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Caenorhabditis elegans as a Model for Free-Living Nematodes in Vectoring Human
Pathogenic Bacteria to Fruits and Vegetables
(Under the Direction of LARRY R. BEUCHAT)

A microbivorous free-living nematode, *Caenorhabditis elegans*, was studied to determine its potential role as a vector for preharvest contamination of fruits and vegetables. Propensity of the nematode to be attracted to strains of *Escherichia coli* O157:H7, serotypes of *Salmonella*, and strains of *Listeria monocytogenes* was investigated. The nematode was attracted to all test strains and serotypes and survived and reproduced in the presence of these bacteria for up to 7 days. The potential role free-living nematodes may play in vectoring *S. Poona* to cantaloupe rind was also investigated. *C. elegans*, and perhaps, other free-living nematodes may play a significant role in the preharvest dispersal of incidental human pathogens in soil to fruits and vegetables and treatment with chemical sanitizers may not be effective in reducing populations of *S. Poona*.

INDEX WORDS: *Caenorhabditis elegans*, *Salmonella*, *E. coli* O157:H7, *Listeria monocytogenes*, free-living nematodes, fruits and vegetables

CAENORHABDITIS ELEGANS AS A MODEL FOR FREE-LIVING NEMATODES IN
VECTORIZING HUMAN PATHOGENIC BACTERIA
TO FRUITS AND VEGETABLES

by

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B.S., Alabama Agricultural and Mechanical University, 2000

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2002

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DEDICATION

To Mom, Kem, Nona, Faye, and Granddaddy

ACKNOWLEDGEMENTS

Many people have supported me throughout my academic career. I cannot begin to express my gratitude for all that they have done. I hope the words printed on this page will give these individuals some indication of how important their being there really meant to. I would like to thank the following people:

Dr. Larry R. Beuchat, for his guidance, patience, and creativity

Dr. Ynes Ortega for serving on my advisory committee and insight

Dr. Phillip L. Williams for the opportunity to work in his lab and support

My mentors back at Alabama A&M University, especially Dr. Lloyd T. Walker, who made me understand the value of hard work and the success that it brings.

The faculty and staff at the Center for Food Safety, especially Barbara B. Adler, Alan Scouten, and Kim Hertz. Dr. Gary L. Anderson for teaching me the basic procedures for maintaining worms in the laboratory.

The current and former students at the Center for Food Safety: Gloria Tetteh, Glenner Richards, Manan Sharma, Wendy Wade, Sarah Holliday, and Megan Lang for their encouragement and support throughout the process.

My family back in Huntsville, AL, whose love, support and encouragement helped me through the hard times. I owe this all to you. To Chris, thanks for being there.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
CHAPTER	
1 INTRODUCTION AND LITERATURE REVIEW	1
2 ATTRACTION OF A FREE-LIVING NEMATODE, <i>CAENORHABDITIS ELEGANS</i> , TO FOODBORNE PATHOGENIC BACTERIA, AND ITS POTENTIAL AS A VECTOR OF <i>SALMONELLA</i> POONA FOR PREHARVEST CONTAMINATION OF CANTALOUPE	38
3 INGESTION OF <i>SALMONELLA</i> POONA BY A FREE-LIVING NEMATODE, <i>CAENORHABDITIS</i> <i>ELEGANS</i> , AND PROTECTION AGAINST TREATMENT WITH SANTITIZERS.....	65
4 SUMMARY AND CONCLUSIONS.....	90

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The consumption of raw fruits and vegetables in the United States has increased rapidly over the past several years. This increase can be in part attributed to desired health benefits associated with the consumption of fresh or minimally processed produce. Nutritionists and health professionals have shown that diets low in fat, high in fiber, and consisting of at least five servings of fruits and vegetables per day are protective against some types of cancer and lessens the risk of heart disease (Rangarajan et al., 2000). An abundance of research during the last decade has shown that a low-fat diet, high in fruits and vegetables, decreases the occurrence of coronary heart disease (Kennedy et al., 1996; Kushi et al., 1995; National Research Council, 1989). Considering that the diet may be responsible for 65% of all cancers, it is important to increase the consumption of fruits and vegetables in order to enhance the potential for reduction of risk of cancer and chronic disease.

Fresh fruits and vegetables are raw agricultural products grown in a natural environment (Delea, 2001). Referred to as raw or fresh produce, they are likely to be sold to consumers in an unprocessed or minimally processed form (U.S. Department of Health Human Services, 1998) or be offered to consumers as ready-to-eat or ready-to-use products (Nguyen-The and Carlin, 1994). Minimally processed fruits and vegetables may be simply trimmed, peeled, sliced/shredded, and washed or disinfected (Francis et al., 1999). The shelf life of minimally processed fruits and vegetables at refrigeration temperatures (2-5°C) ranges from 4-5 days for mixed products and 8-10 days for low-pH salads (Heard, 1999).

Fruits and vegetables can become contaminated with pathogenic bacteria, parasites, and viruses while growing in fields or orchards, or during harvesting, post-harvest handling, processing, and distribution (Beuchat, 1996). Variations in size, shape, surface topography (rough, highly textured, creviced, smooth), presence of entry points (e.g., calyx and stomata) to internal tissues, fragility, and perishability among produce are a challenge to those responsible and for maintaining safety (Cherry, 1999).

Microbiology of Fresh Vegetables

Both Gram-negative and Gram-positive bacteria are present on vegetables at the time of harvest. Gram-negative bacteria of concern in fresh produce include *Aeromonas hydrophila*, some strains of *Escherichia coli*, *Salmonella*, *Shigella*, *Plesiomonas shigelloides*, *Vibrio cholerae*, and *Yersinia enterocolitica*; Gram positive bacteria of public health concern include *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, and *Listeria monocytogenes* (Brackett, 1999). Parasites such as *Cryptosporidium parvum* and *Cyclospora cayetanensis*, and viruses such as hepatitis A and Norwalk, also have been causative agents in outbreaks of human illness associated with produce (Ranagarajan et al., 2000). *Giardia lamblia* has also been implicated in causing illness as a result of consuming raw sliced vegetables (Beuchat, 1996). Infections associated with consumption of fresh fruits and vegetables are primarily those transmitted by a fecal-oral route (NACMCF, 1999).

The survival and growth of microorganisms pathogenic to humans on the surface of unprocessed vegetables depends on the presence of free moisture, relative humidity, temperature, and exposure to sunlight (Nguyen-The and Carlin, 1994). Also, the microbial biota of vegetables reflects the soils in which they are grown (Jay, 2000).

The survival of enteric bacteria in the soil is dependent on level of inoculum, soil type, moisture retention, pH, microbial antagonism, and nutrient availability (Geldreich and Bordner, 1971). Brackett (1999) indicated that some bacteria can survive in soils for months or even years.

Raw vegetables may readily support growth of pathogens once plant cell integrity has been lost as a result of wilting, ageing, or injury such as chopping, shredding, bruising, or juicing (ICMSF, 1998). Minimal processing damages fruit and vegetable tissues, resulting in leakage of cellular fluids containing nutrients and intracellular enzymes (Ahvenainen, 1996). This is of interest since Ackers et al. (1998) showed that most retail stores display their produce above 12°C, a temperature at which many human pathogens can grow. Abdul-Raouf et al. (1993) reported that *E. coli* O157:H7 can grow on shredded lettuce, sliced cucumber, and shredded carrots stored at refrigeration temperature.

The properties of vegetables that influence growth of bacteria include pH, type and amount of organic acids, and the presence of anti-bacterial compounds (Lund, 1992). The pH of vegetables is usually within a range that will support the growth of foodborne bacteria (Lund, 1992). Brackett (1997) stated that the pH of vegetables (5.0-6.0) does not inhibit the growth of most microorganisms. The ability of bacteria to multiply on vegetables is influenced strongly by environmental factors, including the storage temperature, presence of free water, relative humidity, and gaseous environment (Lund, 1992). Vegetables are less often contaminated with foodborne pathogens than are foods of animal origin (Nyguen-the and Carlin, 1994).

The temperature, at which vegetables are harvested, transported, processed, and packaged influences the numbers and types of microorganisms present and also the rate at which they grow (Heard, 1999). Brackett (1987) noted that, among environmental factors, temperature probably has the greatest influence on the growth of microorganisms.

Microbiology of Fresh Fruits

Historically, fruits have had an exceptionally good public health record. Outbreaks of foodborne illness associated with the consumption of fruit occur less frequently than outbreaks associated with other food groups. This may largely be attributed to natural defense mechanisms that many fruits possess (Doyle, 1990). Natural barriers exhibited by fruits inhibit the survival and growth of many pathogenic microorganisms. Natural barriers include the thick skin of many fruits, which is usually removed or thoroughly washed before the fruit is eaten, and antimicrobial substances such as essential oils and/or organic acids that often maintain the $\text{pH} < 4.6$ (Goepfert, 1980). Most fruits are more susceptible to decay by fungi rather than by bacteria because of their low pH (ICMSF, 1996). However, some fully matured fruits have pH values approaching 7 and, once cut to expose the internal flesh to environmental contaminants, can serve as substrates for the growth of bacteria (del Rosario and Beuchat, 1995). Lund (1992) and Brackett (1999) also indicated that some fruits have a pH that is sufficiently high enough to allow the growth of pathogenic bacteria. The pH of most mature fruits and fruit products is < 4.0 . The low pH of many fruits is a major factor that influences the composition of their microflora (Splittstoesser, 1996).

In many foods, acidity is an important preservative factor, either alone or in combination with other inhibitory factors (Ferreira and Lund, 1987). The peel of citrus fruits serves as a natural protectant that prevents microbiological contamination of the interior flesh. Thus, removing the peel eliminates this protective layer and subjects the edible portion to potential microbial invasion and spoilage (Pao et al., 1998). Nutrients in plant tissue fluids released by damaged cells will support survival and growth of microorganisms originally present on the exterior of the fruit. Pao et al. (1998) concluded that exposure of peeled oranges to abusive temperatures can result in considerable growth of foodborne pathogens such as *Salmonella* and *L. monocytogenes* within 12 h. Mishandling, improper storage temperatures, and cross-contamination with other hazardous raw food products are the most common modes of contamination of produce (Bean et al., 1990, 1996; Beuchat, 1996; Knabel, 1995).

Outbreaks of Infections Associated with Fresh Fruits and Vegetables

American consumers enjoy one of the safest supplies of fresh produce in the world. However, over the last several years, documented outbreaks of foodborne illness associated with both domestic and imported fresh fruits and vegetables have increased (U. S. Department of Health Human Services, 1998). Advances in agronomic, processing, preservation, distribution, and marketing technologies have enabled the produce industry to supply nearly all types of high-quality fresh fruits and vegetables to those who desire and are willing to purchase them year round (Beuchat, 1996). Beuchat and Ryu (1997) also listed consumer demand, changes in the produce industry, and social demographics as reasons for changes in patterns of produce consumption and hygiene.

The importation of fruits and vegetables from developing countries may pose a public health problem to the produce industry, since agricultural practices in some countries differ significantly from those practiced in the United States. It has been speculated that imported produce may be less safe than domestically grown produce (Delea, 2001). This is supported by higher populations of *E. coli* (>10/g) found on vegetables imported from tropical countries (Tamminga et al., 1978), which is an indicator of the potential presence of pathogens.

Contamination of fresh produce with pathogenic bacteria, parasites, and viruses can occur at any step in the process from pre-harvest environments to preparation at home by the consumer. Contamination in the field may be a result of poor agricultural practices by the grower and field workers. The grower may use raw or improperly composted manure to fertilize crops. This introduces the potential for contamination by *E. coli* 0157:H7, *Campylobacter jejuni*, *Salmonella*, *L. monocytogenes*, *Cyclospora*, *Cryptosporidium*, *Giardia*, *Toxoplasma*, and viruses. In addition, field workers may not be properly trained concerning proper hygiene for harvesting and handling raw produce. Water used for irrigation or washing produce may be contaminated with human pathogenic microorganisms. There is also the potential for contamination of produce via insects, wild birds, and animals that may be present in the growing area. Produce that comes in contact with soil may also pose a risk for contamination with pathogenic microorganisms that are normal soil inhabitants. Among the pathogenic bacteria in this group are *L. monocytogenes*, *B. cereus*, and *C. botulinum*.

Risk of contamination of fresh produce also exists in processing facilities.

Contamination may result from contact with soiled utensils or equipment surfaces or by using contaminated wash water. Contamination may also occur once the produce is at a food service facility or in the home of the consumer. Inadequate washing produce, cross contamination, or improper storage can lead to increased safety risks.

Outbreaks Caused by Consuming Fresh Vegetables

Investigations of outbreaks associated with fresh produce are often difficult. Fresh produce is obtained from rapidly changing geographic locations, and has a short shelf life, so implicated produce or even produce originating from the same location are rarely available by the time an outbreak has been recognized (Tauxe et al., 1997).

Investigations of outbreaks of human infections associated with fresh produce in the United States have generally revealed that the causative microorganism is widely dispersed in test lots, and contamination of fresh produce is intermittent and at a low level. Therefore, produce-associated outbreaks are typically geographically diffuse and there is a low attack rate (Tauxe et al., 1997). Between 1973 and 1987, among foodborne outbreaks with an identified food vehicle reported to the Centers for Disease Control and Prevention, 2% of outbreaks and 2% of outbreak-associated cases were linked to fresh produce. Between 1988 and 1991, these percentages increased to 5 and 8%, respectively, owing primarily to the number of fruit-associated outbreaks (Tauxe et al., 1997).

Lettuce was the vehicle in three major outbreaks of shigellosis that occurred in Texas in 1983 and 1986 (Martin et al., 1986; Davis et al., 1988). Abdul-Raouf et al. (1993) indicated that lettuce is a biologically plausible vehicle because studies have shown that populations of *E. coli* O157:H7 increased at 12°C and 21°C during storage for up to 14

days. In 1998, California restricted the sale of raw sprouts after three sprout-related outbreaks of *Salmonella* and *E. coli* O157:H7 infections (Thayer and Rajkowski, 1999).

Coleslaw was implicated in the first documented produce-associated outbreak of listeriosis (Schlech et al., 1983). *Listeria monocytogenes* serotype 4b was identified as the causative agent, and the epidemic strain was isolated from coleslaw obtained from the refrigerator of a patient and from two unopened packages obtained from the same manufacturer of coleslaw consumed by the patient (Doyle, 1990). A farmer who supplied cabbage to a regional manufacturer was identified. It was discovered that he raised the cabbage and maintained a flock of sheep that tested positive for listeriosis (Doyle, 1990). Sheep manure was used to fertilize the cabbage, which ultimately resulted in the outbreak of listeriosis in humans.

Outbreaks Caused by Consuming Fresh Fruits

Fresh fruits have not been implicated as often as fresh vegetables in causing outbreaks of infections. Splittstoesser (1996) noted that fruits such as melons may contain enteric pathogens on their rind that can be introduced onto flesh when the fruit is cut. Studies by Lin and Wei (1997) demonstrated that *Salmonella* on the surface of tomatoes is transferred into the tomato flesh during slicing, and will subsequently grow. In June 1991, watermelon was the source of an outbreak of *Salmonella* Javiana among school children in Michigan (Bolstein, 1993). The school children became ill after they consumed watermelon slices served at room temperature over a 3-h period.

***Escherichia coli* O157:H7**

General Characteristics

Escherichia coli O157:H7 was first recognized as a human pathogen following two hemorrhagic colitis outbreaks in 1982 (Riley et al., 1983). It is now recognized as an important cause of bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) in humans (Doyle, 1991). *Escherichia coli* is commonly present in the normal facultative anaerobic microflora of the intestinal tracts of humans and warm-blooded animals (Doyle et al., 1997). The bacterium is characterized as a Gram-negative, facultative anaerobe.

Isolates of *E. coli* are serologically differentiated on the basis of three major surface antigens, viz., the O (somatic), H (flagella), and K (capsule) antigens (Doyle et al., 1997). Six groups of *E. coli* that cause diarrheal illness are categorized into specific groups based on virulence properties, mechanisms of pathogenicity, clinical syndromes, and distinct O:H serogroups. Groups have been designated as enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EaggEC), enteropathogenic (EPEC), and diffusely adherent (DAEC) (Buchanan and Doyle, 1997).

Escherichia coli O157:H7 is included in the EHEC group. All EHEC strains produce compounds cytotoxic to African green monkey kidney (Vero) cells (Doyle et al., 1997), and Shiga toxin (Stx1) and/or Shiga toxin 2 (Stx2), also referred to as verotoxin 1 (VT1) and verotoxin 2 (VT2), respectively (Buchanan and Doyle, 1997). Only those strains that cause hemorrhagic colitis are considered EHEC (Doyle et al., 1997).

Reservoirs and Transmission

Escherichia coli is commonly found in intestinal microbiota of humans and other animals and was, until the late 1950s, recognized as a non-pathogenic cohabitant (Olsvik et al., 1991). The association of *E. coli* O157:H7 with undercooked ground beef and raw

milk has led investigators to assume that cattle are reservoirs for the pathogen (Buchanan and Doyle, 1997). Infections have been most often associated with the consumption of raw or undercooked foods of bovine origin (Doyle et al., 1997). Over the past decade, millions of dollars worth of ground beef contaminated with *E. coli* O157:H7 have been recalled from the market place and destroyed (Zottola, 2001). Transmission of *E. coli* O157:H7 is typically the result of eating foods contaminated with the pathogen or by person to person spread (Neill, 1989). Other foods implicated in *E. coli* O157:H7 infections are raw vegetables, apple cider, cantaloupe, salad dressing, and salami (Doyle et al., 1997).

Illness and Infective Dose

Three principal manifestations of illness have been attributed to *E. coli* O157:H7: hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (Padhye and Doyle, 1992). The initial symptoms of HC generally occur 24-48 h after eating contaminated food (Buchanan and Doyle, 1997). The symptoms are usually a sudden onset of severe crampy diarrhea, which later becomes grossly bloody (Doyle, 1991). Typically there is little fever and the duration of the illness is 2 to 9 days (Doyle, 1991). HUS develops approximately 7 days after an acute diarrheal episode (Neill, 1989), and is the leading cause of acute renal failure in children (Doyle, 1991). Individuals that develop HUS often require dialysis and blood transfusions, and may develop central nervous system disease characterized by frequent seizures and coma (Doyle, 1991). The mortality rate resulting from HUS is 3-5% (Buchanan and Doyle, 1997). TTP, another complication resulting from HC, resembles HUS except that it generally causes less renal damage (Buchanan and Doyle, 1997).

Analysis of foods associated with outbreaks of *E. coli* O157:H7 infections has revealed that the infective dose is low. Some data suggest that this number may be less than a few hundred cells (Doyle et al., 1997). The pathogen causes disease by its ability to adhere to the host cell membrane and then produce Stx1 and/or Stx2 toxins (Doyle et al., 1997).

Survival and Growth

Survival and growth of *E. coli* O157:H7 in foods are dependent on the interaction of various intrinsic and extrinsic factors such as temperature, pH, and water activity (Buchanan and Doyle, 1997). The minimum growth temperature for *E. coli* O157:H7 under otherwise optimum conditions is approximately 8-10°C (Buchanan and Bagi, 1994). Studies have indicated that the organism grows rapidly between 30-42°C in trypticase soy broth, with generation times ranging from 0.49 h at 37°C to 0.64 h at 42°C (Doyle and Schoeni, 1984). Studies conducted by Palumbo et al. (1995) indicate that the maximum temperature for *E. coli* O157:H7 growth is culture medium-dependent; all test strains grew in brain heart infusion (BHI) broth at 45°C, but six out of sixteen strains did not grow in EC broth.

The minimum pH for *E. coli* growth is 4.0-4.5 (Buchanan and Bagi, 1994). Buchanan and Doyle (1997) noted that the minimum pH for growth is dependent on other growth parameters. For example, other stresses raise the minimum pH for growth. When the pH falls below the minimum for growth, *E. coli* O157:H7 populations decline over time (Buchanan and Doyle, 1997). The survival of *E. coli* O157:H7 in low-pH foods is of particular importance. *Escherichia coli* O157:H7 has been shown to survive in apple cider (Zhao et al., 1995) and dry-cured salami (CDC, 1993).

Acid Adaptation and Tolerance

Numerous outbreaks involving low acid foods have led researchers to suggest that *E. coli* O157:H7 may be more tolerant than other serotypes of *E. coli* to acidic conditions. The mechanism of acid tolerance has not been fully elucidated but appears to be associated with a protein(s) that can be induced by exposing the cells to acid conditions (Doyle et al., 1997). Studies done by Leyer et al. (1995) showed that *E. coli* O157:H7 is capable of eliciting an acid-adaptive response, and the expression of this response enhances survival in the presence of lactic acid and in acidified food products such as fermented sausage and apple cider. An outbreak of *E. coli* O157:H7 infection associated with the consumption of apple cider raised concerns regarding the safety of the low-pH products. Prior to the outbreak, apple cider was thought not to support survival and growth of pathogens because of its high acidity and low pH (<4.0) (Doyle et al., 1997). Acid tolerance is probably an important component of virulence for *E. coli* O157:H7 as it may allow a small number of cells to be protected in the gastrointestinal tract and cause illness (Leyer et al., 1995).

Studies conducted by Benjamin and Datta (1995) indicated that many EHEC strains survive for long periods (5 h) in an acidic pH at 37°C, a situation similar to the human gastric environment.

Detection and Isolation

There are several methodologies and procedures for the detection and isolation of *E. coli* O157:H7 in foods. Most conventional procedures are quite tedious and time consuming. The common isolation strategy involves the use of one of several combinations of selective broths and agars, typically with indicators for enzymatic

activities such as sorbitol fermentation (Johnson et al., 1998). Padhye and Doyle (1991) developed the enrichment sandwich ELISA procedure that is rapid, sensitive, and specific for the detection and isolation of *E. coli* O157:H7. Presumptive identification of as few as 0.2 *E. coli* O157:H7 CFU/g of ground beef can be completed in <20 h, and the organism can be isolated and later confirmed within 2 days following presumptive detection (Padhye and Doyle, 1991).

Okrend et al. (1990) described a screening method for detecting and isolating *E. coli* O157:H7 in beef. The method involves using a commercially available reactive disc blot enzyme-linked immunosorbent assay (ELISA) that has a minimum level of sensitivity of 0.6 *E. coli* O157:H7 CFU/g of food, with 0% false-negatives and 2% false-positives. All positive samples must be confirmed, which usually requires 4-5 days, because detection of the O antibody is not specific for enterohemorrhagic *E. coli* O157:H7.

Johnson et al. (1998) compared the BAX screening method with conventional methods for the detection of *E. coli* O157:H7. They suggested that routine culturing, isolation, and recovery of *E. coli* O157:H7 in ground beef are best achieved using a combination of media, not by relying on a single type. The BAX system assay gave positive results for 96.5% of samples that proved positive by any available test in comparison to the conventional methods, which detected only 67% of the positive samples.

Salmonella

General Characteristics

Salmonella is a facultative, anaerobic, Gram-negative rod belonging to the family Enterobacteriaceae (D'Aoust, 1997). It grows optimally at 37°C and catabolizes D-

glucose and other carbohydrates, with the production of acid and gas. *Salmonellae* are oxidase negative and catalase positive, grow on citrate as a sole carbon source, generally produce hydrogen sulfide, decarboxylate lysine, and ornithine, and do not hydrolyze urea (D'Aoust, 1997). The organism is widely distributed in nature, with mammals being their primary reservoirs (Jay, 2000).

Reservoirs and Transmission

Poultry products are the principal reservoirs of *Salmonella* in many countries (D'Aoust, 1997). The feeding habits and close proximity of multiplier breeder, layer, and boiler chickens in rearing facilities encourage the rapid and widespread dissemination of *Salmonella* in poultry houses.

As a result of increased global export of poultry as well as fruits and vegetables in recent years, both have been associated vehicles of human salmonellosis (D'Aoust, 1997). *Salmonellae* have also been isolated from coconut meal, salad dressing, mayonnaise, milk (Jay, 1997), watermelon (Blostein, 1993), and many other foods. Escartin et al. (1989) reported that *Salmonella* is capable of growing on sliced jicama, papaya, and watermelon. Golden et al. (1993) reported that *Salmonella* is capable of rapid and prolific growth on cantaloupe, watermelon, and honeydew incubated at 23°C.

Salmonellosis

Salmonellosis is a term used to describe human and animal infections caused by serotypes of the genus *Salmonella* (Forsythe and Hayes, 1998). Human salmonellosis from non-typhoid strains of *Salmonella* generally occurs as a self-limiting episode of enterocolitis with resolution of symptoms within 5 days of onset of disease, which

appears 8-72 h after contact with the invasive pathogen (D'Aoust, 1991b; D'Aoust, 1997). Symptoms are abdominal pain, nausea, and watery diarrhea with occasional mucus and traces of blood in stools is not uncommon (D'Aoust, 1991b). *Salmonella* can invade the blood stream and thus cause septicaemia. In the more extreme cases, the patient may go into a coma (Forsythe and Hayes, 1998). Susceptibility to infection is highest in infants, elderly people, and immunocompromised individuals (D'Aoust, 1991b). The mortality rate is relatively low, being <1% (Forsythe and Haynes, 1998).

Survival and Growth

Several factors, including pH, salt concentration, temperature, water activity, and nutrient availability in suspending media can synergistically or antagonistically affect the growth of foodborne *Salmonella* (D'Aoust, 1991a). Zhuang et al. (1995) concluded that *Salmonella* can survive and grow on the surface of mature, intact tomatoes held at ambient temperature, and that growth is rapid (reaching 10^8 CFU/g within 24 h) in chopped ripe tomatoes stored at ambient temperature. It has been shown by Lin and Wei (1997) that *Salmonella* will grow in sliced tomatoes.

The minimal growth temperature of *Salmonella* is dependent on food type, temperature, and prevailing pH conditions (D'Aoust, 1991a). Acidity not only affects growth but also contributes to the persistence of salmonellae in refrigerated foods (D'Aoust, 1991a). Asplund and Nurmi (1991) found that citric acid permits the growth of *Salmonella*, even at low pH values. Ferreira and Lund (1992) showed that *Salmonella* can grow in a culture medium at pH 3.8-4.2 when incubated at 30°C or 20°C. The ability of *Salmonella* to grow at low temperatures (psychotrophy) tends to compromise the purported safety of refrigerated foods (D'Aoust, 1991a).

Acid Adaptation and Tolerance

The minimum pH at which *Salmonella* is able to initiate and sustain growth is not well defined but will vary depending on the serotype, temperature of incubation, and the nature and composition of the growth medium (Chung and Goepfert, 1970). *Salmonella* Typhimurium can grow over a wide pH (pH 5- 9) because of physiologically triggered pH homeostasis mechanisms (Foster and Hall, 1991). Foster and Hall (1990) demonstrated that *S. Typhimurium* can be adapted to survive in an pH environment with pH < 5.0. Roering et al. (1999) reported that *S. Typhimurium* DT104 can survive well for 7 days in preservative-free apple cider (pH 3.3-3.5) stored at 4°C.

Salmonella Typhimurium possesses a novel system of acid stress management, including inducible pH homeostasis (Foster and Hall, 1990). Foster Hall (1991) described the induction of acid tolerance as a two-stage process. The process is logical, considering that in nature pH gradient transitions are more likely to be encountered, rather than sharp, severe changes (Foster and Hall, 1991). The first stage, triggered at pH below 6.0, induces synthesis of an acid tolerance response (ATR) -specific homeostasis mechanism, referred to by Foster and Hall (1991) as pre-shock adaptation. The second stage, triggered below pH 4.5, induces synthesis of a different set of proteins which by themselves will not afford protection against extreme low pH, commonly referred to as the acid shock stage of ATR (Foster and Hall, 1991). Acid tolerance response is triggered in *Salmonella* at pH values between 5.5 and 6.0, but protects cells against much lower pH (pH 3.0-4.0) when non-adaptive pH homeostasis normally fails (Foster and Hall, 1991).

Detection and Isolation

Salmonella typically produces acid and gas from glucose but does not utilize lactose or sucrose in triple sugar iron (TSI) agar or in differential media such as brilliant green, xylose lysine deoxycholate, and Hektoen enteric agars (D'Aoust, 1997). Pignato et al. (1995) showed that the Salmosyst-RA method has several advantages over conventional and rapid non-cultural methods. Advantages are that only two media are required in contrast to five media required for conventional methods. In real time, it is comparable to other rapid non-cultural methods, which require 30 to 31 h; also, the method is highly sensitive and specific, and enables the isolation of *Salmonella* that can then be characterized by appropriate phenotypic and genotypic typing methods for epidemiologic investigations.

Chemical and Physical Methods to Reduce Pathogens on Fresh Produce

Effectiveness of Water

Flume and spray washing of raw fruits and vegetables can be successfully used to reduce surface populations of microorganisms. However, Beuchat (1992; 1998) also noted that sterilization by repeated washing, even with sterile water, cannot be achieved because viable microorganisms within tissues of produce remain in place. Ukuku and Sapers (2001) studied the efficacy of sanitizer treatments on *Salmonella* Stanley on cantaloupe and concluded that washing with water alone was not effective in removing surface-adherent cells. Fresh-cut cubes prepared from cantaloupes washed with water were *Salmonella*-positive (Ukuku and Sapers, 2001). Although washing produce in tap water may have some effectiveness in removing soil and other debris, it should not be relied upon to completely remove microorganisms (Beuchat et al., 1998). Interestingly,

washing with tap water is the currently recommended means for consumers to reduce microbial contamination on raw fruits and vegetables.

Effectiveness of Chlorine

The United States Department of Health and Human Services (1998) stated that the effectiveness of an antimicrobial agent depends on its chemical and physical state, treatment conditions (water temperature, acidity [pH], and contact time), resistance of pathogens, and the nature of fruit or vegetable surface. This contention is supported by studies done by Zhang and Farber (1996) showing that the effectiveness of chlorine as a sanitizer against *L. monocytogenes* is influenced not only by the time of exposure and concentration of chlorine, but also by the temperature and type of vegetable used. Results reported by Brackett (1997) using chlorine to eliminate *L. monocytogenes* from fresh produce indicated that although populations were reduced, chlorine was not effective in eliminating the pathogen. Cherry (1999) described the types of microorganisms, their attachment mechanisms, and physical characteristics of fruits and vegetables. The concentration of chlorine used, time taken to process vegetables, and temperature during processing are important factors controlling the effectiveness of chlorine washes (Guerzoni et al., 1996).

Chlorine has been used for many years to treat drinking water and wastewater as well as to sanitize food processing equipment and surfaces in processing environments (Beuchat, 1998). Inhibition of enzymes sensitive to oxidation by chlorine appears to be a mechanism in the inactivation of microorganisms (Beuchat, 1992). Cherry (1999) stated that chlorine is effective in killing 1-2 log CFU/g. Ukuku and Sapers (2001) observed that washing inoculated cantaloupe in 1,000 ppm chlorine for 5 min resulted in a 3.4 log₁₀

CFU/cm² reduction in *S. Stanley*. Research conducted by Jaquette et al. (1996) showed that a 2,000 to 4,000 ppm chlorine soak can reduce populations of *S. Stanley* on alfalfa seeds before germination, but cannot guarantee that sprouts will be free of *Salmonella*. Zhuang et al. (1995) reported that chlorination should be considered as a sanitizer treatment and not as a means of elimination of viable *Salmonella* Montevideo on inoculated tomatoes.

Inaccessibility of hydrochlorous acid to microbial cells in cracks, crevices, pockets, and natural openings on the surface of fruits and vegetables undoubtedly also contributes to the overall lack of effectiveness of chlorine (Beuchat, 1998). Seo and Frank (1999) stated that a major impediment to effective lettuce leaf disinfection is removing or inactivating pathogens that have penetrated damaged surfaces or that are trapped within the stomata.

Effectiveness of Hydrogen Peroxide

Hydrogen peroxide (H₂O₂) has GRAS status for its use in food products as a bleaching agent, oxidizing agent, and antimicrobial agent (Sapers and Simmons, 1998). These researchers reported that treatment with H₂O₂ appears to reduce microbial populations on fresh-cut fruits and vegetables and extends the shelf-life without leaving large amounts of residues or loss of quality. Their study indicated that H₂O₂ treatment reduced the population of fluorescent pseudomonads on mushrooms, zucchini, and cantaloupe by 90% and was similar in effectiveness to chlorine.

Effectiveness of Organic Acid Washes and Sprays

The use of washes and sprays containing organic acids has been useful in the decontamination of beef, poultry, lamb, and pork carcasses (Beuchat, 1992). The effectiveness of organic acids is dependent upon their dissociation constant(s) (pK_a). Due to the acidic pH of the fruits, organic acids are in an active undissociated form.

Pathogenic microorganisms present on the surface of raw produce have the potential to contaminate the interior flesh during slicing. Application of lemon juice to sliced fruit significantly reduces populations of *Shigella* for a short time (Escartin et al., 1989).

The presence of naturally occurring organic acids such as acetic, citric, succinic, malic, tartaric, benzoic, and sorbic acids in fruits hinder the growth of most bacteria (Beuchat, 1998).

Free Living Soil Nematodes (*Caenorhabditis elegans*)

General Characteristics and Anatomy

Caenorhabditis elegans (Figure 1.1) is a, free-living nematode found commonly in soil in many parts of the world. It feeds primarily on bacteria and lives for approximately 2 weeks under optimal conditions (Wood, 1998). *Caenorhabditis elegans* has a typical nematode body structure, with an outer tube that consists of cuticle, hypodermis, neurons, and muscles surrounding a pseudocoelomic space that contains the intestine and gonad (Wood, 1998). The transparency of the body, the constancy of cell number (eutely), and the constancy of cell position from individual to individual are perhaps the most unique characteristics offered by this organism for the study of development (Riddle et al., 1997).

Caenorhabditis elegans has a limited temperature range of approximately 12 to 26°C within which it is viable and fertile (Bargmann and Mori, 1997). It is a simple organism, both anatomically and genetically. The adult hermaphrodite has only 959 somatic nuclei, whereas the adult male has 1031 (Wood, 1998). This soil nematode offers great potential for genetic analysis, partly because of its rapid 3-day life cycle, small size (1.5 mm long, adult), and ease of laboratory cultivation (Riddle et al., 1997). *Caenorhabditis elegans* is a voracious predator that will eat anything that fits into its mouth (Riddle et al., 1997). In the soil, it apparently seeks to consume all available nutrient resources fairly quickly as a means to outgrow its competitors (Riddle et al., 1997). It possesses several advantages as a test model, including simple growth requirements, a rapid generation time with an invariant cell lineage, and the fact that the genetic molecular tools for its manipulation are well defined (Kruz and Ewbank, 2000).

Caenorhabditis elegans is a filter feeder. The worms take in liquid with suspended particles (bacteria) and then spit out the liquid while retaining the particles (Avery and Thomas, 1997). A valve controls the flow of food from the pharynx into the intestine, which terminates in a rectum and anus (Ehrenstein and Schierenberg, 1980). In the laboratory, *C. elegans* feeds on *E. coli* by rapid contraction of pharyngeal muscles that pump the bacteria into the intestine (Darby et al., 1999).

Free-living nematodes live with significant temperature fluctuations, and their ability to tolerate or adapt to thermal changes is probably an important factor in determining distribution (Dusenbery, 1980). It has the ability to survive in an anaerobic environment.

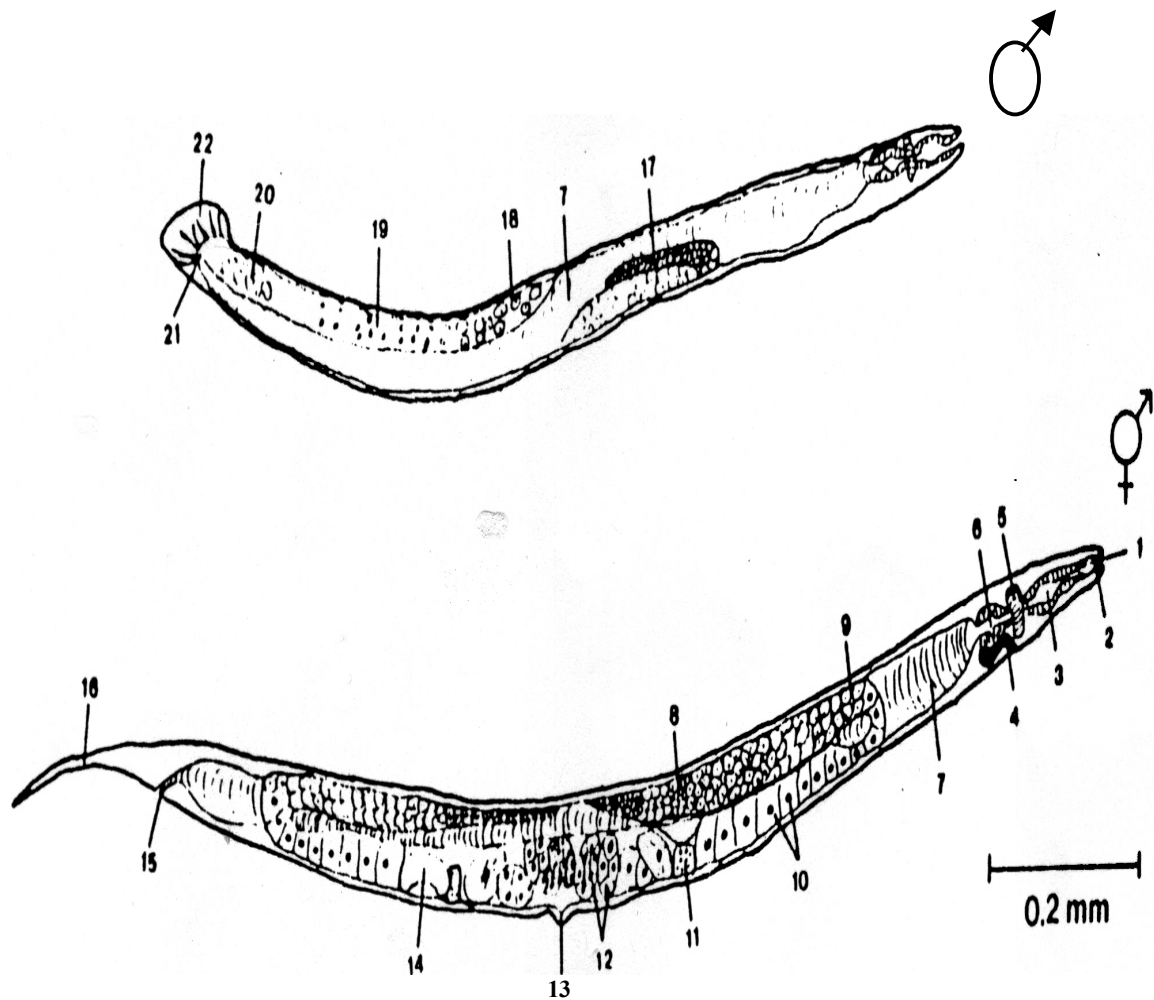


Figure 1.1. Adult *Caenorhabditis elegans*: male: (above), hermaphrodite: (below). (1) Buccal cavity; (2) one of six lips; (3) metacarpus of the pharynx; (4) excretory cell; (5) nerve ring; (6) terminal bulb; (7) intestine; (8) distal arm of the gonad; (9) lopp of the gonad; (10) proximal arm of the gonad with maturing oocytes; (11) spermatheca; (12) uterus with cleaving eggs; (13) vulva; (14) mature oocyte before spermatheca; (15) anus; (16) tail; (17) testis; (18) spermatocytes; (19) vas deferens; (20) mature sperm; (21) cloaca; (22) copulatory bursa with rays and copulatory spicules. (From Ehrenstein and Schierenberg, 1980; reproduced by permission.)

Reproductive Cycle

The life cycle of *C. elegans* is rapid, about 3.5 days at 20°C (Ehrenstein and Schierenberg, 1980). The two sexes, hermaphrodites and males, are each about 1.5 mm in length but differ in appearance as adults (Wood, 1988). Hermaphrodites produce both oocytes and sperm, and can reproduce by self-fertilization. Males, which arise spontaneously at low frequency, can fertilize hermaphrodites; hermaphrodites cannot fertilize each other (Wood, 1988). An hermaphrodite that has not mated lays about 300 eggs during its reproductive life span (Wood, 1988). The typical hermaphrodite produces many more oocysts than sperm and the size of the brood is limited by the number of sperm (Riddle et al., 1998). Juvenile worms hatch and develop through four stages (L1, L2, L3, L4) punctuated by four molts (Wood, 1988; Ehrenstein and Schierenberg, 1980). The times of the molts are approximately 13, 21.5, 29.5, and 41 h after hatching, respectively, for each larval stage (Ehrenstein and Schierenberg, 1980). When the food supply is limited, *C. elegans* enters a dormant survival stage known as the dauerlarva (Cassada and Russell, 1975).

Attraction of C. elegans to Bacteria

The process of response of nematodes to *E. coli* is two-fold, attraction and accumulation. Research conducted by Hosono (1978) indicated that nematodes are not attracted to heated bacteria. *Caenorhabditis elegans* responds to a variety of stimuli, including touch, temperature change, and many different chemical compounds and ions, by moving either toward or away from the stimulus (Wood, 1998). Studies conducted by Grewal and Wright (1992) on the migration of *C. elegans* showed that bacteria affect its pattern of migration on agar plates. The degree of alteration is dependent upon the

species of bacteria. It has been suggested that nematodes might be attracted to younger bacterial colonies as opposed to older colonies (Grewal and Wright, 1992). Studies conducted by Andrew and Nicholas (1976), showed that *C. elegans* was attracted by dense colonies of bacteria, but not by dead bacteria. When the nematode was given the choice of live or dead bacteria, 49 worms migrated into the live bacteria and 7 worms migrated into the dead bacteria (Andrew and Nicholas, 1976). Tracks surrounding the areas of deposition of dead bacteria suggested that the nematodes wandered around these areas, but did not remain for a long period of time in the area.

Darby et al. (1999) studied the lethal effect that *Pseudomonas aeruginosa* has on *C. elegans*. They concluded that when the nematode is placed onto agar plates containing *P. aeruginosa*, pumping became sporadic within seconds and ceased within minutes. Defecation and egg laying also ceased. The locomotion of the nematodes became sluggish, which was sometimes accompanied by twitching. After a 4-h period, most of the worms in contact with *P. aeruginosa* became paralyzed and eventually died (Darby et al., 1999). *Caenorhabditis elegans* has been shown to feed on pathogenic strains of *E. coli*, *L. monocytogenes*, *Shigella*, *Helicobacter pylori*, *Salmonella*, and *Streptococcus bovis* (Darby, 2001). Work conducted by Labrousse et al. (2000) showed that *Salmonella* Typhimurium is capable of infecting and killing *C. elegans*. When *C. elegans* was allowed to feed on *S. Typhimurium*, it displayed a shorter life span and appeared visibly sick before death, but did not show any signs of starvation.

Role of C. elegans in Transmitting Pathogenic Bacteria to Raw Produce

The role that *C. elegans* plays in the transmittal of pathogenic bacteria to raw produce has not been studied. In a survey of vegetables for the presence of amoebae and

Salmonella, Rude et al (1984) recovered nematode eggs and larvae using a n-acconol-ether method. The recovery of nematodes from the fresh vegetables indicates the agronomic conditions and marketing practices may be conducive to the survival of nematodes on fresh produce. This also indicates that if free-living nematodes are present on fresh produce, they may serve as vehicles for contamination with pathogenic bacteria either by surface contact, or via eggs or voided material from their gastrointestinal tract.

Lund (1992) listed insects, fungi, nematodes, animals, birds, rain, hail, and contact with machinery as agents and mechanisms that facilitate entry of spoilage bacteria into produce. The same modes of contamination of raw produce by pathogenic bacteria may also occur. Since free-living nematodes are soil-inhabitants and ingest or come in contact with bacteria in soil used for fruit and vegetable production, there is a need for research to determine what role, if any, free-living nematodes play in contaminating produce that is to be consumed raw.

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CHAPTER 2

ATTRACTION OF A FREE-LIVING NEMATODE, *CAENORHABDITIS ELEGANS*,
TO FOODBORNE PATHOGENIC BACTERIA,
AND ITS POTENTIAL AS A VECTOR OF *SALMONELLA* POONA FOR
PREHARVEST CONTAMINATION OF CANTALOUPE¹

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To be submitted to Journal of Food Protection

ABSTRACT

Caenorhabditis elegans, a free-living nematode, was studied to determine the potential role of free-living nematodes as vectors of preharvest contamination of fruits and vegetables with foodborne pathogens. Propensity of the nematode to be attracted to seven strains of *Escherichia coli* O157:H7, eight serotypes of *Salmonella*, and six strains of *Listeria monocytogenes* was studied. Adult worms (20 - 30) were placed on the surface of K agar midway between a 24-h bacterial colony and 10 µl of uninoculated tryptic soy broth (TSB) positioned 1.5 cm apart. The number of nematodes that migrated to the colony and TSB within 5, 10, 15, and 20 min at 22°C was determined, followed by incubating plates at 37°C for up to 7 days to determine the ability of *C. elegans* to survive and reproduce in bacterial colonies. The nematode was attracted to colonies of all test strains and serotypes. *C. elegans* survived and laid eggs within colonies for up to 7 days. The potential of *C. elegans* to serve as a vector to transport *Salmonella* Poona to cantaloupe rind was also investigated. Worms that had been immersed in a suspension of *S. Poona* were deposited 1 or 3 cm below the surface of soil on which cantaloupe rind was placed. The rind was analyzed for the presence of *S. Poona* after 1, 3, 7, and 10 days at 21°C. The presence of *S. Poona* was more evident on rind positioned on soil beneath which *C. elegans* surface-inoculated with *S. Poona* was initially deposited compared to rind on soil beneath which *S. Poona* alone was deposited. The time required for contamination of rind was longer when it was placed 3 cm above the inoculum, compared to 1 cm. Free-living nematodes may play a role in the preharvest dispersal of incidental human pathogens in soil to raw fruits and vegetables in contact with soil during development and maturation, as evidenced by the behavior of *C. elegans* as a test model.

INTRODUCTION

In recent years, there has been an increase in the number of documented outbreaks of foodborne illness associated with both domestic and imported fresh produce (NACMCF, 1999; Nguyen-the, and Carlin, 2000). Beet leaves, cabbage, carrots, cauliflower, tomatoes, and lettuce are among the salad vegetables that have been reported to occasionally harbor *Aeromonas*, *Bacillus cereus*, *Campylobacter jejuni*, enterotoxigenic *Escherichia coli* and enterohemorrhagic *E. coli* O157:H7, *Salmonella*, *Listeria monocytogenes*, *Shigella*, *Staphylococcus aureus*, *Vibrio cholerae*, *Yersinia enterocolitica*, hepatitis A virus, and Norwalk/Norwalk-like virus (Beuchat, 2002; Lund and Snowden, 2000; NACMCF, 1999; Nguyen-the and Carlin, 2000). Apple cider, orange juice, cantaloupe, raspberries, and strawberries have been associated with outbreaks of disease caused by *Cyclospora cayetanensis*, *Cryptosporidium parvum*, *E. coli* O157:H7, *Salmonella*, hepatitis A, and Norwalk/Norwalk-like virus (Beuchat, 2002).

Outbreaks of salmonellosis in humans have been attributed to the consumption of raw produce such as tomatoes, seed sprouts, watermelon, and cantaloupe (Bolstein, 1993; CDC, 1979, 1991; Taormina et al., 1999; Tauxe, 1997). During June and July 1991, more than 400 laboratory-confirmed cases of *Salmonella* Poona infection occurred in 23 states and Canada (CDC, 1991). Most of the cases were associated with the consumption of cantaloupe from salad bars or in fruit salads. In 2000, cantaloupes imported to the U.S. were implicated as the source of a multi-state outbreak of 46 cases of infection caused by *S. Poona* (Civen, 2002).

Soil is a source of microbial contamination of fruits and vegetables, as evidenced by the isolation of soil-residing pathogenic bacteria from produce (Geldreich and Bordner, 1971). Jay (2000) stated that the microbiota of soil-grown produce may be expected to reflect the microbiota of soils in which they are grown. If there are human enteric pathogens present in the soil, they may be present on the surface of fruits and vegetables, and introduced into the flesh when cut for processing or consumption (Splittstoesser, 1996; Ukuku and Sapers, 2001).

Agents such as insects, fungi, bacteria, nematodes, rain, hail, dust, and mechanical damage may facilitate microbial infection of plants (Lund, 1983; Leben, 1988). Microbivorous nematodes are among the primary grazers of bacteria in soils (Venette and Ferris, 1998). Most nematologists do not attach particular importance to free-living nematodes as vectors of plant pathogens. However, a critical examination of the role nematodes may play in plant or perhaps human diseases has been suggested (Wasilewska and Webster, 1975).

Caenorhabditis elegans, a microbivorous, free-living nematode, has been used extensively in biological studies. Feeding primarily on bacteria, the adult worm lives approximately 2 weeks under optimal environmental conditions (Wood, 1988). *C. elegans* possesses two advantages as a test model to evaluate the role of free-living nematodes in vectoring human pathogens to fruits and vegetables in that it has simple growth requirements and a short generation time (Kruz and Ewbank, 2000). The worm is routinely cultured in the laboratory on *Escherichia coli* OP50, a uracil-deficient non-pathogenic strain that grows slowly on K agar but serves as a nutrient source for growth and reproduction.

C. elegans will ingest nearly anything that fits into its mouth (Riddle et al., 1997). The worms, about 1.5 mm in length, feed on bacteria by taking in water containing suspended particles (including cells) and then spitting out the water while retaining the particles (Avery and Thomas, 1997). *C. elegans* responds to a variety of stimuli, including touch, change of temperature, and many chemical compounds and ions by either moving toward or away from the stimulus (Wood, 1988). It has been used to study host-pathogen interactions (Kurz and Ewbank, 2000). While *C. elegans* is harmless if ingested by humans, it and other free-living nematodes may serve as carriers or vectors of human enteric pathogens. It has been reported that pathogenic enteric bacteria are ingested by free-living nematodes and, while in the gut, are protected against treatment with chlorinated water (Chang et al., 1960). Five to 16% of cells of *Salmonella* Typhosa, *Shigella sonnei*, and *Salmonella* Paratyphi may remain viable in worms present in water supplies and will survive routine chlorination treatment.

The objectives of this study were to determine the propensity of *C. elegans* to migrate toward three human enteric pathogens and cantaloupe juice, as well as its survival and reproductive behavior in the presence of these pathogens. The potential of *C. elegans* as a vector to transport *Salmonella* in soil to the surface of cantaloupe rind was also investigated.

MATERIALS AND METHODS

Maintenance of *C. elegans*. A free-living microbivorous nematode, *C. elegans* (N2, wild type strain), was selected for evaluation because it is easy to culture, serves as a reference for other free-living nematodes, and has been genetically characterized.

The worms were maintained in the laboratory on K agar (pH 6.5), which contains (per liter of deionized water): potassium chloride (2.36 g), sodium chloride (3.0 g), Bacto-peptone (2.5 g), and Bacto agar (17.0 g) (Williams and Dusenbery, 1988). The basal medium was sterilized by autoclaving at 121°C for 15 min, cooled to 47 - 50°C, and supplemented with (per liter of deionized water): 95% cholesterol (1.0 g) (Aldrich, Milwaukee, Wisc.), calcium chloride (11.1 g), and magnesium sulfate (24.7 g), and poured into plastic petri dishes (100 mm in diameter).

E. coli OP50 was grown at 37°C for 24 h in OP50 broth, which contains (per liter of deionized water): sodium chloride (5.0 g) and Bacto-peptone (10.0 g). K agar plates were surface inoculated with 0.1 ml of *E. coli* OP50 culture and incubated at 37°C for 24 h to establish confluent growth. Adult worms were deposited on the K agar surface and incubated at 21°C for up to 3 days before transferring to a fresh plate.

Preparation of nematodes for enteric pathogen assay. K agar plates containing 500 - 1000 eggs, along with adult worms, were washed by depositing 5.0 ml of K medium (broth) (pH 4.8), which contains the same ingredients as K agar, except agar and peptone are omitted (Williams and Dusenbery, 1990) on the surface and gently rubbing. The suspended eggs and worms were aseptically transferred to sterile 15-ml centrifuge tubes. The wash and transfer procedure was repeated to ensure efficient recovery of eggs. Eggs and worms were collected by centrifugation (500 x g, 2 min, 21°C) (IEC Central CL2 benchtop centrifuge, Needham Heights, Mass.) and supernatants were decanted. A pellet from pooled suspensions was resuspended in 10 ml of a solution containing 1% NaOCl and 0.013 M NaOH (pH 12.1) and incubated at 22°C for 15 min to kill all life cycle forms of the worm except the eggs. The suspension was centrifuged (500 x g, 2 min,

21°C), followed by removal of the supernatant. The pellet was resuspended in 10 ml of K medium and the suspension was centrifuged (500 x g, 2 min, 21°C). This step was repeated twice. After the final wash, all but ca. 0.5 ml the supernatant was removed from the tube. One milliliter of K medium was added, followed by depositing 0.1 ml of the egg suspension on the surface of a K agar plate on which a lawn of *E. coli* OP50 had grown. The number of adult worms that developed at 21°C was monitored using a stereomicroscope (Stereomaster, Fisher Scientific, Pittsburgh, Pa.). Worms on plates incubated at 21°C for 3 days were used in experiments to determine their attraction or repulsion to or from foodborne pathogens and to determine their potential role in vectoring of *Salmonella* to cantaloupe juice and rind.

Pathogens used and preparation for *C. elegans* attraction assay. Seven strains of *E. coli* O157:H7, six serotypes of *Salmonella*, and eight strains of *L. monocytogenes* were used. *E. coli* O157:H7 strains were isolated from fecal samples, foods, and infected patients: strain EOO18 (calf fecal isolate), SEA13B88 (unpasteurized apple cider), C7927 (human isolate associated with apple cider outbreak), H1730 (human isolate from an outbreak associated with lettuce), F4546 (alfalfa sprout outbreak), 994 (salami isolate), and 932 (human isolate). The *Salmonella enterica* serotypes used in this study were Montevideo G4639 (from a patient in an outbreak associated with raw tomatoes), Poona (from a patient in an outbreak associated with cantaloupe), Michigan (from cantaloupe), Enteritidis E190-88 (human isolate), Muenchen (from orange juice implicated in an outbreak), Baildon (from a patient in outbreak associated with raw tomato), and Stanley (from a patient in an outbreak associated with alfalfa sprouts). Strains of *L. monocytogenes* used were F8027 (from celery), F8255 (from either a peach or plum),

F8369 (from corn), F8385 (from carrots), G1091 (from coleslaw), and H0222 (from raw potato).

All isolates were grown on tryptic soy agar (TSA, pH 7.3; BBL/ Difco Sparks, Md.) and held as stock cultures at 4°C. Cells were prepared for inocula by growing in 10 ml of tryptic soy broth (TSB, pH 7.3; BBL/ Difco) at 37°C for 24 h. At least two consecutive 24-h transfers to TSB were made before using as inocula for K agar. Each strain of TSB culture (10 µl) was inoculated onto the surface of K agar and allowed to dry for 10 min in a laminar flow hood (class II, type A/B3). The plates were incubated at 37°C for 24 h before using in assays to determine attraction characteristics of *C. elegans*.

Attraction of *C. elegans* to foodborne pathogens. Ten microliters of uninoculated TSB (control) were placed onto the surface of K agar 1.5 cm away from a colony of a pathogen, and plates were allowed to dry in a biosafety hood for 10 min at 21°C to facilitate absorption of water by K agar. A K agar plate on which adult worms had developed was washed with 10 ml of K medium. The suspension was transferred to a sterile 15-ml tube and centrifuged (500 x g, 2 min, 21°C). This step was repeated to reduce the number of *E. coli* OP50 on the worm cuticle. The supernatant was removed and the pellet was resuspended in 1.0 ml of K medium. The worms were allowed to settle to the bottom of the tube for 5 min at 21°C. A suspension (10 µl) containing 20 - 30 worms was deposited onto the center of the K agar plate 0.75 cm away from the test colony and the site on which TSB had been deposited. The number of worms in the inoculum was recorded. The surface tension of the inoculum was carefully broken with a fine-bristle paint brush to facilitate worm movement on the K agar surface. The migration of the worms toward the TSB or colonies of test pathogens was monitored at 5-

min intervals for 20 min. Plates were then incubated at 21°C for up to 7 days to determine if the worms survived and developed through their life cycle in the presence of test pathogens. Observations were made on the movement of the worms, the presence or absence of eggs, and the general location of nematodes on the agar surface. All experiments were replicated three or more times.

Attraction of *C. elegans* to cantaloupe juice. Western (Shipping) cantaloupes (*cucumis melo* L. var. *reticulatus* Naud.) were purchased from a supermarket in Griffin, Ga. The surface of the cantaloupe was not washed nor was it sanitized prior to cutting. The flesh (mesocarp) of each cantaloupe was removed, placed in a sterile stomacher bag, and massaged by hand to release the juice from the tissues. Bags were placed in a Stomacher 400 laboratory blender (Seward Medical Limited, London, UK.) and pummeled for 30 sec at low speed. The juice from the cantaloupe was separated from most of the insoluble tissue by filtering through a coarsely-woven cotton cloth. The pH (Basic pH meter, Denver Instruments, Arvado, Colo.) and °Brix (Refractometer, Spectronic Instruments, Rochester, N.Y.) were measured. A 10-µl sample of cantaloupe juice was placed onto the surface of K agar; uninoculated TSB (control) was placed 1.5 cm away from the cantaloupe juice. The same procedure for preparing the worms for the pathogen attraction/repulsion assay was used to prepare worms for the cantaloupe juice assay. Inoculation and incubation procedures were likewise the same in the two experiments. Using another set of plates, 10 µl of cantaloupe juice instead of uninoculated TSB was deposited 1.5 cm away from a 24-h colony of *S. Poona* on K agar.

The same monitoring procedures used in preceding experiments were used to determine the percent distribution of the worms within 20 min at sites where cantaloupe juice had been deposited and a colony of *S. Poona* had developed. Observations were made on the movement of the worms, the presence or absence of eggs, and the general location of the nematodes on the agar surface. All experiments were conducted three or more times.

Movement of *S. Poona* in soil to cantaloupe rind. Studies were done to determine if *C. elegans* surface-inoculated with *S. Poona* would move through soil and surface contaminate cantaloupe rind. *S. Poona* was grown at 37°C in 10 ml of TSB supplemented with nalidixic acid (50 µg/ml) (TSBN). Cultures (24 h) were harvested by centrifugation (2,000 x g, 15 min, 21°C). Pellets were washed with 10 ml of sterile K medium, collected again by centrifugation, and resuspended in 10 ml of K medium. This suspension served as an inoculum.

Soil (Redi-Earth Peat-Lite Mix, 3CP, The Scotts Company, Columbus, Ohio; pH 6.9) was saturated with water and heat sterilized in a shallow pan at 121°C for 30 min. The sterile soil was stored at 21°C until used in the experiment. Western cantaloupes were purchased from supermarkets in Griffin, Ga. and held at 22°C until used the same day. The surface of the cantaloupe was not washed or sanitized prior to cutting. Cantaloupes were cut into sections and the mesocarp was removed. The rind was further cut into 3.8 x 3.8 cm pieces with a stainless steel knife sterilized by immersing in 70% ethanol. Inoculum (10 µl) of *S. Poona* (7.79 log₁₀ CFU/ml) was placed into sterile glass jars (5.5 cm diam x 6.5 cm high). Soil (1 cm or 3 cm deep) was placed on top of the inoculum and a piece of cantaloupe rind was firmly placed on the surface. Worms grown on K agar were collected and washed twice in K medium before depositing in the K medium

suspension of *S. Poona* (inoculum). In another set of jars, 10 µl of this suspension containing 20 - 30 worms were placed into jars before adding soil (1 cm or 3 cm deep). A piece of cantaloupe rind was firmly placed on top of the soil. A control consisting of a 3-cm layer of soil but no inoculum was also prepared. All jars were covered with parafilm and lids were applied to prevent evaporation of water from the soil.

Jars containing only *S. Poona* or *S. Poona* in combination with *C. elegans* were incubated 1, 3, 7, and 10 days at 21°C before analyzing duplicate samples of soil and cantaloupe rind for the presence of *S. Poona*. Control (uninoculated) samples were analyzed only after incubating 1 day at 21°C. The rind and soil in each jar were separately placed into stomacher 400 bags and 200 ml of lactose broth (pH 6.9, BBL/Difco) supplemented with nalidixic acid (50 µg/L) (LBN) was added. Mixtures were incubated at 37°C for 24 h. A loopful of the LBN was then inoculated into 10 ml of selenite cystine broth (pH 7.0, BBL/Difco) and incubated at 37°C for 24 h, followed by streaking onto bismuth sulfite agar (pH 7.7, BBL/Difco) supplemented with nalidixic acid (50 µg/ml) (BSAN) and incubating at 37°C for 24 h. Presumptive positive colonies of *S. Poona* that formed on BSAN were selected and confirmed by *Salmonella* latex agglutination (Oxoid), triple sugar iron (pH 7.4, BBL/Difco), and lysine iron agar (pH 6.7, BBL/Difco) assays. The presence or absence of *S. Poona* was recorded as either positive (+) or negative (-), respectively. This experiment was replicated four times. Each replicate consisted of three jars.

RESULTS AND DISCUSSION

Attraction of *C. elegans* to foodborne pathogens. The mean number of worms that migrated to colonies of foodborne pathogens or uninoculated TSB (control) after 5, 10,

15, and 20 min at 21°C was used to calculate the percent distribution of *C. elegans*.

The attraction of *C. elegans* to seven strains of *E. coli* O157:H7 is shown in Figure 2.1.

Considering all strains, the percentage of worms that migrated to the pathogen colony was 13 - 48%, 22 - 80%, 28 - 55%, and 31 - 67%, respectively, within 5, 10, 15, and 20 min after inoculation. This compares to 0 - 27%, 0 - 24%, 2 - 22%, and 2 - 22%, respectively, migrating to TSB within 5, 10, 15, and 20 min. Considering all strains, 47% of the worms migrated to colonies within 20 min. More worms were attracted to strain 932 than to the other test strains. The worms were least attracted to strain F4546. The ability of the *C. elegans* to survive and produce eggs in colonies of *E. coli* O157:H7 was monitored over the course of 7 days at 21°C. *C. elegans* produced eggs that eventually hatched and developed into larvae.

Distribution of *C. elegans* to colonies of eight serotypes of *Salmonella* is shown in Figure 2.2. The ranges in percentage of worms attracted to colonies were 34 - 65%, 46 - 79%, 63 - 84%, and 65 - 87%, respectively, within 5, 10, 15, and 20 min after depositing worms on K agar. Ranges in percentages of worms that were associated with uninoculated TSB were 3 - 11%, 4 - 10%, 3 - 8%, and 4 - 7%, respectively, within 5, 10, 15, and 20 min. The highest percentage (87%) of worms migrated to *S. Poona* within 20 min. Considering all serotypes, a mean of 67% of the worms migrated to colonies within 20 min. Survival and reproduction of *C. elegans* in colonies of the eight *Salmonella* serotypes were observed for up to 7 days. Worms produced eggs more rapidly in the colonies of *S. Michigan* than in colonies of other serotypes as observed by the production of numerous eggs within 3 days. The life cycle of *C. elegans* was completed on K agar plates inoculated with each of the eight serotypes of *Salmonella*.

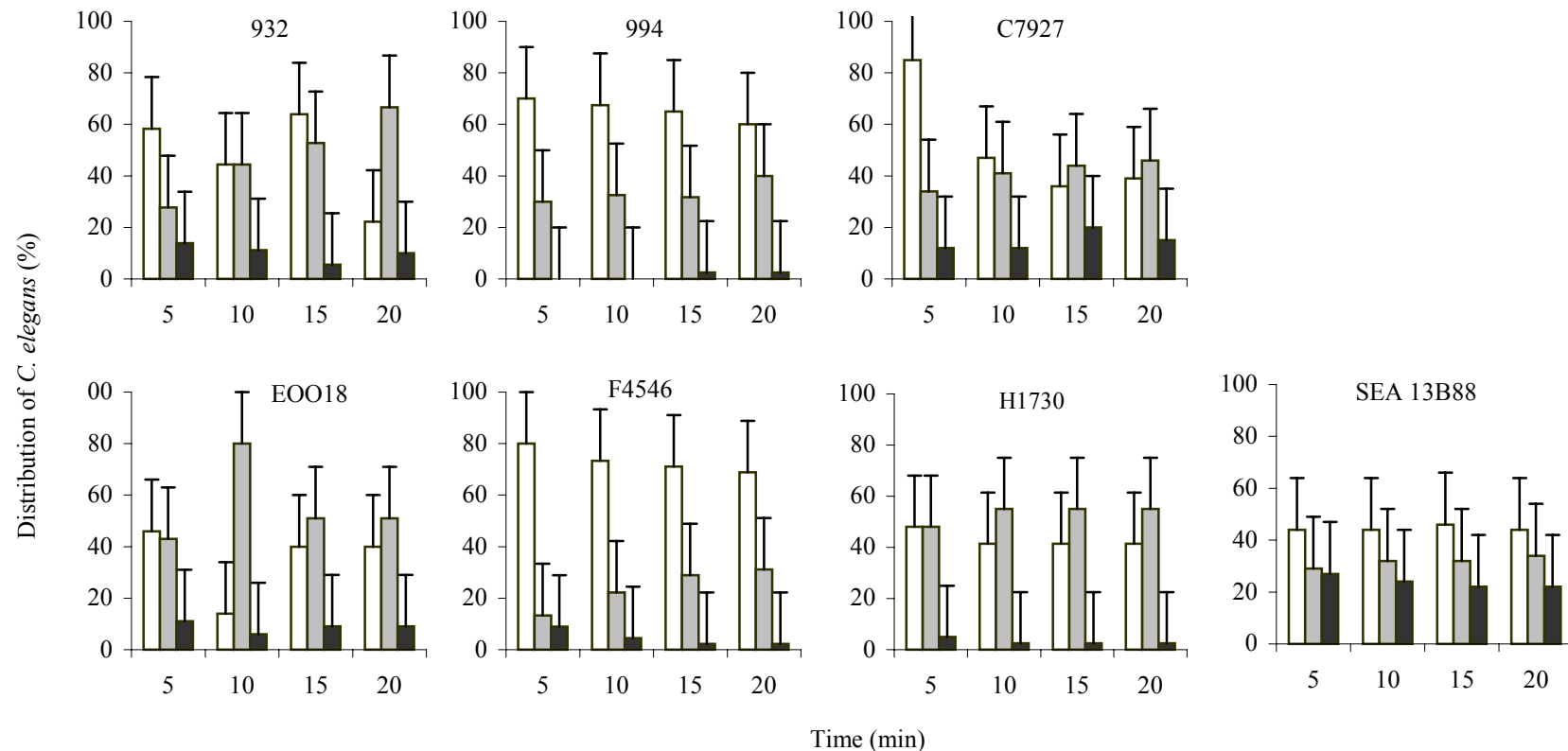


Figure 2.1. Migration of *C. elegans* on K agar to colonies of seven strains of *E. coli* O157:H7 (932, 994, C7927, EOO18, F4546, H1730, and SEA 13B88) and uninoculated TSB. Worms (20 - 30) were deposited 0.75 cm away from inoculation sites. The percentages of worms that migrated to *E. coli* O157:H7 colonies (■), TSB (■), or neither *E. coli* O157:H7 nor TSB (□) within 20 min at 21°C were monitored.

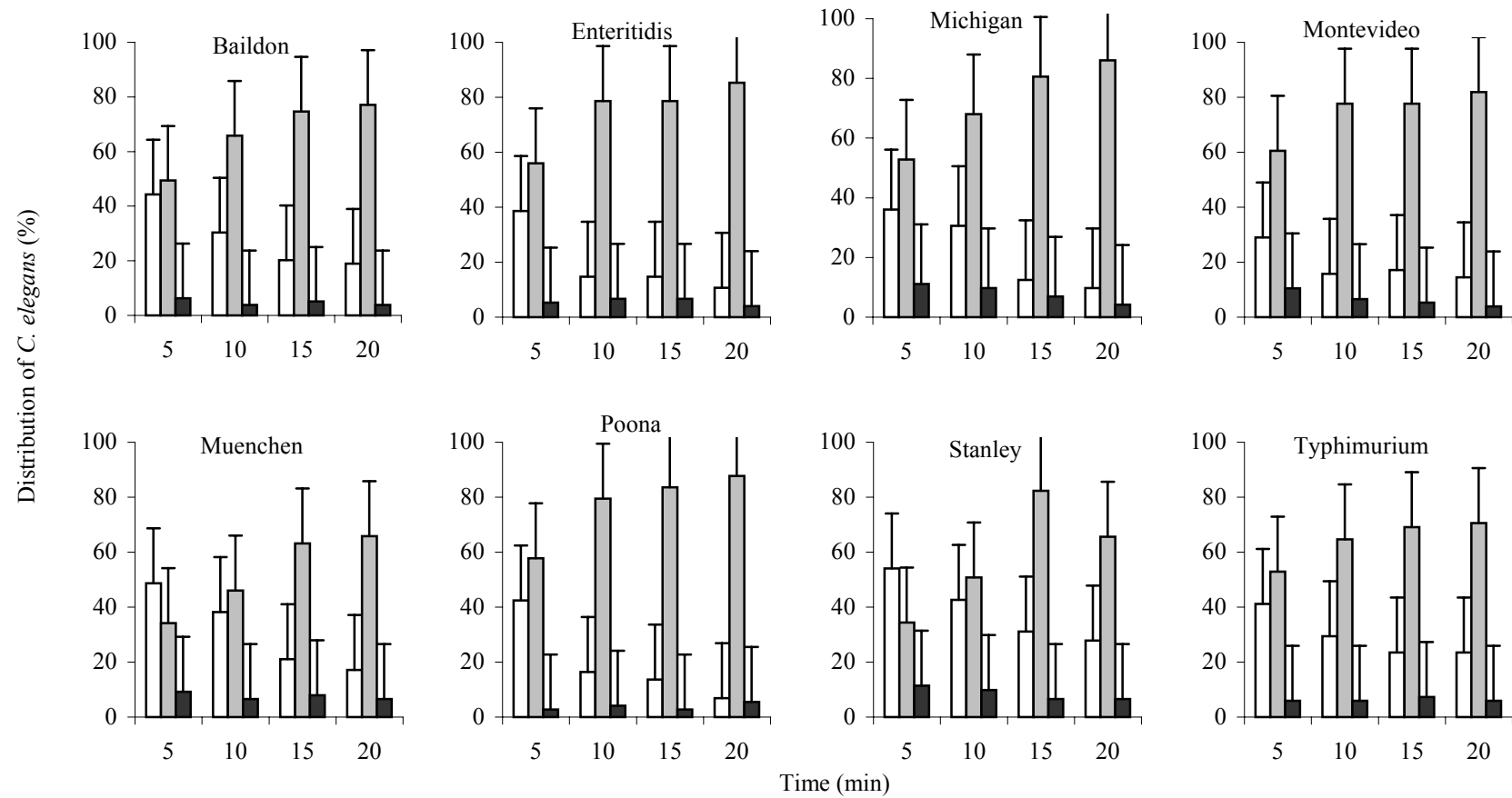


Figure 2.2. Migration of *C. elegans* on K agar to colonies of eight *Salmonella* serotypes (Baidon, Enteritidis, Michigan, Montevideo, Muenchen, Poona, Stanley, and Typhimurium) and uninoculated TSB. Worms (20 – 30) were deposited 0.75 cm away from the inoculation sites. The percentages of worms that migrated to *Salmonella* (■), TSB (■), or neither *Salmonella* nor TSB (□) within 21°C was monitored.

Results of experiments to determine the attraction of *C. elegans* to six strains of *L. monocytogenes* are shown in Figure 2.3. Considering all strains, 8 - 58%, 14 - 82%, 14 - 97%, and 17 - 98%, of the worms, respectively were attracted to *L. monocytogenes* within 5, 10, 15, and 20 min. This compares to 0 - 28%, 0 - 33%, 2 - 38%, and 0 - 34%, respectively, attracted to uninoculated TSB. Considering all strains, 69% of the worms migrated to colonies within 20 min. After 20 min, 98% of the worms were inside the colonies of strain HO222. However, worms appeared to move slower than normal and did not produce a large number of eggs in the presence of this strain. The worms exhibited the lowest affinity to strain F8027. The ability of the worms to survive and produce eggs in colonies formed on K agar by the six strains of *L. monocytogenes* was monitored for 7 days. Worms appeared to move more slowly than normal in colonies of strains 8255 and HO222 within 1 day of inoculation; a large number of worms were either dead or dying.

Attraction of *C. elegans* to cantaloupe juice. Attraction of *C. elegans* to a 24-h colony of *S. Poona* and 10 µl of cantaloupe juice deposited on K agar was studied. The pH of the cantaloupe juice was 6.3 - 6.4 and the °Brix (total dissolved solids) was 8 - 10%. Worms clearly preferred *S. Poona* over cantaloupe juice (Figure 2.4). Only 4 - 5% of the worms were associated with the area containing cantaloupe juice within 20 min. In contrast, 56 - 67% of the worms were attracted to colonies of *S. Poona*.

Figure 2.5 shows the percent of worms distributed in cantaloupe juice and uninoculated TSB. A higher percentage of worms were attracted to TSB than to juice of juice of cantaloupes 2 and 3; however, the reverse migration to TSB and juice of cantaloupe 1 occurred. Attraction of *C. elegans* to cantaloupe juice may be influenced by minor differences in composition of juice.

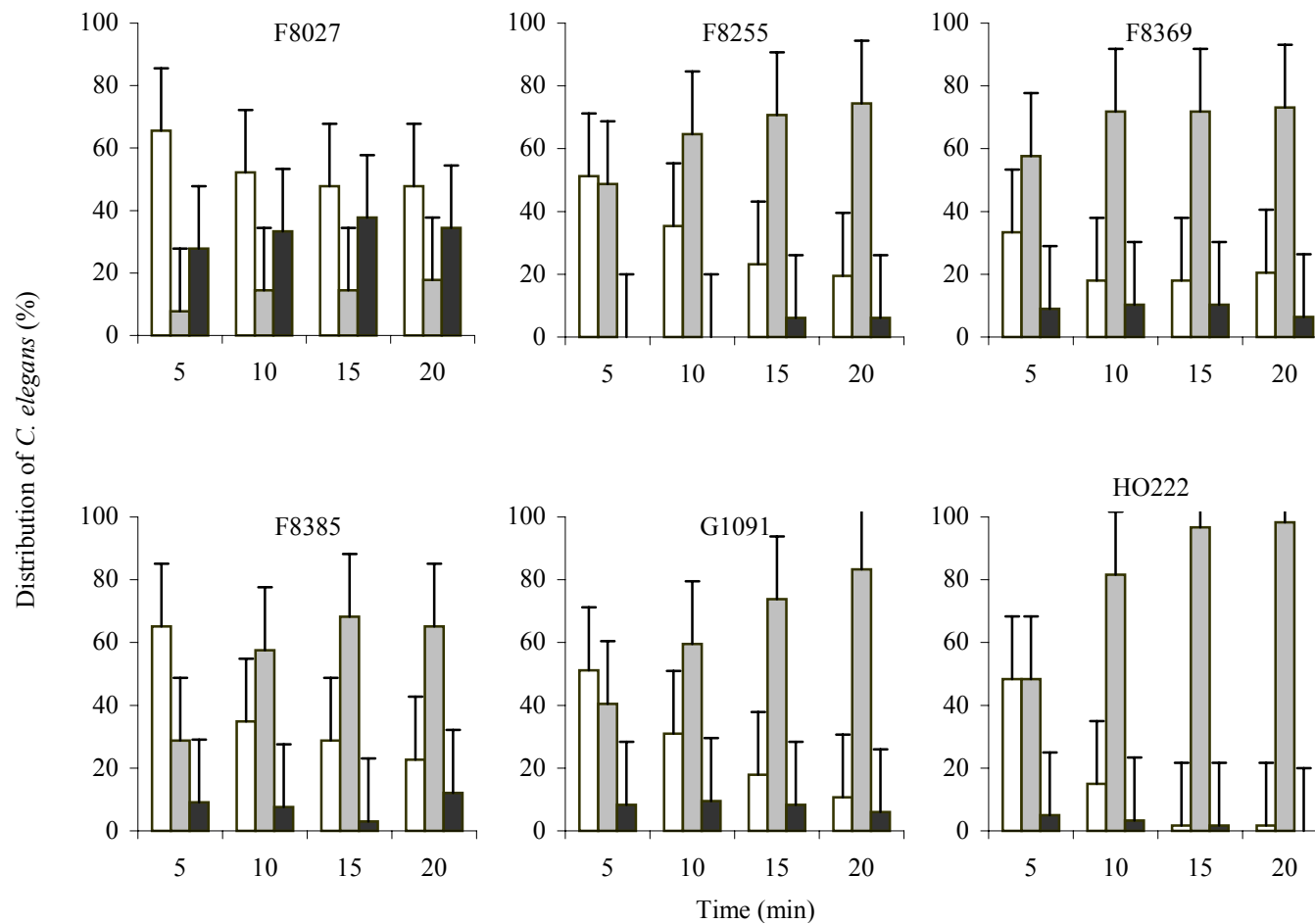


Figure 2.3. Migration of *C. elegans* on K agar to colonies of six strains of *L. monocytogenes* (F8027, F8255, F8369, F8385, G1091, and HO222) and un inoculated TSB. Worms (20 - 30) were deposited 0.75 cm away from inoculation sites. The percentages of worms that migrated to *L. monocytogenes* (■), TSB (■), or neither *L. monocytogenes* nor TSB (□) within 20 min at 21°C were monitored.

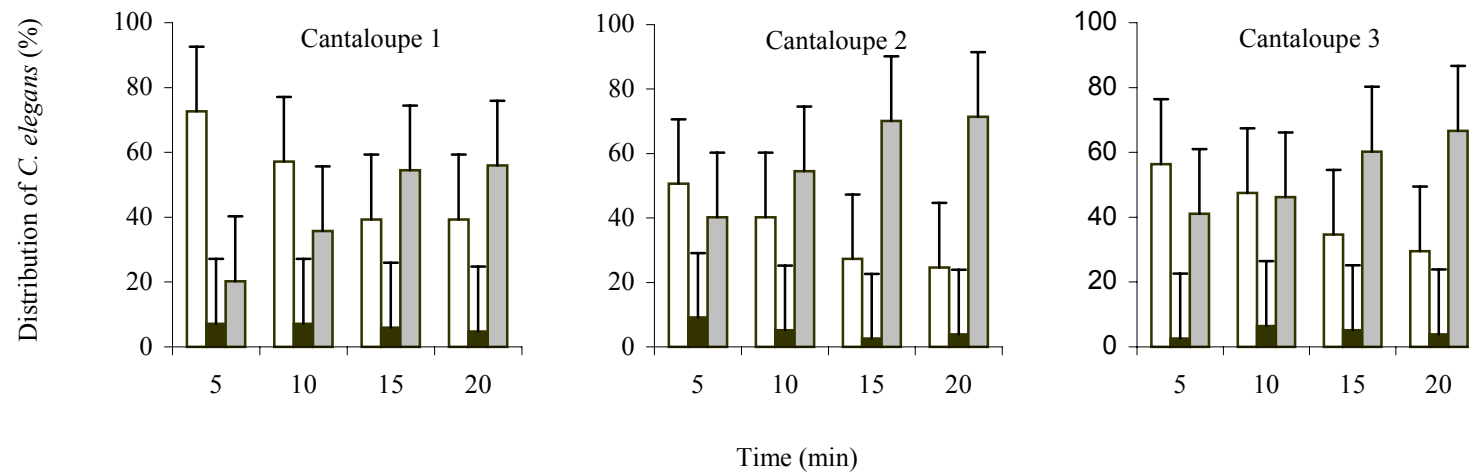


Figure 2.4. Migration of *C. elegans* on K agar to colonies of *S. Poona* and cantaloupe juice. Worms (20 – 30) were deposited 0.75 cm away from colonies and juice. The percentages of worms that migrated to cantaloupe juice (■), *S. Poona* (▒), or neither cantaloupe juice nor *S. Poona* (□), within 20 min at 21°C were monitored.

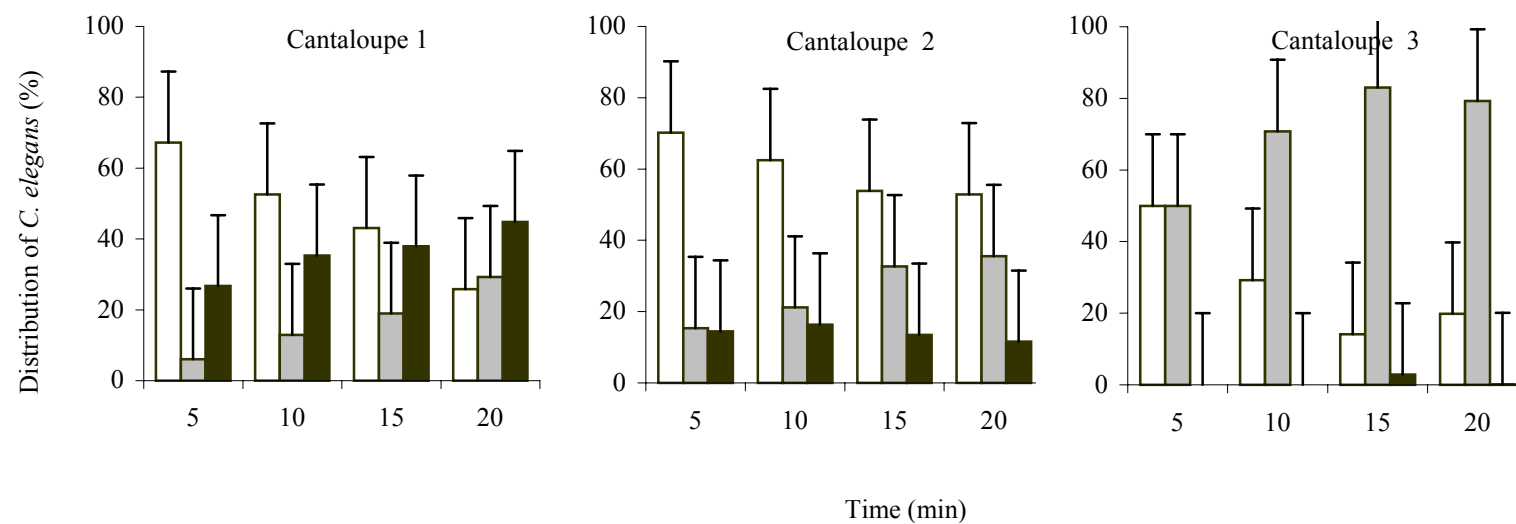


Figure 2.5. Migration of *C. elegans* on K agar to cantaloupe juice and uninoculated TSB. Worms (20 – 30) were deposited 0.75 cm away from TSB and juice. The percentages of worms that migrated to cantaloupe juice (■), TSB (▒), or neither cantaloupe juice nor TSB (□) within 20 min at 21°C were monitored.

Observations on the growth and reproduction of the worms in the presence of cantaloupe juice were also made. Worms deposited onto K agar plates containing *S. Poona* and cantaloupe juice remained in the *S. Poona* colony during incubation at 21°C for 7 days. Worms on plates containing uninoculated TSB and cantaloupe juice appeared to prefer the TSB, although a few were observed in the proximity of the cantaloupe juice. The life cycle of the worms appeared to not be affected by the presence of cantaloupe juice on plates containing TSB and cantaloupe juice. This may suggest that the nematodes are capable of feeding off nutrients present in TSB or cantaloupe juice.

Ability of *C. elegans* to transport *S. Poona* to cantaloupe rind. The ability of *C. elegans* to transport *S. Poona* through soil to the surface of cantaloupe rind was investigated. Results are shown in Table 2.1. Both the cantaloupe rind and soil of control samples were negative for the presence of salmonellae. The time required for cantaloupe rind to test positive for *S. Poona* depended on the depth of soil, i.e., the initial distance between the inoculum and rind, and the presence or absence of *C. elegans*. The presence of *S. Poona* on cantaloupe rind was more evident when rind was placed on soil in jars inoculated with both *S. Poona* and *C. elegans* than on rind placed on soil in jars inoculated only with *S. Poona*. After incubation times of 1, 3, 7, and 10 days, the number of positive cantaloupe rinds placed on top of the 1-cm layer of soil, below which had been deposited a suspension of *C. elegans* and *S. Poona*, was higher than the number of positive rinds placed on a 3-cm layer of soil. This suggests that it took longer for *C. elegans* inoculated with *S. Poona* to migrate from the bottom of the jar containing a 3-cm layer of soil to the cantaloupe rind compared to the time required to migrate through 1 cm of soil. Results show that the incidence of *S. Poona* on cantaloupe rind was increased

Table 2.1. Presence of *Salmonella* Poona in soil and on cantaloupe rind in contact with the surface of soil.

Inoculum	Depth of soil (cm)	Number positive for <i>S. Poona</i> ^a							
		Soil				Cantaloupe rind			
		1	3	7	10	1	3	7	10
Control	3	0				0			
<i>S. Poona</i>	1	12	12	12	12	0	2	4	2
	3	12	12	12	12	1	2	5	2
<i>S. Poona</i> plus <i>C. elegans</i>	1	12	12	12	12	6	7	6	4
	3	12	12	12	12	5	3	4	6

^a Number of samples positive for *S. Poona* out of twelve samples analyzed after 1, 3, 7, and 10 days of incubation at 21°C.

over a 10-day incubation period by the presence of *C. elegans* in soil. The time required for *C. elegans* containing *S. Poona* on its surface to reach the cantaloupe rind is directly affected by the initial distance of soil between the worms and the rind.

The extent of attraction of *C. elegans* to *E. coli* O157:H7 was not unexpected, considering that the nematode is routinely cultured in the laboratory on *E. coli* OP50 as a nutrient source. Attraction of *C. elegans* to *Salmonella* and *L. monocytogenes* in comparatively higher numbers indicates that these pathogens may also serve as nutrient sources. Our observations support earlier work conducted by Grewal and Wright (1992) showing that patterns of migration by *C. elegans* across agar surfaces are influenced by the type of attractant present. Studies conducted by Ward (1973) indicate that *C. elegans* is attracted to cyclic AMP. *C. elegans* has also been reported to be attracted to cations and anions such as Na⁺, Cl⁻, and OH⁻, pyridine, O₂, and CO₂ in borate buffer (pH 8.8), and repelled by CO₂ in phosphate buffer (pH 6.0), D-tryptophan, H⁺, and high osmotic pressure (Dusenberry, 1983). Attractants are produced in abundance when bacteria are in an active growth phase (Grewal and Wright, 1992). However, the degree of alteration of the migration patterns of *C. elegans* depends on the species of bacteria. In our study, *C. elegans* exhibited differences in attraction to three test pathogens. None of the pathogens repelled the worms. The lack of attraction of *C. elegans* to cantaloupe juice may be due to the absence of low concentrations of attractants described by Ward (1973) and Dusenberry (1983).

The ability of bacteria to attract nematodes is influenced by growth conditions. Grewal and Wright (1992) observed that 24-h colonies of *Acinetobacter calcoaceticus* were more attractive to *C. elegans* than were older (48-h) colonies. In a study conducted

by Andrew and Nicholas (1976), it was concluded that *E. coli*, *Pseudomonas fluorescens*, and *P. aeruginosa* were more attractive for *C. elegans* than were *Bacillus subtilis* and *B. mycoides*. *C. elegans* was most attracted to bacteria that produced an alkaline environment in the vicinity of colony development. Khanna et al. (1997) observed that *C. elegans* can tolerate pH in the range of 3.2 - 11.2. Attraction may be based simply on a pH gradient, as *C. elegans* is attracted to high pH (Ward, 1973). The lack of attraction of *C. elegans* to cantaloupe juice may be in part to the reduced pH of the juice (6.3 – 6.4) compared to the pH of K agar (6.5). Grewal and Wright (1992) showed that the attraction of *C. elegans* to bacteria was influenced by conditions used to culture the bacteria.

The potential of *C. elegans* to serve as a vector for the transporting of *Salmonella* in soil to cantaloupe rind has been demonstrated. Detection of *S. Poona* within 1 day on cantaloupe rind initially 1 - 3 cm away from *C. elegans* that had been bathed in a suspension of the pathogen reveals that this nematodes is either attracted to the rind or serendipitously comes in contact with the rind during migration in the soil. Our observations on the lack of attraction of *C. elegans* to cantaloupe juice on K agar would strengthen the latter mode of contamination. Potential cross contamination via this mechanism is probably not limited to cantaloupes. Vectoring *Salmonella* and other pathogens by *C. elegans* and perhaps other free-living nematodes to other fruits and vegetables, especially those in contact with soil during development on the plant, may also occur.

Conditions affecting preharvest transmission of *S. Poona* to cantaloupe and other produce are largely unknown. Studies should be undertaken to better define conditions

that influence attraction of free-living nematodes to fruits and vegetables. Retention of viability of pathogens on and in *C. elegans* and other free-living nematodes in soil and on the surface of fruits and vegetables also needs to be investigated.

ACKNOWLEDGMENTS

The nematode strain used in this work was provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources. The authors also would like to acknowledge Dr. Marc van Iersel, Department of Horticulture, University of Georgia, for supplying the soil used in this experiment. We are also grateful to Mrs. Barbara B. Adler for technical support and assistance.

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CHAPTER 3

INGESTION OF *SALMONELLA* POONA BY A FREE-LIVING NEMATODE,
CAENORHABDITIS ELEGANS, AND PROTECTION AGAINST TREATMENT WITH
SANITIZERS¹

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To be submitted to Journal of Food Protection.

ABSTRACT

Caenorhabditis elegans was selected as a model to study the effectiveness of sanitizers in killing *Salmonella* Poona ingested by free-living nematodes. Adult worms that had fed on *S. Poona* were treated with chlorine, hydrogen peroxide, Sanova[®], Tsunami 200[®], and acetic, citric, and lactic acids. Treatment with 20 µg/ml free chlorine resulted in a significant ($p = 0.05$) reduction ($2.19 \log_{10}$ CFU/worm) in population of *S. Poona* compared to washing worms with water. There was no significant difference in the number of *S. Poona* surviving treatments with 20, 50, 100, 200, or 500 µg/ml chlorine, suggesting that reductions in population were due to inactivation of *S. Poona* on the surface of *C. elegans* but not ingested cells. Treatment with 500 µg/ml chlorine caused the largest reduction ($4.37 \log_{10}$ CFU/worm) compared to washing worms with water. Treatment with Sanova (850 or 1,200 µg/ml), an acidified sodium chlorite sanitizer, caused reductions of 5.74 and 6.34 \log_{10} CFU/worm, respectively, compared to washing worms with water. Treatment with 20 or 40 µg/ml Tsunami 200, a peroxyacetic acid-based sanitizer, resulted in reductions of 4.83 and 5.34 \log_{10} CFU/worm respectively, compared to numbers detected on and in worms washed with water. Among the organic acids evaluated, at a concentration of 2%, acetic acid was least effective in killing *S. Poona* ($1.61 \log_{10}$ CFU/worm reduction, compared to washing with water) and lactic acid was most effective ($5.32 \log_{10}$ CFU/worm reduction). Treatment with up to 500 µg/ml chlorine, 1% hydrogen peroxide, 2,550 µg/ml Sanova, 40 µg/ml Tsunami 200, or 2% acetic, citric, or lactic acids had no effect on the viability or reproductive behavior of *C. elegans*. Treatment with 2% hydrogen peroxide caused death of some of the worms within 1 day.

Treatments were also applied to lettuce inoculated with *S. Poona* or *C. elegans* that had fed on *S. Poona*. Regardless of the drying time (1 or 24 h) after inoculating lettuce with *C. elegans*, the number of *S. Poona* recovered ($6.13 \log_{10}$ CFU/worm and $6.66 \log_{10}$ CFU/worm, respectively) after washing with water and was not significantly different ($\alpha = 0.05$) than the number in the inoculum. Treatment of lettuce inoculated with *C. elegans* with 200 $\mu\text{g/ml}$ chlorine, 850 or 1,200 $\mu\text{g/ml}$ Sanova, or 20 or 40 $\mu\text{g/ml}$ Tsunami significantly reduced the number of *S. Poona* compared to washing lettuce with water but populations recovered from lettuce treated with these concentrations of chemical sanitizers were not significantly different. Our study provides evidence to support the hypothesis that free-living nematodes may serve as vectors of human pathogens and confirms the observation that ingested bacteria are protected from treatment with sanitizers.

INTRODUCTION

An increase in the number of outbreaks of infections associated with consuming fresh produce in recent years is thought to be caused, in part, by changes in agronomic, harvesting, processing, and consumption patterns (Souness and Desmarchelier, 1997). Fruits and vegetables can become contaminated with human pathogenic microorganisms before and during harvesting, during transport, processing, distribution, and marketing, and at sites of preparation in food service and home settings (Beuchat, 1998). Inadequate surface washing and improper storage of produce can lead to growth of pathogenic microorganisms (Sewell and Farber, 2001).

Salmonellosis is among the most frequently reported causes of foodborne diseases of gastroenteritis in the United States (CDC, 1990; NACMCF, 1998; Mead et al., 1999).

Although outbreaks of salmonellosis associated with produce are infrequent compared to outbreaks linked to foods of animal origin, a diverse range in types of fruits and vegetables, including tomatoes, seed sprouts, lettuce, watermelon, and cantaloupe have been implicated (NACMCF, 1998). Several outbreaks of *Salmonella* Poona infections have been associated with consumption of cantaloupe. In 1991, more than 400 cases of infections were linked to cantaloupe that originated in either Texas or Mexico (CDC, 1991). More recently, cantaloupe was implicated in 43 cases of *S. Poona* infection (FDA, 2000). *Salmonella* may survive on preharvest-contaminated cantaloupe rind during subsequent postharvest handling and preparation for consumption (Ukuku and Sapers, 2001).

The surface of cantaloupes and other fruits and vegetables acts as a physical barrier, preventing or greatly minimizing penetration of microorganisms into the interior (Tauxe et al., 1997). However, mechanical damage of cantaloupe during harvesting, washing, subsequent handling, and preparation for consumption may compromise the surface integrity, allowing microorganisms to enter tissues and grow to high numbers if held for sufficient time at non-refrigeration temperature. It has been reported that *Salmonella* present on the surface of cantaloupes (Ukuku and Sapers, 2001) and tomatoes (Lin and Wei, 1997) are transferred to the internal tissues during slicing.

Washing the surface of raw fruits and vegetables with tap water is recommended as a means for consumers to remove soil and microorganisms, but should not be relied upon to disinfect the surface (Beuchat et al., 1998; Beuchat, 1998; Ukuku and Sapers, 2001). The effectiveness of washing produce with a sanitizer for the purpose of killing or removing microorganisms is dependent on the chemical properties of the sanitizer,

contact time, and temperature (Beuchat, 1998; Takeuchi and Frank, 2001), types of microorganisms present and their attachment mechanisms (Sapers et al., 2000), and physical characteristics of fruits and vegetables (Cherry, 1999). Chlorine is the most widely used chemical sanitizer in wash, spray, and flume waters used in the fresh fruit and vegetable industry (Beuchat, 1998; Beuchat and Ryu, 1997). Water containing 50 - 200 µg/ml of free chlorine has been recommended for inactivating of enteric pathogens that might be present on fruits and vegetables (Beuchat, 1998; Hobbs and Gilbert, 1978). Applying acetic, citric, lactic, malic, succinic, or benzoic acids to reduce microbial populations on the surface of fruits and vegetables, followed by further washing with potable water to remove residual acid, also has potential for reducing microbial populations (Beuchat, 1992). Hydrogen peroxide has been suggested as an alternative to chlorine for disinfecting fresh produce and appears to reduce microbial populations without leaving significant residues (Sapers and Simmons, 1998).

The free-living nematode, *Caenorhabditis elegans*, has been used as a model for other free-living nematodes in numerous biological studies. *C. elegans* feeds primarily on bacteria, and the adult worm lives for approximately 2 weeks under optimal environmental conditions (Wood, 1998). The worm is cultured in the laboratory on *Escherichia coli* OP50, a uracil-deficient non-pathogenic strain that serves as a nutrient source for growth and reproduction. *C. elegans* obtains needed nutrients in soil by taking in water containing suspended bacteria and other particles and then spitting out the water while retaining the bacteria (Avery and Thomas, 1997). Studies have indicated that *C. elegans* and perhaps other free-living nematodes may serve as carriers or vectors of human enteric pathogens. Caldwell et al. (2002) showed that *C. elegans* is attracted to

several strains of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes*. Chang et al. (1960) reported that *Salmonella* and *Shigella* ingested by two free-living nematodes, *Cheilobus quadrilabiatu*s and *Diplogaster nudicapitatus*, are protected against inactivation by chlorinated water, and the pathogens survived in the worms' gut for up to 4 days. These nematodes showed resistance to free chlorine and offered protection to ingested pathogens against chlorination.

An objective of the study reported here was to determine the effectiveness of raw vegetable and fruit sanitizers in killing *S. Poona* that had been ingested by *C. elegans*. Lethality of sanitizers to *S. Poona* internalized by *C. elegans* was tested using suspensions of worms *in situ* and also worms applied to lettuce leaves.

MATERIALS AND METHODS

Nematodes used. A free-living nematode, *C. elegans* (N2, wild type strain), was used in the experiment. The worms were cultured on K agar (pH 6.5), which contains (per liter of deionized water): potassium chloride (2.36 g), sodium chloride (3.0 g), Bacto-peptone (BBL/Difco, Sparks, Md.) (2.5 g), and agar (17.0 g) (Williams and Dusenbery, 1988). The basal medium was sterilized by autoclaving at 121°C for 15 min, cooled to 47 - 50°C, and supplemented with (per liter of deionized water): 95% cholesterol (1.0 g) (Aldrich, Milwaukee, Wisc.), calcium chloride (11.1 g), and magnesium sulfate (24.7 g), and poured into plastic petri dishes (100 mm in diameter) (Caldwell et al., 2002).

E. coli OP50 was cultured at 37°C for 24 h OP50 broth, which contains (per liter of deionized water): sodium chloride (5.0 g) and Bacto-peptone (10.0 g). K agar was surface inoculated with 0.1 ml of *E. coli* OP50 culture and incubated at 37°C for 24 h to

establish confluent growth. Adult worms were deposited on the K agar and incubated at 21°C for up to 3 days before transferring to a fresh plate (Caldwell et al., 2002)

Bacterial strain and growth conditions. *Salmonella enteritica* serotype Poona was used. An isolate from a patient in an outbreak associated with consumption of cantaloupe was cultured to grow in tryptic soy broth (pH 7.3, BBL/ Difco) supplemented with 50 µg/ml nalidixic acid (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 24 h. At least two consecutive 24-h loop transfers to 10 ml of TSBN were made before using as a nutrient source for *C. elegans*.

Culturing of nematodes for sanitizer treatments. K agar plates containing 500 - 1000 eggs, along with adult worms, were washed by depositing 5.0 ml of K medium (broth) (pH 4.8), which contains the same ingredients as K agar, except agar and peptone are omitted (Williams and Dusenbery, 1990) on the surface and gently rubbing. Culturing the nematodes for these experiments was conducted following methods described by (Caldwell et al., 2002) in which the egg and worm suspension was transferred to a sterile 15-ml centrifuge tube. Eggs and worms were collected by centrifugation (500 x g, 2 min, 21°C) (IEC Central CL2 benchtop centrifuge, Needham Heights, Mass.) and the supernatant were decanted. The pellet was resuspended in 10 ml of a solution containing 1% NaOCl and 0.013 M NaOH (pH 12.1) and incubated at 22°C for 15 min. The suspension was centrifuged (500 x g, 2 min, 21°C), followed by removal of the supernatant. The pellet was resuspended in 10 ml of K medium and the suspension was centrifuged (500 x g, 2 min, 21°C). After the final wash, all but ca. 0.5 ml the supernatant was removed from the tube. One milliliter of K medium was added, followed by depositing 0.1 ml of the egg suspension on the surface of a K agar plate on

which a lawn of *E. coli* OP50 had grown. The number of adult worms that developed at 21°C was monitored using a stereomicroscope (Stereomaster, Fisher Scientific, Pittsburgh, Pa.). Worms on plates incubated at 21°C for 3 days were used in the following experiments (Caldwell et al., 2002).

Preparation of treatment solutions. Seven chemical solutions were evaluated for their effectiveness in killing *S. Poona* ingested by *C. elegans*. Hydrogen peroxide was tested at concentrations of 0.5, 1.0, and 2.0%. Chlorine (20, 50, 100, 200, and 500 µg/ml) solutions were prepared by combining sodium hypochlorite (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) with 0.05 M phosphate buffer (pH 6.8). The concentration of free chlorine was measured using an amperometric titrator (Hach, Loveland, Colo.). Sanova[®], a sodium chlorite solution, (Alcide Corporation, Redmond, Wash.) at 850, 1200, and 2550 µg/ml, Tsunami 200[®] (Ecolab, St. Paul, Minn.) at 20 and 40 ppm µg/ml, and acetic, citric, and lactic acids, each at 0.5, 1.0, and 2.0% were evaluated. Sterile deionized water was used as a control. All chemical treatment solutions were used within 30 min of preparation.

Ingestion of *S. Poona* by *C. elegans*. *S. Poona* was streaked onto the surface of tryptic soy agar (BBL/Difco) supplemented with nalidixic acid (50 µg/ml) (TSAN, pH 7.3) and incubated at 37°C for 24 h. Adult *C. elegans* grown on K agar inoculated with *E. coli* OP50 was removed by applying 10 ml of K medium to each plate and gently rubbing. The suspension was transferred to a sterile 15-ml tube and centrifuged (500 x g, 2 min, 21°C). The worms were resuspended in 10 ml of K medium and centrifuged again to reduce the number of *E. coli* OP50 on the worm cuticle. The supernatant was removed and the pellet was resuspended in 1.0 ml of K medium at 21°C. The worms were allowed

to settle to the bottom of the tube for 5 min. A suspension (20 μ l) containing 20 - 30 worms was deposited onto the surface of TSAN on which 24-h colonies of *S. Poona* had formed. The worms were allowed to ingest *S. Poona* for ca. 3 h at 22°C before subjecting to sanitizer treatments *in situ* or inoculating onto the surface of lettuce leaves.

Treatment of *C. elegans* with sanitizers. Using a sterile 32-gauge platinum wire attached to the tip of a Pasteur pipette, ten worms that had fed on *S. Poona* for 3 h were removed from the TSAN plates and placed in 20 μ l of sterile deionized water on the tip of a sterile spoonula. The spoonula containing the worms was submerged into 2 ml of chemical treatment solution in a 50-ml centrifuge tube. After 5 min at 21°C, all but ca. 0.5 ml of the treatment solution or water containing the worms was removed, and 4 ml of Dey-Engley (DE) neutralizing broth (pH 7.6) (BBL/Difco) was added. The worms in the suspension were sonicated (Sonicate 450, Danbury, Conn.) using a duty cycle of 25% for 25 sec at 21°C to rupture the cuticle of *C. elegans* and release ingested *Salmonella*. Undiluted samples (0.25 ml in quadruplicate and 0.1 ml in duplicate) of sonicate and duplicate 0.1-ml quantities of suspensions diluted in 0.1% peptone water were surface plated on TSAN. Plates were incubated at 37°C for 24 h before colonies were counted. Presumptive *S. Poona* colonies were picked and confirmed using biochemical tests.

Effect of chemical sanitizers on the behavior of *C. elegans*. In another set of experiments, worms that had ingested *S. Poona* and been treated with the chemical sanitizers but not sonicated were placed back onto a lawn of *E. coli* OP50 on K agar to determine if treatment had an adverse effect on viability and reproduction. After a 5-min treatment, the mixture of chemical solution and DE broth was decanted, leaving the treated worms in ca. 0.5 ml in the bottom of the tube. Worms were placed on the surface

of K agar containing a lawn of *E. coli* OP50. Plates were incubated at 21°C for up to 4 days and worms were observed for characteristic movement and reproductive behavior.

Preparation and inoculation of lettuce. The effectiveness of sanitizers in killing *S. Poona* ingested or not ingested by *C. elegans* and deposited on the surface of lettuce leaves was investigated. Iceberg lettuce (*Lactuca sativa* L.) was purchased from a local supermarket in Griffin, Ga. The core and three outer leaves were removed from the lettuce heads and discarded. A stainless steel template (3.8 x 3.8 cm) was placed on the surface of inner leaves. Pieces of leaves were made by cutting around the perimeter of the template with a sanitized scalpel.

Worms fed on 24-h colonies of *S. Poona* grown on TSAN were prepared as described above. A 20- μ l K medium suspension containing 10 worms was placed on each piece of lettuce and allowed to dry for 1 or 24 h at 37°C before treating with test chemicals.

Experiments were also done to determine the efficacy of sanitizers in killing *S. Poona* not ingested by *C. elegans*. A 24-h TSBN culture of *S. Poona* grown at 37°C was loop-transferred three times before streaking onto TSAN and incubating for 24 h at 37°C. TSAN plates containing colonies of *S. Poona* were flooded with 12 ml of Butterfield's phosphate buffer (pH 7.2) and cells were suspended with a sterile bent glass rod. Ten milliliters of the suspension were centrifuged (2000 x g, 15 min, 22°C) and the pellet was resuspended in 10 ml of 0.1% peptone. The OD₆₁₀ of the suspension was adjusted to 1.27 before depositing 20 μ l on each piece of lettuce, drying for 1 or 24 h at 37°C, and treating with test chemicals.

Treatment of lettuce inoculated with *S. Poona* or *C. elegans* that had ingested *S. Poona*. Each piece of lettuce inoculated with *S. Poona* or *C. elegans* that had ingested *S.*

Poona was placed in 50-ml centrifuge tube. Ten milliliters of chlorinated water (50 and 200 µg/ml), Sanova (850 or 1,200µg/ml), or Tsunami 200 (20 and 40 µg/ml) was added and the mixture was agitated on a platform shaker set at 150 rpm for 3 min at 22°C. Ten milliliters of double-strength DE broth was added and the mixture was homogenized (Polytron PCU11, Brinkmann Instruments, Westbury, N.Y.) at medium speed for 30 sec. The homogenate was then sonicated using a duty cycle of 25% for 25 sec. Undiluted samples (0.25 ml in quadruplicate and 0.1-ml in duplicate) of sonicate and duplicate 0.1-ml quantities of suspensions diluted in 0.1% in peptone water were surface plated on TSAN and XLD agar (BBL/Difco). Plates were incubated for 24 h at 37°C. Twenty milliliters of double-strength lactose broth supplemented with nalidixic acid (50 µg/ml) (LBN) was added and the remaining homogenate/sonicate and incubated at 37° for 24 h. If no presumptive *Salmonella* colonies formed on TSAN on which samples of lettuce inoculated with *C. elegans* that had ingested *S. Poona* had been spread or TSAN and XLD on which samples of lettuce inoculated only with *S. Poona* only had been spread, enriched lactose broth was inoculated into 10 ml of selenite cystine and incubated at 37°C for 24 h, followed by streaking onto bismuth sulfite agar (pH 7.7) (BBL/Difco) supplemented with nalidixic acid (50 µg/ml) (BSAN) and incubating at 37°C for 24 h. Presumptive colonies of *S. Poona* that formed on BSAN were selected and confirmed by biochemical tests.

Statistical analysis. Each experiment was replicated at least three times. Each replicate consisted of three pieces of lettuce. Data were subjected to the Statistical Analysis System (SAS Institute, Cary, N.C.) for analysis of variance and Duncan's multiple range tests to determine significant differences ($\alpha = 0.05$) between mean values.

RESULTS AND DISCUSSION

pH of treatment solutions. Ranges in pH values for chemical sanitizers were: chlorine (pH 6.82 – 6.95), hydrogen peroxide (pH 5.06 – 7.43), Sanova (pH 2.58 – 2.62), Tsunami 200 (pH 3.66 – 3.85), acetic acid (pH 2.66 – 2.98), citric acid (pH 2.50 – 2.83), and lactic (pH 2.16 – 2.52) (Table 3.1). After the addition of DE broth, the pH of treatment solutions ranged from 6.00 (2% lactic acid) to 7.78 (water control). The pH of neutralized chemical solutions would not be expected to adversely affect the viability of *S. Poona* or *C. elegans*. *C. elegans* is tolerant to pH environments in the range of 3.2 – 11.2 (Khanna et al., 1997).

Efficacy of sanitizers in killing *S. Poona* internalized by *C. elegans*. *C. elegans* that had fed on *S. Poona* was suspended in chemical treatment solutions for 5 min, then analyzed for populations of the pathogen that survived. Treatment with 20 µg/ml free chlorine resulted in a significant ($p = 0.05$) reduction ($2.19 \log_{10}$ CFU/worm) in population compared to washing worms with water (Fig 3.1). There was a significantly higher ($\alpha = 0.05$) number of *S. Poona* killed by the treatment with 20 µg/ml chlorine compared to treatment with water. However, there was no significant difference in the number of *S. Poona* surviving treatments with 20, 50, 100, 200, or 500 µg/ml chlorine. Treatment with 500 µg/ml chlorine caused the largest reduction ($4.37 \log_{10}$ CFU/worm) compared to washing worms with water.

A similar trend occurred when worms internalized with *S. Poona* were treated with other chemical sanitizers, i.e., the lowest concentration tested caused a significant ($p =$

Table 3.1. pH of chemical sanitizers before and after adding Dey-Engley broth

Sanitizer	Concentration		pH of treatment solution ^a	
	µg/ml	%	Before adding DE	After adding DE
Chlorine	0		5.30	7.78
	20		6.82	7.70
	50		6.85	7.17
	100		6.86	7.78
	200		6.89	7.79
	500		6.95	7.81
Sanova [®]	0		5.30	7.78
	850		2.58	7.10
	1,200		2.60	7.04
	2,500		2.62	6.71
Tsunami 200 [®]	0		5.30	7.78
	20		3.85	8.18
	40		3.66	8.07
Hydrogen peroxide		0	5.30	7.78
		0.5	5.22	8.55
		1.0	5.06	8.71
		2.0	7.43	8.54
Acetic acid		0	5.30	7.78
		0.5	2.98	7.08
		1.0	2.80	6.39
		2.0	2.66	5.03
Citric acid		0	5.30	7.78
		0.5	2.83	7.95
		1.0	2.69	7.76
		2.0	2.50	7.31
Lactic acid		0	5.30	7.78
		0.5	2.52	7.42
		1.0	2.16	7.12
		2.0	2.16	6.00

^apH of chemical treatment solutions (2 ml) containing ten *C. elegans* internalized with *S. Poona*.

Measurements were made before and after the addition of 4 ml of DE neutralizing broth.

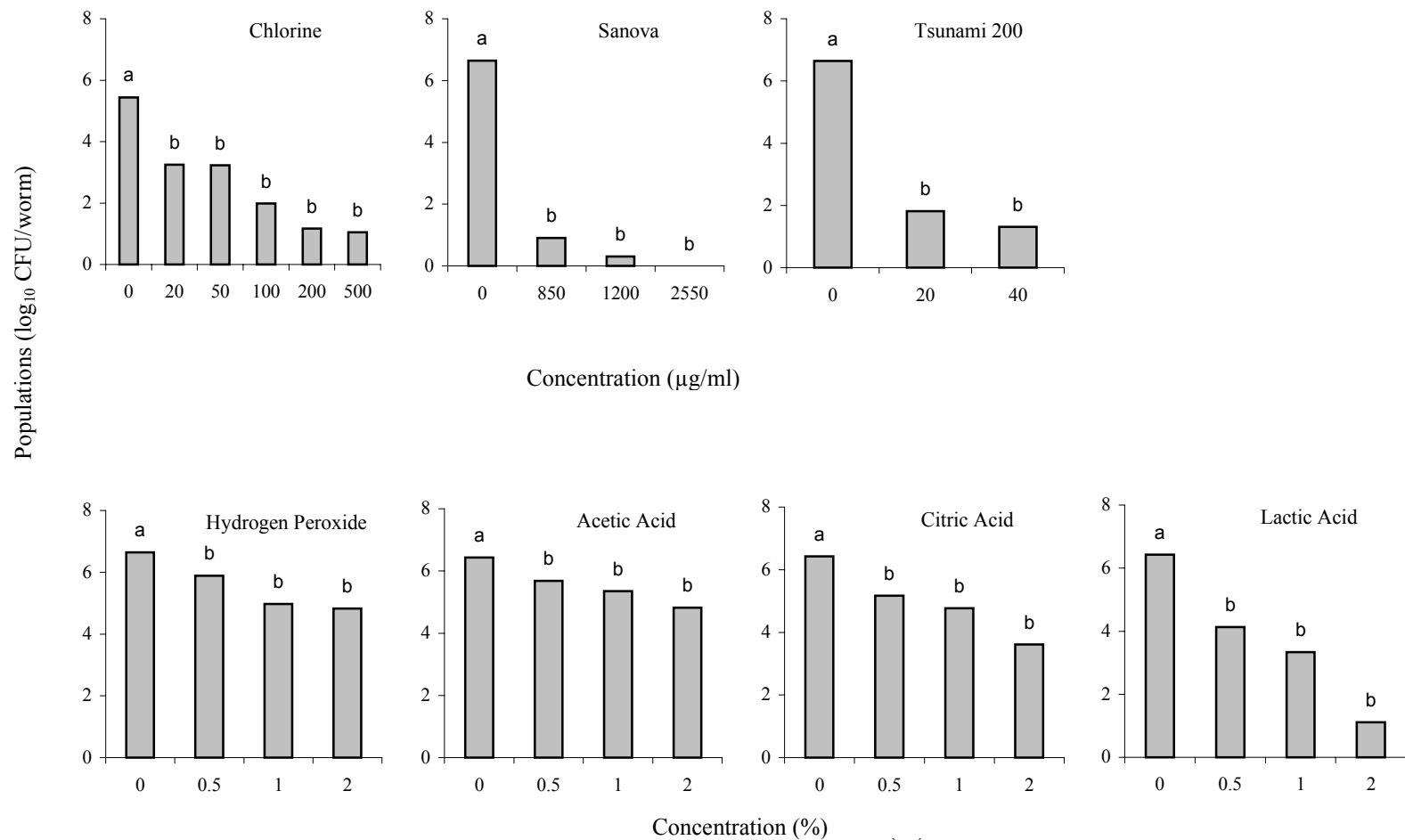


Figure 3.1. Populations of *Salmonella* Poona recovered from *C. elegans* that had fed on the pathogen, followed by treatment with chemical sanitizers. Within each sanitizer, bars noted with a different letter are significantly different ($\alpha = 0.05$).

0.05) reduction in population of *S. Poona* compared to treatment with water but reductions were not significantly increased by treating with higher concentrations (Fig. 3.1). Treatment with 2% hydrogen peroxide reduced the *S. Poona* population by 1.41 log₁₀ CFU/worm compared to treatment with water. Treatment with Sanova[®] was the most effective sanitizer in reducing the populations of *S. Poona*. Treatment with 850 and 1,200 µg/ml of Sanova caused reductions of 5.74 and 6.34 log₁₀ CFU/worm, respectively, compared to washing worms with water. *S. Poona* was not detected in worms treated with 2550 µg/ml of Sanova (< 2 CFU/worm).

Treatment with 20 and 40 µg/ml Tsunami 200, a peroxyacetic acid-based sanitizer, resulted in reductions of 4.83 and 5.34 log₁₀ CFU/worm, respectively, compared to numbers detected on worms washed with water. Among the organic acids evaluated, at a concentration of 2%, acetic acid was least effective in killing *S. Poona* (1.61 log₁₀ CFU/worm reduction, compared to the number surviving in or on worms washed with water) and lactic acid was most effective (5.32 log₁₀ CFU/worm reduction). Treatment with 2% citric acid caused a reduction of 2.82 log₁₀ CFU/worm compared to the number detected in worms washed with water.

Effects of sanitizers on viability and reproductive behavior of *C. elegans*.

Experiments were done to determine if treatment with chemical sanitizers at various concentrations causes lethality or changes in reproductive behavior of *C. elegans* that had ingested *S. Poona*. Treated worms were placed on K agar containing a lawn of *E. coli* OP50, incubated at 21°C, and observed for 4 days. Viability and reproductive behavior was not affected by washing with water. Worms placed onto K agar containing a lawn of *E. coli* OP50 produced eggs within 24 h and the eggs developed into larval stages.

Treatment of worms with up to 500 µg/ml chlorine was not lethal and did not affect the ability of *C. elegans* to produce eggs. Treatment with hydrogen peroxide at concentrations of 0.5 and 1% was not lethal to *C. elegans*; the numbers of worms increased during the 4-day observation period. Treatment with 2% hydrogen peroxide, however, resulted in death of some of the worms within 1 day. Before death, eggs were produced by some of the worms. These eggs hatched and developed into larvae that showed no signs of being affected by the 2% hydrogen peroxide treatment that killed the parent worms. The pH of 2% hydrogen peroxide (7.43) was higher than that of the other solutions of test chemicals but probably did not contribute to lethality. Regardless of concentration in treatment solution, Sanova, Tsunami 200, and acetic, citric, and lactic acids did not appear to have an effect on the viability or reproductive behavior of *C. elegans*. These observations confirm the ability of *C. elegans* to survive exposure to highly acidic environments.

Efficacy of sanitizers in killing *S. Poona* internalized by *C. elegans* and inoculated onto lettuce. The effectiveness of chemical treatments in killing *S. Poona* that had been ingested by *C. elegans* and inoculated onto the surface of lettuce leaves was determined. *Salmonella* was not detected in uninoculated lettuce. The drying time between application of *C. elegans* to lettuce and treatment with water did not cause a significant ($\alpha = 0.05$) reduction in population of *S. Poona*. Regardless of the drying time (1 or 24 h) after inoculation of lettuce, the number of *S. Poona* recovered ($6.13 \log_{10}$ CFU/worm and $6.66 \log_{10}$ CFU/worm, respectively) after washing with water (Table 3.2) was not significantly different ($\alpha = 0.05$) than the number in the inoculum ($6.55 \log_{10}$

Table 3.2. Populations of *Salmonella* Poona recovered from lettuce inoculated with *C. elegans* that had ingested the pathogen, followed by drying 1 h or 24 h at 37°C and treating with chemical sanitizers.

Treatment	Conc. of chemical (µg/ml)	Populations (log ₁₀ CFU/worm) ^a			
		1 h	R ^b	24 h	R
Water (control)	0	A 6.13 A		A 6.66 A	
Chlorine	50	A 6.09 A	0.04	A 5.09 B	1.57
	200	A 5.39 B	0.74	A 5.59 B	1.07
Sanova	850	A 4.36 B	1.77	B 3.14 B	3.52
	1,200	A 3.48 B	2.65	A 3.94 B	2.72
Tsunami 200	20	A 5.55 B	0.58	A 5.09 B	1.57
	40	A 5.33 B	0.80	B 4.08 B	2.58

^aValues within the same column followed by the same letter are not significantly different (" = 0.05). Values in the same row preceded by the same letter are not significantly different (" = 0.05). The population of *S. Poona* was 6.55 log₁₀ CFU/worm before inoculating lettuce and drying for 1 or 24 h.

^bWithin drying time (1 or 24 h), reduction (log₁₀ CFU/worm) compared to washing lettuce with water.

CFU/worm). The number of *S. Poona* recovered from lettuce dried for 1 h and then treated with 50 µg/ml chlorine was not significantly different than the number in the inoculum or the number recovered from inoculated lettuce washed with water. Regardless of the drying time, treatment with 200 µg/ml chlorine, 850 or 1,200 µg/ml Sanova, or 20 or 40 µg/ml Tsunami significantly reduced the number of *S. Poona* in the gut of *C. elegans* compared to washing lettuce with water but populations recovered from lettuce treated with these concentrations of sanitizers were not significantly different.

Efficacy of sanitizers in killing of *S. Poona* inoculated onto lettuce. The efficacy of sanitizers in killing *S. Poona* not ingested by *C. elegans*, but inoculated on the surface of lettuce, was determined. Shown in Table 3.3 are populations of *S. Poona* recovered from lettuce treated with chemical sanitizers. The initial inoculum was 7.56 log₁₀ CFU/ml. After drying 1 or 24 h the populations of *S. Poona* recovered from lettuce by washing with water were 7.18 log₁₀ CFU/ml and 6.45 log₁₀ CFU/ml, respectively. There was a significant reduction in the number of *S. Poona* recovered from lettuce dried for 1 h and washed with water compared to and lettuce dried for 24 h. On balance, populations of *S. Poona* recovered from lettuce treated with water or sanitizers were not significantly affected by drying time.

With the exception of lettuce treated with 50 µg/ml chlorine after drying the inoculum for 24 h, there was a significant decrease ($\alpha = 0.05$) in the number of *S. Poona* recovered from lettuce treated with all concentrations of chemical sanitizers, compared to the number recovered from lettuce treated with water (control). However, within drying time, there were no significant differences in the number of *S. Poona* recovered from

Table 3.3. Populations of *Salmonella* Poona recovered from lettuce inoculated with the pathogen followed by drying 1 or 24 h at 37°C and treating with chemical sanitizers.

Treatment	Conc. of chemical (µg/ml)	Populations (log ₁₀ CFU/ml) ^a			
		1 h	R ^b	24 h	R
Water (control)	0	A 7.18 A		B 6.45 A	
Chlorine	50	A 6.44 B	0.74	A 6.35 A	0.10
	200	A 5.99 B	1.19	B 5.57 B	0.88
Sanova	850	B 4.67 B	2.51	A 5.15 B	1.30
	1,200	A 4.34 B	2.84	A 4.19 B	2.26
Tsunami 200	20	A 5.44 B	1.70	A 5.05 B	1.40
	40	A 5.07 B	2.11	A 5.01 B	1.44

^aValues within the same column followed by the same letter are not significantly different (" = 0.05).

Values in the same row preceded by the same letter are not significantly different (" = 0.05). The population of *S. Poona* was 7.56 log₁₀ CFU/worm before inoculating lettuce and drying for 1 or 24 h.

^bWithin drying time (1 or 24 h), reduction (log₁₀ CFU/worm) compared to washing lettuce with water.

lettuce treated with all concentrations of sanitizers (1 h drying time) or 200 µg/ml chlorine and all concentrations of Sanova and Tsunami (24 h drying time). Treatment with Sanova (1,200 µg/ml) caused the largest reductions in *S. Poona* population (2.84 log₁₀ CFU/ml, 1 h drying time and 2.26 log₁₀ CFU/ml, 24 h drying time). Treatment of lettuce with 50 µg/ml of chlorine was the least effective in reducing *S. Poona* population at both drying times (0.74 log₁₀ CFU/ml for 1 h and 0.10 log₁₀ CFU/ml for 24 h).

Recovery of high numbers of *S. Poona* from the interior and surface of *C. elegans* that had fed on the pathogen was not unexpected. Sonication effectively ruptured the worm's cuticle, releasing *S. Poona* and enabling a composite measurement of both interior and surface populations. The number of cells recovered would depend on the number ingested as well as the extent of digestion by worms. Various enzymes produced internally by *C. elegans* can affect the survival of bacteria during passage through the alimentary canal (Lee, 1965). Earlier work in our laboratory (Caldwell et al., 2002) showed that *C. elegans* is attracted more to colonies of *S. Poona* than to colonies of other *Salmonella enterica* serotypes (Montevideo, Michigan, Enteritidis, Muenchen, Baildon, and Stanley). Chang et al. (1960) determined the number of bacterial cells ingested by free-living nematodes by grinding the worms, followed by plating samples on bismuth sulfite to enumerate *S. Typhosa* and *Salmonella Shigella* agar to enumerate other *Salmonella* and *Shigella*. They observed that 5 - 16% of cells of *S. typhosa*, *S. sonnei*, and *S. paratyphi* remain viable in the worms for up to 4 days and will survive routine treatment with chlorinated water. Smerda et al. (1970) concluded that the free-living nematode, *Pristionchus Iheritieri*, tolerated treatment with chlorine solution (10 µg/ml

for 15 min). Treatment of worms that had ingested *Salmonella typhi* and *S. Wichita* with chlorinated water was not effective in killing these pathogens.

Lupi et al. (1995) suggested that free-living nematodes may release ingested bacteria in viable form, either through defecation or disintegration. This may be a mechanism by which these worms act as vectors to transport bacteria in soil onto the surface of produce. Our study provides evidence that free-living nematodes can harbor a human pathogen, thus serving as a vector of transmission to raw produce. Data also confirm the observation that bacteria ingested by *C. elegans* are protected from sanitizer treatment. The higher lethality of Sanova and Tsunami 200 compared to chlorine reveals the availability of alternative sanitizers to treat produce that may harbor free-living nematodes that have internalized *S. Poona* and, perhaps, other pathogens. Results show the general ineffectiveness of chemical sanitizers in reducing populations of *Salmonella* on lettuce, regardless of whether or not the pathogen is internalized in *C. elegans*.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Soil is a source of microbial contamination of fruits and vegetables, as evidenced by the isolation of soil-residing pathogenic bacteria from produce. The microbiota of soil-grown produce may be expected to reflect the microbiota of soils in which they are grown. If there are human enteric pathogens present in the soil, they may be present on the surface of fruits and vegetables, and introduced into the flesh when cut for processing or consumption. Agents such as insects, fungi, bacteria, nematodes, rain, hail, dust, and mechanical damage may facilitate microbial infection of plants.

The objectives of the research presented in this thesis were:

1. Determine the propensity of *C. elegans* to migrate toward three human enteric pathogens and cantaloupe juice, as well as its survival and reproductive behavior in the presence of these pathogens and juice.
2. Determine the potential of *C. elegans* as a vector to transport *Salmonella* in soil to the surface of cantaloupe rind.
3. Determine the effectiveness of sanitizers in killing *S. Poona* that had been ingested by *C. elegans*.

The extent of attraction of *C. elegans* to *E. coli* O157:H7 was not unexpected, considering that the nematode is routinely cultured in the laboratory on *E. coli* OP50 as a nutrient source. Attraction of *C. elegans* to *Salmonella* and *L. monocytogenes* in comparatively higher numbers indicates that these pathogens may also serve as nutrient sources. Our observations support earlier work showing that patterns of migration by *C. elegans* across the surfaces of agar are influenced by the type of attractant present. However, the degree of alteration of migration patterns of *C. elegans* depends on the species of bacteria. In our study, *C. elegans* exhibited differences in attraction to three

test pathogens. None of the pathogens repelled the worms. The lack of attraction of *C. elegans* to cantaloupe juice may be caused a lack of volatile compounds known to attract free-living nematodes.

The potential of *C. elegans* to serve as a vector for the transporting of *Salmonella* in soil to cantaloupe rind has been demonstrated. Detection of *S. Poona* within 1 day on cantaloupe rind initially 1 - 3 cm away from *C. elegans* that had been bathed in a suspension of the pathogen reveals that this nematode is either attracted to the rind or serendipitously comes in contact with the rind during migration in the soil. The lack of attraction of *C. elegans* to cantaloupe juice on K agar would strengthen the latter mode of contamination. Potential cross contamination via this mechanism may not be limited to cantaloupes. Vectoring *Salmonella* and other pathogens by *C. elegans* and perhaps other free-living nematodes to other fruits and vegetables, especially those in contact with soil during development on the plant, may also occur.

Our study provides evidence to support the hypothesis that free-living nematodes may serve as carriers of human pathogens and confirms the observation that ingested bacteria are protected from chemical treatments. The higher lethality of Sanova and Tsunami 200 compared to chlorine reveals the availability of alternative sanitizers to treat produce that may harbor free-living nematodes that have internalized *S. Poona* and, perhaps, other pathogens. Results provide information on the general lack of efficacy of chemical sanitizers in reducing populations of *S. Poona* on lettuce, regardless of whether or not the pathogen is internalized in *C. elegans*.

Conditions affecting preharvest transmission of *S. Poona* to cantaloupe and other produce are largely unknown. Studies should be undertaken to better define conditions

that influence attraction of free-living nematodes to fruits and vegetables. Retention of viability of pathogens on and in *C. elegans* and other free-living nematodes in soil and on the surface of fruits and vegetables during storage and treatment with sanitizers also needs to be investigated.