POTENTIAL ROLE OF A FREE-LIVING NEMATODE, *CAENORHABDITIS ELEGANS*, IN PREHARVEST CONTAMINATION OF FRUITS AND VEGETABLES WITH *SALMONELLA* NEWPORT

by

STEPHEN JEFFREY KENNEY

(Under Direction the of Larry R. Beuchat)

ABSTRACT

Caenorhabditis elegans, a free-living nematode found in soil, has been shown to ingest human enteric pathogens, thereby potentially serving as a vector for preharvest contamination of fruits and vegetables. Factors affecting the ability of *C. elegans* to harbor pathogens in its gut and transport them to the surface of fruits and vegetables were studied. The effects of temperature and relative humidity on the survival and growth of *Escherichia coli* O157:H7, *Salmonella* Poona, and *Salmonella* Newport were assessed. Populations of ingested pathogens remained constant at 4°C, decreased significantly at 20°C, and increased significantly at 37°C within 3 days. Populations of *S.* Newport decreased by up to 3.44 $\log_{10}$ cfu/worm when infected worms were incubated at 20°C and 33% relative humidity. The efficacy of cleaners commonly used in food processing plants in killing ingested pathogens was influenced by desiccation of worms. Cleaners and sanitizers were more effective in significantly ($P \leq 0.05$) reducing the number of ingested *S.* Newport, but none of the test cleaners or sanitizers eliminated the

pathogen at all temperature/relative humidity combinations. *C. elegans* migrates to bovine manure, turkey manure, composted bovine manure, composted turkey manure, and manure-amended soil inoculated with *S.* Newport, as well as uninoculated lettuce, strawberry, and carrot on an agar medium. *C. elegans* survived and reproduced in turkey and bovine manures, and the presence of *S.* Newport did not adversely affect its behavior. When a 5-cm column of soil containing *C. elegans* was placed on top of bovine manure or bovine manure compost inoculated with *S.* Newport, which in turn was topped with a piece of lettuce, strawberry, or carrot, the pathogen was detected on the surface of the produce within 1 day. The pathogen was rarely detected on produce when *C. elegans* was not present in the soil. In field settings, the incidence of *C. elegans* ingesting human pathogens and transporting them to preharvest fruits and vegetables may be low, but on occasion this phenomenon may occur. Results from these studies reinforce the importance of sanitizing produce prior to consumption.

INDEX WORDS: *Caenorhabditis elegans, Salmonella* Newport, *Escherichia coli* O157:H7, fruits, vegetables, manure, manure compost, relative humidity, sanitizers, contamination

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B.S., University of Florida, 1998

M.S., University of Georgia, 2001

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

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2004

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August 2004

DEDICATION

To Jennifer

ACKNOWLEDGEMENTS

I would like to extend my thanks and sincere gratitude to the following people. Without them, none of this would have been possible.

Dr. Larry Beuchat, for taking me as a student and allowing me to “reenlist” for another 3 years.

Dr. Jinru Chen, Dr. Mark Harrison, Dr. John Maurer, and Dr. Philip Williams for serving on my advisory committee and for their guidance.

The faculty and staff at the Center for Food Safety especially, Alan Scouten, Barbara Adler, David Harrison, David Wilkerson, and Kim Hortz.

The students, both in Athens and Griffin: Manan Sharma, Glenner Richards, Kaye Sy, Jaime Rudolf, Christine Chu, Hannah Barnes, Joshua Gurtler, Jee-Hoon Ryu, and Chow Ming Lee., for your support and encouragement.

My family for their never ending love, support, and encouragement.

Finally, to Jenn, thank you for always being there for me and for daily enduring one of the worst commutes known to mankind so that I could pursue my dream.

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Outbreaks of foodborne illness linked to the consumption of raw or minimally processed fruits and vegetables have increased in recent years. Reasons for these outbreaks have been reviewed (Beuchat, 1998; Beuchat, 2002; Beuchat and Ryu, 1997). While an outbreak may be directly or epidemiologically linked to consumption of a particular type of produce it is often difficult to determine how the contamination event occurred. This is particularly true of plant-based foods that are consumed raw. Good Agricultural Practices (GAPs) demand that precautions be taken to exclude wild and domestic animals from fields where produce destined for the fresh retail market is grown. Nevertheless, animal manure is used as fertilizer and wild animals can be found in or near these fields. Animals may be allowed to graze in fields adjacent to those used for crop production. Human pathogens present in animal excreta may then come in contact with produce through aerosolization, water runoff, or by vectors such as birds, insects, and humans.

Free living, microbivorous nematodes are attracted to and will consume bacteria found in the soil. It is known that these nematodes will consume bacteria pathogenic to humans (Chang *et al.*, 1960; Smerda *et al.,* 1971; Aballay *et al.*, 2000; Garsin *et al.*, 2001; Sifri *et al.,* 2003; Caldwell *et al.,* 2003a; Caldwell *et al.,* 2003b). As nematodes travel in the water phase of moist soil, they may come in contact with preharvest fruits and vegetables, either by attraction or random chance. It is hypothesized that pathogens resident in the gut or on the outer cuticle of infected worms may then be released on the surface of produce. Worms may remain on the surface of produce and, even if the bacterial cells are not released, they may remain viable and cause infection once the produce is consumed.

There have been sporadic reports describing the ability of bacteria, parasites, and viruses to survive in the gut of free-living nematodes after decontamination treatments. Most of these reports conclude by stating that free-living nematodes can potentially vector human pathogens. Until recently, no research has been done to determine the ability of free-living, microbivorous nematodes to vector human pathogens to preharvest fruits and vegetables. A brief review of the

microbiology of produce, manure, and manure composts, with particular associations linking free-living nematodes and foodborne pathogenic bacteria is presented here. Factors affecting the potential of free-living nematodes to vector enteric pathogens to preharvest fruits and vegetables are discussed.

Microbial Contamination of Produce

Consumption of produce and foodborne outbreaks

The current United States Department of Agriculture (USDA) dietary guidelines suggest that five servings of fruits and vegetables be consumed daily (United States Department of Agriculture, 2000a). The per capita consumption of fresh fruits and vegetables increased by 24 – 26% from 1970 to 1999 (USDA, 2000b). Rangarajan *et al.* (2000) suggested that a potential reason for increased consumption of produce is the desire of individuals to eat a healthy diet that is high in fiber and low in fat. Concurrent with rising consumption of fresh produce, there have been increased numbers of foodborne illness outbreaks caused by bacteria, parasites, and viruses that are linked to the consumption of raw or minimally processed fruits and vegetables (Beuchat, 1996; IFT/FDA, 2001). It has been hypothesized that factors such as a more globalized food supply and changes in agronomic practices have been at least partly responsible for the increase in produce-associated outbreaks (Beuchat, 1998; Beuchat and Ryu, 1997; Mead *et al.*, 1999; IFT/FDA, 2001).

Microbiology of produce

There are two categories of microorganisms, those that cause spoilage and those that cause human disease and perhaps also spoilage, that can have a negative impact on produce quality and public health. While fermentation can have a negative impact on some sensory attributes in produce, it is generally considered to be a positive process and thus will not be discussed in this review. Spoilage microorganisms typically alter the sensory quality of produce. In general, spoilage is viewed negatively as produce becomes unsuitable for consumption.

However, extending the shelf life of raw fruits and vegetables by delaying spoilage may permit human pathogens to increase to populations capable of causing disease. For example, asparagus placed in a modified atmosphere environment to extend shelf life has been reported to harbor higher populations of *Listeria monocytogenes* compared to asparagus not exposed to the modified atmosphere storage (Berrang *et al.,* 1989). In this example, it would be more desirable to have the produce spoil prior to the pathogen reaching populations that may cause disease.

Bacteria, yeasts, and molds can be routinely recovered from the surface of raw or minimally processed fruits and vegetables. Populations of these microorganisms can vary widely. Some of the factors that may affect the number of microorganisms recovered include type and variety of produce, growing environment, weather conditions, and proximity to soil (Brackett, 2001). Populations of mesophilic bacteria have been reported to range from 3 to 9 $\log_{10}$ CFU/g of fresh produce (Zagory, 1999). In the apple industry, a fruit is considered sound if bacterial counts are less than 5 $\log_{10}$ CFU per apple (Doores, 1983).

Yeasts, molds, and possibly other spoilage organisms can develop microenvironments that enhance the growth of human pathogens. This is referred to as metabiosis. Fruits and vegetables that have been injured in some manner (e.g., chill injury or mechanical damage) have easy points of entry for spoilage yeasts and molds to access nutrients. Growth of proteolytic yeasts and molds can potentially raise the pH of the infected produce to a level that would permit the growth of human pathogenic bacteria. Proteolytic yeasts and molds have been reported to raise the pH of tomato juice agar (pH = 4.3) and tomato juice (pH = 4.1) to 7.2 and 6.4, respectively (Wade and Beuchat, 2003a). Conditions favorable for growth of *Clostridium botulinum* (Draughon *et al.*, 1988) and *Salmonella* (Wade and Beuchat, 2003b) have been reported to develop in raw tomatoes co-inoculated with proteolytic molds. The impact of yeasts and molds on the growth of bacteria may be less on fruits and vegetables that are near neutral pH. Richards *et al.* (2004) showed that the pH of cantaloupes was altered by the growth of molds, but this did not result in statistically higher populations of *Salmonella* Poona compared to

populations in fruit not coinoculated with mold. The pathogen grew to numbers that would be capable of causing disease in humans, regardless of the presence of molds.

Different microbiota predominates on fresh fruits compared to vegetables. Nutrients from vegetable tissues are capable of supporting the growth of bacteria, yeasts, and molds. The pH of most vegetables is in a range that will support survival and growth of bacteria (Jay, 2000). Since bacteria will often out compete yeasts and molds for nutrient sources, they are more commonly the cause of spoilage in vegetables. Fruits, on the other hand, often have a lower pH than vegetables and therefore are more likely to be spoiled by yeasts and molds rather than bacteria (Jay, 2000).

Contamination of produce with bacteria capable of causing human disease

Much of the concern with microbiological contamination of fresh fruits and vegetables is focused on spoilage microorganisms. Most of the microorganisms not uncommonly found on the surface of fruits and vegetables are not a human health concern; however, some spoilage molds may be mycotoxigenic (Doores, 1983). On occasion, fruits and vegetables become contaminated with bacterial human pathogens (Beuchat, 1996). Gram-negative bacteria of human health concern that have been isolated from raw vegetables include *Aeromonas hydrophilia, Salmonella, Escherichia coli* O157:H7, *Shigella, Vibrio cholerae, Plesiomonas shigelloides,* and *Yersinia enterocolitica;* gram-positive bacteria include *Bacillus cereus, Clostridium botulinum, Clostridium perfringens, Staphylococcus aureus,* and *L. monocytogenes,* (Beuchat, 1998; Brackett 1999).

The concept of fruits and vegetables being contaminated by human pathogenic bacteria is changing. Historically, it was believed that the edible portion of fruits was sterile. Low pH, skin and rind, and naturally occurring antimicrobials were believed to be sufficient to protect the edible tissues of fruits from contamination with human pathogens (Doyle, 1990). Once it was discovered that produce could harbor human pathogens, it was thought that the contamination event occurred during post-harvest handling and processing. This view has shifted to one in

which it is believed that a majority of the contamination of produce occurs before it is harvested (Tauxe, 1997). Although initial contamination of produce may occur in the field, post-process handling is still important as a mechanism for contamination. Improperly handled produce can become damaged and thus a more suitable environment for growth of spoilage and pathogenic bacteria (Brackett, 1994). Damaged produce tissues can exude nutrients, phytoalexins, and other antimicrobials. These exudates may either enhance or retard the growth of pathogens and spoilage microorganisms naturally occurring on produce (Beuchat, 2002).

Contamination of produce with animal parasites

Consumption of surface water, exposure to recreation water, animal-to-person contact, and person-to-person contact have been listed as the major modes of transmission of protozoa to humans (Speer, 1997). Of the protozoan parasites, the apicomplexa group is characterized as requiring a vertebrate host to complete its life cycle and produce infectious cysts (Ortega, 2001). Members of this group that inhabit the intestinal mucosa and cause diarrheal illness in humans include *Cryptosporidium parvum, Cyclospora cayetanensis,* and *Isospora belli*.

Cyclosporiasis has been reported to be associated with raspberries (Centers for Disease Control and Prevention, 1996; 1997b, c), lettuce (Centers for Disease Control and Prevention, 1997c), and basil (Centers for Disease Control and Prevention, 1997d) contaminated with *Cyclospora cayetanensis*. *Toxoplasma gondii,* a coccidial parasite of similar size and shape to *C. cayetanensis*, has been reported to attach the trichomes of raspberries (Kniel *et al.*, 2002). Consumption of unpasteurized apple cider was implicated in an outbreak of cryptosporidiosis (Centers for Disease Control and Prevention, 1997a). The apples were harvested from an orchard that was next to pastureland in which cattle had recently grazed. It is believed that runoff water from the pastureland contaminated the apples prior to harvest. Contamination of food with parasites most likely occurs when the food comes in contact with animal or human feces, contaminated water, or untreated sewage or sludge (Beuchat, 1998).

Trichinella, Ascaris, and *Anisakis*, nematodes that are parasitic to humans, are known to contaminate foods. *Ascaris* does not have an obligate intermediate host and thus can be transmitted to humans via contaminated water or vegetables (Doyle, 2003). *Trichinella* and *Anisakis*, on the other hand, can encyst in the muscle tissue of meat or fish and potentially infect humans when the infected tissue is consumed (Doyle, 2003).

Non-proliferation of protozoan parasites in food, as well as the inability of parasites to grow on culture media, makes them particularly difficult to detect in foods (Jay, 2000). Concentration and staining of parasites are commonly used to detect parasites. This complicates investigations of parasites in foods. It is often difficult to visualize using a microscope how parasites attach to fruits and vegetables, so other techniques must be employed.

Contamination of produce with viruses

Viruses are transmitted from host to host directly (fecal-oral contact) or indirectly (fomites, animals, food, or water). In general, viruses are classified according to mode of transmission, host, type of organ infected, nucleic acid present, structure, and presence or absence of an envelope (Lindsay, 1998).

Viruses such as norovirus and hepatitis A have been associated with large outbreaks of illness in humans. Outbreaks of illness on cruise ships caused by norovirus have been documented (Centers for Disease Control and Prevention, 2002). Despite all of the attention that outbreaks on cruise ships received, it is estimated that 60 – 80% of the outbreaks of norovirus infections occur on land (Centers for Disease Control and Prevention, 2003b). Norovirus was implicated in 22 outbreaks, causing over 2,100 confirmed cases of illness associated with consumption of contaminated fruits and vegetables in 2001 (Centers for Disease Control and Prevention, 2003c). As few as ten norovirus particles are needed to cause illness and it can be transmitted by direct person-to-person contact, consumption of contaminated food or water, airborne droplets of vomitus, and contact with contaminated surfaces (Centers for Disease Control and Prevention, 2003b).

Green onions imported from Mexico were epidemiologically linked to approximately 555 cases of hepatitis A infections in 2003 (Centers for Disease Control and Prevention, 2003). Illnesses were traced to a single restaurant in Monaca, Pennsylvania. Genetic sequences of isolates very similar to the outbreak strain were obtained from patients with hepatitis A in an outbreak linked to green onions in Tennessee, Georgia, and North Carolina (Centers for Disease Control and Prevention, 2003).

Viruses outside of a human host are inert, so replication on food is not a concern. Despite being more acid resistant than bacterial cells, viruses that cause foodborne illness are not more heat resistant (Cliver, 2001). Therefore, viruses are more likely than bacteria to be inactivated upon cooking foods.

Difficulties in decontaminating produce

Once produce becomes contaminated with cells of human pathogenic bacteria it is difficult to disinfect. A variety of decontamination treatments, including high-pressure sprays, brushing, and chemical washes, have been evaluated for their effectiveness in reducing contamination from produce (Parish, 1997; Beuchat *et al.*, 1998, Food and Drug Administration, 1998). None of these proposed methods has been totally effective in removing all viable bacteria from produce (Beuchat, 1998; Annous *et al.*, 2000; Wright *et al.*, 2000).

When a warm piece fruit or vegetable is immersed in a cold liquid (negative temperature differential) a slight vacuum may occur within the produce as internal gasses contract. Under these conditions, bacterial cells have been reported to internalize within tomatoes (Bartz, 1982) and apples (Burnett *et al.*, 2000; Kenney *et al.*, 2001). Bacterial cells located in subsurface tissues of produce may be protected from decontamination treatments by preventing or limiting contact of sanitizers with the bacterial cells. Bacterial cells that remain on the surface of fruits and vegetables may become enmeshed within naturally occurring waxy platelets and subsequently protected from decontamination treatment (Kenney *et al.*, 2001). Growth of *E. coli*

O157:H7 and *Salmonella* can occur on produce to which wax has been applied (Kenney and Beuchat, 2002).

Chlorinated water is commonly used as a sanitizer for fruits and vegetables. A disadvantage of using chlorine is that organic material, light, air, and metals cause inactivation. To be effective and safely used as a sanitizer, chlorine needs to be used at a relatively narrow pH range. Chlorine gas is formed if the pH is decreased to 4.0 or below. The effectiveness of chlorine in eliminating human pathogens is also affected by temperature. At 20°C, the amount of chlorine in the form of hypochlorous acid (HOCl) is 23% and 97% at pH of 6.0 and 8.0, respectively.

Other sanitizers that have been evaluated for their effectiveness in inactivating pathogenic bacteria on produce include hydrogen peroxide (H_2O_2), and chlorine dioxide (ClO_2). These sanitizers are not completely effective in removing bacteria from the surface and subsurface tissues of fruits and vegetables and exhibit unique sanitization characteristics, depending on the nature of the pathogen being targeted as well as characteristics of the fruit and vegetable tissues and juices (Beuchat, 1998).

Hydrogen peroxide (H_2O_2) vapors have been reported to be effective in reducing spoilage microorganisms on grapes, cantaloupes, prunes, cucumbers, green bell pepper, zucchini, and raisins (Sapers and Simmons, 1998). However, *E. coli* was shown to survive on the stem and calyx areas of apples after treatment with 5% H_2O_2 (Sapers *et al*., 2000). A disadvantage of using H_2O_2 as a sanitizer is that it can induce browning in certain fruits and vegetables.

Chlorine dioxide (ClO_2) has some notable advantages over chlorine in that its efficacy is less influenced by pH and organic matter, and it does not tend to form chloramines when in the presence of ammonia (Beuchat, 1998). The need for on-site generation of ClO_2 and the fact that it is unstable above 30°C when exposed to light are some of the disadvantages in its use to disinfect produce. Cells of *E. coli* O157:H7 on injured tissue of green pepper survived treatment with gaseous ClO_2, but the gas was effective in killing the pathogen on the surface of uninjured

skin (Han *et al.,* 2000). More research needs to be conducted to determine factors that influence the effectiveness of ClO_2 in inactivating microorganisms on produce.

Cross protection and adaptation

Bacteria surviving sub-lethal assault may become cross-protected against further disinfection treatments. Bacterial cells grown at acidic or alkaline pH have been reported to be more resistant to disinfection treatments such as heating and further exposure to acidic conditions. Cells of *L. monocytogenes* held for 45 min at pH 12.0, for example, are more resistant to heating than cells held at pH 7.3, but cells exposed to chlorine were more sensitive than control cells to heating (Taormina and Beuchat, 2001). Cells of *Shigella flexneri* and *E. coli* O157:H7 grown under acidic conditions are subsequently more resistant to acidic conditions (Tetteh *et al.*, 2004), sodium lactate, and sodium chloride (Garren, *et al.,* 1998).

In addition to inducing cross protection to sanitizers by pH stress, nutrient availability and growth phase can influence the response of bacteria to disinfection treatments. Starved and stationary phase cells of *E. coli* O157:H7 have been shown to be more acid tolerant than log phase cells in a nutrient rich environment (Arnold and Kaspar, 1995).

These examples demonstrate the need for a process that eliminates pathogens from the surface of fruits and vegetables. Sub-lethal injury can result in cells that are more resistant to subsequent treatments.

Manure, Compost, and Soil as Sources of Enteric Pathogens on Pre-harvest Fruits and Vegetables

Guidelines that recommend the exclusion of domestic and wild animals from fruit and vegetable production areas have been issued by the United States Food and Drug Administration (Food and Drug Administration, 1998). It is acknowledged that it may not be possible to completely exclude animals from these areas, but reasonable effort should be given to ensure compliance with Good Agricultural Practices (GAPs). To comply with GAPs, domestic animals

should be excluded from fields and orchards during the growing season and measures be taken to ensure animal wastes from adjacent fields or storage facilities do not contaminate production areas (Food and Drug Administration, 1998). Ideally, GAPs should be adhered to by all farmers and growers. In reality, however, there will always be a certain percentage of farmers and growers that are unaware of or choose not to follow GAPs. When GAPs are not followed, the potential for preharvest produce to become contaminated with enteric pathogens is increased.

Pathogens in manure

The risk of contaminating preharvest crops with any number of human pathogens is high when untreated or raw manure is applied to fields. Table 1.1 lists human pathogens that have been isolated from various animals. Animals infected with pathogens capable of causing human infections may discharge the pathogen into the environment through its excreta. Human pathogens may be part of the normal microbiota of the animal (e.g., *Campylobacter* in chicken) and presence of the pathogen in the feces of the animal would be expected. Enteric pathogens may cause illness in wild and domestic animals as well as in humans. Acutely ill animals can be treated and excretion of the pathogenic microorganism can be controlled. Asymptomatic animals may shed pathogens in their feces (Cray and Moon, 1995). This situation is probably the most difficult to deal with because when an animal does not display any outward signs of illness, it is likely that no action will be taken to control the shedding of the pathogen in feces of the infected animal.

Verotoxin-producing *E. coli* was found in 24% of slurry, farmyard manure, and sewage sludge in France (Vernozy-Rozand *et al.,* 2002). In a survey of 14 dairy herds from 11 states, Zhao *et al.* (1995) found that populations of *E. coli* O157:H7 ranged from 3 – 5 $\log_{10}$ CFU/g feces in cows harboring the pathogen. In another study, it was determined that cattle infected with *Campylobacter* shed the pathogen at a rate of 1.84 – 4.52 CFU/g feces (Stanley *et al.* 1998). Various factors may influence the number of bacteria shed by animals. For example, *E. coli* O157:H7 is shed at higher numbers by cattle during late summer and early fall than in other

Table 1.1. *Human pathogens isolated from domestic and wild animals*

Animal	Pathogen	Reference
Cattle	*Escherichia coli* O157:H7	Borczyk *et al.*, 1987
	Salmonella	Davis *et al.*, 1999
	Listeria monocytogenes	Siragusa *et al.*, 1993
	Campylobacter jejuni	Beach *et al.*, 2002
	Cryptosporidium parvum	Tanriverdi *et al.*, 2003
Poultry	*Salmonella*	Oosterom, 1991
	L. monocytogenes	Suihko *et al.,* 2002
	C. jejuni	Kelley *et al.*, 1998
	Yersinia enterocolitica	Kelley *et al.,* 1998
Pigs	*E. coli* O157:H7	Tutenel *et al.,* 2003
	Salmonella	Rose, *et al.*, 2002
	L. monocytogenes	Kanuganti *et al.,* 2002
	C. jejuni	Nesbakken *et al.*, 2003
	Y. enterocolitica	Nesbakken *et al.,* 2003
Sheep	*E. coli* O157:H7	Kudva *et al.,* 1996
	Salmonella	Sandberg *et al*., 2002
	L. monocytogenes	Barbuddhe *et al.,* 2000
	Campylobacter	Erganis *et al.,* 2002
	Y. enterocolitica	Bin-Kun *et al.,* 1994
Deer	*E. coli* O157:H7	Keene *et al.,* 1997
	C. parvum	Skerrett and Holland, 2001
Dogs	*Salmonella*	Rinkinen *et al.*, 2003
	C. jejuni	Rinkinen *et al.*, 2003
	Cryptosporidium	Miller *et al.*, 2003

seasons (Van Donkersgoed, *et al.*, 1999; Elder *et al.*, 2000) while *Campylobacter* displays no seasonal variation in shedding rates in cattle (Stanley *et al.*, 1998).

Depending on storage conditions, foodborne pathogenic bacteria may survive for extended periods of time in manure or manure-amended soil. *E. coli* O157:H7 has been reported to survive for up to 56 days in bovine feces stored at 22°C (Wang, 1996). Kudva *et al.* (1998) reported that they recovered *E. coli* O157:H7 from ovine feces stored for 100 days at 4 or 10°C.

E. coli O157:H7 was isolated from manure-amended soil stored at 5, 15, and 21°C for up to 77, >226, and 231 days, respectively (Jiang *et al.*, 2002). Populations of *E. coli* O157:H7 declined more rapidly when inoculated into unheated manure-amended soil compared to manure-amended soil that had been autoclaved (Jiang *et al.*, 2002). Rainfall can leach *E. coli* O157:H7 from manure and transport it through the soil profile (Gagliardi and Karns, 2000). Simulated rainfall at a rate of 1.65 cm/h was applied to sandy loam and silty clay loam soils inoculated with *E. coli* O157:H7. Leachate samples were analyzed hourly to determine populations of the pathogen. Numbers of *E. coli* O157:H7 recovered from the leachate remained near the inoculum level for at least 8 h. This indicates that the microorganism was multiplying to maintain high numbers in the leachate. Additionally, the pathogen was recovered up to 10 cm below manure applied on the surface of soil (Gagliardi and Karns, 2000).

Handling of manure

Collection of manure from cattle for use as fertilizer is limited to barns, feed lots, and open lots. Manure from animals grazing on pastureland is not used as compost. Cattle manure is collected and handled in either liquid (slurry) or solid form. Manures with higher moisture content (i.e., slurries) are not as feasible, both economically and technically, for use in composting because large amounts of dry amendments must be added to reach the optimum moisture content (Kashmanian and Rynk, 1996).

Poultry manure intended for use as fertilizer is handled in a much different manner. Feces from the birds are mixed with the bedding (wood chips) and thus have a higher dry matter content. Poultry manure is high in nitrogen and, even when combined with litter, materials high

in carbon may need to be added to obtain the proper carbon-to-nitrogen (C:N) ratio before application to soil.

Feces from several animals are routinely mixed together to produce a batch of manure for application as a fertilizer and soil amendment. When mixed, feces from a single infected animal can contaminate an entire batch. To minimize the chance of recontaminating manures treated to eliminate pathogens, it is recommended that untreated manure not be added to batches of treated manure (Food and Drug Administration, 1998).

The size of animal production facilities and proximity to cropland can influence the manner in which the manure is handled. Smaller facilities with few animals may not have the space to process the excreta into compost. In this case, manure may be transported directly from the building housing animals and applied to the field without treating to eliminate pathogens. Poultry farms are reported to be less likely to compost manures because poultry farms tend to grow less feed at the location where animals are matured compared to cattle farms and are less likely to have cropland where manure or compost can be applied (Kashmanian, 1996).

Time and space are two requirements needed for proper composting of manure. Farmers that do not have the time or space to properly compost manure may use alternative treatments to reduce or eliminate pathogens. Chicken manure can be dried to 10% moisture content and then exposed to 1% (manure wet weight) ammonia gas for 72 h. *Salmonella* Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* were reduced, respectively, by 8, 8, and 4 $\log_{10}$ CFU/g of manure treated in this manner (Himathongkham and Riemann, 1999). Drying of the manure for the same time without gassing resulted in less than 2 $\log_{10}$ CFU/g reductions for each of the pathogens. Storage of manure below a_w 0.89 at 20°C can result in up to 6 $\log_{10}$ CFU/g reduction of salmonellae within 8 days (Himathongkham *et al.*, 1999a).

Without any treatment, populations of pathogens will usually decline in manure over time. This is useful for farmers that do not have the space required to make the large heaps or windrows needed for composting. *E. coli* O157:H7 and *S.* Typhimurium had decimal reduction times of 6 – 21 days in manure and 2 – 35 days in manure slurry held at 4, 20, or 37°C

(Himathongkham *et al* 1999b). During storage the pH at the manure surface increased by 1.5 – 2 units and the oxidation-reduction potential of the manure declined to below –200 mV, but these changes did not appear to affect the rate of pathogen destruction (Himathongkham *et al.*, 1999b).

Compost

Organic matter can be decomposed and converted into a material that can be used as organic fertilizers, soil amendments, or potting media through a process known as composting. The desire to reduce water pollution and to encourage sustainable agriculture has increased the popularity of composting in recent years (Kashmanian and Rynk, 1996). Once composted, nutrients are less likely to be carried into surrounding areas by surface runoff.

To prepare compost, the organic matter is gathered together, usually placed in piles or windrows, and bacteria and fungi are allowed to carry out degradative processes. For rapid degradation, the C:N ratio should be ca. 30:1 and the moisture content should be 40 – 60%. Since manures are high in nitrogen, carbon-rich materials such as peanut hulls, straw, dry leaves, paper, and cardboard may be combined with the manure to achieve the proper C:N ratio. Small particle size and ample oxygen are also important to enhance the breakdown of organic materials. As organic matter is broken down, heat is produced. It is this heat that gives the added benefit of reducing or eliminating human pathogens, weed seeds, or fly larvae that may be present prior to composting (Kashmanian and Rynk, 1996; Pell, 1997).

There are four distinct stages (mesophilic, thermophilic, cool-down, and maturation) of the composting process. Different microorganisms predominate at each stage. When degradative processes are most active (thermophilic stage), temperatures can reach 66°C. Bacilli usually dominate during the thermophilic stage and fungi, streptomycetes, and yeasts often are not detected (Ryckeboer *et al.,* 2003). As long as the pile is turned to keep it adequately aerated, the thermophilic stage can last for 3 – 4 weeks. It is during this time that the pathogens are destroyed. The diversity of microbial populations increases as the pile cools and matures.

Gram-positive and gram-negative bacteria can be detected and *Aspergillus* and *Mucor* are among the most common molds (Ryckeboer *et al.*, 2003).

Composting of bovine manure has been reported to reduce *E. coli* O157:H7 from 7 $\log_{10}$ CFU/g to undetectable levels when the temperature is maintained at 50°C for 1 – 2 weeks (Jiang *et al,* 2003). In another study, a laboratory-scale composting system was used to inactive 7 $\log_{10}$CFU of rifampicin-resistant *E. coli* O157:H7/g and *Salmonella* Enteritidis/g when compost was held at 45°C for 96 h (Lung *et al.,* 2001). Variations in the effectiveness of the compost in destroying pathogens may be due to the amendments added to manures to achieve the proper C:N ratio. In the study reported by Jiang *et al.* (2003) wheat straw, cottonseed meal, and ammonium sulfate were used as manure amendments, while in the study reported by Lung *et al.* (2001) cypress sawdust was used as an amendment. Woods such as cypress and redwood are rot resistant and may contain antimicrobial compounds that are lethal or inhibit growth of microorganisms.

Manure in agriculture

The application of manure to arable cropland can pose a significant risk of contaminating preharvest fruits and vegetables with human pathogenic bacteria, parasites, and viruses. In conjunction with increasing organic cropland acreage, there has been an increase in the amount of manure and manure composts applied. Human bacterial pathogens have been isolated from soils that have had an annual application of cattle manure (de Freitas *et al.*, 2003). In the same study, the presence of fecal coliforms in hog manure-amended soils was reported. Populations of Enterobacteriaceae were 10-fold higher in soil amended with cattle manure compared to soil amended with hog manure. The biomass in soils amended with manure is higher than that in soils to which no manure or inorganic fertilizers have been applied (Peacock *et al.*, 2001). In addition, the use of manure as a fertilizer has been shown to improve soil quality by reducing phytopathogens, increasing soil organic matter and total carbon, and lowering the bulk density (Bulluck *et al.*, 2002).

Climactic conditions at the time of manure application can affect how long pathogenic microorganisms will persist in soil. *S.* Typhimurium in manure has been reported to persist in soils for longer periods of time when applied at warm temperatures (> 20°C) compared to cool (< 10°C) temperatures (Natvig *et al.*, 2002). *S.* Typhimurium did not survive repeated freeze-thaw cycles in the soil and this suggests that manure applied in late fall may not increase the risk of contaminating vegetables planted the next spring (Natvig *et al.*, 2002).

The United States Food and Drug Administration Center for Food Safety and Applied Nutrition (CFSAN) has issued guidelines for the use of manure and manure compost on fields used to grow fruits and vegetables destined for the fresh produce market (Food and Drug Administration, 1998). These guidelines suggest that manures be actively and passively treated to reduce or completely eliminate enteric pathogens prior to application. It is also suggested that measures be taken to exclude wild and domestic animals from fresh produce fields, vineyards, and orchards during the growing season.

Soil composition

Soil composition can have a major influence on movement of bacteria. In sandy soils, the capillaries between sand particles are of sufficient size to allow movement of bacterial cells (Bitton *et al.*, 1974). Only limited movement of bacteria occurs in heavy soils because capillaries present tend to be inside aggregates, and their size excludes entrance of bacteria (Bitton *et al.*, 1974). Organic matter and clay particles are believed to have the greatest influence on the movement of bacteria because of adsorption of cells to negatively charged surfaces in the soil column (Mawdsley *et al.*, 1995).

Nematodes

It has been estimated that there are approximately 500,000 different species of nematodes (Dusenbery, 1980). This is in the same magnitude as the number of insect species. Nematodes constitute a large proportion of the microorganisms naturally occurring in soil and some play an

important role in nutrient cycling by decay of organic material. Other nematodes are parasitic to humans and can be transmitted in foods. Included in this group are *Trichinella, Ascaris,* and *Anisakis.* Soil nematodes consist of two major groups, those that are parasitic to plants and those that are free-living, i.e., derive nutrients in the water phase of soil. Human parasitic nematodes are not the focus of research described in the following chapters and will not be reviewed here. Instead, this review will focus on free-living nematodes found in the soil.

Classification of nematodes

Classifying nematodes (worms) has proved to be difficult and problematic. Yeats *et al.* (1993) suggested that nematodes be classified into eight groups, based largely on feeding behavior:

1. Plant feeding (seven sub divisions)
2. Fungal feeding
3. Bacterial feeding
4. Substrate ingestion
5. Animal predators (two sub divisions)
6. Unicellular eucaryote feeding
7. Dispersal or infective stages of animal parasites
8. Omnivorous

Worms in the same genus are usually classified in the same feeding group, but exceptions do occur. The majority of species within the *Aphelenchoides* and *Ditylenchus* genera are fungal feeders; however, *Aphelenchoides ritzemabosi* and *Ditylenchus dipsaci* are plant pathogens (Yeates, 1993). Another weakness of this classification system is that different developmental stages of a worm may exist in different feeding groups. Juveniles of the monochids are bacterivorous, while the adults are carnivorous (Yeates, 1987). Using this classification system, *Caenorhabditis elegans* is categorized as a bacterial feeding nematode.

Nematodes can also be classified using a system based on life strategy characteristics. Rhabditid nematodes, such as *C. elegans,* are usually considered to be opportunists (r-strategists) that are attracted to locations of high microbial populations, e.g., manure (Bongers and Bongers, 1998). Another characteristic of the Rhabditids is that they have a short generation time (ca. 1 week), but a large number of small eggs are produced. At the other extreme are the persisters (K-strategists). Persisters tend to have long generation times (months), produce a few large eggs, and do not respond well upon exposure to rapidly changing food resources (Bongers and Bongers, 1998). It has been proposed that nematodes be categorized according to a colonizer-persister (cp) scale (cp1 – cp5), where cp1 would be r-strategists and cp5 would represent K-strategists. *C. elegans* would be classified as a cp1 nematode.

These classification systems described above are adequate, but each has drawbacks. Bongers and Bongers (1998) presented a third classification system that merged the two systems. Under the new system, *C. elegans* would be classified as a bacterial feed colonizer (Ba-1).

General characteristics of Caenorhabditis elegans

C. elegans (Figure 1.1), a microbivorous, free-living nematode, lives in the interstitial spaces of soils in temperate regions. It has a short (3 days at 20°C) life cycle, small size (ca. 1 mm as adult), transparent body, invariant number of cells (eutely), and invariant cell position (Riddle *et al.*, 1997). In 1998, *C. elegans* became the first multicellular organism to have its entire genome mapped (Hope, 1999). Mapping of the genome revealed that many of the biological mechanisms of *C. elegans* are conserved across the animal kingdom (Hope, 1999). For these reasons, *C. elegans* is ideally suited for study as a model system for many animals, including humans. Despite the advantages of the using *C. elegans* as an experimental model, it is rarely studied in field experiments in part because of its lack of impact on higher microorganisms or plants.

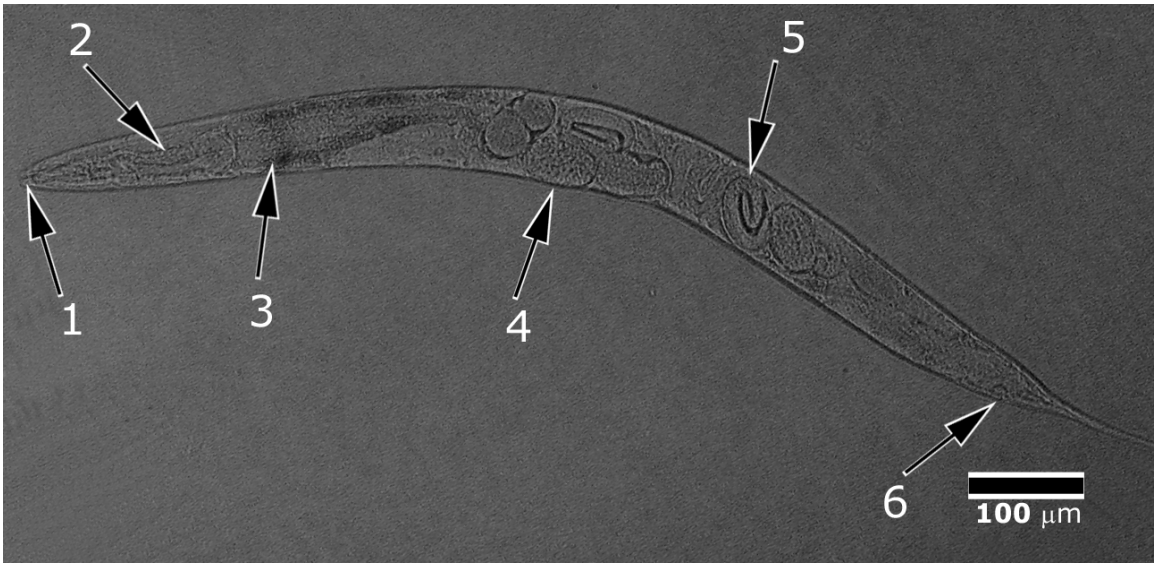

Figure 1.1 Micrograph of adult form of *Caenorhabditis elegans.* (1) Mouth; (2) Pharynx; (3) Intestine; (4) Egg; (5) Immature worm; (6) Anus

When compared to nematode parasites of animals, soil nematodes tend to be comparatively short (< 6.0 mm) and thin (< 0.01 mm diameter) (Ritz and Trudgill, 1999). Their small size allows for relatively free movement between soil particles to find food and a mate. Thus, compacted soil may not be able to accommodate some of the larger species of soil nematodes.

Nematodes, in general, will grow over a wide range of temperatures (Dusenbery, 1980). Bargmann and Mori (1997) reported that wild type *C. elegans* reproduces within a relatively narrow temperature range of 12 - 26°C. The temperature at which *C. elegans* is incubated influences the rate of maturation of the worm. Worms mature 2.1 and 1.3 times faster when incubated at 25 and 21°C, respectively, than those incubated at 16°C (Stiernagle, 1999).

Once a *C. elegans* egg hatches, the juvenile worm will progress through four larval stages (L1, L2, L3, and L4) with each stage being punctuated by a molt (Wood, 1988; Ehrenstein and Schierenberg, 1980). Molts at 20°C occur approximately 13, 22, 30, and 41 h after hatching, respectively, for each of the larval stages L1, L2, L3, and L4 (Ehrenstein and Schierenberg, 1980).

There are few requirements for growth and reproduction of *C. elegans* in the laboratory. Requirements include a bacterial population of 8 $\log_{10}$ CFU/ml, high concentrations of sterols

(not provided by bacteria), a humid environment, and aeration (Dusenbery, 1980; Hope 1999). Sub-optimal bacterial populations (<8 $\log_{10}$ CFU/ml) will decrease the rate at which *C. elegans* reproduces. As a bacterial population increases to greater than 8 $\log_{10}$ CFU/ml, it will take longer for *C. elegans* to begin reproducing at its optimal rate (Venette and Ferris, 1998).

C. elegans is separated into two sexes, hermaphrodite and male, that are made up of 959 and 1031 somatic cells, respectively (Wood, 1988). The majority of adult worms are hermaphroditic, with males spontaneously arising at a rate of 1/500 worms (Wood, 1988). Hermaphroditic nematodes are morphologically female but they have an ovotestis and produce sperm prior to oocyte production (Evans, 1998). Under optimal conditions, rapid reproduction of *C. elegans* is facilitated by the self-fertilization of hermaphrodites. This allows completion of the reproductive cycle without having to find a mate. Hermaphroditic *C. elegans* cannot fertilize each other, but a male can fertilize hermaphrodites. Sperm produced by males out competes sperm produced by hermaphrodites. Eggs fertilized by male sperm will produce males and hermaphrodites at an equal rate; however, eggs fertilized by hermaphrodite sperm will only produce hermaphroditic offspring (Hope, 1999).

C. elegans will continue to increase in number as long as the food supply is sufficient. However, once the food supply is depleted, the *C. elegans* will enter a survival life stage at the time of the L2/L3 larval molt. This stage is referred to as the dauer larva. The dauer is non-feeding and specially adapted to survive adverse conditions such as desiccation and lack of food for several months (Wood, 1988). Most of the time the dauer will remain motionless, but it will respond to a touch stimulus by moving away rapidly. It is not uncommon for worms in the dauer state to climb objects projecting out of the agar, to stand on their tails, and wave their head (Riddle, 1998). Riddle (1998) suggested that this behavior is to facilitate attachment of the dauer to passing insects that may then transport the worm to a more suitable environment. As environmental conditions become more favorable for growth and survival of *C. elegans* the dauer molts to become a L4 larva. Dispersal of the dauer favors hermaphroditic reproduction, as only one worm is needed to start a new generation (Riddle *et al.,* 1997).

C. elegans *attractants*

C. elegans responds to chemicals in air and water. It has been suggested that the worm uses chemical attractants in water for short-range chemotaxis to food sources and that volatiles in the air are used for longer-range attraction to food sources (Bargmann and Mori, 1997). The response of wild-type and mutant worms has been compared and it was determined that chemical attraction sensory receptors are located in the head of the worm (Ward, 1973). The same researcher determined that *C. elegans* is attracted to cAMP, cGMP, the anions, Cl^-, Br^-, I^-, the cations Na^+, Li^+, K^+, Mg^+, and by alkaline pH.

Age, viability, and growth conditions of bacteria influence the attractiveness of the bacteria to *C. elegans*. In general, the worm is more readily attracted to young (24 – 48 h) bacterial colonies than to older (96 – 192 h) colonies (Grewal and Wright, 1992). The extent of behavioral alteration is dependant upon the bacterial species. For instance, *Acinetobacter calcoaceticus* var. *antratus* attracts *C. elegans* within 192 h, but worms are not attracted to *Serratia liquefaciens* colonies at the same age (Grewal and Wright, 1992).

C. elegans is less attracted to *E. coli* killed by immersion in boiling water than to live cells (Hosono, 1978). Worms were placed on an agar medium with suspensions of live and heat-treated cells of *E. coli* OP50. A majority of the worms were associated with the live bacteria within 10 min, but few worms were observed in the suspension of dead cells. Worms that fed on dead cells had a slightly slower rate of growth, but laid eggs that hatched normally compared to worms that fed on intact cells (Hosono, 1978). *E. coli* killed by irradiating with ultra violet light or autoclaving were similarly unattractive to *C. elegans* (Andrew and Nicholas, 1976). Bacterial cells that were killed *in-situ* by chloroform gas remained attractive to *C. elegans* (Grewal and Wright, 1992). It was hypothesized that actively growing bacteria produce and excrete substances that are attractive to *C. elegans*. They also stated that gradients of attracting substances could occur in agar around bacterial colonies. These substances would not have time to accumulate in agar when cells are freshly transferred, but could eventually accumulate and attract worms, even after the death of the bacteria.

Bacteria isolated from plant tissues and the soil rhizosphere can be excellent sources of nutrients for free-living nematodes. Populations of *Diplogaster lheritieri* have been reported to increase by at least 700-fold after 17 days of feeding on *Comamonas testosterone,* pseudomonads, and A*grobacterium* (Kimpinski and Sturz, 1996). The presence of a high-quality food source in rhizospheres may be partially responsible for the higher numbers of nematodes recovered from this area of soil (Griffiths, 1994).

Feeding habits

C. elegans consumes bacteria voraciously. It is believed that this is a mechanism used by the nematode to quickly outgrow its competition for nutrients (Riddle *et al.*, 1997). Bacteria suspended in liquid are ingested and filtered out, and the liquid is expelled by nematodes (Avery and Thomas, 1997). Bacteria are retained in the pharynx. The terminal bulb of the pharynx is where the grinding mechanism is located. The lumenal surface of the muscle cells in the terminal bulb secretes a thick, rigid cuticle that constitutes the grinding mechanism (Avery and Thomas, 1997). Some bacterial cells, after passing through the grinding mechanism, may remain viable and be voided by the worm (Bird and Ryder, 1993). The defecation cycle of *C. elegans* is approximately 45 sec and is regular and periodic over time (Avery and Thomas, 1997). It does not appear that the rate at which ingested solids are defecated is influenced by temperature; however, food sources high in water cause a lengthening of the defecation cycle.

There are a wide variety of bacterial genera on which *C. elegans* has been reported to feed. In the laboratory, *C. elegans* is routinely cultured on *Escherichia coli* OP50. The worm, however, will consume bacteria that are pathogenic to certain forms of the life cycle. *Enterococcus faecalis* and *Enterococcus faecium,* for example, are lethal to the eggs and hatchlings of *C. elegans*; but only *E. faecium* will kill adults (Garsin *et al.,* 2001). This behavior is useful in monitoring the survival, growth, and reproductive behavior of adult worms fed on a specific bacterium. By allowing *C. elegans* to feed on *E. faecalis* after a treatment is applied the investigator can be assured that the adult worms received the treatment. *Staphylococcus aureus*

(Sifri *et al.,* 2003), *Streptococcus pneumoniae* (Garsin *et al.,* 2001), *Streptococcus pyogenes* (Jansen *et al.*, 2002), *Brukholderia pseudomallei* (O'Quinn *et al.*, 2001), and *Pseudomonas aeruginosa* (Tan *et al,* 1999) have also been reported to be pathogenic to *C. elegans.*

Various bacterial species cause lethality to *C. elegans* in different ways. *S. pyogenes* kills *C. elegans* by producing hydrogen peroxide (Jansen *et al.* 2002) while *P. aeruginosa* poisons the worms with cyanide (Gallagher and Manoil, 2001). Dead streptococci do not produce hydrogen peroxide and are not lethal to *C. elegans* because hydrogen peroxide is quickly degraded in the environment (Jansen *et al.,* 2002). *P. aeruginosa*, on the other hand, produces cyanide that can accumulate and persist in the environment. Colonies of *P. aeruginosa* can kill *C. elegans* even after the bacteria have been inactivated (Gallagher and Manoil, 2001).

C. elegans is not attracted to all species of bacteria. In fact, the worm is repelled by *Bacillus megaterium* (Andrew and Nicholas, 1976). The authors offered no explanation, other than *B. megaterium* being gram-positive, as to why the worm was repulsed.

Studies on the consumption of bacteria by *C. elegans* have focused on microorganisms of clinical or agricultural concern. More recently, studies have been undertaken to determine if *C. elegans* is attracted to foodborne bacteria known to cause human diseases. Test microorganisms have included numerous strains of *E. coli* O157:H7, and *L. monocytogenes*, and several serotypes of *Salmonella* (Caldwell *et al*. 2003b). *C. elegans* was placed on an agar medium and movement of the worm between the bacterial colonies or a suspension of cells was monitored. None of the microorganisms evaluated in these studies repelled *C. elegans*, but the worm had different behavioral responses to various bacteria. For example, some strains of *E. coli* O157:H7 were more attractive than others to *C. elegans* (Caldwell *et al.,* 2003b). *C. elegans* was comparatively more attracted to *Salmonella* and *L. monocytogenes* than to *E. coli* O157:H7. It was hypothesized that any of these bacteria may be used by free-living nematodes as nutrient sources.

Anderson *et al.* (2003) examined an avirulent strain of *Salmonella* Typhimurium, *Listeria welshimeri,* and *Bacillus cereus* as surrogates for enteric pathogens to determine their ability to

attract *C. elegans*. All of the surrogates attracted *C. elegans* and more than 90% of the worms were associated with bacteria within 16 min of inoculating an agar surface. Tracking of worms for 48 h showed that they would move into and out of colonies of *L. welshimeri* and *B. cereus*, but tended to remain associated with colonies of *S.* Typhimurium and *E. coli.* The gram reaction of bacteria may influence how attractive they are to free-living nematodes. Gram-positive bacteria, such as *L. welshimeri* and *B. cereus*, are less attractive to *C. elegans* and in some cases gram-positive bacteria will repel the worm (Andrews and Nicholas, 1976). Exceptions to this theory do occur, as was observed with *L. monocytogenes*. Gram-positive bacteria may not be as nutritionally adequate as are gram-negative bacteria for nematodes. Worms feeding on gram-positive bacteria have been reported to have slightly delayed development (Anderson *et al.*, 2003) and produce fewer eggs (Grewal, 1991).

Some ingested bacterial cells may pass through the grinder in nematodes unharmed. Ingested bacteria may persist and proliferate within the gut. *S.* Typhimurium (Aballay *et al.*, 2000) and *E. faecalis* (Garsin *et al.,* 2001) are known to establish persistent infections and proliferate in the gut of *C. elegans*. Bacteria ingested by free-living nematodes can be dispersed by worms over several hours. *E. coli*, avirulent *S.* Typhimurium, *L. welshimeri,* and *B. cereus* were dispersed over a 3 h period by worms that had fed on a 24-h lawn of the bacteria (Anderson *et al.*, 2003). Numbers of viable cells excreted appear to be at least partially influenced by the level of attraction of *C. elegans* to a particular bacterial species. For example, the nematode consumed comparatively fewer cells of *B. cereus* than the other test microorganisms, and when placed on bacteria-free agar, the lowest number of foci per worm per unit time was observed with *B. cereus*. Reduced attraction of free-living nematodes to certain species of bacteria can reduce the potential for it of being vectored by the worms. Bacteria that colonize the gut of worms, on the other hand, can potentially be dispersed over longer periods of time and over larger areas.

Nematodes infected with human pathogens may transmit the pathogens to progeny and uninfected nematodes. Adult worms can perish with viable eggs in their gut. As the progeny

mature they can eat their way out of the dead worm. It is hypothesized that juvenile worms would consume human pathogens that may be present in the gut of the adult worm. Other mechanisms of release may result in dispersal of ingested pathogens into the environment. When the cuticle of a worm is ruptured, the gut contents may be released. Nearby worms may consume bacterial cells excreted by infected worms or released by ruptured worms. Depending on the environmental conditions, if these bacterial cells are not immediately consumed by another nematode, they may proliferate and potentially be ingested and infect many more nematodes. In a simulated tidally influenced environment, significant regrowth of *E. coli* and enterococci but not *C. perfingens* in soil has been reported to occur with tides (Desmarais *et al.*, 2002). A similar phenomenon may occur in agricultural soils with periodic rainfall or irrigation.

Behavior of nematodes in soil

Free-living nematodes such as *C. elegans* play an important role in nutrient cycling. Application of cattle slurry to soil has been shown to cause increases in populations of free-living nematodes and nitrogen mineralization (Opperman *et al.,* 1993; Bouwman and Zwart, 1994). Opperman *et al.* (1993) suggested that nitrogen mineralization was stimulated by the activities of nematodes in the soil. In soil void of nematode predators, the rate of mineralization was reduced because populations of bacterivorous nematodes were allowed to grow unchecked and they consumed more bacteria than would have been consumed in the presence of predators.

Higher populations of nematodes have been observed in soil amended with semi-liquid manure than in untreated soil (Dmowska and Kozlowska, 1988). Manure was applied to fields and at rate of 0, 1000, 2000 and 3000 l/km^2 and soils were analyzed for populations for a spectrum of nematodes. Free-living nematodes were recovered in the highest numbers in soils to which manure had been applied in amounts of 1000 l/km^2 or more (Dmowska and Kozlowska, 1998). Microbivorous nematodes may be attracted to the higher populations of bacteria that are found in manure-amended soils.

Soil nematodes can make significant contributions to nitrogen content in soil. A survey of plots in several different countries showed that each month nematodes contributed 2 – 27% of available nitrogen (Ekschmitt *et al.*, 1999). This contribution to soil fertility enhances plant growth. Grasslands where high amounts of microbivorous nematode activity was observed had greater nutrient uptake by the vegetation compared to areas with little or no nematode activity (Bardgett *et al*, 1999).

Soil composition can influence how nematodes move toward food sources. Microbivorous nematodes will move in a linear manner toward a food source in the soil matrix (Anderson *et al.,* 1997a). The foraging strategy of nematodes is one to avoid "traps" in the soil such as dead ends where the food source would quickly be consumed (Anderson *et al.,* 1997b). In a structurally homogenous environment such as nutrient agar, a certain number of nematodes will forage for alternative food sources even when food is readily available (Young *et al.*, 1996). In the soil, nematodes will move toward a food source and may stay near that source until it is depleted (Young *et al,* 1998).

Movement and reproduction of soil nematodes can be influenced by how soil amendments are added. *C. elegans*, for example, was more uniformly distributed in sand that had a humus-litter mixture added that created small areas of organic matter compared to when the mixture was added to sand and there were large areas containing organic matter (Mikola and Sulkava, 2001). When large patches of organic matter were present, *C. elegans* tended to be enmeshed in the organic matter and few worms could be isolated from the sand matrix (Mikola and Sulkava, 2001).

Toxicants such as Cu, Pb, and Cd that may be present in soils can influence the movement and feeding of *C. elegans*. *C. elegans* exhibits decreased movement and feeding when exposed to Cu and Pb for 4 h (Boyd *et al.,* 2003). Exposure to Cd for 4 h did not affect movement of *C. elegans,* but feeding was significantly less than that of unexposed worms.

Difficulties in eliminating human pathogens in nematodes

Once ingested by a nematode, microorganisms capable of causing disease in humans have increased resistance to some types of chemicals used to sanitize raw fruits and vegetables. The outer cuticle of a nematode provides a physical barrier to protect ingested microorganisms against external application of sanitizers. Researchers have reported survival characteristics of viruses, shigellae (Chang *et al.*, 1960), and salmonellae (Chang *et al.*, 1960; Smerda *et al.*, 1971) ingested by free-living nematodes after treatment with chlorinated water. Coxsackie A9 and Echo 97 viruses and *Salmonella* Typhosa*, Salmonella* Paratyphi*, Salmonella* Typhimurium, *Shigella sonnei,* and *Shigella dysenteriae* were recovered from 100% of free-living nematodes treated with 0 – 95 µg/ml chlorine for up to 120 min (Chang, *et al*. 1960). Treatment of *Pristionchus lheritieri* with 10 µg/ml chlorine for 15 min was effective in inactivating 6.7 and 52.5% of ingested *Salmonella* Wichita and *Salmonella typhi*, respectively (Smerda *et al.*, 1971). More recently, Caldwell *et al.* (2003) demonstrated the inability of chlorine and sanitizers to eliminate *Salmonella* Poona ingested by *C. elegans*. The overall effectiveness of chlorine and other sanitizers in eliminating ingested pathogens from the gut of nematodes can be influenced by temperature, contact time, concentration, and the chemical properties of the sanitizer.

C. elegans remains viable in environments with pH in the range of 3.2 to 11.2 (Khanna *et al.*, 1997). It has been demonstrated that *C. elegans* can survive in highly acidic environments (pH 2.2 – 2.7) for at least 5 min without adversely affecting its reproductive behavior (Caldwell *et al.* 2003a). Survival of the worm in low-pH environments suggests that the cuticle of the worm remains intact. To be effective at killing human pathogens in the gut of an infected worm, sanitizers must be able to degrade or otherwise penetrate the cuticle of the worm so that the active form of the sanitizer comes in contact with the pathogen.

In the laboratory, an alkaline NaOCl (pH 13.0) solution is used to surface sterilize eggs of *C. elegans*, kill all life-forms of the nematode except the eggs, and synchronize the maturation of the nematode. On occasion, laboratory cultures of *C. elegans* may become contaminated with microorganisms other than *E. coli* OP50. An alkaline hypochlorite solution can be used to

eliminate contaminants and recover clean stock cultures of *C. elegans* for further experiments. The extreme alkaline pH of this solution may degrade the cuticle of the worm, facilitating contact of the chlorine with bacteria in the gut of the worm.

Treatment of *C. elegans* with a 2% hydrogen peroxide solution resulted in the death of adult worms within 1 day (Caldwell *et al.,* 2003a). Some worms were able to lay eggs prior to death but the resulting progeny showed no outward signs of being adversely affected.

In general, soil-borne nematodes are sensitive to desiccation. Worms deposited on the surface of post-harvest fruits and vegetables may become desiccated within a short time. The carcass of the infected nematode may be removed during the cleaning and sanitizing process, or remain on the surface of the produce until it is consumed. Desiccated worms may be less effective at protecting ingested bacteria from sanitizers compared to live worms. As the worm enters a higher moisture environment, the carcass may begin to absorb the sanitizer, which may eventually come in contact with microorganisms remaining in the gut. If the relative humidity is high enough, autolysis of worms may occur. As the worm decays its cuticle may disintegrate and gut contents would be released into the environment. If this occurs while the worm is on the surface of fruits or vegetables, dispersal of microorganisms surviving in the gut of the worm would occur. Once produce becomes contaminated, regardless of the mechanism, it is difficult to sanitize (Beuchat, 1998).

Vectoring of human bacterial pathogens to preharvest fruits and vegetables

The possibility that plant-parasitic nematodes can vector human pathogenic bacteria onto produce remains remote. A study done to determine if tomato plants grown in soil infested with *Meloidogne incognita* could internalize *Salmonella enterica* revealed that the pathogen was not taken up by plant tissues. The pathogen was not detected in roots, galls, stems and leaves of plants over a period of 4 weeks after inoculation (Beuchat *et al.,* 2003).

It remains unclear if free-living nematodes such as *C. elegans* are attracted to pre-harvest fruits and vegetables or if they come in contact with the produce by random chance. *C. elegans*

is attracted to *S.* Poona more readily than to cantaloupe juice (Caldwell *et al.,* 2003b). Cantaloupe juice deposited on the surface of agar contained only 4 – 5% of *C. elegans* inoculated onto the agar compared to 56 – 67% of worms associated with an inoculum containing *S.* Poona within 20 min. In the same study, a higher percentage of worms tended to be attracted to tryptic soy broth than to cantaloupe juice samples. This does not exclude the possibility that free-living nematodes may be attracted to other types of fruits or vegetables.

It is known that infected nematodes can transport at least one foodborne pathogen through the soil matrix and eventually deposit it on the surface of produce (Caldwell *et al.*, 2003b). An inoculum of *S.* Poona was placed at the bottom of glass jars. A suspension of 20 – 30 worms was deposited in the inoculum. The inoculum was covered with 1 or 3 cm of sterile soil and a piece of cantaloupe rind was placed on top. A separate set of jars not containing *C. elegans* was also prepared. Jars were sealed and incubated at 20°C for up to 10 days. *S.* Poona was detected on cantaloupe rind 1 –3 cm from the inoculum within 1 day when *C. elegans* was present (Caldwell *et al,* 2003b). In nearly all cases, *S.* Poona was detected on more cantaloupe rind samples when *C. elegans* was present than when the worm was not present.

Rude *et al.* (1984) conducted a 2-year survey of salad vegetables in wholesale and retail markets to determine the presence of nematodes, amoebae, and *Salmonella.* Vegetables included in the survey were cucumbers, cabbage, lettuce, celery, carrots, radishes, tomatoes, mushrooms, cauliflower, and spinach. Carrots and radishes contained more then twice the number of larval nematodes compared to other types of produce. *Salmonella* was detected on 8% of the produce samples tested.

Pseudomonas fluorescens biovar *reactans*, an antagonist to the bacterial blotch pathogen (*Pseudomonas tolaasii*) in mushrooms, has been reported to be vectored by *C. elegans* (Grewal 1990). Higher populations of *P. fluorescens* biovar *reactans* than *P. tolaasii* were routinely isolated from the gut of *C. elegans.* Mushrooms grown in the presence of *C. elegans* that had ingested *P. fluorescens* biovar *reactans* were infected less often by *P. tolaasii* than mushrooms not grown in the presence of the worm. It is likely that *P. tolaassii* is out competed by *P.*

fluorescens in the gut of the worm and that the worm sheds viable cells on mushroom sporophores.

In addition to bacteria, human parasites and viruses are ingested by *C. elegans*. *C. elegans* can ingest 100 – 200 *Cryptosporidium parvum* oocystes within 2 h, but the worm does not ingest *Cyclospora cayetanensis* oocysts (Huamanchay *et al.*, 2004). It was suggested that the larger size of *C. cayetanensis* oocysts (8 – 10 μm) compared to *C. parvum* (4 – 6 μm) may be the reason that *C. cayetanensis* was not ingested by *C. elegans*. It is possible, however, that other free-living nematodes are capable of ingesting *Cyclospora* oocysts. Ingested oocysts from live worms that were released by grinding and from whole, desiccated worms were infective to mice (Huamanchay *et al*., 2004). A free-living nematode isolated from city water was reported to ingest Coxsackie A9, a virus capable of causing disease in humans, and protect the virus from chlorination treatments (Chang *et al,* 1960). Lower numbers of the virus were found in nematodes compared to numbers of bacterial cells. Despite the difficulty worms had ingesting the virus, compared to *S. typhosa* and *Shigella sonnei,* Coxsackie A9 virus survived for longer periods of time in the gut of nematodes (Chang *et al.*, 1960).

CONCLUSIONS AND FURTHER RESEARCH NEEDS

C. elegans is attracted to manures that have high populations of bacteria, some of which may be pathogenic to humans. It is known that microbivorous nematodes will voraciously ingest bacteria, parasites, and viruses. Once ingested, foodborne pathogens may be harbored in the gut of the worm for extended periods of time and potentially protected from sanitizer treatments.

Ingestion of pathogens by free-living nematodes can be influenced by many factors. Toxicants in the soil can reduce the activity of free-living nematodes, which can retard nutrient cycling. Temperature, amount of rainfall, and application of manure, composts, and chemicals to cropland are some of the other factors that may influence nematode activity.

The exclusion of microbivorous nematodes from cropland soil would clearly have a detrimental effect on the soil quality. Therefore it would not be practical to remove them from

soil in which fruits and vegetables are grown. The other option is to prevent soils from becoming contaminated with pathogens. It is unrealistic to think that human pathogens can be completely excluded from soil, but strict adherence to FDA guidelines and GAPs can minimize the risks of pre-harvest fruits and vegetables becoming contaminated.

Further research needs to be conducted to determine the potential of free-living nematodes to vector human pathogens to preharvest fruits and vegetables. The objectives of studies conducted in this dissertation research project for the purpose of providing some of this information were as follows:

1. To determine the persistence of *Escherichia coli* O157:H7, *Salmonella* Poona, and *Salmonella* Newport in the gut of *C. elegans* as affected by temperature and relative humidity, and to determine if infected worms transmit *S.* Newport to progeny and uninfected worms.
2. To evaluate the efficacy of cleaners and sanitizers in killing *S.* Newport in the gut of *C. elegans*.
3. To determine if *C. elegans* migrates to and survives and reproduces in manure, manure composts, and manure-amended soil.
4. To determine if *C. elegans* can transport *S.* Newport from manure or manure compost through soil and deposit the pathogen on the surface of fruits and vegetables.

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CHAPTER 2

PERSISTENCE OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA ENTERICA* SEROTYPE NEWPORT, AND *SALMONELLA ENTERICA* SEROTYPE POONA IN THE GUT OF A FREE-LIVING NEMATODE, *CAENORHABDITIS ELEGANS,* AND TRANSMISSION TO PROGENY AND UNINFECTED NEMATODES[1]

[1]Kenney, S. J., Anderson, G. L., Williams, P. L., Millner, P. D., and Beuchat, L. R. To be submitted to *International Journal of Food Microbiology.*

Abstract

A study was undertaken to determine the persistence of *Escherichia coli* O157:H7 and salmonellae in the gut of a free-living nematode, *Caenorhabditis elegans*, as affected by temperature and relative humidity and to determine if infected worms transmit *Salmonella enterica* serotype Newport to progeny and uninfected worms. Worms were fed cells of *E. coli* OP50, *E. coli* O157:H7, and salmonellae followed by incubating at 4, 20, and 37°C for up to 5 days. Initial populations of ingested pathogens significantly increased by up to 2.93 $\log_{10}$ cfu/worm within 1 day at 20°C on K agar and remained constant for an additional 4 days. When worms were deposited on Bacto agar, populations of ingested pathogens remained constant at 4°C, decreased significantly at 20°C, and increased significantly at 37°C within 3 days. Worms fed *E. coli* OP50 or *S.* Newport were incubated at 4 or 20°C at relative humidities of 33, 75, or 98% to determine survival characteristics of ingested bacteria. Fewer cells of the pathogen survived incubation at 33% relative humidity compared to higher relative humidities. Populations of ingested *E. coli* OP50 and *S.* Newport decreased by up to 1.65 and 3.44 $\log_{10}$ cfu/worm, respectively, in worms incubated at 20°C and 33% relative humidity. Placement of adult worms labeled with green fluorescent protein (gfp) that had ingested gfp-labeled *S.* Newport with uninfected wild type worms on K agar resulted in transfer of the pathogen to gut of wild type worms. *S.* Newport was isolated from *C. elegans* two generations removed from exposure to the pathogen. Results of these studies show that *C. elegans* may serve as a temporary reservoir of foodborne pathogens and perhaps as a vector for contaminating preharvest fruits and vegetables.

1. Introduction

Consumption of raw and minimally processed fruits and vegetables has increased substantially in the United States during the past two decades (Mead et al., 1999; NACMCF, 1999; Putnam et al., 2000). Concurrent with this increase, outbreaks of human microbial infections associated with the consumption of raw produce have increased (IFT/FDA, 2001; Beuchat, 2002). These outbreaks have raised interest in identifying preharvest and postharvest sources from which raw and minimally processed fruits and vegetables can become contaminated with microorganisms capable of causing human diseases.

Cropland soil may become contaminated with human pathogenic bacteria in a variety of ways (Nicholson et al., 2000). Application of raw or improperly composted manure (Food and Drug Administration, 1998), contaminated irrigation water, runoff from pastureland, or excreta from wild animals that inhabit cropland have been suggested as vehicles through which soil and preharvest produce may become contaminated with microorganisms capable of causing human diseases (Williams and Dusenbery, 1990; Centers for Disease Control and Prevention, 1997; Cody et al. 1999). Depending upon environmental conditions, the population density of microorganisms within the soil matrix changes and is not homogeneous (Williams and Dusenbery, 1998). Higher populations of microfauna are known to reside in the rhizosphere of plants compared to areas in the soil distant from plant roots (Khanna et al., 1997).

Free-living, bacterivorous nematodes are attracted to areas in soil in which large populations of bacteria are present (Opperman et al., 1993). *Caenorhabditis elegans*, a free-living nematode, has been reported to feed on human pathogenic bacteria such as *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella enterica* serotype Poona (Caldwell et al., 2003b), *Salmonella enterica* serotype Typhimurium (Aballay et al., 2000), *Bacillus cereus*

(Anderson et al., 2003), and *Staphylococcus aureus* (Sifri et al., 2003), as well as on *Pseudomonas aeruginosa* (Mahajan-Miklos et al., 1999; Tan et al., 1999) and *Enterococcus faecalis* (Ewbank, 2002). Ingestion of *S.* Typhimurium (Aballay et al., 2000), *S. aureus* (Sifri et al., 2003), and *P. aeruginosa* (Mahajan-Miklos et al., 1999) shortens the life span of *C. elegans.*

Bacterial cells ingested by *C. elegans* have been shown to remain viable after treatment of worms with acidic sanitizers at concentrations lethal to planktonic cells (Caldwell et al., 2003a). Solutions containing high concentrations of NaOH and NaOCl are used to kill juvenile and adult nematodes and to surface sterilize their eggs without loss of viability, indicating that active components in these solutions can penetrate or compromise the cuticle of the adult worm. *C. elegans* is relatively sensitive to drying. Worms that may be present on the surface of postharvest fruits and vegetables may become desiccated and would be expected to die but bacteria in their gut may remain viable. The cuticle of the dead worm may serve as a barrier to prevent or inhibit produce sanitizers from coming in contact with ingested bacteria, thus enabling them to survive the sanitizing process.

We hypothesize that free-living nematodes may ingest human enteric pathogens present in soil matrices and harbor them in their gut. Ingested pathogens may then remain in the gut and be protected against environmental stresses imposed by desiccation or sanitizers used to decontaminate raw fruits and vegetables, even after the worm has died. The primary objectives of this study were to confirm that *C. elegans* ingests *E. coli* O157:H7 and salmonellae and to determine persistence characteristics of the pathogens in the gut after ingestion. The effects of temperature and relative humidity on survival and growth of ingested cells was investigated. Transmission of ingested *S. enterica* serotype Newport to adult progeny of *C. elegans* and to

uninfected worms was studied. The efficacy of an alkaline NaOCl solution in eliminating cells of pathogens ingested by worms and on the surface of eggs was examined.

2. Materials and methods

2.1. *Maintenance of* C. elegans

A wild type (N2) and a green fluorescent protein (gfp) labeled strain (PD4792) of *C. elegans* were used. Worms were maintained 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; BBL/Difco, Sparks, Md.), and agar (17.0 g) (Williams and Dusenbery, 1998). *Escherichia coli* OP50, an avirulent strain routinely used as a feed source for *C. elegans*, was cultured at 37°C for 24 h in OP50 broth (Brenner, 1974), which contains (per liter of deionized water): sodium chloride (5.0 g) and Bacto peptone (10.0 g). The K agar was surface inoculated with 0.1 ml of a 24-h culture of *E. coli* OP50 and incubated at 37°C for 24 h to establish confluent growth. Approximately 50 adult worms were deposited on the surface of K agar and incubated at 20°C for up to 3 days prior to transferring worms to a fresh K agar plate with a 24-h lawn of *E. coli* OP50, *E. coli* O157:H7, or *S.* Newport and *Salmonella enterica* serotype Poona.

2.2. *Preparation of* C. elegans *for enteric pathogen assay*

The surface of ten K agar plates, each containing 500 – 1000 eggs, together with 30 – 50 adult worms, was washed by depositing 5 ml of sterile K medium (Williams and Dusenbery, 1990) and gently rubbing with a sterile bent glass rod. The suspended eggs and worms were aseptically transferred to a sterile 15-ml centrifuge tube. The wash and transfer procedure was repeated to enable efficient harvesting of eggs. Eggs and worms were collected by centrifugation (500 x *g*, 2 min) and supernatants were removed using a pipette. Worms and eggs in a pellet from pooled suspensions were resuspended in 10 ml of 0.013 M NaOH solution

containing 1% NaOCl (pH 13.0) and incubated at 20°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) and the supernatant was removed. Worms and eggs in the pellet were resuspended in 10 ml of K medium and centrifuged again. The supernatant was removed and the eggs and dead worms were resuspended in K medium. The suspension (0.1 ml containing 400 – 600 eggs) was deposited on the surface of a K agar plate on which a lawn of *E. coli* OP50 had formed, followed by incubation at 20°C for 3 days. This procedure ensures that all worms used in assays are of the same age (synchronized culture). Adult worms were used in assays to confirm that they will ingest pathogenic bacteria and, once ingested, persistence in the gut as affected by temperature and relative humidity.

2.3. *Bacteria used and preparation for ingestion and persistence assays*

Two strains of *E. coli* O157:H7 (SEA13B88 and E0018), two serotypes of *Salmonella enterica* (Poona and a multidrug-resistant strain of Newport), and *E. coli* OP50 (control) were evaluated. All strains were adapted to grow in tryptic soy broth (TSB, pH 7.3; BBL/Difco) supplemented with 50 µg/ml nalidixic acid (TSBN). Nalidixic acid-adapted cells were grown in 10 ml of TSBN at 37°C for 24 h. Cultures were transferred twice to 10 ml of TSBN by loop inocula at successive 24-h intervals. Inoculum (0.1 ml of a 24-h culture) was surface plated on K agar supplemented with 50 µg/ml nalidixic acid (KN agar) and incubated at 37°C for 24 h to produce a lawn of cells. Three-day-old adult worms from synchronized culture grown on K agar were placed on the surface of KN agar plates individually containing lawns of *E. coli* OP50 or a test pathogen and allowed to feed on cells for 3 h at 20°C. K medium (10 ml) was deposited on the surface of plates and worms and bacteria were suspended using a sterile glass rod. Suspensions were placed in sterile 15-ml centrifuge tubes, centrifuged (500 x *g*, 2 min),

resuspended in K medium, and centrifuged again. The supernatant was decanted and 100 – 200 worms were placed on K agar plates containing a lawn of nalidixic acid-sensitive *E. coli* OP50 cells and incubated at 20°C for up to 120 h. Nalidixic acid-sensitive *E. coli* OP50 was used to facilitate detection of nalidixic acid-resistant pathogens on tryptic soy agar (BBL/Difco) supplemented with 50 µg/ml nalidixic acid (TSAN) on which worms that had ingested nalidixic acid-adapted pathogens would be eventually placed. Sensitive *E. coli* OP50 cells would not be expected to grow on TSAN but resistant cells of pathogens voided by the worm would be expected to form colonies.

At 24-h intervals, 10 worms were removed from each plate, washed in 10 ml of K medium, and centrifuged (500 x *g*, 2 min) to remove most of the nalidixic acid-sensitive *E. coli* OP50 cells from the outer cuticle. Washed worms were suspended in 10 ml of sterile 0.1% peptone water and 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 bacteria. Sonicate was serially diluted in 0.1% peptone and surface plated (0.1 ml in duplicate) on TSAN. Plates were incubated at 37°C for 24 h before colonies of test pathogens were counted.

2.4. *Persistence of* E. coli *OP50 and* S. enterica *serotype Newport in* C. elegans *as affected by temperature*

Worms were fed on lawns of nalidixic acid-resistant *E. coli* OP50, *E. coli* O157:H7, *S.* Poona, and *S.* Newport on K agar for 3 h at 20°C as described above. Worms (100 – 200) were removed from KN agar containing pathogens or *E. coli* OP50 using the procedure described above, washed, placed on the surface of uninoculated Bacto agar (2.0%, wt/vol) (BBL/Difco), and incubated at 4, 20, or 37°C for up to 72 h. Populations of ingested pathogens and *E. coli* OP50 were determined at 24-h intervals as described above.

2.5. Persistence of E. coli *OP50 and* S. enterica *serotype Newport in* C. elegans *as affected by relative humidity*

C. elegans (100 – 200 worms) was fed on a lawn of nalidixic acid-adapted *E. coli* OP50 or *S.* Newport on TSAN for 24 h at 20°C. Worms were harvested from the TSAN surface by flooding plates twice with 5 ml of K medium, collecting by centrifugation, and washing twice with K medium as described above. Washed worms (100 – 200 worms) in 0.5 ml of K medium were placed on the surface of uninoculated K agar and the K medium was allowed to absorb into agar at 20°C for 1 h. Relative humidities of 33, 75, or 98 % inside 1.7-liter tubs were achieved by depositing 300 ml of saturated magnesium chloride, sodium chloride, or disodium phosphate, respectively, beneath an elevated surface of a platform on which paper discs (6 mm diameter) inoculated with *C. elegans* that had ingested test bacteria would be eventually placed. A suspension (5 µl) containing 10 worms in sterile 0.1% peptone water was placed on each disc. The inoculated discs were placed in tubs with known relative humidities and sealed by applying a lid. Worms were incubated in tubs at 4 or 20°C for 24 h before analyzing for populations of bacterial cells. Each disk was aseptically transferred to a sterile 15-ml centrifuge tube containing 10 ml of 0.1% peptone water, and sonicated as described above. The suspension was spread plated on TSAN as described above. Plates were incubated at 37°C for 24 h before colonies were counted.

2.6. Transmission of ingested S. enterica *serotype Newport to progeny of* C. elegans

C. elegans was fed on a lawn of nalidixic acid-adapted *S.* Newport on KN agar for 3 h at 20°C. Worms were harvested by flooding plates with K medium, collected by centrifugation, and washed twice with K medium before placing, one per plate, on uninoculated Bacto agar and incubating at 20°C for 96 h. Ten adult worms were removed from the Bacto agar using a sterile

platinum wire, suspended in 10 ml of sterile 0.1% peptone water, and sonicated as described above. Sonicate (0.25 ml in quadruplicate) was surface plated on bismuth sulfite agar (BSA; BBL/Difco) supplemented with 50 µg/ml nalidixic acid (BSAN) and TSAN. Plates were inverted and incubated at 37°C 24 h. Presumptive *S* Newport colonies counted and randomly selected for confirmation using latex agglutination (FT 0203A; Oxoid Ltd., Basingstoke, Hampshire, U.K.). Concurrently, individual adult progeny were aseptically transferred to uninoculated plates of Bacto agar and incubated at 20°C for 96 h. Ten second-generation worms were analyzed for the presence of ingested *S* Newport as described above.

2.7. Transmission of ingested S. *Newport to uninfected* C. elegans

Approximately 50 *C. elegans* with their pharynx labeled with green fluorescent protein (gfp) were fed on a lawn of gfp-transformed *S. enterica* serotype Newport (SN78gfp) on TSA supplemented with 100 µg/ml ampicillin (TSAA) for 72 h at 20°C. Worms were removed from the plate, washed, and centrifuged as described above before depositing ca. 50 on TSAA. Approximately 50 wild type *C. elegans* were removed from K agar plates on which they had fed on *E. coli* OP50 72 h at 20°C. The wild type worms were washed and centrifuged using the same procedure described for gfp-labeled nematodes. Wild type worms (ca. 50) were deposited on the surface of the same TSAA plate containing the gfp-labeled worms. Plates were incubated at 20°C for 24 h.

Sterile, molten Bacto agar (0.5 ml) was placed on a sterile glass microscope slide, and allowed to solidify and cool to 20°C for 1 h. Ten microliters of 37% formalin (Sigma Chemical Co., St. Louis, Mo.) was deposited on the agar pad. A sterile platinum wire was used to remove ten worms from the TSAA. Worms were placed in the formalin on the agar pad, allowed to fix for 5 min, covered with a coverslip, and sealed with melted valap (equal parts petroleum jelly,

lanolin, and paraffin). Wild type worms were examined microscopically for the presence of ingested gfp-transformed *S. enterica* serotype Newport.

2.8. Transmission of ingested S. enterica *serotype Newport to uninfected* C. elegans

Approximately 50 *C. elegans* with their pharynx labeled with green fluorescent protein (gfp) were fed on a lawn of gfp-transformed *S. enterica* serotype Newport (SN78gfp) on TSA supplemented with 100 µg/ml ampicillin (TSAA) for 72 h at 20°C. Worms were removed from the plate, washed, and centrifuged as described above before depositing ca. 50 on TSAA. Approximately 50 wild type *C. elegans* were removed from K agar plates on which they had fed on *E. coli* OP50 72 h at 20°C. The wild type worms were washed and centrifuged using the same procedure described for gfp-labeled nematodes. Wild type worms (ca. 50) were deposited on the surface of the same TSAA plate containing the gfp-labeled worms. Plates were incubated at 20°C for 24 h.

Sterile, molten Bacto agar (0.5 ml) was placed on a sterile glass microscope slide, and allowed to solidify and cool to 20°C for 1 h. Ten microliters of 37% formalin (Sigma Chemical Co., St. Louis, Mo.) was deposited on the agar pad. A sterile platinum wire was used to remove ten worms from the TSAA. Worms were placed in the formalin on the agar pad, allowed to fix for 5 min, covered with a coverslip, and sealed with melted valap (equal parts petroleum jelly, lanolin, and paraffin). Wild type worms were examined microscopically for the presence of gfp-transformed *S. enterica* serotype Newport in their gut.

2.9. Survival of ingested E. coli *OP50 and* S. enterica *serotype Newport as affected by treatment of worms with alkaline NaOCl*

This experiment was done to confirm the effectiveness of an alkaline NaOCl solution in killing *E. coli* OP50 and *S.* Newport ingested by *C. elegans* and on the surface of worms and

eggs. Approximately 50 wild type *C. elegans* were fed on lawns of nalidixic acid-adapted *E. coli* OP50 or *S.* Newport on TSAN for 24 h at 20°C. K medium was deposited on the surface of TSAN and suspensions of worms were removed and collected by centrifugation as described above. All but 0.5 ml of the K medium was removed from the centrifuge tube. Worms in the pellet were resuspended in 10 ml of 0.013 M NaOH solution containing 1% NaOCl or 0.1% peptone (control) and held at 20°C for 15 min. The suspension was centrifuged (500 x *g*, 2 min) and the supernatant was removed using a sterile pasteur pipette. Worms and eggs were resuspended in 10 ml of K medium and centrifuged again. A suspension (0.1 ml containing 400 – 600 eggs) was deposited on the surface of a TSAN plate and incubated at 37°C for 24 h. TSAN plates were examined for the presence of *E. coli* OP50 and *S.* Newport.

2.10. Statistical analysis

All experiments were replicated three times. Data were analyzed using the general linear models procedure of the Statistical Analysis Software (SAS Institute, Cary, N.C.). Significant differences ($P \leq 0.05$) between mean values were determined using Duncan's multiple range test.

3. Results

The initial populations of ingested pathogens (2.52 – 3.20 $\log_{10}$ cfu/worm) significantly ($P \leq 0.05$) increased by up to 2.93 $\log_{10}$ within 1 day after removal from respective feed sources and remained constant for an additional 4 days on K agar incubated at 20°C (Table 2.1). Populations of ingested nalidixic acid-adapted *E. coli* OP50 (control) recovered from worms incubated on K agar remained constant for 5 days. There were no significant ($P > 0.05$) differences in populations of the two strains of *E. coli* O157:H7 or between *S.* Newport and *S.*

Poona on each day of analysis.

Table 2.1. Populations (log_{10} cfu/worm) of *Escherichia coli* OP50, *E. coli* O157:H7, *Salmonella enterica* serotype Newport, and *S. enterica* serotype Poona recovered from *Caenorhabditis elegans* incubated at 20°C on K agar with *E. coli* OP50 for up to 5 days.

	Populations (log_{10} cfu/worm)[a]					
Microorganism	Day 0	1	2	3	4	5
Escherichia coli OP50	5.20 a	5.16 a	5.13 a	5.09 a	5.16 a	4.72 a
E. coli O157:H7 (SEA13B88)	2.81 b	5.20 a	5.35 a	5.41 a	5.35 a	5.32 a
E. coli O157:H7 (E0018)	2.52 b	5.45 a	5.33 a	5.39 a	5.31 a	5.37 a
Salmonella enterica serotype Newport	2.81 b	5.17 a	4.95 a	5.15 a	5.41 a	5.27 a
S. enterica serotype Poona	3.20 c	5.25 b	5.51 a	5.36 ab	5.40 ab	5.48 ab

[a]Mean values in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$).

Compared to day 0, populations of ingested test pathogens did not differ by more than 0.47 log_{10} cfu/worm when worms that had fed on pathogens were subsequently incubated on Bacto agar at 4°C for up to 3 days (Table 2.2). At 20°C, populations of ingested pathogens decreased significantly within 1 or 3 days. Populations recovered from worms incubated at 37°C significantly increased ($P \leq 0.05$) within 1 day and remained constant for two additional days.

Placement of *C. elegans* on K agar for 1 h at 20°C did not significantly ($P > 0.05$) reduce the number of ingested *E. coli* OP50 and *S.* Newport (Table 2.3). Initial populations (0 h) of *E. coli* OP50 and *S.* Newport ingested by *C. elegans* decreased by up to 1.65 and 3.44 log_{10} cfu/worm, respectively, when inoculated discs were incubated at 20°C and 33% relative humidity for 24 h.

Table 2.2 Populations (log_{10} cfu/worm) of *Escherichia coli* OP50, *E. coli* O157:H7, *Salmonella enterica* serotype Newport, and *S. enterica* serotype Poona recovered from *Caenorhabditis elegans* incubated on uninoculated Bacto agar at 4, 20, or 37°C for up to 3 days.

Temp.		Population (log_{10} cfu/worm)[a]			
(°C)	Microorganism	Day 0	1	2	3
4	*Escherichia coli* OP50	2.47 b	3.06 ab	3.45 a	3.03 ab
	E. coli O157:H7 (SEA13B88)	2.77 a	2.41 a	2.68 a	2.57 a
	E. coli O157:H7 (E0018)	2.91 a	2.97 a	2.78 a	3.02 a
	S. enterica serotype Newport	2.63 a	3.04 a	2.91 a	2.85 a
	S. enterica serotype Poona	3.69 a	3.22 a	3.72 a	3.31 a
20	*E. coli* OP50	2.47 ab	2.77 a	1.88 c	2.05 bc
	E. coli O157:H7 (SEA13B88)	2.77 a	0.87 c	1.96 b	2.05 b
	E. coli O157:H7 (E0018)	2.91 a	2.51 ab	2.45 ab	2.42 b
	S. enterica serotype Newport	2.63 a	1.06 c	1.89 b	1.76 b
	S. enterica serotype Poona	3.69 a	2.36 b	2.11 b	1.37 c
37	*E. coli* OP50	2.47 b	4.64 a	5.14 a	5.11 a
	E. coli O157:H7 (SEA13B88)	2.78 c	4.23 ab	4.73 a	4.00 b
	E. coli O157:H7 (E0018)	2.91 b	4.95 a	5.01 a	4.97 a
	S. enterica serotype Newport	2.63 b	4.56 a	4.74 a	4.66 a
	S. enterica serotype Poona	3.69 b	4.61 a	4.71 a	5.13 a

[a]Mean values in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$).

Table 2.3. Populations (log_{10} cfu/worm) of *Escherichia coli* OP50 and *Salmonella enterica* serotype Newport recovered from *Caenorhabditis elegans* incubated at 4 or 20°C and 33, 75, or 98% relative humidity for 24 h.

			Population (log_{10} cfu/worm)[a]			
Microorganism	Temp (°C)	Relative humidity (%)	Before placing on K agar[b]	After 1 h on K agar (0 h)[c]	After drying for 24 h[d]	R[e]
Escherichia coli OP50	4	33	2.79 a	2.05 ab	a 0.48 b	1.57
		75	2.79 a	2.05 a	a 1.48 a	0.57
		98	2.79 a	2.05 a	a 1.02 a	1.03
	20	33	2.79 a	2.05 ab	a 0.40 b	1.65
		75	2.79 a	2.05 ab	a 0.61 b	1.44
		98	2.79 a	2.05 ab	a 0.47 b	1.58
Salmonella enterica serotype Newport	4	33	3.21 a	3.44 a	b 0.72 b	2.72
		75	3.21 a	3.44 a	b 1.35 b	2.09
		98	3.21 a	3.44 a	a 3.30 a	0.14
	20	33	3.21 a	3.44 a	b <0.04 b	3.44
		75	3.21 a	3.44 a	a 1.42 b	2.02
		98	3.21 a	3.44 a	a 1.37 b	2.07

(continued)

(Table 2.3, continued)

[a]Mean values in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$). Within microorganism and storage temperature, mean values in the same column that are not preceded by the same letter are significantly different ($P \leq 0.05$).

[b]Values are for worms analyzed prior to placing on K agar for 1 h.

[c]Values are for worms analyzed after incubating on K agar for 1 h, i.e., at the time worms were placed on paper discs (0 h).

[d]Worms were analyzed 24 h after placing on discs and incubating at 4 or 20°C at 33, 75, or 98% relative humidity.

[e]Reduction in population of microorganism compared to population detected in *C. elegans* before drying for 24 h.

The number of *S.* Newport that survived in worms exposed to 33 or 75% relative humidity at 4°C or 33% relative humidity at 20°C was significantly ($P \leq 0.05$) lower than the number surviving exposure to higher relative humidities at respective temperatures.

Cells of *S.* Newport labeled with gfp were detected in the gut of wild type *C. elegans* progeny after contact for 24 h with gfp-labeled *C. elegans* (Fig 1). This shows that worms that had fed on *S.* Newport subsequently infected worms not previously exposed to the pathogen. Cells of nalidixic acid-resistant *S.* Newport were isolated from all adult *C. elegans* analyzed from two successive generations removed from exposure to the pathogen.

An interesting phenomenon in this series of studies was the observed presence of gfp-labeled juvenile worms inside infected wild type worms (Fig 2). Also, worms were observed

feeding on the gut contents of other worms with ruptured cuticles. The gut probably contained viable bacterial cells that had been ingested by the worm.

No *E. coli* OP50 or *S.* Newport cells were recovered from worms or eggs treated with the alkaline NaOCl solution.

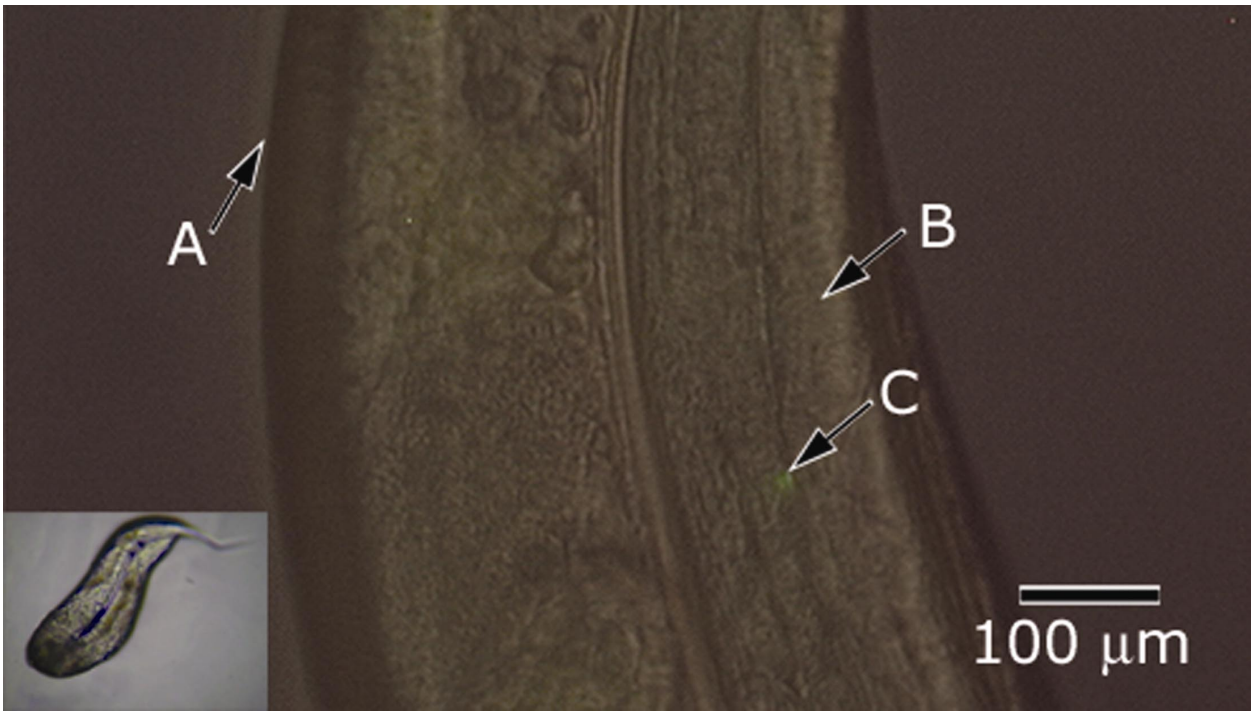

Figure 2.1. Photomicrograph of wild type *C. elegans.* Inset shows general orientation of *C. elegans*. A, cuticle of posterior portion of worm; B, pharynx; C, gfp-labeled *S. enterica* serotype Newport in gut slightly posterior of pharynx.

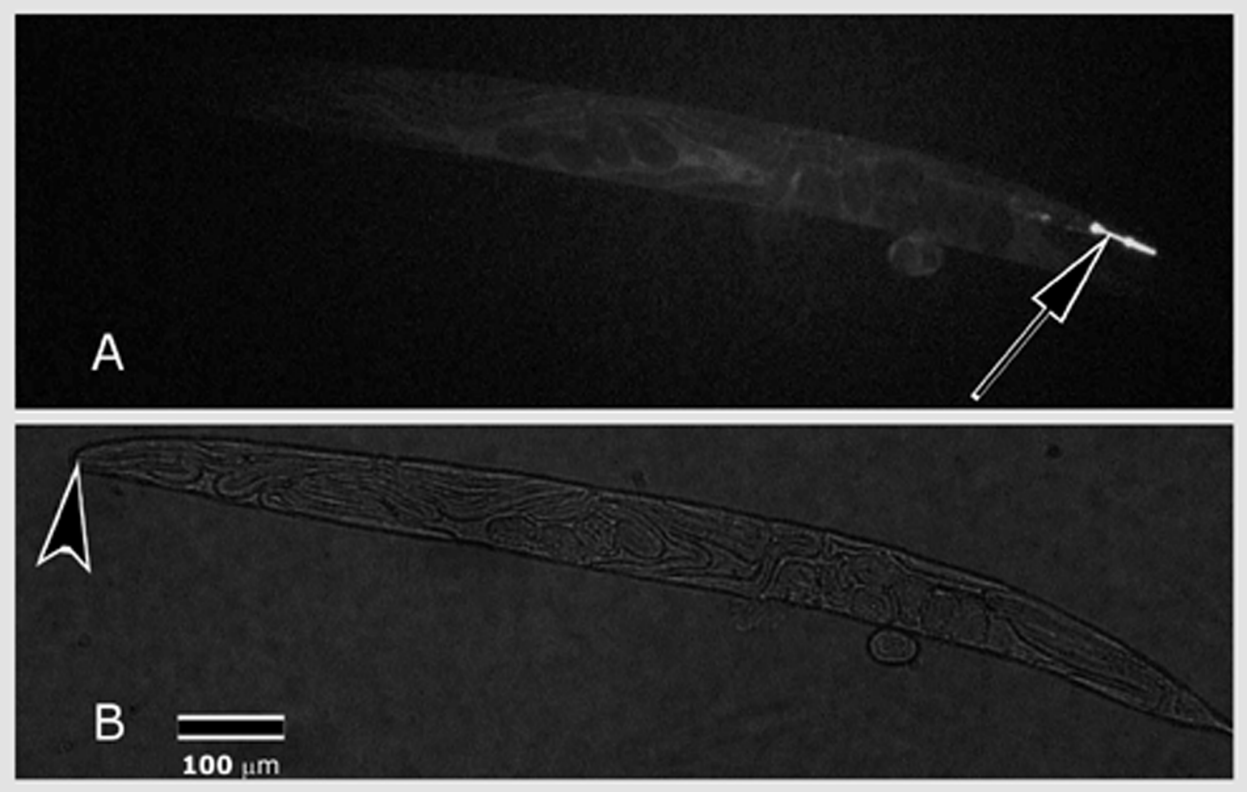

Figure 2.2. Fluorescent (A) and phase contrast (B) photomicrographs of wild type *C. elegans*. Arrow points to a gfp-labeled juvenile worm in the gut of an adult wild type *C. elegans*. Arrowhead points to the oral cavity of the wild type worm.

DISCUSSION

Results indicate that once *C. elegans* has ingested enteric pathogens, progeny may remain infected until they are voided of the pathogens or environmental conditions become unsuitable for the bacteria to survive. Transmission of bacteria from infected *C. elegans* to its progeny may occur as a result of excretion of viable cells into the environment, by discharge of cells into the environment as a result of rupture of the cuticle, or by juvenile worms eating their way out of a infected adult worm, thereby ingesting bacteria in the process. If the environmental conditions

are favorable, excreted or discharged bacteria may grow. Growth of bacteria may, in turn, attract uninfected worms that may ingest pathogens, thus repeating the cycle of infection.

Worms that have ingested *Salmonella* have been reported to have a decreased life span (Aballay et al., 2000). The time to death for 50% (TD_{50}) of the worms ranged from 3.7 – 9.0 days after ingesting various serotypes of *S. enterica* compared to 9.9 days after ingesting *E. coli* OP50. The reduced life span of worms that carry foodborne pathogens may have little impact on their ability to contaminate preharvest fruits and vegetables. Caldwell *et al.* (2003b) showed that *C. elegans* vectors *S.* Poona to cantaloupe rind in contact with soil within 1 day of ingesting the pathogen.

Populations of ingested pathogens increased when worms were incubated on TSAN at 20°C; however, reduced numbers of cells were recovered from worms incubated on Bacto agar at 20°C. TSAN is rich in nutrients. Bacto agar, on the other hand, is devoid of nutrients required for bacterial growth, and was used only as a physical support for the worms and to prevent them from desiccating. Bacteria from the cuticle surface or excreted from the worms would be expected to grow on the TSAN but not on Bacto agar. Bacterial cells may grow on TSAN at a rate faster than they are consumed by *C. elegans.* Aballay *et al.* (2000) reported that *S.* Typhimurium populations in the intestinal lumen of *C. elegans* increased when the worm was placed on nutrient agar.

C. elegans survived at 4°C but did not move on the Bacto agar surface to forage for bacteria. All test bacteria could be recovered from worms incubated at 4°C for at least 3 days. Metabolic processes associated with digestion in *C. elegans* would be much slower or non existent at 4°C than at 20°C. This would suggest that cells ingested by worms prior to exposure to refrigeration temperatures would be less likely to be affected by digestive processes.

Pathogens ingested by *C. elegans* that may contaminate the surface of postharvest produce would be protected at refrigeration temperatures. Produce in the retail market is not uncommonly displayed at ambient temperature (ca. 20°C), which could also protect ingested pathogens, even after death of the worms.

Worms incubated at 20°C are in their most active state and will consume most bacteria that they come in contact with rather quickly. Since Bacto agar is nutrient deficient and would not support the growth of bacteria, *C. elegans* probably used bacterial cells as a nutrient source, resulting in a decrease in populations during the 3-day incubation period. *C. elegans* does not survive at 37°C. The increase in bacterial populations in worms incubated on Bacto agar at 37°C is attributed to an increase in the availability of nutrients for growth as the worms undergo autolysis.

It is unclear how the labeled worm became internalized in the gut of the wild type worm. A possibility would be that the juvenile worm entered the adult wild type worm through a rupture in the cuticle. Juvenile worms in the carcass of a dead adult worm may be a normal occurrence; however, lack of phenotypic differences in the wildtype worms make it difficult to determine if juveniles were progeny of a dead worm or from another worm. Worms that were observed feeding on the gut contents of dead worms were probably attracted to viable bacterial cells that had been ingested preceding rupture of the cuticle. In this way, one infected worm could infect many previously uninfected worms. It is also possible that the wild type worm ingested an egg from a transgenic worm that then developed into a juvenile. Studies have shown *C. elegans* is capable of ingesting rigid objects smaller than 5 μm in diameter (Boyd et al., 2003). *C. elegans* eggs are ca. 30 μm in diameter but the eggs may be pliable enough allow ingestion by adult worms.

At both 4 and 20°C, incubation of *C. elegans* at 33% relative humidity resulted in a significant decrease ($P \leq 0.05$) in the number of *S.* Newport recovered. Worms would be more desiccated when exposed to 33% relative humidity compared to 75 or 98% relative humidity. Largest reductions in *E. coli* OP50 and *S.* Newport were observed when worms were incubated at 20°C and 33% relative humidity. Autolysis of worms would occur at a more rapid rate at 20°C than at 4°C. At 20°C, bacteria that have been injured by desiccation may have increased sensitivity to degradative enzymes released by dead worms and bacteria.

In the laboratory, an alkaline NaOCl solution is used to surface sterilize eggs and to separate eggs from other life stages of *C. elegans*. Other disinfectants have been reported to unsuccessfully eliminate bacteria that may be present in the gut of the worm (Chang et al., 1960; Smerda et al., 1970; Caldwell et al., 2003a). In our study, bacterial colonies were not formed on TSAN plates on which worms treated with a 1% alkaline NaOCl solution were incubated at 37°C for 24 h. This suggests that the NaOCl solution is effective in penetrating the cuticle of the worm and inactivating the bacteria in the gut. The pH of the solution was 13.0, a value at which *C. elegans* cannot survive (Khanna et al. 1997). When sanitizing produce, a NaOCl solution is most effective at a pH of 6.0 – 7.5 (Beuchat, 1998).

Results indicate that *E. coli* O157:H7 and salmonellae can survive for an extended time after ingestion by *C. elegans*. It is plausible that worms infected with bacteria capable of causing disease in humans may come into contact with pre-harvest fruits and vegetables and contaminate their surfaces by excreting pathogens. Attachment of infected worms to the surface of produce may be followed by death of the worms but ingested pathogens may survive and be physically protected against treatment with sanitizers, rendering sanitization more difficult.

Acknowledgment

This study was supported in part by the USDA-ARS National Alliance for Food Safety and Security. The *C. elegans* strains used in this work were provided by the Caenorhabditis Genetic Center, which is funded by the NIH National Center for Research Resources.

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CHAPTER 3

EFFECTIVENESS OF CLEANERS AND SANITIZERS IN KILLING *SALMONELLA* NEWPORT IN THE GUT OF A FREE-LIVING NEMATODE, *CAENORHABDITIS ELEGANS*[1]

[1]Kenney, S. J., Anderson, G. L., Williams, P. L., Millner, P. D., and Beuchat, L. R. To be submitted to *Journal of Food Protection.*

ABSTRACT

Caenorhabditis elegans, a free-living nematode found in soil, has been shown to ingest human enteric pathogens, thereby potentially serving as a vector for preharvest contamination of fruits and vegetables. A study was undertaken to evaluate the efficacy of cleaners and sanitizers in killing *Salmonella enterica* serotype Newport in the gut of *C. elegans*. Adult worms were fed nalidixic acid-adapted cells of *Escherichia coli* OP50 (control) or *S.* Newport for 24 h, washed, placed on paper discs, and incubated at 4 or 20°C and relative humidites of 33 or 98% for 24 h. Two commercial cleaners (Enforce® and K Foam Lo®) and four sanitizers (2% acetic acid, 2% lactic acid, Sanova®, and chlorine [50 and 200 μg/ml]) were applied to worms for 0, 2, or 10 min. Populations of *E. coli* and *S.* Newport (CFU/worm) in untreated and treated worms were determined by sonicating worms in 0.1% peptone and surface plating suspensions of released cells on tryptic soy agar containing nalidixic acid. Populations of *S.* Newport in worms exposed to 33 or 98% relative humidity at 4°C or 33% relative humidity at 20°C were significantly ($P \leq 0.05$) lower than the number surviving exposure to 98% relative humidity at 20°C. In general, treatment of desiccated worms with cleaners and sanitizers was effective in significantly ($P \leq 0.05$) reducing the number of ingested *S.* Newport. Results indicate that temperature and relative humidity influence the survival of *S.* Newport in the gut of *C. elegans,* and cleaners and sanitizers may not eliminate the pathogen.

INTRODUCTION

Outbreaks of foodborne illness associated with the consumption of raw or minimally processed fruits and vegetables have increased in recent years (*4, 15, 20*). These outbreaks have raised interest in determining mechanisms through which preharvest and postharvest produce becomes contaminated with enteric pathogens.

Application of animal manure to cropland soil as a fertilizer is not an uncommon practice. Human pathogenic bacteria have been isolated from soils to which bovine manure had been applied (*12*). The microbial profile of soil in which fruits and vegetables are grown or come in contact can have an impact on the postharvest quality and safety of the produce (*5, 16*). This was illustrated when apples that had fallen from trees were used to make cider that was implicated in outbreaks of cryptosporidosis and *Escherichia coli* O157:H7 infection (*10, 11, 19*). It is believed that runoff water from cattle pastureland contaminated the apples. The skin of tomatoes in contact with soil containing *Salmonella* can become contaminated with the pathogen (*14*).

Large microbial populations in the soil matrices, such as those amended with manure, have been reported to attract free-living nematodes (*21*). *Caenorhabditis elegans*, a free-living, microbivorous nematode found in the soil of temperate regions, has been reported to ingest *E. coli* O157:H7, *Listeria monocytogenes* (*2, 8*), several serotypes of *Salmonella enterica* (*1, 8, 17*), *Bacillus cereus* (*2*), and *Staphylococcus aureus* (*24*). Depending on environmental conditions, bacteria may persist within the gut of *C. elegans* for several days after consumption (*1, 17*). Release of pathogens as a result of rupturing of the cuticle or defecation are ways that infected nematodes can contaminate the soil environment. *C. elegans* can transport ingested pathogens to the surface tissues of produce in contact with soil. *Salmonella enterica* serotype Poona, placed

in soil 1 or 3 cm away from cantaloupe rind, was detected on rind in contact with the soil containing *C. elegans* within a shorter time compared to rind on soil void of the nematode (*8*).

In general, free-living nematodes are sensitive to desiccation. Worms in soil, on the surface of root crops, or on aerial parts of plants as a result of postharvest handling can quickly become desiccated. Carcasses of desiccated worms may harbor viable bacteria that were ingested by the worm prior to death. The cuticle of a worm may provide a physical barrier between bacteria resident in the gut and commercial cleaners and sanitizers used to remove soil or decontaminate equipment and produce. Salmonellae (*7, 9*) and *Shigella sonnei* (*9*) have been reported to survive sanitizer treatments in nematodes. Bacterial cells surviving environmental stresses imposed by desiccation, commercial cleaners, and sanitizers may remain on the produce until ingested by the consumer.

Commercial cleaners and sanitizers used by the produce industry may contain surfactants to aid in the release of microorganisms, and perhaps also nematodes, from the surface of produce. Nematodes may subsequently become resident in or on produce contact areas such as water baths, belts, tables, and sorters in processing facilities. Pathogens from a single worm released onto processing equipment could theoretically contaminate large amounts of produce. The plausibility of this series of events happening on a commercial level can be more easily assessed if the effectiveness of cleaners and sanitizers in killing pathogens ingested by nematodes is known.

A study was undertaken to determine the effectiveness of two commercial cleaners and four sanitizers in killing *E. coli* OP50 and *S.* Newport in the gut of *C. elegans*. The effectiveness of these treatments in killing planktonic cells of *E. coli* OP50 and *Salmonella enterica* serotype Newport was also evaluated.

MATERIALS AND METHODS

Maintenance of *C. elegans*. A wild type (N2) strain of *C. elegans* was used. Worms were maintained 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; BBL/Difco, Sparks, Md.), and agar (17.0 g) (*26*). *E. coli* OP50, an avirulent strain routinely used as a feed source for *C. elegans*, was cultured 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) (*6*). The K agar was surface inoculated with 0.1 ml of a 24-h culture of *E. coli* OP50 and incubated at 37°C for 24 h to establish confluent growth. Approximately 50 adult worms were deposited on the surface of K agar and incubated at 20°C for up to 3 days.

Preparation of *C. elegans* for enteric pathogen assay. Three-day adult worms were transferred to a fresh K agar plate with a 24-h lawn of *E. coli* OP50 or *S.* Newport. The surface of ten K agar plates, each containing 500 – 1000 eggs, and 30 – 50 adult worms, was washed by depositing 5 ml of sterile K broth (*27*) and gently rubbing with a sterile bent glass rod. The suspended eggs and worms were aseptically transferred to a sterile 15-ml centrifuge tube. The wash and transfer procedure was repeated to enable efficient harvesting of eggs. Eggs and worms were collected by centrifugation (500 x *g*, 2 min) and the supernatant was removed using a pipette. Worms and eggs in a pellet from pooled suspensions were resuspended in 10 ml of 0.013 M NaOH solution containing 1% NaOCl (pH 12.97) and incubated at 20°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) and the supernatant was removed. Worms and eggs in the pellet were resuspended in 10 ml of K broth and centrifuged again. The supernatant was removed and the eggs and dead worms were resuspended in K broth. The suspension (0.1 ml containing 400 – 600 eggs) was deposited

on the surface of a K agar plate on which a lawn of *E. coli* OP50 had formed, followed by incubation at 20°C for 3 days. This procedure ensured that all worms used in assays were of the same age. Adult worms were used in assays to confirm that they will ingest pathogenic bacteria and, once ingested, to determine the efficacy of cleaners and sanitizers in killing *E. coli* OP50 and *S.* Newport in the gut as affected by temperature and relative humidity.

Preparation of cleaner and sanitizer solutions. Seven chemical solutions used or having potential for use as cleaners or sanitizers in the produce industry were evaluated for their effectiveness in killing *E. coli* OP50 and *S.* Newport ingested by *C. elegans* (Table 3.1). K Foam Lo (CK Enterprises, Inc., Lee's Summit, Mo.), an ethylene glycol monobutyl ether solution, and Enforce (Ecolab, St. Paul, Minn.), an alkaline chlorine solution, were tested at 100% of the manufacturers' recommended working concentration. Sanova, an acidified sodium chlorite sanitizer (Alcide Corporation, Redmond, Wash.) and acetic and lactic acids, each at 2%, were evaluated. Chlorine solutions were prepared by combining sodium hypochlorite (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) with 0.05 M potassium phosphate buffer (pH 6.8). The concentration of free chlorine was measured by using a DR/820 colorimeter (Hach, Loveland, Colo.). Sterile distilled water was used as a control. All chemical treatment solutions were used within 30 min of preparation.

Bacteria used and preparation of worms for desiccation assays. *E. coli* OP50 (control) and a multidrug resistant strain of *S.* Newport were evaluated. Both strains were adapted to grow in tryptic soy broth (TSB, pH 7.3; BBL/Difco) supplemented with nalidixic acid (50 µg/ml) (TSBN). Nalidixic acid-resistant *E. coli* OP50 and *S.* Newport were used to facilitate their detection on tryptic soy agar (BBL/Difco) supplemented with nalidixic acid (50 µg/ml) (TSAN). Sensitive *E. coli* OP50 cells remaining on the surface or in the gut of the worm as a

TABLE 3.1. *Treatments evaluated for killing of* Escherichia coli OP50 *and* Salmonella *Newport ingested by* Caenorhabditis elegans.

Code	Treatment	Conc.	pH[b]
Control			
A	Peptone water	0.1%	6.1
Cleaner			
B	K Foam Lo	100%[a]	11.1
C	Enforce	100%[a]	12.3
Sanitizer			
D	Sanova	1200 µg/ml	2.4
E	Acetic acid	2%	2.4
F	Lactic acid	2%	2.0
G	NaOCl	50 µg/ml	6.9
H	NaOCl	200 µg/ml	6.9

[a]100% of manufacturers' recommended working concentration.

[b]pH of a working concentration of the treatment solution at 20°C.

result of feeding before exposure to nalidixic acid-resistant cells would not be expected to grow on TSAN but resistant cells would form colonies. Nalidixic acid-resistant cells were grown in 10 ml of TSBN at 37°C for 24 h. *E. coli* OP50 and *S.* Newport were transferred twice to 10 ml of

TSBN by loop inocula at successive 24-h intervals. Inoculum (0.1 ml of a 24-h culture) was surface plated on K agar supplemented with 50 µg/ml nalidixic acid (KN agar) and incubated at 37°C for 24 h to produce a lawn of cells. Three-day-old adult worms from synchronized culture grown on K agar with lawns of nalidixic-acid sensitive of *E. coli* OP50 were placed on the surface of KN agar plates individually containing lawns of *E. coli* OP50 or *S.* Newport and allowed to feed on cells for 24 h at 20°C. K broth (10 ml) was deposited on the surface of plates and worms and bacteria were suspended using a sterile glass rod. Suspensions were placed in sterile 15-ml centrifuge tubes, centrifuged (500 x *g*, 2 min), resuspended in K broth, and centrifuged again. Washed worms (100 – 200 worms) in 0.5 ml of K broth were placed on the surface of uninoculated K agar and the K broth was allowed to absorb into agar at 20°C for 1 h. These worms were used in assays to test the effectiveness of cleaners and sanitizers in killing ingested *E. coli* OP50 and *S.* Newport.

Atmospheric desiccation of worms and treatment with cleaners and sanitizers. Relative humidities of 33 or 98 % inside 1.7-liter containers were achieved by depositing 300 ml of saturated solutions of magnesium chloride or disodium phosphate, respectively, beneath an elevated surface of a platform on which inoculated paper discs (6 mm diameter) would be eventually placed. A suspension (5 µl) containing 10 worms was prepared using a platinum wire to remove worms from surface of K agar and placing in sterile 0.1% peptone water. Each disc was inoculated with a suspension of worms. The inoculated discs were placed in containers with 33 or 98% relative humidity and sealed by applying a lid. Worms were incubated at 4 or 20°C for 24 h before applying cleaner or sanitizer treatment and analyzing for populations of test bacteria. Initial populations of test bacteria were determined by analyzing a suspension of worms prior to incubation. Each disk was aseptically transferred to a sterile 15-ml centrifuge

tube containing 5 ml of distilled water (control), 0.1% peptone (control), cleaner, or sanitizer at 20°C. After 2 or 10 min, 5 ml of Dey-Engley neutralizing broth (BBL/Difco) was added to each tube. The disc and water or treatment solution were sonicated (Sonicate 450, Danbury, Conn.) using a duty cycle of 25% for 25 s at 21°C to rupture the cuticle of *C. elegans* and release ingested bacteria. Sonicate was serially diluted in sterile 0.1% peptone and surface plated (0.1 ml in duplicate) on TSAN containing 0.1% sodium pyruvate (TSANP). Plates were incubated at 37°C for 24 h before presumptive colonies of *E. coli* OP50 or *S.* Newport were counted. Random colonies were confirmed using API 20E miniaturized diagnostic kits (bioMerieux Vitek, Inc., Hazelwood, Mo.) and *Salmonella* latex agglutination assay (FT 0203A; Oxoid Ltd., Basingstoke, Hampshire, U.K.), respectively.

Treatment of planktonic cells with cleaners and sanitizers. *E. coli* OP50 and *S.* Newport were grown in 10 ml of TSBN at 37°C for 24 h, then incubated at 4°C or 20°C for an additional 24 h to approximate environmental conditions that cells ingested by *C. elegans* were subjected to in studies involving desiccated worms. Cell suspensions were centrifuged at 2000 x *g* for 10 min. Supernatants were decanted and cells were resuspended in 10 ml of sterile 0.1% peptone water. Centrifugation and resuspension of cells was repeated two times before treating with distilled water (control), cleaners, or sanitizers as described above, with the exception that sonication was omitted. Undiluted and diluted suspensions of control and treated cells were spread plated on TSANP agar and incubated at 37°C for 24 h. Presumptive colonies of *E. coli* OP50 and *S.* Newport were enumerated and confirmed as described above.

Statistical analysis. Each experiment was replicated three times. Data were analyzed using the general linear models procedure of the Statistical Analysis Software (SAS Institute,

Cary, N.C.). Significant differences ($P \leq 0.05$) between mean values were determined using Duncan's multiple range test.

RESULTS AND DISCUSSION

Effectiveness of cleaners and sanitizers on killing *E. coli* OP50 and *S.* Newport ingested by *C. elegans.* Mean populations of *E. coli* OP50 and *S.* Newport recovered from untreated *C. elegans* and from worms treated in distilled water (control), cleaners, or sanitizers are shown in Table 3.2. With the exception of worms that had fed on *S.* Newport and were incubated at 33% relative humidity before treatment for 10 min, the initial number of *E. coli* OP50 and *S.* Newport recovered from worms before incubation at 4°C was not significantly ($P > 0.05$) different than the number recovered from worms treated with distilled water or peptone water (treatment A). Neither organism would be expected to grow at 4°C or 33% relative humidity, so the increase in population of *S.* Newport is considered as an aberrant data point.

Significant increases ($P \leq 0.05$) in *E. coli* OP50 and *S.* Newport populations occurred in worms incubated at 20°C and 98% relative humidity, as evidenced by numbers of both organisms recovered from worms treated with water or 0.1% peptone for 10 min. Bacteria present in the gut of dead worms apparently utilized nutrients released by the autolysis. This is supported by a study that showed bacterial populations increasing in carcasses of *C. elegans* incubated at 37°C on nutrient-void Bacto agar (*17*). Degradative enzyme activity would be higher at 20°C than at 4°C. Bacterial cells in worms incubated at 20°C and 33% relative humidity may have been stressed by a reduction in a_w or are more sensitive to the degraditive enzymes promoting autolysis, resulting in their death or inability to grow. Incubation at 20°C and 98% relative humidity would not be expected to inhibit the growth of *E. coli* OP50 or *S.* Newport.

TABLE 3.2. *Populations (log_{10} cfu/worm) of* Escherichia coli *OP50 and* Salmonella *Newport recovered from* Caenorhabditis elegans *incubated at 4 or 20°C and relative humidities of 33 or 98% for 24 h.*

				Populations (log_{10} cfu/worm)[a]																	
			Treat-		Treatment[b]																
	Temp.	Relative humidity	ment time		Distilled																
Microorganism	(°C)	(%)	(min)	Initial	water	A	B	C		D		E		F		G		H			
E. coli OP50	4	33	2	1.53 a	1.12 a	1.59 a	1.30 a	0.16 a		<0.04 b	(0)	<0.04 b	(0)	0.01 b		0.01 b		<0.04 b	(0)		
			10	1.53 a	1.42 a	1.45 a	0.89 b	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	0.01 c		<0.04 c	(0)		
		98	2	1.53 b	1.82 a	2.01 a	1.52 b	0.01 c		<0.04 c	(1)	2.65 a		<0.04 c	(0)	0.23 c		<0.04 c	(0)		
			10	1.53 a	1.81 a	1.83 a	0.45 b	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	0.01 c		<0.04 c	(0)	<0.04 c	(0)		
	20	33	2	1.53 b	0.47 c	0.60 c	0.41 cd	0.01 d		<0.04 d	(0)	<0.04 d	(0)	2.75 a		<0.04 d	(0)	<0.04 d	(0)		
			10	1.53 a	0.83 b	0.67 b	0.59 b	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)		
		98	2	1.53 a	1.00 b	1.18 b	1.03 b	<0.04 c	(0)	<0.04 c	(0)	2.86 a		<0.04 b	(0)	<0.04 c	(0)	<0.04 c	(0)		
			10	1.53 b	2.44 a	2.47 a	2.38 a	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)		
S. Newport	4	33	2	1.55 ab	1.52 ab	2.76 a	0.17 b	<0.04 b	(0)	0.01 b		0.77 b		<0.04 b	(0)	0.55 b		1.35 ab			
			10	1.55 b	2.15 a	2.26 a	0.01 c	<0.04 c	(0)	<0.04 c	(0)	0.21 c		<0.04 c	(0)	0.19 c		<0.04 c	(0)		
		98	2	1.55 b	1.98 b	2.03 b	0.43 c	0.01 c		<0.04 c	(1)	0.21 c		<0.04 c	(0)	0.40 c		0.17 c			
			10	1.55 a	1.97 a	1.95 a	0.01 c	<0.04 c	(0)	<0.04 c	(0)	0.11 c		<0.04 c	(0)	0.03 c		<0.04 c	(1)		
	20	33	2	1.55 a	0.98 ab	1.00 ab	0.10 c	<0.04 c	(0)	<0.04 c	(0)	0.55 cb		0.03 c		0.15 c		0.79 cb			
			10	1.55 a	1.12 ab	1.17 ab	0.01 c	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	<0.04 c	(0)	0.93 abc		<0.04 c	(0)		
		98	2	1.55 cb	4.44 a	4.62 a	0.42 d	<0.04 d	(0)	0.01 d	(1)	2.35 b		<0.04 d	(0)	2.11 cb		1.91 cb			
			10	1.55 b	4.57 a	4.93 a	0.01 d	<0.04 d	(0)	<0.04 d	(0)	1.33 bc		<0.04 d	(0)	0.74 bcd		0.48 cd			

[a]Mean values in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$); the minimum detection limit was 0.04 CFU/worm.

Numbers in parenthesis following values indicate the number of positive samples out of three analyzed.

[b]Refer to Table 1 for list of treatments

Overall, K Foam Lo (treatment B) was the least effective in killing *E. coli* OP50 and *S.* Newport in *C. elegans*. Up to a 1.12 $\log_{10}$ CFU/worm reduction in *E. coli* OP50, compared to the initial population, was observed in worms immersed in this cleaner. Treatment of worms that had been held at 4°C and 33% relative humidity for 10 min or worms held at 4°C and 98% relative humidity for 2 or 10 min with K Foam Lo caused significant reductions in populations of *E. coli* OP50 compared to treatment with distilled water or 0.1% peptone (treatment A). K Foam Lo was ineffective in killing *E. coli* OP50 in worms that had been held at 20°C and 33 or 98% relative humidity. In a study evaluating the lethality of seven commercially available alkaline cleaners in killing *E. coli* O157:H7, it was demonstrated that K Foam Lo was the least effective (*23*). Considering all combinations of incubation temperature, relative humidity, and treatment time, except for worms incubated at 4°C and 33% relative humidity and treated for 2 min, mean populations of *S.* Newport were significantly reduced ($P \leq 0.05$) by up to 4.92 $\log_{10}$ CFU/worm compared to mean populations detected in worms treated with distilled water or peptone water.

Treatments C (Enforce), D (Sanova), and F (2% lactic acid) were the most effective among test cleaners and sanitizers in eliminating test microorganisms ingested by *C. elegans*. Enforce is a commercially available cleaner and has the highest pH (12.3) of all products evaluated. Sanova, an acidified sodium chlorite-based sanitizer with a pH of 2.4, had the same pH as 2% acetic acid (treatment E); however, Sanova was much more effective in killing ingested bacterial cells. This indicates that factors other than pH play a role in the lethality of cleaners and sanitizers to *E. coli* OP50 and *S.* Newport lodged in the gut of *C. elegans*. *C. elegans* can survive in a pH range of 3.2 – 11.8 for at least 96 h (*18*).

Two organic acids (treatments E and F) were evaluated in this study. Treatment with 2% acetic acid (treatment E) reduced the number of *S.* Newport to a level below the detection limit

(0.04 $\log_{10}$ CFU/worm) in 1 of 8 treatment combinations compared to 7 of 8 treatment combinations for 2% lactic acid (treatment F). This agrees with results reported by Caldwell *et al.* (*7*) in which higher initial populations of ingested *S.* Poona were reduced by up to 1.61 $\log_{10}$ CFU/worm treated with 2% acetic acid compared to reductions of 5.32 $\log_{10}$ CFU/worm when treated with 2% lactic acid. Other studies have shown acetic acid to be more effective than lactic acid in killing *E. coli* O157:H7 (*13, 22*) and *Shigella flexneri* (*25*). At a working concentration of 2%, the pH of lactic acid (2.0) is lower than that of acetic acid (2.4). The lower pH may be partially responsible for the increased effectiveness of lactic acid in killing *E. coli* OP50 and salmonellae in the gut of *C. elegans*.

Solutions of NaOCl containing free chlorine at 50 µg/ml (treatment G) and 200 µg/ml (treatment H) were more effective in reducing *E. coli* OP50 populations than *S.* Newport populations. Compared to the number of *E. coli* OP50 recovered from worms treated with water or peptone, treatment with both concentrations of chlorine caused significant ($P \leq 0.05$) reductions in counts, regardless of previous desiccation conditions or treatment time. Populations were reduced to undetectable levels in worms that had been subjected to 5 of 8 temperature/relative humidity/treatment time combinations, regardless of the concentration of chlorine in treatment solution. In contrast, *S.* Newport was recovered from desiccated worms that had been exposed to all combinations of incubation temperature and relative humidity after treatment with 50 or 200 µg/ml chlorine for 2 min. With one exception (20°C, 33% relative humidity), compared to treatment with water or peptone for 10 min, significant reductions in populations of *S.* Newport were achieved by treating worms with both concentrations of chlorine for 10 min. In the presence of organic matter, the efficacy of chlorine in killing bacteria is greatly diminished. The treatment solutions in which desiccated worms were immersed contain

little or no organic matter other than that in the worms and the bacteria within them. This study represents a best-case scenario in terms of testing the effectiveness of chlorine in killing bacteria ingested by worms. The ability of chlorine to kill ingested bacteria would be further diminished if worms were in environments such as produce processing facilities or on the surface of produce because of the presence of large amounts of organic matter in wash waters and in produce tissues. For disinfection of produce using chlorine, contact times of no more than 1 – 2 min are commonly used (*3*). A chlorine concentration of 200 μg/ml is the upper limit used by the produce industry, but most processors use substantially lower concentrations. For chlorine to be effective at killing bacteria located in the gut of *C. elegans* and perhaps other free-living nematodes, the concentration of free chlorine in wash water would have to be closely monitored and contact times may have to be increased. To be effective in eliminating bacteria from the gut of *C. elegans,* treatments must penetrate the cuticle of the worm or otherwise disrupt the integrity of the cuticle and come into contact with the target cells. Enforce and Sanova both contain bactericidal constituents that apparently have a synergistic effect with pH. The cuticular permeability of desiccated *C. elegans* may increase as the pH of the environment deviates from neutral, allowing bactericides in cleaners and sanitizers to penetrate the cuticle and come in contact with ingested cells.

Effectiveness of cleaners and sanitizers on killing planktonic *E. coli* OP50 and *S.* Newport cells. Mean populations of planktonic cells of *E. coli* OP50 and *S.* Newport recovered after treatment with distilled water (control), 0.1% peptone, cleaners, and sanitizers are shown in Table 3.3. Initial mean populations of test microorganisms were not significantly ($P > 0.05$) influenced by treatment of cells that had been held at 4°C or 20°C for 24 h in distilled water or peptone (treatment A).

TABLE 3.3 *Populations (log_{10} cfu/worm) of planktonic* Escherichia coli *OP50 and* Salmonella *Newport recovered from cells incubated at 4 or 20°C for 24 h.*

Microorganism	Temp (°C)	Treat-ment time (min)	Initial	Populations (log_{10} cfu/worm)[a]													
				Distilled water	Treatment[b]												
					A	B	C		D		E	F	G		H		
E. coli OP50	4	2	5.46 a	5.17 a	5.25 a	2.93 c	0.54 e		<0.04 e	(0)	4.41 b	2.00 d	<0.04 e	(0)	<0.04 e	(0)
		10	5.46 a	5.15 a	5.22 a	0.95 c	<0.04 d	(0)	<0.04 d	(0)	4.33 b	1.02 cd	<0.04 e	(0)	<0.04 e	(0)
	20	2	5.46 a	5.22 a	5.21 a	3.37 c	1.22 e		<0.04 f	(0)	4.38 b	1.93 d	<0.04 f	(0)	<0.04 f	(0)
		10	5.46 a	5.27 a	5.14 a	1.52 c	0.73 de		<0.04 e	(0)	4.41 b	1.02 cd	<0.04 e	(0)	<0.04 e	(0)
S. Newport	4	2	4.47 a	4.64 a	4.55 a	2.63 c	<0.04 b	(0)	<0.04 d	(0)	4.23 b	2.41 c	<0.04 d	(0)	<0.04 d	(0)
		10	4.47 a	4.66 b	4.63 a	0.38 b	<0.04 b	(0)	<0.04 b	(0)	4.11 a	0.43 b	<0.04 b	(0)	<0.04 b	(0)
	20	2	4.47 ab	4.62 a	4.58 a	2.29 c	0.53 d		<0.04 e	(0)	4.17 b	2.16 c	<0.04 e	(0)	<0.04 e	(0)
		10	4.47 a	4.77 a	4.63 a	0.63 cd	0.91 bc		<0.04 d	(0)	4.21 a	1.50 b	<0.04 d	(0)	<0.04 d	(0)

[a]Mean values in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$); the minimum detection limit was 0.04 CFU/worm. Numbers in parenthesis following values indicate the number of positive samples out of three analyzed.

[b]Refer to Table 3.1 for list of treatments

E. coli OP50 and *S.* Newport were not detected by direct plating or enrichment after treatment with Sanova (treatment D), 50 µg/ml chlorine (treatment G), or 200 µg/ml chlorine (treatment H). The two cleaners, K Foam Lo (treatment B) and Enforce (treatment C), were less effective in killing the two microorganisms. Considering the incubation temperature preceding treatment as well as treatment time, K Foam Lo significantly ($P \leq 0.05$) reduced populations of *E. coli* OP50 and *S.* Newport by up to 4.27 $\log_{10}$ CFU/worm and 4.25 $\log_{10}$ CFU/worm, respectively, compared to reductions caused by treating cells with 0.1% peptone. While reductions were significant, both test microorganisms were detected by direct plating. In general, compared to reductions in populations caused by K Foam Lo, significantly greater reductions of *E. coli* OP50 were achieved by treating cells with Enforce.

Treatment with 2% lactic acid (treatment F) was significantly ($P \leq 0.05$) more effective than treatment with 2% acetic acid (treatment E) in reducing populations of *E. coli* OP50 and *S.* Newport. Acetic acid was the least effective chemical treatment evaluated in killing planktonic cells of both test microorganisms.

Treatment with cleaners and sanitizers may be more effective in killing or releasing ingested bacterial cells from the gut of desiccated worms than from live worms. Placement of desiccated worms in an environment with a higher moisture content, relative to the worms, would result in absorption of water. Bactericidal compounds in the water would come in contact with bacterial cells in the gut of the worm. Worms not desiccated before exposure to cleaners or sanitizers would absorb less water, and therefore less bactericidal constituents. During the process of desiccation and rehydration the cuticlar structure of the nematode may become compromised, thereby resulting in increased exposure of the gut contents to cleaners and

sanitizers and a higher level of lethality compared to that achieved by treating worms that are not desiccated.

In summary, none of the commercial cleaners and sanitizers evaluated were effective in killing all cells of *S.* Newport ingested by *C. elegans* using all test conditions. The use of cleaners and sanitizers in combination with other treatments to kill bacteria ingested by nematodes is an important step in reducing pathogens on produce contact surfaces and raw or minimally processed fruits and vegetables destined for the retail market.

Acknowledgment

This study was supported in part by the USDA-ARS National Alliance for Food Safety. The *C. elegans* strain used in this work was provided by the Caenorhabditis Genetic Center, which is funded by the NIH National Center for Research Resources.

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CHAPTER 4

MIGRATION OF *CAENORHABDITIS ELEGANS* TO MANURE AND MANURE COMPOST AND POTENTIAL VECTORING OF *SALMONELLA ENTERICA* SEROTYPE NEWPORT TO FRUITS AND VEGETABLES[1]

[1]Kenney, S. J., Anderson, G. L., Williams, P. L., Millner, P. D., and Beuchat, L. R. To be submitted to *Applied and Environmental Microbiology*.

ABSTRACT

A study was done to determine if a free-living, bacterivorous nematode, *Caenorhabditis elegans*, migrates to bovine manure, turkey manure, composted bovine manure, composted turkey manure, and manure-amended soil inoculated with *Salmonella enterica* serotype Newport. Movement of the worm to lettuce, strawberries, and carrots was also studied. *C. elegans* was placed on the surface of agar 3.5 cm away from ca. 0.5 g of manure, composted manure, or manure-amended soil inoculated with *S. enterica* serotype Newport, as well as uninoculated soil. In another study, a piece of lettuce, strawberry, or carrot was placed on agar 1.8 cm away from bovine manure or bovine manure compost and uninoculated soil. Movement of the nematode toward test samples on the agar surface was monitored for up to 30 min at 20°C. *C. elegans* moved most rapidly to turkey manure and strawberries, with 35% and 60% of worms, respectively, being associated with samples within 30 min. Survival and reproduction of *C. elegans* in test materials was not affected by the presence of *S. enterica* serotype Newport. The ability of *C. elegans* to transport *S. enterica* serotype Newport through soil and contaminate produce was investigated. Bovine manure and bovine manure compost (1 g) inoculated with *S. enterica* serotype Newport (8.6 $\log_{10}$ CFU/g) were separately placed in the bottom of a glass jar (2.5 cm diameter) and covered with a layer of soil (5 cm) inoculated (50 worms/g) or not inoculated with *C. elegans*. A piece (0.5 – 0.7 g) of lettuce, strawberry, or carrot was placed on top of the soil. Jars were sealed and incubated at 20°C for up to 10 days. In the system using soil inoculated with *C. elegans, S. enterica* serotype Newport initially in bovine manure was detected on the surface of lettuce, strawberry, and carrot samples within 3, 1, and 1 days, respectively. The pathogen was detected on lettuce, strawberry, and carrot within 1, 7, and 1 days, respectively, when present in bovine manure compost. With one exception (strawberries, 7

days, compost inoculated with *S. enterica* serotype Newport), the pathogen was not detected on the produce over the 10-day study when *C. elegans* was not present in the soil. Results suggest that *C. elegans* that ingests *S. enterica* serotype Newport or carries it as an external contaminant may transport the pathogen to the surface of preharvest fruits and vegetables. The incidence of this phenomenon may be low in a field setting, but observations reinforce the importance of sanitizing produce prior to consumption.

INTRODUCTION

Numerous outbreaks of foodborne illness associated with the consumption of raw or minimally processed fruits and vegetables contaminated with human enteric pathogens have been documented (4, 12, 18). It is often difficult to determine if contamination is a preharvest or postharvest event. These outbreaks have raised interest in identifying processes through which preharvest fruits and vegetables can become contaminated with foodborne pathogens.

Escherichia coli O157:H7, *Listeria monocytogenes* (2, 7), several serotypes of *Salmonella enterica* (1, 7, 14), *Bacillus cereus* (2), and *Staphylococcus aureus* (21) have been reported to be ingested by *Caenorhabditis elegans,* a free-living, bacterivorous nematode found in the soils of temperate regions. The cuticle of live or dead intact worms may provide a physical barrier to protect bacterial cells present in the gut against chemical cleaners and sanitizers applied to processing equipment and some types of raw fruits and vegetables (6, 14). Chang *et al.* (8) reported that chlorinated water is ineffective at killing salmonellae, *Shigella sonnei,* and viruses in the gut of nematodes isolated from water. It is hypothesized that pathogens surviving disinfection treatments may be protected by the cuticle of worms and could remain on the surface of produce or on processing equipment for extended times.

It is not uncommon for animal manure and manure compost to be applied to cropland soil as fertilizers. *E. coli* O157:H7 can survive in bovine manure-amended soil held at 21°C for at least 193 days (13). The application of manure and manure compost to soil may attract nematodes in the soil that feed on bacteria. Free-living, microbivorous nematode populations have been reported to increase in soils to which cattle manure slurry has been applied (19). The extent to which various types of manure and manure composts are incorporated into the soil can influence populations of nematodes. Sand homogeneously amended with a humus-litter mixture

has been reported to support higher populations of *C. elegans* compared to sand containing isolated patches of the humus-litter mixture (17).

It is hypothesized that free-living nematodes such as *C. elegans* and possibly other genera may ingest human pathogens occasionally found in the soil and transport these pathogens through the soil matrix. As a worm migrates through soil it may come in contact with external tissues of plants, either by attraction mechanisms or by random chance. *Salmonella enterica* serotype Poona was detected on cantaloupe rind in contact with soil infested with *C. elegans* within a shorter time compared to rind on soil not containing the nematode (7). If infected worms reside on the surface of produce, they may cause contamination by excreting pathogens or as a result of rupturing of the cuticle and releasing the gut contents.

Manure and manure composts originating from various animals can have markedly different chemical composition and physical properties. Compared to bovine manure, for example, turkey manure tends to contain more ammonia. Composts generally have lower moisture content than raw manures. The origin and properties of manures and manure composts may affect the survival and reproduction of *C. elegans* and other free-living nematodes, and perhaps their role as vectors of foodborne pathogens.

To date, minimal research has been conducted to determine conditions that may affect the movement of *C. elegans* to fruits and vegetables. Differences in attraction behavior of *C. elegans* may be influenced by the type and amount of volatile chemical compounds released by fruits and vegetables. Various exudates and volatiles released by fruits and vegetables may attract or repel nematodes and other biota in the soil in different ways. Free-living nematodes would be more likely to come in contact with preharvest fruits and vegetable to which they are attracted.

A study was undertaken to determine if *C. elegans* is attracted to bovine manure, turkey manure, composted bovine manure, composted turkey manure, and manure-amended soil inoculated with *Salmonella enterica* serotype Newport. Survival and reproduction of *C. elegans* in the same matrices not inoculated with *S.* Newport were investigated. Movement of *C. elegans* to lettuce, strawberries, and carrots on an agar medium and the ability of the nematode to transport *S. enterica* serotype Newport in soil to the surface of produce was also studied.

MATERIALS AND METHODS

Maintenance of *C. elegans.* A transgenic green fluorescent protein (gfp) labeled strain (PD4792) of *C. elegans* was used. This stain expresses strong fluorescence in the pharynx region. Worms were maintained 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; BBL/Difco, Sparks, Md.), and agar (17.0 g) (22). *E. coli* OP50, a nonpathogenic strain routinely used as a feed source for *C. elegans*, was grown at 37°C for 24 h in OP50 broth (5), which contains (per liter of deionized water): sodium chloride (5.0 g) and Bacto peptone (10.0 g). The K agar was surface inoculated with 0.1 ml of a 24-h culture of *E. coli* OP50 and incubated at 37°C for 24 h to establish confluent growth. Approximately 50 adult worms were deposited on the surface of K agar and incubated at 20°C for up to 3 days prior to age synchronization of worms.

Preparation of *C. elegans* for reproduction and migration experiments. The surface of ten K agar plates, each containing 500 – 1000 eggs, and 30 – 50 adult worms, was washed by depositing 5 ml of sterile K medium (23) and gently rubbing with a sterile bent glass rod. The suspended eggs and worms were aseptically transferred to a sterile 15-ml centrifuge tube. The wash and transfer procedure was repeated to enable efficient harvesting of eggs. Eggs and

worms were collected by centrifugation (500 x *g*, 2 min) and the supernatant was removed using a pipette. Worms and eggs in a pellet from pooled suspensions were resuspended in 10 ml of 0.013 M NaOH solution containing 1% NaOCl (pH 13.0) and incubated at 20°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) and the supernatant was removed. Worms and eggs in the pellet were resuspended in 10 ml of K medium and centrifuged again. The supernatant was removed and the eggs and dead worms were resuspended in K medium. The suspension (0.1 ml containing 400 – 600 eggs) was deposited on the surface of a K agar plate on which a lawn of *E. coli* OP50 had formed, followed by incubation at 20°C for 3 days. This procedure ensured that all worms used in assays were of the same age. Adult worms were used in reproduction and migration experiments.

Bacteria used and preparation of manures and composts for assays. A multidrug resistant strain of *Salmonella enterica* serotype Newport was adapted to grow in tryptic soy broth (TSB, pH 7.3; BBL/Difco) supplemented with nalidixic acid (50 µg/ml) (TSBN). Nalidixic acid-adapted *S. enterica* serotype Newport was used to facilitate its detection with minimal background interference on tryptic soy agar (TSA; BBL/Difco) supplemented with nalidixic acid (50 µg/ml) (TSAN). Nalidixic acid-sensitive bacteria naturally occurring in soil, manures, and manure composts and *E. coli* OP50 cells remaining on the surface or in the gut of the worm as a result of feeding would not be expected to grow on TSAN but nalidixic acid-adapted *S. enterica* serotype Newport would form colonies. Nalidixic acid-adapted *S. enterica* serotype Newport was grown in 10 ml of TSBN at 37°C for 24 h. The pathogen was transferred twice to 10 ml of TSBN by loop inoculum (ca. 10 µl) at successive 24-h intervals. Cells were collected by centrifugation (2,000 x *g*, 10 min), resuspended in 10 ml of sterile deionized water, collected

again, and resuspended in 10 ml of deionized water. This suspension served as an inoculum for *C. elegans* attraction experiments.

Turkey manure mixed with litter, turkey manure compost, separated bovine solids (bovine manure), and bovine manure compost were supplied by the United States Department of Agriculture, Agricultural Research Service (Beltsville, Md). Soil (Redi-Earth Peat-Lite Mix, 3CP, The Scotts Company, Columbus, Ohio) (pH 6.9) was saturated with water and combined with the turkey manure or bovine manure (9:1, w/w). Samples (40 g) of manures, manure composts, and manure-amended soils were placed in individual quart Ziploc® storage bags (S.C. Johnson & Son, Inc., Racine, Wisc.) and inoculated with 2 ml of a washed cell suspension of *S. enterica* serotype Newport to give 8.55 $\log_{10}$ CFU/g. Bags containing inoculated samples were sealed and vigorously shaken for 30 sec to uniformly distribute the pathogen. Before using in assays, samples were incubated at 20°C for 24 h.

Populations of nalidixic acid-adapted *S. enterica* serotype Newport in samples incubated at 20°C for 0, 1, 3, 5, and 7 days were determined. Samples (2.2 g) were individually placed in a stomacher 80 bag (Seward Medical Ltd., London, U.K.) containing 19.8 ml of sterile 0.1% peptone water. Bags containing slurries were pummeled in a stomacher 80 (Seward Medical) for 60 sec at high speed. Undiluted samples (0.25 ml in quadruplicate and 0.1 in duplicate) and serially diluted samples (0.1 ml in duplicate) were spread plated on TSAN and incubated at 37°C for 24 h. Presumptive *S. enterica* serotype Newport colonies were enumerated and random colonies were confirmed using a *Salmonella* latex agglutination assay (Oxoid, Basingstoke, U.K.).

Survival and reproduction of *C. elegans*. Manures, manure composts, and manure-amended soil were prepared as described above. Samples (2.2 g) inoculated with *S. enterica*

serotype Newport, as well as uninoculated soil, manures, manure composts, and manure-amended soil samples, were individually placed in 35 mm diam. x 10 mm deep petri dishes. Ten worms suspended in 10 µl of K medium were deposited on top of each sample. The petri dish was covered, placed in a tub, and sealed by applying a lid. Samples were incubated for 1, 3, 5, and 7 days at 20°C before analyzing for populations of *C. elegans*. Each sample was placed in a sterile 50-ml centrifuge tube containing 10 ml of Ludox® TM-30 colloidal silica (Sigma-Aldrich, St. Louis, Mo.) and the mixture was centrifuged (500 x *g*, 2 min). Supernatant containing worms was decanted into a petri dish (100 mm diam. x 15 mm deep) and examined with a dissecting microscope to determine if numbers of *C. elegans* declined (-), did not change (nc), or increased (+ or ++ [>2000%]) compared to initial populations. All experiments were replicated three times.

Migration of *C. elegans* to manure, manure compost, and manure-amended soil inoculated with *S. enterica* serotype Newport, and to uninoculated soil. Four plastic rings (2 cm diam., 0.2 cm long) were placed on the surface of TSAN equidistant (ca. 5 cm) from each other around the perimeter of a petri dish (100 x 15 mm). Samples (ca. 0.5 g) of uninoculated soil and bovine manure, bovine manure compost, and bovine manure-amended soil inoculated with *S. enterica* serotype Newport were placed in the four rings; the same procedure was done using uninoculated soil and turkey manure, turkey manure compost, and turkey manure-amended soil inoculated with *S. enterica* serotype Newport. Forceps were used to remove the rings, leaving samples on the surface of the agar.

A K agar plate containing a lawn of *E. coli* OP50 on which adult worms had developed was flooded with 10 ml of K medium. The suspension was transferred to a sterile 15-ml tube and centrifuged (500 x *g*, 2 min). 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 20°C. A suspension (10 µl) containing 20 – 30 worms was deposited onto the surface of the TSAN agar plate 3.5 cm from each of the four sites where test samples had been placed. The number of worms in the inoculum was recorded. The surface tension of the inoculum was carefully broken with a sterile fine-bristle paint brush to facilitate worm contact with the TSAN agar surface. Migration of worms toward the test samples was monitored at 5-min intervals for 30 min. This experiment was replicated five times.

Migration of *C. elegans* to lettuce, strawberries, and carrots on TSAN. Lettuce (*Lactuca sativa* L.), strawberries (*Fragaria* x *ananassa* Duchesne), and carrots (*Daucus carota* L.) were purchased from a supermarket in Griffin, Ga. and held at 20°C until used in experiments the same day. Circular (1.5 cm diam.) pieces were cut from produce with no. 9 cork borer which had been sterilized by immersing in 70% ethanol. The circular piece of produce, skin side down, was placed on the surface of TSAN. Two plastic rings (2 cm diam., 0.2 cm long) were also placed on the surface of TSAN at positions ca. 5 cm apart to form the points of an equilateral triangle that included the site of the piece of produce. One ring was filled with either *S. enterica* serotype Newport inoculated bovine manure or bovine manure compost, both inoculated with *S. enterica* serotype Newport, and the other ring was filled with uninoculated soil. The rings were removed from the agar using a forcep and *C. elegans* (20 – 30 worms) suspended in K medium was placed on the surface of the agar plate ca. 1.8 cm from each test sample. The surface tension of the suspension was broken and attraction of worms to test samples was monitored as described above. This experiment was replicated three times.

Migration of *S. enterica* serotype Newport in soil to lettuce, strawberries, or carrots. Circular (1.5 cm diam.) pieces were cut from produce with no. 9 cork borer sterilized by

immersing in 70% ethanol. Bovine manure (1 g) or bovine manure compost (1 g), both inoculated with *S. enterica* serotype Newport as described above, were separately placed into a sterile 20-ml disposable scintillation jar (Fisher Scientific). Soil (9 g) inoculated with *C. elegans* (ca. 50 worms/g) or not inoculated with the worm was placed on top of the manure or compost inoculated with *S. enterica* serotype Newport (5 cm deep). A piece of lettuce, strawberry, or carrot was firmly placed on top of the soil. In another set of jars, bovine manure, bovine manure compost, and soil were prepared as described above, except lettuce, strawberry, and carrot samples were not placed on top of the soil. Jars were covered with parafilm and lids were applied to prevent evaporation of water. Samples were incubated for 1, 3, 5, 7, and 10 days at 20°C before analyzing produce or the top layer of soil (1 cm deep) for the presence of *S. enterica* serotype Newport.

Pieces of lettuce, strawberry, carrot, or ca. 2 g of soil removed from the top 1 cm layer were separately placed into a stomacher 400 bag and 100 ml of lactose broth (pH 6.9, BBL/Difco) supplemented with nalidixic acid (50 μg/ml) (LBN) was added. Mixtures were incubated at 37°C for 24 h. A loopful of LBN was inoculated into 10 ml of Rappaport-Vassiliadis enrichment broth (RV) (pH 5.2, Oxoid) and incubated at 42°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. Randomly selected presumptive-positive colonies of *S. enterica* serotype Newport that formed on BSAN were confirmed by *Salmonella* latex agglutination assays. The presence or absence of *S. enterica* serotype Newport was recorded as either positive (+) or negative (–), respectively. This experiment was replicated four times.

Statistical analysis. Each experiment was replicated at least three times. Data were analyzed using Statistical Analysis Software (SAS Institute, Cary, N.C.). Significant differences ($P \leq 0.01$) between values were determined using t-test.

RESULTS AND DISCUSSION

Survival and reproduction of *C. elegans.* Populations of *C. elegans* remained unchanged (10 worms/sample) for 1 day at 20°C in manures, manure compost, manure-amended soil, and soil inoculated or not inoculated with *S. enterica* serotype Newport, but increased or decreased, depending on the test material between 1 and 7 days. *Salmonella* has been reported to slightly shorten the life span of *C. elegans* (1); however, any detrimental effect *S. enterica* serotype Newport had on the survival of the worm in our study was not evident. The presence of *S. enterica* serotype Newport in samples did not affect the survival or reproduction of worms. Populations of *C. elegans* were highest in turkey manure and bovine manure incubated for 7 days at 20°C. Manures had the highest moisture contents (62.2 – 79.8%) of all products examined. Microbial populations and profiles in fresh manure can change rapidly. Manures may have nutrients that can be readily utilized by bacteria to reproduce and maintain population size, despite predation by *C. elegans*. Younger bacterial cells may have been more attractive to *C. elegans*, resulting in an increase in the rate of reproduction. It has been reported that *C. elegans* is more readily attracted to young (24 – 48 h) bacterial colonies than to older (96 – 192 h) colonies on agar media (11). Attraction of *C. elegans* to bacteria is also dependant on the bacterial species. *Acinetobacter calcoaceticus* var. *antratus* attracts *C. elegans* within 192 h, but the worm is not attracted to *Serratia liquefaciens* at the same age (11). Anderson *et al*. (2) also

observed different levels of attraction of *C. elegans* to surrogates of enteric pathogens of the same physiological age.

Populations of *C. elegans* began to decline in turkey manure compost and bovine manure compost after 1 day at 20°C. Few (<10 viable worms/sample) or no juvenile worms were observed in composts at all subsequent sampling times. The moisture content of the turkey manure compost (31.5 – 36.2%) and bovine manure compost (59.6 – 61.9%) may have been insufficient to enable survival or support reproduction of *C. elegans.* Populations of *S. enterica* serotype Newport, together with bacteria naturally present in composts, would have been sufficient to support reproduction of *C. elegans*. Soil to which compost has been added for the purpose of growing fruits and vegetables would likely have a higher moisture content than the compost tested in this study. Periodic rainfall or irrigation will increase the moisture content of soils and can contribute to bacterial survival and growth (9). Simulated rainfall at a rate of 1.65 cm/h was applied to sandy loam and silty clay loam soils inoculated with *E. coli* O157:H7 (9). Hourly analysis of leachate samples revealed that numbers of *E. coli* O157:H7 recovered remained near the inoculum level for at least 8 h. This indicates that the microorganism was multiplying to maintain high numbers in the leachate. The moist environment of soil and compost-amended soil would promote the growth of bacteria to be used as a nutrient source by *C. elegans.*

C. elegans increased from an initial population of 10 worms/2.2 g sample in manure-amended soil, to ca. 20 worms/2.2-g sample between 1 and 3 days, to ca. 50 worms/2.2-g sample between 3 and 5 days, and remained constant between 5 and 7 days. The increase was not as large as in manures. The worms may have been more closely associated with the manure particles and to nutrients in the manure than to soil particles in the manure-amended soil.

Results are in agreement with those reported by Mikola and Sulkava (17) showing that *C. elegans* populations were higher in humus-litter than in sand in a heterogeneous mixture.

C. elegans is not commonly observed outside of the laboratory environment, possibly due to its lack of economic impact on agricultural crops. *Diploscapter* sp., reportedly found in a range of agricultural habitats (20), has a higher thermal tolerance compared to *C. elegans* (16). Lawns of *S. enterica* serotype Poona, *E. coli* O157:H7, and *L. monocytogenes* can support survival and reproduction of *Diploscapter* on agar media and in manures (10). The worm was also able to disperse pathogenic bacteria after it was exposed to the pathogens in soil.

Migration of *C. elegans* to manure, manure compost, and manure-amended soil inoculated with *S. enterica* serotype Newport and to uninoculated soil. The mean number of worms that migrated to test materials inoculated with *S. enterica* serotype Newport or uninoculated soil after 5, 10, 15, 20, 25, and 30 min at 20°C was used to calculate the percent distribution of *C. elegans.* Attraction of *C. elegans* to test samples is shown in Fig. 4.1. Regardless of the origin of manures and composts, *C. elegans* was associated with these materials in greater numbers than to uninoculated soil. The largest percentage of *C. elegans* was observed in turkey manure. In general, worms that entered samples of turkey manure or turkey manure-amended soil remained associated with these matrices for the duration of the 30-min incubation time. At the end of the 30-min test period, 45.4% of the worms initially deposited on the plates were detected in turkey manure.

Turkey manure had the strongest odor of materials tested and it is possible that *C. elegans* is attracted to one or more of the aromatics, e.g., ammonia, released by the manure. Turkey manure, turkey manure compost, bovine manure, and bovine manure compost had ammonia concentrations of 5.39, 0.01, 1.45, and 0.89 mg/kg dry weight, respectively. Fewer numbers of

C. elegans were observed in soil amended with turkey manure than in turkey manure. Within 5 min of depositing worms on the agar surface, 7 – 8% of worms on agar had entered turkey manure-amended soil compared to 23 – 37% of worms in manure.

Compared to turkey manure and turkey manure compost, *C. elegans* was much less often associated with bovine manure and bovine manure compost. Fewer than 10% of the worms were associated at any incubation time. More *C. elegans* were located in or near bovine compost than other test materials between 5 and 10 min, but equal or higher numbers of *C. elegans* were observed in bovine manure after 10 min. A maximum of 7% of the worms were associated with the soil amended with bovine manure over the 30-min test period.

Migration of *C. elegans* to lettuce, strawberries, and carrots. The mean number of worms that migrated to lettuce, strawberries, or carrot, bovine manure or bovine manure compost inoculated with *S. enterica* serotype Newport, or uninoculated soil after 5, 10, 15, 20, 25, and 30 min at 20°C was used to calculate the percent distribution of *C. elegans.* The percent distribution of *C. elegans* in produce, bovine manure, and soil is shown in Fig 4.2. *C. elegans* migrated more often to manure inoculated with *S. enteric* serotype Newport and to produce rather than uninoculated soil. In the presence of bovine manure, *C. elegans* was less likely to migrate to lettuce. No more than 10% of worms were associated with lettuce at any given time during the 30-min test period. Numbers of *C. elegans* in manure increased slightly between 10 and 30 min. At 25 and 30 min, the percentage of *C. elegans* in manure was higher than the percentage migrating to lettuce. *C. elegans* was strongly attracted to strawberries in preference to inoculate bovine manure or uninoculated soil. The number of *C. elegans* associated with the

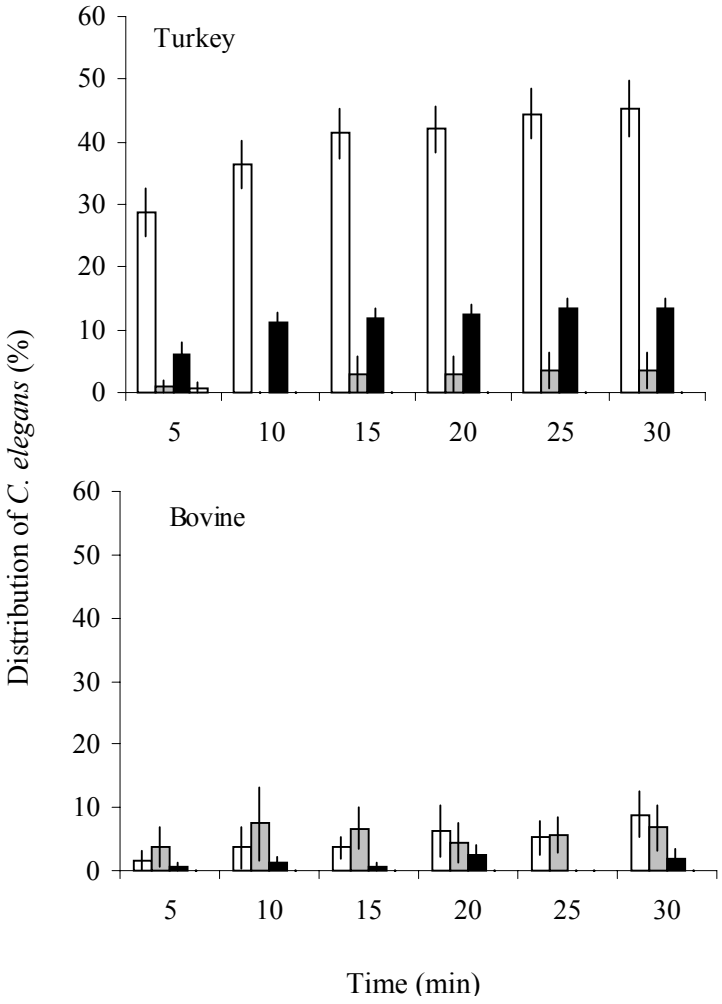

Figure 4.1. Migration of *C. elegans* on TSAN on which turkey or bovine manure, manure compost, and manure-amended soil inoculated with *S. enterica* serotype Newport and uninoculated soil were deposited. Worms (20 – 30) were deposited 2.5 cm away from samples. The percentages of worms distributed manure (open bars), manure compost (shaded bars), in manure amended soil (solid bars), or uninoculated soil (hatched bars) within 30 min at 20°C were monitor.

strawberry increased over the 30 min sampling time. Moderate numbers of *C. elegans* migrated to carrots.

The percent distribution of *C. elegans* in produce, bovine manure compost, and soil is shown in Fig 4.3. At all incubation times except 10 min, a higher percentage of *C. elegans* were located in the bovine manure compost than were associated with lettuce. Lower numbers of *C. elegans* were observed in bovine manure compost (Fig 4.3) compared to bovine manure (Fig 4.2). This resulted in a lower percentage of worms being associated with strawberry when manure was present on the agar compared to when manure compost was present. Strawberry samples were highly aromatic and this likely contributed to the strong attraction of *C. elegans*. Worms were moved more slowly to carrot than to lettuce or strawberries. A large percentage of worms did not move to bovine manure compost or soil on plates also containing carrot.

Caldwell *et al.* (7) showed that *C. elegans* is more attracted to colonies of *S. enterica* serotype Poona on agar than to cantaloupe juice. *S. enterica* serotype Poona may have released compounds that preferentially attracted *C. elegans*. In our study, bovine manure and bovine manure compost inoculated with *S. enterica* serotype Newport, uninoculated soil, and each type of produce were placed on the surface of agar not more than 5 min before depositing worms. This procedure reduced the time that compounds from the samples entered the air within the covered petri dishes or could be absorbed by the agar. Results clearly indicate that *C. elegans* migrates preferentially to strawberries than to other test materials.

Migration of *S. enterica* serotype Newport in soil to lettuce, strawberries, or carrots. Numbers of lettuce, strawberry, and carrot samples positive for the presence of *S. enterica* serotype Newport after incubation for 0, 1, 3, 5, 7, and 10 days at 20°C on the top of soil under which bovine manure or bovine manure compost inoculated with *S. enterica* serotype Newport

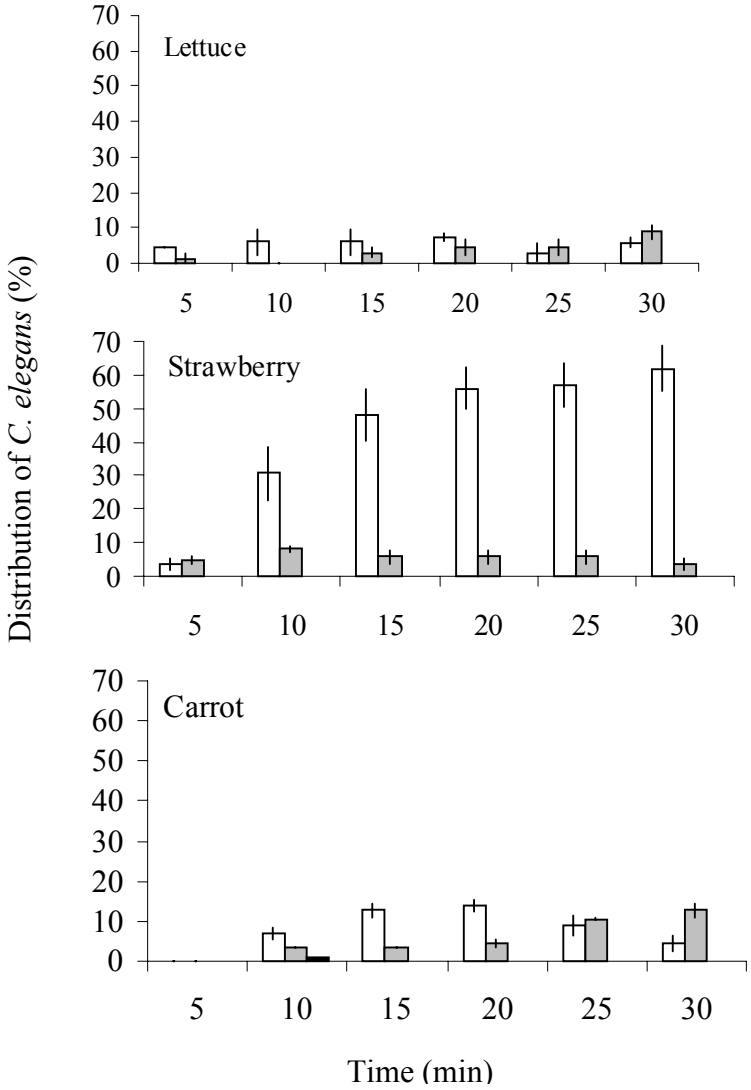

Figure 4.2. Migration of *C. elegans* on TSAN on which lettuce, strawberry, carrot, bovine manure inoculated with *S. enterica* serotype Newport, and uninoculated soil were deposited. Worms (20 – 30) were deposited 1.8 cm away from samples. The percentages of worms distributed in produce (open bars), manure (shaded bars), and uninoculated soil (solid bars) within 30 min at 20°C were monitored.

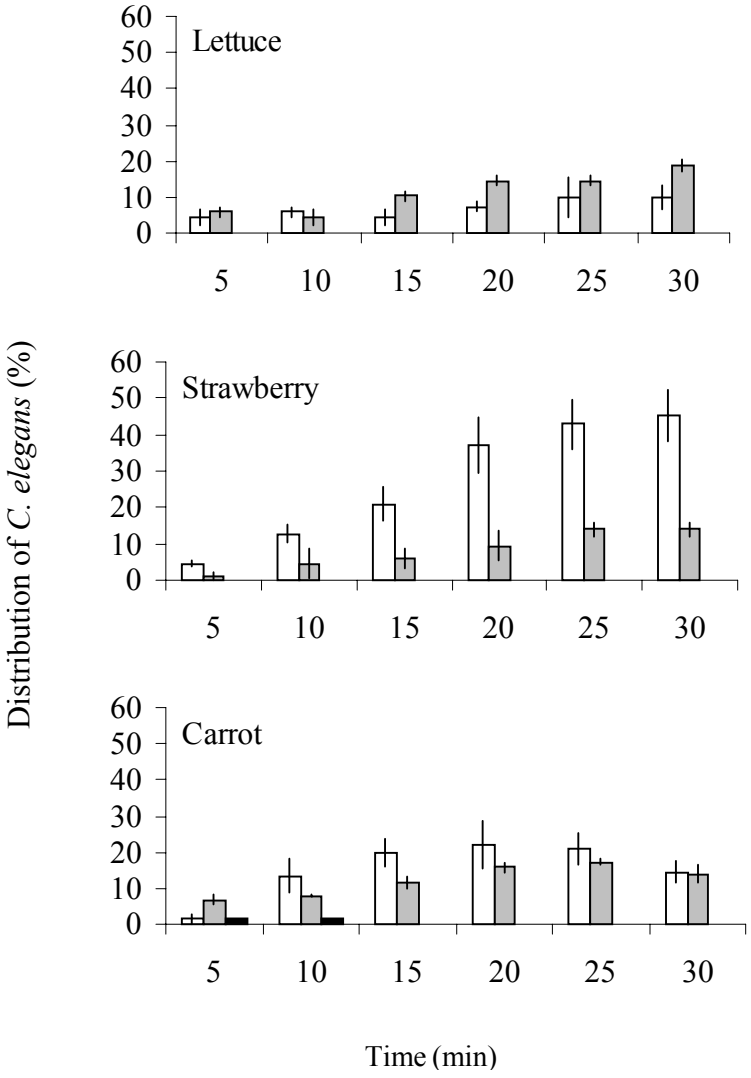

Figure 4.3. Migration of *C. elegans* on TSAN on which lettuce, strawberry, carrot, bovine manure compost inoculated with *S. enterica* serotype Newport, and uninoculated soil were deposited. Worms (20 – 30) were deposited 1.8 cm away from samples. The percentages of worms distributed in produce (open bars), bovine manure. compost (shaded bars), and uninoculated soil (solid bars) within 30 min at 20°C were monitored.

had been placed are shown in Table 4.1. The presence of *C. elegans* in soil significantly ($P \leq 0.01$) contributes to the contamination of produce with the pathogen. *S. enterica* serotype Newport was detected on the surface of lettuce, strawberry, and carrot within 3, 1, and 1 days, respectively, when *C. elegans* was present in soil layered on top of manure inoculated with the pathogen and within 1, 7, and 1 days when the worm was present in soil layered on top of manure compost inoculated with *S. enterica* serotype Newport. With one exception (strawberries, 7 days, compost inoculated with *S. enterica* serotype Newport), the pathogen was not detected on the produce over the 10-day study when *C. elegans* was not present in the soil.

When produce was not placed on top of the soil, *S. enterica* serotype Newport was detected in the top 1 cm layer of only 2 of 80 samples analyzed over the 10-day incubation period. One soil sample tested positive for the pathogen after 7 days and 1 sample was positive after 10 days. *C. elegans* fed *E. coli* O157:H7 prior to being added to turkey compost amended and unamended soils has been reported to contaminate the soils with detectable amounts of the pathogen within 4 days (3).

Results indicate that *C. elegans* transports *S. enterica* serotype Newport initially in manure and compost for distances of 5 cm to produce in contact with the surface of soil. Attraction of *C. elegans* to strawberries appears to be stronger than attraction to lettuce or carrot, which is in agreement with observations in attraction experiments using agar. Preharvest fruits and vegetable that attract *C. elegans* would theoretically have a higher probability of becoming contaminated with salmonellae and perhaps other enteric pathogens that may be present in soil than produce having lower attractant properties. Whether free-living, bacterivorous nematodes act as vectors of enteric pathogens in field settings is not known, but observations do indicate

TABLE 4.1. Presence of *Salmonella enterica* serotype Newport in soil and on lettuce, strawberry, or carrot in contact with the surface of soil.

Material inoculated with *S. enterica* serotype Newport	Presence of *C. elegans* in soil	Number of samples positive for *S. enterica* serotype Newport																			
		Lettuce[a]					Strawberry[a]					Carrot[a]					No Produce[b]				
		1	3	5	7	10	1	3	5	7	10	1	3	5	7	10	1	3	5	7	10
Bovine manure	–	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0	0	0	0	0
	+	0/0	2/1	0/0	0/0	0/0	1/1	0/0	2/0	0/1	2/2	0/1	0/0	0/0	0/1	1/1	0	0	0	1	0
Bovine manure compost	–	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/1	0/0	0/0	0/0	0/0	0/0	0/0	0	0	0	0	0
	+	1/1	0/0	0/0	0/0	0/0	0/0	1/0	1/0	1/1	2/2	0/1	1/0	1/1	1/1	1/1	0	0	0	0	1

[a]Number of samples positive for *S. enterica* serotype Newport out four samples of soil and out of four samples of lettuce, strawberry, and carrot analyzed (2 g of soil/piece of produce) after incubating for 1, 3, 5, 7, and 10 days at 21°C.

[b]Number of samples of soil from top 1-cm layer positive for *S. enterica* serotype Newport out of four samples analyzed.

that *C. elegans* can serve as a vector of *Salmonella* to preharvest produce and reinforces the importance of sanitizing produce prior to consumption.

Acknowledgment

This study was supported in part by the USDA-ARS National Alliance for Food Safety and Security. The *C. elegans* strain used in this work was provided by the Caenorhabditis Genetic Center, which is funded by the NIH National Center for Research Resources.

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CHAPTER 5

SUMMARY AND CONCLUSION

Studies were undertaken to determine the role of *Caenorhabditis elegans* in contaminating preharvest fruits and vegetables with foodborne pathogenic bacteria. Specific objectives of these studies were:

1. To determine the persistence of *Escherichia coli* O157:H7, *Salmonella* Poona, and *Salmonella* Newport in the gut of *C. elegans* as affected by temperature and relative humidity, and to determine if infected worms transmit *S.* Newport to progeny and uninfected worms.
2. To evaluate the efficacy of cleaners and sanitizers in killing *S.* Newport in the gut of *C. elegans*.
3. To determine if *C. elegans* migrates to and survives and reproduces in manure, manure composts, and manure-amended soil.
4. To determine if *C. elegans* can transport *S.* Newport from manure or manure compost through soil and deposit the pathogen on the surface of fruits and vegetables.

Results reveal that *C. elegans* will ingest *E. coli* O157:H7, *S.* Poona, and *S.* Newport. These pathogens can persist in the gut of the worm for several days, depending upon temperature and relative humidity. Infected worms may transmit pathogens to uninfected worms by a rupture in the cuticle, excretion of the pathogen into the environment, or by some other means. If conditions are suitable, excreted cells of the pathogen may replicate and be ingested by previously uninfected worms, thus repeating the cycle of infection.

The outer cuticle of nematodes may provide a physical barrier between ingested bacteria in the gut of the worm and chemical sanitizers. The effectiveness of cleaners and sanitizers in removing *S.* Newport from the gut of *C. elegans* is influenced by

temperature and relative humidity at which the worm was held at prior to treatment. Significantly more cells of *S.* Newport survived storage at 20°C and 98% relative humidity compared to 33% or 98% relative humidity at 4°C or 33% relative humidity at 20°C. Cleaners and sanitizers were generally most effective in reducing the numbers of ingested *S.* Newport in desiccated worms. Desiccated worms may have ruptures in their cuticles that provide portals of entry of antimicrobial constituents when exposed to cleaners and sanitizers. This would result in increased contact of ingested bacteria with antimicrobial agents.

C. elegans will migrate towards bovine manure, turkey manure, bovine manure compost, turkey manure compost, and manure-amended soil inoculated with *S.* Newport. In addition, the worm will migrate to lettuce, strawberries, and carrots. Of these products, the worms migrated to most rapidly to turkey manure and strawberries. The rate of reproduction of *C. elegans* was highest in manures. In general, the worm did not survive or reproduce well when placed in manure compost. This may have been due to the low moisture content.

In soil inoculated with *C. elegans, S.* Newport initially in bovine manure or bovine manure compost ca. 5 cm away from lettuce, strawberry, or carrot was detected on the surface of these produce within 1 day. The pathogen was rarely detected on the surface of produce samples when *C. elegans* was not present in the soil. Fruits such as strawberries, which are attractive to *C. elegans*, may have a higher probability of coming in contact with *C. elegans* compared to produce that are less attractive to the worm.

In the field, the incidence of *C. elegans* contaminating preharvest fruits and vegetables is probably low. However, the possibility that an infected worm can

contaminate preharvest produce does exist. This underscores the importance of sanitizing produce prior to consumption.