

ABSTRACT

JOHNSTON, LYNETTE MARIE. The microbial quality and safety of produce. (Under the direction of Dr. Lee-Ann Jaykus.)

The proportion of foodborne illnesses associated with fresh produce has increased over the last decade. Economically effective and practical programs to reduce the risk of contamination are limited by gaps in our knowledge of the microbiological quality and safety of produce. In an effort to address this concern, the objectives of the first three studies were (i) to determine the quality and safety of produce sampled from the farm and packing sheds by enumerating microbial indicator organisms and testing for various pathogens; (ii) to examine the routes of microbial contamination in domestic and imported produce, and identify areas of potential contamination throughout production and processing; and (iii) to characterize the antimicrobial resistance profiles of *Enterococcus* spp. among fresh produce. Furthermore, in response to recent governmental testing recommendations for the seed sprout industry, the final study seeks to improve rapid detection methods of foodborne pathogens associated with raw sprouts.

For the first study, produce samples were collected from the southern United States throughout production and processing and assayed by enumerative tests for total aerobic bacteria, total coliforms, total *Enterococcus*, and *E. coli*. These samples were also analyzed for the presence of *Salmonella*, *Listeria monocytogenes*, and *E. coli* O157:H7. Microbial levels remained relatively constant throughout processing for most of these products; however, for cilantro and parsley, total coliform levels increased during the packing process. For cantaloupe, microbial levels significantly increased from field through processing. Only *Salmonella* was detected among the produce samples.

The second study analyzed the antibiotic resistance profiles of *Enterococcus faecium* and *E. faecalis* isolated from a subset of the product samples collected during the first study, all harvested in the southern U.S. *E. faecium* and *E. faecalis* isolates were screened for antibiotic resistance profiles using a panel of seventeen antibiotics. Of human clinical importance, *E. faecium* strains had a much higher prevalence of resistance to ciprofloxacin, tetracycline, and nitrofurantoin than *E. faecalis*. *E. faecalis* strains had a low prevalence of resistance to antibiotics used to treat *E. faecalis* infections of both human and animal clinical relevance. Thirty-four percent of the isolates had multiple drug resistance patterns, excluding intrinsic resistance.

The third study evaluated the microbiological quality and safety of domestic and imported produce samples collected from packing sheds in the southern U.S., including microbial loads on environmental surface swabs. Antibiotic resistance profiles of *E. faecium* and *E. faecalis* strains isolated from the produce samples was also analyzed. Enumerative tests included total aerobic bacteria (APC), total coliforms, total *Enterococcus*, and *E. coli*. Produce samples were also analyzed for the presence of *Salmonella*, *Listeria monocytogenes*, *Shigella*, and *E. coli* O157:H7. The levels of microbial indicators remained constant in most cases, with increases in several commodities, throughout the wash process within the packing sheds. The degree of environmental contamination from swabs was similar to that found for fresh produce. No *Salmonella*, *Shigella*, or *E. coli* O157:H7 were detected from the produce samples. However, three samples were found positive for *L. monocytogenes*. Overall, a low degree of antibiotic resistance was found among *Enterococcus* isolates.

The purpose of final study was to develop a simple method to pre-concentrate pathogens from sprouts and spent irrigation water to facilitate the direct (without prior

cultural enrichment) detection of pathogens using the polymerase chain reaction (PCR). Alfalfa sprouts and spent irrigation water were seeded with *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7 in the range of 10^{-1} to 10^6 CFU/g or ml. The samples were then pre-concentrated by centrifugation, and the resulting precipitate was processed for DNA isolation, PCR amplification, and amplicon confirmation by Southern hybridization. Using primers targeting the *inv A* gene for serovar Typhimurium and the *stx* genes of *E. coli* O157:H7, it was possible to detect both pathogens in alfalfa sprouts at seeding levels as low as 10^1 CFU/g. PCR detection limits for both pathogens from spent irrigation water were 10^{-1} CFU/ml, the equivalent of 10^2 CFU/liter.

Taken together, this research contributes to a body of knowledge that will assist in the design of effective intervention strategies for the lowering the risk of foodborne disease associated with fresh produce.

THE MICROBIAL QUALITY AND SAFETY OF PRODUCE

by

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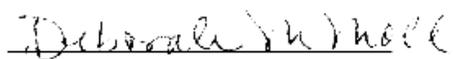
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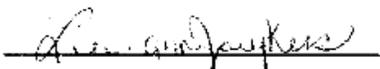
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DEDICATION

To my family and in loving memory of my dad

BIOGRAPHY

Lynette Johnston was born on November 21, 1975. She grew up in Nazareth, Texas, on a family-owned dairy farm. In 1998, she received her B.S. in Food Science at Texas Tech University, and upon graduation, she worked as a production supervisor at Leprino Foods in Roswell, NM for approximately two years. She began her graduate studies at NC State in 2000, and was actively involved in the Food Science Club. After obtaining her Ph.D., Lynette has taken a post doc position at the College of Veterinary Medicine at NC State, and looks forward to starting a family.

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CHAPTER 1
LITERATURE REVIEW

**THE EPIDEMIOLOGY OF PRODUCE-ASSOCIATED OUTBREAKS OF
FOODBORNE DISEASE**

1.1. INTRODUCTION

The food supply in the United States is considered one of the safest in the world. In fact, recent reports indicate that from 1996 to 2003 the estimated incidence of several important foodborne diseases in the U.S., including those caused by *Escherichia coli* O157:H7, *Salmonella*, *Campylobacter*, and *Cryptosporidium*, have declined (CDC 2004). Regardless, in the U.S. alone, it is estimated that 76 million persons contract foodborne illness each year, with an associated 325,000 hospitalizations and 5,000 deaths (Mead et al., 1999).

The epidemiology, or the occurrence and distribution, of foodborne disease in a population, is the result of complex interactions among environmental, cultural, and socioeconomic factors (Potter et al., 1996). As new foods and those from alternative sources become available, new opportunities for transmission of foodborne disease often follow. Other factors which contribute to the dynamics of foodborne disease include changes in human demographics and behavior, as well as new food production and processing technologies. Some infectious agents have been either newly described or newly associated with foodborne transmission routes (Hedberg et al., 1994; Altekruze et al., 1997; Tauxe 1997). Examples such as *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Cyclospora cayetanensis* are illustrative of this phenomenon.

Fresh produce can be a vehicle for the transmission of bacterial, parasitic, and viral pathogens capable of causing human illness. Although low, the proportion of U.S. foodborne illness associated with both domestic and imported fresh fruits and vegetables has increased over the last several decades. In fact, the median number of reported produce-associated outbreaks increased from two outbreaks per year in the 1970s, to 16 per year in the 1990s (Sivapalasingam, 2004). In the decade between 1970 and 1980, 0.7% of reported outbreaks were associated with produce; however, in the 1990's, fresh produce was associated with over 5% of reported foodborne outbreaks (Olsen et al., 2000; Sivapalasingam, 2004). The majority of these were caused by pathogens transmitted by fecal-oral routes (Table 1.1). Produce outbreaks also accounted for an increased proportion of foodborne illnesses among all outbreaks, increasing from 1% (708 of 68,712 cases) in the 1970s to 12% (8,808 of 74,592 cases) in the 1990s (Sivapalasingam, 2004). This increase is illustrated in Figure 1.1 by comparing the relative rates of yearly total foodborne cases and produce-associated cases from 1989 through 1997 to those occurring in 1988. Specifically, between 1992 and 1996, a significant increase in the number of produce-associated cases occurred.

1.2. FACTORS AFFECTING THE EPIDEMIOLOGY OF PRODUCE-ASSOCIATED OUTBREAKS

Over the last 15 years, our knowledge of foodborne disease epidemiology has evolved at the same time the fresh fruit and vegetable industry has undergone significant changes. Modifications in agronomic practices, processing and packaging technologies, along with global marketing strategies have allowed the fresh produce industry to supply consumers with a wide variety of products year-round. Some of these same technologies and

practices have also introduced an increased risk for human illness associated with pathogenic microorganisms. Furthermore, changes in population demographics, food consumption patterns, and increased awareness due to stepped-up epidemiologic surveillance programs and increased media attention have contributed to better documentation of produce-associated foodborne disease.

The proportion of the population in industrialized countries with heightened susceptibility to severe foodborne infections has increased due to demographic changes. In the United States, a growing segment of the population is immunocompromised due to infection with human immunodeficiency virus (HIV) or underlying chronic disease. For example, reported rates of salmonellosis, campylobacteriosis, and listeriosis were higher among HIV-infected persons than among those not infected with HIV (Altekruse et al., 1994). *Salmonella* (and possibly *Campylobacter*) infections are more likely to be severe, recurrent, or persistent in this population (Altekruse et al., 1994). The elderly are also at an increased risk to foodborne infections (Altekruse et al., 1997). According to the U.S. Census Bureau (1998), over the course of the next 25 years, the age structure of the world's population will continue to shift, with older age groups making up an increasingly larger share of the total. For example, during the 1998-2025 period, the world's elderly population (ages 65 and above) will more than double while the world's youth (population under age 15) will grow by 6 percent, and the number of children under age 5 will increase by less than 5 percent. As a result, world population will become progressively older during the coming decades.

Notable changes have occurred in the surveillance of foodborne disease. The Foodborne Diseases Active Surveillance Network (FoodNet) is the principal foodborne

disease component of the U.S. Centers for Disease Control and Prevention (CDC) Emerging Infections Program (EIP). FoodNet is a collaborative project between the CDC, ten EIP sites (represented by various health departments in the states of California, Colorado, Connecticut, Georgia, New York, Maryland, Minnesota, New Mexico, Oregon, and Tennessee), the U.S. Department of Agriculture (USDA), and the U.S. Food and Drug Administration (FDA). The total population of the 2003 catchment was 37.6 million people, or 13.8% of the total U.S. population. Foodborne infections monitored in the FoodNet program include those caused by *Salmonella*, *Shigella*, *Campylobacter*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Vibrio* spp., and the parasites *Cryptosporidium* and *Cyclospora*. It must be noted that FoodNet is a disease surveillance program, and except for limited case control studies, the program cannot conclusively link these infections to foodborne transmission, nor can it routinely identify the food vehicle associated with the diseases under surveillance. Increased surveillance through programs, such as FoodNet, along with the National Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet), a network of laboratories in state health departments, CDC, and food regulatory agencies, has contributed to an overall increased awareness of foodborne illness and its effect on public health (MMWR, 1999a).

Due to the health benefits of fresh produce, public health officials have recently begun to recommend increased consumption of fruits and vegetables. For example, the USDA's *Food Guide Pyramid* has increased the recommended servings of produce, to five to nine per day, as part of a healthier diet to potentially reduce the risks of heart disease and certain cancers (DHHS, 2000). At the same time, the World Health Organization (WHO) has developed a global initiative advising sufficient consumption of fruits and vegetables as part

of a regular diet, stating that up to 2.7 million lives could be saved annually with adequate fresh produce consumption. According to this WHO report, inadequate fruit and vegetable intake is estimated to cause about 31% of heart disease and 11% of strokes worldwide (WHO, 2003).

Consequently, the produce market has seen a remarkable increase in demand over the last ten years. From 1992 to 2001, U.S. consumption of fresh fruits and vegetables increased from 296.7 pounds to 322.4 pounds per capita annually (USDA, 2001), topping out in 2000 at an increase of 8.7% (Table 1.2). Interestingly, if U.S. consumers were to meet the recommendations of USDA health officials, fruit consumption would more than double. Specifically, consumption of citrus, melons, and berries would need to rise by 150%, and consumption of other fruits would need to rise 114% to meet these recommendations. Fulfilling this increased demand with domestic production alone would imply a three to four million acre increase in planted area. Although consumption of vegetables as a group would have to rise only 10%, average diets would need to include more than four times as many dark-green leafy and deep-yellow vegetables (Blisard, 2002).

Along with the significant rise in consumption of fresh produce, major shifts in consumer consumption trends have forced changes in the marketing of these commodities. For instance, as a consequence of the demand for variety, the number of produce items offered by retailers doubled from 173 to 345 different products during the period from 1987 to 1997 (Supermarket Business, 1999; Dimitri, 2003). One explanation for the diversity of product selection is the growing consumer preference for foods that are convenient, minimally processed, and containing fewer preservatives (De Roeber, 1999). For example, a sharp increase in convenience items such as precut, or minimally processed lettuce, has risen

from 1% to 15% of total sales within the past fifteen years (Handy et al., 2000). Minimally processed fruits and vegetables may pose increased risks of foodborne pathogens. There has also been a growing trend toward food consumption outside the home and an increase in the popularity of salad bars. Larger volumes of intact or minimally processed fruits and vegetables are being shipped from central locations and distributed over vast geographical areas to diverse markets.

Growing consumer demand for organic produce, once thought of only as a niche market, has also contributed to market transformation. In 2000, more organic food was purchased from conventional markets than from any other venue (Dimitri, 2002). In the U.S., freshness, taste, and quality rank among the top reasons for organic produce purchases. In fact, from 1997 to 2001, the number of acres dedicated to the production of organic fruits and vegetables increased by over 30%. It has been suggested that because of the agronomic practices used in organic farming, conventionally-produced fruits and vegetables may be microbiologically safer (Mukherjee et al., 2004). However, there is little comprehensive research in this area. A recent study in Minnesota analyzed fresh fruit and vegetables from 32 organic farms and 8 conventional farms to examine differences in microbiological indicator levels as well as the prevalence of select pathogens (i.e., *E. coli*, *Salmonella*, and *E. coli* O157:H7). These investigators found that organic produce was more susceptible to fecal contamination. For example, organic produce samples from farms that used manure or compost aged less than 12 months had an *E. coli* prevalence 19 times higher than product from farms that used manure aged more than 12 months. Nonetheless, pathogens were rarely found in either organic or conventionally produced items (Mukherjee et al., 2004).

Consumers are currently able to purchase a vast array of produce items year-round, many of which were once thought of only as seasonal. Between 1980 and 2001, fresh vegetable imports increased by over 250%, while fresh fruit imports increased by 155% (Clemens, 2004). Along with a rise in the sales of imported produce, an increasing number of foodborne disease outbreaks have been linked to imported products. In addition to recent outbreaks associated with imported cantaloupe and green onions (CDC, 2002b; CDC, 2003) several other cases of foodborne illness have been associated with produce originating from other countries. In 1996, a total of 1,465 cases of cyclosporiasis were reported from 20 states, the District of Columbia, and two Canadian provinces. Epidemiologic investigations found that the illnesses were due to the consumption of raspberries imported from Guatemala (Herwaldt et al., 1997). In March 1997, a total of 153 cases of hepatitis A were reported in Calhoun County, Michigan; subsequent epidemiological investigation implicated strawberries from Mexico as the source of the illnesses (CDC, 1997a). Furthermore, a 1999 multistate outbreak of *Salmonella* Newport infection was associated with the consumption of mangoes imported from a single farm in Brazil (Sivapalasingam et al., 2003). Despite these reports, little is known about the relative importance of imported product as compared to domestic produce when considering the overall burden of produce-associated foodborne disease.

1.3. PATHOGENS ASSOCIATED WITH FRESH PRODUCE

Epidemiologic evidence demonstrates a relationship between some foodborne pathogens and specific commodities. For example, the predominant reservoir for *Salmonella enterica* serovar Enteritidis is contaminated raw eggs, while *E. coli* O157:H7 infection is often associated with the consumption of contaminated, improperly cooked ground beef.

Although fresh produce is not the most common vehicle for the transmission of the majority of foodborne pathogens, fruits and vegetables have gained notoriety as occasional vehicles of a wide array of pathogens, including bacterial, viral and protozoan agents.

Numerous pathogens have been isolated from a wide variety of fresh fruits and vegetables. Table 1.3 lists various pathogens found in produce from research studies performed in several countries. Detection rates ranged from 0% to well over 50%. It is important to note the number of samples in each study significantly varied. Although not all of the pathogens have been associated with produce-related foodborne disease outbreaks, they are all capable of causing illness. A wide variety of these are of considerable public health significance including *Shigella* spp., enterotoxigenic and enterohemorrhagic *E. coli*, *Campylobacter* spp., *L. monocytogenes*, *Y. enterocolitica*, *Bacillus cereus*, *Clostridium botulinum*, enteric viruses, and parasitic protozoa such as *C. cayetanensis*, *Giardia lamblia*, and *C. parvum*.

Figure 1.2 illustrates reported produce-associated outbreaks in the U.S. of known etiology by produce group and etiologic agent from 1990 to 2002. A specific etiologic agent was identified for 187 produce-associated outbreaks during this 13-year span. Among these outbreaks, 102 (55%) were caused by bacteria; 68 (36%) were caused by viruses; and 17 (1%) were caused by parasites. Among the bacterial agents, *Salmonella* accounted for 60% of outbreaks, while pathogenic *E. coli* was responsible for 25% of bacterial outbreaks. Norovirus caused a majority of viral outbreaks, accounting for over 80%. It must be noted that through improved surveillance and detection methods, the apparent prevalence of norovirus has increased. *Cyclospora* caused the majority (65%) of protozoan produce-associated outbreaks. Over 40% of the outbreaks were caused by salads (including lettuce

and tomatoes), while fruit and fruit salads comprised 13% of the outbreaks. Melons, including cantaloupe, honeydew, and watermelon also represent 12% of produce-associated outbreaks, while sprouts comprised 10% of the outbreaks. Table 1.4 lists examples of recent produce-associated outbreaks.

1.3.1. *Salmonella*

Salmonella is the second most common cause of bacterial foodborne disease in the United States, following *Campylobacter*. Although the number of reported *Salmonella* cases actually increased between 2000 and 2002, recent preliminary reports have suggested an overall decrease in their number since then (CDC, 2004b.). Birds, reptiles, amphibians, and mammals are natural reservoirs of *Salmonella*, and several surveys have reported the presence of various *Salmonella* serotypes on certain produce items. A study done in Italy in the 1970s reported *Salmonella* to be present on 72% (64/89) of fennel samples and 68% (82/120) of lettuce samples (Ercolani, 1976). In a more recent FDA survey of over 1000 imported produce samples originating from 21 countries, 35 samples were confirmed positive for *Salmonella* (FDA, 2001a). The contaminated items included cantaloupe (8/151), celery (1/84), cilantro (16/171), culantro (6/12), lettuce (1/116), parsley (2/84), scallions (1/180), and strawberries (1/143). The organism has also been detected on beet leaves, cardoon, cabbage, cauliflower, eggplant, peppers, endive, and spinach (Beuchat, 1996b).

Several outbreaks of salmonellosis in the United States have involved fresh fruits, particularly imported cantaloupe. In 1990, a large outbreak of salmonellosis, affecting at least 245 people in 30 states, was associated with the consumption of cantaloupe served at salad bars (Ries et al., 1990). Two deaths were reported and it was estimated that 25,000 individuals were eventually infected (CDC, 1991). During June and July of 1991, more than

400 laboratory-confirmed infections of *Salmonella enterica* serovar Poona reported in 23 states and Canada were linked to the consumption of contaminated cantaloupe. Thereafter, three high-profile multistate outbreaks of serovar Poona associated with eating cantaloupe imported from Mexico occurred during the spring of consecutive years between 2000 and 2002. Outbreaks were first identified by the California Department of Health Services (2000 and 2001) and the Washington State Department of Health (2002) and involved residents of 12 states and Canada (CDC, 2002b). Following these outbreaks, the FDA conducted farm investigations in Mexico, issued press releases to warn consumers, placed implicated farms on detention, and conducted sampling surveys of imported cantaloupe.

Acidic foods, such as orange juice, that were once thought to be an unlikely vehicle of *Salmonella*, have recently been associated with outbreaks. For instance, 62 visitors to a large theme park in Orlando contracted salmonellosis following the consumption of unpasteurized orange juice (Cook et al., 1998). Unpasteurized apple cider and apple juice have also been associated with outbreaks of *Salmonella* Typhimurium (CDC, 1975). Laboratory-based studies have shown that some *Salmonella* strains can survive in orange juice at detectable levels for 27 days (pH 3.5 product) and 73 days (pH 4.4 product) (Parish et al., 1997).

1.3.2. *Escherichia coli* O157:H7

Enterohemorrhagic *E. coli* was first recognized as a human pathogen in 1982 when it was identified as the cause of two outbreaks of hemorrhagic colitis (Wells et al., 1983). Since then, sporadic infections and outbreaks have been reported from many parts of the world, including North America, Australia, Asia, Western Europe, and Africa. Undercooked or raw hamburger (ground beef) has been implicated in most of the

documented outbreaks, however *E. coli* O157:H7 has also been found on other products, including contaminated produce items. For example, Zepeda-Lopez and coworkers (1995) detected *E. coli* O157:H7 on 25%, 19.5%, and 20% of cabbage, cilantro, and coriander, respectively. On the other hand, several studies have reported the absence of *E. coli* O157:H7 on produce (Lin et al., 1996; Little et al., 1999; FDA, 2001a; FDA, 2003). It should be noted that in those studies in which *E. coli* O157:H7 contamination was absent, sample sizes ranged from 51 to over 1000 produce items.

Seed sprouts are considered a high risk produce item because seeds are sometimes contaminated with enteric pathogens and the high temperature and humidity of the sprout germination process is conducive to the proliferation of these organisms. The first reported outbreak of *E. coli* O157:H7 infection associated with eating alfalfa sprouts occurred in the summer of 1997. In this outbreak, a total of 60 cases from 16 counties were reported to the Michigan Department of Community Health; simultaneously, the Virginia Department of Health received reports of 48 cases. Isolates from both the Michigan and Virginia outbreaks were compared using a molecular subtyping method (Pulsed Field Gel Electrophoresis, PFGE); the analysis revealed that the isolates were identical and both outbreaks were associated with alfalfa seeds originating from the same supplier and lot (CDC, 1997d). The world's largest reported *E. coli* O157:H7 outbreak occurred in Japan during 1996 and was linked to the consumption of white radish sprouts. Approximately 6,000 school children were infected and 17 people died. Interestingly, during May through August of that same year, approximately 10,000 cases of *E. coli* O157:H7 infection occurring in at least 14 separate disease clusters were reported in Japan (Watanabe et al., 1999). In the following

year, white radish sprouts were once again implicated in a Japanese outbreak of *E. coli* O157:H7 which affected a total of 126 people (Gutierrez, 1997).

Along with *Salmonella* spp., *E. coli* O157:H7 has been implicated in outbreaks involving the consumption of unpasteurized fruit juices. In 1991, the first confirmed outbreak of *E. coli* O157:H7 associated with apple cider occurred in Massachusetts, affecting 23 people (Meng et al., 2001). In 1996, three outbreaks of *E. coli* O157:H7 were associated with unpasteurized apple juice, the largest of which occurred in the western United States and Canada, with 71 confirmed cases and one death (CDC, 1996a). The source of the pathogen was suspected to be animal manure since “drops” or apples that had fallen on the ground during season were used to produce the cider. While the definitive source of contamination is still not clear, further investigation of the 1991 outbreak revealed that cattle grazed in a location adjacent to the orchard and could have served as the source of fecal contamination (Besser et al., 1993).

Researchers have demonstrated that *E. coli* O157:H7, as well as some *Salmonella* strains, can withstand the low pH (3.3 – 4.2) characteristic of many juices (Dingman, 2000; Koodie et al., 2001; Janes et al., 2002). As a result, the FDA mandated the use of Hazard Analysis and Critical Control Point (HACCP) principles for juice processing (FDA, 2001c). Manufacturers are also required to use processes that achieve a 5-log reduction in the numbers of the most resistant pathogen in their finished products, effectively resulting in pasteurization (FDA, 2001b).

1.3.3. *Shigella*

In 1999, the WHO estimated that *Shigella* spp. were responsible for over 166 million illnesses annually (Kotloff et al., 1999). This organism is also the third leading cause of

bacterial foodborne outbreaks in the United States (Mead et al., 1999), infecting 450,000 people each year with no significant change in the occurrence of reported cases over the last six years. This pathogen is not indigenous to foods, but rather introduced through the fecal-oral route by contact with human excreta; fecally-contaminated water and poor hygiene of infected food handlers are the most common causes of contamination of fresh produce.

Shigella spp. have been isolated from several produce items. In FDA 1000-sample surveys of imported and domestic produce, *Shigella* was isolated from less than 2.5% and 0.5% of the samples, respectively; commodities that were occasionally contaminated included cantaloupe, celery, lettuce, parsley, and scallions (FDA, 2001a; FDA, 2003). In 1985, Saddik et al. found *Shigella* in one of 57 salad green samples obtained from Egyptian retail outlets (Saddik et al., 1985). Although only reported occasionally, several foodborne outbreaks of shigellosis have been associated with raw produce, including green onions (Cook, 1995), iceberg lettuce (Frost et al., 1996), and uncooked baby maize (Molbak and Neimann, 1998). In the summer of 1998, eight outbreaks of *Shigella sonnei*, occurring in Minnesota, California, Massachusetts, and Florida, as well as in Ontario and Alberta, Canada were linked to chopped, uncooked, curly parsley. In this case, molecular subtyping revealed that strains from the seven outbreaks for which isolates were available shared the same PFGE pattern, indicating a common source (CDC, 1999b).

1.3.4. *Listeria monocytogenes*

While the causative agent of listeriosis was discovered more than 70 years ago, the significance of the foodborne route of transmission has only been recognized within the last 20 years. In fact, *L. monocytogenes* came to the forefront as an emerging pathogen after a 1981 outbreak of listeriosis in Nova Scotia, Canada which was traced back to the

consumption of contaminated coleslaw (Schlech III et al., 1983). Thirty-four perinatal and seven adult listeriosis cases initiated the epidemiological investigation. Coleslaw obtained from a patient's refrigerator was positive for the epidemic strain, *L. monocytogenes* serotype 4b, which was the same strain isolated from the patients' blood. Further investigation led to the identification of a cabbage grower whose farming practices provided sufficient opportunities for the introduction of *L. monocytogenes* in the food chain. In this case, the cabbage was grown in fields fertilized with raw sheep manure, and interestingly, two of the farmer's sheep had previously died of listeriosis.

L. monocytogenes is a major public health concern because of its severe disease manifestations (meningitis, septicemia, and abortion), high case-fatality rate (approximately 20 to 30%), long incubation period (one to six weeks for severe cases), and predilection for immunocompromised individuals. In 2000, the CDC included listeriosis among its list of notifiable diseases (CDC, 2002d). The estimated incidence of *Listeria* infections shows considerable variation, but did not change significantly between 1998 – 2003, with the number of cases ranging from 94 to 114 during this period (CDC, 2004b).

L. monocytogenes is different from other foodborne pathogens for several reasons. First, it can persist under diverse environmental conditions, including reduced pH and relatively high salt concentrations (Lou and Yousef, 1999). The pathogen is also microaerobic and is able to grow at very low temperatures (2° to 4°C) (Swaminathan, 2001). *L. monocytogenes* is found in soil and water and is widely distributed among animals, humans, and, particularly, plant vegetation (Beuchat, 1996a).

While *L. monocytogenes* has been isolated from a wide variety of raw and ready-to-eat meat products, it can also be associated with produce items. In 1994, Arumugaswamy

and coworkers surveyed various food products from a Malaysian market and found that 6 of 7 samples of bean sprouts and 5 of 22 leafy vegetable samples were contaminated with *L. monocytogenes* (Arumugaswamy et al., 1994). The prevalence of *L. monocytogenes* during production and postharvest processing of cabbage was recently examined in farms and packing sheds in south Texas (Prazak et al., 2002). The pathogen was isolated from 26 of 855 (3%) of the total samples. Twenty isolates originated from cabbage samples that were obtained from farms and packing sheds, while three isolates were from water samples. Additionally, three isolates were obtained from environmental sponge samples taken from packing shed surfaces. *L. monocytogenes* can grow on fresh produce stored at refrigeration temperatures, as has been demonstrated for asparagus, cauliflower, and broccoli held at 4°C (Berrang et al., 1989). The pathogen has also been reported to grow on lettuce at 5°C (Steinbrugge et al., 1988; Berrang et al., 1989). Growth of *L. monocytogenes* can occur on the surface of tomatoes held at 21°C, but not at 10°C (Beuchat and Brackett, 1991). Interestingly, carrot juice has been reported to have an inhibitory effect on *L. monocytogenes* growth (Beuchat and Brackett, 1990; Beuchat et al., 1994; Beuchat and Doyle, 1995).

1.3.5. *Campylobacter*

Before 1972, when methods were first reported for its isolation from feces, *Campylobacter* was believed to be primarily an animal pathogen causing abortion and enteritis in sheep and cattle. Since then, epidemiological studies have shown that *Campylobacter jejuni* and *C. coli* together are the leading cause of bacterial diarrheal illness in the U.S. Raw milk and undercooked foods of animal origin are recognized as the primary vehicles for infection, although the potential for cross-contamination of fresh produce during the preparation of poultry and other meats is significant (Nachamkin, 2001).

Park and Sanders (1991) studied the occurrence of thermotolerant campylobacters in fresh vegetables sold at supermarkets and farmers' markets in Canada (Park and Sanders, 1992). Out of a total of 1,564 samples of 10 vegetable types, *Campylobacter* was detected on spinach (3.3%), lettuce (3.1%), radish (2.7%), green onion (2.5%), parsley (2.4%), and potatoes (1.6%). Interestingly, *Campylobacter* was found among only those samples collected from outdoor markets with no microbial decontamination step. Produce samples collected from supermarkets and outdoor markets which had been thoroughly washed with chlorinated water were all negative for *Campylobacter*.

Produce is an infrequent vehicle of *Campylobacter* enteritis and very few incidents have occurred in which vegetables have been involved. Only one outbreak of *Campylobacter* enteritis associated with the contamination of fruits or vegetables was reported for the period 1973-1987, while another *Campylobacter* outbreak due to fresh produce was reported during 1989 (Bean, 1996; Bean, 1990). An outbreak of campylobacteriosis due to the ingestion of a cabbage-beef stew by school children was reported in Germany. Since the stew was cooked, cross-contamination with *C. jejuni* was a likely contributing factor (Steffen, 1986). Similarly, cross-contamination of salad lettuce by raw chicken led to a small outbreak of campylobacteriosis in an Oklahoma restaurant in 1996 (CDC, 1998b). Harris (1986) analyzed the dietary histories of individuals who had campylobacteriosis and determined that out of the several vegetable items surveyed, only mushrooms were significantly associated with *C. jejuni* enteritis cases.

1.3.6. Other Potential Bacterial Pathogens

Aeromonas strains can be present in drinking, fresh, saline and brackish waters, as well as in sewage. The organism is considered ubiquitous, having been found in a wide

range of seafoods, meats, and poultry. Several studies have isolated *Aeromonas* spp. from produce. For example, Szabo et al. (2000) found *Aeromonas* spp. in over 50% (66/120) lettuce samples. While no produce-associated outbreaks with this organism have been reported, several characteristics of the *Aeromonas* genus suggest its potential as an infectious agent (Llopis et al., 2004, Martins et al., 2002). *Aeromonas* spp. can grow rapidly on raw vegetables and seed sprouts at refrigeration temperatures (Harris, 2003).

Yersinia enterocolitica has also been found in a wide variety of environments, including the intestinal tract of various mammalian species, as well as in birds, frogs, fleas, oysters, flies, and fish (Cover and Aber, 1989) Like *L. monocytogenes*, *Y. enterocolitica* can grow at refrigeration temperatures, making it a potential concern for food manufacturers. *Y. enterocolitica* has been isolated from many foods, including fresh produce. Szabo et al. (2000) detected *Y. enterocolitica* in 71 of 120 (59%) packaged lettuce samples; however, the strains isolated were not pathogenic. Another study reported a 4% prevalence (27/673) of *Yersinia* spp. on ready-to-eat vegetables; of the 27 strains isolated in this study, 18 were *Y. enterocolitica* (Lee et al., 2004).

1.3.7. Human Enteric Viruses

Hepatitis A virus (HAV) and the noroviruses are the most commonly documented viral agents to contaminate food. Because of their low infective dose, many foodborne viral infections are transmitted via infected food handlers. HAV is primarily transmitted by the fecal-oral route, either by person-to-person contact or by ingestion of food or water contaminated with human feces (Fiore 2004). Between 1980 and 2001, an average of 25,000 cases of HAV were reported annually to the CDC, however when corrected for asymptomatic infections and underreporting, this number probably exceeds 250,000 (CDC, 2002a).

Fortunately, foodborne transmission accounts for only about 5% of HAV cases of known etiology (Mead et al., 1999).

The cause of most reported foodborne hepatitis A outbreaks has been infected food handlers who contaminate the product at the point of sale or those who prepare food for social events. A single HAV-infected food handler can transmit the virus to dozens or even hundreds of persons and cause a substantial public health problem. A common theme for such outbreaks includes the presence of an HAV-infected food handler who worked while viremic (two weeks before to one week after the onset of symptoms) and who had contact with ready-to-eat foods; subsequently, the appearance of secondary cases occurs among other food handlers and patrons who ate product contaminated by the index case (Fiore, 2004).

Hepatitis A outbreaks also have been associated with the consumption of fresh produce contaminated during cultivation, harvesting, processing, or distribution. Outbreaks involving a food item that was contaminated before distribution are particularly difficult to identify as the product might be widely distributed geographically before recognition of the first cases. Low attack rates are common probably because contamination is only found in a small proportion of the distributed food.

Several recent clusters of hepatitis A infection occurred in the fall of 2003 in four states, including Tennessee, Georgia, North Carolina, and Pennsylvania. Raw or undercooked green onions served in restaurants were the implicated source, with disease manifested in over 900 people, three of whom died. The nucleic acid sequences of the Pennsylvania outbreak strains were very similar to sequences obtained from persons involved in hepatitis A outbreaks in the other three states. Raw green onions from three farms in Mexico have since been implicated in the Tennessee and Georgia outbreaks (CDC, 2003).

Noroviruses (formerly known as the Norwalk-like viruses) are regarded as the most common of the foodborne viruses, and are the most significant cause of acute nonbacterial gastroenteritis in both children and adults. Transmitted by the fecal-oral route, the two most likely ways by which to become exposed to noroviruses are through person-to-person contact or the consumption of contaminated food or water. Food items implicated in norovirus outbreaks include molluscan shellfish and ready-to-eat (RTE) foods that become contaminated by human handling, such as fruit salad, raspberries, cake icing and deli meat. Noroviruses have also been associated with produce outbreaks. For instance, an outbreak caused by norovirus occurred in December of 1979 at a luncheon banquet (Griffin et al., 1982). Among all the foods served, consumption of green salad was epidemiologically associated with the disease which affected 63 persons. Contamination by infected food handlers was thought to be the likely source of the virus. In April of 1998, an outbreak of viral gastroenteritis linked to the consumption of imported frozen raspberries occurred in Helsinki, Finland (Ponka et al., 1999). It was suspected that the raspberries, imported from Eastern European countries, were contaminated through the use of fecally-impacted waters, either during irrigation or by post-harvest sprays which were applied immediately before freezing.

1.3.8. Parasitic Protozoa

In 1999, Mead et al. estimated that foodborne transmission of parasitic agents accounted for over 350,000 cases of illness annually in the U.S. alone (Mead et al., 1999). From 1993 to 1997, 19 foodborne outbreaks of parasitic etiology were reported to the CDC, resulting in a total of 2,325 cases (CDC, 2000b). The environmental routes of transmission

for the parasitic protozoa include water, soil, and food which become contaminated by contact with animal or human fecal matter (Slifko et al., 2000).

Cyclospora cayetanensis, *Giardia lamblia*, and *Cryptosporidium parvum* are the most common human enteric protozoan infections. Outbreaks of cyclosporiasis have occurred in the last decade and have been associated with the consumption of fresh raspberries, basil, mesclun lettuce, and snow peas (CDC, 2004a; Herwaldt, 2000). In the mid-to-late 1990's, several multistate outbreaks of cyclosporiasis were associated with the consumption of raspberries imported from Guatemala. An outbreak in the summer of 1997, with 57 reported clusters of cyclosporiasis and 341 cases in Northern Virginia, Washington D.C., and Baltimore, Maryland, was associated with the consumption of basil (CDC, 1997b; Herwaldt, 2000). Non-human animal species serve as reservoir hosts of *C. cayetanensis*. In contrast to other foodborne enteric pathogens, *Cyclospora* oocysts are not immediately infective after excretion but require a period of days to weeks in favorable environmental conditions in order to sporulate and become infectious. Therefore, person-to-person transmission or transmission via infected foodhandlers is considered unlikely (Herwaldt, 2000). Anecdotal evidence from outbreak investigations suggests that the infective dose for *Cyclospora* is low (Herwaldt, 2000).

Outbreaks of giardiasis have been associated with the consumption of fruit salad (two outbreaks), iceberg lettuce (one outbreak), and salad (one outbreak) (Juranak, 2005 – personal communication) An outbreak caused by *Giardia lamblia* occurred in a cafeteria at a corporate office building for which an asymptomatic food handler was the probable source of contamination to raw sliced vegetables (Mintz et al., 1993). In 1997, an outbreak of cryptosporidiosis in Washington which caused 54 laboratory-confirmed illnesses was linked

to the consumption of green onions. In this instance, the onions reportedly had not been washed by either the supplier or the restaurant where they were served (CDC, 1998a). Two outbreaks of cryptosporidiosis were also associated with the consumption of apple cider (Juranek, 2005).

1.3.9. Spore-forming bacteria

The contamination of fruits and vegetables with spores of *Bacillus cereus*, *Clostridium botulinum*, or *Clostridium perfringens* is not uncommon. Harmon et al. (1987) isolated *Bacillus cereus* from 83% (33/40) of mung bean sprout samples collected from a health food store (Harmon et al., 1987) and *B. cereus* and *C. perfringens* were isolated from over one-third (34/100) of assorted vegetables collected from retail outlets in the U.K. (Roberts et al., 1982). However, spore-forming bacteria become a public health threat only when produce is handled in a way that will support the germination of spores and growth of vegetative cells, which is not a common practice.

Nevertheless, there have been disease outbreaks in fresh produce associated with contamination by these organisms. In 1993, *C. perfringens* caused an outbreak affecting 48 people in Ontario Canada, for which the probable vehicle was salad. In 1989, an outbreak of botulism in New York was caused by the consumption of chopped garlic in oil. The product had been made between 1985 and 1987, contained no preservatives, and was kept at room temperature for approximately three months after purchase prior to opening. This outbreak was traced back to the same processor implicated in a 1985 *C. botulinum* outbreak (Morse et al., 1990). In response, the Food and Drug Administration took steps to prevent a recurrence by requiring the use of microbial inhibitors or acidifying agents such as phosphoric or citric acid in vegetable tubers or roots cooked or coated in oil (Morse et al., 1990).

1.4. FACTORS CONTRIBUTING TO PRODUCE CONTAMINATION

Fresh produce may become contaminated with pathogens at any point during cultivation, harvesting, processing, distribution, or preparation. In 1998, the FDA, USDA, and CDC published voluntary guidelines to address produce safety issues entitled “*The Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables*” (FDA et al., 1998). The purpose of the Guide was to provide a framework for the identification and implementation of practices likely to decrease the risk of pathogen contamination in fresh produce from production, packaging, and transport based on Good Agricultural Practices (GAPs) and Good Manufacturing Practices (GMPs). Table 1.5 refers to the major sources of pathogen contamination that can occur during both pre-harvest and post-harvest phases. While there are numerous potential sources of contamination along the farm to fork continuum, several central themes emerge from the document and these will be discussed in the following sections.

1.4.1. The Use of Animal Manure and Biosolids

Animal wastes are commonly recycled to agricultural land and provide an economical and environmentally sustainable means of disposal. While animal manures have beneficial fertilizer value to field crops, the use of animal manure has resulted in the contamination of produce with pathogenic bacteria, viruses, and parasites. Animal manure may harbor a plethora of pathogens, including but not limited to *Salmonella* spp, *Campylobacter* spp., *E. coli* O157:H7, *L. monocytogenes*, *Giardia* spp., and *C. parvum* (Mawdsley et al., 1995). Although frequently used as a crop fertilizer in the developing world, in the U.S. excess animal manure may be spread on land only in the vicinity of animal or produce farms, but not directly on land intended for fruit and vegetable production. According to a USDA survey,

organic sources of fertilizers are not commonly used by conventional fruit and vegetable growers (Suslow, 2003).

Several studies have reported extended survival of pathogens in animal manure (Himathognkham et al., 1999; Lung et al., 2001; Jiang et al., 2002; Islam et al., 2004). In 1998, Kudva et al. reported that *E. coli* O157:H7 survived in ovine manure for as long as 21 months (Kudva et al., 1998). Furthermore, an ongoing study in the U.K. has found that *E. coli* O157, *Salmonella*, and *Campylobacter* survived in stored manure slurries for up to three months, with *L. monocytogenes* surviving for up to six months. Following land application of inoculated manure, *E. coli* O157:H7, *Salmonella* and *Campylobacter* survived in sandy and clay loam soil for up to one month, and *L. monocytogenes* survived for more than one month. However, these same investigators found that pathogens could not be detected after one week in solid manure heaps where temperatures greater than 55°C were obtained, supporting the use of high-temperature composting as an effective measure to reduce the microbial load in solid manure (Nicholson et al., 2004).

The listeriosis outbreak in the Maritime Provinces of Canada (Schlech III et al., 1983) is a significant example of the importance of proper use of fertilizer. A review of agronomic practices revealed that the implicated product was produced by a farmer who also maintained a flock of sheep. Both composted and raw sheep manure had been applied to fields in which the cabbage was grown. It has been reported that sheep manure from flocks with known cases of “circling disease” (listeriosis) may contain viable *L. monocytogenes* cells (Bojsen-Moller, 1972). In this particular case, ovine manure may have been the source of the pathogen to the cabbage crop. Furthermore, cold storage of the cabbage from the last harvest in October through the winter and early spring provided conditions under which *L.*

monocytogenes could thrive. An *E. coli* O157:H7 outbreak in Montana affecting 70 people was due to the consumption of lettuce, possibly contaminated from irrigation runoff or compost used for fertilization. In this case, it was found that cattle had access to the source of the irrigation water that may have contaminated the lettuce (Ackers et al., 1998). Islam et al. (2004) studied the fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated irrigation water and manure composts (Islam et al., 2004) and found that the pathogen could survive in the soil for over 200 days, regardless of contamination mode. Furthermore, *Salmonella* persisted for 84 and 203 days on radishes and carrots, respectively.

1.4.2. Water Quality

During production, irrigation and surface run-off waters can be sources of pathogenic microorganisms that contaminate fruits and vegetables in the field. The use of water during production includes irrigation and the application of pesticides and fertilizer. Typical sources of agricultural water are surface water from rivers, streams, irrigation ditches, and open canals; impounded water such as ponds, reservoirs, and lakes; locally collected water such as cisterns and rain barrels; groundwater from wells; and municipal supplies. The Guatemalan raspberries implicated in the 1996 cyclosporiasis outbreak were believed to have become contaminated by water used in insecticide and fungicide sprays (Herwaldt et al., 1997). In addition, although the source of irrigation water may influence the safety and quality of fresh produce, the type of irrigation method is also important. For example, overhead irrigation has been shown to have a higher probability of contaminating produce than drip or furrow irrigation methods (Suslow et al., 2001).

Most fruits and vegetables receive some sort of processing before being distributed to commercial locations. Within the packing house, water quality is one of the key issues in maintaining a safe product. In the 1998 outbreaks of *Shigella sonnei* associated with Mexican parsley, field investigations revealed that the packing shed used unchlorinated municipal water in a hydrocooler to chill the product immediately after harvest. Furthermore, the water was recirculated, and this same water was used to produce the ice for transport packaging (CDC 1999). Taken together, these practices facilitated the survival of the organism and promoted widespread cross-contamination of parsley.

1.4.3. Worker Hygiene

During harvest, one of the most important factors in preventing the contamination of fresh produce is worker hygiene. The potential for transferring fecal contamination to product is increased in the absence of sanitary hand-washing facilities in the production area. The FDA suggests that packing sheds educate workers on the importance of good hygiene, as promoted through the use of GAPs and GMPs. Several HAV outbreaks have been associated with poor worker hygiene. For instance, a 1983 outbreak of hepatitis A associated with contaminated strawberries was apparently caused by infected food handlers picking the berries (Reid and Robinson, 1987). Over 100 people became ill in Florida after consuming lettuce which had been handled by an HAV-infected food handler who practiced poor hygiene (Lowry et al., 1989).

1.4.4. Environmental Sources

The production environment itself may also be the source of pathogens (Luechtefeld et al., 1980; Lee et al., 1982; Fenlow, 1985; Quessy and Messier, 1992). For instance, both

insects and wild birds are important vectors in the transfer of fecal material (Geldreich, 1964). Allowing domestic animals access to field crops and orchards may result in contamination of produce and subsequent human infection with enteric pathogens. In the 1998 *E. coli* O157:H7 outbreak linked to unpasteurized apple cider, the juice was traced back to an orchard in which cattle were kept prior to harvest and from which “drops” or fallen apples were used to produce the cider (Tamblyn et al., 1999). An inspection following a *Salmonella* Hartford outbreak associated with the consumption of unpasteurized orange juice at a popular theme park revealed signs of environmental contamination, including a poorly sealed processing room which may have facilitated unrestricted access by feral rodents, birds, and amphibians (Cook et al., 1998).

Some of the pathogens which can contaminate vegetables are natural inhabitants of the growing environment. Soil that has not been contaminated with feces is generally not a source of enteric microorganisms. However, spores of *C.*, *C. perfringens*, and *B. cereus* have been isolated from soils free of fecal contamination. *L. monocytogenes*, an environmentally ubiquitous organism, has been found in soil and decaying vegetation (Beuchat, 1996a). Twenty-seven strains of *L. monocytogenes* were isolated from soil and decaying corn, soybean plants, and wild grasses taken from 19 sites in the Netherlands (Welshimer and Donker-Voet, 1971; Beuchat and Ryu, 1997) The survival of enteric organisms in soil is dependent on factors such as soil type, moisture retention, pH, microbial activity, nutrient availability, and inoculum level. A French study reported that *L. monocytogenes* was able to survive in chalky and peaty soil for well over one year when incubated at 20° C, whereas at 4° C, the pathogen survived up to 1500 days (Picard-Bonnaud et al., 1989).

1.5. RECENT PRODUCE-ASSOCIATED OUTBREAKS: LESSONS LEARNED

1.5.1. Cyclosporiasis and Guatemalan Raspberries

Prior to 1996, *Cyclospora* caused sporadic cases of traveller's diarrhea in developed countries and its disease was considered one of several occurring among the immunocompromised and children in the developing world (Shields and Olson, 2003). In fact, the majority of documented cases of cyclosporiasis in North America were in overseas travelers and only four clusters of cases, affecting 91 people, had been reported before 1996 (Huang et al., 1995; Carter et al., 1996; Koumans et al., 1996; Herwaldt, 2000). However, in the spring of 1996, an increase in cyclosporiasis cases was reported to the CDC and Health Canada which subsequently focused attention on this little-known pathogen (CDC, 1996b; CDC, 1996c; Chew et al., 1996; Colley, 1996; Fleming et al., 1996; Herwaldt, 2000; Katz et al., 1996; Neamatullah et al., 1996; CDC, 1997e). Over 1,400 cases of cyclosporiasis were reported, which were epidemiologically linked to the consumption of Guatemalan raspberries.

In the late 1980s, raspberries were introduced into the Guatemalan market as an export crop (Herwaldt, 2000). By the mid-1990's, exports of Guatemalan raspberries were increasing rapidly, escalating 113% from 1995 to 1996. The Guatemalan spring growing season was essentially over by the time raspberries were recognized as the vehicle of the outbreak in 1996, and no regulatory action was taken. After the 1996 outbreak, the CDC and the FDA investigated Guatemalan farming practices, however the mode of contamination was never definitively established. The investigators' leading hypothesis was that the raspberries became contaminated by exposure to agricultural water, specifically when the

berries were sprayed with insecticides, fungicides, and fertilizers that had been mixed with water (Hoge et al., 1993; Rabold et al., 1994; Huang et al., 1995; Herwaldt, 2000).

In response to this outbreak, the FDA provided technical assistance and suggested the application of GAPs, GMPs, and Standard Sanitation Operating Procedures. In addition, the Guatemalan Berry Commission (GBC) was developed and voluntarily implemented control measures on farms that focused on improving employee hygiene, water quality, and sanitation. In the spring of 1997, the commission allowed only farms which were classified as low risk to export raspberries to the U.S. (CDC, 1997e). However, despite these measures, another multistate outbreak of cyclosporiasis, linked to Guatemalan raspberries occurred in the United States and Canada in 1997, with 1,012 reported cases (Herwaldt et al., 1999; Herwaldt, 2000). After consultation with the FDA, the GBC voluntarily stopped exporting raspberries to the U.S. Interestingly, importation to Canada continued, and in 1998 a multi-cluster outbreak of cyclosporiasis linked to the consumption of Guatemalan raspberries occurred in Ontario, Canada, affecting over 300 people (Herwaldt, 2000).

This series of outbreaks was unique because it was caused by an obscure pathogen, involved prolonged illness, and had widespread exposure with many cases of illness. These large outbreaks alerted public health officials to the hazards associated with a pathogen once thought to be problematic only in the developing world. They also illustrated how such a problem can be transferred to developed countries through food importation. Indeed, it has become increasingly apparent that global intervention strategies are needed to assure produce safety. Unfortunately, the impact of these outbreaks was not limited to public health; Guatemalan farmers suffered substantial economic losses as well. It was estimated that

ceasing raspberry exports to the U.S. in mid-season resulted in a loss of \$10 million in income for Guatemalan industry (Powell, 1998).

1.5.2. Hepatitis A and Mexican Strawberries

Many hepatitis A outbreaks occur as the result of a single infected food handler at a single food establishment. Sporadically, widespread foodborne outbreaks of HAV are associated with the contamination of product before distribution; furthermore, the source of infection cannot be identified in approximately 50% of reported cases of hepatitis A infection (CDC, 1999c; CDC, 2000a; Fiore, 2004). In 1997, the epidemiologic investigation of an outbreak and several sporadic cases of HAV in multiple states revealed a single, pre-distribution contamination source. This particular investigation was facilitated by new molecular biology detection and typing methods (CDC, 1997a).

Between January and May of 1997, a total of 258 cases of hepatitis A were reported in Michigan and Maine. Of these, 242 (94%) occurred among school employees and students in 23 Michigan schools, with 39 additional cases reported from 13 schools in Maine (Shields and Olson, 2003). The incidence peaked during March and then declined rapidly, suggesting a common source. Case-control studies in both states indicated that the frequency of eating school lunch was associated with an increased likelihood of illness, and food items containing frozen strawberries were the likely vehicles of infection.

The strawberries implicated in this outbreak were grown in Mexico, processed and frozen at a California facility, and distributed commercially through the USDA school lunch programs. Trace back information indicated that 22 lots of frozen strawberries processed in April and May of 1996 were sent to Michigan and Maine school lunch programs. Regulatory agency inspection of both processing and school facilities did not reveal a likely source of

contamination. Investigation of three Mexican growing sites showed inadequate hygiene and toilet facilities. The only hand-washing facilities were on trucks which circulated through the fields, and the pickers did not wear gloves when removing the strawberry stems with their fingernails. Additionally, only a few slit latrines were available for field workers' use.

Other states also received strawberries from the same lot and reported sporadic cases of hepatitis A infection. These states included Tennessee (two cases), Arizona (nine cases), Wisconsin (five cases), and Louisiana (four cases). In order to determine if the sporadic cases were related to the Michigan and Maine outbreaks, viral nucleic acid obtained from clinical specimens was sequenced. Amplified regions of the viral RNA obtained from all the Michigan patients tested had identical sequences; the sequences of 8 of 10 of the Maine patients matched those of the Michigan patients. Sequence analysis further determined that the five cases in Wisconsin, two of the Louisiana cases, seven from Arizona, and one of the two patients in Tennessee were identical to the Michigan strain. In this case, sequencing a part of the viral genome detected in outbreak cases in Michigan and Maine, along with sporadic cases in other states, confirmed the epidemiologic evidence indicating that the outbreak originated from a common source exposure.

1.5.3. *Escherichia coli* O157:H7 in Apple Juice

Produce-associated outbreaks have been responsible for important new food safety regulations within the past 10 years. *E. coli* O157:H7, once considered an obscure pathogen, has challenged clinicians, alarmed food producers, and transformed public perception about food safety. Outbreaks of *E. coli* O157:H7 and *Salmonella* associated with the consumption of contaminated juices have dramatically altered the production and processing of these food products. Since 1990, at least six reported outbreaks of *E. coli* O157:H7 infection in North

America were associated with unpasteurized apple cider (Health, 1999). Five of these outbreaks occurred in the U.S. over a three-year period (Besser et al., 1993; CDC, 1997c; Luedtke and Powell, 2002).

In the past, it was assumed that unpasteurized fruit juices were unlikely to harbor or support the growth of pathogens, primarily due to the low pH of the product. However, research has confirmed that *E. coli* O157:H7 can survive for a relatively long time in fruit juices. In response to these outbreaks, FDA proposed two regulations to improve the safety of fresh and processed juices. In 1998, FDA began mandating the use of warning labels on all unpasteurized juices or juices not otherwise treated to control disease-causing pathogens. In January of 2001, FDA published final regulations requiring all domestic and foreign fruit and vegetable processors to use HACCP approaches to prevent, reduce, or eliminate microbiological hazards in juices. Large companies had one year after publication of the regulation to implement HACCP; small companies were required to comply in two years and very small companies within three years. Specifically, the rule stipulated that juice manufacturers in the U.S. implement a process able to achieve a 5- \log_{10} reduction of the most resistant pathogen(s) of concern in their product (FDA, 2001).

1.6. CONCLUSIONS

Advanced production practices, new packaging technologies, global marketing, and ever-changing consumer lifestyles have forced public health officials to respond to new and increasing microbial threats from the consumption of fresh produce. Production and processing technologies in the produce industry have evolved to meet consumer demands at the same time that epidemiologic surveillance tools for foodborne disease have also advanced. The 1998 hepatitis A outbreak associated with the consumption of strawberries

represented one of the first instances in which molecular analyses were used to supplement an ongoing epidemiologic investigation. Indeed, federal public health systems have initiated significant active surveillance programs and engaged multiple relevant state and local health constituencies, as illustrated in programs such as the PulseNet and FoodNet. Voluntary preventive and control measures such as Hazard Analysis and Critical Control Point (HACCP) are being implemented not only at a national level, but also internationally. The 1996 outbreak of *Cyclospora cayetenensis* associated with the consumption of Guatemalan raspberries not only added to concern about new and emerging pathogens, but was also an example of how the food safety sector must be prepared to control and react globally in order to keep the U.S. food supply safe. However, at the same time, health officials and regulators are reminded that the epidemiology of foodborne disease, particularly infections associated with fresh produce, is constantly changing and there are new challenges to face and lessons to learn. Research continues to examine the ecology, growth, and survival of pathogens on fresh produce. The food safety sector has demonstrated its ability to keep up with changes in the produce industry and will continue to respond with new technologies and regulations in order to maintain a safe and wholesome food supply.

Table 1.1. Examples of fecally-associated pathogens

Bacteria	Viruses	Parasites
Animal		
<i>Aeromonas</i> spp.	Coronavirus	<i>Cryptosporidium parvum</i>
<i>Bacillus anthracis</i>	Norovirus*	
Enterohemorrhagic <i>Escherichia coli</i>	Rotavirus	
<i>Listeria monocytogenes</i>		
<i>Salmonella</i> spp.		
<i>Yersinia enterocolitica</i>		
<i>Campylobacter</i>		
Human		
<i>Shigella</i>	Hepatitis A	<i>Cryptosporidium parvum</i>
<i>Salmonella</i> spp.	Norovirus	<i>Giardia lamblia</i>
<i>Campylobacter</i>	Rotavirus	<i>Cyclospora cayetanensis</i>
Enterohemorrhagic <i>Escherichia coli</i>	Astrovirus	<i>Entamoeba histolytica</i>

* Ability of norovirus to cross species barrier is still unknown

Table 1.2. Per capita consumption (lbs) of fresh fruits and vegetables in the U.S. (Data Source: Fruit and Tree Nut Situation and Outlook Report, USDA, 2004)

Year	Fruits	Vegetables
1992	122.8	173.9
1993	123.5	180.7
1994	124.9	186.5
1995	122.5	180.9
1996	126.2	185.9
1997	129.4	190.1
1998	128.8	186.5
1999	129.6	191.3
2000	127.2	200.4
2001	125.8	196.6

Table 1.3. Detection rate of various foodborne pathogens in representative produce items

Pathogen	Reservoir	Produce	Detection Rate (%)	Reference
<i>Aeromonas hydrophila, caviae</i>	Fresh, stagnant, estuarine, or brackish water	Chicory salads, lettuce, salad mix, watercress, endive, carrots	48 - 100	Marchetti et al. 1992, Nguyen-the, C. and Carlin F., 2000
<i>Bacillus cereus</i>	Frequently isolated from soil, growing plants	Alfalfa, mung bean, and wheat sprouts, broccoli	12 - 92.9	Harmon et al., 1987, Splittsoesser et al., 1983, Thunberg 2002
<i>Campylobacter</i>	Zoonotic; isolation includes poultry, cattle, sheep, rodents, horses, pigs	Lettuce, parsley, mushrooms, green onion, potatoes, spinach, radish	0.6 - 1.5	Park and Sanders 1992, Little et al. 1999, Doyle and Schoeni, 1986
<i>Clostridium botulinum</i>	Soil, sediments of streams, lakes, and coastal waters, intestinal tracts of fish and mammals, in the gills and viscera of crabs and other shellfish	Salad mix, shredded cabbage, chopped green pepper	0.3 - 0.7	Lilly, et al., 1996, Notermans et al. 1989
<i>Clostridium perfringens</i>	Soil, dust, intestinal tract of humans and animal	Mixed raw vegetables	34	Nguyen-the, C. and Carlin F., 2000
<i>Cryptosporidium</i>	Human and animal intestines, surface waters	Lettuce, Cilantro, Carrots, Cucumber, Radish, Tomato	1.3 - 8.7	Monge and Chinchilla, 1996
<i>Escherichia coli</i> O157:H7	Intestinal tract of cattle and other domestic animals and wildlife	Mixed raw vegetables, Cilantro, Corriander, celery	19 - 20	Nguyen-the, C. and Carlin F., 2000, Beuchat, 1996
<i>Giardia</i> spp.	Surface waters	Mixed raw vegetables	13	Nguyen-the, C. and Carlin F., 2000

Table 1.3. (cont.) Detection rate of various foodborne pathogens in representative produce items

Pathogen	Reservoir	Produce	Detection Rate (%)	Reference
<i>Listeria monocytogenes</i>	Decaying vegetation, Surface waters, soil, feces of animals	Mung bean sprouts, Lettuce, Prepacked salads, Salad mix, mixed vegetables, broccoli, cabbage, coleslaw, cucumber, green pepper, leafy vegetables, mushrooms, potatoes, radish, tomatoes, field cress	1.1 - 86	Nguyen-the, C. and Carlin F., 2000, Arumugaswamy et al., 1994, Odumeru et al., 1997, Francis et al., 1999, Szabo et al., 2000, Harvey and Gilmore, 1993, Sizmur and Walker, 1988, Beuchat, 1996, Lin et al., 1996, de Simon et al., 1992, Wong et al., 1990, MacGowan et al., 1994, Francis et al., 1999, Heisick et al., 1989
<i>Salmonella</i>	Poultry, eggs, swine, water, soil, insects	Cantaloupe, Strawberries, mung bean sprouts, alfalfa seeds, lettuce, salad greens, mixed raw vegetables, chili, cilantro, culantro, parsley, artichoke, cabbage, cardoon, cauliflower, celery, eggplant, endive, fennel, green onions, spinach, beet leaves	0.4 - 72	FDA 2001, Madden 1992, Splittstoesser et al., 1983, Beuchat 1996, Jerngklinchan and Saitanu, 1993, Ercolani 1976, Tamminga et al. 1978, Saddik et al. 1985, Lin et al. 1996, Garcia-Villanova et al. 1987, Rude et al., 1985, Al-Hindawi and Rished, 1979, Tauxe et al. 1997, Mukherjee A. et al. 2004
<i>Shigella</i>	Humans	Cantaloupe, Lettuce, Salad greens, Salad mix, Parsley, Celery, Green Onion	1.1 - 2.4	FDA 2001a, Saddik et al. 1985,
<i>Staphylococcus aureus</i>	Humans and animals	Alfalfa, mixed, and onion sprouts, Parsley, Carrots, Radish, lettuce	5.1 - 28.0	Prokopowich and Blank 1991, Beuchat 1996, Thunberg 2002
<i>Yersinia enterocolitica</i>	Intestinal tract of species, birds, frogs, fish, flies, soil, surface waters	Lettuce, Prepacked salad, mixed raw vegetables, watercress	1 - 100	Szabo et al. 2000, Brocklehurst et al. 1987, Darbas et al. 1985, Buechat 1996, Tassinari et al. 1994

Table 1.4. Examples of recent outbreaks of foodborne disease associated with fruits and vegetables

Pathogen	Year	Food Source	No. of States	No. of Cases	Food Origin	Comments	Reference
Bacterial							
<i>Escherichia coli</i> O157:H7	1996	Unpasteurized Apple Juice	8, Canada	70	U.S.	Dropped apples used, apple orchard near deer and cattle. Incorrect use of disinfectant during wash.	CDC 1996a; Cody et al., 1999
<i>Escherichia coli</i> O157:H8	1996	Mesclun	2	49	U.S.	Contaminated lettuce was traced to a single grower; cattle found near lettuce fields	Hilborn et al., 1999
<i>Escherichia coli</i> O157:H9	1998	Alfalfa Sprouts	1	8	California and Nevada	Contaminated seeds; sprouts were traced to single sprouter	Taormina et al., 1999; Harris, 2001
<i>Salmonella</i> Enteritidis	2000	Citrus Juices	Multistate	14	California	Gallon-sized containers of domestic citrus juices were implicated in the outbreak	Harris, 2001
<i>Salmonella</i> Kottbus	2001	Alfalfa Sprouts	4	24	California	Traceback investigation identified a single sprout producer as the source of the contaminated sprouts; sample of seed lot yielded <i>S. Kottbus</i> ; seeds were imported from Australia	CDC, 2002c

Table 1.4 cont'd. Example of recently reported outbreaks of foodborne disease associated with fruits and vegetables (continued from previous page)

Pathogen	Year	Food Source	No. of States	No. of Cases	Food Origin	Comments	Reference
<i>Salmonella</i> Poona	2002	Cantaloupe	10, Canada	58	Mexico	Possible sources of contamination included irrigation water contaminated with sewage, cleaning and cooling produce with <i>Salmonella</i> -contaminated water, poor hygiene of harvesters and processors, pests in packing facilities, and inadequate cleaning and sanitizing of equipment	CDC, 2002b
<i>Salmonella</i> Newport	2002	Tomatoes	22	404	Mid-Atlantic Region, U.S.	All tracebacks implicated the same tomato packing shed	Kretsinger et al., 2003
<i>Shigella flexneri</i> 6A	1994	Green onions	2	72	Mexico	Possible contamination during harvest or packaging	Tauxe, 1997
<i>Shigella sonnei</i>	1998	Parsley	4	310	Mexico	Poor water quality in packing shed and poor worker hygiene; in restaurant, product was chopped and left at room temperature	CDC, 1999

Table 1.4 cont'd. Examples of recent outbreaks of foodborne disease associated with fruits and vegetables

Viruses							
Hepatitis A	2003	Green onions	4	>900	Mexico	Raw green onions from three firms in Mexico have been implicated in the Tennessee and Georgia outbreaks	CDC, 2003
Hepatitis A	1997	Strawberries	4	242 (+14 suspect)	Mexico	Possible contamination during harvesting	CDC, 1997a
Parasites							
<i>Cryptosporidium parvum</i>	1996	Apple Juice	1	20 (+11 suspect)	New York	Dairy farm nearby; E. coli was detected in well water indicating fecal contamination	CDC, 1997c
<i>Cyclospora cayetanesis</i>	1999	Blackberries	Ontario, Canada	104	Guatemala, likely	Source of contamination unknown	Herwaldt, 2000
<i>Cyclospora cayetanesis</i>	2004	Snow Peas	Pennsylvania	96	Guatemala	Source of contamination unknown	CDC, 2004a
<i>Cyclospora cayetanesis</i>	2004	Basil/Mesculin Spring Mix Salad	Illinois, Texas	95	Not yet known	Source of contamination unknown	FDA, 2004

Table 1.5. Sources of pathogenic microorganisms on fresh fruits and vegetables

Preharvest	Postharvest
Feces	Feces
Soil	Human handling
Irrigation water	Harvesting equipment
Water used to apply fungicides, insecticides	Transport containers
Green or inadequately composted manure	Wild and domestic animals
Dust	Insects
Wild and domestic animals	Dust
Insects	Wash and rinse water
Human handling	Processing equipment
	Ice
	Transport vehicles
	Improper storage

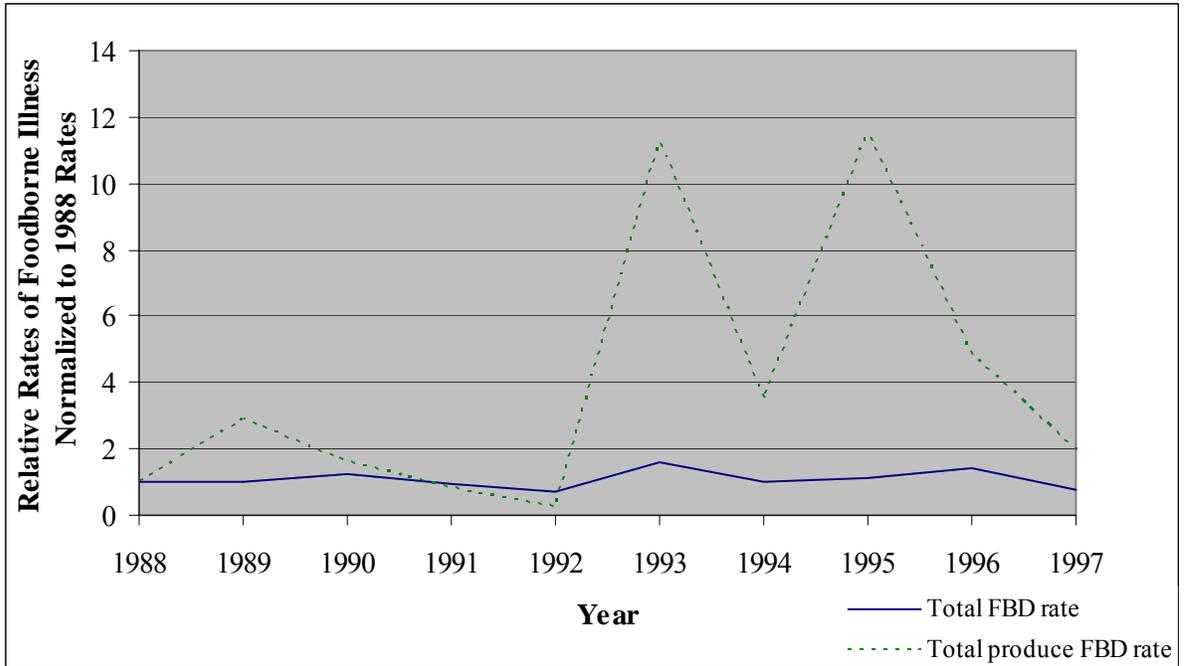
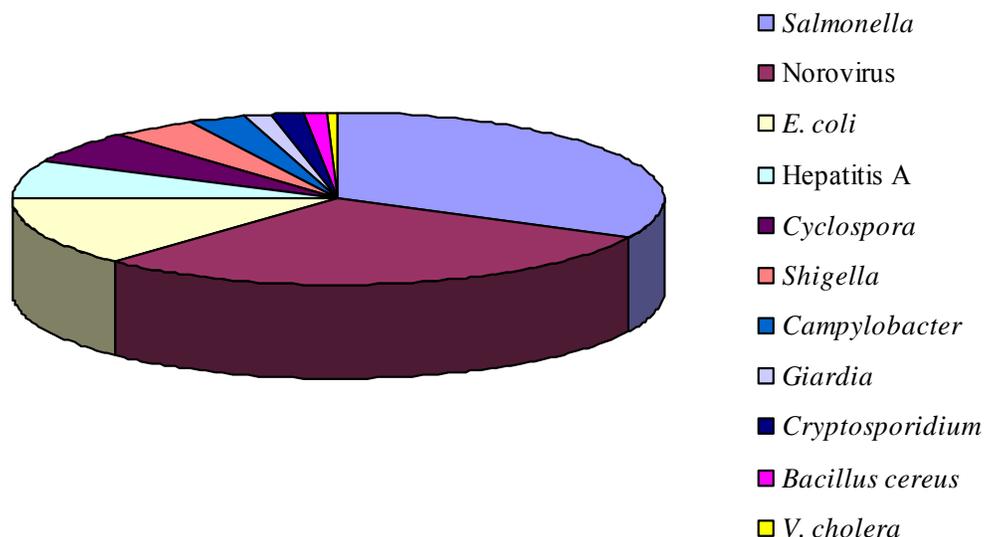


Figure 1.1. Relative rates of total foodborne disease (FBD) cases and produce-associated cases of FBD compared with 1988 on CDC surveillance data (CDC, 2000b).

Proportion of Outbreaks by Etiologic Agent



Proportion of Outbreaks by Produce Group

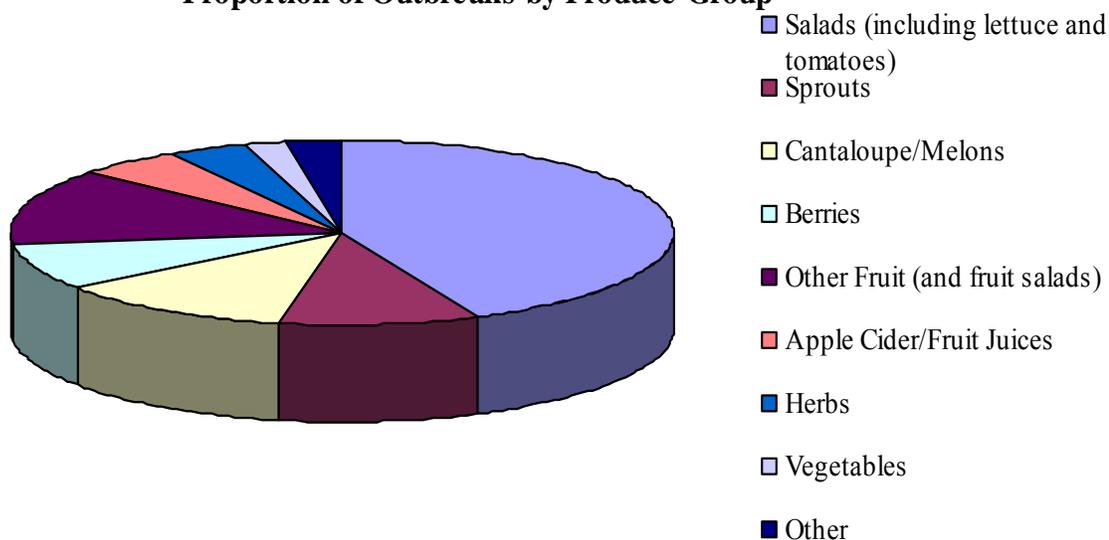


Figure 1.2. Reported produce-associated outbreaks of known etiology, U.S. 1990-2002 by etiologic agent and by produce group (Data Source: CDC Foodborne Outbreak Surveillance System, www.cdc.gov)

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CHAPTER 2

A FIELD STUDY OF THE MICROBIOLOGICAL QUALITY AND SAFETY OF FRESH PRODUCE

2.1 ABSTRACT

The U.S. Centers for Disease Control and Prevention (CDC) has reported that the proportion of foodborne disease outbreaks associated with fruits and vegetables has increased over the last decade. The objective of this study was to examine the routes of microbial contamination in produce, and identify areas of potential contamination throughout production and processing. As part of a broader study aimed at identifying specific production and processing practices associated with microbial contamination of produce, we report here the levels of bacterial indicator organisms and the prevalence of select pathogens in produce samples collected from the southern U.S. A total of 398 produce samples, consisting of leafy greens, herbs, and cantaloupe, were collected throughout production and processing and assayed by enumerative tests for total aerobic bacteria (APC), total coliforms, total *Enterococcus*, and *E. coli*. These samples were also analyzed for the presence of *Salmonella*, *Listeria monocytogenes*, and *E. coli* O157:H7. Microbiological methods were based on those recommended by the U.S. Food and Drug Administration as per the Bacteriological Analytical Manual. For all leafy greens and herbs, geometric mean indicator levels ranged from 4.5 log₁₀ to 6.2 log₁₀ CFU/g (APC); less than 1 log₁₀ to 4.3 log₁₀ CFU/g (coliforms and *Enterococcus*); and less than 1 log₁₀ to 1.5 log₁₀ CFU/g (*E. coli*). Indicator levels remained relatively constant throughout processing for most of these products; however, for cilantro and parsley, total coliform levels increased during the packing process.

For cantaloupe, microbial levels significantly increased from field through processing, with ranges of 6.4 log₁₀ to 7.0 log₁₀ CFU/g (APC); 2.1 log₁₀ to 4.3 log₁₀ CFU/g (coliforms); 3.5 log₁₀ to 5.2 log₁₀ CFU/g (*Enterococcus*); and less than 1 log₁₀ to 2.5 log₁₀ CFU/g (*E. coli*). The prevalence of pathogens for all samples was 0%, 0%, and 0.7% (3/398) for *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella*, respectively. This study demonstrates that each step from production to consumption may affect the microbial load of produce and reinforces government recommendations for ensuring a high quality product.

2.2 INTRODUCTION

The fresh fruit and vegetable industry has been a rapidly evolving market over the last two decades. In the United States, there has been a growing awareness of the health benefits associated with the consumption of fresh produce and this has contributed to a \$36.2 billion increase in retail and foodservice sales from 1987 to 1997 (FDA, 2001). Furthermore, retailers' demand for year-round fresh produce has helped sustain the growing international trade market, assuring consistent supplies to consumers during the off-season (Jerardo, 2003). Despite the nutritional and economic benefits of fresh produce, there have also been issues of public health concern. While fruits and vegetables were associated with 0.5% to 4.2% of foodborne disease outbreaks between 1988 and 1997, the U.S. Centers for Disease Control and Prevention (CDC) reported that the proportion of foodborne disease outbreaks associated with fruits and vegetables doubled between 1973 and 1987 and again between 1988 and 1991 (CDC, 1996; CDC, 2000; Tauxe et al., 1997). During this time period, several changes were taking place, including the discovery of newly identified pathogens, the improvement of diagnostic methods, as well as the advancement of foodborne disease surveillance systems (Sewell and Farber, 2001).

A broad variety of fresh produce items have been linked to various pathogens, including cantaloupe, herbs, and leafy greens (Beuchat, 1996; Sewell and Farber, 2001). The majority of well-characterized outbreaks have been caused by bacteria, namely *Salmonella*, *E. coli* O157:H7, *Shigella*, and *Listeria monocytogenes*, while a few outbreaks have also been linked to viruses and parasites such as hepatitis A virus, noroviruses, and *Giardia lamblia*, respectively (Beuchat, 1996; Ponka et al., 1999).

There are many factors which may contribute to microbial contamination throughout production and processing of fresh produce (Beuchat, 1996). These include the use of contaminated irrigation or process water, the use of biosolids or manure for fertilization, poor worker hygiene, and poor equipment sanitation. In an effort to improve the safety of produce, the U.S. federal agencies responsible for food safety (i.e., U.S. Food and Drug Administration(FDA) and the U.S. Department of Agriculture (USDA)) published voluntary guidelines in 1998 entitled “*Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables*” (FDA, 1998). The Guide’s primary purpose was to provide a framework for the identification and implementation of practices likely to decrease the risk of pathogenic microbiological contamination of produce, based on Good Agricultural Practices (GAPs) and Good Manufacturing Practices (GMPs). Although the Guide provides general knowledge about potential pathways by which produce may become contaminated with pathogens, systematic studies to identify critical points through the production to consumption continuum where contamination may occur are lacking.

In an effort to address these data needs, we sought to identify and further understand routes for potential microbial contamination of produce throughout production and processing. The objectives of this particular study were three-fold: (i) to monitor the

microbiological quality of fresh produce from the field through the packing process; (ii) to evaluate the prevalence of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* on fresh produce; and (iii) to identify differences in microbiological quality between various produce items and over the course of production and processing. The data reported here are part of a larger study to determine specific farm and on-site processing practices that may be associated with microbial contamination of produce.

2.3 MATERIALS AND METHODS

2.3.1 Sample collection

The sampling site, located in the Southern United States, included 13 farms and 5 packing sheds. Samples were collected between November 2000 and May 2002. Target commodities included produce items that are mostly consumed raw, except for collards and mustard greens (Table 2.1). Samples were taken in a sequential manner, following the same crop from harvest throughout processing and packaging. Samples designated as “field” included midseason crops, harvest samples, and samples collected at point of entry to the packing shed. Samples designated as “wash tank” and “rinse” were taken immediately following the wash and rinse step, respectively, at the packing shed. Samples labeled “Box” were collected from boxes just prior to distribution. Cantaloupe samples were also taken directly off of the conveyor belt between the rinse step and box for distribution.

Two sets of composite samples (400-600 g each) of every produce commodity were obtained from each location using hands protected by sterile, disposable gloves. Samples were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI). One of these composite sets was used for enumerative analyses and was numerically and alphabetically coded by the

collection technicians to assure anonymity. At the request of our Scientific Advisory Committee, the other composite sample (intended for pathogen assay) was unmarked and could therefore not be traced back after testing. All samples were immediately shipped on ice to our location at North Carolina State University by overnight courier. Microbial analyses were initiated within 24 h of sample collection.

2.3.2 Microbial Indicator Analysis

Unless otherwise stated, all media were obtained from Becton Dickinson Laboratories (Sparks, MD). Twenty-five g subsamples were weighed and diluted 1:10 in 0.1% peptone buffer. Cantaloupe samples were prepared by trimming rind (less than 0.5 cm deep) from melons with a sanitized paring knife and removing all visible mesocarp material. After homogenizing for 2 min at 230 rpm in a Stomacher[®] 400 (Seward, Norfolk, UK), samples were subsequently processed for enumeration of total aerobic bacteria (aerobic plate count, APC), total coliforms, total *Enterococcus*, and *E. coli*. Assays for total aerobic bacteria, coliforms, and *E. coli* were done using Aerobic Count Plate Petrifilm[™] and Coliform/*E. coli* Petrifilm[™] plates (3M, Saint Paul, MN, USA), respectively (Feng et al., 2002). Total enterococci were enumerated using KF Streptococcal agar (Hartman, et al., 1992).

2.3.3 Pathogen Analysis

Three subsamples of 25 g each, originating from the composite sample intended for pathogen detection, were weighed and prepared for *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7 assays using the FDA Bacteriological Analytical Manual (BAM) methods (Andrews et al., 2003; Feng and Weagant, 2002; Hitchins, 2003). For *Salmonella* detection, samples were homogenized in 225 ml of lactose broth, followed by incubation at 37°C for 24

h. One ml of the lactose pre-enrichment broth was then transferred to tetrathionate and selenite cysteine broths and incubated at 37°C. After 18-24 h, samples were streaked to xylose lysine desoxycholate, bismuth sulfite, and hektoen enteric agar. Two or more typical colonies were then transferred to lysine iron agar and triple sugar iron agar slants, followed by *Enterobacteriaceae* MICRO-ID[®] (Remel, Lenexa, KS) for the generic identification of *Salmonella*. Presumptive *Salmonella* isolates were sent to the College of Veterinary Medicine at North Carolina State University for Vitek (bioMerieux, Hazelwood, MO) identification and subsequently shipped to the National Veterinary Services Laboratories (Ames, Iowa) for serotyping.

For *L. monocytogenes* detection, 25 g produce samples were incubated in *Listeria* Enrichment Broth at 30°C for 24 to 48 h. *Listeria* spp. were then isolated using Oxford agar and Lithium Chloride-Phenylethanol-Moxalactam (LPM) agar, supplemented with esculin and ferric ammonium citrate (Sigma Chemical Company, St. Louis, MO). Typical colonies were analyzed for beta-hemolysis on 5% sheep blood agar (Remel, Lenexa, KS), and colonies displaying beta-hemolysis were streaked on blood agar for the CAMP test, followed by *Listeria* MICRO-ID (Remel, Lenexa, KS) for speciation.

For *E. coli* O157:H7 detection, 25g produce samples were first enriched in 225 ml of EHEC enrichment broth at 37°C for 24 h followed by plating on sorbitol-MacConkey agar, supplemented with potassium tellurite and cefixime (Dynal[®], Lake Success, NY). At least two presumptive colonies were screened for the presence of the O:157 antigen using the commercial Prolex *E. coli* O:157 latex test reagent kit (Pro-Lab Diagnostics, Round Rock, TX).

2.3.3 Statistics

Statistical analyses, including geometric means, standard deviations, ranges, and medians were done using Sigma Plot version 8.0 (SPSS, Chicago, IL). One-way ANOVA tests were performed using Tukey comparisons to derive statistical differences ($P < 0.05$) of microbial levels between all sampling locations. In an effort to avoid under- and over-representation of sample counts, when enumerative results fell below the assay limit of detection, they were assigned a value halfway between zero and the assay detection limit (Shumway et al., 1989; Garg et al., 1990).

2.4 RESULTS

2.4.1 Sample Collection

A total of 398 produce samples were collected between November 2000 and May 2002, originating from 13 farms and 5 packing sheds (Table 2.1). Over 80% of the produce items collected consisted of cantaloupe (23%), cilantro (24%), mustard greens (18%), and parsley (20%). Due to sampling limitations, smaller numbers of other produce items (arugula, collards, spinach, and dill) were collected.

2.4.2 Microbiological Quality of Produce

Table 2.2 displays the \log_{10} geometric means and standard deviations for various measures of microbiological quality, including total aerobic mesophiles, total enterococci, total coliforms, and *E. coli*. These numbers are given for each commodity, irrespective of sample location. Total aerobic bacteria ranged from a geometric mean of 4.5 to 6.6 \log_{10} CFU/g. *Enterococcus* levels ranged from 1.3 to 4.3 \log_{10} CFU/g, with cantaloupe and mustard greens having the highest levels. Geometric mean total coliform counts ranged from

1.0 to 3.4 log₁₀ CFU/g. Overall geometric mean *E. coli* counts were quite low for most produce items (≤ 1.0 log₁₀ CFU/g) and highest for cantaloupe (1.5 log₁₀ CFU/g).

In order to identify critical points of contamination, further data analysis was done to compare microbial levels on produce associated with specific sampling locations. Figures 2.1 through 2.4 show changes in microbial levels from the field and throughout the packing shed process for cilantro, parsley, mustard greens, and cantaloupe, respectively. Due to increased sample representation from these four commodities, this separate data analysis was limited to these commodities only. Each box and whiskers plot displays the 10th, 25th, 50th, 75th and 90th percentile, and the geometric mean. For cilantro (Figure 2.1) total APC levels increased from the field and throughout packing, with mean ranges of 5.7 log₁₀ in the field to 6.7 log₁₀ CFU/g in the samples obtained from boxes ready for distribution. *Enterococcus* levels remained consistently low, with levels ranging from 1.7 to 2.3 log₁₀ CFU/g. There was a statistically significant (P<0.05) increase in total coliforms (approximately 1.4 log₁₀) from harvest through packing, with a rise occurring mainly at the rinse step. The levels of *E. coli* on cilantro were extremely low, typically below the lower limit of detection (<10 CFU/g).

In contrast, parsley showed a slight increasing trend throughout processing for APC, enterococci, and total coliforms (Figure 2.2). The APC levels increased approximately 1.0 log₁₀ CFU/g within the packing shed, from a mean of 5.2 log₁₀ CFU/g at point of entry to 6.1 log₁₀ CFU/g in the samples ready for distribution. This increase occurred at the rinse step, with APC levels remaining stable thereafter. Enterococci levels increased from a geometric mean of 2.1 log₁₀ CFU/g from the field to 3.1 log₁₀ CFU/g at the rinse step. Total coliform levels doubled after the rinse when compared to the product at point of entry. Like cilantro, the levels of *E. coli* were quite low, usually falling below the lower limit of detection.

The microbial levels on mustard greens, including APC, enterococci, coliforms, and *E. coli*, did not change significantly from the field through the packing process (Figure 2.3). However, there was no indication that packing shed steps, such as water rinsing, reduced the microbial load on this product.

Concentrations of total enterococci, total coliforms, and *E. coli* on cantaloupes increased from harvest through packing (Figure 2.4). Aerobic plate counts remained constant from production and throughout the packing process, with a mean range of 6.4 log₁₀ at point of entry to nearly 7.0 log₁₀ CFU/g in the distribution box. A statistically significant (P<0.05) increase in total enterococci (approximately 1 log₁₀) occurred between the rinse step and the conveyor belt. Total coliforms showed the same trend, with levels doubling at the conveyor belt step. Interestingly, *E. coli* levels increased substantially from 0.8 log₁₀ CFU/g for samples taken from the field to 2.5 log₁₀ CFU/g for samples ready for retail distribution. As with enterococci and coliforms, these increases appeared to occur at the conveyor belt step.

2.4.3 Pathogen Detection in Fresh Produce

All samples were analyzed for the presence of *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella*. *Listeria monocytogenes* and *E. coli* O157:H7 were not detected in any of the produce items tested (0/398). However, *Salmonella enterica* serovar. Montevideo was detected on three cantaloupe samples, resulting in a prevalence of 0.8% for all produce items and 3.3% for cantaloupe alone.

2.5 DISCUSSION

The objectives of this study were to monitor the microbiological quality of various fresh produce items from the field through the packing process and to evaluate the prevalence

of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* on fresh produce. In so doing, we sought to supplement previous survey data by examining possible routes of contamination and identifying potential control points through production and processing.

Overall, the microbial quality of cilantro, parsley, and mustard greens was excellent. In most cases, statistically significant increases in microbial load did not occur during the packing process, and the levels of *E. coli*, which suggest fecal contamination, were extremely low. Moreover, no pathogens were detected in any of these produce items, either from the field or from the packing shed. These results are similar to those presented in the FDA's Survey of Domestic Produce published in 2003 (FDA, 2003), which also failed to find *E. coli* O157:H7 in either cilantro or parsley. In addition, the FDA survey reported a low prevalence of *Salmonella* among leafy greens, with 0/90 parsley samples testing positive for *Salmonella* and only 1/85 (1.2%) cilantro samples positive for this pathogen.

The results of our study indicate that microbial loads on cantaloupes increased significantly during the packing process. For total enterococci and coliforms, significant increases occurred between the rinse step and the conveyor belt, suggesting contaminated equipment surfaces. A significant increase in *E. coli* levels occurred between the rinse and conveyor belt, and again between the conveyor belt and the final distribution box. Cantaloupes possess characteristics which create challenges for maintaining a microbiologically sound product. The surface topography of cantaloupe, known as the *netting*, may favor microbial attachment and complicate efforts aimed at reducing surface contamination. Furthermore, the pH range of the fruit itself (6.1-7.1) is suitable for microbial growth. Another potential concern is the waxing procedure, the purpose of which is to replace natural waxes that may have been removed during washing and rinsing of the melon,

thereby improving appearance and reducing shrinkage or water loss (Park, H.J.). Based on the strong attachment characteristics of bacteria, particularly *Salmonella* (Ukuku and Fett, 2002), and the physical characteristics of the netting material, the wax may potentially provide a barrier to further removal of microorganisms which might occur during washing at the retail and consumer level. Three of 90 (3.3%) cantaloupe samples in our study were contaminated with *Salmonella enterica* serovar. Montevideo. This is similar to data reported in the FDA Domestic Produce Survey which reported 4 of 164 (2.4%) cantaloupe samples positive for *Salmonella* (FDA, 2003). Furthermore, a recent study by Castillo, et al. (2004) reported the low prevalence of *Salmonella* contamination on cantaloupe during production. Among 950 domestic cantaloupes, 5 (0.5%) were found to be contaminated with *Salmonella*, and 1 of 300 (0.3%) Mexican cantaloupes were found to contain the pathogen.

In general, our data is consistent with other studies that have examined microbial levels on fresh produce items. Several investigators have reported similar levels of total aerobic bacteria on leafy green vegetables collected from both production and retail establishments (Garg, et al., 1990; King et al., 1991; Ruiz et al., 1987; Stewart et al., 1978). For example, Ruiz et al. (1987) examined the levels of microbiological indicators on several types of leafy vegetables and herbs, including spinach, cabbage, lettuce and parsley. Total aerobic bacteria levels ranged from 10^5 to 10^7 CFU/g on field samples, while levels on retail samples ranged from 10^4 to 10^6 CFU/g. However, the coliform and *E. coli* levels on leafy greens and herbs reported in our study were 2 to 3 \log_{10} CFU/g lower than those reported by Ruiz et al. (1987) in their study.

Interestingly, only a few studies have characterized the change in microbial levels throughout the production, processing, and packaging of fresh produce. In an early study,

Geldreich and Bordner (1971) reported a significant increase in the fecal coliform load for both root crops and leafy vegetables from field to market. As with our results on various microbiological indicators, Prazak et al. (2002) found that packing sheds provided a suitable environment for the survival and proliferation of *Listeria* spp., particularly conveyor belts, where cross-contamination between processing surfaces and cabbage may occur. Likewise, Gagliardi et al. (2003) concluded that a significant amount of contamination on cantaloupe occurs at the packing shed (during processing and washing) rather than in the field or during harvest. Another study found the frequency of *E. coli* among Mexican cantaloupes to increase at the packing shed, supporting the idea that the practice of washing melons after harvesting may increase the chance of fecal coliform contamination (Castillo et al., 2004). If a limited number of products are contaminated, contamination may be spread over the entire lot during washes, such as water dips which are commonly used in produce packing sheds (Beuchat, 1998).

In general, these studies, along with the results presented here, suggest that microbiological levels may either increase or originate in the packing shed phase, perhaps impacting the shelf life of the product. However, attempts to correlate increased levels of microorganisms with spoilage have given conflicting results. High microbial counts found on unstored lettuce were related to a short shelf life. However, there was a negative correlation between product quality and bacterial counts for shredded endive (Nguyen-the and Carlin, 2000). Consequently, it may not be appropriate to assume that high microbial counts on some produce items in this study are indicative of low quality or reduced shelf-life. Furthermore, the health significance of high levels of APC, coliforms and enterococci on produce is also not clear. Some coliforms (*Klebsiella*) are commonly associated with

vegetable produce and can multiply under favorable environmental conditions (Knittel et al., 1977).

Produce packing sheds often rely on a wash procedure after harvest to remove soil and debris, to reduce microbial levels, and to potentially increase the shelf life or quality of products. The use of sanitizers in the packing shed is perceived as an essential strategy to maintain clean wash and rinse water (FDA, 1998; Zagory, 1999). For cilantro and parsley, we observed an increase in the level of total coliforms subsequent to the wash step (Figs 1C and 2C). In both cases, the increase occurred during rinsing. While chlorine has been proven to be an effective disinfectant for drinking and recreational waters and an effective surface disinfectant, it is less effective for reducing microbial loads on produce items. Previous studies have reported that chlorinated wash water will generally reduce microbial loads on produce by only one to two \log_{10} units (Beuchat, 1998). Senter et al. (1987) reported that chlorine had little effect on reducing microbial load on tomatoes. While Beuchat and Brackett (1990) found chlorine (200 to 250 $\mu\text{g}/\text{mL}$) to be initially effective in reducing microbial loads on lettuce, after several days of storage microbial levels increased significantly, and no significant differences could be found between microbial populations on lettuce washed with chlorinated water versus unchlorinated water. Li et al. (2001) found that treatment of lettuce with 20 ppm chlorine at either 20° C or 50° C did not result in significantly greater reductions in populations of *E. coli* O157:H7 compared to treatments in water without chlorine. The relative ineffectiveness of chlorine as a disinfectant for produce items is evident in our study as well. For example, even though the majority of packing sheds in our study used chlorine in wash water (data not shown), the results for mustard

greens, herbs, and cantaloupe suggest that the use of chlorine did not reduce the microbial load on these products.

For chlorine to be an effective bactericide, several factors must be considered. Concentrations must be monitored continuously to ensure the maintenance of effective levels. Furthermore, there are several important process and product parameters, including pH, presence of organic matter, and the type of wash procedure. For instance, chlorine is more effective at an acidic pH, and its reactivity is quickly saturated in the presence of organic matter. Several packing sheds that we worked with processed cantaloupe by submerging an entire bulk load of melons into chlorinated wash water tanks. In this case, it becomes extremely important to consistently replace the wash water to prevent potential cross-contamination and to maintain effective levels of chlorine.

Equipment sanitation is another important consideration in controlling microbial contamination. The conveyor belt material used in many packing sheds consists of an abrasive, brush-like material, which may be difficult to thoroughly clean. We also saw carpeted surfaces in these sheds which would be very difficult to clean and would be reservoirs for microbes. Cantaloupe samples collected from conveyor belts indicated that microbial levels increased at this stage in the packing shed. It is not clear whether these increases were due to contact with the conveyor belt or possibly due to contact with workers hands during sorting and grading before packing.

Even though packing sheds offer manageable ways of cleaning and packing produce under controlled conditions, the concept of field packing is worth revisiting for some products. Systematic studies comparing the quality of field-packed cantaloupes versus those packed in sheds are lacking. Packing in the field could potentially decrease exposure to post-

harvest sources of contamination, such as dirty rinse water, contact with dirty equipment and/or additional human handling.

While adherence to the “*Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables*” can address produce quality and safety issues during growing, harvesting, sorting, packing, and distribution, our study reinforces the frequently cited concept that every step from production to consumption will affect the microbial load of produce. In fact, our results emphasize the importance of thorough sanitation measures in production, and particularly during the packing shed phase. In conclusion, this study supports the use of proper GAPs and GMPs, as referred to by the governmental guide, since sanitation is such a critical component of ensuring a safe and wholesome product.

Government recommendations have established an important and necessary foundation for production and processing sanitation; however, the results from this study clearly indicate a need for the reevaluation of processing techniques. Ultimately, it is in the interest of individual growers and packers to examine their own processes and incorporate strategies for maintaining high quality produce.

Table 2.1. Summary of produce samples collected from each production and packing shed location

Commodity	N (%)	Field	Wash Tank	Rinse	Conveyor Belt	Box
Arugula	15 (4)	9	3	NA ^a	NA	3
Cantaloupe	90 (23)	36	3	15	18	18
Cilantro	94 (24)	49	12	12	NA	21
Collards	12 (3)	6	NA	3	NA	3
Dill	12 (3)	6	NA	3	NA	3
Mustard Greens	70 (18)	31	3	18	NA	18
Parsley	78 (20)	36	9	15	NA	18
Spinach	27 (7)	18	3	3	NA	3
Total	398	191	33	69	18	87

^aNA This step was not included in the process and/or no samples were

Table 2.2. Microbial loads in various produce commodities expressed as log₁₀ mean ± standard deviation

Produce Item	APC	Enterococci	Total Coliforms	<i>E. coli</i>
Arugula	5.8 ± 1.0	2.1 ± 1.3	3.4 ± 1.2	0.7 ± 0.0
Cantaloupe	6.6 ± 1.0	4.1 ± 1.2	3.0 ± 1.3	1.5 ± 1.1
Cilantro	6.1 ± 1.1	1.9 ± 1.2	1.8 ± 1.2	0.8 ± 0.5
Collards	4.5 ± 1.0	1.3 ± 0.6	1.0 ± 0.7	0.7 ± 0.0
Dill	5.4 ± 0.6	3.6 ± 0.8	2.9 ± 1.0	0.7 ± 0.0
Mustard Greens	6.2 ± 1.0	4.3 ± 1.3	2.4 ± 1.3	1.0 ± 0.9
Parsley	5.6 ± 1.0	2.5 ± 1.0	2.3 ± 1.1	1.0 ± 0.2
Spinach	5.8 ± 1.0	2.1 ± 0.9	1.5 ± 0.8	0.7 ± 0.0

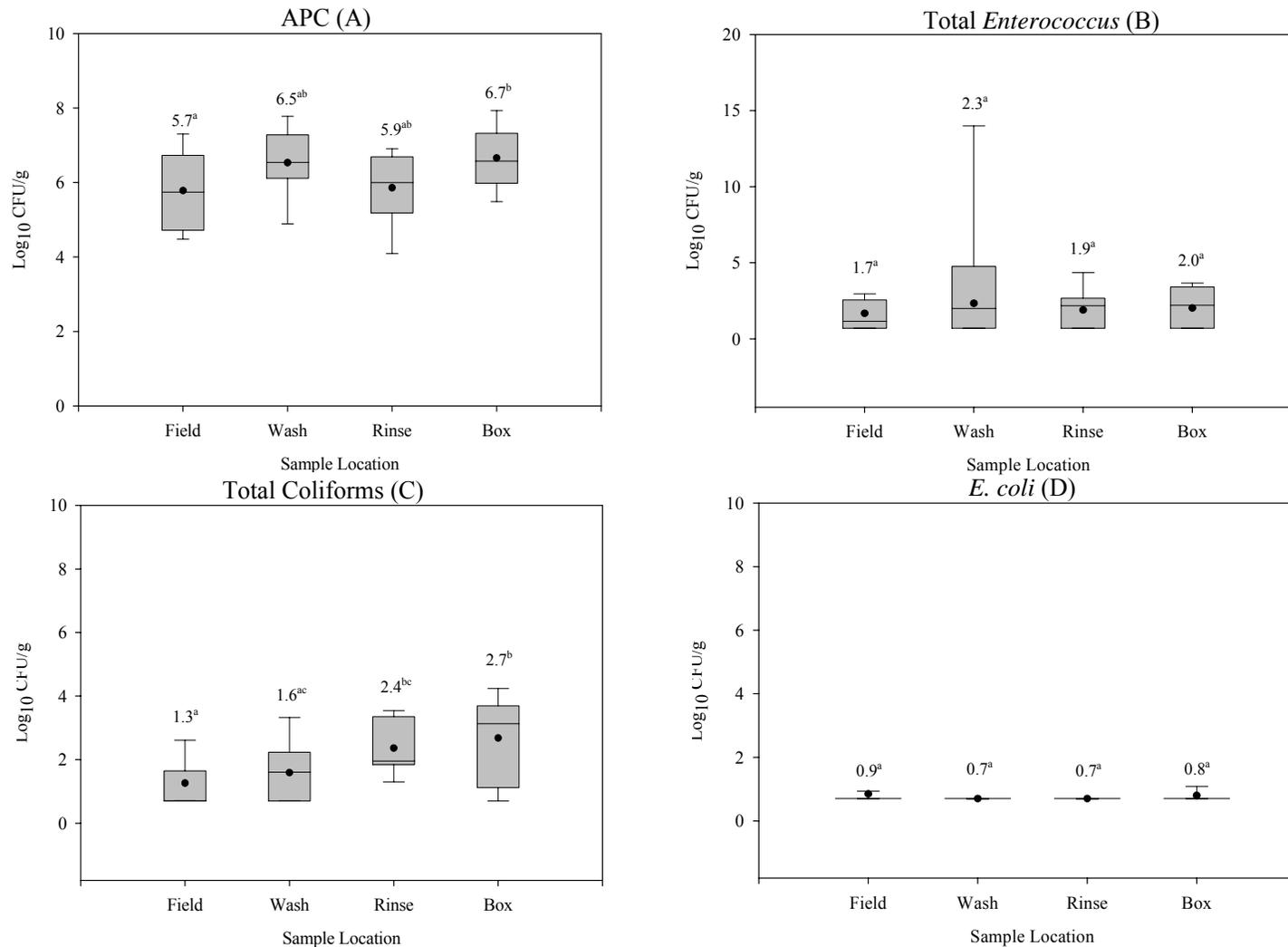


Figure 2.1. Aerobic plate count (A), total *Enterococcus* (B), total coliforms (C), and *E. coli* (D) levels from cilantro collected from the field and various steps throughout the packing shed. The box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles. The number above each box plot indicates the geometric mean, also indicated by the black circle. In instances when superscript letters differ between sampling locations, this indicates significant differences among the log₁₀ means ($p < .05$).

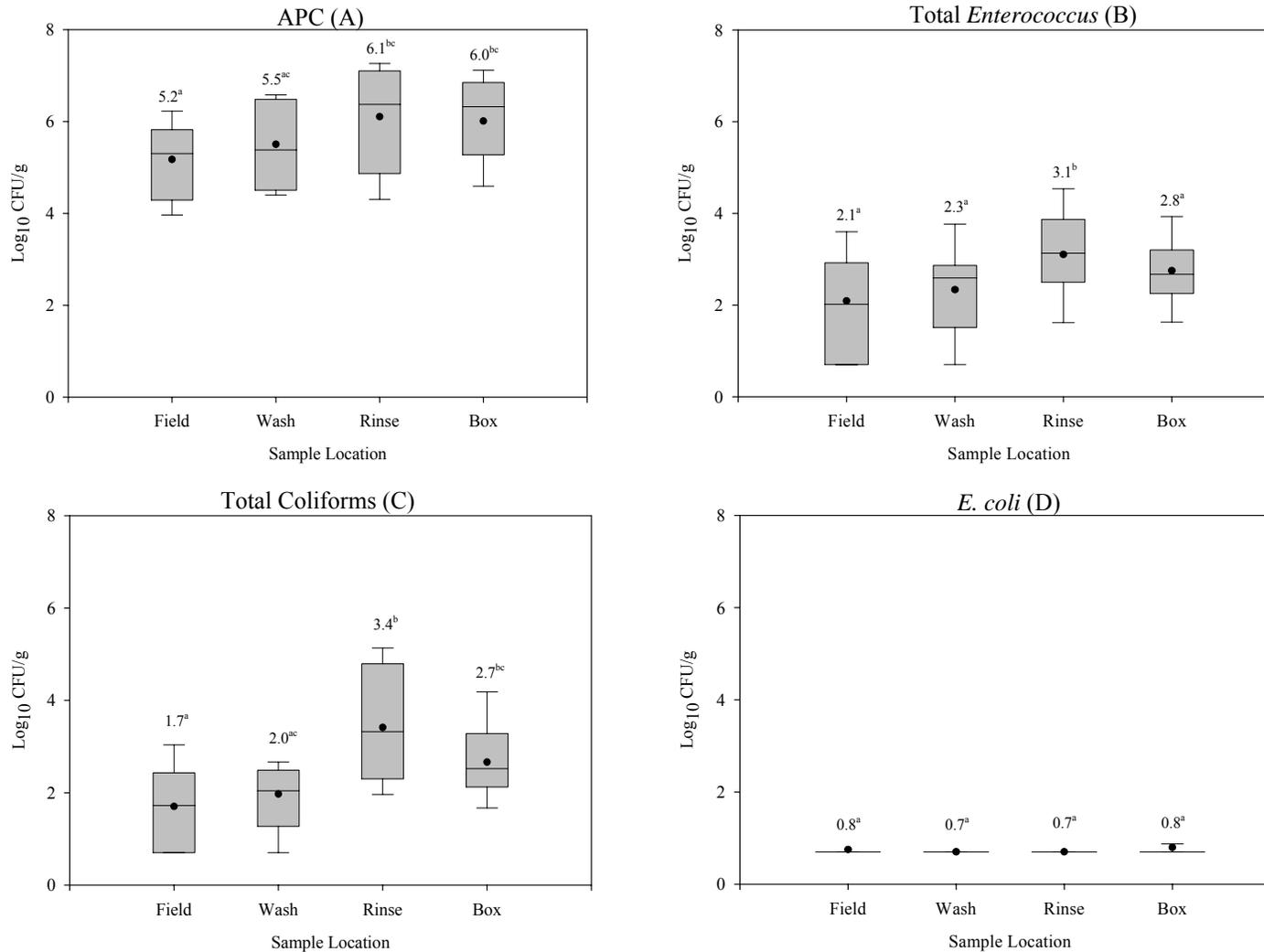


Figure 2.2. Aerobic plate count (A), total *Enterococcus* (B), total coliforms (C), and *E. coli* (D) levels from parsley collected from the field and various steps throughout the packing shed. The box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles. The number above each box plot indicates the geometric mean, also indicated by the black circle. In instances when superscript letters differ between sampling locations, this indicates significant differences among the log₁₀ means (p < .05).

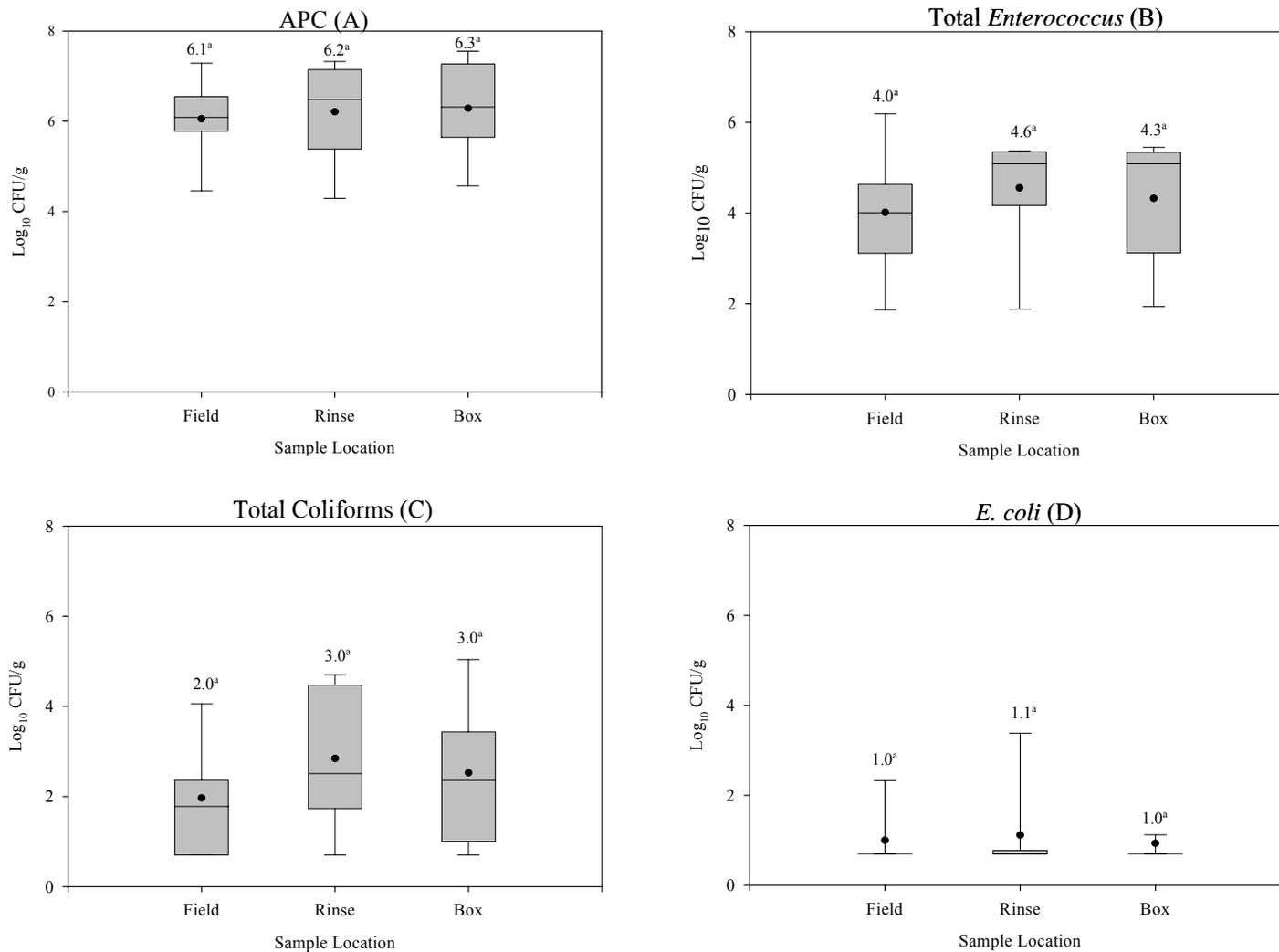


Figure 2.3. Aerobic plate count (A), total *Enterococcus* (B), total coliforms (C), and *E. coli* (D) levels from mustard greens collected from the field and various steps throughout the packing shed. The box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles. The number above each box plot indicates the geometric mean, also indicated by the black circle. In instances when superscript letters differ between sampling locations, this indicates significant differences among the log₁₀ means ($p < .05$).

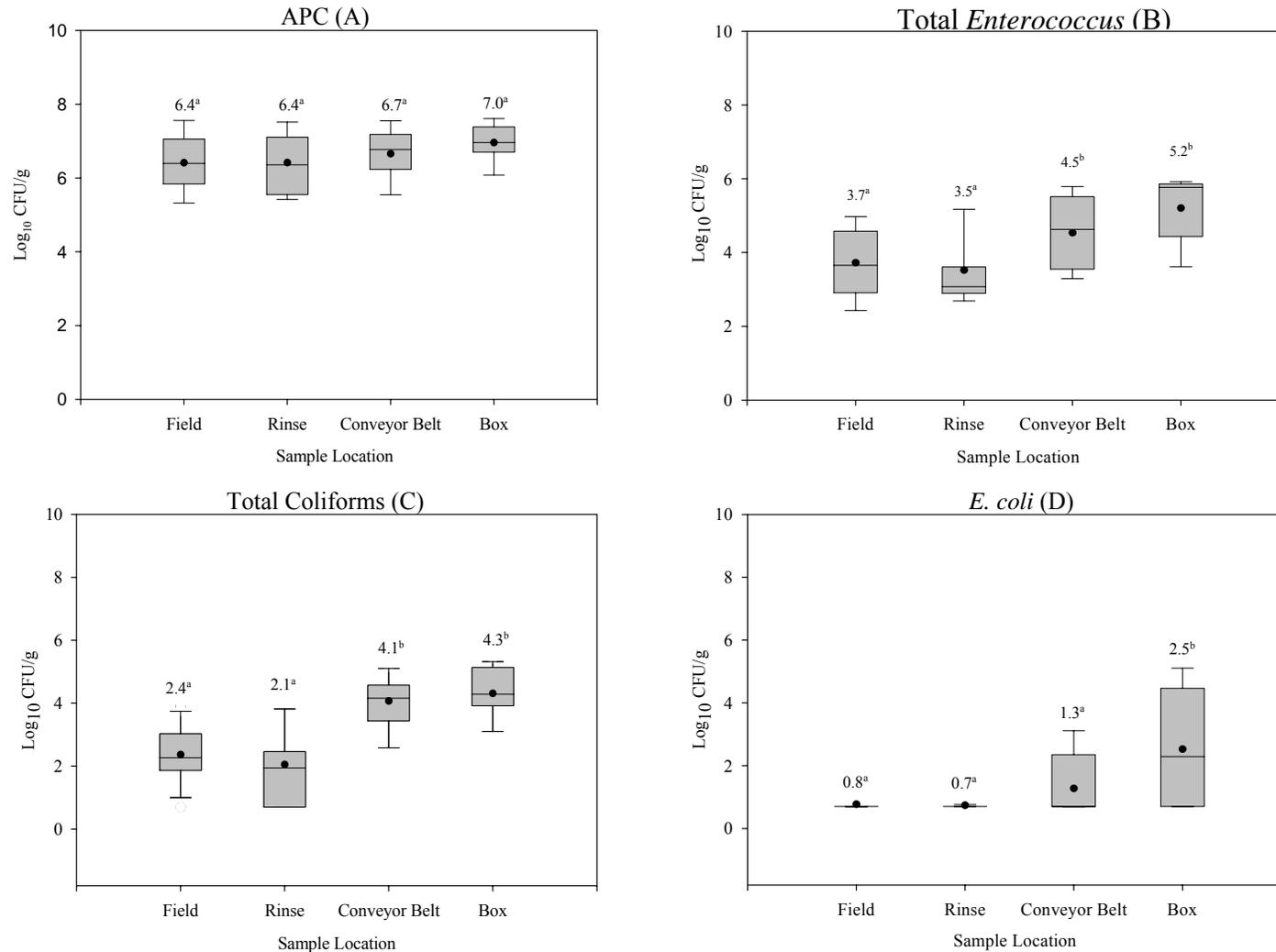


Figure 2.4. Aerobic plate count (A), total *Enterococcus* (B), total coliforms (C), and *E. coli* (D) levels from cantaloupe collected from the field and various steps throughout the packing shed. The box plot indicates the 10th, 50th, and 90th percentiles. The number above each box plot indicates the geometric mean, also indicated by the black circle. In instances when superscript letters differ between sampling locations, this indicates significant differences among the log₁₀ means ($p < .05$).

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CHAPTER 3

ANTIMICROBIAL RESISTANCE OF *ENTEROCOCCUS* SPECIES ISOLATED FROM PRODUCE

3.1 ABSTRACT

The purpose of this study was to characterize the antibiotic resistance profiles of *Enterococcus* species isolated from fresh produce harvested in the southwestern United States. Among the 185 *Enterococcus* isolates obtained, a total of 97 (52%) were *E. faecium*; 38 (21%) were *E. faecalis*; and 50 (27%) were other *Enterococcus* species. Of human clinical importance, *E. faecium* strains had a much higher prevalence of resistance to ciprofloxacin, tetracycline, and nitrofurantoin than *E. faecalis*. *E. faecalis* strains had a low prevalence of resistance to antibiotics used to treat *E. faecalis* infections of both clinical and of agricultural relevance, excluding its intrinsic resistance patterns. Thirty-four percent of the isolates had multiple drug resistance patterns, excluding intrinsic resistance. Data on the prevalence and types of antibiotic resistance in *Enterococcus* species isolated from fresh produce may be used to describe baseline antibiotic susceptibility profiles associated with *Enterococcus* spp. isolated from the environment. The data collected may also help elucidate the role of foods in the transmission of antibiotic resistant strains to human populations.

3.2 INTRODUCTION

Enterococcus species are ubiquitous, commensal inhabitants of the gastrointestinal tract of humans and animals. They are frequently isolated from environmental sources such as soil, surface waters, and raw plant and animal products, where their intrinsic ruggedness

allows them to persist and spread in the environment. Once viewed as a genus of minimal clinical impact, enterococci, particularly *Enterococcus faecium* and *Enterococcus faecalis*, have surfaced as organisms of importance due to the emergence of multiple drug resistant strains that are currently responsible for approximately 12% of all nosocomial infections in the United States (Kühn et al., 2000; Linden and Miller, 1999). Furthermore, their ability to acquire antibiotic resistance through transfer of plasmids and transposons, chromosomal exchange, or mutation presents a significant challenge for therapeutic measures (Mundy et al., 2000).

Antibiotic resistant strains of *Enterococcus* have been isolated from raw foods (Giraffa, 2002) and some believe that water and food are possible vectors of strain transmission to human intestinal flora (Witte, 2000). Of recent concern includes the potential development of environmental reservoirs of antibiotic resistance in farmland. Specifically, the application of untreated irrigation water or manure slurry to croplands could result in the spread of resistance to indigenous soil bacteria through horizontal transfer, which could in turn transfer resistance back to animals or humans via crops (Nwosu, 2001).

While the prevalence and transmission of antibiotic resistance among bacteria associated with food animals has been well documented, research regarding resistance profiles of bacteria isolated from raw produce is lacking (Aarestrup, 2002; Hayes et al., 2003; Thal et al., 1995; van den Bogaard, 2000). A few studies examining the prevalence of resistance among Gram-negative microorganisms isolated from produce exist, although results are conflicting. Hamilton-Miller and Shah (2001) characterized the antibiotic susceptibility of enterobacterial flora of salad vegetables, finding a high degree of resistance to ampicillin and the first- and second-generation cephalosporins. Alternatively, a Finnish

study found that members of the *Enterobacteriaceae* family isolated from vegetables were highly susceptible to the antibiotics studied, and multi-drug resistant strains were generally not identified (Österblad et al., 1999). Prazak et al. (2002) studied the resistance patterns among *Listeria monocytogenes* isolates from cabbage farms, in which 98% of the isolates were resistant to at least two drugs and 85% were found to be resistant to penicillin. However, the prevalence and patterns of antibiotic resistance among *Enterococcus* strains isolated from fresh vegetables is not yet well understood.

This study was undertaken as a supplement to a larger project, the purpose of which was to determine the prevalence of select microorganisms in fresh produce harvested from the southwestern United States. In this study, we report on the isolation, identification, and antibiotic susceptibility profiles for members of the *Enterococcus* genus isolated directly from these fresh produce samples.

3.3 ISOLATION, IDENTIFICATION, AND ANTIBIOTIC RESISTANCE PROFILES OF *E. FAECALIS* AND *E. FAECIUM*

The sampling site, located in the southwestern United States, included 13 farms and 5 packing sheds. All samples were obtained between January and May 2002. A total of 304 produce samples were collected throughout production and processing and consisted of a variety of leafy greens, herbs, and cantaloupe. Composite samples of approximately 200 g were obtained using hands protected by sterile, disposable gloves and placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI). These were then immediately shipped on ice to our location at North Carolina State University by overnight courier. All microbial analyses were initiated within 24 hours of sample collection.

The cultural methods used were recommended by the U.S. Food and Drug Administration, Center for Veterinary Medicine (Hayes et al., 2003; Simjee et al., 2002; Wagner, Personal Communication, 2002). After 24 hours at 37°C, a representative colony for each different morphology, generally 2-3 per sample, showing esculin hydrolysis (darkened colony with black halo) was purified and screened for hemolysis by streaking on 5% sheep blood agar (Remel, Lenexa, KS). The colonies were then screened at the genus level by PCR-based assays and at the species level by the Vitek system (Vitek 32, GPI panel, bioMerieux, Hazelwood, MO). For PCR, DNA was extracted using the Ultra Clean™ microbial DNA isolation kit (Mo Bio Laboratories, Inc., Solana, CA) in accordance with manufacturer recommendations. Primers were directed to the *tuf* gene (forward primer TACTGACAACCATTTCATGATG, reverse primer AACTTCGTCACCAACGCGAAC), yielding a 112 base pair product (Ke et al., 1999). Two µl of DNA were added to a 98 µl mixture containing 1X PCR buffer, 200 µM each dNTP, 2.5 units of *AmpliTaq* polymerase, 3.0 mM MgCl₂, and 1 µM of each primer. The PCR mixtures were subjected to pre-denaturation at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. A 7-minute final elongation step at 72°C then concluded the PCR assay. Two positive controls, *E. faecalis* ATCC 29212 and *E. faecium* ATCC 19434 were used. Isolates producing an amplicon band of the appropriate size by agarose gel (3%) electrophoresis were considered presumptively positive for the *Enterococcus* genus and were sent on for Vitek speciation to the Clinical Microbiology Laboratory in the College of Veterinary Medicine at North Carolina State University.

Strains identified as *E. faecium* or *E. faecalis* were screened for antibiotic susceptibility using the microdilution broth method with Mueller-Hinton media (TREK

Diagnostics, Westlake, OH) as outlined by the National Committee on Clinical Laboratory Standards (NCCLS) (NCCLS, 2000). A customized Gram-positive panel of seventeen antibiotics with various concentration ranges (TREK Diagnostics), identical to that used in the National Antimicrobial Resistance Monitoring System (NARMS 2001) program, was used in this study. The antibiotics and their concentration ranges were as follows: bacitracin, 8 to 128 IU/ml; chloramphenicol, 2 to 32 µg/ml; erythromycin, 0.5 to 8 µg/ml; bambarmycin (Flavomycin), salinomycin, vancomycin, quinuprotistin/dalfopristin, and lincomycin, 1 to 32 µg/ml; penicillin, 0.5 to 16 µg/ml; tetracycline, 4 to 32 µg/ml; tylosin tartrate, 0.25µg/ml; ciprofloxacin, 0.12 to 4 µg/ml; linezolid, 0.5 to 8 µg/ml; nitrofurantoin, 2 to 128 µg/ml; kanamycin and gentamicin, 128 to 1028 µg/ml; streptomycin, 512 to 2048 µg/ml. Minimal inhibitory concentrations (MIC) were determined manually by assessing each antibiotic and strain combination for growth. Isolates were categorized as susceptible, intermediate, or resistant, based on the NCCLS interpretive standards, where applicable (NCCLS, 2001). The MIC values, based on NCCLS breakpoints, were as follows: chloramphenicol and vancomycin, ≥ 32 µg/ml; erythromycin and linezolid, ≥ 8 µg/ml; penicillin and tetracycline, ≥ 16 µg/ml; Q/D and ciprofloxacin, ≥ 4 µg/ml; nitrofurantoin, ≥ 128 µg/ml; gentamicin, >500 µg/ml; streptomycin, $>1,000$ µg/ml (NCCLS, 2001). Differentiation among susceptibility and resistance are based on pharmacological, clinical, and microbiological criteria. Unfortunately, both pharmacological and clinical data are lacking for most antibiotics used as growth promoters (Butaye et al., 2003). Therefore, in instances where NCCLS standards were not available, a quantitative evaluation of resistance was done through calculation of MIC₅₀ and MIC₉₀ (NCCLS 2000, 2001). Control strains included *E. faecalis* ATCC 29212 (vancomycin-susceptible) and ATCC 51299 (vancomycin-

resistant) (Hayes et al., 2003; NCCLS, 2000). Profiles from different isolates collected from the same sample that differed by less than two dilutions for at least one antimicrobial MIC were considered duplicates. Consequently, only a single isolate was included for subsequent analysis.

3.4 PREVALENCE AND ANTIBIOTIC RESISTANCE PATTERNS OF *E. FAECALIS* AND *E. FAECIUM* ISOLATES FROM PRODUCE

The distribution of produce samples and *Enterococcus* species is shown in Table 3.1. Mustard greens, parsley, and cantaloupe represented nearly 70% (208) of the total produce items collected. At least one *Enterococcus* strain was isolated from over half of these samples. Among the 185 *Enterococcus* isolates obtained from all of the samples, a total of 97 (52%) were *E. faecium*; 38 (21%) were *E. faecalis*; and 50 (27%) were other *Enterococcus* species. Ninety-one percent of the *E. faecium* isolates and 32% of the *E. faecalis* isolates were resistant to at least one of the antibiotics tested, excluding intrinsic resistance. A summary of resistance profiles is provided in Table 3.2.

3.5 INHERENT RESISTANCE

The treatment of *Enterococcus* infections is limited by the intrinsic resistance among enterococci. In general, enterococci show intrinsic resistance to cephalosporins, lincosamides, and many synthetic β -lactams, such as the penicillinase-resistant penicillins (Giraffa, 2002; Preston and Drusano, 1999). *Enterococcus* species are also resistant to low levels of aminoglycosides, due to the decreased uptake of this antibiotic class (Giraffa, 2002). In this study, a majority of the *E. faecium* and *E. faecalis* isolates showed inherent resistance patterns which were consistent with previous studies in farm animals and pets (Butaye, 2001; Chow and Shlaes, 1999). For instance, both *E. faecium* and *faecalis* had intrinsic resistance

to bacitracin, i.e. 90% of the isolates were inhibited at concentrations greater than 128 IU/ml. *E. faecium* also had a MIC₉₀ of greater than 32 µg/ml for flavomycin. Consistent with reported data (Jones et al., 1998; Schouten et al., 1999), a majority (97%) of *E. faecalis* isolates were resistant to quinupristin/dalfopristin when speciation was based on PCR alone, less so (87%) if speciation was based on Vitek. Results also showed that isolates were resistant to low levels of lincomycin.

3.6 RESISTANCE RELEVANT TO ANIMAL AGRICULTURE

It has been suggested that the overuse of antibiotics in livestock production may provide an environmental reservoir of antibiotic resistance (Sengeløv et al., 2002). Among the panel of seventeen antibiotics screened in this study, seven are used in animal feed for growth promotion. These include bacitracin, flavomycin, penicillin, salinomycin, tetracycline, lincomycin, and tylosin (Miller Publishing Co., 2000). Both *E. faecium* and *E. faecalis* demonstrated a high degree of susceptibility to salinomycin, lincomycin, and tylosin. As mentioned above, *E. faecium* is intrinsically resistant to flavomycin; however, *E. faecalis* isolates were susceptible to flavomycin (MIC₉₀ = 4 µg/ml). Less than 10% of the *E. faecium* isolates were resistant to penicillin, and all *E. faecalis* isolates were susceptible to penicillin. Twenty-nine percent of the *E. faecium* and no *E. faecalis* isolates were resistant to tetracycline. Erythromycin is also used in livestock production, specifically for therapeutic purposes in chickens and turkeys (Miller Publishing Co., 2000). In this case, 10% of the *E. faecium* isolates were resistant to erythromycin, while only 3% of the *E. faecalis* isolates were resistant. We can generally conclude that there was not a high degree of resistance to the antibiotics commonly used in animal agriculture among the *Enterococcus* isolates

collected in this study, especially for *E. faecalis*. However, *E. faecium* demonstrated a higher degree of resistance to tetracycline (29%).

3.7 RESISTANCE RELEVANT TO HUMAN MEDICINE

All of the antibiotics used in the NARMS 2001 panel are of importance to human therapeutic use except for tylosin tartrate, salinomycin, and flavomycin (Yu et al., 1999). Penicillin, vancomycin, aminoglycosides, chloramphenicol, ciprofloxacin, and quinupristin/dalfopristin all have been used in the treatment of enterococcal infections either in combination therapy, for optimal killing, or else monotherapeutically (Chow and Shlaes, 1999). Synergistic treatment includes the use of an aminoglycoside, with the addition of a cell wall-active agent, such as vancomycin or penicillin (Chow and Shlaes, 1999). From the present study, an extremely low level of resistance was found to the aminoglycosides (*E. faecium* 3%, *E. faecalis* 0%), vancomycin (*E. faecium* 0%, *E. faecalis* 0%), and penicillin (*E. faecium* 7%, *E. faecalis* 0%). Chloramphenicol, also used synergistically in documented cases, was shown to inhibit a majority of all isolates; 5% of *E. faecium* strains and only 3% of *E. faecalis* strains were resistant to chloramphenicol (Chow and Shlaes, 1999).

According to Chow and Shlaes (1999), enterococcal infections of less severity have been treated with a single antibiotic. Among the NARMS 2001 panel, an example of such an antibiotic is ciprofloxacin, for which 27 (28%) of the *E. faecium* strains and 2 (5%) of the *E. faecalis* strains were found to be resistant. Quinupristin/dalfopristin can also be used for the treatment of *E. faecium* infections in humans, and in our study, 16 (16%) isolates were found to be resistant and 48 (49%) were intermediately resistant to these drugs. There is evidence that during the therapeutic use of quinupristin/dalfopristin for *E. faecium* bacteremia, superinfection of *E. faecalis* can occur, posing concerns regarding such a high proportion of

E. faecium resistance to these drugs (Chow and Shlaes, 1999). Finally, and consistent with the literature (Stein and Havlichek, 1999), we found that all *E. faecalis* strains were susceptible to nitrofurantoin, a drug frequently used for the treatment of *E. faecalis* urinary tract infections. When the data are taken together, there was a relatively low prevalence of resistance to most of the drugs used in clinical treatment of enterococcal infections in humans, especially for *E. faecalis*.

3.8 MULTIPLE DRUG RESISTANCE

Fifty-nine (61%) of the *E. faecium* isolates and 4 (11%) of the *E. faecalis* isolates showed multi-drug resistance, i.e., resistance to two or more drugs, although no specific patterns of multi-drug resistance were readily apparent. In general, the *E. faecium* isolates had a greater degree of multi-drug resistance than did the *E. faecalis* isolates. Twenty-five percent of the *E. faecium* strains had simultaneous resistance to three or more drugs. As previously mentioned, significant resistance was found among the *E. faecium* isolates to ciprofloxacin, tetracycline, and nitrofurantoin. Interestingly, at least one of these clinically important antibiotics was represented in over 75% of the multi-drug resistant *E. faecium* strains, suggesting the possibility of gene linkage, although this was not confirmed in our study. For all *E. faecium* and *E. faecalis* isolates, 24 (18%) multi-drug resistant strains were resistant to ciprofloxacin, 22 (16%) strains were resistant to tetracycline, and 21 (16%) strains were resistant to nitrofurantoin. Six (6%) *E. faecium* strains were found to be simultaneously resistant to all three antibiotics.

3.9 CONCLUSIONS

Multiple drug resistant strains of *E. faecalis* and *E. faecium* have been increasingly associated with nosocomial infections. Of particular interest has been the potential for foods as a vehicle for transmission of these strains to humans, or alternatively, as a reservoir for horizontal transfer between strains. This might be considered credible since once ingested, enterococci can survive gastric passage, multiply, and colonize the gastrointestinal tract for a significant amount of time (Sørensen et al., 2001). Indeed, there is strong epidemiological evidence to link the use of antibiotics in human medicine and animal agriculture with the presence of resistant strains in animal products. In most cases where high rates of resistance have been shown to occur in food and humans, there is also a link to drug use in animals, conferring cross-resistance from among avoparcin and vancomycin (van den Bogaard and Stobberingh, 2000; Witte, 2000). In general, the prevalence of antibiotic resistant enterococci in farm animals and their meat is high (>60%) (Giraffa, 2002). Moreover, Hayes et al. (Hayes et al., 2003), applying the same panel of antibiotics used in our study, reported that resistant *Enterococcus* spp. commonly contaminate retail meat and their resistance patterns reflect the use of antimicrobial agents in the production of such products. Comparatively, the patterns of resistance to antibiotics are similar between Hayes et al. (Hayes et al., 2003) and this study, however, the prevalence (or degree) of antibiotic resistance in produce is lower than that found in retail meats.

In our study, resistance patterns differed among species of the *Enterococcus* genus. Overall, *E. faecium* was found to have a higher prevalence of resistance among the panel of antibiotics, particularly tetracycline, ciprofloxacin, and nitrofurantoin, while *E. faecalis* isolates had a relatively lower prevalence of resistance to antibiotics of both clinical and agricultural relevance, excluding its inherent resistance to quinupristin/dalfopristin. A high

percentage of the *E. faecium* isolates were found to be resistant to multiple drugs, an issue that contributes to the challenge of therapeutic measures. While *Enterococcus* resistance to glycopeptides is among current clinical concerns, the absence of vancomycin-resistant enterococci in the present study suggests that raw produce does not contribute to the dissemination of vancomycin resistance.

The present study provides evidence that can be used in subsequent risk assessment exercises to elucidate the role of raw produce in the dissemination of antibiotic resistance to human populations. The findings indicate that while fresh produce items do harbor strains of enterococci that are resistant to many commonly used antibiotics, the resistance patterns are not significantly different than those reported for *Enterococcus* strains isolated from animal products such as poultry and pork. However, animal products are usually cooked prior to consumption, which should theoretically inactivate most of the native microflora, including enterococci, in those products prior to consumption. Fresh produce, in many instances, is consumed without a terminal heating step. Clearly, the role of food in the transmission of these strains is a question for which there is no clear answer. However, data such as those presented here offer evidence that should be helpful in the identification of future studies and initiatives aimed at reducing the public health burden of antibiotic resistant pathogens.

Table 3.1. Number of produce samples and the number of *Enterococcus faecium* and *Enterococcus faecalis* isolates from each commodity

Commodity (n=304)	n	n* (%)	n'	Number of Isolates		
				<i>E. faecium</i> (n=97)	<i>E. faecalis</i> (n=38)	Other <i>Enterococcus</i> spp.
Celery	20	1 (5)	1	1 (100)	0	0
Cilantro	25	4 (16)	5	2 (40)	0	3
Mustard Greens	70	39 (56)	56	29 (52)	7 (13)	20
Spinach	12	2 (17)	2	0	0	2
Collards	12	4 (33)	4	2 (50)	0	2
Parsley	48	24 (50)	31	19 (61)	2 (6)	10
Dill	12	9 (75)	11	9 (82)	0	2
Cabbage	15	12 (80)	18	11 (61)	4 (22)	3
Cantaloupe	90	48 (53)	57	24 (42)	25 (44)	8

n = number of samples

n* = number of samples with *Enterococcus* spp.

n' = total number of *Enterococcus* isolates

Table 3.2. Antibiotic resistance profiles among *E. faecium* and *E. faecalis* isolates from produce

Antibiotic	<i>E. faecium</i> (n=97)				<i>E. faecalis</i> (n=38)			
	Resistant (%)	Intermediate (%)	MIC ₅₀	MIC ₉₀	Resistant (%)	Intermediate (%)	MIC ₅₀	MIC ₉₀
Bacitracin	NA	NA	>128	>128	NA	NA	128	>128
Chloramphenicol	5 (5)	5 (5)	-	-	1 (3)	2 (5)	-	-
Erythromycin	10 (10)	73 (75)	-	-	1 (3)	26 (68)	-	-
Flavomycin	NA	NA	>32	>32	NA	NA	1	4
Penicillin	7 (7)	NA	-	-	0	NA	-	-
Salinomycin	NA	NA	1	2	NA	NA	1	2
Quinupristin/dalfopristin	16 (16)	48 (49)	-	-	37 (97)	1 (3)	-	-
Tetracycline	28 (29)	3 (3)	-	-	0 (0)	0	-	-
Vancomycin	0	5 (5)	-	-	0	0	-	-
Lincomycin	NA	NA	16	32	NA	NA	32	32
Tylosin Tartrate	NA	NA	2	8	NA	NA	1	4
Ciprofloxacin	27 (28)	22 (23)	-	-	2 (5)	8 (21)	-	-
Linezolid	0	9 (9)	-	-	0	2 (5)	-	-
Nitrofurantoin	23 (24)	55 (57)	-	-	0 (0)	2 (5)	-	-
Kanamycin	NA	NA	256	1024	NA	NA	128	128
Gentamicin	0	NA	-	-	0	NA	-	-
Streptomycin	3 (3)	NA	-	-	0	NA	-	-

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CHAPTER 4

A FIELD STUDY OF THE MICROBIOLOGICAL QUALITY AND SAFETY OF FRESH PRODUCE OF DOMESTIC AND IMPORTED ORIGIN

4.1 ABSTRACT

Produce is responsible for an increasingly larger proportion of all foodborne disease outbreaks. In particular, the globalization of the food supply may introduce new food safety risks and allow widespread distribution of contaminated food, particularly produce. The objectives of this study were to: (i) compare the overall quality of domestic and imported produce throughout the packing process; (ii) examine changes in microbiological quality of both domestic and imported produce at each stage of production and processing; and (iii) evaluate the prevalence of selected pathogens on fresh produce. Furthermore, we sought to characterize the antibiotic resistance profiles of *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from fresh produce. A total of 466 produce and swab samples were collected from various locations in packing sheds in the southern US from November 2002 through December 2003. These samples were assayed by enumerative tests for total aerobic bacteria (APC), total coliforms, total *Enterococcus*, and *E. coli*. Produce samples were also analyzed for the presence of *Salmonella*, *Listeria monocytogenes*, *Shigella*, and *E. coli* O157:H7. All microbiological methods used were those recommended by the U.S. Food and Drug Administration. A total of 112 *E. faecium* and *E. faecalis* isolates were further screened for antibiotic resistance profiles using a panel of seventeen antibiotics. Overall, the microbiological quality of fresh produce ranged from 4.0 to 7.9 log₁₀ CFU/g (APC); less than 10 CFU/g to 4.5 log₁₀ CFU/g (coliforms); less than 10 CFU/g to 4.0 log₁₀ CFU/g (*E.*

coli); and less than 10 CFU/g to 5.4 log₁₀ CFU/g (*Enterococcus*). Despite increased attention to the role of imported produce in foodborne disease, this study does not support the assumption that domestic produce is of higher microbial quality than imported produce. No *Salmonella*, *Shigella*, or *E. coli* O157:H7 were detected from the 466 produce samples tested. However, three domestic cabbage samples were found to be positive for *L. monocytogenes*. Of the *Enterococcus* isolates, *E. faecium* had a higher degree of resistance to antibiotics in general, while *Enterococcus* spp. isolated from imported produce had a higher degree of antibiotic resistance when compared to strains isolated from produce samples of domestic origin.

4.2 INTRODUCTION

Fresh fruits and vegetables continue to be a public health concern from the standpoint of food safety. In the last several decades, the proportion of foodborne outbreaks associated with produce rose from 0.7% of outbreaks in the 1970s to 6% of outbreaks in the 1990s. Likewise, produce-associated outbreaks were responsible for a higher proportion of all foodborne disease cases, from 1% (708 of 68,712) of cases in the 1970s to up to 12% (8,808 of 74,592) of cases in the 1990s (Sivapalasingam et al., 2004)

As the overall consumption of fresh produce has increased within the past decade, the amount of produce imported into the U.S. market has also increased. Between 1980 and 2001, fresh vegetable imports increased by over 250%, while fresh fruit imports increased by 155% (Clemens, 2004). Furthermore, imports account for 25% of the total volume of produce currently sold by U.S. supermarkets, and this number is projected to increase to over 30% in five years (Ceullar, 2003). The globalization of the food supply may introduce new food safety risks and the potential widespread dissemination of contaminated food. For

example, there were multi-state outbreaks of *Salmonella* serotype Poona associated with the consumption of Mexican cantaloupe during the spring of consecutive years from 2000 to 2002 (CDC, 2002). In March 1997, an outbreak of hepatitis A due to the consumption of contaminated Mexican strawberries affected over 200 schoolchildren and teachers (CDC, 1997). Additionally, in 1996, nearly 1500 cases of cyclosporiasis were reported to the Centers for Disease Control and Prevention (CDC) and Health Canada, apparently due to ingestion of imported raspberries (CDC, 1996).

The U.S. Food and Drug Administration is responsible for the safety of imported produce; however, it does not have legal authority to ensure that international exporters have food production and inspection systems equivalent to those recommended by the United States. In an effort to improve the safety of domestic and imported produce, the FDA and the USDA published voluntary guidelines in 1998, entitled “*Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables*” (FDA, 1998). The Guide’s primary purpose was to provide a framework for the identification and implementation of practices likely to decrease the risk of pathogenic microbiological contamination in produce through the steps of production, packaging, and transport, based on both Good Agricultural Practices (GAPs) and Good Manufacturing Practices (GMPs).

Although the Guide provides general knowledge about potential pathways by which produce can become contaminated with pathogens, systematic studies to identify critical points where contamination occurs are lacking. In response to the increasing recognition of produce as a food safety concern, a plethora of recent research has focused on developing effective decontamination methods. However, to fully benefit from the ongoing mitigation research, fruit and vegetable producers, processors, retailers, and consumers must first

recognize the sources of microbial contamination and how to prevent introduction of microbial contamination at each stage in the food production and transport chain.

Besides the growing concern about the safety and quality of both domestic and imported produce, *Enterococcus* species have surfaced as opportunistic pathogens of importance due to the emergence of multiple drug resistant strains that are currently responsible for approximately 12% of all nosocomial infections in the U.S. (Kühn et al., 2000; Linden, 1999). *Enterococcus* species are ubiquitous, commensal inhabitants of the gastrointestinal tract of humans and animals. They are frequently isolated from environmental sources such as soil, surface waters, and raw plant and animal products, where their intrinsic ruggedness allows them to persist and spread in the environment. Of particular concern may be the possibility of environmental reservoirs of antibiotic resistance. In the case of produce, the application of untreated irrigation water or manure slurry to croplands could result in the transfer of antibiotic resistance genes to indigenous soil bacteria and pathogenic bacteria, which could in turn transfer resistance back to animals or humans via crops (Nwosu, 2001; Sengeløv, 2002). While the prevalence and transmission of antibiotic resistance among bacteria associated with food animals has been well documented, data on antibiotic resistance profiles of bacteria isolated from raw produce is lacking (Aarestrup, 2002; Hayes, 2003; Thal et al., 1995; van den Bogaard, 2000).

In an effort to examine microbial contamination in specific areas within the packing shed used to prepare domestic and imported produce for distribution, the objectives of this study were to: (i) compare the overall quality of domestic and imported produce throughout the packing process; (ii) examine changes in microbiological quality of both domestic and imported produce at each stage of production and processing; and (iii) evaluate the

prevalence of select pathogens on fresh produce. Furthermore, we also characterized the antibiotic resistance profiles of naturally occurring *E. faecium* and *E. faecalis* strains isolated from fresh produce items for seventeen clinical antibiotics routinely used in human medicine and animal production.

4.3 MATERIALS AND METHODS

4.3.1 Produce sample collection

The sampling site, located in the southern United States, included 8 packing sheds and 11 types of produce. All samples were obtained between November 2002 and December 2003. Target commodities included produce items that are mostly consumed raw with a few exceptions (Table 4.1). Samples were taken in a sequential manner, following the same crop through processing and packaging. Samples designated as “bin” were taken prior to processing, while samples labeled as “wash tank” and “rinse” were taken from the packing sheds immediately following the wash and rinse step, respectively. Samples labeled “box” were collected from boxes just prior to distribution. Cantaloupe samples were also taken from the conveyor belt between the rinse step and final distribution box and were labeled as “conveyor belt”. In most cases, the imported produce we were able to sample in our study was not washed in the US processing shed prior to packing.

Two sets of composite samples (400-600 g each) of every produce commodity were obtained from each location using hands protected by sterile, disposable gloves. Samples were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) and were immediately shipped on ice to our laboratory at North Carolina State University by overnight courier. Microbial analyses were initiated within 24 hours of sample collection.

4.3.2 Environmental swab collection

Environmental surface swabs were taken throughout the packing shed at the same locations where produce samples were collected. At each location, a 10x10 cm² area was swabbed using RediSwab™ with 10 ml of letheen broth (International BioProducts, Bothell, WA). The swab tubes were immediately placed on ice and shipped overnight to North Carolina State University. Microbial analysis of swabs was initiated within 24 hours of sample collection.

4.3.3 General microbiological quality

Unless otherwise stated, all media was obtained from Becton Dickinson Laboratories (Sparks, MD). Twenty-five g subsamples of produce samples were weighed and diluted 1:10 in 0.1% peptone buffer. Cantaloupe samples were prepared by trimming rind (less than 0.5 cm deep) from melons with a sanitized paring knife and removing all visible mesocarp material. After homogenizing for 2 min at 230 rpm in a Stomacher® 400 (Seward, Norfolk, UK), samples were subsequently processed for enumeration of total aerobic bacteria (APC), total coliforms, total *Enterococcus*, and *E. coli*. Assays for total aerobic bacteria, coliforms, and *E. coli* were done using Aerobic Count Plate Petrifilm™ and Coliform/*E. coli* Petrifilm™ (3M, Saint Paul, MN, USA), respectively (Feng, 2000). Total enterococci were enumerated using KF Streptococcal agar (Hartman et al., 1992).

4.3.4 Pathogen detection

Four subsamples of 25 g each, originating from the composite produce sample intended for pathogen detection, were weighed and prepared for *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7 assays using FDA Bacteriological Analytical Manual

(BAM) standard methods (Andrews, 2003; Feng, 2002; Hitchins, 2003). For *Salmonella* detection, samples were homogenized in 225 ml of lactose broth, followed by incubation at 37°C for 24 h. One ml of the lactose pre-enrichment broth was then transferred to tetrathionate and selenite cysteine broths and incubated at 37°C. After 18-24 h, samples were streaked to xylose lysine desoxycholate, bismuth sulfite, and hektoen enteric agar. When present, two or more typical colonies were transferred to lysine iron agar and triple sugar iron agar slants, followed by *Enterobacteriaceae* MICRO-ID[®] (Remel, Lenexa, KS) for the generic identification of *Salmonella*. Presumptive *Salmonella* isolates were sent to the College of Veterinary Medicine at North Carolina State University for Vitek (bioMerieux, Hazelwood, MO) identification.

For *L. monocytogenes* detection, 25 g produce samples were incubated in *Listeria* Enrichment Broth at 30°C for 24 to 48 h. *Listeria* spp. were then isolated using Oxford agar and Lithium Chloride-Phenylethanol-Moxalactam (LPM) agar, supplemented with esculin and ferric ammonium citrate (Sigma Chemical Company, St. Louis, MO). Typical colonies were analyzed for beta-hemolysis on 5% sheep blood agar (Remel, Lenexa, KS), and hemolytic colonies were streaked on blood agar for the CAMP test, followed by *Listeria* MICRO-ID (Remel, Lenexa, KS) for speciation.

For *E. coli* O157:H7 detection, 25g produce samples were first enriched in 225 ml of EHEC enrichment broth at 37°C for 24 h followed by plating on sorbitol-MacConkey agar, supplemented with potassium tellurite and cefixime (Dynal[®], Lake Success, NY). At least two presumptive colonies were screened for the presence of the O:157 antigen using the commercial Prolex *E. coli* O:157 latex test reagent kit (Pro-Lab Diagnostics, Round Rock, TX).

Shigella detection also followed FDA Bacteriological Analytical Manual (BAM) methods. Twenty-five gram produce sample were first enriched in 225 ml of *Shigella* broth, supplemented with 0.5 µg/ml of novobiocin, under anaerobic conditions at 42°C for 20 h. Samples were then plated on MacConkey agar for 20 h at 37°C, followed by inoculation onto Triple Sugar Iron agar slant and *Enterobacteriaceae* MICRO-ID for analysis of biochemical reactions (Remel, Lenexa, KS).

4.3.5 Statistics

Statistical analyses, including geometric means, standard deviations, ranges, and medians were done using Sigma Plot version 8.0 (SPSS, Chicago, IL). One-way ANOVA tests were performed, using Tukey comparisons to derive statistical differences ($P < .05$) of microbial levels between all sampling locations. In an effort to avoid under- and overrepresentation of sample counts, when enumerative results fell below the assay limit of detection, they were assigned a value halfway between zero and the detection limit (Shumway et al., 1989; Garg et al., 1990).

4.3.6 Isolation, identification, and antibiotic resistance profiles of *E. faecalis* and *E. faecium*

Enterococcus isolation, speciation, and antibiotic resistance were done only on 141 samples obtained between September 2003 and December 2003. These produce samples were collected throughout production and processing and consisted of a variety of leafy greens, herbs, and cantaloupe.

The *Enterococcus* culture methods used were recommended by the U.S. Food and Drug Administration, Center for Veterinary Medicine (Johnston and Jaykus, 2004). For *Enterococcus* isolation, 25-g subsamples of each produce item were enriched in 225 ml of

Enterococcosel broth (Becton Dickinson and Co., Sparks, MD) for 48 hours at 45°C and subsequently streaked for preliminary identification on Enterococcosel agar (Becton Dickinson). After 24 hours at 37°C, a representative colony for each morphology showing esculin hydrolysis (darkened colony with black halo) was purified and screened for hemolysis by streaking on 5% sheep blood agar (Remel, Lenexa, KS). The colonies were then screened at the genus level by PCR-based assays and at the species level by the Vitek system (Vitek 32, GPI panel, bioMerieux, Hazelwood, MO). For PCR, DNA was extracted using the Ultra Clean™ microbial DNA isolation kit (Mo Bio Laboratories, Inc., Solana, CA) in accordance with manufacturer recommendations. Primers were directed to the *tuf* gene (forward primer 5¹ – TACTGACAACCATTCATGATG - 3¹, reverse primer 5¹ – AACTTCGTCACCAACGCGAAC - 3¹), yielding a 112 base pair product (Ke et al., 1999). Two µl of DNA were added to a 98 µl mixture containing 1X PCR buffer, 200 µM each dNTP, 2.5 units of *AmpliTaq* polymerase, 3.0 mM MgCl₂, and 1 µM of each primer. The PCR mixtures were subjected to pre-denaturation at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. A 7-minute final elongation step at 72°C then concluded the PCR assay. Isolates producing an amplicon band of the appropriate size by agarose gel (3%) electrophoresis were considered presumptively positive for the *Enterococcus* genus and were sent on for Vitek speciation to the Clinical Microbiology Laboratory in the College of Veterinary Medicine at North Carolina State University.

Strains identified as *E. faecium* or *E. faecalis* were screened for antibiotic susceptibility using the microdilution broth method with Mueller-Hinton media (TREK Diagnostics, Westlake, OH) as outlined by the National Committee on Clinical Laboratory

Standards (NCCLS) (NCCLS, 2000). A customized Gram-positive panel of seventeen antibiotics with various concentration ranges (TREK Diagnostics), identical to that used in the National Antimicrobial Resistance Monitoring System (NARMS, 2001) program, was used in this study. The antibiotics and their concentration ranges were as follows: bacitracin, 8 to 128 IU/ml; chloramphenicol, 2 to 32 µg/ml; erythromycin, 0.5 to 8 µg/ml; bambarmycin (Flavomycin), salinomycin, vancomycin, quinuprotistin/dalfopristin (Q/D), and lincomycin, 1 to 32 µg/ml; penicillin, 0.5 to 16 µg/ml; tetracycline, 4 to 32 µg/ml; tylosin tartrate, 0.25µg/ml; ciprofloxacin, 0.12 to 4 µg/ml; linezolid, 0.5 to 8 µg/ml; nitrofurantoin, 2 to 128 µg/ml; kanamycin and gentamicin, 128 to 1028 µg/ml; streptomycin, 512 to 2048 µg/ml. Minimal inhibitory concentrations (MIC) were determined manually for each antibiotic and strain combination. Isolates were categorized as susceptible, intermediate, or resistant based on the NCCLS interpretive standards, where applicable (NCCLS, 2001). Differentiation between susceptibility and resistance are based on pharmacological, clinical, and microbiological criteria. Unfortunately, both pharmacological and clinical data are lacking for most antibiotics used as growth promoters . Therefore, in instances where NCCLS standards were not available, a quantitative evaluation of resistance was done through calculation of MIC₅₀ and MIC₉₀ (NCCLS, 2000 and 2001). Control strains included *E. faecalis* ATCC 29212 (vancomycin-susceptible) and ATCC 51299 (vancomycin-resistant) (NCCLS, 2000). Profiles from different isolates collected from the same sample that differed by less than two dilutions for at least one antimicrobial MIC were considered duplicates. Consequently, only a single isolate was included for subsequent analysis.

4.4 RESULTS

4.4.1 Sample Collection

A total of 466 produce samples were collected from nine domestic packing sheds from November 2002 through December 2003. Among the 466 samples, 310 were produced at domestic locations and 129 were imported from Mexico. Ten types of produce items were collected, including green swiss chard, turnip greens, collards, cabbage, kale, cilantro, parsley, dill, cantaloupe, and broccoli. To facilitate data analysis, the produce commodities were classified into four categories, i.e., leafy greens, herbs, melons, and vegetables. Table 4.1 specifies the number of samples of each product type, stratified by sample origin (domestic vs. imported), and indicates the sampling locations within the packing shed. A total of 466 environmental swabs were taken throughout the packing process at the same time and location as the produce samples.

4.4.2 Domestic Produce Results

To examine changes in microbiological quality during the packing process, we calculated the mean bacterial indicator concentrations for each of the produce categories (leafy greens, herbs and cantaloupe) at key stages in the packing process (Figures 4.1-4.3). Only domestic produce was included in these analyses because the small number of imported samples. For leafy greens (Figure 4.1), which included Swiss chard, turnip greens, and kale, the concentrations of microbiological indicators were generally quite low, and there was no statistically significant change in the levels of any microbial group during the packing process. For domestic herbs, which included parsley and cilantro, a similar trend was observed (Figure 4.2). The microbiological quality of cantaloupe, however, did change over the course of processing (Figure 4.3) as we have reported previously (Johnston et al., 2005). Average total aerobic bacteria (APC) levels remained relatively unchanged, despite a

washing step, however there was a significant increase ($p < 0.05$) in APC concentrations when comparing samples collected from the conveyor belt before the wash step to samples collected from the final box for distribution. Average coliform levels ranged from $1.79 \log_{10}$ CFU/g to $3.91 \log_{10}$ CFU/g through packing, with a statistically significant increase (nearly $2 \log_{10}$ CFU/g, ($p < 0.05$)) between the field samples entering the shed and the subsequent processing steps. On average, *E. coli* levels increased over two \log_{10} CFU/g throughout the packing process for cantaloupe. Like *E. coli*, a statistically significant increase ($p < 0.05$) in *Enterococcus* levels on cantaloupe was observed between the samples taken from the conveyor belt after wash and those taken from the box just prior to distribution.

4.4.3 Environmental Swab Results

Because we had observed degradation in microbiological quality of some produce groups during shed processing, we wanted to examine whether surfaces in the packing shed, such as conveyor belts and sorting tables, could be a source of contamination that could be transferred to the produce during contact. Swabs of environmental surfaces in the packing sheds were collected at the same time as the produce samples and were examined for each produce category (i.e., leafy greens, herbs, and cantaloupe) Figures 4.4-4.6 summarize the results from environmental swabs collected during processing of domestic produce where we had more data because domestically produced samples received considerably more handling in the packing shed phase than imported produce. In most cases, the levels of microbial indicators were quite low, with no statistically significant differences between locations within the shed. There was a significant change in APC counts on swabs collected during the processing of domestic leafy greens (Fig 4.4a) and cantaloupe (Fig 4.6a). Environmental surfaces used during cantaloupe processing had higher microbial loads, particularly

coliforms, *E. coli*, and enterococci, than surfaces used to process other commodities. Interestingly, this trend is similar to that found for the produce samples.

4.4.4 Comparison of Domestic and Imported Produce Quality

When comparing the overall microbiological quality of domestic versus imported produce in general, in almost all instances, samples of domestic produce had either higher or equivalent microbial loads compared to samples of their counterpart imported produce items. We compared APC, coliforms, *E. coli*, and *Enterococcus* levels between domestic and imported herbs and cabbage because we had a sufficient number of samples of these produce commodities from both sources (Tables 4.2 and 4.3). Although both domestic and imported cantaloupe were sampled, the numbers of imported cantaloupe samples were too low to make reliable statistical comparisons due to an embargo on Mexican cantaloupes at the time. Mean APC, *E. coli*, and *Enterococcus* levels on samples of imported herbs entering the shed were significantly lower ($p < 0.05$) than the levels of the same bacteria on samples of domestic herbs entering the shed (Table 4.2). Samples of imported herbs from the final box for distribution had significantly lower levels of coliforms, *E. coli*, and *Enterococcus* than samples of domestic herbs from the final box for distribution. Furthermore, as noted for the domestic samples, the microbial quality of the imported herbs either stayed the same or degraded during processing. *E. coli* levels on the imported herbs increased significantly ($p < 0.05$) between samples entering the shed and samples of herbs from the final box for distribution.

A similar comparison was made between domestic and imported cabbage samples (Table 4.3). In this case, mean levels of total aerobic bacteria, total coliforms, and *Enterococcus* did not differ markedly between samples of imported cabbage and samples of

domestic cabbage. However, levels of *E. coli* were significantly higher ($p < 0.05$) on samples of domestic cabbage collected from the conveyor belt, with a mean value of $1.31 \log_{10}$ CFU/g compared with $0.70 \log_{10}$ CFU/g on samples of imported cabbage collected from the conveyor belt. Again, we observed that *E. coli* levels significantly increased ($p < 0.05$) as the imported cabbage moved from the conveyor belt to the final box for distribution. In contrast, samples of the domestic cabbage had significantly lower ($p < 0.05$) total coliforms, *E. coli* and enterococci levels as the cabbage moved from the point of shed entry to the final box for distribution (Table 4.3).

Although the sample size was small ($N = 36$ domestic, 6 imported), the level of total coliforms and *E. coli* differed significantly ($p < 0.05$) between imported and domestic cantaloupe samples. Of note, *E. coli* levels were higher for domestically produced cantaloupe by about two \log_{10} CFU/g (data not shown).

4.4.5 Pathogen Detection in Fresh Produce

We also evaluated the prevalence of select pathogens which have been associated with fresh produce. All 466 produce samples were analyzed for the presence of *L. monocytogenes*, *E. coli* O157:H7, *Salmonella*, and *Shigella*. *E. coli* O157:H7, *Salmonella*, and *Shigella* were not detected in any of the produce items tested (0/466). Three of 43 domestic cabbage samples tested positive for *L. monocytogenes*, resulting in a prevalence of 1% for all produce items combined and 7% for cabbage alone.

4.4.6 Antibiotic Resistance of Enterococci Isolated from Produce

The final part of the study sought to characterize the antibiotic resistance profiles of *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from fresh produce. The

Enterococcus antibiotic susceptibility work was done on a subsample of the produce samples collected in this study. In total, 141 domestic and imported produce samples from seven packing sheds were screened for *Enterococcus* species and subsequent antibiotic resistance typing of isolates (Table 4.4). A total of 128 *Enterococcus* species were isolated, of which 45 (35%) were identified as *E. faecalis* and 67 (52%) were identified as *E. faecium*. The isolates obtained from domestic origin were from cilantro (n = 42) and parsley (n = 9). *Enterococcus* isolates of imported origin were from broccoli (n = 15), cabbage (n = 45), and cilantro (n = 30). We then characterized the overall resistance profiles of both *E. faecium* and *E. faecalis* to 17 antibiotics (Table 4.5). In general, both *E. faecium* and *E. faecalis* demonstrated inherent resistance to several antibiotics, which is consistent with published literature (Johnston and Jaykus, 2004; Giraffa, 2002; Chow, et al. 1999). With the exception of this intrinsic resistance, the overall degree of antibiotic resistance among both species was quite low. *E. faecium* showed notable resistance to ciprofloxacin (28%), tetracycline (24%), and erythromycin (22%). Three percent of the *E. faecium* isolates were also found to be resistant to penicillin. *E. faecalis* demonstrated an extremely low level of resistance to all antibiotics tested. Among the isolates found to have to resistance to at least one antibiotic (not including those inherent resistances), a majority were found to be resistant to a single antibiotic. Among the *E. faecium* isolates, 16 of 65 (25%) were found to be resistant to more than one antibiotic. Two of 47 (4%) *E. faecalis* isolates were found to have multi-resistance to the antibiotics.

When the antibiotic resistance profiles of *E. faecium* and *E. faecalis* isolates were stratified by sample origin (domestic vs. imported), a few trends became apparent. While the resistance profiles for *E. faecium* from domestic and imported samples were very similar for

most antibiotics, interestingly, 19 (37%) of the isolates from imported samples were resistant to ciprofloxacin, while all *E. faecium* isolates of domestic origin were susceptible to this particular antibiotic. For *E. faecalis*, most isolates, whether of domestic or international origin, had similar resistance profiles, although we only obtained a small number of *E. faecalis* isolates (n=6) from imported produce samples (data not shown).

4.5 DISCUSSION

4.5.1 Microbial quality of produce

The purpose of this study was to compare the overall quality of domestic and imported produce sampled through the packing process and to evaluate the prevalence of selected pathogens on fresh produce. The levels of various microbiological indicators associated with shed surfaces and equipment, as evaluated by environmental sampling, was also assessed. Additionally, the degree of susceptibility to seventeen antibiotics used routinely in humans and animals was also evaluated for strains of *E. faecium* and *E. faecalis* isolated directly from a subset of the produce samples.

Microbial levels increased significantly during packing for cantaloupe. Most notably, average *E. coli* levels increased by two log₁₀ CFU/g from the beginning to the end of processing for this commodity. This is similar to previous results from a comparable study (Johnston et al., 2005). *E. coli* levels from domestic cantaloupe samples were also significantly higher than those from imported cantaloupe, which may suggest a higher degree of fecal contamination within domestic packing sheds. In many cases, there was no significant change in the microbiological quality of domestic leafy greens and herbs, during the packing process, and the levels of *E. coli*, which suggests fecal contamination, were

extremely low. Overall, the microbial quality of produce, both of international and domestic origin, was excellent. In general, our data is consistent with other studies that have examined microbial levels on fresh produce items. Several investigators have reported similar levels of total aerobic bacteria on leafy green vegetables collected from both production and retail establishments (Garg, 1990, King, 1991, Ruiz, 1987, Stewart, 1978). Ruiz et al. (1987) examined the levels of microbiological indicators on several types of leafy vegetables and herbs, including spinach, cabbage, lettuce and parsley. Total aerobic bacteria levels ranged from 10^5 to 10^7 CFU/g from field samples, while levels in retail samples ranged from 10^4 to 10^6 CFU/g.

Microbiological quality of fresh produce is a concern not only from a food safety perspective, but also because of resultant losses due to decreased product shelf-life. An estimated 30% of produce is lost due to microbial spoilage between the time of harvest and consumption (Beuchat, 1992). While multiple steps within production affect the microbial quality of incoming produce, the microbial loads remaining on produce after post-harvest operations must also be considered. The efficacy and practicality of procedures available for produce disinfection are extremely important. All of the packing sheds targeted in this study used a wash procedure, which in many cases included chlorine. The main purpose of the washing procedure was to remove soil and debris from the product, with the added benefit of removing and/or inactivating some microorganisms. Washing is almost always done by dipping or spraying. While chlorine is usually added at levels ranging from 5 to 250 mg/liter, the ability of chlorine to inactivate microorganisms is limited; the literature indicates that microbial reductions on the surface of produce items from chlorine washes range from 1 to 2 \log_{10} only (Beuchat, 1998).

We did not isolate *Salmonella*, *Shigella*, or *E. coli* O157:H7 from the fresh produce sampled in this study. This low pathogen prevalence is consistent with other published studies. For example, both the FDA Domestic and Imported Produce surveys found an extremely low prevalence of pathogen contamination. Of 1028 domestic samples, 99% were found to be free of *Shigella*, *Salmonella*, and *E. coli* O157 (FDA, 2003). Likewise, the study of imported produce found 96% of 1000 samples to be free of the same pathogens (FDA, 2001). However, in our study, three of 43 (7%) domestic cabbage samples were positive for *L. monocytogenes*. The prevalence of *L. monocytogenes* during production and post-harvest processing of cabbage was recently examined in farms and packing sheds in south Texas (Prazak et al., 2002). The investigators found *L. monocytogenes* in 3% (26 of 855) of cabbage, water, and environmental samples, and they concluded that contact with packing shed surfaces may be a source of contamination, highlighting the importance of equipment sanitation.

Based on the results of the environmental swab samples collected in this study, the overall sanitation of equipment was good, and most microbial loads were low. In most cases, there was no statistically significant difference in microbial loads when comparing various packing shed locations. Also, *E. coli* levels were extremely low, usually below detectable limits (<10 CFU/g). A notable exception was cantaloupe processing, for which a substantial proportion of swab samples collected from surfaces where cantaloupe were handled had quantifiable *E. coli* levels. Our study design was not able to determine whether the surface contamination resulted in the transfer of greater microbial loads to the cantaloupe during processing or whether the surfaces become contaminated from contact with cantaloupe that were already contaminated. Because of the recent occurrence of outbreaks associated with

contaminated cantaloupe (CDC, 2002), better understanding and control of contamination during cantaloupe processing is a critical research need.

Consumers are now able to purchase a vast array of produce items year-round, many of which were once thought of only as seasonal items. Along with an increase in sales of imported produce, an increasing number of outbreaks have been linked to these products, as illustrated by recent U.S. outbreaks associated with imported green onions and cantaloupe (CDC, 2002, 2003). These outbreaks and others, have given rise to speculation that imported produce is more likely to be contaminated with pathogens than domestically grown produce. Whether this is true has yet to be ascertained. Based on the results presented here, generally, the imported produce we tested had lower microbial loads than the domestic produce. However, our sample size, particularly for imported product, was quite small (n = 156), and given an expected low prevalence of pathogen contamination, larger and more comprehensive studies are needed to determine the frequency and magnitude of pathogen contamination on produce and identify higher risk produce types. Furthermore, studies of agricultural practices on farms that export produce to the US would be valuable to ascertain the sources of contamination and how to prevent it.

4.5.2 Antibiotic resistance of *Enterococcus* isolates

In this study, both *E. faecalis* and *E. faecium* strains isolated directly from produce were found to have a relatively low level of resistance to all antibiotics tested, with the exception of inherent resistance. For *E. faecium*, notable resistance was found against erythromycin (22%), quinupristin/dalfopristin (28%), tetracycline (24%), and ciprofloxacin (28%), all of which are important in human medicine. In particular, quinupristin/dalfopristin and ciprofloxacin have been used to treat enterococcal infections, with ciprofloxacin

frequently used treat those infections of lesser severity. Overall, *E. faecalis* was susceptible to most antibiotics, having resistance exceeding 5% to only erythromycin, tetracycline, and nitrofurantoin.

Interestingly, in almost all cases, *E. faecium* and *E. faecalis* isolates originating from samples of international origin had a higher degree of antibiotic resistance than did strains isolated from produce samples of domestic origin. For example, *E. faecium* isolated from imported produce was more often resistant to ciprofloxacin (37% vs. 0%) than those isolates of domestic origin. Differences in antibiotic resistance between strains of domestic and imported origin may be due to differences in production practices, such as fertilization techniques, irrigation water sources, and soil history. These variables were not further characterized in this study, and the health implications of these antibiotic resistant isolates for consumers is not known.

4.6 CONCLUSIONS

The manner in which fruits and vegetables are handled at harvest, and shortly thereafter, affect their microbiological quality. This is significant because the microbial load on produce at the time of harvest may be carried over to the final product at consumption if there are no effective intervention processes in place between harvest and consumption. Accordingly, sanitation and wash procedures within the packing shed have an extremely important impact on the overall quality of fresh produce. Adherence to the “*Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables*” can address produce quality and safety issues during growing, harvesting, sorting, packing, and distribution, and our study reinforces the frequently cited concept that every step from

production to consumption will affect the microbial load on produce. Despite increased attention to the role of imported produce in foodborne disease, this study does not support the assumption that domestic produce is of higher microbial quality than imported produce.

Table 4.1. Number of domestic and imported produce commodities classified by produce type

Sample Type	Commodity	n	Sample Locations
Leafy Greens (N=175)			
Domestic (N=109)	Green Swiss Chard	9	Bin, Merry Go Round, Box
	Turnip Greens	33	Bin, Merry Go Round, Box
	Collards	15	Bin, Merry Go Round, Box
	Cabbage	43	Trailer, Conveyor Belt, Box
	Kale	9	Bin, Merry Go Round, Box
Imported (N=66)	Cabbage	66	Bin, Conveyor Belt, Box
Herbs (N=222)			
Domestic (N=165)	Cilantro	93	Bin, Wash Tank, Merry Go Round, Box
	Parsley	63	Bin, Wash Tank, Merry Go Round, Box
	Dill	9	Bin, Merry Go Round, Box
Imported (N=57)	Cilantro	48	Bin, Merry Go Round, Box
	Parsley	9	Bin, Box
Melons (N=42)			
Domestic (N=36)	Cantaloupe	36	Conveyor Belt (before wash), Wash
			Conveyor Belt (after wash), Box
Imported (N=6)	Cantaloupe	6	Box
Vegetables (N=27)			
Imported (N=27)	Broccoli	27	Box

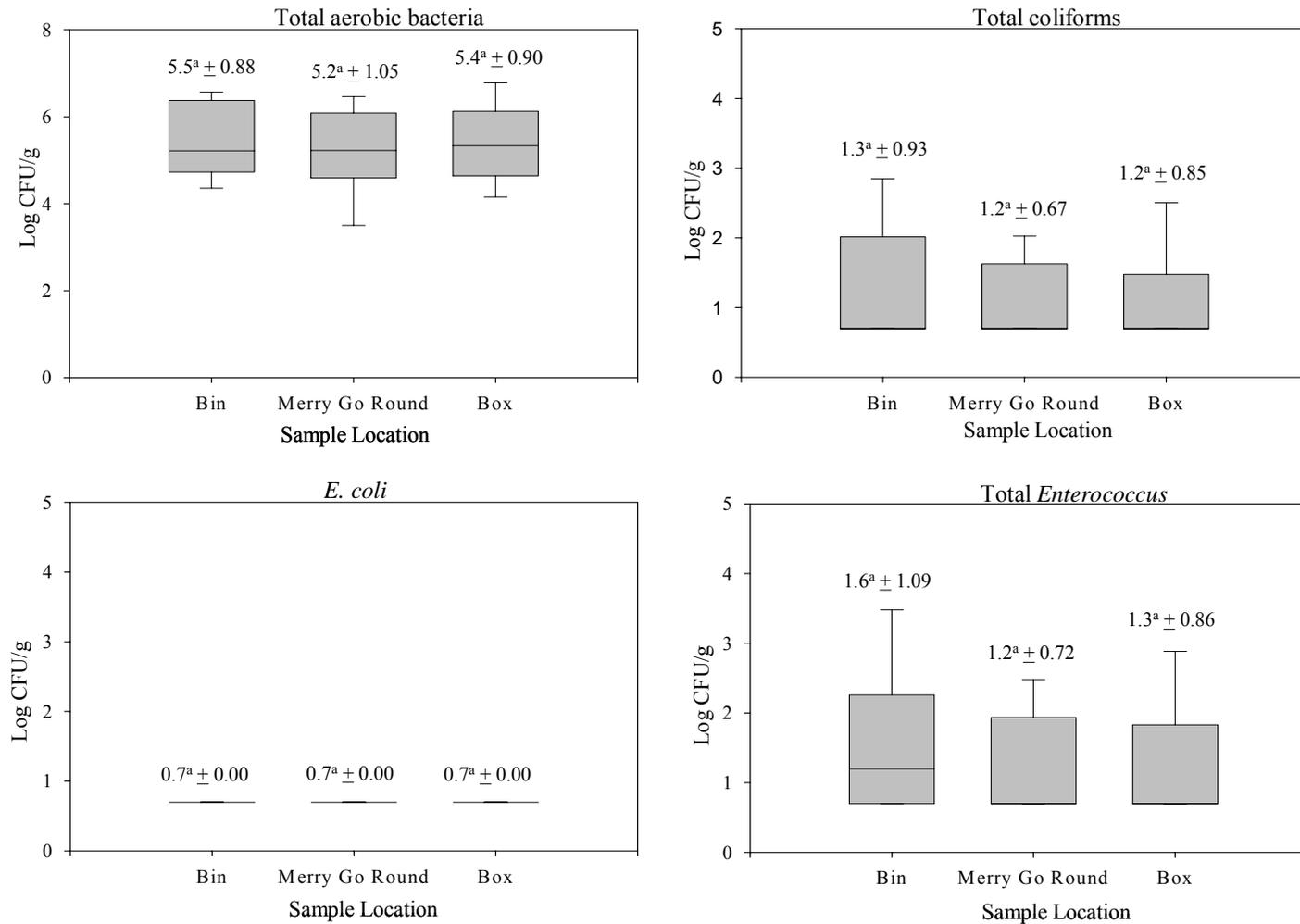


Figure 4.1. Domestic leafy greens: total aerobic bacteria, total coliforms, *E. coli*, and total *Enterococcus* levels collected from various steps throughout the packing shed. The box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles. The number above each box plot indicates the geometric mean. Superscript letters indicate significant differences among the log₁₀ means. Means that share the same superscript letter are not significantly different from each other; means with different superscript letters are significantly different ($P < 0.05$).

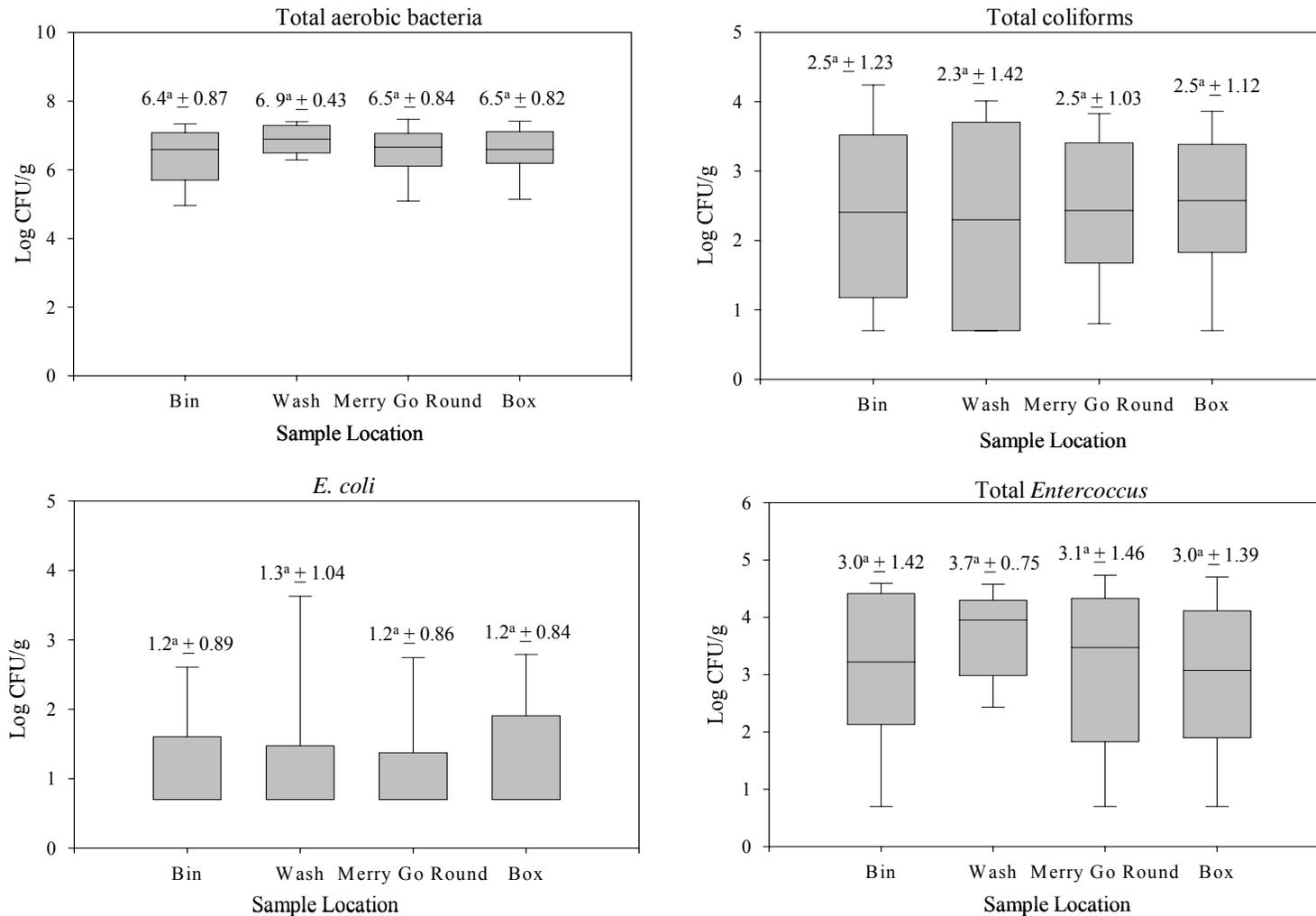


Figure 4.2. Domestic herbs: total aerobic bacteria, total coliforms, *E. coli*, and total *Enterococcus* levels collected from various steps throughout the packing shed. The box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles. The number above each box plot indicates the geometric mean. Superscript letters indicate significant differences among the log₁₀ means. Means that share the same superscript letter are not significantly different from each other; means with different superscript letters are significantly different (P < 0.05).

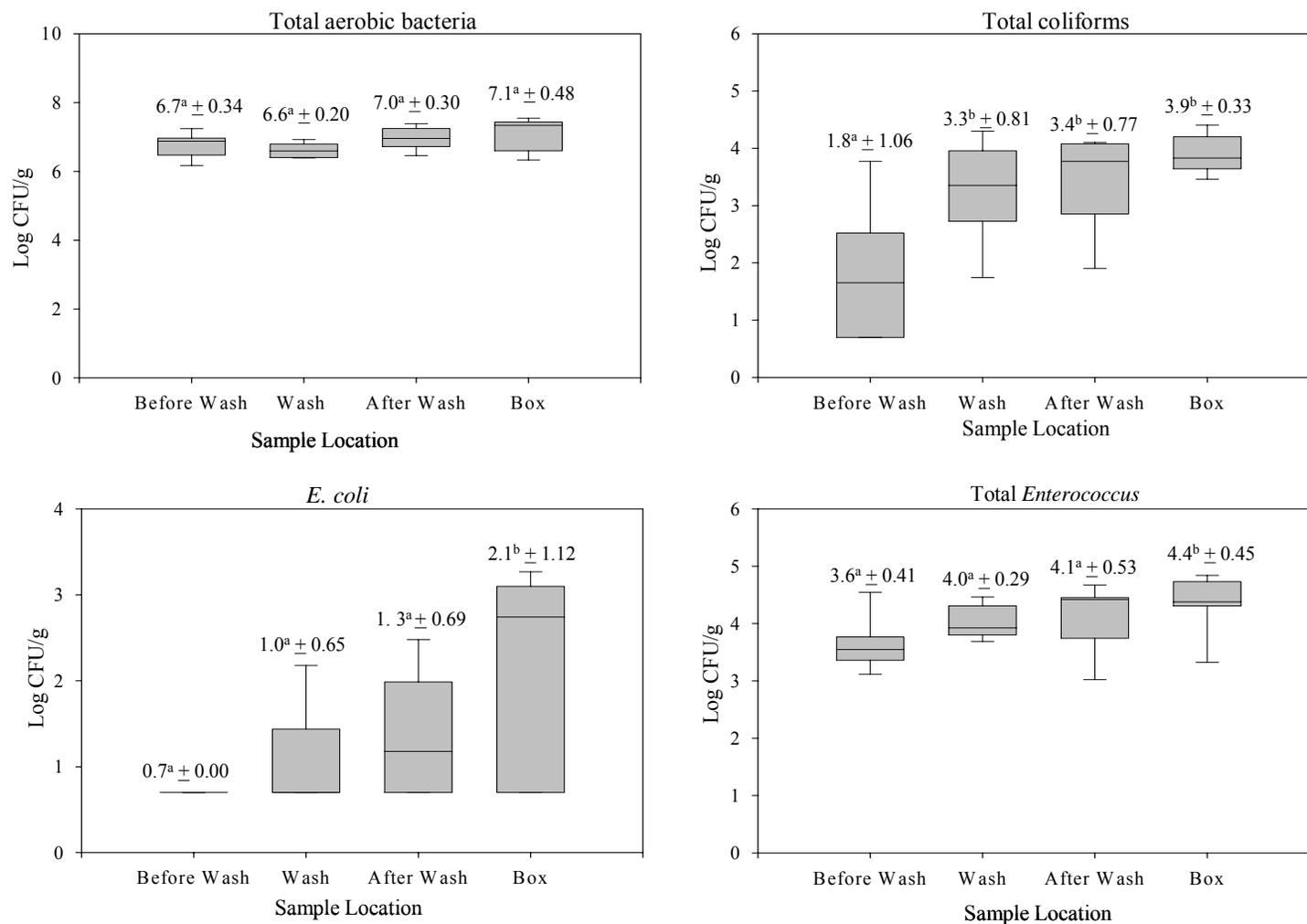


Figure 4.3. Domestic cantaloupe: total aerobic bacteria, total coliforms, *E. coli*, and total *Enterococcus* levels collected various steps throughout the packing shed. The box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles. The number above each box plot indicates the geometric mean. Superscript letters indicate significant differences among the log₁₀ means. Means that share the same superscript letter are not significantly different from each other; means with different superscript letters are significantly different (P < 0.05).

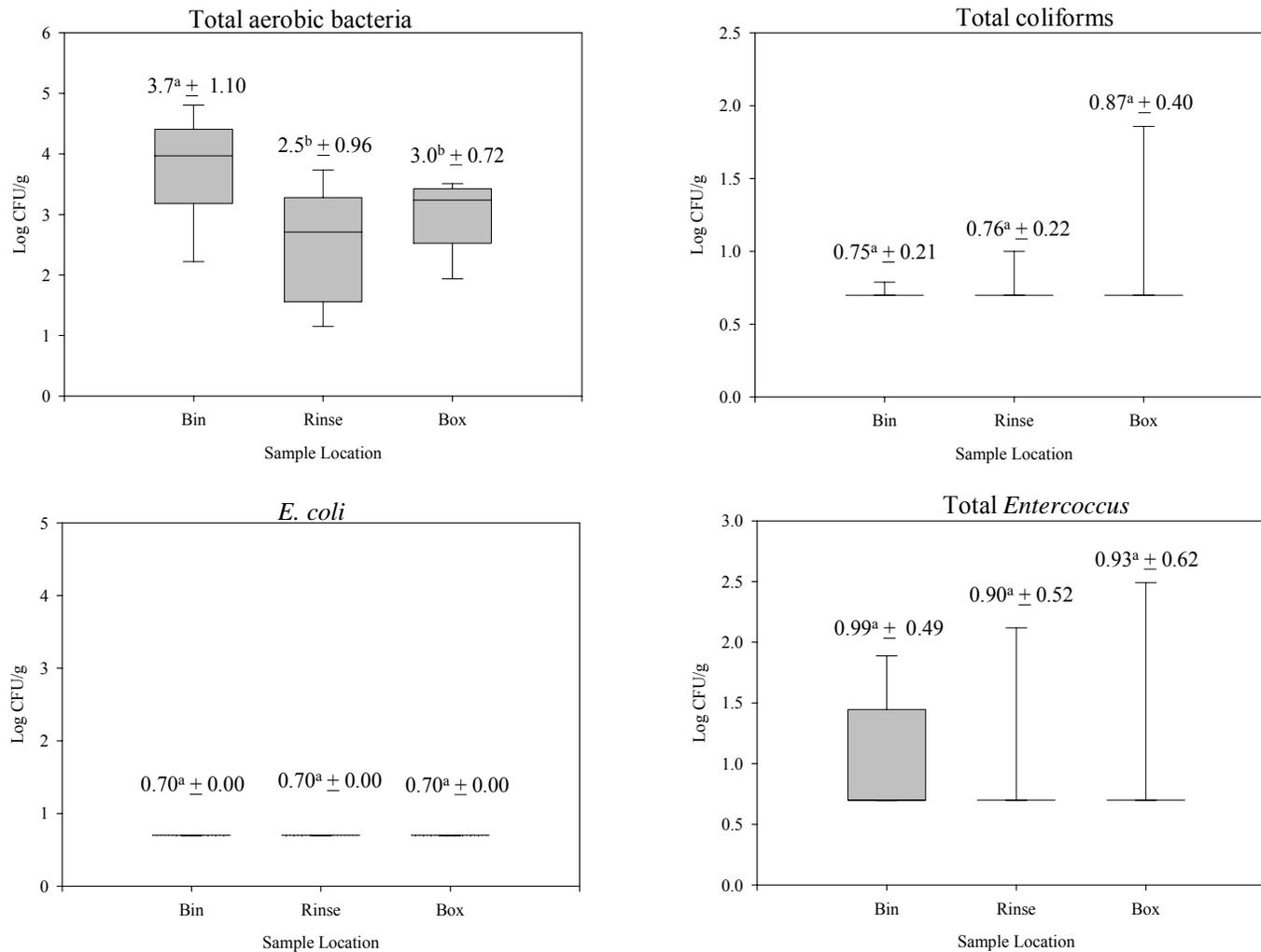


Figure 4.4. Environmental swabs from domestic leafy greens: total aerobic bacteria, total coliforms, *E. coli*, and total *Enterococcus* levels collected from various steps throughout the packing shed. The box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles. The number above each box plot indicates the geometric mean. Superscript letters indicate significant differences among the log₁₀ means. Means that share the same superscript letter are not significantly different from each other; means with different superscript letters are significantly different (P < 0.05).

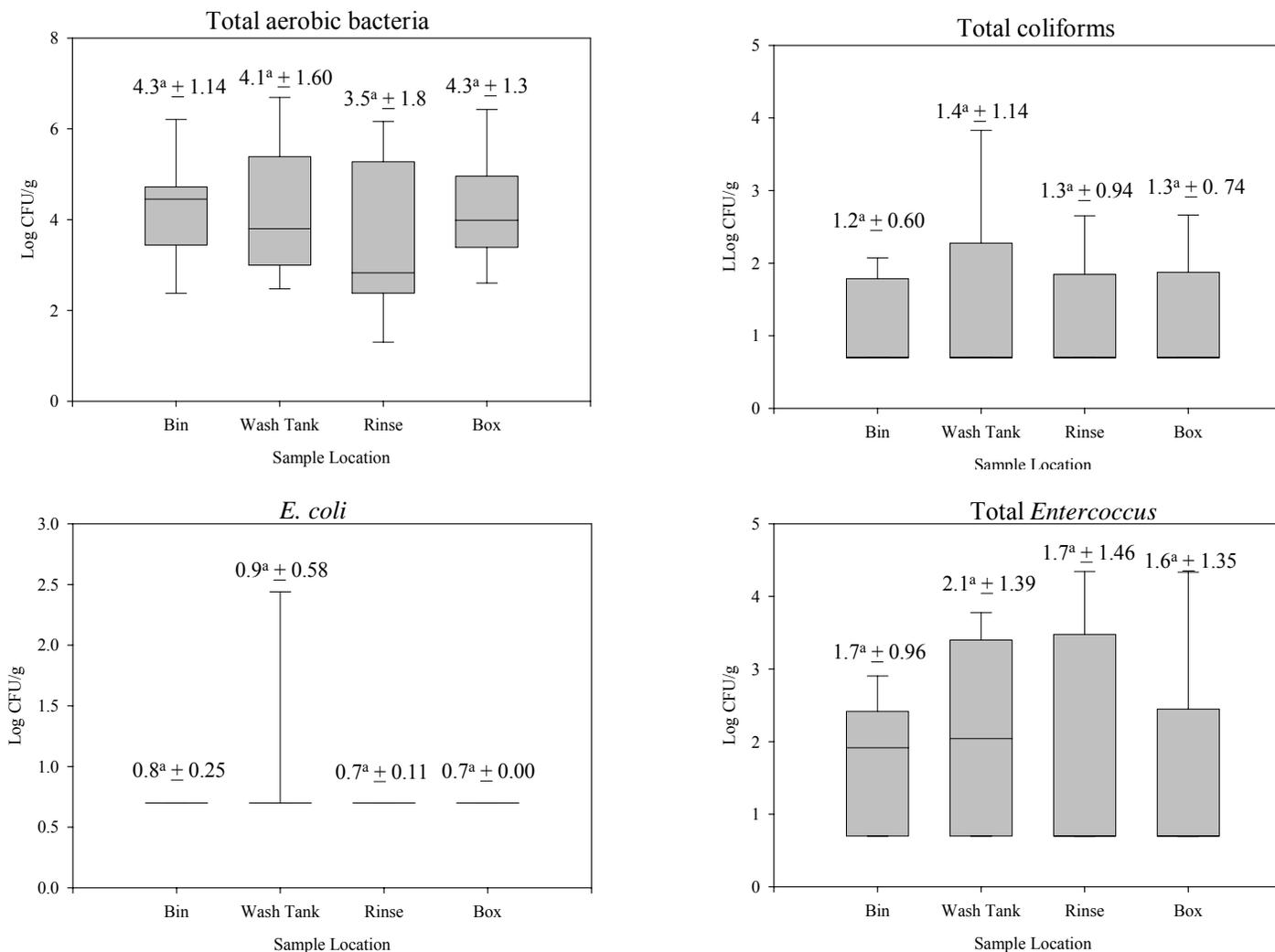


Figure 4.5. Environmental swabs from domestic herbs: total aerobic bacteria, total coliforms, *E. coli*, and total *Enterococcus* levels collected from various steps throughout the packing shed. The box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles. The number above each box plot indicates the geometric mean. Superscript letters indicate significant differences among the log₁₀ means. Means that share the same superscript letter are not significantly different from each other; means with different superscript letters are significantly different (P<0.05).

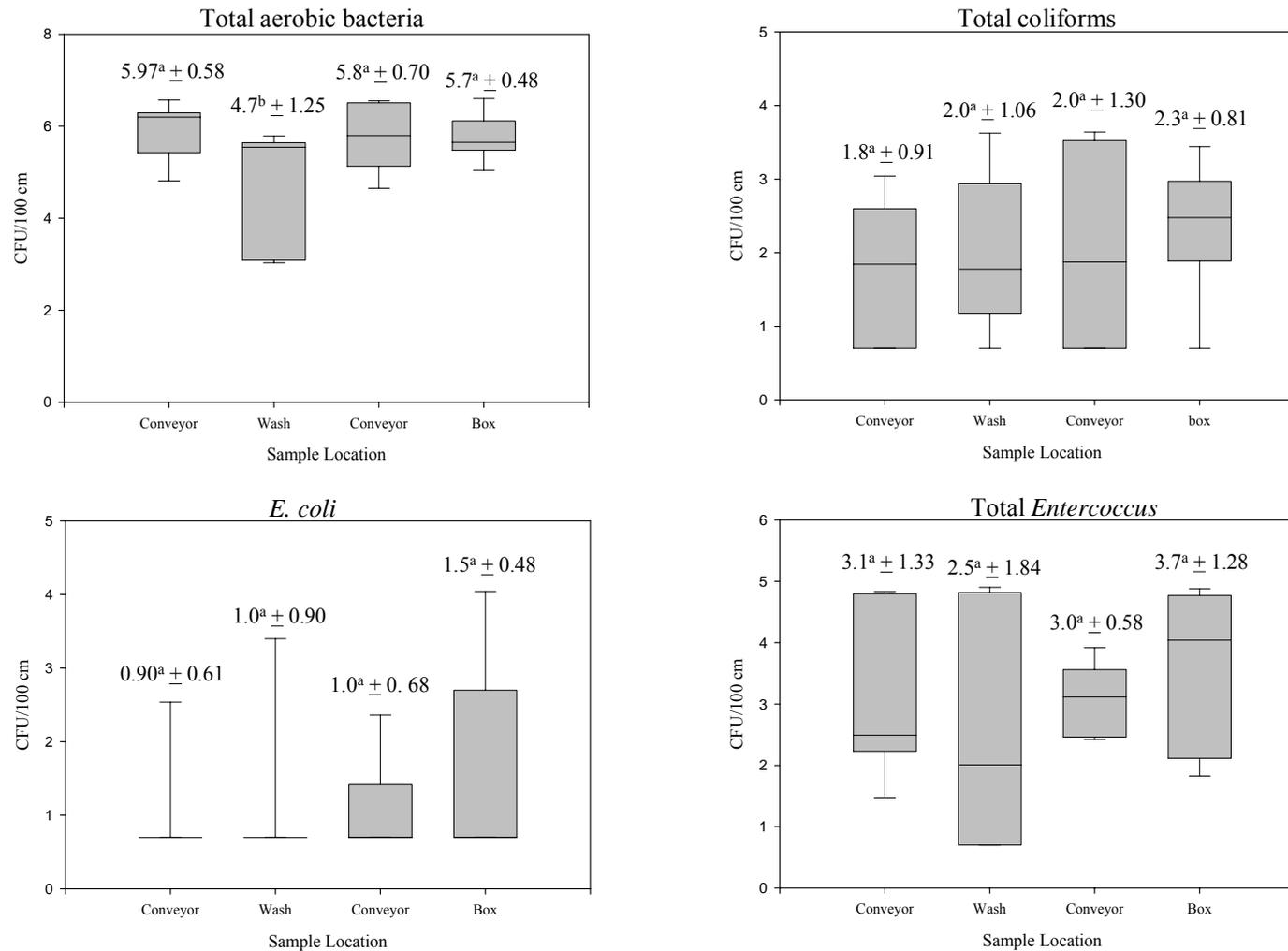


Figure 4.6. Environmental swabs from domestic cantaloupe: total aerobic bacteria, total coliforms, *E. coli*, and total *Enterococcus* levels collected from various steps throughout the packing shed. The box plot indicates the 10th, 25th, 50th, 75th, and 90th percentiles. The number above each box plot indicates the geometric mean. Superscript letters indicate significant differences among the log₁₀ means. Means that share the same superscript letter are not significantly different from each other; means with different superscript letters are significantly different (P < 0.05).

Table 4.2. Comparison of the levels of various microorganisms of domestic and imported herbs within packing sheds

Produce	Range (Mean \pm SD) Log CFU/g			
	Samples Received	Wash	Rinse	Samples for Distribution
Imported				
APC	5.95-6.09 (6.03 \pm 0.07) ^{a*A**}	NA	NA	5.68-7.50 (6.64 \pm 0.41) ^{bH}
Coliforms	1.60-2.29 (1.97 \pm 0.35) ^{cC}	NA	NA	0.70-4.32 (1.75 \pm 0.91) ^{eI}
<i>E. coli</i>	0.70-0.70 (0.70 \pm 0.00) ^{dD}	NA	NA	0.70-1.93 (0.84 \pm 0.32) ^{eK}
Enterococci	2.13-2.40 (2.25 \pm 0.14) ^{fF}	NA	NA	0.70-4.04 (2.26 \pm 0.96) ^{fM}
Domestic				
APC	4.61-7.48 (6.44 \pm 0.81) ^{gB}	6.12-7.43 (6.87 \pm 0.43) ^g	4.19-7.85 (6.59 \pm 0.71) ^g	4.42-7.71 (6.50 \pm 0.82) ^{gH}
Coliforms	0.70-4.48 (2.56 \pm 1.22) ^{hC}	0.70-4.11 (2.22 \pm 1.44) ^h	0.70-4.22 (2.51 \pm 1.05) ^h	0.70-4.37 (2.54 \pm 1.15) ^{hJ}
<i>E. coli</i>	0.70-3.79 (1.26 \pm 0.91) ^{iE}	0.70-4.01 (1.31 \pm 1.04) ⁱ	0.70-3.85 (1.20 \pm 0.88) ⁱ	0.70-3.19 (1.27 \pm 0.86) ^{iL}
Enterococci	0.70-5.29 (3.05 \pm 1.47) ^{jG}	2.30-4.74 (3.71 \pm 0.75) ^j	0.70-5.37 (3.13 \pm 1.46) ^j	0.70-5.42 (3.09 \pm 1.42) ^{jN}

* Lower case superscript denotes statistically significant difference (p<0.05) between sample locations among origin of each sample type

** Upper case superscript denotes statistically significant difference (p<0.05) between sample origins among sample locations of each sample type

Table 4.3. Comparison of the levels of various microorganisms of domestic and imported cabbage within packing sheds

Produce	Range (Mean \pm SD) Log CFU/g		
	Samples Received	Conveyor Belt	Samples for Distribution
Imported			
APC	NA	6.42-6.81 (6.63 \pm 0.20) ^{a*A**}	5.30-7.46 (6.33 \pm 0.49) ^{aF}
Coliforms	NA	1.95-3.20 (2.42 \pm 0.68) ^{bB}	0.70-3.48 (1.82 \pm 0.95) ^{bH}
<i>E. coli</i>	NA	0.70-0.70 (0.70 \pm 0.00) ^{cC}	0.70-3.23 (0.86 \pm 0.49) ^{dI}
Enterococci	NA	3.71-4.23 (4.00 \pm 0.27) ^{eE}	0.70-4.43 (3.06 \pm 1.04) ^{eJ}
Domestic			
APC	5.38-6.46 (6.08 \pm 0.36) ^f	3.95-6.36 (5.61 \pm 0.75) ^{fA}	4.33-6.40 (5.80 \pm 0.56) ^{fG}
Coliforms	1.70-3.24 (2.53 \pm 0.53) ^g	0.70-2.28 (1.92 \pm 0.55) ^{ghB}	0.70-3.42 (1.43 \pm 0.83) ^{hH}
<i>E. coli</i>	0.70-2.81 (2.10 \pm 0.75) ^j	0.70-2.48 (1.31 \pm 0.62) ^{jkD}	0.70-3.53 (0.96 \pm 0.66) ^{kI}
Enterococci	2.48-4.58 (4.10 \pm 0.72) ^m	2.57-4.58 (4.02 \pm 0.68) ^{mE}	1.00-4.45 (3.07 \pm 1.01) ^{nJ}

* Lower case superscript denotes statistically significant difference ($p < 0.05$) between sample locations among origin of each sample type

** Upper case superscript denotes statistically significant difference ($p < 0.05$) between sample origins among sample locations of each sample type

Table 4.4. *Enterococcus* isolates by commodity type

Commodity (N=141)	# of Domestic	# of Imported	# of Enterococcus Isolates (%)	Number of Isolates (%)		
				<i>E. faecium</i>	<i>E. faecalis</i>	Other <i>Enterococcus</i> spp.
Broccoli	0	15	7 (5)	6 (4)	0 (0)	1 (1)
Cabbage	0	45	35 (25)	27 (19)	3 (2)	5 (4)
Cilantro	42	30	77 (55)	29 (21)	38 (27)	10 (7)
Parsley	9	0	9 (6)	3 (2)	6 (4)	0 (0)

Table 4.5. Antibiotic resistance profiles among *E. faecium* and *E. faecalis* isolates from produce

Antibiotic	<i>E. faecium</i> (n=67)			<i>E. faecalis</i> (n=45)		
	Resistant (%)	MIC ₅₀	MIC ₉₀	Resistant (%)	MIC ₅₀	MIC ₉₀
Bacitracin	NA	>128	>128	NA	128	>128
Chloramphenicol	1 (1)	-	-	0 (0)	-	-
Erythromycin	15 (22)	-	-	4 (9)	-	-
Flavomycin	NA	>32	>32	NA	2	>32
Penicillin	2 (3)	-	-	2 (4)	-	-
Salinomycin	NA	1	2	NA	1	4
Q/D	19 (28)	-	-	45 (100)	-	-
Tetracycline	16 (24)	-	-	3 (7)	-	-
Vancomycin	0 (0)	-	-	0 (0)	-	-
Lincomycin	NA	16	32	NA	32	32
Tylosin Tartrate	NA	2	8	NA	1	4
Ciprofloxacin	19 (28)	-	-	1 (2)	-	-
Linezolid	0 (0)	-	-	0 (0)	-	-
Nitrofurantoin	1 (1)	-	-	5 (11)	-	-
Kanamycin	NA	256	1024	NA	128	128
Gentamicin	1 (1)	-	-	1 (2)	-	-
Streptomycin	0 (0)	-	-	0 (0)	-	-

*NA, not applicable

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CHAPTER 5

A SIMPLE METHOD FOR THE DIRECT DETECTION OF SALMONELLA AND E. COLI O157:H7 FROM RAW ALFALFA SPROUTS AND SPENT IRRIGATION WATER USING PCR

5.1 ABSTRACT

Recognizing that raw seed sprouts are an important cause of foodborne disease, the U.S. Food and Drug Administration is now recommending that either spent irrigation water or final product be screened for *Salmonella* spp. and *Escherichia coli* O157:H7 as a means of assuring the safety of product intended for consumption. In an effort to streamline such testing efforts, the purpose of this study was to develop a simple method to pre-concentrate pathogens from sprouts and spent irrigation water to facilitate the direct (without prior cultural enrichment) detection of pathogens using the polymerase chain reaction (PCR). Alfalfa sprouts and spent irrigation water were seeded with *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7 in the range of 10^{-1} to 10^6 CFU/g or ml, respectively. Samples were blended (sprouts only) and then centrifuged at relatively high speed to sediment the total bacterial population. The precipitate was processed for DNA isolation, PCR amplification, and amplicon confirmation by Southern hybridization. Mean pathogen recoveries after centrifugation ranged from 96-99% for both pathogens in both matrices. Using primers targeting the *inv A* gene for serovar Typhimurium and the *stx* genes of *E. coli* O157:H7, it was possible to detect both pathogens in alfalfa sprouts at seeding levels as low as 10^1 CFU/g. PCR detection limits for both pathogens from spent irrigation water were 10^{-1} CFU/ml, the equivalent of 10^2 CFU/liter of water. Spent irrigation water,

since it is rather constitutionally simple, is particularly well suited for prior bacterial concentration using simple centrifugation steps. This study demonstrates progress toward a rapid, inexpensive, and sensitive method for the detection of pathogens associated with this commodity, and one that is relevant to current industrial practices and needs.

5.2 INTRODUCTION

Raw seed sprouts have been recognized as significant vehicles for the transmission of foodborne illness within the last ten years. Since 1996, the U.S. Food and Drug Administration (FDA) has responded to 27 outbreaks, accounting for over 1,500 cases of salmonellosis and *Escherichia coli* O157:H7 infection, all of which were associated with raw or lightly cooked sprouts (FDA, 2004). Most notably, a large outbreak of over 6,000 culture-confirmed cases of *E. coli* O157:H7 occurred in Japan in 1996, in which illnesses were strongly linked to the consumption of radish sprouts (National Institute of Infectious Diseases and infectious Diseases Control Division, 1997). More recently, alfalfa sprouts produced at a single facility were implicated as the cause of a *Salmonella enterica* serovar Kottbus outbreak, affecting 32 patients in four states (Arizona, California, Colorado and New Mexico) (CDC, 2002).

Within the fresh produce category, sprouts present a unique challenge because the conditions that promote sprouting of the seed (e.g., temperature, humidity, available nutrients) also promote the growth of bacteria, including pathogens. To date, numerous investigators have examined the efficacy of disinfectant treatments to lower the risk of foodborne disease associated with raw sprouts (Thayer et al., 2003; Kim et al., 2003; Wade et al., 2003; Weissinger et al., 2001; Beuchat et al., 2001; Himathongkham et al., 2001). However, none of these have completely eliminated pathogen contamination on seeds, which

is the most likely initial source of contamination (NACMCF, 1999). Sprout producers are therefore advised to conduct microbiological testing from each production lot to ensure that contaminated product is not distributed. Specifically, this entails the testing of spent irrigation water (i.e., irrigation water that has flowed over and through the sprouts) or the seed sprouts themselves, for the pathogens *Salmonella* and *E. coli* O157:H7. These methods stipulate the testing of 100 ml of irrigation water (or 25 g of sprouts) at or after 48 hours from the start of the sprouting process. The assay requires an overnight cultural enrichment step followed by detection using cultural, immunological, or molecular methods. The subsequent detection methods are not sensitive enough to detect pathogens in irrigation water unless the prior enrichment step is done (FDA, 1999), thereby necessitating a test that takes at least 24 hours to obtain presumptively positive results.

While much attention and expectation has been placed on molecular amplification-based methods as applied to the detection of foodborne pathogens, their performance has remained approximately the same as second generation methods such as ELISA and DNA hybridization. This is because of various sampling and matrix issues, including the need to test large sample volumes (≥ 25 ml or g) as compared to small amplification volumes; residual food components which inhibit molecular amplification reactions; and low levels and sporadic contamination with pathogens. Additionally, particularly in foods such as sprouts, rapid detection of microbial pathogens at the low levels that might be anticipated in naturally contaminated product is confounded by our inability to detect minute signals amid the noise presented by food components, including competing microflora. It has frequently been suggested that the rapid detection of specific pathogens could be enhanced by the separation, concentration, and purification of the agent(s) from the sample matrix prior to

applying nucleic acid amplification. Methods such as centrifugation, filtration, and immunomagnetic separation have all been reported in this regard. While these methods may improve the sensitivity of detection, none has broad applicability for all foods and the methods need to be optimized for each product or product/pathogen combination (Stevens and Jaykus, 2004; Lantz et al., 1994). In this paper, we report a prototype method to process sprouts and spent irrigation water to concentrate bacteria prior to applying PCR to their detection. In so doing, we demonstrate that *Salmonella* and *E. coli* O157:H7 can be detected from alfalfa sprouts and spent irrigation water without prior cultural enrichment at relatively low levels of contamination.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial cultures and media

Pathogen cultures were labeled with specific antibiotic resistance to facilitate their enumeration from among natural background microflora in the cultural (enumerative) analyses. A previously reported *Salmonella enterica* serovar Typhimurium LT2 strain (MA1567), resistant to kanamycin, was used in the assays (El Hanafi and Bossi, 2000). *E. coli* O157:H7 ATCC 43895 was genetically engineered for resistance to chloramphenicol and tetracycline using methods of transformation. Briefly, for competent cell preparation, 100 ml of Luria Broth (LB) (Difco Laboratories, Sparks, MD, USA) were inoculated with 1 ml of an overnight culture of ATCC 43895. Cells were grown at 37°C with shaking to an optical density of 600 nm. Cells were then centrifuged and washed twice in 100 ml of saline and once in 50 ml of 10% glycerol. The pellet was resuspended in 250 µl of 10% glycerol, aliquoted and stored at -80°C. For electroporation, 50 µl of cells were thawed on ice and

mixed with 1 μ l of a miniprep solution of pACYC184, a plasmid containing the *tet* and *cat* genes, encoding tetracycline and chloramphenicol resistance, respectively (courtesy of Dr. Frederik Boccard, Centre National de la Recherche Scientifique, Gif sur Yvette, France). This mixture was placed in a pre-chilled electroporation cuvette (0.2 cm inter-electrode gap) and a single electric pulse (Bio-Rad Gene Pulser) was applied (2.5 kV peak voltage, 25 μ F capacitance, and 200 Ω resistance). Cells were then recovered in 1 ml of LB broth. After 1 h incubation at 37°C, cells were spread on LB agar containing 20 μ g/ml chloramphenicol, for selection of transformed cells. Random transformants were chosen (a total of four), screened on Brain Heart Infusion broth (Difco) supplemented with chloramphenicol (20 μ g/ml) and tetracycline (10 μ g/ml) (BHI-CT) and a single transformant showing normal growth patterns on the antibiotic-supplemented BHI was chosen for subsequent assays.

To prepare inocula for seeding studies, bacterial cultures were propagated aerobically overnight at 37°C in BHI broth supplemented with kanamycin (50 μ g/ml) (BHI-K) for *Salmonella*, and in BHI-CT for *E. coli* O157:H7. Ten-fold serial dilutions were done in 0.1% peptone (Difco) to reach approximate seeding densities ranging from 10² to 10⁸ CFU/ml. For the recovery and enumeration of *Salmonella*, pour plates of serial dilutions were done using Sorbitol MacConkey agar (Difco) supplemented with kanamycin (50 μ g/ml) (SMAC-K) while for *E. coli* O157:H7, Violet Red Bile Agar (Difco), supplemented with chloramphenicol (20 μ g/ml) and tetracycline (10 μ g/ml) (VRBA-CT) was used. In enumerative assays, inoculated plates (both SMAC-K and VRBA-CT) were incubated for 24 h at 37°C prior to counting.

5.3.2 Inoculation of sprouts and spent irrigation water

Sprouts were inoculated using the work of Beuchat et al. (2001) as a guide (Beuchat et al., 2001). Alfalfa sprouts obtained from a local retail outlet were aliquoted into 100 g portions and submerged in one liter of desired inoculum level (10^8 to 10^2 CFU/liter), which was suspended in 0.1% peptone buffer. Following gentle agitation for 5 min, sprouts were then removed from the inoculum, and placed in a single layer under a laminar flow hood for approximately 45 min to facilitate inoculum draining (Beuchat, et al., 2001). Inoculum levels on sprouts were confirmed by serial dilution plating on SMAC-K and VRBA-CT for *Salmonella* and *E. coli* O157:H7, respectively. Spent irrigation water from alfalfa sprout production was obtained courtesy of a local sprouting facility and frozen in one liter aliquots at -20° C. Before experiments, aliquots were thawed and inoculated with a 1 ml suspension of each pathogen to reach levels ranging from 10^2 - 10^6 CFU/ml.

5.3.3 Concentration of pathogenic bacteria in alfalfa sprouts and spent irrigation water

The sample pre-treatment and concentration scheme is outlined in Fig. 1. Briefly, this included a homogenization step (sprouts only) followed by centrifugation, DNA extraction and purification, and PCR amplification and hybridization. In the case of sprouts, 25 g subsamples of seeded product were weighed and homogenized in 225 ml of 0.1% peptone buffer with 2.5% Tween 80 to which was added 10 g polyethylene glycol (PEG) 8000 (Fisher Scientific) to obtain a final concentration of 4% PEG. In order to remove large matrix-associated particulates, a filter stomacher bag (Nasco, Ft. Atkinson, WI, USA) was used. Samples were homogenized in a Stomacher[®] 400 (Seward, Norfolk, UK) at 260 rpm for 2 min at room temperature. The resulting filtrate was collected and centrifuged at $9,100 g$ (Sorvall RC-5B, Dupont Company, Wilmington, DE, USA) for 10 min at 4° C. Because of

rotor size limitations, we chose to process 400 ml subsamples of spent irrigation water. For sample pre-concentration in this case, the entire 400 ml sample was centrifuged at 9,100 g for 20 min at 4°C. The supernatant resulting from centrifugation of both sprouts and water was decanted and the remaining pellet (~ 0.5 to 2.0 g) was resuspended in 0.1% peptone buffer with 2.5% Tween 80 to make a 1:10 dilution.

Enumerative assays were done on both the discarded supernatant and the pathogen-containing pellet to evaluate bacterial load. Percent recovery was calculated based on direct plating of the pellet and on loss to the supernatant, as previously described (Lucore et al., 2000). Specifically, the former was calculated as follows: % recovery = (total population in pellet after centrifugation) x 100/(total population in sample before centrifugation), while the latter was expressed as % recovery = (100 - % loss to supernatant). All experiments were done in triplicate. Statistical comparisons included the analysis of variance and the Tukey-Kramer multiple comparisons test on the percent recovery data using the INSTAT 2 Statistical Analysis Package (GraphPad Software, San Diego, CA, USA).

5.3.4 DNA extraction

The precipitates resulting from the centrifugation step were resuspended in Plant DNAzol[®] (Invitrogen, Carlsbad, CA, USA) for subsequent DNA extraction. The procedure uses a guanidine-detergent lysing solution to hydrolyze RNA and a chloroform extraction step to remove pigments and insoluble plant debris. For alfalfa sprouts, the pellet (~ 0.5 g) was extracted with 3 ml of Plant DNAzol[®] reagent, while the pellet (~ 2.0 g) from spent water was extracted in 4 ml of this reagent. The extraction process was carried out according to manufacturer's instructions which briefly included a chloroform extraction, centrifugation for 10 min at 12,000 g, followed by DNA precipitation in 2 ml of 100% ethanol. The pellet

resulting after centrifugation at 5,000 g for 4 min was washed twice with ethanol and solubilized in 80 µl of sterile deionized water. Additional purification of the DNA was achieved by centrifugation through a QIAshredder[®] (QIAGEN Inc., Valencia, CA, USA) column for 2 min at 11,750 g (Sair et al., 2003) and the final filtrate was retained for nucleic acid amplification. For both alfalfa sprouts and spent water, the inhibitory effect of plant components was evaluated by dilution series PCR as previously reported (Lucore et al., 2000; Taylor et al., 2005).

5.3.5 PCR amplification

PCR reactions were done using the GeneAmp[®] PCR kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Each 100 µl reaction contained 1x PCR buffer, 200 µM each dATP, dCTP, dTTP, and dGTP, 2.5 units AmpliTaq DNA polymerase, and 2 µl of DNA extract. For *Salmonella*, primers SAL3 (5'-GCTGCGCGCGAACGGCGAAG-3') and SAL4 (5'-TCCCGGCAGAGTTCCCATT-3') targeting the virulence gene *inv A* were used (Manzano et al., 1998) under conditions of 2.0 mM magnesium chloride. Amplification was done in a Perkin-Elmer DNA Thermal Cycler (Perkin Elmer, Norwalk, CT, USA) and consisted of one cycle of 95°C for 5 min, followed by 35 cycles of 95°C for 90 sec, 58°C for 80 sec, 72°C for 2 min, and a final extension of 72°C for 7 min.

For *E. coli* O157:H7, degenerate sequence primers MK1 (5'-TTTACGATAGACTTCTCGAC-3') and MK2 (5'-CACATATAAATTATTTTCGCTC-3') which amplify both *stx1* and *stx2* gene sequences were used (Karch and Meyer, 1989). In this case, amplification was optimized at 3.0 mM magnesium chloride with the addition of 0.5 mM of dithiothreitol. PCR amplification conditions consisted of one cycle at 95°C for 5

min, followed by 35 cycles of 94°C for 1 min, 48°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 7 min. Ten µl of each amplification product was separated by gel electrophoresis on 1.5% agarose, stained with ethidium bromide, and visualized by ultraviolet light. Expected amplicon sizes were 389 bp and the 227 bp for *Salmonella* and *E. coli* O157:H7, respectively.

5.3.6 Southern blot hybridization

PCR products were transferred to nylon membranes (Roche, Indianapolis, IN, USA) using the method of Southern (Sambrook et al., 1989) and hybridized with a digoxigenin (DIG)-labeled DNA probe (DIG Oligonucleotide Tailing Kit, Roche), according to manufacturer's instructions. The internal oligoprobe sequences used for confirmation of amplicon identity were 5'-TTTGTGAACTTTATTGGCGG-3' for *Salmonella* (Manzano et al., 1998) and 5'-CAGGCGCGTTTTGACCATCTT-3' for *E. coli* O157:H7 (Karch and Meyer, 1989). Hybridization was done using the ExpressHyb Hybridization Solution (BD Biosciences Clontech, Palo Alto, CA, USA) at 55°C (*Salmonella*) and 60°C (*E. coli* O157:H7) and 50 pmol of DIG-labeled probe. The membranes were washed in series at room temperature and detection was achieved using the DIG Nucleic Acid Detection Kit (Roche), which is based on an enzyme-catalyzed colorimetric reaction using 5-bromo-4-chloro-3 indoyl phosphate and nitroblue tetrazolium salt as the reagents to produce an insoluble blue precipitate.

5.4 RESULTS

5.4.1 Optimization of sample pre-concentration for sprouts

In this study, we purposely used pathogen cultures that were labeled with specific antibiotic resistance to facilitate their enumeration from among natural background microflora in the cultural (enumerative) analyses. In all cases, the antibiotic supplementation of recovery medium effectively inhibited the growth of indigenous microflora while allowing the growth of the target pathogen (data not shown). In order to remove large food particulates, 25 g samples of alfalfa sprouts were homogenized in a two-partition Whirlpak™ filter bag. Preliminary enumerative assays demonstrated that there was no statistically significant difference in microbial levels when comparing the filtrate from the filter stomacher bag to the homogenate produced in a conventional stomacher bag (data not shown). This indicated that the microbial concentration in the filtrate was representative of the population in the sample at large and hence the filter bags were used in all subsequent sprout assays.

For recovery experiments, we sought to evaluate bacterial recoveries after centrifugation by direct plating of both the pellet and the discarded supernatant. In all instances, mean bacterial loss to discarded supernatants never exceeded 5% (Table 5.1). However, if the centrifuged pellet was resuspended in 0.1% peptone alone, few pathogens could be recovered by direct plating of the pellet, presumably due to clumping. By adding 4% PEG to the initial homogenate, and by manipulating the composition of the peptone reconstitution buffer to include 2.5% Tween 80, we were able to obtain virtually 100% recovery of *Salmonella* when calculations were based on direct plating of the pellet (Table 5.1). These protocol changes were incorporated in all subsequent experiments.

5.4.2 Recoveries from optimized concentration method

Using the optimized method, alfalfa sprouts and spent irrigation water were inoculated at levels of 10^6 , 10^4 , and 10^2 CFU/g or ml, respectively, and processed for bacterial concentration using centrifugation. When based on loss to discarded supernatants, the recovery of both pathogens from alfalfa sprouts ranged from 96-99% and there were no statistically significant differences based on initial inoculum level. In all cases, <4% of the input *Salmonella* and <2% of the input *E. coli* O157:H7 were lost to discarded supernatants (Table 5.2). When recoveries were calculated based on direct plating of the centrifuged pellet, the values were more variable, with recoveries ranging from 78 - 121% for *Salmonella* and 95% - 134% for *E. coli* O157:H7 (Table 5.2). The centrifugation step resulted in a 50-fold sample volume concentration, reducing the 25-g sample to a pellet size of 0.5 g. After DNA extraction, an additional six-fold sample concentration was obtained; in all, the 25-g sample was concentrated 300-fold to a final volume of 80 μ l.

Similar to the sprout samples, when spent irrigation water was processed by centrifugation, recoveries for both pathogens also ranged from 96-99% when calculated based on loss to discarded supernatants, with loss to supernatant never exceeding 3% for both pathogens and no statistically significant differences between inoculum levels (Table 5.2). When the pellet was directly plated, *Salmonella* recoveries ranged from 62% - 105%, while those for *E. coli* O157:H7 ranged from 80% - 118% (Table 5.2). The centrifugation of 400 ml of spent irrigation water resulted in an average pellet size of 2.0 g, representing a 200-fold sample volume reduction. When combined with the DNA extraction step, an additional 2.5-fold volume reduction was obtained; in all, the 400 ml water sample was reduced 5000-fold to a final volume of 80 μ l.

5.4.3 Detection by PCR and Hybridization

In an effort to ascertain the overall PCR and hybridization detection limits of the combined concentration-detection method, alfalfa sprouts and spent irrigation water were seeded with serial dilutions (10^6 to 10^0 CFU/g and 10^6 to 10^{-1} CFU/ml, respectively) of *Salmonella* Typhimurium or *E. coli* O157:H7, processed by centrifugation, DNA extraction and purification, and PCR amplification followed by Southern hybridization. For *Salmonella*, detection was possible at initial inoculum levels of 10^1 CFU/g, which would correspond to 10^2 CFU/25 g, for 2 of the 3 samples tested (Fig. 5.2A). Detection limits for *E. coli* O157:H7 from sprouts were the same at 10^1 CFU/g (10^2 CFU/25 g), with 1 of 3 replicates found to be positive for the pathogen (Fig. 5.2B). At initial inoculum levels of 10^2 CFU/g and above, all seeded sprout samples were positive by PCR and subsequent hybridization for both pathogens.

Interestingly, when the method was applied to the detection of these pathogens in seeded spent irrigation water, detection limits were much improved over those observed for seeded raw alfalfa sprouts (Fig. 5.3A). Specifically, gel electrophoresis indicated a detection limit of 10^2 CFU/ml for *Salmonella*, however we could confirm amplicons in one of the three samples by hybridization at the lowest level of inoculum used, which corresponded to less than 10^{-1} CFU/ml (10 CFU/400 ml). Consistent detection in all replicates was found at initial inoculum levels of $\geq 10^1$ CFU/ml (10^3 CFU/400 ml). Similar results were obtained for *E. coli* O157:H7 (Fig. 5.3B).

5.5 DISCUSSION

The purpose of this study was to develop a direct (free of cultural enrichment) method to detect *Salmonella* and *E. coli* O157:H7 from alfalfa sprouts and spent irrigation water

using PCR. The approach used simple centrifugation and filtration methods to concentrate the pathogens and prepare the matrix for DNA extraction, thereby removing matrix-associated inhibitory compounds and providing sample size reduction with recovery of viable bacterial cells. Overall, the approach was successful, as we were able to achieve detection limits of 10^1 CFU/g for both pathogens from seeded alfalfa sprouts, and detection limits as low as 10^{-1} CFU/ml (10 CFU/400 ml) from seeded spent irrigation water.

It is well known that bacteria can be pre-concentrated by simple centrifugation, and this step is frequently applied when preparing pure cultures as inocula in seeding studies, or when preparing cultural enrichments for detection by PCR. Using centrifugation to concentrate pathogens from the food matrix is more difficult, but has nonetheless been done. The most extensive work has been done in the dairy matrix, and several studies have included centrifugation steps in an effort to improve the detection of various pathogens from dairy products (Makino et al., 1995; Meyer et al., 1991; Wegmüller et al., 1993; Stevens and Jaykus, 2004). A common theme in all these studies is the need to optimize the matrix preparation method prior to its reliable use. Even though alfalfa sprouts or spent irrigation water might be considered compositionally less complex than a meat or cheese matrix, optimization of the sample concentration method was nonetheless necessary to ensure reliable matrix preparation with recovery of most of the input bacterial cells. The value of centrifugation is further described by Sharpe (2004), who noted that this process not only increases target cell number, but by separating the bacteria from the food components, results in reduced sample volume and improved signal-to-noise ratio (Sharpe, 2004). In the case of sprouts, using filtration to remove large particulates that would otherwise co-sediment with the bacteria helped reduce matrix-associated inhibition. Based on the work of Wu et al.

(2001), the use of surfactants was incorporated in the resuspension of the pellet following centrifugation. Several studies have shown that aggregates and biofilms may harbor on plant surfaces (Fett, 2000; Fett and Cooke, 2005; Warriner et al., 2003), and we hypothesize that aggregation is indeed the reason why recoveries from directly plating of the pellet were lower and more variable. Tween 80 alters the surface tension of the micro-environment and may aid in the release of aggregates, thereby alleviating potential clumping of the bacteria. In our study, the addition of Tween 80 to the final resuspension buffer improved recoveries when calculations were based on the direct plating of the pellet obtained after centrifugation. We also supplemented the sprout samples with PEG, in which we speculate that it promotes protein precipitation, with the bacteria in the food suspensions co-precipitating in a manner similar to organic flocculation (Sobsey et al., 1975). Additional sample optimizations included the use of a chloroform extraction during DNA preparation, and the QiaShredder® column, both of which facilitated the removal of residual food-related inhibitors. Careful attention to PCR amplification conditions, including the addition of dithiothreitol (DTT) and second-generation *Taq* DNA polymerase also reduced the chance of matrix-associated non-specific amplification which might otherwise impact overall assay detection limits (Stevens and Jaykus 2004).

Although nucleic acid amplification methods are increasingly being applied to pathogen detection in foods, detection limits rarely exceed 10^2 to 10^3 CFU/ml or g (Bej and Mahbubani, 1994; Swaminathan and Feng, 1994; Wilson, 1997). In fact, when investigators report detection limits ≤ 10 CFU/ml or g, it is almost always after cultural enrichments ranging from 8 to 48 hours in length. For instance, Liming and Bhagwat (2004) could detect 4 CFU/25 g alfalfa sprouts using real-time PCR after an 18 hour enrichment period (Liming

and Bhagwat, 2004). In another study, investigators reported on the detection *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes* at initial inoculum levels of 10 CFU/25 g alfalfa sprouts, but in most cases, a 24 hour enrichment step was needed to achieve these detection limits (Strapp et al., 2003). Likewise, detection limits as low as 1 CFU per 25 g of alfalfa sprouts, for the pathogens *Salmonella* and *L. monocytogenes*, were obtained using the PCR-based BAX system, again after a 24-h pre-enrichment (Shearer et al., 2001). Fratamico and Bagi (2001) found that an 18 hour cultural enrichment enhanced the sensitivity of the TaqMan[®] real-time PCR assay when applied to the detection of *E. coli* O157:H7 from spent irrigation water collected during alfalfa sprouting (Fratamico and Bagi, 2001). Recently, several investigators conducted studies to improve the detection of *Salmonella* and *E. coli* O157:H7 from seed sprouts and spent irrigation water using novel biosensor technologies. In these studies, the detection limits reported were equal to if not better than these previous detection assays designed for sprouts and spent irrigation water. For instance, Kramer and Lim (2004) found that they could directly detect a minimum of 10⁵ CFU/ml of *Salmonella* Typhimurium in spent irrigation water using the so-called RAPTOR technology (Kramer and Lim, 2004). However, another study (Muhammad-Tahir and Alocilja, 2004) reported detection limits similar to ours in that they could detect 78 CFU/g *E. coli* O157:H7 in alfalfa sprouts through the use of a disposable polyaniline biosensor which was based on an electrochemical sandwich immunoassay (Muhammad-Tahir and Alocilja, 2004). Interestingly, novel rapid methods such as these biosensors required pre-concentration of the target from the sample matrix to optimize the likelihood that the organism would be captured and detected. Lampel et al. (2000) also reported the use of concentrated food washes which were subsequently aliquoted onto FTA filters for direct DNA extraction and subsequent

detection of several foodborne pathogens (Lampel et al., 2000). They specifically reported PCR detection limits around 50 CFU/ml for *Shigella flexneri* from several produce items, including alfalfa sprouts. These studies confirm the value of sample concentration and purification in improving the detection limits of a variety of different rapid detection approaches.

Of all the matrices that might be amenable to prior sample pre-concentration and rapid detection, spent irrigation water from sprout production could be considered an exceptional candidate for the following reasons: (i) this is a high risk product with respect to foodborne pathogen contamination; (ii) a water-based matrix is compositionally simple, particularly when compared to other foods; and (iii) there is a federal recommendation that spent irrigation water from sprout production lots be screened for pathogens (FDA, 1999). Our method is simple and sensitive; in fact, because of the particularly low detection limits reported here, sprouters could potentially begin sampling earlier than the required 48 hours, which may be appropriate. For instance, Stewart et al. (2001), reporting on the growth of *E. coli* O157:H7 during the alfalfa sprouting process, found that low levels of the pathogen in seeds ($1.86 \log_{10}$ CFU/g) could increase as much as $3 \log_{10}$ during the first day of sprouting (36); Howard and Hutcheson (2003) and Stewart et al. (2001) reported similar results for *Salmonella* serotypes (15, 37). Even though the FDA and Fu et al. (2001) cite a lower pathogen load in water as compared to product as a potential disadvantage to testing spent irrigation water (FDA, 1999; Fu et al., 2001) this may be compensated for by the additional sensitivity gained by sample pre-concentration. Also, our method was applied to a sample size even larger than that recommended for testing (400 ml vs. 100 ml of spent irrigation water), allowing for greater sample representation and further assay sensitivity by virtue of

the larger sample size. Finally, since the sprouting process is in essence a cultural amplification step, the issue of detecting dead rather than viable pathogens is of less concern for this commodity when compared to other food products.

Strategies for the improvement of pathogen detection must be based on the widespread need within the food industry of sensitive, faster, simpler, and more inexpensive tests. There is clearly a need for better detection approaches as applied to raw seed sprout production. Current “rapid” assays require prior cultural enrichment; subsequent cultural confirmation is always necessary to confirm presumptively positive samples. Although not described in this study, by linking PCR detection to hybridization using any number of the “real-time” approaches, this assay could be designed to provide confirmed pathogen detection in less than one day. The sample pre-concentration method reported in this paper is crucial to this approach as it allows the laboratory to forego prior cultural enrichment and still achieve good detection limits. Indeed, this method provides further support that in some cases, we may be able to forego cultural enrichment in favor of more direct detection approaches.

Table 5.1. Comparison of 2.5% Tween 80, 4% PEG, and 0.1% peptone buffer on the recovery of *Salmonella* Typhimurium (10^6 CFU/g) from alfalfa sprouts

	0.1 % Peptone Buffer	2.5% Tween	2.5% Tween and 4.0%PEG
% Recovery (\pm SD) - based on loss to supernatant	95.3 \pm 6.50 ^a	99.1 \pm 0.32 ^a	96.6 \pm 0.41 ^a
% Recovery-pellet (+ SD) - based on direct plating of pellet	13.3 \pm 1.16 ^b	66.94 \pm 31.43 ^b	115.1 \pm 11.00 ^c

Table 5.2. Mean recovery of pathogens from artificially-contaminated alfalfa sprouts and spent irrigation water

	<i>Salmonella</i> Typhimurium		<i>E. coli</i> O157:H7	
	% Recovery (\pm SD) - based on loss to supernatant	% Recovery-pellet (+ SD) - based on direct plating of pellet	% Recovery (\pm SD) - based on loss to supernatant	% Recovery-pellet (+ SD) - based on direct plating of pellet
Alfalfa sprouts				
10 ⁶ CFU/g	96.6 \pm 0.55 ^a	121.2 \pm 4.10 ^b	98.6 \pm 0.52 ^a	62.0 \pm 14.30 ^b
10 ⁴ CFU/g	98.7 \pm 0.63 ^a	78.0 \pm 5.62 ^c	98.7 \pm 1.01 ^a	105.5 \pm 10.64 ^a
10 ² CFU/g	96.2 \pm 0.52 ^a	97.3 \pm 5.91 ^a	97.3 \pm 1.23 ^a	83.7 \pm 9.30 ^{ab}
Spent water				
10 ⁶ CFU/ml	99.1 \pm 0.21 ^a	95.2 \pm 6.91 ^a	97.7 \pm 0.62 ^a	80.0 \pm 12.71 ^{ab}
10 ⁴ CFU/ml	95.8 \pm 1.10 ^a	105.3 \pm 16.23 ^a	98.6 \pm 0.72 ^a	105.5 \pm 4.95 ^{ac}
10 ² CFU/ml	97.9 \pm 0.82 ^a	134.5 \pm 16.31 ^b	98.4 \pm 0.61 ^a	118 \pm 17.02 ^{ac}

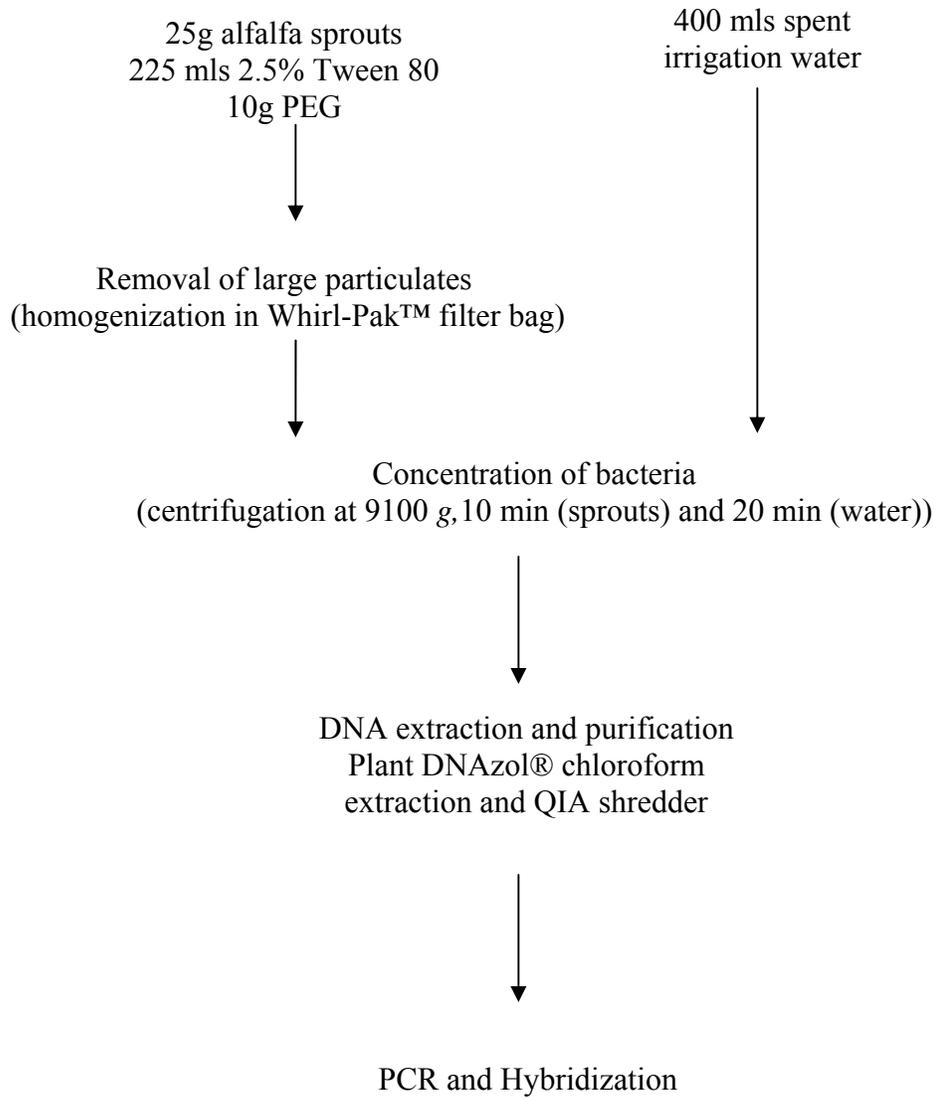


Figure 5.1. Flow diagram of sample concentration purification and detection

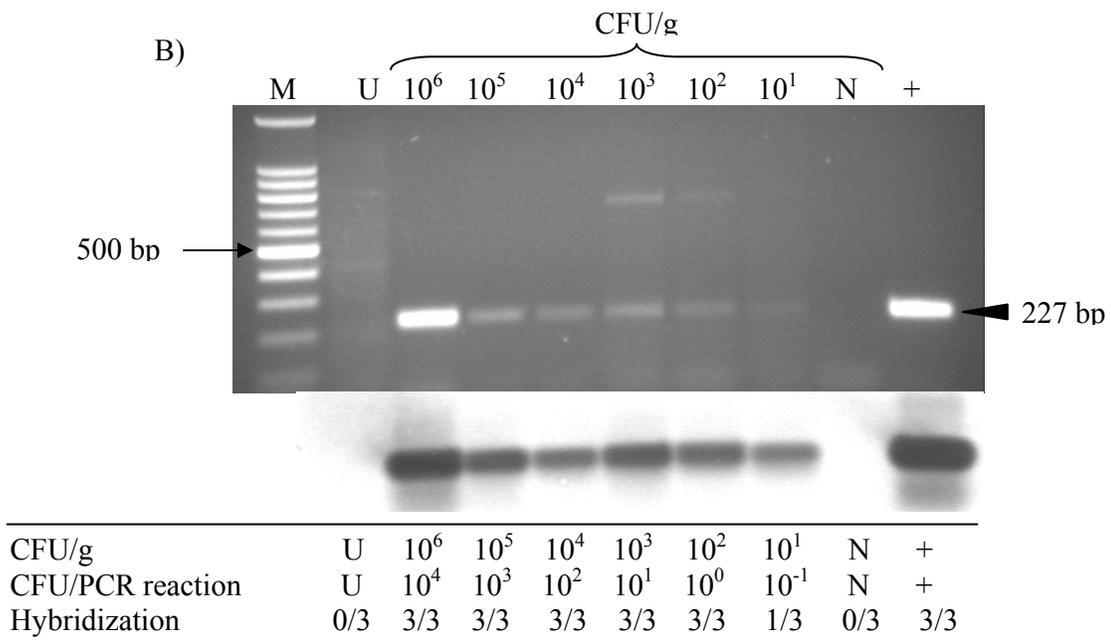
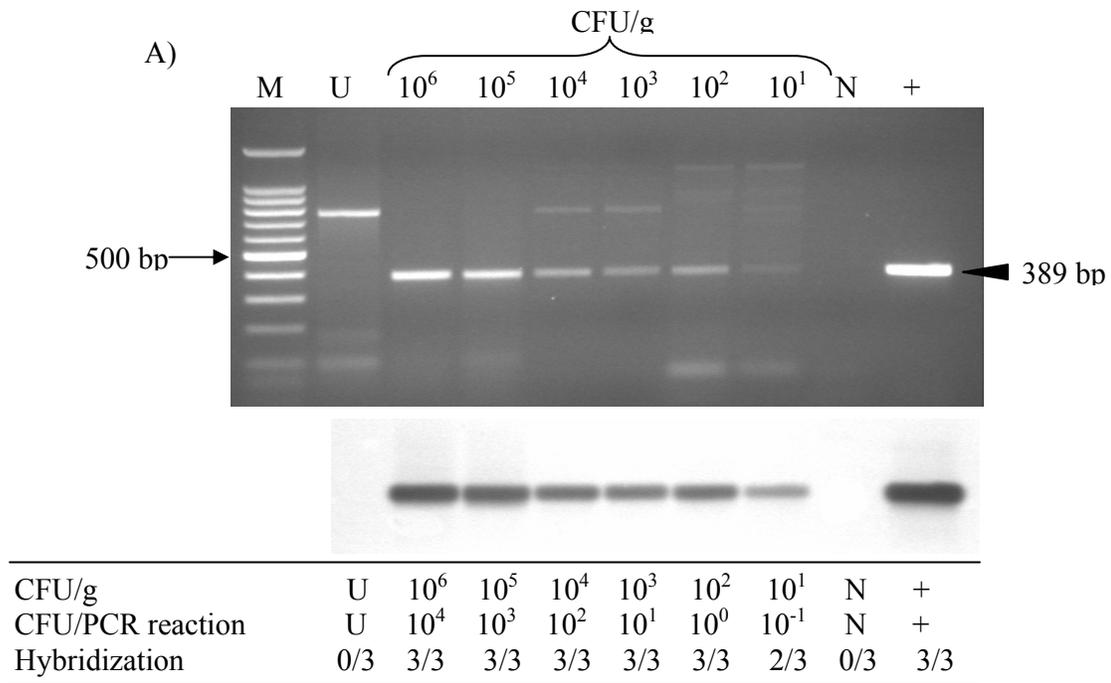


Figure 5.2. Detection of *Salmonella enterica* serovar. Typhimurium (A) and *E. coli* O157:H7 (B) in artificially contaminated sprouts after centrifugation. Twenty-five gram samples were inoculated with 10^6 to 10^1 CFU per gram and processed for bacterial concentration followed by DNA isolation, PCR amplification, and Southern hybridization. Lanes: M, marker; U, uninoculated sample; N, PCR mix without DNA template; +, positive control, whole cells from pure culture.

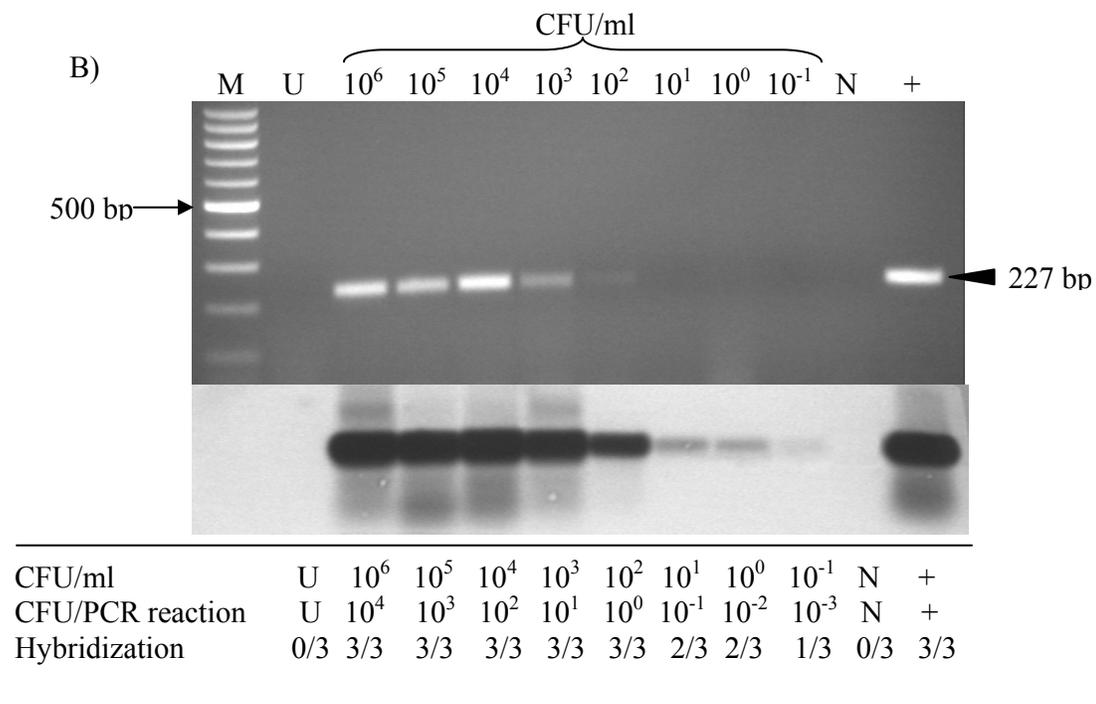
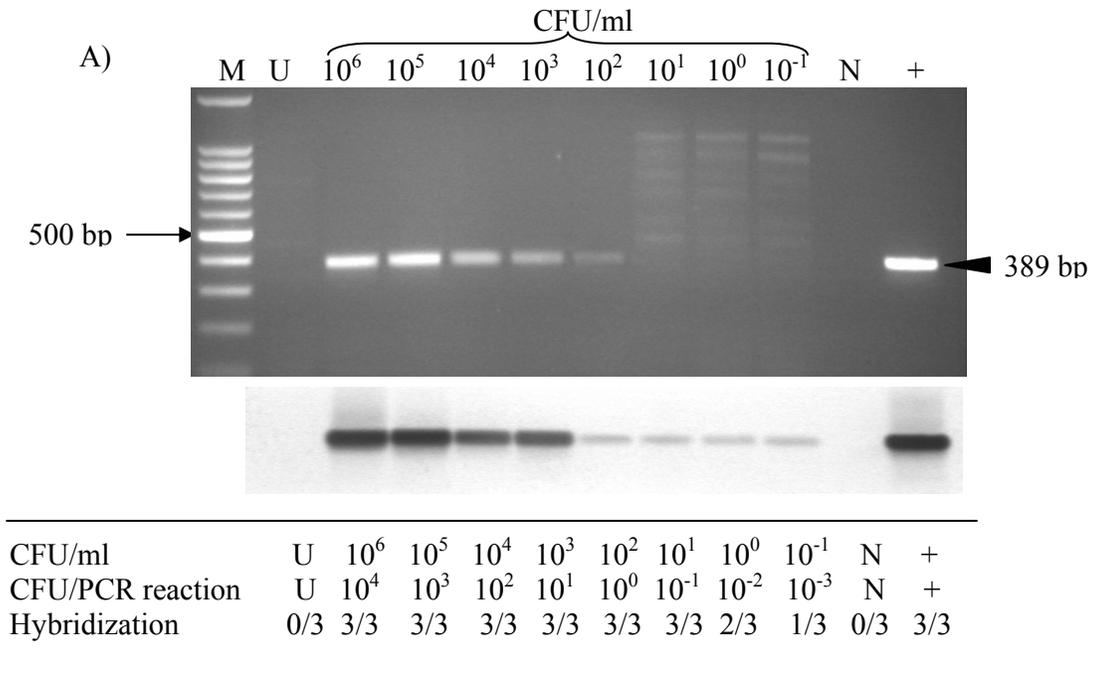


Figure 5.3. Detection of *Salmonella enterica* serovar. Typhimurium (A) and *E. coli* O157:H7 (B) in artificially contaminated spent irrigation water after centrifugation. Four hundred milliliter samples were inoculated with 10⁶ to 10⁻¹ CFU per gram and processed for bacterial concentration followed by DNA isolation, PCR amplification, and Southern hybridization. Lanes: M, marker; U, uninoculated sample; N, PCR mix without DNA template; +, positive control, whole cells from pure culture.

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CHAPTER 6

CONCLUSIONS

The fresh produce industry has experienced significant changes within the last decade. At the same time, fruits and vegetables have received much attention within the food safety sector, as the proportion of foodborne outbreaks associated with produce has increased. Numerous investigators have published information concerning the epidemiology of produce-associated illness, as well as various mitigation strategies that might prove to be effective within the industry to reduce the risk of foodborne illness. Concurrently, the U.S. government has taken steps to provide the industry with recommendations based on laboratory studies. However, there is a need to understand the reality of production and processing practices in order to establish economically effective and practical advice for the industry, which is limited by gaps in our knowledge of the microbiological quality and safety of produce. It is only with an accurate understanding of microbiological hazards to the produce industry that we can effectively reduce and/or control contamination. Along with the need for field studies, there is a need for improved rapid methods which are capable of detecting foodborne pathogens at low levels. The U.S. FDA has made specific recommendations to the seed sprout industry that producers conduct microbiological testing of spent irrigation water or the seed sprouts themselves for *Salmonella* and *E. coli* O157:H7. The final study focused on an effort to streamline such testing efforts, by developing a method to pre-concentrate pathogens from sprouts and spent irrigation water to facilitate the

direct (without prior cultural enrichment) detection of pathogens using the polymerase chain reaction (PCR).

The first and third study focused on the microbial levels of produce from farms and packing sheds. Results from these studies showed that levels of microbial indicators, including total bacteria, coliforms, enterococci, and *E. coli* remained constant, and in some cases, increased during the processing phase. Interestingly, a majority of the packing sheds used chlorine during the wash steps. Within the third study, samples of imported produce were compared to the microbiological quality of domestic produce. In most cases, imported samples were found to be of higher microbial quality than domestic samples. Microbial levels from environmental swab were quite low, with no statistically significant differences between locations within the shed. Furthermore, *Enterococcus* spp. isolated from both studies revealed an overall low resistance to antibiotics of both animal and human relevance.

Adherence to governmental recommendations can address produce quality and safety issues during growing, harvesting, sorting, packing, and distribution, and our study reinforces the frequently cited concept that every step from production to consumption will affect the microbial load of produce. Consequently, the use of thorough sanitation measures in production, and particularly during the packing shed phase is important. Government recommendations have established an important and necessary foundation for production and processing sanitation through GAPs and GMPs; however, the results from this study clearly indicate a need for the reevaluation of processing techniques. Ultimately, it is in the interest of individual growers and packers to examine their own processes and incorporate strategies for maintaining high quality produce.

In the final study, a method was developed to pre-concentrate pathogens from sprouts and spent irrigation water to facilitate the direct (without prior cultural enrichment) detection of pathogens using PCR. Alfalfa sprouts and spent irrigation water were seeded with *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7 in the range of 10^{-1} to 10^6 CFU/g or ml. Samples were blended (sprouts only) and then centrifuged at relatively high speed to sediment the total bacterial population. The precipitate was processed for DNA isolation, PCR amplification, and amplicon confirmation by Southern hybridization. Mean pathogen recoveries after centrifugation ranged from 96-99% for both pathogens in both matrices. Using primers targeting the *inv A* gene for serovar Typhimurium and the *stx* genes of *E. coli* O157:H7, it was possible to detect both pathogens in alfalfa sprouts at seeding levels as low as 10^1 CFU/g. PCR detection limits for both pathogens from spent irrigation water were 10^{-1} CFU/ml, the equivalent of 10^2 CFU/liter.

Spent irrigation water, since it is rather constitutionally simple, is particularly well suited for prior bacterial concentration using simple centrifugation steps. This study demonstrates progress toward a rapid, inexpensive, and sensitive method for the detection of pathogens associated with this commodity, and one that is relevant to current industrial practices and needs. With future optimization including “real-time” detection strategies, this method provides further support that in some cases, we may be able to forego cultural enrichment in favor of more direct detection approaches.