80 mg/ml acetic acid throughout the 300-sec exposure period (Fig. 3.1). The population was reduced by ca. 4.6 $\log_{10}$ cfu/ml. Fluorescence intensity of suspensions treated with acetic acid for 300 sec was not significantly higher (P > 0.05) than the fluorescence intensity of the negative control, suggesting that although over 4 $\log_{10}$ cfu of viable *E. coli* O157:H7 EGFP$^+$ per ml of suspension remained, EGFP lost its ability to fluoresce and no signal was detected. Fluorescence intensity of individual cells throughout the 300-sec exposure period was unstable, but significantly (P # 0.05) lower than intensities of cells treated with water, chlorine, or hydrogen peroxide. At 120 sec of exposure, cellular fluorescence intensity was not significantly different (P > 0.05) than that of the negative control. These observations indicate that EGFP cannot be used as a label to estimate numbers of viable or total *E. coli* O157:H7 cells following treatment with 80 mg/ml acetic acid. A slight signal was detected from *E. coli*

O157:H7 EGFP$^-$ cells using CSLM, indicating that cells exhibited some degree of autofluorescence.

Each sanitizer employed in this study influenced fluorescent intensities of *E. coli* O157:H7 EGFP$^+$ differently. EGFP must meet certain criteria to be used to detect either viable or total (both viable and dead) *E. coli* O157:H7 cells in produce tissues following treatment. To use to label all cells, the EGFP chromophore must remain intact and be functional upon cell death. In addition, the intensity of fluorescence must be greater than that of the autofluoresence of the surrounding tissue. To be used to detect viable cells, fluorescence of cells must cease upon death, as a result of a denatured chromophore. In both cases, cells labeled with EGFP should ideally behave similarly upon exposure to several types of sanitzers if comparisons are to be made in their efficacies of killing cells on and in produce tissues. The results of this study indicate that EGFP would not be an acceptable fluorescent label of *E. coli* O157:H7 cells in studies dealing with surface treatments applied to produce.

3.1. Comparison of stains to differentiate viable and dead E. coli *O157:H7 cells on apples*

E. coli O157:H7 cells on apple cuticle specimens stained with propidium iodide were difficult to discern due to their overall weak fluorescence intensity in comparison to the autofluorescence from the underlying tissue. Upon binding with nucleic acids, propidium iodide is reported to have a 20- to 30-fold fluorescence enhancement (Roth et al., 1997). Fluorescence intensities of SYTOX Orange and SYTOX Green, in contrast, increase by >500 fold when bound to nucleic acids (Roth et al., 1997; Yan et al., 2000). Both the SYTOX stains effectively stained dead cells on apple cuticles, providing an intensely fluorescent label which could be easily distinguished from background fluorescence. Poor staining of *E. coli* cells by propidium iodide, compared to SYTOX Green, has been reported (Mortimer et al., 2000) to result from an efflux

pump that removes of ethidium bromide, a molecule structurally similar to propidium iodide.

A limitation of using SYTOX Orange in conjunction with Alexa Fluor 488-Ab was evident. CSLM analysis of *E. coli* O157:H7 cells labeled with these two stains revealed that cross-signal was detected between photomultiplier 1 (detecting Alexa Fluor 488-Ab) and photomultiplier 2 (detecting SYTOX Orange), even when the distance between emission filters of the two photomultipliers was maximized. This observation is explained by principles associated with Stoke's shift (Abramowitz, 1993). When fluorescent molecules are excited with light of a specific wavelength, they radiate light of another, longer wavelength. This difference between excitation light and fluorescent light is referred to as Stoke's shift. Thus, fluorescent light emitted from cells stained by Alexa Fluor 488-Ab was detected by photomultiplier 2, designated to detect emitted light from cells stained with SYTOX Orange, resulting in the possibility of over-estimating the number of dead cells on the cuticles of apples following treatment with sanitizer. The use of SYTOX Green and Alexa Fluor 594-Ab (Fig. 3.2) circumvented this problem. Although a cross-signal between the photomultiplier detecting SYTOX Green and the photomultiplier detecting Alexa Fluor 594-Ab occurred after maximizing ranges of emitted light collected by both, the influence of this cross-signal would not affect numbers of dead cells detected, since cells stained with SYTOX Green were labeled with Alexa Fluor 594-Ab as well. The majority of the *E. coli* O157:H7 cells detected on the cuticle of apples treated with 0 (water) μg/ml chlorine were dead. Cell death likely occurred from desiccation resulting from the 18-h incubation period after inoculation and prior to treatment. Background autofluorescence intensity detected by CSLM differed, depending on the degree of accumulation of natural cutin on apple surfaces and the number of fluorescent *E. coli* O157:H7 cells located within visual fields.

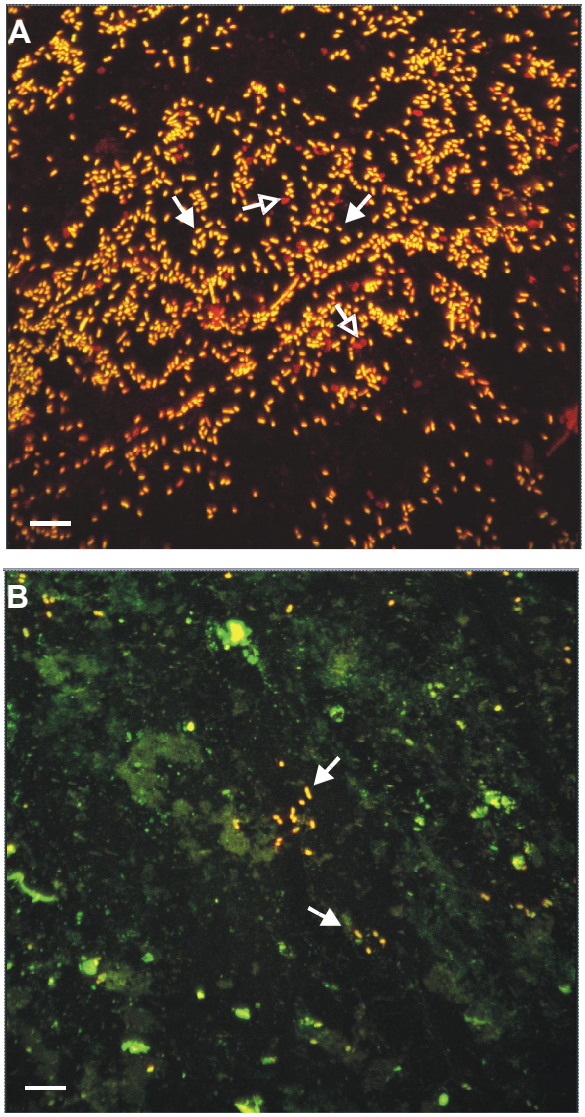


Figure 3.2. CSLM photomicrographs showing influence of treatment with 0 (A), and 200 (B) µg/ml active chlorine on viability of *E. coli* O157:H7 cells attached taapple cuticle. SYTOX Green was used to stain dead cells and Alexa Fluor 594-Ab was used to label viable and dead (total) cells. Yellow cells are dead (closed arrowheads) and red cells are viable (open arrowheads). Bar = 10 µm.

There are some limitations to using membrane impermeable nucleic acid stains in determining the efficacy of sanitizers in killing bacterial cells. Leakage of nucleic acids upon the loss of membrane integrity after exposure to chlorine or other potentially lethal chemicals may cause a reduction in the number of available dye binding sites (Komanapalli and Lau, 1996). In addition, investigations have suggested that these stains may underestimate the fraction of dead cells within starved populations of *E. coli* as a result of inefficient binding of the stain to damaged nucleic acids within cells (Lebaron et al., 1998; Mortimer et al., 2000). Consideration must also be given to the time allowed for bacterial attachment to produce surfaces, which leads to cell death as a result of desiccation. Appropriate controls must be incorporated into these investigations to account for natural death of cells.

In conclusion, this study demonstrates that EGFP is not an acceptable fluorescent label to detect viable or total *E. coli* O157:H7 cells following treatment with sanitizers. In addition, we observed that the use of SYTOX Green in conjunction with Alexa Fluor 594-Ab to stain dead and total *E. coli* O157:H7 cells, respectively, attached to apple cuticles performed better than propidium iodide or SYTOX Orange and Alexa Fluor 488-Ab to detect dead and viable cells. This technique holds promise for successful use in studies seeking to differentiate viable and dead *E. coli* O157:H7 cells on other types of produce following surface treatment with sanitizers.

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CHAPTER 4

DIFFERENTIATION OF VIABLE AND DEAD *ESCHERICHIA COLI* O157:H7 CELLS ON AND IN APPLE STRUCTURES AND TISSUES FOLLOWING CHLORINE TREATMENT[1]

[1]Burnett, Scott L., and Larry R. Beuchat. Submitted to Journal of Food Protection

ABSTRACT

Confocal scanning laser microscopy (CSLM) was used to differentiate viable and non-viable cells of *Escherichia coli* O157:H7 on and in raw apple tissues following treatment with water and 200 or 2000 ppm active chlorine solution. Whole, unwaxed Red Delicious cv. apples at 25°C were inoculated by dipping in a suspension of *E. coli* O157:H7 (8.48 $\log_{10}$ CFU/ml) at 4°C, followed by treatment in water or chlorine solution (21°C) for 2 min. The number of dead cells on and in apples was distinguished from live cells by treating tissue samples with SYTOX green nucleic acid stain. Viable and dead cells were then labeled with an antibody conjugated with a fluorescent dye (Alexa Fluor 594). The percentage of viable cells on the apple surface as well as at various depths in surface and internal structures was determined. The mean percentage of viable cells located at each site after treatment with water or chlorinated water was in the following order, which also reflects the order of protection against inactivation: floral tube wall (20.5%) > lenticels (15.0%) > damaged cuticle surrounding puncture wounds (13.0%) > intact cuticle (8.1%). The location of viable cells within tissues was dependent upon the structure. With the exception of lenticels, the percentage of viable cells increased as depth into CSLM stacks increased, indicating that cells attached to subsurface structures were better protected against inactivation with chlorine than were cells located on exposed surfaces. Further research is warranted to investigate the efficacy of other chemical sanitizers as well as the influence of surfactants and solvents in combination with sanitizers for efficacy in removing or killing *E. coli* O157:H7 lodged in protective structures on the surface and within apples tissues.

INTRODUCTION

Unpasteurized apple cider is the product of milled, pressed apples, and is traditionally consumed during harvest in autumn months in apple-growing regions. Since 1974, ten outbreaks of human infections caused by *Escherichia coli* O157:H7, *Salmonella*, or *Cryptosporidium* have been associated with the consumption of fresh apple cider, resulting in 683 documented cases and two deaths (*11*). These outbreaks have raised interest in determining the efficacy of decontamination treatments applied to apples intended for the unpasteurized cider market.

Recent surveys of cider manufacturing practices in three states (*14, 17, 20*) and one Canadian province (*10*) indicate that less than half of the producers sanitize apples prior to milling. Active chlorine concentrations ranging from 50 to over 300 ppm active chlorine are used as a treatment to reduce numbers of microorganisms on apple surfaces. Research has shown, however, that significant populations of viable *E. coli* O157:H7 cells remain attached to apple tissues after the application of these treatments (*3).* In an investigation of the efficacy of a commercial flatbed brush washer in combination with several chemical treatments in reducing *E. coli* from the surface of Golden Delicious apples, Annous et al. *(1)* observed that none of the washing treatments tested significantly reduced the population. The authors suggested that this may have been due to the failure of washing agents to reach inaccessible regions of apples near the calyx and stem, where bacteria have been demonstrated to reside *(4, 6)*.

Buchanan et al. (*4*) attributed the uptake of cells into the core of whole apples for the meager reductions of *E. coli* O157:H7 resulting from treatment with 200 ppm active chlorine. Investigators have offered several explanations for the limited effectiveness of chlorine in killing *E. coli* O157:H7 associated with apple surfaces. Wisniewsky et al. (*18*) and Wright et al. (*19*) surmised that bacteria located within pores, crevices, and cracks on the apple surface might be protected from the action of

sanitizers. The elimination of active chlorine due to contact with organic material (i.e., apple tissue) has also been cited (*3*).

Infiltration of pathogenic bacteria into internal structures and tissues of apples *(5, 6, 8*), lettuce (*15, 16, 17*), and tomatoes (*2*) has been reported. Using confocal scanning laser microscopy (CSLM), viable *E. coli* O157:H7 cells have been observed within stomata and internalized into cut tissue of lettuce leaves after treatment with a 20 ppm chlorine solution (*15*). Pao and Brown *(12)* determined that treatment of citrus fruits inoculated with *E. coli* using sodium orthophenylphenate did not significantly reduce populations of the bacterium compared to treatment with water. More than 2 $\log_{10}$ cfu/cm^2 *E. coli* remained attached to fruit surfaces following treatment, suggesting that bacterial cells were located within tissues where they were afforded protection from physical removal by the wash treatment and disinfection by the sanitizer.

To date, research investigations describing the role of specific structures of apples such as lenticels, the intact cuticle, damaged cuticle, and the floral tube to protect *E. coli* O157:H7 from treatment with chlorine have not been described. In the study reported here, CSLM was used to detect and quantify viable *E. coli* O157:H7 cells attached to apple surfaces and within structures and tissues following treatment with 0, 200, or 2000 ppm active chlorine.

MATERIALS AND METHODS

Strain used and preparation of inoculum. *E. coli* O157:H7 SEA13B88, isolated from an outbreak associated with unpasteurized apple cider, was obtained from Dr. R. L. Buchanan at the U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition, Washington, D.C. The strain, maintained at 4°C on tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich.) slants, was cultured at 37°C in 10 ml of tryptic soy broth (TSB, pH 7.3) (Difco). Two successive 24-h loop transfers to 10 ml of

TSB were made prior to a final loop transfer into 100 ml of TSB. Following static incubation at 37°C for 24 h, cells were harvested by centrifugation (2,000 x *g*, 15 min, 21°C), rinsed with 10 ml of sterile 0.1% (wt/vol) peptone water, recollected by centrifugation, and resuspended in 10 ml of 0.1% peptone water. This suspension served as an inoculum.

Inoculation of apples. Unwaxed, Red Delicious cv. apples were obtained from a local supermarket and stored at 4°C until used. Apples of similar shape and size, free of obvious bruises, cuts, and other assaults, were selected for inoculation. Apple cuticle and cortex pH were measured with a flat surface pH electrode (Fisher Scientific, Pittsburgh, Pa.) and pH meter (Denver Instruments, Arvado, Colo.). Immediately before inoculating, the surfaces of one set (n = 9) of apples were punctured (3.5 mm length x 0.5 mm width x 2.0 mm depth) with a sterile stainless steel surgical blade tip at five locations between the apex and the base. A second set of apples (n = 6) was not punctured prior to inoculation. Each apple, tempered at 25°C, was placed in a sterile polyethylene bag containing 10 ml of the inoculum and 290 ml of 0.1% peptone water at 4°C. Air was removed from the bags, which were then heat sealed to ensure complete immersion of apples in the inoculum. Each bag containing an apple immersed in diluted inoculum was placed in an ice bath (2°C) for 20 min. Apples were then removed from the inoculum and placed under a laminar flow hood (class II, type A/B3) for 2 h to enhance attachment of *E. coli* O157:H7 to apple surfaces.

Preparation of chlorine solutions. Solutions containing 200 and 2000 ppm active chlorine were prepared by diluting a stock solution of 4% sodium hypochlorite (Sigma - Aldrich, St. Louis, Mo.) in 0.05 M potassium phosphate buffer (pH 6.8, 21°C). A working solution containing 4000 ppm active chlorine was prepared by titrating with 0.0451 N phenylarsine oxide using an amperometric titrator (Hach, Loveland, Colo.) and diluted appropriately with 0.05 M potassium phosphate buffer to prepare 200 and 2000 ppm solutions used to treat apples.

Treatment of apples and microbiological analysis. Inoculated apples were individually placed in resealable polytethylene bags (17.8 cm x 23.3 cm) to which 300 ml of 0 (sterile, filtered [0.20 μm pore size] distilled water), 200, or 2000 ppm active chlorine solution was added. Bags were sealed and shaken moderately by hand (12 cm arch) for 2 min, followed by decanting solutions. To bags containing apples intended for CSLM analysis (3 apples/set), 300 ml of 0.2% (wt/vol) sodium thiosulfate (Sigma - Aldrich) was added to neutralize remaining active chlorine. Apples were submerged without agitation for 30 s before the solution was decanted. The apples were removed from bags, placed in sterile plastic baskets, and dried under the laminar flow hood for 30 min. To bags containing apples to be analyzed for populations of *E. coli* O157:H7 (6 apples/set), 100 ml of 0.1% peptone water buffered with 0.5 M potassium phosphate buffer (pH 6.8) containing 0.2% sodium thiosulfate was added. Apples were rubbed by hand for 1 min, followed by vigorous shaking for 1 min to facilitate removal of cells from apple surfaces. Apples were removed from bags with sterile stainless steel tongs and placed on a sanitized cutting board in preparation for removal of the core.

To determine the efficacy of water, 200, or 2000 ppm chlorine in reducing populations of *E. coli* O157:H7 within cores of apples, the following procedure was followed. To minimize carriage of cells from the external surface of apples to the core during removal, the apple skin surrounding the open calyx was sanitized with a Kimwipe (Kimberly-Clark, Roswell, Ga.) wetted with 70% ethanol. A stainless steel knife sanitized with 70% ethanol was used to remove the apex end of the apple, while leaving the calyx and apple skin immediately surrounding it intact. The core was then removed from the apple with a sterile stainless steel apple corer. One set of cores (n = 6) was treated by injecting 0.3 ml of 0, 200, or 2000 ppm active chlorine solution (21°C) into the floral tube just behind the flower sepals with a sterile 21 ga. needle and syringe. After a 2-min treatment, cores were placed in stomacher bags containing 100 ml of 0.1% peptone water buffered with 0.05 M potassium phosphate (pH 6.8)

containing 0.2% sodium thiosulfate. Bags were placed in a Stomacher 400 laboratory blender (Seward Medical Limited, London, UK) and pummeled for 1 min at medium speed. Cores not receiving the injection treatment were also placed in stomacher bags containing diluent, and pummeled as described above.

Surface wash suspensions and core stomachates were analyzed for populations of *E. coli* O157:H7 by serially diluting (9:1) in 0.1% peptone water and plating (0.1 ml) in duplicate on sorbitol MacConkey agar (SMA) (Oxoid, Basingstoke, Hampshire, UK). Presumptive colonies formed on SMA were counted after incubating plates for 24 h at 37°C and three colonies representing each combination of treatment and suspension were selected for confirmation by latex agglutination reaction (Oxoid).

Preparation of stains. Dead *E. coli* O157:H7 cells attached to apples were detected by treatment with SYTOX green nucleic acid stain (SYTOX green) (Molecular Probes, Eugene, Ore.). Stock solution, maintained at -30°C, was diluted in 10 mM Tris 1 mM EDTA (pH 7.1) (Sigma) at a working concentration of 2.5 μM. Viable and dead *E. coli* O157:H7 cells were subsequently labeled with affinity purified antibody to *E. coli* O157:H7 (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) conjugated with a fluorescent dye, Alexa Fluor 594 (Molecular Probes, Eugene, Ore.), using a protein labeling kit supplied by the manufacturer. The labeled antibody (Alexa-Ab) solution was stored at 4°C in the dark until used. Working solutions were made by diluting the Alex-Ab 1:100 in 1% (wt/vol) bovine serum albumin (Sigma) in 0.01 M potassium phosphate buffer containing 0.85% saline (PBS, pH 7.2, 21°C).

Procedure for preparing specimens for CSLM analysis. *E. coli* O157:H7 cells on and within the intact cuticle, the damaged cuticle surrounding puncture wounds, lenticels, the wall of the floral tube, and seed locules following treatment with water or chlorine were examined. Apples were placed on a sanitized cutting board in a laminar flow hood. Five 1 cm x 1 cm sections, three of which contained puncture wounds, were removed from the surface of each apple with a sterile stainless steel scalpel and placed

in a sterile petri plate. Samples of the floral tube wall were prepared by aseptically removing the core of apples as described with a sterile stainless steel corer and slicing the core cylinder transversely above and below the seed locules with a sterile knife. The middle section containing the seeds and the section containing the floral tube were aseptically cut longitudinally, and sections of the seed locule and floral tube wall were removed using a sterile stainless steel blade and a sterile forceps.

To stain dead cells, samples (7/apple/treatment) were placed in sterile glass vials containing 2.0 ml of SYTOX green stain (21°C) and incubated on a rotary shaker (45 rpm) for 10 min. After rinsing (rotary shaker, 45 rpm) in sterile, filtered (0.2 μm pore size) distilled water (21°C) in sterile glass vials for 3 min, samples were transferred to 1.0 ml of Alexa-Ab solution and incubated on a rotary shaker (45 rpm) for 30 min. Samples were rinsed as described above, placed in sterile petri plates, and dried in a laminar flow hood for 10 min.

Mounts of apple specimens for microscopic analysis were prepared by placing 0.3 ml of silica gel (Dow Corning, Midland, Mich.) inside a plastic collar (2.8 mm high x 1.9 cm internal diam) attached to a clean microscope slide with a rapid curing, general purpose epoxy adhesive (Devcon, Riviera Beach, Fla.). Stained apple samples were placed on the silica gel, followed by applying a drop of filtered (0.45 μm pore size) glycerol. A coverslip was then placed on the apple specimen with gentle downward pressure to facilitate adherence to the silica gel.

Visualization and image analysis with CSLM. Samples were analyzed using a Leica TCS SP II confocal scanning laser microscope (Leica Lasertechnik, Wetzler, Germany), which is equipped with adjustable bandwidths for detecting fluorescence wavelengths. Fluorescence of dead and total cells was detected after exciting with 50 mW Ar/Kr laser at 488 nm and a 1 mW Gre/Ne laser at 543 nm, repectively. Samples were scanned using a Leica DMR upright microscope (Leica) equipped with a 100x oil immersion objective (N.A. = 1.4). SYTOX green fluorescence was detected from 510

to 530 nm and concurrently the Alexa-Ab emission from 610 to 690 nm. Both photomultipliers were adjusted to the same sensitivity. Fields containing at least five *E. coli* O157:H7 cells at each surface or subsurface location were scanned, and at least three image stacks were recorded for each structure and treatment combination. Data from CSLM were collected in the form of a series of 2-D images representing optical sections through each specimen. These serial images are collectively referred to as the image stack. Optical sections in this study were collected at 1-µm steps along the z-axis. The fluorescence signal from *E. coli* O157:H7 cells in tissues was detected in multiple and adjacent optical sections. To prevent counting the same cell more than once, cells detected in the first three optical sections of image stacks were counted, whereas the second group of three optical sections was disregarded, and so on. Because mounts of apple tissue were not always perpendicular to the laser beam when scanned, depth is reported along the z-axis in CSLM stacks, not the actual tissue depth. The percentage of viable cells located at selected groups of three optical sections was determined by the formula: % viable cells = [1 – (number of dead cells / number of total cells)] x 100. Selected CSLM series of slices were stacked using Leica software and formatted using CorelDRAW 9.0.

Statistical analysis. All experiments were performed in triplicate. In experiments investigating populations of *E. coli* O157:H7 on apple surfaces and in cores, each replicate consisted of six apples, making a total of eighteen apples analyzed for each combination of test parameters. In experiments using CSLM, each replicate consisted of three apples, making a total of nine apples analyzed for each combination of test parameters. Data were subjected to the Statistical Analysis System (SAS; SAS Institute, Cary, N.C.) for analysis of variance with Duncan's multiple range tests to determine if significant differences ($P \leq 0.05$) in populations or percent viable cells of *E. coli* O157:H7 existed between mean values.

RESULTS

Recovery of *E. coli* O157:H7 from apples by surface plating. The mean population in the dip inoculum used in all experiments was 8.48 ("0.35) $\log_{10}$ CFU/ml. Results from experiments in which populations of *E. coli* O157:H7 on the surface and in cores of apples were detected following treatments with water or chlorinated (200 and 2000 ppm chlorine) water are summarized in Table 4.1. Surface treatment of punctured and non-punctured apples with 200 ppm active chlorine resulted in only 0.08 and 0.57 $\log_{10}$ CFU/cm^2 reductions in *E. coli* O157:H7, respectively, compared to the control. Reductions of 0.57 and 0.98 $\log_{10}$ CFU/cm^2 on the surface of apples with and without punctures, respectively, were achieved by treating apples with 2000 ppm chlorine. A significant reduction (P # 0.05) in surface population was observed when punctured apples were treated with the 2000 ppm chlorine solution compared with both the 200 ppm chlorine treatment and the control, but no significant differences among counts were observed on apples that were not punctured. The presence of puncture wounds on the apple surface did not influence the number of *E. coli* O157:H7 recovered following each water or chlorine treatment.

High numbers (6.82 $\log_{10}$ CFU/core) of *E. coli* O157:H7 were detected in the core of apples treated with water. Populations of the pathogen in cores of control (not injected) apples were reduced only by 0.12 and 0.23 $\log_{10}$ CFU/core by treatment with 200 and 2000 ppm, respectively, indicating that high numbers of viable cells remained. Injecting chlorine solutions into the floral tube following surface treatment with 200 or 2000 ppm chlorine reduced populations of the pathogen by 0.28 and 0.31 $\log_{10}$ CFU, respectively. Within treatment, the number of CFU recovered was not significantly different in non-injected and injected cores.

CSLM analysis and percent viability. Results from CSLM analysis to determine the presence, location, and viability of *E. coli* O157:H7 cells associated with

Table 4.1. *Populations of* E. coli *O157:H7 recovered from the surface and cores of whole apples following treatment with 0, 200, or 2000 ppm chlorine*

Treatment (ppm chlorine)	Population[a]			
	Log_{10} CFU/cm^2 of surface		Log_{10} CFU/core	
	With punctures[b]	Without punctures	Not Injected	Injected[c]
0 (control)	a 4.82 a	a 5.34 a	a 6.82 a	a 6.71 a
200	a 4.74 a	a 4.77 a	a 6.70 ab	a 6.43 b
2000	a 4.25 b	a 4.36 a	a 6.59 b	a 6.40 b

[a] Within surface or within core, mean values in the same row not preceded by the same letter are significantly different ($P \# 0.05$). Mean values in the same column not followed by the same letter are significantly different ($P \# 0.05$).

[b]Five punctures (35 mm length x 0.5 mm width x 20 mm depth) per apple were applied using a sterile surgical blade tip immediately before inoculating.

[c]Cores of apples treated with 0, 200, or 2000 ppm chlorine were injected with 0.3 ml of 0, 200, or 2000 ppm chlorine, respectively, using a 21 ga. needle and syringe. After 2 min, cores were stomached in 0.1% peptone.

the intact cuticle, damaged cuticle surrounding puncture wounds, lenticels, and the wall of the floral tube are summarized in Table 4.2. Cells attached to the intact cuticle of apples remained largely unprotected from chlorine treatments. The percentage of cells located on the cuticle at a depth of 0 - 14 µm following treatment with 0 ppm (water) that were viable was 24.3%. Treatment with 200 or 2000 ppm active chlorine resulted in a significant (P # 0.05) decrease in percent viable cells to #0.1% of the total number of cells detected. The percent of cells attached to the cuticle of apples that remained viable after treatment with water increased as depth into CSLM image stacks increased. Percentages of viable cells at 0 - 2, 6 - 8, and 12 - 14 µm in cuticles of apples treated with water was 17, 20, and 57%, respectively. Treatment with 200 ppm chlorine resulted in 0.1% viable cells at 0 - 2 µm in cuticles. No viable cells were detected in from 6 - 14 µm or 0 - 14 µm in cuticles of apples treated with 200 or 2000 ppm, respectively. Image analysis of stacks (Fig. 4.1) shows that higher numbers of viable cells were located within cuticular cracks and junctions between wax platelets, which are more frequently scanned at greater depths than on the uniform, smooth areas of the cuticle. Cells located in image stacks near the cuticle surface would be more exposed to environmental stresses such as desiccation during the 2-h drying period following inoculation, thus resulting in death. In addition, cells at these locations were not afforded protection against chlorine treatment.

Damaged cuticle surrounding puncture wounds protected *E. coli* O157:H7 cells from chlorine treatment (Fig. 4.2). Compared with the percentage of viable cells located on the intact cuticle of apples treated with 200 and 2000 ppm (0.1% and 0%, respectively), populations of viable cells associated with the cut or torn edges of puncture wounds were higher (10.6% and 6.2%, respectively), but not significantly different (Table 4.2). With increasing depths of stacks up to 20 µm into puncture wounds, a trend toward lower percentages of viable cells occurred (Fig. 4.3). At a depth of 24 - 26 µm in damaged cuticle, compared to the 18 - 20 µm sections, however,

Table 4.2. *Percentage of viable* E. coli *O157:H7 cells located in apple structures following treatment with 0, 200, or 2000 ppm active chlorine*[a]

Apple structure	Depth (μm)	Mean population (cells/region)	Percent viable cells after treatment with chlorine (ppm)[b]		
			0	200	2000
Intact cuticle	0 - 14	212	a 24.3 a	b 0.1 a	b 0.0 b
Damaged cuticle[c]	0 - 26	111	a 22.1 a	ab 10.6 a	b 6.2 ab
Lenticel	0 - 14	81	a 22.8 a	a 12.9 a	a 9.2 ab
Floral tube wall	0 - 20	63	a 32.8 a	ab 16.7 a	b 11.9 a

[a]Determined by confocal scanning laser microscopy image analysis.

[b]Mean values in the same column not followed by the same letter are significantly different ($P \# 0.05$). Mean values in the same row not preceded by the same letter are significantly different ($P \# 0.05$).

[c]Broken cuticle surrounding puncture wounds made on apple surfaces prior to inoculation.

Figure 4.1. *CSLM photomicrographs showing the influence of treatment of apples with 0 (A, 13-μm depth), 200 (B, 20-μm depth), and 2000 (C, 7-μm depth) ppm active chlorine on the viability of* E. coli *O157:H7 cells attached to the intact cuticle. Yellow to orange cells are dead (closed arrowheads) and red cells are viable (open arrowheads). Bar = 10 μm.*

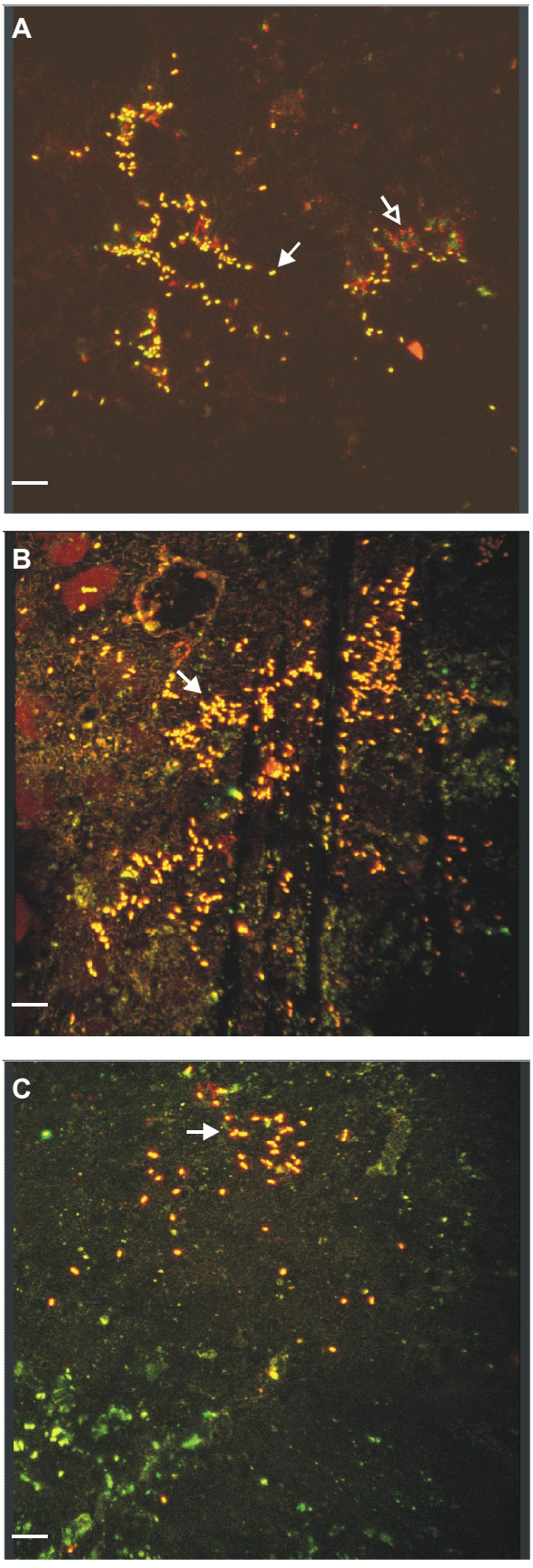
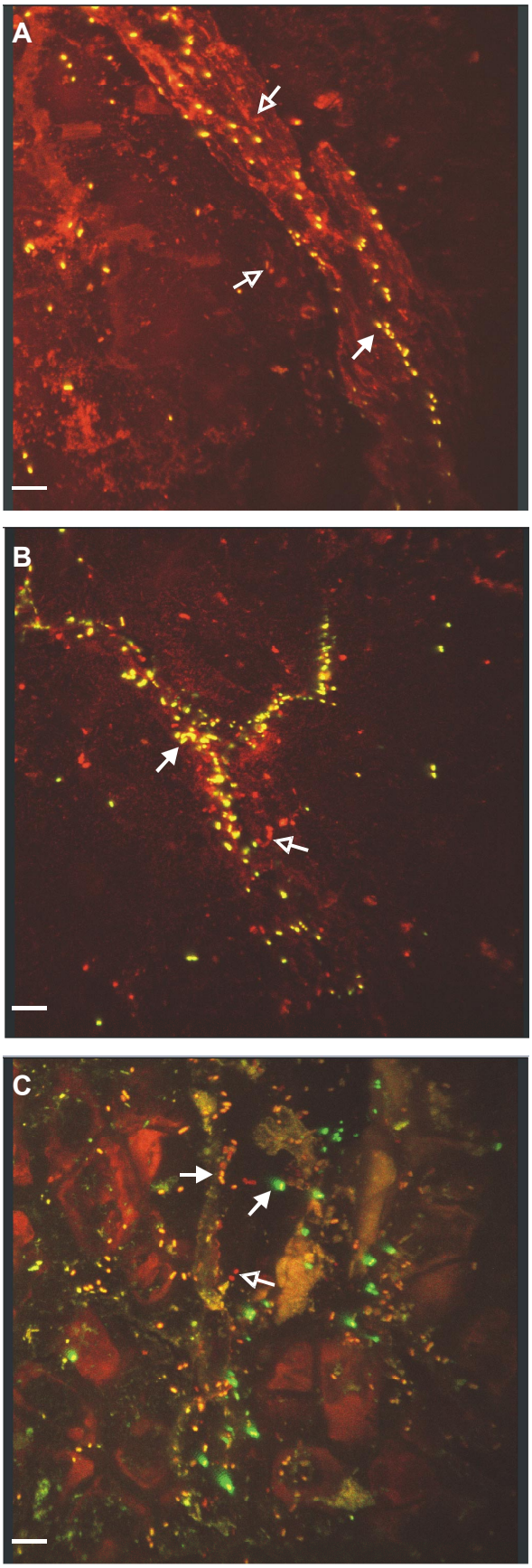

A
B
C

Figure 4.2. *CSLM photomicrographs showing the influence of treatment of apples with 0 (A, 25-μm depth), 200 (B, 32-μm depth), and 2000 (C, 25-μm depth) ppm active chlorine on the viability of* E. coli *O157:H7 cells attached to damaged tissue surrounding puncture wounds. Green-yellow to orange cells are dead (closed arrowheads) and red cells are viable (open arrowheads). Bar = 10 μm.*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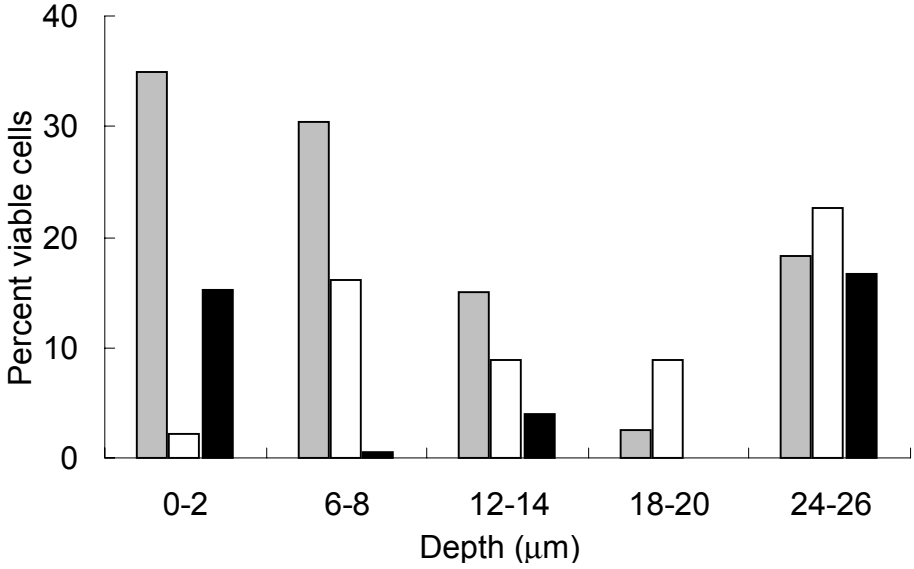


Figure 4.3. *Percentage of viable* E. coli *O157:H7 cells attached to damaged cuticle surrounding puncture wounds of apples following treatment with 0 (shaded bars), 200 (open bars), or 2000 (solid bars) ppm active chlorine as influenced by depth into CSLM stacks.µm.*

a marked increase in the percentage of viable cells was detected in apples, regardless of treatment. *E. coli* O157:H7 cells were detected in damaged tissue surrounding puncture wounds to depths of 24 - 26 µm. Independent of surface treatments, percent viability of cells at this depth was higher than at 12 - 14 and 18 - 20 µm within image stacks.

The percentages of viable cells located in lenticels following treatment with 200, and 2000 ppm were 22.8, 12.9, and 9.2%, respectively (Table 4.2). Although these values are not significantly different, the trend suggests that cells located within lenticels are better protected from chlorine than cells on or in the intact cuticle, since treatment with 200 or 2000 ppm chlorine resulted in significantly higher percentages of viable cells in lenticels at depths of 0 - 14 µm compared to percentages in the intact cuticle at the same depth. In lenticels on apples treated with 0 or 200 ppm chlorine, the percentage of viable cells decreased as depth into stacks in the 0 - 14 µm range increased (Fig. 4.4). The percentage of viable cells in lenticels on apples treated with 2000 ppm chlorine was higher (11.0%) at 6 - 8 µm than at 12 - 14 µm (5.3%). The topography of lenticels is extremely irregular and may contribute to anomalies in trends in observations. Viable and non-viable of cells associated with lenticels on apples treated with 0, 200, or 2000 ppm active chlorine are shown in Fig. 4.5.

Cells attached to the wall of the floral tube, within the calyx of apples, were protected against inactivation with 200 or 2000 ppm chlorine (16.7% and 11.9% viable, respectively), when compared to the percentage of live cells located on the intact cuticle (0.1% and 0%, respectively) (Table 4.2). In contrast, 32.8% of cells associated with the floral tube wall were viable after treatment with water. As depth into image stacks of the floral tube wall increased, the percentage of viable cells within stacks increased (Fig. 4.6). At 0 - 2 µm within stacks, 31.3, 0, and 13.6% of cells were viable after treatment with 0, 200, and 2000 ppm chlorine, respectively, compared with 63.0, 35.5, and 21.3%, respectively, at 18 - 20 µm. CSLM photomicrographs revealed the presence

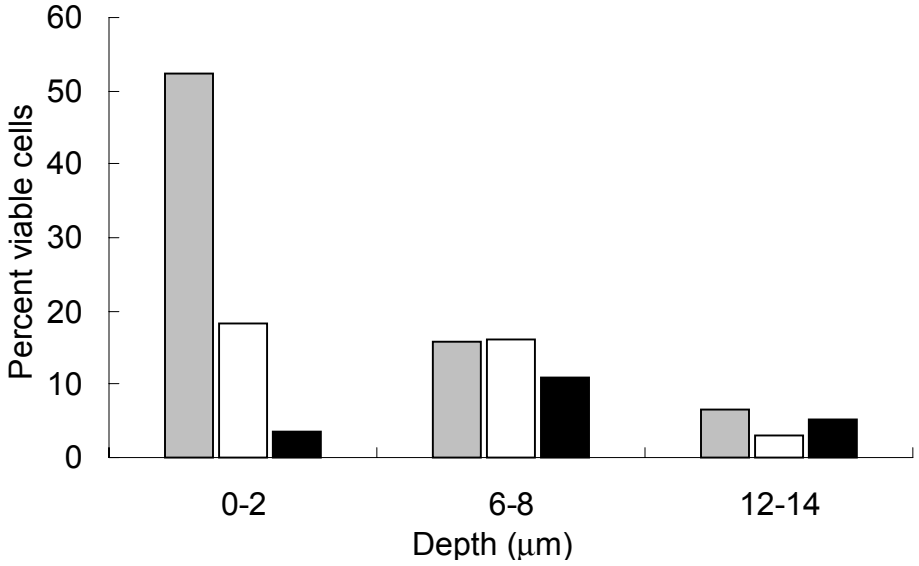
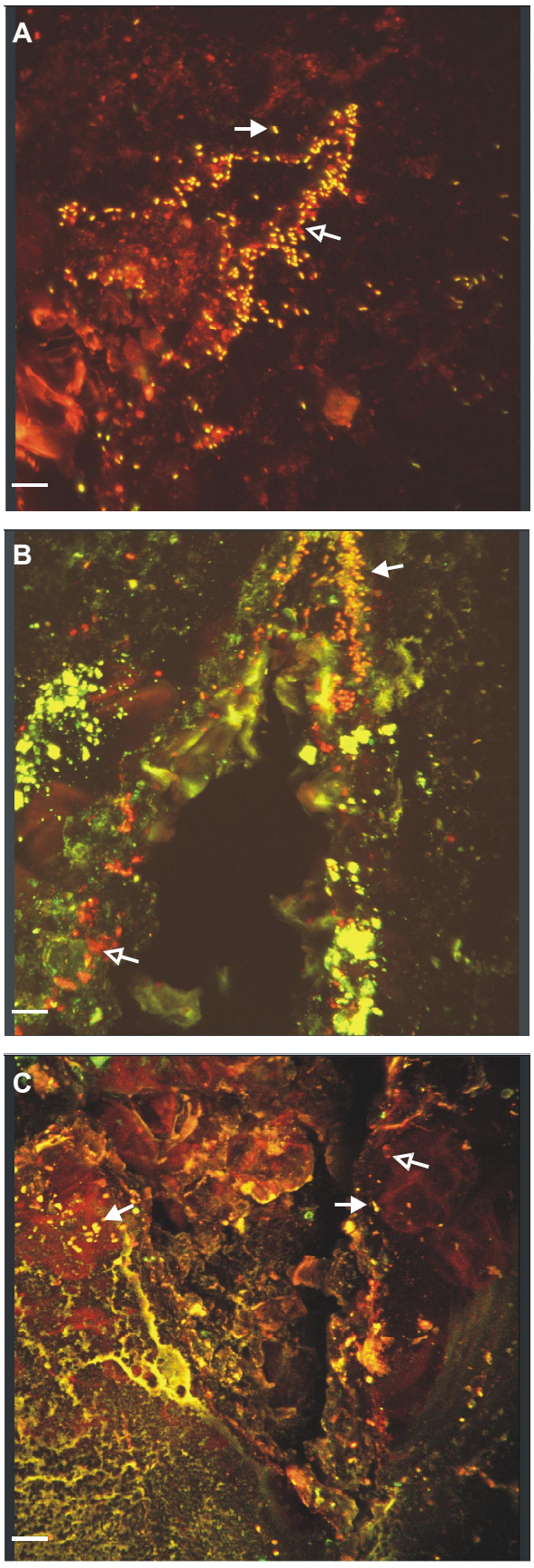


Figure 4.4. *Percentage of viable* E. coli *O157:H7 cells attached to lenticels of apples following treatment with 0 (shaded bars), 200 (open bars), or 2000 (solid bars) ppm active chlorine as influenced by depth into CSLM stacks.*

Figure 4.5. *CSLM photomicrographs showing the influence of treatment of apples with 0 (A, 25-µm depth), 200 (B, 24-µm depth), and 2000 (C, 34-µm depth) ppm active chlorine on the viability of* E. coli *O157:H7 cells attached to lenticels. Yellow to orange cells are dead (closed arrowheads) and red cells are viable (open arrowheads). Bar = 10 µm.*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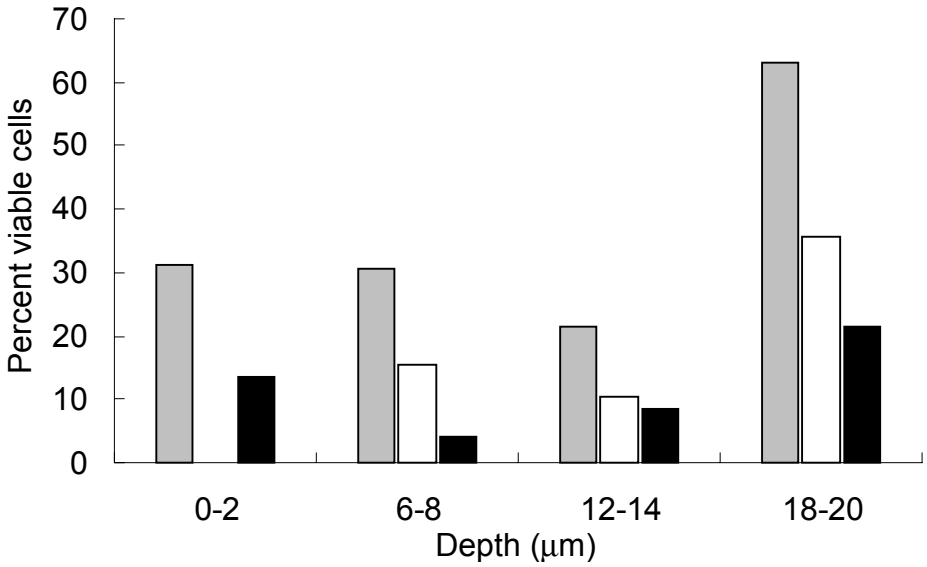
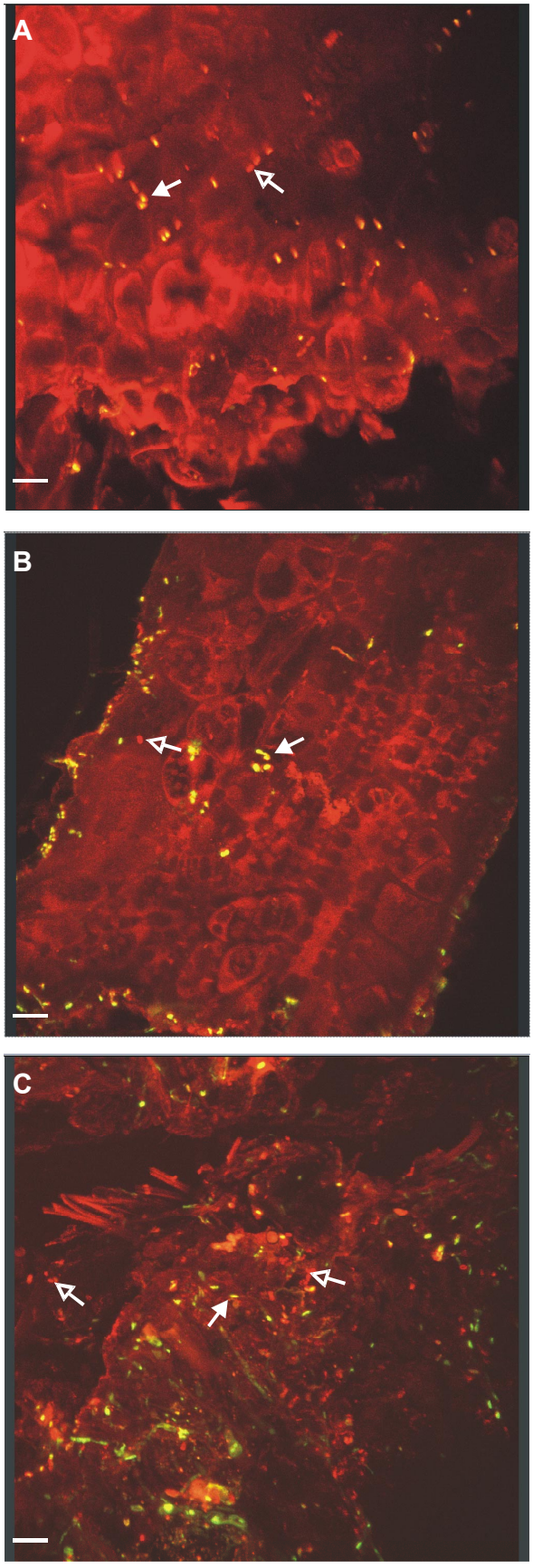


Figure 4.6. *Percentage of viable* E. coli *O157:H7 cells attached to the floral tube wall of apples following treatment with 0 (shaded bars), 200 (open bars), or 2000 (solid bars) ppm active chlorine as influenced by depth into CSLM stacks.*

Figure 4.7. *CSLM photomicrographs showing the influence of treatment of apples with 0 (A, 26-μm depth), 200 (B, 24-μm depth), and 2000 (C, 32-μm depth) ppm active chlorine on the viability of* E. coli *O157:H7 cells attached to the wall of the floral tube. Yellow to orange cells are dead (closed arrowheads) and red cells are viable (open arrowheads). Bar = 10 μm..*


A
B
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of viable and dead cells at depths of 24 - 32 μm in the floral tube wall of apples treated with 0, 200, or 2000 ppm chlorine (Fig. 4.7A, 4.7B, and 4.7C, respectively).

Attachment and disinfection protection from the seed locules was examined. Of the seed locule samples examined by CSLM (n = 17), 44% harbored *E. coli* O157:H7. The mean number of cells associated with this structure, however, was 2.5 cells, preventing quantitative analysis.

Considering all surfaces, structures, and tissues examined, the mean percentage of viable cells located at each site after treatment with water or chlorine was in the order of the floral tube wall (20.5%) > lenticels (15.0%) > damaged cuticle surrounding puncture wounds (13.0%) > intact cuticle (8.1%), which also reflects the general order of protection against inactivation with chlorine.

DISCUSSION

This study confirms that the apple core is a structure that can harbor high numbers of *E. coli* O157:H7 when apples are inoculated by immersion in a suspension of the pathogen. Viability of cells residing within the core is not markedly reduced by treatment of apples with 200 or 2000 ppm chlorine. Results of CSLM analysis support evidence presented by others *(1, 4, 5, 13*) that the core of the apple is an area of great concern with regard to infiltration of bacteria and the resulting inaccessibility to contact with sanitizers. Injecting cores with chlorinated water had little effect on killing cells, indicating that high numbers of cells were located in the cavity of apples between the stem and the cuticle immediately surrounding it. Moreover, neither surface treatment nor recovery method (i.e., rubbing and washing) were effective in inactivating or removing cells from cores. Thus, the low numbers of viable *E. coli* O157:H7 cells that may have been present in puncture wounds after treatment, rubbing, and washing would not markedly influence the number recovered from the entire apple because high

numbers of viable cells remained in the stem end and calyx area after treatment. CSLM analysis also indicates that the damaged cuticle surrounding puncture wounds provides cells protection against inactivation by chlorine or removal by rubbing and washing.

Significant reductions in percentages of viable cells attached to the floral tube wall of apples following surface treatment with 2000 ppm were detected compared to reductions on apples washed with water, indicating that active chlorine penetrated through the calyx to inactivate cells residing in the floral tube. The suffusion of the chlorine treatment solution into the floral tube may be influenced by the condition of the flower sepals. Fully intact, rigid sepals would inhibit or prevent infiltration of cell suspension in the inoculum and penetration of chlorine solution or water into the floral tube, whereas damaged or missing sepals may not prevent these processes. Neutralization of active chlorine resulting from contact with apple tissues within the floral tube might account for the observation of increased percent viability of cells at a depth 18 - 20 μm into stacks.

A reduction in number of viable *E. coli* O157:H7 cells located at increasing depths into lenticels was observed in all apples subjected to all treatments. Ursolic acid, a triterpene known to be a major constituent of natural wax formed on the surface apples (*8*), has been reported to have a bactericidal effect on *E. coli* (*6*). Loosely packed wax platelets, especially those located near the openings of lenticels, offer a large surface area for contact between wax components and treatment solutions. *E. coli* O157:H7 cells within these structures may be exposed to solubilized ursolic acid in addition to chlorine. Depending on the topography of the lenticel, cells located 0 - 2 μm from the lenticel surface are likely to be more susceptible to the surface treatment. As the depth of attached cells increases, contact with ursolic acid may result in lethality. The topography of lenticels is extremely irregular, consisting of numerous crests and crevices within which pockets can harbor *E. coli* O157:H7. These discontinuous

structures may account for anomalies observed in the trends of percentages of viable cells located at various depths in lenticels.

Puncture wounds were made in apples before inoculation to determine if *E. coli* O157:H7 attached to the cut tissue was afforded protection against surface treatments. In addition to the apple skin, parenchyma tissue, located under the cuticle, was disrupted upon the creation of wounds. Thus, there existed, along this damaged tissue, a pH gradient from the cuticle (pH 7.0) to the parenchyma tissue (pH 4.1). On damaged cuticle from apples treated with 200 ppm, at a depth of 0 - 2 µm, only 2.1% of the *E. coli* O157:H7 cells were viable. Cells located within this region were more susceptible to inactivation by chlorine. As the depth into stacks increased to 6 - 8 µm, an increase in percent viability was observed. At this depth, cells may be protected from the chlorine treatment, but not exposed to an acidic environment sufficient to cause death. At 12 - 14 and 18 - 20 µm within stacks, a slight decrease in percent viability was observed, perhaps indicative of the effects of the low pH in these microevironments in addition to limited or no contact with hypochlorous acid. Regardless of treatment, however, the populations of viable cells increased at a depth of 24 - 26 µm, where active chlorine had not penetrated and cells, while experiencing stress due to the low pH, remained viable.

This study demonstrates that *E. coli* O157:H7 cells attached to the floral tube wall, lenticels, and damaged cuticle and tissue surrounding puncture wounds may evade disinfection of whole apples treated with 2000 ppm active chlorine. Further investigation is warranted to study the influence of other chemical treatments, including surfactants and solvents in combination with other antimicrobials, for their efficacy in inactivating or removing *E. coli* O157:H7 and other pathogens that may be present on apples and other raw produce. In addition, observations made on the Red Delicious cultivar need to be confirmed by investigating decontamination treatments applied to

other apple cultivars used in the manufacture of fresh apple cider or intended for the fresh produce market.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Outbreaks of disease caused by *E. coli* O157:H7 associated with the consumption of unpasteurized apple cider have raised interested among scientists to develop more efficacious methods to remove or kill pathogens associated with apple tissues. A limitation in the effectiveness of sanitization treatments in reducing populations of pathogens on produce surfaces is the likelihood that microorganisms are able to infiltrate tissues and are thus afforded protection from sanitizers.

Confocal scanning laser microscopy (CSLM) was used to characterize sites of attachment of *E. coli* O157:H7 cells on whole apples, to determine the influence of temperature differentials on the extent on infiltration of cells into apple tissues, and to differentiate viable and non-viable cells of the pathogen on apple tissues following treatment with water and 200 or 2000 ppm active chlorine solution. Dead *E. coli* O157:H7 cells on apple tissues were detected using SYTOX Green, a membrane impermeable nucleic acid stain, and both dead and viable cells were detected using an affinity purified antibody to *E. coli* O157:H7 tagged with a fluorescent dye, Alexa Fluor 594.

Results showed preferential cellular attachment of *E. coli* O157:H7 cells to discontinuities in the waxy cuticle on the surface and to damaged tissue surrounding puncture wounds, where the pathogen was observed at depths up to 70 μm below the skin surface. Attachment to lenticels was sporadic, but occasionally observed at depths of up to 40 μm. Infiltration through the floral tube and attachment to seeds, cartilaginous pericarp, and internal trichomes were observed in all apples examined. A negative temperature differential enhanced infiltration of *E. coli* O157:H7 cells into five of the eight structures analyzed.

The mean percentage of viable cells located at each site after treatment with water or chlorinated water was in the following order, which also reflects the order of protection against inactivation: floral tube wall (20.5%) > lenticels (15.0%) > damaged cuticle surrounding puncture wounds (13.0%) > intact cuticle (8.1%). The location of

viable cells within tissues was dependent upon the structure. With the exception of lenticels, the percentage of viable cells increased as depth into CSLM stacks increased, indicating that cells attached to subsurface structures were better protected against inactivation with chlorine than were cells located on exposed surfaces. Results suggest that *E. coli* O157:H7 cells attached to internal core structures or within subsurface tissues may evade treatment of up to 2000 ppm active chlorine. Further research is warranted to investigate the efficacy of other chemical santizers as well as the influence of surfacants and solvents in combination with sanitizers for efficacy in removing or killing *E. coli* O157:H7 lodged in protective structures on the surface and within apple tissues.