EVALUATION OF INOCULATION METHOD AND INOCULUM DRYING TIME FOR THEIR EFFECTS ON SURVIVAL AND RECOVERY OF FOODBORNE PATHOGENS INOCULATED ONTO RAW VEGETABLES AND TREATED WITH CHLORINE

by

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(Under the Direction of Larry R. Beuchat)

ABSTRACT

This study evaluated methods for inoculation and inoculum drying time on survival and recovery of foodborne pathogens on the surface of tomatoes, iceberg lettuce, and parsley. Five-strain mixtures of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* were applied to produce by dip, spot, or spray inoculation. Inocula were dried for 1 or 24 h at 22°C on tomatoes or 2 h at 22°C and 2 h followed by 22 h at 4°C on lettuce and parsley before being treating with water or chlorine (200 µg/ml). Results indicate that inoculation method, drying time, and treatment affect survival and/or recovery of pathogens inoculated onto the surface of tomatoes, lettuce, and parsley. It is recommended that spot inoculation be used for applying pathogens to produce and drying times be 24 h at 22°C for tomatoes, and 2 h at 22°C followed by 22 h at 4°C for lettuce and parsley before chlorine treatment is applied.

INDEX WORDS: *Escherichia coli* O157:H7, *Salmonella*, *Listeria monocytogenes*, inoculation method, drying time, tomatoes, lettuce, parsley, chlorine

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DEDICATION

To my mom and dad for instilling in me the value of education and to my husband, Edward, for all his love and support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

In 1991, the United States and the National Cancer Institute implemented the national campaign, “5 a Day for Better Health”, aimed at increasing consumption of fruits and vegetables. In response to this campaign, the American consumer has adopted a healthier diet consisting of more fruits and vegetables than in the past (Hedberg et al., 1994). Consumers are also eating more varieties of fruits and vegetables, benefiting from year round availability of produce that until recently were considered seasonal (EC, 2002). In 1998, consumers can could choose from an average of 345 different produce items in a typical supermarket, compared to 173 in 1987 (Litwak, 1998). Globalization of the produce industry has made this possible, along with changing horticultural practices (EC, 2002). From 1987 to 1997, the fresh produce market has increased from $34.6 billion in retail and foodservice sales to $70.8 billion. (Kaufman et al., 2000). However with this increased growth new challenges concerning microbiological hazards associated with fresh fruits and vegetables have surfaced (Beuchat, 2001).

According to data complied by the Centers for Disease Control and Prevention (CDC) the number of produce-associated outbreaks remained constant between 1988 - 1992 and 1992 - 1997; however, the number of cases of foodborne disease associated with produce increased 5-fold during this same period (Bean et al., 1997; Olsen et al., 2000). This may be due in part to increased consumption of contaminated produce. Of greatest concern is the number of cases of enteric infections implicating produce. According to data compiled by the CDC, between 1992 – 1997, *Escherichia coli* O157:H7 and *Salmonella* accounted for approximately 61% of the outbreaks associated with the consumption of produce (Olsen et al., 2000).

This literature review focuses on examining the importance of fresh produce, specifically vegetables, as vehicles in the transmission of human pathogens and their association with outbreaks of foodborne disease. The need to establish a standardized protocol for determining the survival characteristics and enumerating bacterial pathogens on fresh produce will be discussed.

Foodborne Illness Associated with Produce

According to data released in 2002 by the CDC, there are approximately 76 million cases of foodborne illness each year, which cause 325,000 hospitalizations, 5,000 deaths, and a multibillion-dollar economic damage. The majority of outbreaks of foodborne illness have been historically associated with undercooked meat, poultry, seafood, or unpasteurized milk (Tauxe, 1997). Recently, however, new vehicles of foodborne disease have emerged. One important feature of these vehicles is that contamination of implicated products usually occurs in the early stages of production, in contrast to past views that the majority of contamination of food products was thought to occur just before consumption (Tauxe, 1997). Fresh produce is a commodity that historically has been thought to be microbiologically safe except for occasions where cross-contamination from products of animal origin has occurred. However, this view has changed in recent years as the number of outbreaks of foodborne illness linked to fresh produce has increased (Bean and Griffin, 1990; CDC, 1990; CDC, 2000).

Globalization of the food market, along with a shift from small producers to mass production and complex distribution networks, has had an effect on the epidemiology of foodborne illness outbreaks. In the past, outbreaks usually involved well defined groups with high attack rates and short incubation periods (Hedberg et al., 1994). However, with more produce coming from fewer producers, outbreaks are now not limited to a well-defined group or a specific location and have low attack rates (Hedberg et al., 1994; NACMCF, 1999). This makes it more difficult to trace back to the source of contamination responsible for the outbreak. Moreover, the exposure to contaminated food can be subtle. For instance, garnishes like green onions on a baked potato or parsley on the side of plates that may go unrecognized by the consumer, but have been associated with foodborne illness (NACMCF, 1999). Complicating the issue further is that in the cases of produce related outbreaks, it is difficult to pin point the source of contamination, i.e,. the source of contamination may involve looking at windblown sources and possible problems that may be caused by flooding or other weather related problems (Guzewich and Salsbury, 2001). In these situations it is more complicated to find and correct the problem than it is to find a sick person or animal and provide medicine.

Fresh fruits and vegetables are susceptible to contamination by a wide variety of pathogenic microorganisms (NACMCF, 1999). Therefore strong associations between pathogens and produce have not been established. This is unlike outbreaks associated with dairy and meat products where there is likely one or two microorganisms are strongly associated with a certain food, e.g., *E. coli* O157:H7 with ground beef or *Salmonella* and *Camplylobacter* with chicken. Table 1.1 lists characteristics of the microorganisms most commonly associated with outbreaks of foodborne illness outbreaks linked to fresh produce.

Table 1.1. Characteristics of pathogenic microorganisms linked to produce related foodborne illness outbreaks.[a]

Microorganism	Incubation period	Symptoms	Infectious dose	Source
Bacterial Species				
Clostridium botulinum	12 to 36 h	Nausea, vomiting, fatigue, dizziness, dryness of mouth and throat, muscle paralysis, difficulty swallowing, double or blurred vision, drooping eyelids, and breathing difficulties	Intoxication growth and toxin production in food	Soil, lakes, streams decaying vegetation reptiles
Escherichia coli O157:H7	2 to 5 d	Bloody diarrhea, abdominal pain, can lead to hemolytic uremic syndrome, kidney failure, and throbocytopenia purpura especially in children and the elderly	10 to 1000 cells	Animal feces especially deer, cattle, and human, cross - contamination from raw meat and contaminated water used in processing of produce
Salmonella spp.	18-72 h	Abdominal pain, diarrhea, chills, fever, nausa, and vomiting	10 to 100,000 cells	Animal and human feces; cross-contamination from raw meat, poultry, or eggs; contaminated water used in processing of produce
Shigella	1 to 3 d	Abdominal pain, diarrhea, fever, and vomiting	As few as 10	Human feces

(Continued)

Table 1.1 (continued)

Microorganism	Incubation period	Symptoms	Infectious dose	Source
Listeria monocytogenes	1 d to 5 or more wk	Febrile gastroenteritis in healthy in healthy adults, may lead to spontaneous abortion or stillbirth in pregnant women; severe septicemia and meningitis in neonates and immunocompromised adults; mortality may be 20 to 40%	Unknown	Soil, food processing environments
Parasitic Species				
Cryptosporidium spp.	1 to 12 d	Profuse watery diarrhea, abdominal pain, anorexia, and vomiting	As few as 30	Animal and human feces
Cyclospora spp.	1 to 11 d	Watery diarrhea, nausea, anorexia, and abdominal cramps	Unknown, probably low	Unknown at this time
Viruses				
Hepatitis A	25 to 30 d	Fever, malaise, anorexia, nausea abdominal pain, jaundice, and dark urine	10 to 50 viral particles	Human feces and urine
Norwalk/Norwalk-like	12 to 48 h	Vomiting diarrhea, malaise, fever, nausea, and abdominal cramps	Unknown, probably low	Human feces, vomitus

[a] Adapted from IFT (2001).

Given the national trend towards consumption of fresh fruits and vegetables, outbreaks are likely to occur more frequently in the future (Hedberg et al., 1994). Shown in Table 1.2 are examples of outbreaks of foodborne illness associated with vegetables from 1990 to 2000. Although only one outbreak of vegetable-associated listeriosis was reported during this period, there have been outbreaks reported in the past. In 1981, an outbreak of listeriosis was attributed to coleslaw containing *Listeria monocytogenes* in which the implicated food product was cabbage (Schlech et al., 1983). Other vegetables implicated in outbreaks of listeriosis include lettuce, celery, and tomatoes (Ho et al., 1986).

Although increased consumption of fresh produce is not directly correlated with the increased number of outbreaks of foodborne illness outbreaks linked to produce, it is one factor, along with others such as improved surveillance and changes is social demographics (Beuchat, 2002). Globalization of the food market, may be another factor impacting the safety of fresh produce (Beuchat, 1996; Brackett, 1999; Beuchat, 2002).

Microbial Contamination of Produce

A significant number of the bacteria present on freshly harvested vegetables are those that are present on vegetables in the field (Lund, 1992). Vegetables crops are grown in environments open to multiple sources of contamination, the main sources being air, soil, and water (Nguyen-the and Carlin, 2000). The relative importance of these sources depends on the structural entity of the plant, e.g., leaves have the greatest exposure to air, while root crops have a greater exposure to soil (ICMSF,1999).

Table 1.2. Examples of foodborne illness associated with fresh vegetables from 1990 – 2002.

Vegetable	Pathogen	Number of cases	Number of deaths	Year	Location	Reference
Basil	*Cyclospora cayatenensis*	>308	0	1997	U.S.[a]	CDC, 1997a
	Cyclospora cayatenensis	59	Unknown	1999	U.S.	Guzewich and Salsbury, 2001
Cabbage	*Vibrio cholerae*	UK	71	1991	Peru	Swerdlow et al., 1992
Carrots (shredded)	*Escherichia coli*	168	0	1993	U.S.	CDC, 1994; Wei et al., 1995; Tauxe, 1997
Cilantro	*Salmonella* Thompson	76	0	1999	U.S.	Campbell et al., 2001
Coleslaw	*E. coli* O157:H7	33	0	1998	Indianapolis	Griffin and Tauxe, 1999
Corn	*Listeria monocytogenes*	1566	0	1997	Italy	Paolo et al. 2000
Green onions	*Shigella flexneri* 6A	72	0	1994	U.S.	Tauxe, 1997
	Cryptosporidium parvum	54	0	1997	Washington	CDC, 1998a
Lettuce	*Shigella sonnei*	110	0	1994	Norway Sweden Spain, U.K.	Kapperund et al., 1995
	E. coli O157:H7	70	0	1995	U.S.	Ackers et al., 1998

(Continued)

Table 1.2. (continued)

Vegetable	Pathogen	Number of cases	Number of deaths	Year	Location	Reference
Lettuce	*Campylobacter jejuni*	14	0	1996	Oklahoma	CDC, 1998
Lettuce (iceberg)	*E. coli* O157:H7	30	0	1995	Maine	CSPI, 2000
	E. coli O157:H7	23	0	1995	Canada	Preston et al., 1997
Lettuce (mesclun)	*E. coli* O157:H7	49	0	1996	U.S.	Hilborn et al., 1999; Tauxe, 1997
	Cyclospora cayatenensis	>91	0	1997	Florida	Herwaldt and Beach, 1999
Lettuce (romaine)	*E. coli* O157:H7	21	0	1995	Idaho	CSPI, 2000
Mamey	*S.* Typhi	13	0	1998-1999	U.S.	Lund and Snowdon, 2000
Parsley	*Shigella sonnei*	>400	0	1998	U.S.	CDC, 1999
Salad	*E. coli*	2	0	1998	California	Griffin and Tauxe, 1999
Sprouts (alfalfa)	*S.* Bovismorbificans	595	0	1994	Sweden Finland	Ponka et al., 1995
	S. Newport	154	0	1995	Denmark	Feng, 1997

(Continued)

Table 1.2. (continued)

Vegetable	Pathogen	Number of cases	Number of deaths	Year	Location	Reference
Sprouts (alfalfa)	*S*. Stanley	>272	0	1995	U.S.,Canada, Finland	Mahon et al., 1997
	S. Newport	133	0	1995-1996	BritishColumbia Canada, U.S. Denmark	Feng, 1997
	S. Kottbus	23	0	2001	U.S.	CDC, 2002
	S. Montevideo *S*. Meleagridis	>500	1	1996	California[a]	Taormina et al.,1999
	S. Meleagridis	124	0	1997	Canada	Feng, 1997
	S. Infantis *S*. Anatum	109	0	997	U.S.	Feng, 1997 Taormina et al.,1999
	E. coli O157:H7	108	0	1997	U.S.	CDC, 1997b
	S. Senftenberg	52	0	1997-1998	U.S.	Toarmina et al., 1999
	E. coli O157:NM	8	0	1998	California	Taormina et al., 1999
Sprouts (bean)	*S*. Enteritidis	27	0	2000	Netherlands	van Duynhoven et al., 2002
Sprouts (radish)	*E. coli* O157:H7	6561	3	1996	Japan	Michino et al., 1999
	E. coli O157:H7	126	0	1997	Japan	Feng, 1997

(Continued)

Table 1.2. (continued)

Vegetable	Pathogen	Number of cases	Number of deaths	Year	Location	Reference
Tomatoes	*S.* Javiana	174	0	1990	U.S.	Tauxe, 1997; Beuchat, 1996b
	S. Montevideo	84	0	1993	U.S.	Lund and Snowdon, 2000
	S. Baildon	86	Unknown	1993	U.S.	Cummings et al., 2001
Tomatoes (diced)	Hepatitis A	92	0	1994	Arkansas	Lund and Snowdon, 2000

[a] If more than one state involved in an outbreak, then the location is listed as U.S.

Human activities can also influence the microbial composition of vegetables (ICMSF, 1998; Beuchat, 1996; Nguyen-the and Carlin, 2000). The result may be the control of microorganisms in situations where pesticides are applied to control insects, the pesticides may be detrimental to the microorganisms as well (ICMSF, 1998). Cultivation of plants by hand or mechanically can introduce and/or distribute microorganisms to areas not previously contaminated (ICMSF, 1998).

The presence of microorganisms on harvested vegetables depends on their capacity to survive and multiply on the plant before harvest (Nguyen-the and Carlin, 2000). Studies have shown that the number mesophilic bacteria on vegetables ranges from 10^2 to 10^8 cfu/g, depending on the type of vegetable (Nguyen-the and Carlin, 2000). The highest populations are normally found on salad sprouts, whereas the lowest population occur on vegetable and fruits or on the inner leaves of cabbage (Nguyen-the and Carlin, 2000). The mean populations of molds on fresh vegetables range from 10^3 to 6.7×10^4 cfu/g (ICMSF, 1998). This range reflects a unique combination of composition and physical characteristics of vegetables, growing and harvesting practices, cooling temperatures, and environments that dictate the ability of microorganisms to attach and survive (NACMFC, 1999). Intrinsic properties that affect bacterial growth on vegetables include pH, type of organic acids, availability of nutrients, and the presence of anti-bacterial compounds (Lund, 1992). There are also vast differences in surface morphology, internal tissue composition, and metabolic activities of leaves, stems, florets, fruits, roots, and tubers that provide a wide range of ecological niches that can selectively support specific groups or species of microorganisms (Beuchat, 2002). Extrinsic factors that influence bacterial growth on vegetables include storage temperature, the amount of free water, relative humidity, and the gaseous environment (Lund, 1992). The most prevalent types of microorganisms associated with vegetables are those responsible for spoilage (Beuchat, 2002).

The association of microorganisms with fresh produce is not limited to the external surfaces of produce. They can also be found associated with the internal tissues of fresh produce. The ability of microorganisms to be internalized in the tissue of produce depends on the type and variety of crop (Samish et al., 1962). They found high populations of bacteria internalized in cucumbers were found close to the periphery, and lower populations were found in the central core. In tomatoes, high populations were found close to the stem scar and central core, and lower populations where found in the periphery. The process by which microorganisms become internalized in the tissues of fresh produce can vary, but regardless of how they become internalized, those microorganisms that are able to persist usually survive as harmless commensals (Samish et al., 1963). However, occasionally pathogens can be introduced onto and into produce through various routes throughout the pre- and post-harvest system (Beuchat, 2002; Brackett, 1999; Heard, 1999: NACMCF, 1999).

Generally, fresh vegetables that have not been exposed to human or animal wastes do not contain human pathogens (ICMSF, 1998). Potential sources of microbial contamination during the pre-harvest period include soil, feces, irrigation water, water used to apply fungicides and insecticides, dust, insects, inadequately composted manure, wild and domestic animals, and human handling (Beuchat, 2002; Brackett, 1999). The sources of contamination in two foodborne outbreaks of *Shigella* dysentery associated with lettuce have been epidemiologically linked to pre-harvest contamination (Martin et al., 1986; Kapperud et al., 1995). Martin et al. 1986, stated that contamination of the lettuce occurred as a result of application of contaminated irrigation water from incompletely treated sewage, fertilization with sewage sludge or manure contaminated with human feces, or accidental flooding of agricultural land with polluted water after a heavy period of rain. The source of the contamination in a second outbreak was thought to have occurred due to use of partially-treated sewage effluent to fertilize the lettuce or to a sewer leak in a warehouse where the lettuce was stored (Kapperud et al., 1995). Free-range chickens and manure from cattle in a farm processing shed may have been sources of *E. coli* O157:H7 in water used to irrigate lettuce associated with an outbreak of infections in 1996 (Hilborn, et al., 1999). Wachtel et al. (2002), also concluded that irrigation water containing pathogenic microorganisms can be a source of contamination of produce. Gagliardi et al. (2003), examined on farm and post harvesting processing conditions for cantaloupe and found high levels of fecal coliforms and fecal enteroccoci present in furrow soil and in standing water in fields as well as contamination of water in an irrigation pound adjacent to fields used for crop production, indicating possible sources of the fecal coliforms found on the cantaloupes. The potential for water to be vehicle of transmission is not surprising since contamination of water with pathogenic *E. coli*, *Salmonella*, *Vibrio cholerae*, *Shigella*, *Cryptosporidium parvum*, *Giardia lamblia*, *Cyclsospora cayetanensis*, *Toxiplasma gondii*, Norwalk-like viruses, and Hepatitis A virus has been documented (FDA, 1997).

One factor that can influence the microbial quality of fresh produce, but is often overlooked, is the history of the land (Brackett, 1999). Land that has been used to graze livestock and wild animals is more likely to be contaminated with enteric pathogens

(Tauxe, 1997). History of flooding can also be a potential source of contamination of agricultural land used for produce production. Contamination of soil can occur when floodwater covers areas where animals have grazed. This can cause the floodwater to become polluted with animal waste, which can be carried downstream and contaminate croplands (Brackett, 1999). The flooding of croplands with contaminated water may deposit microorganisms that can remain for months and years after flooding (Beuchat and Ryu, 1997).

Soil also has the potential to be a source of contamination, because it is a natural reservoir for human pathogens such as *L. monocytogenes* and *Clostridium botulinium* (Beuchat, 1996). *Listeria* was found in 108 of 115 (93.9%) sewage samples tested in a study in the United Kingdom; untreated sewage, treated sewage, and sludge were tested (MacGowan, et al., 1994). This study also revealed that 20 of 136 (14.7%) soil samples were positive for *Listeria* spp. Guo et al., (2002) showed the ability of *Salmonella* to survive in soil under laboratory conditions at 22 ± 1°C for up to 45 days. This may indicate that if contamination of soil occurs, pathogens may be able to persist for an extended period of time. The ability of *Salmonella* to persist may increase exposure of produce to pathogens and therefore increasing the probability for contamination. This study also showed that contaminated soil is a potential source of contaminates on tomatoes in contact with soil.

The use of animal manure as a crop fertilizer can be a potential source of pathogen contamination of fresh produce. The agricultural advantages of using animal manure as a source of nitrogen is well established. Likewise the ability of manure to transfer pathogens to crops and cause human disease is also recognized. The accepted mode of preventing the spread of pathogens from manure to produce is by not using raw or untreated manure (IFT, 2001). However, this does not prevent raw manure from being used. In a survey by the USDA (2001) it reported that of all the manure users, 22% of fruit growers and 15% of vegetable growers used untreated manure. The use of untreated manure can impact large areas of land if contaminated run-off or irrigation waters become contaminated and are then applied to crops. The prevalence and type of pathogens in manure varies greatly depending on the animal source and the health of the animal (IFT, 2001).

Further evidence of the potential for contamination of produce from the pre-harvest environment is presented in a study by Janisiewicz et al. (1999). They determined that fruit flies can act as vehicles for the transfer *E. coli* O157:H7 from one apple to another. It is not hard to imagine this situation in the field where various types of insects could serve as vehicles for cross contamination of crops in adjacent fields.

In addition to surface contamination of produce with pathogens, the potential for pathogens to become internalized in tissues should also be considered as a mode of contamination. Beuchat and Brackett (1991) studied the ability of *L. monocytogenes* to survive after being inoculated in to raw tomatoes and processed tomato products. They determined that *L. monocytogenes* was unable to grow in tomatoes, but was able to survive, although decreasing in population when stored for 20 days at 10 and 21°C. The ability of the pathogen to survive was affected by temperature, as seen by a significant decrease in the rate of death at 10°C compared to 21°C. Zhuang et al. (1995) described the survival characteristics of *Salmonella* Montevideo in raw tomatoes. They determined that populations of *S.* Montevideo in tomatoes increased when stored for 22 h at 20 or 30°C.

Poor worker hygiene is also a possible cause of contamination of produce. Investigations of outbreaks of foodborne illness investigators have shown that employees failed to wash their hands after using the bathroom and instances where sick employees were allowed to work (Guzewich and Salsbury, 2001).

Post-harvest contamination sources include feces, human handling, harvesting equipment, transport containers, wild and domestic animals, insects, dust, rinse water, ice, transport vehicles, and processing equipment (Beuchat, 2002). In 1986, an outbreak of shigellosis was traced back to lettuce shredded at a processing plant after intact lettuce from the same supplier was not found to be contaminated (Davis et al., 1988). An investigation into the outbreak suggested the contamination occurred via a food handler and that the method of processing had been the source of cross contamination (Davis et al., 1988). In an investigation into the effects of processing on total microbial counts, Garg et al. (1990) found that shredders and slicers used in processing can be responsible for dispersing microorganisms. Water can also be a source of contamination in the post-harvest environment. Untreated water used in a hydrocooling system and to make ice that was used for packing parsley was the likely source of contamination in an outbreak that occurred at 7 locations (CDC, 1999). The study by Gagliari et al. (2003) also showed in their study of pre and postharvest conditions for cantalopes the presence of fecal coliforms and fecal enteroccoci increased in samples after post harvest processing. The increase in the presence of fecal coliforms and fecal enteroccoci was attributed to a primary washing tank or hydrocooler. Wachtel and Charkowski (2002) found that one piece of contaminated lettuce has the potential to contaminate all other pieces of lettuce processed as the same time. They found that combining of one piece of inoculated lettuce with uninoculated lettuce in water resulted in contamination of 100% of the uninoculated lettuce.

Internalization of pathogens from contaminated wash water is another source of post-harvest contamination (Burnett et al., 2000). Several researchers have described the potential for contaminated flume and wash water as likely vehicles of bacterial ingress of produce tissues including those of apples, oranges, and grapefruit (Beuchat et al., 1998; Buchanan et al., 1999). Burnett et al. (2000) determined that attachment or infiltration of *E. coli* O157:H7 on and into apples was greater under conditions where a negative temperature differential existed. In the post-harvest environment the potential for a negative temperature differential exists in instances where wash water has a lower temperature than the produce. If water is contaminated, there is greater potential for internalization of the pathogens into produce.

Development of a Standardized Method

Fresh or minimally processed produce is not subjected to treatments designed to prevent survival or growth of pathogens (Tauxe, 1997). The need to develop a method(s) to detect and enumerate human pathogens on fresh or minimally processed produce is therefore critical. Much research has been done to evaluate the efficacy of sanitizers in removing or killing pathogens on fresh produce and to determine conditions affecting survival and growth during storage. However, the results from these studies are difficult to compare because of the numerous methodologies being employed and because of incomplete reporting of these methods (Beuchat, et al., 2001). Variations in procedures include the way inocula are prepared and applied to produce, conditions for treatment and storage, the procedure for retrieving and enumerating pathogens, and how counts are reported (Beuchat et al., 2001). Table 1.3 gives examples of methodologies used to study the growth and enumeration of pathogenic microorganisms on fresh vegetables.

In order to evaluate sanitizers for their effectiveness in killing or removing pathogens, there first must be uniformity in the decision of which microorganism(s) to use and the method to prepare cultures. As evident in Table 1.3, there is not only great variation in the number of strains used in different studies, but also in the buffer used for inoculation. The use of standard, well-characterized, reference strains would greatly benefit the assessment of a basic method (Beuchat et al., 2001). This is because there are differences in genetic and phenotypic characteristics within different species in regard with their ability to adhere, colonize, and grow in unique environmental niches. In order to mimic more closely the microflora of fresh produce, strains should be selected that were isolated from produce or patients with illness associated with the consumption of produce. It would also be advantageous to use more than one strain of a particular pathogen. Beuchat et al. (2001) advocates using a cocktail consisting of at least five strains of the same pathogen. The reason for this is that if there may be differences in the ability of strains to survive, grow, or resist treatment with sanitizers (Beuchat et al., 2001). In situations

Table 1.3. Examples of methods used in studies to evaluate conditions affecting growth and the removal of pathogenic microorganisms from fresh vegetables.

Produce									
Type	Cut or Whole	Inoculation method	No. of strains	Buffer	Drying method	Sample weight (g)	Sample volume (ml)	Unit	Reference
Asparagus	Whole	Spot/0.1 ml	5,6[a]	Peptone	2-3 h	100	100	CFU/ml	Park and Beuchat, 1999
	Whole	Dip/1 min	2	KP buffer[b]	Drained	50	200	CFU/g	Berrang et al., 1989
Broccoli	Sliced	Dip/1 min mixed	3	Peptone	Drained 1 min	25	225	CFU/g	Richert et al., 2000
	Fresh cut	Dip/1 h	1,1,1	Peptone	Not done	25	225	CFU/g	Wang et al.,2001
	Whole	Dip/1 min	2	KP buffer	Drained	50	200	CFU/g	Berrang et al., 1989
Cabbage	Shredded	Dip/15 min mixed	1	BPB[c]	Drained	50	450	CFU/g	Satchell et al., 1990
Cucumber	Sliced	Dip/1 min mixed	5	SDW[d]	Drained	50	200	CFU/g	Abdul-Raouf et al., 1993
	Sliced	Dip/1 min mixed	3	Peptone	Drained 1 min	25	225	CFU/g	Richert et al., 2000
	Whole	Dip/1 min mixed	3	Peptone	Drained 1 min	25	100	CFU/ml	Richert et al., 2000
Endive	Leaves	Dip/10 min	3	Sterile water	AP[e]	Leaves	6	CFU/g	Carlin et al., 1996;
Green pepper	Sliced	Dip/1 min mixed	3	Peptone	Drained 1 min	25	225	CFU/g	Richert et al., 2000
	Whole	Dip/1 min mixed	3	Peptone	Drained 1 min	whole	100	CFU/ml	Richert et al., 2000
	Cut	Spot/0.1 ml	1	TSB[f]	2 h	whole	50	CFU/g	Han et al., 2001a
	Cut	Spot/0.1 ml	1	TSB	2 h	5	50	CFU/5g	Han et al., 2001b
Lettuce	Shredded	Dip/1 min mixed	5	SDW	Drained	50	200	CFU/g	Abdul-Raouf et al., 1993
	Shredded	10 ml/300 g	1	TSBN	NA	25	225	CFU/ml	Weissinger et al., 2000
	Leaves	Dip/10 min	1	Sterile water	AP	1 Leaf	6	CFU/g	Li et al., 2001
	Leaves	Dip/1 min mixed	6,4,5	KP buffer[d]	18-22 h	50	50	CFU/cm^2	Beuchat et al., 2001
	Leaves	Spot/0.5 ml	5	Peptone Feces slurry	16-18 h	50	200	CFU/g	Beuchat et al., 1999

(Continued)

Table 1.3 (continued)

Produce									
Type	Cut or Whole	Inoculation method	No. of strains	Buffer	Drying method	Sample weight (g)	Sample volume (ml)	Unit	Reference
Lettuce	Leaves	dip/1 min shaken	3	SDW	1 h	10	90	CFU/g	Singh et al., 2002
	Leaves	Drop/0.01 ml	3	SDW	1 h	10	90	CFU/g	Singh et a., 2002
	Leaves	Sprinkle/1 ml	3	SDW	1 h	10	90	CFU/g	Singh et al., 2002
Mixed Salads	Cut	Injected	1	BHIB[g]	NA	25	225	CFU/g	Garccia-Gimeno et al., 1996
Tomato	Cut	Spot/0.1 ml	3	Sterile saline	Not done	20	180	CFU/g	Asplund and Nurmi, 1991
	Chopped	1ml/50 g	1	KP buffer	NA	50	50	CFU/g	Zhuang et al., 1995
	Chopped	10 ml/1000 g	2	KP buffer	NA	50	200	CFU/g	Beuchat and Brackett., 1991
	Diced	10 ml/300 g	1	Tomato juice	NA	25	225	CFU/ml	Weissinger et al., 2000
	Whole	Dip/1 min mixed	6,5,4	KP buffer	18-22 h	Whole	20	CFU/cm^2	Beuchat et al., 2001
	Whole	Dip	2	KP buffer	Not done	50	200	CFU/g	Beuchat et al., 1991
	Whole	Spot/0.025 ml	1	SDW	12 h	Spot	5	CFU/ml	Lukaski et al., 2001
	Whole	Spot/0.05 ml	5	PBS[h]	2 h	Whole	20	CFU/tomato	Guo et al., 2002
	Whole	Spot/0.1 ml	5,5,5	Peptone	1 h	Whole	50	CFU/ml	Venkitanaraynan et al., 2000
	Whole	Dip/2 min mixed	1	KP buffer	4 h	Whole	20	CFU/cm^2	Zhuang et al., 1995
	Whole	Spot/0.05 ml	5	5% HS[i]	75-105 min	Whole	195	CFU/tomato	Harris et al., 2001

[a] More than one genus of bacteria used; numbers indicate the strains used in each genus specific cocktail.

[b] Potassium Phosphate Buffer.

[c] Butterfields Phosphate Buffer

[d] Sterile Deionized Water.

[e] Absorbent Paper.

[f] Tryptic soy broth

[g] Tryptic soy broth supplemented with nalidixic acid

[h] Brain Heart Infusion Broth.

[I] Phosphate Buffered Saline.

[j] 5% Horse Serum.

where differences occur, the more robust strains will survive. The use of a single strain that has not been evaluated against other strains for its ability to withstand chemical decontamination treatments has the potential to give an inaccurate view of the sanitizer's effectiveness in reducing populations of pathogens.

The method for preparing cultures and the carrier used to apply the inoculum can have an effect on the ability of microorganism to attach on the surface of produce. The most common carriers used to prepare cell suspensions for inoculation of produce are 0.1% peptone and potassium phosphate buffer; however, various other carriers have been used. The limitation of using these carriers is that they do not simulate the highly organic nature of carriers in which pathogens are more likely trapped in or on contaminated produce (Beuchat et al., 2001). The most common sources of contamination are dust, aerosols, rain water, irrigation water, sewage, soil, feces, decayed plant material, contact surfaces, and workers at any point from harvest to consumption (Beuchat, 1996). Considering these modes of contamination the most suitable carrier should contain organic material. The presence of organic material in the inoculum could protect microorganisms against exposure to sanitizers and provide nutrients for growth that are not present in inorganic salt's. Buffers with salts and other chemicals could, in fact, be detrimental to the cells (Beuchat et al., 2001). These compounds may injure the cells, making them more susceptible to decontamination treatments or altering their ability to persist on produce during storage.

Three methods of inoculating microorganisms onto fresh produce were in survival and elimination studies are spray inoculation, dip inoculation, and spot inoculation (Beuchat, 2002). The utility of any of the three methods varies, depending on application. For instance, if the suspected point of contamination in a commercial situation is an immersion process, then dipping the produce in the test cell suspension may be an appropriate means of inoculation (Beuchat et at., 2001). Spray inoculation resembles situations where contamination of produce could occur through the use of contaminated irrigation water, or if contaminated water is used in a hydrocooling system. The major problem with the utility of both the dip and spray inoculation methods is that the numbers of cells that actually are applied or adhere to a sample are unknown (Beuchat et al., 2001; Harris et al., 2001). The use of either a spray or dip method requires that more samples be analyzed, than with the spot inoculation method, to account for the large variability of inoculum that can occur among samples (Beuchat, 2001). The large variation in inoculum population can also lead to inaccurate evaluations of the efficiency of recovery or changes in populations during storage or after treatment with sanitizers or wash solutions (Beuchat et al., 2001). In the spot inoculation method, a known number of cells is applied in a known volume of inoculum to one or more locations on the vegetable (Beuchat et al., 2001; Harris et al., 2001). The utility (or limitation) of this method is that it only represents contamination that would occur from a point source, for example soil, workers hands, or contact with equipment (Beuchat et al., 2002).

There are various methodologies for drying the inoculum on the produce, although these methods have not been exclusively studied (Table 1.3). Beuchat et al. (2001) found that populations of *E. coli* O157:H7 greatly diminished when tomatoes were held at 22°C for more than 3 h after inoculation, but populations of *Salmonella* and *L. monocytogenes* were unaffected. If populations in inocula are determined by direct plating on an appropriate agar medium, then the number of microorganisms that actually undergo treatment could be significantly lower thereby leading to an overestimation of the efficiency of the sanitizer, or an underestimation of the survival of a pathogen. In situations where inoculated produce is drained, not subjected to drying, or dried for an insufficient amount of time, the microbial cells may not have adhered to the produce. In these situations, the efficacy of sanitizers may indicate that they are more effective than they actually are. Not allowing the inoculum to dry prior to storage will provide free water and could enhance the growth of pathogens on produce that normally would not support growth.

The lack of uniformity among sampling methods makes it difficult to compare results between research studies and to establish industry guidelines. However, it is unrealistic to believe that one method will be applicable for all produce, because there are large differences in sizes, shapes, and surface morphologies of fresh fruits and vegetables. For example, knowledge that populations of microorganisms on tomatoes are greater on the stem scar and central core (Samish et al., 1963) gives investigators a more confined surface area to evaluate the efficiency of decontamination treatments. This necessitates the use of a specific method with the understanding that modifications will be necessary to accommodate natural variations in produce.

There are various methods for detecting and enumerating pathgens on fresh produce. Though the optimum protocol for detecting and enumerating pathogens may differ, depending on the produce and pathogen being analyzed, there is a need to standardize various aspects of the method. Beuchat et al. (2001) suggest that the composition and pH of the diluent used along with a standardized ratio of diluent to sample be consistent within each fruit and vegetable group. Other aspects of detecting and enumerating of pathogens on fresh produce that they suggest be standardized are time and temperature used for preparing samples for plating or enrichment.

There are a multitude of complexities in reporting the efficacy of sanitizers for fresh produce. The diversity in types of fresh produce, naturally occurring microflora, and presence of organic matter necessitates that a sufficient number of replicates be incorporated into any experiment designed to test the efficacy of sanitizers (Beuchat et al., 2001). Also, previous methods of reporting reductions in populations in terms of CFU/g or CFU/cm^2 do not always provide accuracy in reporting reductions in populations on fresh produce (Beuchat et al., 2001). This is due to the vast variations in weight-surface area ratios of different types of produce. The development of a standardized method for reporting the efficacy of sanitizers that accommodates the variations between produce would benefit comparative assessment among laboratories.

Research Needs

Although, much attention has been given to survival, growth, and removal of pathogens on fresh produce, there are still key elements regarding methodologies that need to be addressed. The affects of different sample preparation methods have been evaluated (Burnett and Beuchat, 2001), but there are other aspects to sample analysis that need to be investigated. For example the composition of the wash fluid used in survival and sanitizer efficacy studies should be evaluated to eliminate differences in elution properties.

The effect of inoculation (dip, drop, and sprinkle) and washing methods have been studied (Singh et al., 2002). However, terminology used makes it difficult to compare results with other researchers who have used spot and spray method of inoculation. This study found that there is an effect of inoculation method on the recovery of pathogens from lettuce. One pathogen, *E. coli* O157:H7, on one produce, lettuce, was studied in order to develop a standardized method for testing the efficacy of sanitizers. Studies should be done that examine more than one pathogen on more than one produce type.

The effects of drying methods on the retention of cell viability and attachment on produce has been examined, but not extensively studied. The evaluation and selection of reference strains, and their subsequent characterization with regard to sensitivity or resistance to certain chemical treatments and drying procedures would greatly facilitate the move toward a establishing a standard method for determining the efficacy of sanitizers for reducing populations of pathogens from fresh produce. The utility of various inoculation methods has been reported; however, these methods need to be evaluated in order to establish a standard method that will be of value in determining the efficacy of sanitizers.

The objective of this research reported in this thesis was to develop a standard method for inoculating the surface of different vegetables with three pathogenic bacteria. The relationship between time of inoculation and retrieval on the viability and recoverability of pathogens was investigated. The effectiveness of chlorine to remove or kill pathogenic bacteria on the surface of raw vegetables was evaluated.

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CHAPTER 2

EVALUATION OF INOCULATION METHOD AND INOCULUM DRYING TIME FOR THEIR EFFECTS ON SURVIVAL AND RECOVERY OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA*, AND *LISTERIA MONOCYTOGENES* INOCULATED ONTO RAW TOMATOES, LETTUCE, AND PARSLEY AND TREATED WITH CHLORINE

Abstract

A study was undertaken to evaluate methods for applying inoculum and drying time after inoculation on survival and recovery of foodborne pathogens from the surface of raw, ripe tomatoes, iceberg lettuce leaves, and parsley. Five-strain mixtures of *Escherichia coli* O157:H7, *Salmonella*, or *Listeria monocytogenes* were applied to tomatoes, lettuce, and parsley by dip, spot, or spray inoculation methods. Inocula were dried for 1 or 24 h at 22°C on tomatoes or 2 h at 22°C followed by 22 h at 4°C on lettuce and parsley before treating with water (control) or chlorine (200 µg/ml). Significantly ($\alpha = 0.05$) higher populations (CFU/tomato, lettuce leaves, and parsley) of *E. coli* O157:H7 and *Salmonella* were recovered from produce inoculated by dipping compared to spot or spray inoculation. This is attributed to larger numbers of pathogens adhering to produce subjected to dip inoculation. Populations of *E. coli* O157:H7 and *Salmonella* recovered from tomatoes and lettuce inoculated by spot and spray methods was not significantly different. Populations of *E. coli* O157:H7 and *Salmonella* recovered from parsley inoculated by spot inoculation was significantly higher than populations recovered by spray inoculation. Significantly different populations of *L. monocytogenes* were recovered from tomatoes (dip > spot > spray) and lettuce (dip > spray > spot). Populations of *L. monocytogenes* recovered from dip inoculated parsley were significantly higher than those recovered from spot- or spray-inoculated parsley, which were not significantly different from each other. Populations of pathogens recovered from tomatoes were significantly higher when inocula were dried for 1 h compared to 24 h. Populations recovered from lettuce and parsley after drying inocula for 2 h at 22°C were significantly higher or equal to populations recovered after drying for 2 h at 22°C

followed by 22 h at 4°C. Significant differences (water > chlorine) were observed in populations of all pathogens recovered from treated tomatoes, lettuce, and parsley, regardless of inoculation method and drying time. Results indicate that inoculation method, drying time, and treatment affect survival and/or recovery of foodborne pathogens inoculated onto the surface of tomatoes, lettuce, and parsley. It is recommended that spot-inoculation with a drying time of 24 h at 22°C for tomatoes and 2 h at 22°C followed by 22 h at 4°C be used for lettuce and parsley be used to determine the efficacy of chlorine and other sanitizers in killing foodborne pathogens on tomatoes, lettuce and parsley

Introduction

In 1991, the National Cancer Institute implemented the national campaign, "Five a Day for Better Health," aimed at increasing consumption of fruits and vegetables. Due in part to the success of this campaign and to globalization of the produce market, which has increased the variety of fresh fruits and vegetables available to consumers year-round, there has been an increase in per capita consumption of fresh fruits and vegetables in the United States in the past decade *(7).* With this increase, a concurrent increase in foodborne disease outbreaks associated with consumption of produce has occurred. According to data complied by the Centers for Disease Control and Prevention (CDC) the number of produce-associated outbreaks remained constant between 1988 - 1992 and 1992 - 1997; however, the number of cases of foodborne disease associated with produce increased five-fold during this same period (*4, 22*). This may be due in part to increased consumption of contaminated produce. Of greatest concern is the number of cases of enteric infections implicating produce. According to data compiled by the CDC, between 1992 – 1997, *Escherichia coli* O157:H7 and *Salmonella* accounted for approximately 61% of the outbreaks associated with the consumption of produce (*22)*. Outbreaks of enteric infections have been associated with several fresh fruits and vegetables, including tomatoes (*5, 12, 13, 21, 28)*, lettuce (*2, 16, 24)*, and parsley (*11*).

Contamination of raw produce with pathogenic microorganisms can occur at any of several points from the field through the time of consumption. Given sufficient time and appropriate environmental conditions, pathogens can grow to populations exceeding 10^7 CFU/g of tomatoes (*30, 31, 33).* Much work has been done to define conditions that result in contamination of produce and subsequent growth of pathogens during storage (*1,* *3, 26).* Researchers have evaluated the effectiveness of a wide range of chemical sanitizers and physical means for decontamination of fresh produce *(6, 10, 18, 27, 34).* Despite efforts to evaluate the efficacy of produce sanitizers, however, results of studies done in different laboratories are difficult to compare because of the numerous variations in methodologies employed and incompleteness in describing results *(8).*

The lack of uniformity in methods of treatment used to treat produce and enumerate of microorganisms surviving treatment makes it difficult to assess the effectiveness of sanitizers and establish industry recommendations and guidelines. In 1997, in response to this situation, the U. S. Environmental Protection Agency assembled a Scientific Advisory Panel to discuss criteria for the development of a standard method for evaluating fresh produce sanitizers *(15).* The development of a standard method would eliminate variations in methodologies used in various laboratories, thereby enabling a comparison of pathogen reductions resulting from treatment with sanitizers *(9).* Recognizing that it is not realistic that one method can be used for all fruits and vegetables, because of the large differences in size, shape, and surface morphology, the goal would be to develop a basic method with the understanding that modifications would be necessary to accommodate natural variations in produce surface morphology *(9).*

An objective of the study reported here was to evaluate procedures for inoculating *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* onto the surface of tomatoes, lettuce, and parsley, with the goal of selecting an inoculation procedure to be used in a standard method. Dip, spot, and spray inoculation were evaluated. Another objective was to examine the time between application of inoculum and analysis of produce on the viability and retriveability of pathogens. Inocula applied to tomatoes, lettuce, and parsley were subjected to two drying times followed by treatment with water (control) or chlorine (200 µg/ml), then analyzed for number of surviving cells.

Materials and Methods

Selection of test strains. Mixtures of five strains of each pathogen isolated from produce or feces of humans with infections associated with consuming produce were used. The strains studied and their sources were as follows: Enterohemorrhagic *Escherichia coli* O157:H7 strains LJH557 (apple cider), SEA-13B88 (human feces, apple cider-associated outbreak), CDC-658 (human feces, cantaloupe-associated outbreak), H1730 (human feces, lettuce-associated outbreak), and F4546 (human feces, alfalfa sprout-associated outbreak); *Salmonella enterica* serotypes Agona (alfalfa sprouts), Baildon (human feces, tomato-associated outbreak), Gaminara (orange juice), Michigan (cantaloupe-associated outbreak), and Montevideo (human feces, tomato-associated outbreak); and *Listeria monocytogenes* strains G1091 (serotype 4b; human feces, coleslaw-associated outbreak), F8027 (serotype 4b; celery), F8255 (serotype 1/2b; peach and plum), F8369 (serotype 1/2a; corn), and HO222 (serotype 1/2a; potato). Test strains of *E. coli* O157:H7 and *Salmonella* were grown in tryptic soy broth (TSB, pH 7.3; BBL/Difco, Sparks, Md.) supplemented with 50 µg/ml nalidixic acid (TSBN). Although gram-positive bacteria are minimally affected by nalidixic acid, *L. monocytogenes* was grown in brain heart infusion broth (BHIB, pH. 7.4; BBL/Difco) also supplemented with 50 µg/ml nalidixic acid (BHIBN). This procedure greatly minimized the formation of colonies formed by microorganisms naturally present on produce, thus facilitating detection of colonies produced by test pathogens on or in recovery media supplemented with nalidixic acid. Nalidixic acid was sterile filtered and added after autoclaving and cooling broth media.

Test for cross-strain inhibition. Each strain of test pathogen was examined for its ability to inhibit growth of all other test strains of the same pathogen. Pathogens were grown in 10 ml of TSBN for 24 h at 37°C. Cultures of each strain were cross streaked on tryptic soy agar (TSA, pH 7.3; BBL/Difco) supplemented with nalidixic acid (50 µg/ml) (TSAN) and incubated for 24 h at 37°C. Plates were examined for inhibition of growth at junctions of cross streaks of all combinations of test strains of each pathogen.

Produce evaluated. Vine-ripened tomatoes (*Lycopersicon esculentum* Mill. 'Rutgers') to which wax or mineral oil had not been applied were used in an attempt to simulate field and post-harvest handling conditions to which tomatoes are exposed prior to waxing or oiling at a commercial level. A sample unit consisted of a tomato weighing 150 ± 2g. Tomatoes were purchased at a farmers' market, Griffin, GA and stored at 22 ± 2°C for a maximum of 2 days before use in experiments.

Iceberg lettuce (*Lactuca sativa*) was purchased at a supermarket in Griffin, GA and stored at 4°C for a maximum of 2 days before use in experiments. A sample unit consisted of three 9 x 9-cm pieces of lettuce leaves (total weight was 16 - 20 g; surface area was approximately 486 cm^2). The core and 2 - 4 outer leaves of each lettuce head were removed before each 9 x 9-cm piece of internal leaf was cut with a sterile scalpel using a stainless steel template to standardize dimensions. Care was taken to minimize the amount of surface tissue damage.

Parsley (*Petroselinum crispum*) was purchased at the State Farmers' market in Forest Park, GA and stored for a maximum of 2 days at 4°C before use in experiments.

Twenty-four hours prior to inoculation, 20 ± 1 g of parsley leaves and leaf stems, were bound in bunches with a rubber band approximately 1 cm from the end of the stems. Samples were stored at 4°C for up to 24 h before holding at 22°C for 30 min prior to inoculation.

Preparation of inocula. Five-strain mixtures of each pathogen were used as inocula. Stock cultures of *E. coli* O157:H7 and *Salmonella* were streaked on TSAN. Stock cultures of *L. monocytogenes* were streaked on brain heart infusion agar (BHIA, pH 7.4; BBL/Difco) supplemented with 50 µg/ml nalidixic acid (BHIAN). Cultures were incubated at 37°C for 24 h before picking colonies of *E. coli* O157:H7 and *Salmonella* to inoculate into 10 ml of TSBN or colonies of *L. monocytogenes* to inoculate into 10 ml of BHIBN. Cultures were incubated at 37°C and transferred twice at 24-h intervals using a loop (ca. 10 µl). Prior to use, 0.2 ml of culture was inoculated into each of two 200-ml volumes of either TSBN or BHIBN in 500-ml Erlenmeyer flasks and incubated at 37°C for 24 h. Cells of each strain cultured in each flask were collected by centrifugation (4,000 x *g*, 15 min, 21°C), washed in 100 ml of sterile 0.1% peptone, and resuspended in 100 ml of sterile 5% horse serum (Sigma Chemical Co., St. Louis, Mo.). Two 100-ml suspensions of each pathogen were combined to give 1 L of each five-strain mixture containing approximately equal populations of each strain.

Inoculation of tomatoes. Three methods were used to inoculate tomatoes with five-strain mixtures of *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes*.

Dip inoculation. Inoculum (5 L at 22 ± 2°C) of each pathogen was prepared by combining 1 L of cell suspension prepared as described above with 4 L of sterile 5% horse serum in a 20-L stainless steel container (stockpot). Twenty-four tomatoes (22 ±

2°C) were placed in a metal screen basket fabricated to fit inside the stockpot and submerged in the inoculum with gentle agitation for 1 min. All tomatoes in each replicate experiment were immersed and removed from the inoculum at the same time. Inoculated tomatoes were placed on a wire screen elevated 14 cm above the work surface in a laminar flow biosafety hood to facilitate drying.

Spot inoculation. Tomatoes (22 ± 2°C) were placed stem-end down on waxed paper placed on the work surface inside a biosafety hood. Within a 3-cm-diameter circle on the surface of the blossom end of the tomato, 50 μl of a five-strain inoculum of a test pathogen prepared as described for dip inoculation was applied using a micropipettor, with care taken to avoid applying inoculum on the blossom scar. To prevent the inoculum from running off the side of the tomato and to facilitate drying, small, approximately equal volumes of inoculum were applied at 10 - 15 locations.

Spray inoculation. Inoculum prepared as described for the dip inoculation was applied to tomatoes (22 ± 2°C) using a TLC reagent sprayer (model No. 422530-0050, Kontes Glass Company, Vineland, N.J.). The carrier gas was nitrogen at approximately 2 psi. The sprayer was attached to a ring stand in a horizontal position 11 cm above the base in a biosafety hood. Tomatoes were placed stem-end-down on a piece of waxed paper placed on the base of a ring stand. The distance between the sprayer nozzle and the tomato surface was 5 - 6 cm, depending on the size and shape of each tomato. Approximately 50 μl of the inoculum was applied to each tomato by spraying for 2 sec.

Inoculation of lettuce. Three methods were also used to inoculate lettuce with five-strain mixtures of *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes.*

Dip inoculation. Inoculum (5 L at 22 ± 2°C) of each pathogen was prepared by combining 1 L of cell suspension prepared as described above with 4 L of sterile 5% horse serum in an 11-L stainless steel container (mixing bowl). Twenty-four three-piece samples of lettuce (72 pieces total) at (22 ± 2°C) were placed in a metal screen strainer (35-cm-diameter) and submerged in the inoculum with gentle agitation for 1 min. All pieces of lettuce were immersed and removed from the inoculum at the same time. Inoculated pieces of lettuce were placed in a single layer on a wire screen 14 cm above the work surface in a laminar flow biosafety hood to facilitate drying.

Spot inoculation. Seventy-two pieces of lettuce (24 3-piece samples) were placed in a single layer on wire screens elevated 14 cm above the work surface in a laminar flow biosafety hood. Fifty microliters of inoculum prepared as described for dip inoculation was applied to the surface of each piece of lettuce, such that every sample was inoculated with 150 µl. The inoculum was applied in small approximately equal volumes at 5 - 10 locations to facilitate drying.

Spray inoculation. Inoculum was applied using the same TLC reagent sprayer apparatus described above for spray inoculation of tomatoes. The sprayer was attached to a ring stand in a horizontal position 6 cm above the base in a biosafety hood. Each piece of lettuce was placed on a piece of waxed paper placed on the base of the ring stand. The distance between the sprayer nozzle and the lettuce surface was 6 cm. Approximately 50 µl of inoculum prepared as described for dip inoculation was applied to each piece of lettuce by spraying for 2 sec, thereby applying a total of sample 150 µl to each sample. Inoculated leaves were transferred to wire screens and placed 14 cm above the work surface in a laminar flow biosafety hood to dry.

Inoculation of parsley. Three methods were also used to inoculate parsley with five-strain mixtures of *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes.*

Dip inoculation. Inoculum (5 L at 22 ± 2°C) of each pathogen was prepared by combining 1 L of cells suspension prepared as described above with 4 L of sterile 5% horse serum in a 54 x 43 ½ x 13-cm (length x width x height) Nalgene tray. Twenty-four bunches (samples) of parsley (22 ± 2°C) were submerged in the inoculum and gently agitated for 1 min. All samples of parsley were immersed and removed from the inoculum at the same time. After inoculation, rubber bands used to secure parsley leaves in each sample were removed and leaves were placed in single layer on a wire screen 14 cm above the work surface in a laminar flow biosafety hood to facilitate drying.

Spot inoculation. Samples of parsley were placed on a wire screens elevated 14 cm above the work area in a laminar flow biosafety hood. Inoculum (150 µl) prepared as described for dip inoculation was applied to the parsley leaves. The inoculum was applied in small approximately equal volumes at 10 - 15 locations to facilitate drying.

Spray inoculation. Inoculum was applied using a TLC reagent sprayer apparatus described above for spray inoculation of tomatoes and lettuce. The sprayer was attached to a rind stand in a horizontal position 11 cm above the base in a biosafety hood. Each sample of parsley was placed on a piece of waxed paper placed on the base of the ring stand. The distance between the sprayer nozzle and parsley was approximately 6 cm. Approximately 150 µl of inoculum prepared as described for dip inoculation was applied to each parsley sample by spraying for 6 sec. Inoculated parsley samples were placed on wire screens 14 cm above the work surface in a laminar flow biosafetly hood to facilitate drying.

Drying (attachment) time. After tomatoes were inoculated by dip, spot, or spray methods, they were held in a biosafety hood to 'dry' the inoculum for 1 h at 22 ± 2°C or at static conditions for 24 ± 1 h at 22 ± 2°C before separately being placed, blossom end down, in separate quart-sized Ziplock Bags® (S. C. Johnson, Racine, Wisc.), treating with sterile deionized water (control) or chlorine (200 µg/ml) solution, and subjecting to microbiological analysis.

After lettuce pieces were inoculated by dip, spot, or spray methods, they were held in a biosafety hood to 'dry' the inoculum for 2 h at 22 ± 2°C. Each sample consisting of three pieces was then placed into a 400-ml stomacher® bag (Seward Medical, Ltd., London, U.K.). Lettuce was treated with water (control) or chlorine (200 µg/ml) solution, then analyzed for populations of test pathogen, or held at 4°C for 22 h before treating and subjecting to analysis.

After parsley samples were inoculated by dip, spot, or spray methods, they were held for 2 h at 22 ± 2°C in a biosafety hood to 'dry' the inoculum. Each sample consisting of 20 ± 1 g of parsley was then placed into 400-ml stomacher bag. Rubber bands securing parsley samples that had been spot or spray inoculated were removed prior to placing parsley in a 400-ml stomacher bag. Parsley was treated with water (control) or chlorine (200 µg/ml) solution, then analyzed for populations of a test pathogen, or held at 4°C for 22 h before treating and subjecting to analysis.

Procedure for treating produce. Each produce type subjected to all combinations of inoculation method and inoculum drying time was treated with chlorinated water or deionized water (control).

Tomatoes. To each inoculated tomato in a Ziplock bag, 200 ml of sterile deionized water (control) or chlorine (200 µg/ml) solution was added. The chlorine solution was prepared by combining NaOCl (Aldrich, Milwaukee, Wisc.) with 0.05 M potassium phosphate buffer (pH 6.8, 22 ± 2°C). The free chlorine content was determined with an amperometric titrator (Hach, Ames, Iowa) immediately before use. Each bag containing a tomato submerged in 200 ml of water or chlorine solution was placed in a 1-L beaker secured on a rotary shaker and agitated at 150 rpm for 5 min. Each tomato was then aseptically transferred to a second bag and 20 ml of Dey-Engley broth (DE broth, pH 7.6; BBL/Difco) was added. Each tomato was hand rubbed for 1 min before analyzing the DE broth for presence (by enrichment) and/or population of test pathogen. Water and chlorine treatment solutions were also analyzed for populations of test pathogens.

Lettuce. To each lettuce sample (three 9 x 9-cm pieces), in a 400-ml stomacher bag, 200 ml of sterile deionized water (control) or chlorine (200 µg/ml) solution was added. Each bag containing a lettuce sample was placed in a wire basket that was attached to a rotary shaker and agitated at 150 rpm for 5 min. Bags were positioned such that the entire sample was completely submerged in the water or chlorine solution. After treating lettuce for 5 min, water or chlorine solution were decanted into sterile beakers and 100 ml of DE broth was added to each bag. Lettuce and DE broth were pummeled in a stomacher (Seward Medical. Ltd.) for 1 min at normal speed before DE broth was analyzed for the presence (by enrichment) and/or populations of test pathogens. Water and chlorine treatment solutions were also analyzed for populations of test pathogens.

Parsley. To each parsley sample (20 ± 1 g) in a 400-ml stomacher bag, 200 ml of sterile deionized water (control) or chlorine (200 µg/ml) solution was added. Each bag was placed in a wire basket that was attached to a rotary shaker and agitated at 150 rpm for 5 min. Bags were positioned such that the entire sample was completely submerged in the water or chlorine solution. After treating parsley for 5 min, water or chlorine solution were decanted into sterile beakers and 100 ml of DE broth was added to each bag. Parsley and DE broth were pummeled in a stomacher (Seward Medical. Ltd.) for 1 min at normal speed before DE broth was analyzed for the presence (by enrichment) and/or populations of test pathogens. Water and chlorine treatment solutions were also analyzed for populations of test pathogens.

Microbiological analyses. Populations (CFU/ml) of test pathogens in inocula were determined. Single- and mixed-strain suspensions of each pathogen in sterile 5% horse serum were serially diluted in sterile 0.1% peptone. Duplicate 0.1-ml samples of diluted suspensions of *E. coli* O157:H7 and *Salmonella* were surface plated on TSAN supplemented with 0.1% pyruvic acid (TSANP). Pyruvic acid was added to recovery media because it has been shown to enhance the recovery of injured cells (*17, 32*). Solutions of pyruvic acid and nalidixic acid were filter-sterilized and added to the molten agars (45 - 50°C) before pouring into Petri plates. Diluted *E. coli* O157:H7 suspensions were also surface plated on sorbitol MacConkey agar (SMAC, pH 7.4; Oxoid, Basingstoke, U.K.) containing 50 µg/ml nalidixic acid and 0.1% pyruvic acid (SMACNP). *Salmonella* suspensions were serially diluted in 0.1% peptone and surface plated (0.1 ml in duplicate) on bismuth sulfite agar (BSA, pH 7.0; BBL/Difco) supplemented with 50 µg/ml nalidixic acid (50 µg/ml) and 0.1% pyruvic acid (BSANP).

Serially diluted suspensions of *L. monocytogenes* were surface plated (0.1 ml in duplicate) on brain heart infusion agar (BHIA, pH 7.4; BBL/Difco) supplemented to contain 50 µg/ml nalidixic acid and 0.1% pyruvic acid (BHIANP) and modified Oxford medium (MOX, pH 7.0; Oxoid), also supplemented with 50 µg/ml nalidixic acid and 0.1% pyruvic acid (MOXNP). Surface inoculated TSANP, SMACNP, and BSANP plates were incubated at 37°C for 24 ± 2 h; inoculated BHIANP and MOXNP plates were incubated for 48 ± 2 h at 37°C before colonies were counted.

Uninoculated tomatoes, lettuce, and parsley. Uninoculated produce (four samples per experiment) was analyzed for the presence of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*. One tomato or lettuce sample (three 9 x 9-cm pieces) was placed in a Ziploc® bag or a 400-ml stomacher bag, respectively, containing 200 ml of double modified tryptic soy broth (dmTSB) (*23*), lactose broth (BBL/Difco), or Listeria enrichment broth (Oxoid) supplemented with nalidixic acid (50 µg/ml) and pyruvic acid (0.1%) to detect *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*, respectively, by enrichment. Bags containing produce and dmTSB or lactose broth enrichments were incubated for 24 h at 37° C. Bags containing produce and Listeria enrichment broth were incubated for 48 h at 37°C. The enrichment broths were then streaked onto SMACNP, BSANP, or MOXNP and incubated as described above. Confirmation tests were not necessary because presumptive colonies of test pathogens were not detected on uninoculated produce.

Inoculated tomatoes. To determine the population (CFU/tomato) of each test pathogen on inoculated tomatoes (four per replicate experiment) before treatment with water or chlorine solution, 20 ml of DE broth (BBL/Difco) was added to a quart-sized

Ziploc bag containing an inoculated tomato. Each tomato was hand rubbed for 1 min before DE broth was plated on recovery media. Undiluted samples (0.1 ml in duplicate or 0.25 ml in quadruplicate) and samples (0.1 ml in duplicate) serially diluted in 0.1% peptone were surface plated on TSANP and SMACNP to enumerate *E. coli* O157:H7, TSANP and BSANP to enumerate *Salmonella*, and BHIANP and MOXNP to enumerate *L. monocytogenes.* Plates were incubated for 24 h at 37°C for *E. coli* O157:H7 and *Salmonella* and for 48 h at 37°C for *L.* monocytogenes. Presumptive colonies (10 - 20 per replicate experiment) were selected at random for confirmation by agglutination and biochemical tests. *E. coli* O157:H7 was confirmed using the O157 latex agglutination test (Oxoid). *Salmonella* was confirmed using the *Salmonella* latex agglutination test (Oxoid). Both pathogens were subjected to biochemical tests using the API 20E diagnostic kit (BioMerieux Vitek Inc., Hazelwood, Mo.). *L. monocytogenes* was confirmed using the API Listeria diagnostic kit (BioMerieux Vitek Inc.).

The presence and/or populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in treatment solutions (water or chlorinated water) and DE broth from treated tomatoes were determined. Samples were serially diluted in 0.1% peptone water, plated on appropriate media, and incubated as described above before presumptive colonies were counted and subjected to confirmation tests.

To each bag containing a tomato inoculated with *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes* and 20 ml of DE broth, 200 ml of dmTSB, lactose broth, or Listeria enrichment broth, respectively, each supplemented with of nalidixic acid (50 µg/ml) and pyruvic acid (0.1%), were added. Bags containing enrichment broth and a tomato inoculated with *E. coli* O157:H7 or *Salmonella* were incubated at 37°C for 24 h. Bags containing enrichment broth and a tomato inoculated with *L. monocytogenes* were incubated at 37°C for 48 h. Enrichment broths containing tomatoes inoculated with *E. coli* O157:H7 and *L. monocytogenes* were streaked onto SMACNP and MOXNP, respectively. Plates were incubated for 24 h or 48 h, respectively, at 37°C before examining for presumptive colonies of *E. coli* O157:H7 or *L. monocytogenes.* Enrichment broth (0.1 ml) containing tomatoes inoculated with *Salmonella* was inoculated into 10 ml of selenite cystine broth (BBL/Difco) and incubated at 37°C for 24 h. Broth was streaked onto BSANP and incubated at 37°C for 24 h. Presumptive colonies isolated from the enrichment broths on SMACNP, MOXNP, and BSANP were confirmed as described above.

Inoculated lettuce. To determine the population (CFU/lettuce sample) of a test pathogen on lettuce (four samples per replicate experiment) before treatment, 100 ml of DE broth was added to each 400-ml stomacher bag containing a lettuce sample (three 9 x 9-cm pieces). Lettuce and DE broth were pummeled in a stomacher for 1 min at normal speed before analyzing the DE broth for the populations of pathogens. Undiluted samples (0.1 ml in duplicate or 0.25 ml in quadruplicate) and samples (0.1 ml in duplicate) serially diluted in 0.1% peptone were surface plated on TSANP and SMACNP to enumerate *E. coli* O157:H7, TSANP and BSANP to enumerate *Salmonella*, and BHIANP and MOXNP to enumerate *L. monocytogenes.* Plates were incubated as described above for the analysis of tomatoes before presumptive colonies were counted and subjected to confirmation tests.

The presence and/or populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in treatment solutions (water or chlorinated water) and DE broth from treated lettuce samples were determined. Samples were plated and incubated as described above before presumptive colonies were counted and subjected to confirmation tests.

To each bag containing a lettuce sample inoculated with *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes* and 100 ml of DE broth, 100 ml of double strength dmTSB, lactose broth, or Listeria enrichment broth, respectively, each containing nalidixic acid (100 µg/ml) and pyruvic acid (0.2%), was added. Bags containing enrichment broth and lettuce inoculated with *E. coli* O157:H7 or *Salmonella* were incubated at 37°C for 24 h. Bags containing enrichment broth and lettuce inoculated with *L. monocytogenes* were incubated at 37°C for 48 h. Because pathogens could be enumerated by direct plating of DE broth, lettuce was subjected to enrichment only in the first replicate experiment.

Inoculated parsley. To determine the population (CFU/parsley sample) of a test pathogen on parsley (four samples per replicate experiment) before treatment, 100 ml of DE broth was added to each 400-ml stomacher bag containing a parsley sample (20 ± 1 g). The sample and DE broth were pummeled in a stomacher for 1 min at normal speed before analyzing the DE broth for the populations of pathogens. Undiluted samples (0.1 ml in duplicate or 0.25 ml in quadruplicate) and samples (0.1 ml in duplicate) serially diluted in 0.1% peptone were surface plated on TSANP and SMACNP to enumerate *E. coli* O157:H7, TSANP and BSANP to enumerate *Salmonella*, and BHIANP and MOXNP to enumerate *L. monocytogenes.* Plates were incubated as described above for the analysis of tomatoes before presumptive colonies were counted and subjected to confirmation tests.

The presence and/or populations of *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes* in treatment solutions (water or chlorinated water) and DE broth from treated parsley samples were determined. Samples were plated and incubated as described above before presumptive colonies were counted and subjected to confirmation tests.

To each bag containing a parsley sample inoculated with *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes* and 100 ml of DE broth, 100 ml of double strength dmTSB, lactose broth, or Listeria enrichment broth, respectively, each containing nalidixic acid (100 µg/ml) and pyruvic acid (0.2%), was added. Bags containing enrichment broth and parsley inoculated with *E. coli* O157:H7 or *Salmonella* were incubated at 37°C for 24 h. Bags containing enrichment broth and parsley inoculated with *L. monocytogenes* were incubated at 37°C for 48 h.

Statistical analyses. The experimental design consisted of three pathogens (*E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*), three types of produce (tomatoes, lettuce, and parsley), three inoculation procedures (dip, spot, and spray), two inoculum-drying times (1 and 24 h for tomatoes; 2 and 22 h for lettuce and parsley), two treatment solutions (water [control] and chlorine [200 µg/ml]), and two recovery media for each pathogen. Each experiment was replicated three times and each replicate consisted of four produce samples.

The experiment consisted of a split-split-split plot design. An individual batch of produce was initially divided into three equal batches for three different inoculation methods. Each batch for a given inoculation method was divided equally between two drying times and then further subdivided into three equal four-sample batches, one to remain untreated and two to undergo treatment. Each individual produce sample was assayed for presence (by enrichment) and/or population of test pathogens using two direct plating media.

Data were subjected to SAS (Statistical Analysis Systems Institute, Cary, N.C.) for analysis of variance and Duncan's multiple range tests. Significant differences between mean values are presented at $\alpha = 0.05$.

Results and Discussion

Cross-strain inhibition tests revealed that, within pathogen, none of the strains inhibited the growth of other strains. Mixtures of strains in inocula did not present a problem that might be associated with loss of viability caused by strain interaction.

Table 2.1 shows the populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in suspensions used to inoculate tomatoes, lettuce, and parsley. The populations of each strain or serotype in each inoculum were approximately equal, as was the number of cells in inocula applied to each type of produce.

Table 2.1. Populations of test pathogens in 24-h cultures and five–strain or five-serotype inocula.

			Population ($\log_{10}$ CFU/ml)		
Pathogen	Strain/serotype	Recovery medium	Tomato	Lettuce	Parsley
E. coli O157:H7	SEA-13B88	TSANP	9.45 – 9.72	8.52 – 9.18	8.53 – 9.42
	LJH-557		8.76 – 9.18	8.57 – 8.83	8.32 – 9.39
	CDC-658		8.34 – 9.09	8.39 – 8.75	8.16 – 9.37
	F4546		9.68 – 10.10	9.13 – 9.65	8.81 – 9.39
	H1730		8.87 – 9.05	8.14 – 8.75	8.92 – 9.24
	inoculum[a]		7.61 – 8.79	7.61 – 8.75	8.19 – 8.40
Salmonella	Agona	TSANP	8.95 – 9.82	8.57 – 9.40	9.17 – 9.91
	Baildon		8.85 – 9.34	8.86 – 9.64	8.66 – 9.00
	Gaminara		8.57 – 9.86	9.10 – 9.66	8.51 – 9.13
	Michigan		8.90 – 9.50	8.53 – 8.86	8.84 – 9.12
	Montevideo		9.10 – 9.31	8.93 – 9.15	9.07 – 9.61
	inoculum		8.03 – 8.75	7.77 – 8.72	7.98 – 8.64
L. monocytogenes	G1091	BHIANP	8.76 – 9.38	8.64 – 9.29	9.47 – 9.70
	H0222		8.86 – 9.44	8.55 – 9.45	9.18 – 9.50
	F8255		8.62 – 9.34	8.52 – 9.05	9.11 – 9.69
	F8369		8.59 – 8.92	8.90 – 9.29	8.77 – 9.25
	F8027		8.90 – 9.60	8.73 – 9.10	8.47 – 9.51
	inoculum		8.11 – 8.91	7.80 – 8.91	8.29 – 8.54

[a] Inocula applied to produce contained approximately equal populations of each strain/or serotype in 5% horse serum.

Recovery of pathogens from tomatoes. Table 2.2 shows the number of *E. coli* O157:H7 recovered from water and chlorine (200 µg/ml) solutions on TSANP after treating tomatoes and from DE broth after washing inoculated, untreated tomatoes as well as from DE broth after washing tomatoes as affected by inoculation method and drying time. Mean values were converted to $\log_{10}$ CFU/tomato. Our inability to recover cells from chlorine solutions (detection limit was 1 CFU/2 ml [100 CFU/tomato]) indicates that cells removed from tomatoes were killed. Within inoculation method and drying time, with the exception of tomatoes spray inoculated and dried for 24 h, significantly ($\alpha = 0.05$) lower populations were detected on tomatoes treated with chlorine solution compared to tomatoes treated with water. There was no statistical difference in populations of *E. coli* O157:H7 recovered from DE broth after washing untreated tomatoes that had been inoculated by dip, spot, or spray methods and dried for 1 h. Drying inocula for 24 h resulted in significant differences (dip > spot > spray) in populations recovered from untreated tomatoes inoculated using three inoculation methods. Within drying time, significantly higher populations were recovered from dipped tomatoes subjected to water or chlorine treatment compared to spot- or spray-inoculated tomatoes subjected to the same treatments; populations recovered from spot- or spray-inoculated tomatoes treated with water or chlorine were not significantly different. Populations of *E. coli* O157:H7 recovered from untreated tomatoes and tomatoes treated with water after drying for 1 h were significantly higher than those recovered from tomatoes dried for 24 h, regardless of method of inoculation. The number of *E. coli* O157:H7 recovered from tomatoes treated with chlorine was not significantly affected by drying time. The pathogen was not detected (< 10 CFU/tomato)

Table 2. 2. Populations of *E. coli* O157:H7 recovered from tomatoes.

			Population ($\log_{10}$ CFU/ ml or tomato)[a]					
			Treatment solution		DE broth			
Inoculation method	Drying time (h)	Treatment[b]	CFU/ml	CFU/tomato (± SD)[c]	CFU/ml	CFU/tomato (± SD)[d]	Reduction[e]	En[f]
Dip	1	None	nd[g]	nd	5.28	6.58 (0.26) a^1 a^2 a^3		
		Water	4.18	6.48 (0.37) a^1 a^2 a^3	4.32	5.62 (0.32) a a b	0.96	
		Chlorine	<-0.30	< 2.00 a a b	1.48	2.78 (0.85) a a c	3.80	
	24	None	nd	nd	3.59	4.89 (0.56) a b a		
		Water	2.38	4.68 (0.55) a b a	3.31	4.61 (0.47) a b a	0.28	
		Chlorine	<-0.30	< 2.00 a a b	1.83	3.13 (1.19) a a b	1.76	1/2
Spot	1	None	nd	nd	4.84	6.14 (0.40) a a a		
		Water	3.42	5.72 (0.41) ab a a	2.38	3.68 (0.48) b a b	2.46	
		Chlorine	<-0.30	< 2.00 a a b	<-0.30	1.00 b a c	> 5.14	3/12
	24	None	nd	nd	2.74	4.04 (0.26) b b a		
		Water	1.60	3.90 (0.30) ab b a	0.60	1.78 (0.62) b b b	2.26	2/2
		Chlorine	<-0.30	< 2.00 a a b	<-0.30	< 1.00 b a b	> 3.04	3/12
Spray	1	None	nd	nd	4.89	6.18 (0.42) a a a		
		Water	3.03	5.33 (0.55) b a a	2.83	4.13 (0.38) b a b	2.05	
		Chlorine	<-0.30	< 2.00 a a b	0.24	1.31 (0.57) b a c	4.87	2/5
	24	None	nd	nd	1.57	2.87 (0.31) c b a		
		Water	0.86	3.16 (0.93) b b a	0.37	1.67 (0.62) b b b	1.20	2/5
		Chlorine	<-0.30	< 2.00 a a a	0.52	1.59 (1.35) b a b	1.28	3/10

(Continued)

Table 2.2. (continued)

[a] Treatment solutions (water or chlorinated water) and Dey-Engley (DE) wash broth were analyzed for numbers ($\log_{10}$ CFU/ml) of *E. coli* O157:H7 by direct plating; values were converted to $\log_{10}$ CFU/tomato. The detection limit was 1 CFU/2 ml of treatment solution (100 CFU/tomato) or 1 CFU/2 ml of DE wash (10 CFU/tomato).

[b] Tomatoes were not treated (none) with water or chlorine, or treated with 200 ml of deionized water or chlorinated (200 µg/ml) water for 5 min, then washed in 20 ml of DE broth for 1 min.

[c] Mean values ($\log_{10}$ CFU/tomato) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[d] Mean values ($\log_{10}$ CFU/tomato) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[e] Within inoculation method and drying time, reduction ($\log_{10}$ CFU/tomato) compared to the population recovered from tomatoes not treated (none) with water (control) or chlorine (200 µg/ml).

[f] Number of treated, washed tomatoes positive for *E. coli* O157:H7, as detected by enrichment (En), out of the number of tomatoes analyzed by enrichment. Tomatoes on which *E. coli* O157:H7 was detected by direct plating of DE broth were not analyzed by enrichment.

[g] nd, not determined.

by direct plating DE broth after washing spot-inoculated tomatoes treated with chlorine, regardless of drying time, but enrichment revealed its presence on 3 of 12 tomatoes subjected to each drying time.

Populations of *Salmonella* recovered on TSANP from water and chlorine (200 µg/ml) solutions after treating inoculated tomatoes and from DE broth after washing tomatoes are listed in Table 2.3. Populations recovered from water used to treat tomatoes that had been dip inoculated and allowed to dry for 1 h were significantly higher than the population from tomatoes that had been spray-inoculated and allowed to dry for 1 h. The number of *Salmonella* recovered from water after treatment of spot-inoculated tomatoes dried for 1 h was not significantly different than populations recovered from dip- or spot-inoculated tomatoes. Populations recovered from water after treatment of tomatoes dried for 1 h were significantly higher than those recovered after drying for 24 h, regardless of inoculation method. Within inoculation method and drying time, significantly lower populations were detected in chlorinated water than in water after treating tomatoes. Populations recovered from DE broth from untreated tomatoes were significantly different (dip > spot > spray) when inoculum was dried for 1 h. Within drying time, populations recovered from dip-inoculated tomatoes treated with water or chlorine were significantly higher than populations recovered from spot- or spray-inoculated tomatoes. Within inoculation method, significant reductions in populations occurred between 1 and 24 h of drying inoculum. Populations recovered from tomatoes treated with chlorine were significantly lower than populations recovered from tomatoes treated with water, regardless of inoculation method or drying time.

Table 2.3. Populations of *Salmonella* recovered from tomatoes.

			Population ($\log_{10}$ CFU/ml or tomato)[a]					
			Treatment solution		DE broth			
Inoculation method	Drying time (h)	Treatment[b]	CFU/ml	CFU/tomato (± SD)[c]	CFU/ml	CFU/tomato (± SD)[d]	Reduction[e]	En[f]
Dip	1	None	nd[g]	nd	6.14	7.44 (0.22) a^1 a^2 a^3		
		Water	4.40	6.70 (0.47) a^1 a^2 a^3	5.02	6.32 (0.38) a a b	1.12	
		Chlorine	<-0.30	< 2.00 a a b	2.37	3.67 (0.61) a a c	3.77	
	24	None	nd	nd	4.75	6.05 (0.71) a b a		
		Water	3.11	5.41 (0.40) a b a	4.08	5.38 (0.31) a b a	0.67	
		Chlorine	<-0.30	< 2.00 a a b	2.02	3.32 (1.12) a a b	2.73	2/2
Spot	1	None	nd	nd	5.11	6.42 (0.27) b a a		
		Water	3.56	5.86 (0.34) ab a a	3.02	4.32 (0.41) b a b	2.10	
		Chlorine	<-0.30	< 2.00 a a b	<-0.30	< 1.00 b a c	> 5.42	5/12
	24	None	nd	nd	3.72	5.02 (0.19) ab b a		
		Water	2.53	4.83 (0.20) a b a	1.75	3.05 (0.50) b a b	1.97	
		Chlorine	<-0.30	< 2.00 a a b	-0.28	1.02 (0.06) b a c	4.00	2/12
Spray	1	None	nd	nd	4.55	5.85 (0.39) c a a		
		Water	2.72	5.02 (0.53) b a a	3.05	4.35 (0.73) b a b	1.50	
		Chlorine	<-0.30	< 2.00 a a b	-0.25	1.05 (0.13) b a c	4.80	10/12
	24	None	nd	nd	2.70	4.00 (0.68) b b a		
		Water	1.56	3.86 (0.66) b b a	1.30	2.60 (0.41) b b b	1.40	
		Chlorine	<-0.30	< 2.30 a a b	-0.25	1.05 (0.18) b a c	2.95	4/12

(Continued)

Table 2.3. (continued)

[a] Treatment solutions (water or chlorinated water) and Dey-Engley (DE) wash broth were analyzed for numbers ($\log_{10}$ CFU/ml) of *Salmonella* by direct plating; values were converted to $\log_{10}$ CFU/tomato. The detection limit was 1 CFU/2 ml of treatment solution (100 CFU/tomato) or 1 CFU/2 ml of DE wash (10 CFU/tomato).

[b] Tomatoes were not treated (none) with water or chlorine, or treated with 200 ml of deionized water or chlorinated (200 µg/ml) water for 5 min, then washed in 20 ml of DE broth for 1 min.

[c] Mean values ($\log_{10}$ CFU/tomato) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[d] Mean values ($\log_{10}$ CFU/tomato) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[e] Within inoculation method and drying time, reduction ($\log_{10}$ CFU/tomato) compared to population recovered from tomatoes not treated (none) with water (control) or chlorine (200 µg/ml).

[f] Number of treated, washed tomatoes positive for *Salmonella*, as detected by enrichment (En), out of the number of tomatoes analyzed by enrichment. Tomatoes on which *Salmonella* was detected by direct plating DE broth were not analyzed by enrichment.

[g] nd, not determined.

Table 2.4 lists populations of *L. monocytogenes* recovered on BHIANP from water and chlorine solutions after treatment of tomatoes as well as DE broth after washing tomatoes. Within drying time, populations recovered from water after treating dipped tomatoes were significantly higher than populations recovered from spot- or spray inoculated tomatoes, which were not significantly different from each other. Regardless of inoculation method, the number of *L. monocytogenes* recovered from water after treatment of tomatoes on which inoculum was dried for 24 h was not significantly different. The population recovered from water after treatment of tomatoes on which spot inoculum had dried for 24 h was not significantly different than the population recovered from spot-inoculated tomatoes dried for 1 h. The extended drying time resulted in significant reductions in populations recovered from dip- or spray-inoculated tomatoes. Populations of *L. monocytogenes* recovered from DE broth after washing untreated tomatoes differed from populations of *E. coli* O157:H7 and *Salmonella* in that significantly higher numbers were recovered from spray-inoculated tomatoes than from spot-inoculated tomatoes. Although approximately equal numbers of *L. monocytogenes* were recovered from spot- and spray-inoculated tomatoes treated with chlorine, enrichment revealed that more tomatoes receiving spray inoculum were positive for the pathogen than were spot-inoculated tomatoes. Overall, higher populations were recovered from dip-inoculated tomatoes compared to spot- or spray-inoculation, regardless of drying time or treatment. Populations recovered from spray-inoculated tomatoes dried for 24 h were equal or significantly higher than those recovered from spot-inoculated tomatoes. Treatment with chlorine resulted in significant reductions in populations compared to treatment with water, regardless of the inoculation method or

Table 2.4 Populations of *Listeria monocytogenes* recovered from tomatoes.

			Population ($\log_{10}$ CFU/ml or tomato)[a]					
			Treatment solution		DE broth			
Inoculation method	Drying time (h)	Treatment[b]	CFU/ml	CFU/tomato (± SD)[c]	CFU/ml	CFU/tomato (±SD)[d]	Reduction[e]	En[f]
Dip	1	None	nd[g]	nd	6.16	7.46 (0.29) a[1] a[2] a[3]		
		Water	5.01	7.31 (0.23) a[1] a[2] a[3]	5.37	6.67 (0.24) a a b	0.79	
		Chlorine	<-0.30	< 2.00 a a b	2.42	3.72 (0.80) a a c	3.74	
	24	None	nd	nd	5.33	6.63 (0.27) a b a		
		Water	3.64	5.94 (0.40) a b a	4.51	5.81 (0.14) a b b	0.82	
		Chlorine	<-0.30	< 2.00 a a b	1.27	2.58 (0.59) a a c	4.05	1/1
Spot	1	None	nd	nd	5.07	6.37 (0.11) c a a		
		Water	3.84	6.14 (0.12) b a a	2.76	4.06 (0.66) c a b	2.31	
		Chlorine	<-0.30	< 2.00 a a b	-0.28	1.02 (0.06) b a c	5.35	4/11
	24	None	nd	nd	4.53	5.83 (0.17) b b a		
		Water	3.23	5.53 (0.42) a a a	2.05	3.35 (0.61) c a b	2.48	
		Chlorine	<-0.30	< 2.00 a a b	<-0.30	< 1.00 b a c	> 4.83	6/12
Spray	1	None	nd	nd	5.55	6.85 (0.31) b a a		
		Water	3.91	6.21 (0.31) b a a	3.96	5.26 (0.33) b a b	1.59	
		Chlorine	<-0.30	< 2.00 a a b	-0.14	1.16 (0.22) b a c	5.69	9/11
	24	None	nd	nd	4.62	5.92 (0.30) b b a		
		Water	3.36	5.66 (0.17) a b a	3.64	4.94 (0.50) b a b	0.98	
		Chlorine	<-0.30	< 2.00 a a b	<-0.30	< 1.00 b a c	> 4.92	10/12

(Continued)

Table 2.4. (continued)

[a] Treatment solutions (water or chlorinated water) and Dey-Engley (DE) wash broth were analyzed for numbers ($\log_{10}$ CFU/ml) of *L. monocytogenes* by direct plating; values were converted to $\log_{10}$ CFU/tomato. The detection limit was 1 CFU/2 ml of treatment solution (100 CFU/tomato) or 1 CFU/2 ml of DE wash (10 CFU/tomato).

[b] Tomatoes were not treated (none) with water or chlorine, or treated with 200 ml of deionized water or chlorinated (200 µg/ml) water for 5 min, then washed in 20 ml of DE broth for 1 min.

[c] Mean values ($\log_{10}$ CFU/tomato) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[d] Mean values ($\log_{10}$ CFU/tomato) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[e] Within inoculation method and drying time, reduction ($\log_{10}$ CFU/tomato) compared to population recovered from tomatoes not treated (none) with water (control) or chlorine (200 µg/ml).

[f] Number of treated, washed tomatoes positive for *L. monocytogenes*, as detected by enrichment (En), out of the number of tomatoes analyzed by enrichment. Tomatoes on which *L. monocytogenes* was detected by direct plating of DE broth were not analyzed by enrichment.

[g] nd, not determined.

inoculum drying time. Higher numbers of chlorine-treated, spray-inoculated tomatoes were positive for *L. monocytogenes* by enrichment than were chlorine-treated, spot-inoculated tomatoes.

Data indicate that the method of inoculation can result in differences in numbers of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* adhering to raw tomatoes. Higher populations of pathogens were recovered from tomatoes that were dipped in cell suspensions compared to application of inoculum by spot or spray methods. The dip method mimics field or packinghouse situations in which the entire surface of tomatoes may be exposed to contaminated irrigation water or dump tank water. Recognizing that microorganisms may infiltrate the stem scar tissue and blossom ends of tomatoes fruits, however, avoiding these areas by using spot or spray inoculation may give an incomplete evaluation when testing the efficacy of sanitizers in killing or removing cells located at these sites. A major drawback to the dip method is that, compared to the spot and spray methods, there is no control of the volume of inoculum adhering to the tomato. This results in an unknown number of cells applied by dip inoculation. Consequently, the number of cells killed by a given sanitizer treatment cannot be calculated. Though the volume of inoculum could theoretically be ”controlled” by standardizing the size of the tomato as well as all morphological structures on it’s surface, this would be a formidable and unrealistic goal. More control of the number of cells applied to each tomato is afforded by spot and spray inoculation. The spot method of inoculation allows for a consistent volume of inoculum to be applied without the greater risk of human error that can be associated with the spray method. Applying a known, consistent, volume of inoculum is advantageous in that one can determine with more accuracy the reduction in pathogen population, either by removal or cell death, resulting from treatment with a sanitizer. However, spot inoculation of tomatoes with a low number of cells or a low volume containing a high numbers of cells may not be useful in determining the efficacy santizers because after treating tomatoes that were spot inoculated and treated with chlorine (200 µg/ml) no pathogens were detected by direct plating above the detection limit of 10 CFU/tomato. Enrichment of these tomatoes failed to detect pathogens from spot inoculated tomatoes treated with chlorine.

Results also show that the amount of time between inoculation and retrieval of cells can affect the viability and recovery of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* from tomatoes. Overall, higher populations of pathogens were recovered from tomatoes on which inocula were dried for 1 h compared to 24 h. The greater reduction in populations detected after 24 h is attributed in part to cell death and/or injury caused to desiccation.

Recovery of pathogens on lettuce. Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* recovered from inoculated lettuce are shown in Tables 2.5, 2.6, and 2.7, respectively. Values reflect number of *E. coli* O157:H7 and *Salmonella*, and *L. monocytogenes* recovered on BHIANP. Similar to results from studies on tomatoes, no cells were recovered from chlorine solution after treatment of lettuce, which indicates that cells removed from lettuce were killed. Within drying time and treatment, populations of pathogens detected in water after treating dip-inoculated lettuce were significantly higher ($\alpha = 0.05$) than populations in treatment water from spot- or spray-

Table 2.5. Populations of *E. coli* O157:H7 recovered from lettuce samples.

			Population ($\log_{10}$ CFU/ml or lettuce sample)[a]				
			Treatment solution		DE broth		
Inoculation method	Drying time (h)	Treatment[b]	CFU/ml	CFU/lettuce sample (± SD)[c]	CFU/ml	CFU/lettuce sample (± SD)[d]	Reduction[e]
Dip	2	None	nd[f]	nd	6.62	8.62 (0.27) a^1 a^2 a^3	
		Water	6.17	8.47 (0.52) a^1 a^2 a^3	6.18	8.18 (0.27) a a b	0.44
		Chlorine	<-0.30	< 2.00 a a b	5.52	7.52 (0.45) a a c	1.10
	24	None	nd	nd	6.67	8.67 (0.25) a a a	
		Water	5.92	8.22 (0.26) a a a	6.27	8.27 (0.32) a a b	0.40
		Chlorine	<-0.30	< 2.00 a a b	5.43	7.43 (0.30) a a c	1.24
Spot	2	None	nd	nd	5.16	7.16 (0.18) b a a	
		Water	4.75	7.05 (0.31) b a a	4.27	6.27 (0.23) c a b	0.89
		Chlorine	<-0.30	< 2.00 a a b	3.74	5.74 (0.36) b a c	1.42
	24	None	nd	nd	5.15	7.15 (0.21) b a a	
		Water	4.63	6.93 (0.23) b a a	4.31	6.31 (0.30) b a b	0.84
		Chlorine	<-0.30	< 2.00 a a b	3.70	5.70 (0.38) b a c	1.45
Spray	2	None	nd	nd	5.31	7.31 (0.36) b a a	
		Water	4.62	6.92 (0.26) b a a	4.72	6.72 (0.19) b a a	0.59
		Chlorine	<-0.30	< 2.00 a a b	3.56	5.56 (0.61) b a b	1.75
	24	None	nd	nd	5.10	7.10 (0.27) b a a	
		Water	4.33	6.63 (0.94) b a a	4.64	6.64 (0.26) b b b	0.46
		Chlorine	<-0.30	< 2.00 a a b	3.70	5.70 (0.57) b a c	1.40

(Continued)

Table 2.5. (continued)

[a] Treatment solutions (water of chlorinated water) and Dey-Engley (DE) wash broth were analyzed for numbers ($\log_{10}$ CFU/ml) of *E. coli* O157:H7 by direct plating; values were converted to $\log_{10}$ CFU/lettuce sample (three 9 x 9 cm pieces). The detection limit was 1 CFU/2 ml of treatment solution (100 CFU/lettuce sample) or 1 CFU/2 ml of DE wash (10 CFU/lettuce sample).

[b] Lettuce samples were not treated (none) with water or chlorine, or treated with 200 ml of deionized water or chlorinated (200 µg/ml) water for 5 min, then washed in 100 ml of DE broth for 1 min.

[c] Mean values ($\log_{10}$ CFU/lettuce sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different

[d] Mean values ($\log_{10}$ CFU/lettuce sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different

[e] Within inoculation method and drying time, reduction ($\log_{10}$ CFU/lettuce sample) compared to the population recovered from lettuce samples not treated (none) with water (control) or chlorine (200 µg/ml).

[f] nd, not determined.

Table 2.6. Populations of *Salmonella* recovered from lettuce samples.

			Population (log$_{10}$ CFU/ml or lettuce sample)[a]				
			Treatment solution		DE broth		
Inoculation method	Drying time (h)	Treatment[b]	CFU/ml	CFU/lettuce sample (± SD)[c]	CFU/ml	CFU/lettuce sample (± SD)[d]	Reduction[e]
Dip	2	None	nd[f]	nd	6.92	8.92 (0.20) a^1 a^2 a^3	
		Water	6.12	8.42 (1.36) a^1 a^2 a^3	6.71	8.71 (0.20) a a a	0.21
		Chlorine	<-0.30	< 2.00 a a b	5.83	7.83 (0.31) a a b	1.09
	24	None	nd	nd	6.96	8.96 (0.21) a a a	
		Water	6.04	8.34 (0.28) a a a	6.67	8.67 (0.29) a a b	0.29
		Chlorine	<-0.30	< 2.00 a a b	5.55	7.55 (0.19) a a c	1.41
Spot	2	None	nd	nd	5.55	7.55 (0.18) b a a	
		Water	4.97	7.27 (0.20) b a a	4.99	6.99 (0.20) b a b	0.56
		Chlorine	<-0.30	< 2.00 a a b	3.94	5.94 (0.55) b a c	1.61
	24	None	nd	nd	5.51	7.51 (0.16) b a a	
		Water	4.83	7.13 (0.21) b a a	4.90	6.90 (0.17) b a b	0.61
		Chlorine	<-0.30	< 2.00 a a b	3.66	5.66 (0.55) b b c	1.85
Spray	2	None	nd	nd	5.65	7.65 (0.23) b a a	
		Water	4.92	7.22 (0.32) b a a	5.27	7.27 (0.35) b a b	0.38
		Chlorine	<-0.30	< 2.00 a a b	4.05	6.05 (0.25) b a c	1.60
	24	None	nd	nd	5.43	7.43 (0.16) b a a	
		Water	4.63	6.93 (1.15) b a a	5.12	7.12 (0.27) b b a	0.31
		Chlorine	<-0.30	< 2.00 a a b	3.85	5.85 (0.51) b a b	1.58

(Continued)

Table 2.6. (continued)

[a] Treatment solutions (water of chlorinated water) and Dey-Engley (DE) wash broth were analyzed for numbers ($\log_{10}$ CFU/ml) of *Salmonella* by direct plating; values were converted to $\log_{10}$ CFU/lettuce sample (three 9 x 9-cm pieces). The detection limit was 1 CFU/2 ml of treatment solution (100 CFU/lettuce sample) or 1 CFU/2 ml of DE wash (10 CFU/lettuce sample).

[b] Lettuce samples were not treated (none) with water or chlorine, or treated with 200 ml of deionized water or chlorinated (200 µg/ml) water for 5 min, then washed in 100 ml of DE broth for 1 min.

[c] Mean values ($\log_{10}$ CFU/lettuce sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.
[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different
[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different

[d] Mean values ($\log_{10}$ CFU/lettuce sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.
[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different
[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different

[e] Within inoculation method and drying time, reduction ($\log_{10}$ CFU/lettuce sample) compared to the population recovered from lettuce samples not treated (none) with water (control) or chlorine (200 µg/ml).

[f] nd, not determined.

Table 2.7. Populations of *Listeria* monocytogenes recovered from lettuce samples.

			Population ($\log_{10}$ CFU/ ml or lettuce sample)[a]				
			Treatment solution		DE broth		
Inoculation method	Drying time (h)	Treatment[b]	CFU/ml	CFU/lettuce sample (± SD)[c]	CFU/ml	CFU/lettuce sample (± SD)[d]	Reduction[e]
Dip	2	None	nd[f]	nd	6.89	8.89 (0.29) a^1 a^2 a^3	
		Water	6.21	8.51 (0.31) a^1 a^2 a^3	6.52	8.52 (0.21) a a b	0.37
		Chlorine	<-0.30	< 2.00 a a b	5.73	7.73 (0.39) a a c	1.16
	24	None	nd	nd	6.91	8.91 (0.24) a a a	
		Water	5.71	8.01 (0.39) a b a	6.47	8.47 (0.30) a a b	0.44
		Chlorine	<-0.30	< 2.00 a a b	6.70	7.70 (0.19) a a c	1.21
Spot	2	None	nd	nd	5.38	7.37 (0.21) c a a	
		Water	4.63	6.93 (0.45) c a a	4.50	6.50 (0.39) c a b	0.87
		Chlorine	<-0.30	< 2.00 a a b	3.59	5.58 (0.64) b a c	1.79
	24	None	nd	nd	4.91	6.91 (0.16) c b a	
		Water	4.46	6.76 (0.25) b a a	4.50	6.50 (0.57) c a a	0.41
		Chlorine	<-0.30	< 2.00 a a b	3.77	5.76 (0.53) b a b	1.15
Spray	2	None	nd	nd	5.64	7.64 (0.23) b a a	
		Water	4.93	7.23 (0.33) b a a	5.21	7.21 (0.30) b a b	0.43
		Chlorine	<-0.30	< 2.00 a a b	3.98	5.98 (0.42) b a c	1.66
	24	None	nd	nd	5.19	7.19 (0.26) b b a	
		Water	4.38	6.68 (0.41) b a a	5.03	7.03 (0.37) b a a	0.16
		Chlorine	<-0.30	< 2.00 a a b	3.66	5.66 (0.51) b a b	1.53

(Continued)

Table 2.7. (continued)

[a] Treatment solutions (water of chlorinated water) and Dey-Engley (DE) wash broth were analyzed for numbers ($\log_{10}$ CFU/ml) of *L.monocytogenes* by direct plating; values were converted to $\log_{10}$ CFU/lettuce sample (three 9 x 9-cm pieces). The detection limit was 1 CFU/2 ml of treatment solution (100 CFU/lettuce sample) or 1 CFU/2 ml of DE wash (10 CFU/lettuce sample).

[b] Lettuce samples were not treated (none) with water or chlorine, or treated with 200 ml of deionized water or chlorinated (200 µg/ml) water for 5 min, then washed in 100 ml of DE broth for 1 min.

[c] Mean values ($\log_{10}$ CFU/lettuce sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.
[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different
[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different

[d] Mean values ($\log_{10}$ CFU/lettuce sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.
[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different
[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different

[e] Within inoculation method and drying time, reduction ($\log_{10}$ CFU/lettuce sample) compared to the population recovered from lettuce samples not treated (none) with water (control) or chlorine (200 µg/ml).

[f] nd, not determined.

inoculated lettuce. With the exception of lettuce dip-inoculated with *L. monocytogenes* (Table 2.7), within inoculation method, the number of pathogens detected in water or chlorinated water after treatment of lettuce was not affected by drying time. Populations of all three pathogens recovered from DE broth after washing dip-inoculated lettuce were significantly higher than populations recovered from spot- or spray-inoculated lettuce, regardless of drying time or treatment. Significantly higher populations of *E. coli* O157:H7 (Table 2.5) and *L. monocytogenes* (Table 2.7) were detected in water after treatment of spray-inoculated lettuce dried for 2 h compared to spot-inoculated lettuce and in wash water from untreated lettuce spray-inoculated with *L. monocytogenes* and dried for 2 h compared to untreated spot-inoculated lettuce. Within inoculation method and treatment, drying time did not significantly affect the number of *E. coli* O157:H7 (Table 2.5) or *Salmonella* (Table 2.6) recovered from lettuce in DE broth. However, within lettuce spot-inoculated or spray-inoculated with *L. monocytogenes* (Table 2.8), significantly higher populations were detected in DE broth from lettuce on which inoculum was dried for 2 h versus 24 h. Within inoculation method and drying time, significantly lower populations of all three pathogens were recovered from lettuce treated with chlorine compared to treatment with water. In some instances, washing with water did not result in a significant reduction in populations compared to populations recovered from untreated lettuce.

Results indicate that the method of inoculation can result in different numbers of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* adhering to lettuce. Higher populations of pathogens were recovered from dip-inoculated lettuce compared to lettuce that was spot- or spray-inoculated. The dip method facilitated infiltration of cut edges and stomata on the surface of the lettuce, whereas spot- and spray-inoculation minimized distribution of cells at these sites. Overall, the spot method affords more control over the volume of inoculum, and therefore the number of cells applied, compared to the dip and spray methods. Although care was taken with the spray method to place the inoculum on the middle of the piece of lettuce leaf, it was difficult to avoid delivery of some of the inoculum on the cut edges. This would make it difficult to use spray-inoculation as a standard method of inoculation, as distribution of some of the cells in the cut edges of the lettuce leaf rather than on the surface of the lettuce leaf would affect the efficacy of sanitizers and efficiency of recovering of surviving cells. Spot-inoculation allows for a known consistent volume of inoculum to be applied to a confined location, thereby enabling for a more accurate determination of the reduction in pathogen population, either by removing or killing cells.

Results also indicate that drying time after inoculation had little or no affect on the viability or recoverability of *E. coli* O157:H7, *Salmonella*, and *L.monocytogenes* from lettuce. Populations recovered after drying inoculum for 2 h compared to 24 h were similar and in most cases not significantly different, regardless of method of inoculation.

Recovery of pathogens on parsley. Populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* recovered from inoculated parsley are shown in Tables 2.8, 2.9, and 2.10, respectively. Values reflect numbers of *E. coli* O157:H7 and *Salmonella* recovered on TSANP and *L. monocytogenes* recovered on BHIANP. Similar to results from tomato and lettuce samples, pathogens were not recovered from chlorine solution after treatment of parsley, which indicates that cells removed from parsley were killed.

Table 2.8. Populations of *E. coli* O157:H7 recovered from parsley.

			Population ($\log_{10}$ CFU/ ml or parsley sample)[a]					
			Treatment solution		DE broth			
Inoculation method	Drying time (h)	Treatment[b]	CFU/ml	CFU/parsley sample (± SD)[c]	CFU/ml	CFU/parsley sample (± SD)[d]	Reduction[e]	En[f]
Dip	2	None	nd[g]	nd	6.99	8.99 (0.34) a^1 a^2 a^3		
		Water	6.69	8.99 (0.49) a^1 a^2 a^3	6.20	8.22 (0.25) a a b	0.77	
		Chlorine	<-0.30	2.00 a a b	4.73	6.73 (0.30) a a c	2.26	
	24	None	nd	nd	6.76	8.76 (0.25) a a a		
		Water	6.71	9.01 (0.40) a a a	6.23	8.23 (0.18) a a b	0.53	
		Chlorine	<-0.30	2.00 a a b	4.58	6.58 (0.20) a a c	2.18	
Spot	2	None	nd	nd	4.83	6.83 (0.23) b a a		
		Water	4.33	6.63 (0.21) b a a	3.74	5.74 (0.23) b a b	1.09	
		Chlorine	<-0.30	2.00 a a b	1.44	3.44 (1.38) b a c	3.39	2/2
	24	None	nd	nd	4.36	6.36 (0.31) b a a		
		Water	4.20	6.50 (0.40) b a a	3.73	5.73 (0.43) b a a	0.63	
		Chlorine	<-0.30	2.00 a a b	0.97	2.97 (1.08) b a b	3.39	
Spray	2	None	nd	nd	3.36	5.36 (0.33) c a a		
		Water	2.89	5.19 (0.43) c a a	2.87	4.87 (0.39) c a a	0.49	
		Chlorine	<-0.30	2.00 a a b	0.50	2.50 (1.20) b a b	2.86	4/4
	24	None	nd	nd	3.24	5.24 (0.37) c a a		
		Water	2.43	4.73 (0.48) c a a	2.62	4.62 (0.41) c a a	0.62	
		Chlorine	<-0.30	2.00 a a b	0.43	2.43 (1.30) b a b	2.81	3/4

(Continued)

Table 2.8. (continued)

[a] Treatment solutions (water or chlorinated water) and Dey-Engley (DE) wash broth were analyzed for numbers ($\log_{10}$ CFU/ml) of *E. coli* O157:H7 by direct plating; values were converted to $\log_{10}$ CFU/parsley sample. The detection limit was 1 CFU/2 ml of treatment solution (100 CFU/parsley sample) or 1 CFU/2 ml of DE wash (10 CFU/parsley sample).

[b] Parsley samples were not treated (none) with water or chlorine, or treated with 200 ml of deionized water or chlorinated (200 µg/ml) water for 5 min, then washed in 100 ml of DE broth for 1 min.

[c] Mean values ($\log_{10}$ CFU/parsley sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[d] Mean values ($\log_{10}$ CFU/parsley sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[e] Within inoculation method and drying time, reduction ($\log_{10}$ CFU/parsley sample) compared to the population recovered from parsley samples not treated (none) with water (control) or chlorine (200 µg/ml).

[f] Number of treated, washed parsley samples positive for *E. coli* O157:H7, as detected by enrichment (En), out of the number of parsley samples analyzed by enrichment. Parsley samples on which *E. coli* O157:H7 was detected by direct plating of DE broth were not analyzed by enrichment.

[g] nd, not determined.

Table 2.9. Populations of *Salmonella* recovered from parsley.

			Population ($\log_{10}$ CFU/ ml or parsley sample)[a]					
			Treatment solution		DE broth			
Inoculation method	Drying time (h)	Treatment[b]	CFU/ml	CFU/parsley sample (± SD)[c]	CFU/ml	CFU/parsley sample (± SD)[d]	Reduction[e]	En[f]
Dip	2	None	nd[g]	nd	7.13	9.13 (0.37) a^1 a^2 a^3		
		Water	6.45	8.76 (0.36) a^1 a^2 a^3	6.61	8.61 (0.46) a a a	0.52	
		Chlorine	<-0.30	2.00 a a b	5.42	7.42 (0.61) a a b	1.71	
	24	None	nd	nd	6.93	8.93 (0.41) a a a		
		Water	6.41	8.72 (0.43) a a a	6.49	8.49 (0.26) a a a	0.44	
		Chlorine	<-0.30	2.00 a a b	5.34	7.34 (0.35) a a b	1.59	
Spot	2	None	nd	nd	4.89	6.89 (0.24) b a a		
		Water	4.27	6.57 (0.11) b a a	4.16	6.16 (0.26) b a b	0.73	
		Chlorine	<-0.30	2.00 a a b	0.31	2.31 (1.18) b a c	4.58	4/4
	24	None	nd	nd	4.53	6.53 (0.26) b a a		
		Water	3.93	6.23 (0.27) b a a	4.09	6.09 (0.24) b a a	0.44	
		Chlorine	<-0.30	2.00 a a b	0.26	2.26 (1.11) b a b	4.27	2/2
Spray	2	None	nd	nd	4.50	6.50 (0.45) c a a		
		Water	3.73	6.03 (0.31) c a a	4.14	6.14 (0.37) b a a	0.36	
		Chlorine	<-0.30	2.00 a a b	0.50	1.50 (0.87) b a b	5.00	8/8
	24	None	nd	nd	4.19	6.19 (0.23) b a a		
		Water	3.44	5.74 (0.25) c a a	3.77	5.77 (0.24) c a a	0.42	
		Chlorine	<-0.30	2.00 a a b	0.24	2.24 (1.40) b a b	3.95	3/3

(Continued)

Table 2.9. (continued)

[a] Treatment solutions (water or chlorinated water) and Dey-Engley (DE) wash broth were analyzed for numbers ($\log_{10}$ CFU/ml) of *Salmonella* by direct plating; values were converted to $\log_{10}$ CFU/parsley sample. The detection limit was 1 CFU/2 ml of treatment solution (100 CFU/parsley sample) or 1 CFU/2 ml of DE wash (10 CFU/parsley sample).

[b] Parsley samples were not treated (none) with water or chlorine, or treated with 200 ml of deionized water or chlorinated (200 µg/ml) water for 5 min, then washed in 100 ml of DE broth for 1 min.

[c] Mean values ($\log_{10}$ CFU/parsley sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[d] Mean values ($\log_{10}$ CFU/parsley sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[e] Within inoculation method and drying time, reduction ($\log_{10}$ CFU/parsley sample) compared to the population recovered from parsley samples not treated (none) with water (control) or chlorine (200 µg/ml).

[f] Number of treated, washed parsley samples positive for *Salmonella*, as detected by enrichment (En), out of the number of parsley samples analyzed by enrichment. Parsley samples on which *Salmonella* was detected by direct plating of DE broth were not analyzed by enrichment.

[g] nd, not determined.

Table 2.10. Populations of *Listeria monocytogenes* recovered from parsley.

			Population ($\log_{10}$ CFU/ ml or parsley sample)[a]					
			Treatment solution		DE broth			
Inoculation method	Drying time (h)	Treatment[b]	CFU/ml	CFU/parsley sample (± SD)[c]	CFU/ml	CFU/parsley sample (± SD)[d]	Reduction[e]	En[f]
Dip	2	None	nd[g]	nd	7.26	9.26 (0.26) a^1 a^2 a^3		
		Water	6.73	9.04 (0.35) a^1 a^2 a^3	6.59	8.39 (0.18) a a b	0.87	
		Chlorine	<-0.30	2.00 a a b	4.80	6.80 (0.27) a a c	2.46	
	24	None	nd	nd	7.03	9.03 (0.23) a a a		
		Water	6.27	8.57 (0.39) a a a	6.20	8.20 (0.31) a a b	0.83	
		Chlorine	<-0.30	2.00 a a b	4.82	6.82 (0.35) a a c	2.21	
Spot	2	None	nd	nd	5.21	7.21 (0.18) b a a		
		Water	4.58	6.88 (0.15) b a a	4.52	6.52 (0.20) b a a	0.69	
		Chlorine	<-0.30	2.00 a a b	1.91	3.91 (1.17) b a b	3.30	1/1
	24	None	nd	nd	4.78	6.78 (0.17) b b a		
		Water	4.22	6.52 (0.30) b a a	4.25	6.25 (0.17) b a b	0.53	
		Chlorine	<-0.30	2.00 a a b	1.42	3.42 (0.98) b a c	3.36	0/1
Spray	2	None	nd	nd	5.28	7.28 (0.27) b a a		
		Water	4.49	6.79 (0.36) b a a	4.89	6.89 (0.25) b a a	0.39	
		Chlorine	<-0.30	2.00 a a b	2.24	4.24 (0.74) b a b	3.04	
	24	None	nd	nd	4.73	6.73 (0.45) b b a		
		Water	3.92	6.23 (0.47) b b a	4.34	6.34 (0.27) b b a	0.39	
		Chlorine	-0.22	2.07 (0.15) a a b	1.99	3.99 (0.63) b a b	2.74	

(Continued)

Table 2.10. (continued)

[a] Treatment solutions (water or chlorinated water) and Dey-Engley (DE) wash broth were analyzed for numbers ($\log_{10}$ CFU/ml) of *L. monocytogenes* by direct plating; values were converted to $\log_{10}$ CFU/parsley sample. The detection limit was 1 CFU/2 ml of treatment solution (100 CFU/parsley sample) or 1 CFU/2 ml of DE wash (10 CFU/Parsley sample).

[b] Parsley samples were not treated (none) with water or chlorine, or treated with 200 ml of deionized water or chlorinated (200 µg/ml) water for 5 min, then washed in 100 ml of DE broth for 1 min.

[c] Mean values ($\log_{10}$ CFU/parsley sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[d] Mean values ($\log_{10}$ CFU/parsley sample) in columns were analyzed for significant differences ($\alpha = 0.05$):

[1] First column of letters: Within drying time and treatment, values not followed by the same letter are significantly different.

[2] Second column of letters: Within inoculation method and treatment, values not followed by the same letter are significantly different.

[3] Third column of letters: Within inoculation method and drying time, values not followed by the same letter are significantly different.

[e] Within inoculation method and drying time, reduction ($\log_{10}$ CFU/parsley sample) compared to the population recovered from parsley sample not treated (none) with water (control) or chlorine (200 µg/ml).

[f] Number of treated, washed parsley samples positive for *L. monocytogenes*, as detected by enrichment (En), out of the number of parsley samples analyzed by enrichment. Parsley samples on which *L. monocytogenes* was detected by direct plating of DE broth were not analyzed by enrichment.

[g] nd, not determined.

Within drying time and treatment, the number of *E. coli* O157:H7 (Table 2.8) and *Salmonella* (Table 2.9) recovered from parsley was in the overall order of dip > spot > spray inoculation. Based on inoculation method, within drying time and treatment, the order of recovery of *L. monocytogenes* from parsley was dip > spot = spray (Table 2.10). Overall, higher populations of *L. monocytogenes* survived drying for 2 h at 22◦C compared to drying for 2 h at 22◦C followed by 22 h at 4◦C. Higher populations of all three pathogens were recovered from parsley treated with water compared to chlorine.

Data indicate that inoculation method affects the numbers of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* adhering to parsley. Higher populations of cells were recovered from parsley that was dip-inoculated compared to parsley spot- or spray-inoculated. Dip-inoculation allows for inoculum to come into contact with more surface area than the spot- or spray-inoculation methods. Unlike the spot- and spray-inoculation methods, dip inoculum came into contact with the cut ends of the parsley leaf stem, which may facilitate adherence or uptake of cells by cut tissues. Overall, the spot inoculation method affords more control over the volume of inoculum applied and the surfaces to which the inoculum is applied. Although care was taken to apply the inoculum to the leaf portion of parsley, some of the inoculum may not have adhered to the parsley, but rather passed through the spaces between leaves. This potential for variation in volume of inoculum applied makes spray-inoculation less desirable for use in a standard method for testing the efficacy of sanitizers. Spot-inoculation enables a known consistent volume of inoculum to be applied to a specific area of the parsley sample.

Data also indicate, similar to observations on lettuce, that drying time after inoculation had little or no affect on the viability or recoverability of pathogens from parsley. Populations recovered after drying inoculum for 2 h compared to 24 h were similar and in most cases not significantly different, regardless of method of inoculation.

Overall effects of test parameters. Shown in Table 2.11 are overall comparisons of populations of pathogens recovered from produce as affected by each test parameter. Numbers of test pathogens recovered from tomatoes inoculated by the dip method were significantly ($\alpha = 0.05$) higher than numbers recovered from tomatoes inoculated by spot and spray methods. In the dip inoculation method, more inocula came in contact with a larger surface area than resulted from either spot or spray methods, thereby increasing the number of sites available for attachment of a larger number of cells. Care was taken in the spot and spray methods to avoid placing the inocula on the stem scar, as previous studies (*20, 33*) have shown the porous stem scar tissue on tomatoes to be more susceptible than skin to infiltration of microorganisms. When dip inoculated, cells in suspensions that came in contact with the stem scar tissue would be expected to be retained in higher numbers than on tomato skin. The spot and spray methods of inoculation afford more control of the amount of inoculum applied. A known volume of inoculum can be placed on a selected site on the surface of produce. Populations of *E. coli* O157:H7 or *Salmonella* recovered from tomatoes after inoculation by spot and spray methods were not significantly different. Significantly ($\alpha = 0.05$) different populations (dip > spot > spray) were recovered from tomatoes inoculated with *L. monocytogenes* using the three test methods. Populations of all test pathogens

Table 2.11. Overall comparisons of populations of foodborne pathogens recovered from tomatoes, lettuce, or parsley as affected by test parameter.

		Population ($\log_{10}$ CFU/tomato, lettuce, or parsley)[a]		
Produce	Test parameter	*E. coli* O157:H7	*Salmonella*	*L. monocytogenes*
Tomato	Inoculation method			
	Dip	4.44 a	5.26 a	5.26 a
	Spot	2.82 b	3.37 b	4.10 b
	Spray	2.81 b	3.07 b	3.55 c
	Drying time			
	1 h	4.02 a	4.41 a	4.66 a
	24 h	2.69 b	3.40 b	3.97 b
	Treatment			
	None	4.88 a	5.67 a	6.32 a
	Water	3.45 b	4.21 b	4.94 b
	Chlorine (200 µg/ml)	1.76 c	1.82 c	1.68 c
	Recovery medium			
	TSANP	3.51 a	--	--
	SMACNP	3.21 b	--	--
	TSANP	--	4.00 a	--
	BSANP	--	3.81 b	--
	BHIANP	--	--	4.46 a
	MOXNP	--	--	4.18 b
Lettuce	Inoculation method			
	Dip	8.13 a	8.44 a	8.37 a
	Spot	6.39 b	7.76 b	6.44 c
	Spray	6.51 b	6.89 b	6.78 b
	Drying time			
	2 h	7.00 a	7.44 a	7.27 a
	24 h	7.00 a	7.29 b	7.13 b
	Treatment			
	None	7.67 a	8.00 a	7.82 a
	Water	7.07 b	7.61 b	7.37 b
	Chlorine (200 µg/ml)	6.24 c	6.48 c	6.40 c
	Recovery medium			
	TSANP	7.98	--	--
	SMACNP	6.57	--	--
	TSANP	--	7.48	--
	BSANP	--	7.21	--
	BHIANP	--	--	7.01
	MOXNP	--	--	6.91

(Continued)

Table 2.11. (continued)

Produce	Test parameter		Population ($\log_{10}$ CFU/tomato, lettuce, or parsley)[a]		
			E. coli O157:H7	*Salmonella*	*L. monocytogenes*
Parsley	Inoculation method				
		Dip	7.91 a	8.32 a	8.12 a
		Spot	5.15 b	5.04 b	5.93 b
		Spray	4.17 c	4.72 c	5.76 b
	Drying time				
		2 h	5.83 a	6.07 a	6.85 a
		24 h	5.65 a	5.98 b	6.40 b
	Treatment				
		None	6.90 a	7.36 a	7.73 a
		Water	6.24 b	6.88 b	7.13 b
		Chlorine (200 µg/ml)	4.12 c	3.85 c	4.92 c
	Recovery medium				
		TSANP	5.79	--	--
		SMACNP	5.29	--	--
		TSANP	--	6.38	--
		BSANP	--	6.02	--
		BHIANP	--	--	6.52
		MOXNP	--	--	6.15

[a] Samples consisted of one tomato, three 9 x 9-cm pieces of lettuce leaf or 20 g of parsley. Within produce, test parameter, and pathogen, mean values not followed by the same letter are significant.

recovered from tomatoes after drying inoculum for 1 h were significantly higher than populations recovered after drying for 24 h. *E. coli* O157:H7 was the least resistant to drying, with a decrease of 1.33 $\log_{10}$ CFU/tomato compared to decreases in populations of 1.01 $\log_{10}$ CFU/tomato and 0.69 $\log_{10}$ CFU/tomato for *Salmonella* and *L. monocytogenes,* respectively, between 1 and 24 h. This may be due in part, to greater resistance to desiccation of the gram-positive cell wall of *L monocytogenes* (*29).* This concurs with observations reported by Beuchat et al. (*8*), showing greater reductions in populations of *E. coli* O157:H7 compared to *Salmonella* and *L. monocytogenes* inoculated onto tomatoes and dried for 2 h at 22 ± 2°C. Populations of all pathogens recovered from tomatoes treated with chlorine (200 µg/ml) were significantly lower than populations recovered from tomatoes treated with water. The largest reduction (4.64 $\log_{10}$ CFU/tomato), compared to untreated tomatoes, occurred on tomatoes inoculated with *L. monocytogenes*, and treated with chlorine. Significantly higher numbers of pathogens were recovered on non-selective media (TSANP or BHIANP) than on the selective media (SMACNP, BSANP, and MOXNP).

Higher populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* were recovered from lettuce inoculated by dipping in cell suspension compared to spot and spray inoculation methods (Table 2.11). Unlike the spot and spray methods, cut edges of the lettuce leaf, which have a greater propensity for the uptake of microbial cells, compared to the surface of the lettuce leaf (*25),* were exposed to the dip inoculum. Populations of *E. coli* O157:H7 and *Salmonella* recovered from lettuce inoculated by spot or spray methods were not significantly different. As observed with tomatoes, however, significantly ($\alpha = 0.05$) different populations (dip > spray > spot) were recovered from lettuce inoculated with *L. monocytogenes*. Populations of *E. coli* O157:H7 recovered from lettuce on which inocula had dried for 2 h at 22 ± 2°C, compared to drying for 2 h at 22 ± 2°C followed by 22 h at 4°C, were not significantly different. Populations of *Salmonella* and *L. monocytogenes* recovered from lettuce on which inocula were dried for 2 h at 22°C were significantly higher than populations recovered from lettuce after drying inocula for an additional 22 h at 4°C. Reductions in populations of these pathogens between the two drying times, however, were only 0.15 and 0.14 $\log_{10}$ CFU/lettuce sample, respectively. Other studies have also recorded little or no change in populations of pathogens recovered from inoculated lettuce stored at refrigerated temperatures for 24 h (*14, 19*). Significant reductions of 0.83, 1.13, and 0.97 $\log_{10}$ CFU/lettuce sample for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*, respectively, were achieved by treatment with chlorine compared to water.

Higher populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* were recovered from parsley inoculated by dipping in cell suspensions compared to spot and spray inoculation methods (Table 2.11). Unlike the spot and spray methods in which inoculum was applied only to the uncut leaf portion of the parsley samples, inoculum came in contact with both the leaf portion and stems including the cut ends of the parsley that was dip inoculated. Populations of *E. coli* O157:H7 and *Salmonella* recovered from parsley by the three inoculation methods were significantly ($\alpha = 0.05$) different (dip > spot > spray). Populations of *L. monocytogenes* recovered from parsley inoculated by spot and spray methods were not significantly different from each other, but were significantly lower than populations recovered from dip inoculated parsley. Like populations of *E. coli* O157:H7 recovered from lettuce, no significant difference was seen between parsley dried for 2 h at 22°C, compared to parsley dried for 2 h at 22°C and followed by 22 h at 4◦C. Populations of *Salmonella* and *L. monocytogenes* recovered after 2 h at 22°C were significantly higher than populations recovered from samples dried for 2 h at 22°C and followed by 22 h at 4°C. Significant reductions of 2.12, 3.03, and 2.21 $\log_{10}$ CFU/parsley sample for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*, respectively, resulted from treatment with chlorine compared to treatment with water. The largest reduction (3.51 $\log_{10}$ CFU/parsley sample), compared to untreated parsley, occurred on parsley, inoculated with *Salmonella* and treated with chlorine.

Higher populations of all three pathogens were recovered from lettuce and parsley using non-selective media versus selective media in the first replicate experiment. Since background microflora did not interfere with counting colonies by direct plating of samples on TSANP or BHIANP, selective media were not used in the second and third replicate experiments. For this reason, mean values for populations recovered from lettuce and parsley on non-selective and selective media were not subjected to statistical analysis. Because higher or equal populations of pathogens were recovered on non-selective media (TSANP or BHIANP) compared to selective media (SMACNP, BSANP, or MOXNP) for tomatoes, lettuce, and parsley only data obtained using non-selective media were presented in Tables 2.2-2.10.

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CHAPTER 3

SUMMARY, CONCLUSIONS, AND RECCOMMENDATIONS

This study investigated the effects of inoculation method and drying time as factors to be considered in developing a standard method to measure the efficacy of sanitizers in killing foodborne pathogens on salad vegetables. Recoverability of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* inoculated onto tomatoes, lettuce, and parsley followed by treatment with chlorine was evaluated.

Results show that the method of inoculation affects the number of cells that adhere to and are recovered from tomatoes, lettuce, and parsley. The spot inoculation method affords more control over the volume of inoculum and number of cells applied to produce compared to the dip and spray methods, thereby enabling a more accurate measurement of reduction in pathogen population resulting from removal or death upon treatment with chlorine.

The time between inoculation and treatment should be considered when developing a standardized method for testing the efficacy of sanitizers. Reductions in populations of pathogens during drying on tomatoes, lettuce, and parsley are attributed in part to different temperatures at which produce was stored during the drying period and to substantial differences in surface morphology and structures that may influence the attachment, infiltration, and survival of bacterial cells.

A standardized method for testing the efficacy of sanitizers should be developed such that comparisons between a standard sanitizer, e.g. chlorine at 200 µg/ml, and the test sanitizer can be made. If treatment of produce with a standard sanitizer results in reductions in populations of pathogens to very low or undetectable levels, then the relative efficacy of the test sanitizer, which may be more lethal, than the standard sanitizer cannot be determined.

In this study pathogens were not recovered from tomatoes that were spot inoculated and treated with chlorine. In order to enable comparisons to be made between 200 µg/ml chlorine, a standard sanitizer, and a potentially more effective test sanitizer, modifications of the conditions used to spot inoculate tomatoes should be made. Higher levels of inoculum could remedy this situation. Beuchat et al. (2001) showed that under different inoculum preparation methods, i.e. higher population levels but the same volume of inoculum (50 µl), treatment with chlorine at 200 µg/ml did not reduce populations of pathogens to undetectable limits. It is recommended that spot inoculation be used in a standard method for testing the efficacy of sanitizers. However, for produce samples with smooth surface morphology similar to tomatoes the inoculum should be prepared as in the study by Beuchat et al., 2001. Because of differences in surface morphology, modifications of inoculum preparation are not needed for lettuce and parsley.

A standard method should ideally include an inoculum drying time that allows complete removal of water from the inoculum carrier, without loss of cell viability due to desiccation. Drying time will depend on the surface morphology of the produce being tested. Based on the results of this study an inoculum drying time of 24 h at 22°C is recommended for tomatoes; drying for 2 h at 22°C followed by 22 h at 4°C is recommended for lettuce and parsley. These drying times are recommended because they mimic some post-harvesting storage conditions to which produce may be subjected.

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