POTENTIAL ROLE OF DIPLOSCAPTE*R*, A FREE-LIVING NEMATODE, AS A VECTOR OF PATHOGENIC BACTERIA TO PRE-HARVEST FRUITS AND VEGETABLES IN SOIL

by

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(Under the Direction of PHILLIP L. WILLIAMS)

ABSTRACT

Diploscapter, a free-living soil nematode commonly found in compost, sewage and agricultural soil within the United States, was studied to determine its role as a vehicle of pathogenic bacteria to pre-harvest produce. *Diploscapter*'s ability to survive in the presence of pathogenic bacteria on agar media, in soil, compost and cow manure was investigated. Worms survived and reproduced in environments containing *Salmonella enterica* serotype Poona, enterohemorrhagic *Escherichia coli* O157:H7, and *Listeria monocytogenes*. Attraction of *Diploscapter* to pathogenic bacteria was studied at 10, 20, 30, and 60 minutes and 24 h to assess colonization of bacterial colonies. *Diploscapter*'s potential to disperse pathogenic bacteria after exposure to agar media and soil environments inoculated with pathogenic bacteria, including compost and manure was investigated. Results exhibit the potential for *Diploscapter* to disperse pathogenic bacteria after exposure in different environments.

INDEX WORDS: *Diploscapter*, *Escherichia coli* 0157:H7, *Salmonella* Poona *Listeria monocytogenes*, fruits and vegetables, soil, free-living nematodes

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DEDICATION

To my mother, Wanda Mae Davidson Gibbs
1954 - 1988

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TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION

Within the past decade, human bacterial infections associated with the consumption of fresh produce have occurred in numbers large enough to prompt scientists to investigate potential sources of produce contamination (41). Healthier living is of rising concern among many individuals in the United States. Association between minimally processed fruits and vegetables and improved health, has encouraged many consumers to increase their daily consumption of fresh produce. Not only are fruit and vegetable intake believed to prevent certain cancers, but also daily exercise that includes cardiovascular activity has been linked to decreasing the risk of developing heart disease (33). With increased intake of produce, the risk of developing bacterial infection strappingly coincides (14). Improper practices involving harvesting, agronomic practices, processing, distribution, and ultimately consumption have played significant roles in contributing to increased human bacterial infections (11).

Anthropogenic processes involved with pre-harvest produce contamination are of concern related to the soil (9) and attention has turned towards bacterial-feeding nematodes (16). Nematodes are believed to be the most bountiful soil meso-fauna, having free-living, bacterial-feeding species that may serve as vectors for spreading bacteria capable of causing human disease. In soil, free-living nematodes are attracted to areas of high biological activity (top 5 cm of soils), including areas to which animal manure has been applied and presumably ingest bacteria as a nutrient source (16).

Previous research involving human pathogens has centered on *Ceanorhabditis elegans,* but due to *Diploscapter* sp. being more common to agricultural soils in the U.S., attention has turned to the latter species (16). Reports on numerous species of the genus *Diploscapter* have shown this particular nematode more common in an array of agricultural habitats (47). Little is known about *Diploscapter's* behavior in the soil environment and its ability to feed on various

bacteria, which has prompted further investigation. Agricultural practices involving the application of compost, manure, and poorly treated irrigation water are common playgrounds for *Diploscapter* (3,20). The reproductive cycle and feeding behavior of free-living nematodes are believed to potentially be important factors in the distribution of pathogenic bacteria in pre-harvest soil environments (16). Possibilities as such and the lack of documented research concerning *Diploscapter* imply that it is imperative to gain insight on its behavior and role in agricultural soil as it pertains to pre-harvest contamination of fruits and vegetables with pathogenic bacteria harmful to human health.

This thesis is an initial investigation of the potential role of *Diploscapter* sp. as a vehicle for vectoring pathogenic bacteria to fresh fruits and vegetables. Chapter 2 provides an overview of the literature related to this topic and Chapter 3 presents the research approach and findings. *Diploscapter*'s survival / reproduction and attraction to three types of pathogenic bacteria (*Salmonella enterica* serotype Poona, enterohemorrhagic *Escherichia coli* O157:H7, and *Listeria monocytogenes*) on agar was assessed. Additional work evaluating survival was performed using soil, cow manure, and turkey compost (or various combinations). Studies also were performed on the organism's ability to disperse the pathogens.

The final section of this thesis, Chapter 4, provides an overview of the conclusions derived from this study.

CHAPTER 2

LITERTURE REVIEW

Foodborne illness is an increasing concern among consumers in the United States. Potential factors associated with these illnesses are being studied with hopes of better understanding possible sources of contamination. This study addresses the possible interactions between three strains of pathogenic bacteria and one minimally studied bacterial feeding nematode, *Diploscapter*.

Foodborne Outbreaks

Pathogenic microorganisms are part of a broad group associated with human bacterial infections resulting from food consumption (4). In recent years, fresh produce has had an astounding increased demand among consumers in the United States (14). It is believed that numerous constituents of fruits and vegetables may reduce the risk of several human health ailments such as: coronary heart disease (33), breast cancer (55), lung cancer (26), colon and rectal cancer (43, 53), male bladder cancer (42), urothelial cancer (58), and increased immune response to Non-Hodgkin's Lymphoma among women (59).

The U.S. has access to one of the safest supplies of fresh produce internationally. Unfortunately, the past decade has brought with it numerous outbreaks of human bacterial infections having ties to fruits and vegetables (52). Publicized foodborne illnesses have been linked to cantaloupe, tomatoes, lettuce, celery, cucumber, mushrooms, watermelon, carrots, strawberries, alfalfa sprouts, and orange and apple juice (14). From 1993 – 1997, 2751 outbreaks of foodborne disease were reported. Among the outbreaks, at least 75%, which were of known etiology, was determined to be of pathogenic bacterial origin (19).

Although potential contributing factors have yet to be determined in virtually all cases, a number of notable causes have been suggested for these foodborne diseases. They include: contamination with domestic and wild animal fecal matter, contaminated water, the use of

untreated manure or sewage, lack of field sanitation, poorly sanitized transportation vehicles, and handler contamination. Nearly every step from production to consumption impacts the microbiological safety of fresh produce (9). Pre-planting events can affect bacteriological quality and safety of final products. These events include: choice of growing location, the history of the land, which is a factor often ignored, land that has had livestock grazing, and flooding history. Floodwaters have the potential to carry animal waste and contaminants downstream, where they flood over croplands (11). Deposited microorganisms on flooded croplands have been reported persistent for months or years after flooding events (11). Enteric pathogens are more likely to contaminate croplands that have been exposed directly to animal waste (51). *Salmonella* and *Listeria monocytogenes* are reported to survive for months in sewage sludge that can be applied to agricultural soils (56). Farmers are encouraged to take note of past exposure to livestock manure pre-choosing cropland (11).

One reason of particular importance when attempting to determine causes of human bacterial infection is increased salad bar usage and meals eaten outside the home. From 1988-1996, yearly consumption of fresh fruits and vegetables increased by almost 20 pounds per person in the U.S. (14) and between 1973 and 1992 the Center for Disease Control reported double the number of produce related outbreaks. During the summer of 1996, an outbreak of more than 6000 cases of *E. coli* O157:H7 sickened 4000 young children, climaxing with 4 deaths. A foodborne outbreak caused by *E. coli* O157:H7 contaminated sprouts occurred in the U.S. between 1995-1998, which reported more than 1200 cases. These events and others like it have prompted the U.S. Food and Drug Administration (FDA) to investigate these matters. (14)

Nearly every type of fruit and vegetable has the potential to become contaminated with bacterial pathogens, but the simple presence of a pathogenic bacterium does not mean illness will

always result. Even though a variety of products have had pathogenic bacteria isolated from them, very few have been confirmed as a vehicle of foodborne illness (11). Even though fresh produce may harbor microorganisms such as pseudomonad's or *Listeria monocytogenes*, proper refrigeration temperatures limits growth of most pathogenic microorganisms. Usually modified atmospheres are efficient in maintaining quality of fresh produce, but the effects on microorganisms are inconsistent. To an extent, chemical disinfectants reduce initial bacterial contamination, but irradiation seems to be more efficient (45). Produce having pH range of 3.9 to 4.5, are reported to retard growth of enteric pathogens such as *E. coli* O157:H7. Adjustment to stress environments can result in a pathogen becoming better suited for survival and growth and can become more virulent. *E. coli* O157:H7 (22) and *Salmonella* are known to adapt to reduced pH and exhibit increased tolerance to stressful environments (38). The pH of several vegetables, melons, and soft fruit skin or flesh is at least 4.6 or greater. Thus, being suitable for pathogenic bacteria growth. Post-harvest growth of fungi in the subsurface can alter the pH of plant tissues, which permits pathogenic bacterial growth (14). All produce is believed to potentially be a place of protection for pathogens (7). In terms of the recent outbreaks of human bacteria infection *Salmonella, E.coli* O157:H7, and *L. monocytogenes* (7) are among a relative few of significant importance in fresh produce (11).

***Escherichia coli* O157:H7**

Escherichia coli bacteria were first discovered in 1885 by a German bacteriologist named Theodor Escherich and were linked to infant gastroenteritis and diarrhea. The name of the discovered bacteria was changed from *Bacterium coli* to *Escherichia coli* in honor of Dr. Escherich's historic public health discovery. *Escherichia coli* is a gram-negative rod shaped bacteria found in the gastrointestinal tract of all warm-blooded animals and is usually considered

harmless. Nevertheless, several strains are capable of causing gastroenteritis (40). *E. coli* O157:H7 was first identified in 1982 as a cause of hemorrhagic colitis, and has been recognized as an important agent of food-borne disease (30). It is now considered an important cause of bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) in humans.

Three surface antigens differentiate isolates of *E. coli*. They are, the H (flagella), O (somatic), and K (capsule) antigens (23). Those groups of *E. coli* that cause diarrhea illness are categorized based on virulence properties, pathogenic mechanisms, quantifiable syndromes, and specific O:H serogroups. *E. coli* O157:H7 is included in the enterohemorrhagic (EHEC) group, which produces Vero toxin 1 and 2 (VT1 and VT2) that damage intestinal lining, resulting in hemorrhagic colitis or bloody diarrhea (13,24).

Foods associated with *E. coli* O157:H7 infection have revealed a low infectious dose, which in some cases has been reported to be a little over a hundred cells. (24) Symptoms are usually sudden and result in severe cramps coinciding with diarrhea becoming relentlessly bloody between 24 – 48 hours after ingestion of contaminated products. Illness usually last 2 – 9 days, and is accompanied by little to no fever. (23) Hemolytic Uremic Syndrome (HUS) usually develops around 7 days after an acute incident of diarrhea. It is a leading cause of renal failure in children (23), and has a mortality rate of 3 – 5 percent. (13,24). Hemolytic Uremic Syndrome cases often require kidney dialysis and blood transfusions. In some cases, the central nervous system is affected and can lead to coma.

Although other means of transmission have been documented, the most common source of outbreaks of *E. coli* O157:H7 infection has been ground beef (31,51). Dairy cattle have been identified as a principal reservoir of *E. coli* O157:H7 (54). Studies show *E. coli* O157:H7 can survive in feces for extended periods of time and retain its ability to produce Vero toxins.

Therefore, bovine feces are potential vehicles for transmitting *E. coli* O157:H7 to cattle, food, and the environment. (54) In nonaerated manure piles, *E. coli* O157:H7 has been shown to survive for more than one year (36). The largest reported outbreak of *E. coli* O157:H7 infection in North America was at a "Jack in the Box" in Washington State in 1993 from undercooked ground beef (19).

Other foods associated with outbreaks of *E coli* O157:H7 infection include salad bar items and salami (31,51). Like most non-spore forming bacteria, *E. coli* O157:H7 is not heat resistant and proper cooking practices should decrease the risk of consuming contaminated products. However, fruits and vegetables are often consumed uncooked. Studies have shown *E. coli O157:H7* populations to increase on shredded lettuce, sliced cucumber, and shredded carrot stored at 21 degrees C, but an unknown factor associated with carrots was noted to possibly inhibit the growth of *E. coli* O157:H7. An eventual decrease in *E. coli* O157:H7 populations in food samples stored at 21 degrees C was attributed to the toxic effect of accumulated acids. (1)

Salmonella

Salmonella is a group of bacteria that cause diarrhea illness in humans. For over a century, *Salmonella* has been reported to cause numerous human related bacterial infections (Salmonellosis) (19). *Salmonella* is a very large group of rod-shaped gram-negative bacteria consisting of more than 2000 serotypes (41). Primary reservoirs for *Salmonella* are mammals, but poultry are more common. (21) Over 800,000 cases of *Salmonella* and 5000 deaths are reported each year, which reportedly has prevalence in children, immunocompromised, and the elderly.

Salmonellosis is the second leading cause of foodborne illness in the U.S. (41). Being that *Salmonella*'s route of transmission is fecal-oral, food or water contaminated with feces has

the potential to spread the organism to new host (41). Symptoms include diarrhea, fever, and abdominal cramps that develop 12 to 72 hours after infection. Illness usually last 4 to 7 days and recovery usually doesn't require treatment. Contaminated foods are normally of animal origin, such as poultry, milk, and eggs (19), but vegetables are becoming more of a potential threat as a result of handling, processing, and pre-harvest factors.

Temperature, pH, water activity, and nutrient availability affect the growth of *Salmonella*. Food type, pH conditions, and acidity affect growth of *Salmonella* in refrigerated conditions. Studies show *Salmonella* to survive at low pH conditions in the presence of citric acid (5). Studies also show *Salmonella* to survive in manure and on vegetable surfaces stored in ambient temperatures (60). One author indicated *Salmonella* is capable of rapid and abundant growth on watermelon, cantaloupe flesh, and honeydew incubated at 23 degrees C (29). Persistence of *Salmonella* in refrigerated foods has been attributed to acidity (21).

Both conventional and organic farmers commonly apply bovine manure as a fertilizer to cropland. Produce grown organically represented 2% of retail produce in the U.S. in 2000. (44) In a 401-sample survey of raw fruits and vegetables collected in retail markets, a reported 66% affected by bacterial soft rot were positive for *Salmonella* colonies. Among those fruits and vegetables tested were cantaloupe and tomatoes. Reported bacterial counts were 10-fold higher when produce such as carrots, potatoes, and peppers were exposed to *Salmonella* enterica serotype *Typhimurium* compared to just *Salmonella* sp. (7)

Listeria monocytogenes

Listeria monocytogenes is a gram-positive bacterium motile by means of flagella. Studies suggest 1-10% of humans could be intestinal carriers of *L. monocytogenes*. At least 37 mammalian species, including domestic and feral, several species of birds, and possibly some

species of fish and shellfish have been found to be carriers of *L. monocytogenes*. *Listeria monocytogenes* has been isolated from soil, silage, and other environmental sources and is believed to be quite resilient as well as resist lethality from freezing, drying, and heat amazingly well for a bacterium that does not form spores. (39). *Listeria monocytogenes* contamination has been largely associated with the consumption of dairy products, beef, pork, poultry, and seafood. On the other hand, an increasing amount of data support and suggest that salad vegetables, like lettuce, cucumber, onion, radish, and tomatoes have a high incidence of *L. monocytogenes* (39).

The group of disorders caused by *L. monocytogenes* is known as Listeriosis. The symptoms of listeriosis include meningitis, encephalitis, and cervical infections in pregnant women, which are believed to possibly result in miscarriages. They are usually preceded by influenza-like symptoms that include fever, nausea, vomiting, and diarrhea (39).

From October 1987 to August 1988, tests were conducted on 10 types of fresh produce from two Minneapolis, MN area supermarkets to isolate *Listeria*. *Listeria monocytogenes* was isolated from cabbage, mushrooms, potatoes, radishes, with potatoes and radishes having significant amounts of L. *monocytogenes* present. (32) *Listeria monocytogenes* is commonly found in the environment among decaying vegetation, soil, sewage, and feces of animals (7). Onset of listeriosis is unknown, but is believed to range from a few days to three weeks, which may include gastrointestinal symptoms in approximately 12 hours. The strain of *Listeria* and susceptibility of the victim are attributed contributors to listeriosis. Its ability to grow at temperatures as low as 3 degrees C permits multiplication in refrigerated foods. (39) Cases of listeriosis in humans are believed to be a result of consuming produce contaminated by manure ruminants (7).

Scientists suggest *Listeria monocytogenes* to be potentially the most prevalent disease-causing microorganism in soil (8). *Listeria* has shown saprophytic tendencies as a result of its frequent isolation in areas where decayed corn, soybean plants, and wild grasses are found (9). The reports of *Listeria* in sewage, in many instances, have demonstrated higher counts than other bacteria like *Salmonella*. Application of sludge containing *Listeria* and *Salmonella* to soil shows *Listeria* to survive longer, having duration of 7 weeks (9). As compared to *E. coli* O157:H7, *L. mononcytogenes* acid tolerance at 37 degrees C was a much lower tolerance to low pH, which is believed to imply a higher infective dose.

Soil and Nematodes

Soil is a dynamic, naturally occurring body possessing properties due to the collective effects of climate and biotic activity on parent material modified by time and topography. There are four major components of soil: mineral matter (sand, silt, & clay) organic matter, water, and air. Each of these components plays a vital role in soil biotic activity (12). Isolation of pathogenic bacteria from fruits and vegetables grown at or near soil surfaces has cited soil as a source of contamination of pre-harvest crops (28). The presence of enteric pathogens in soil has the potential to contaminate the surfaces of resident crops and can be introduced into the produce after processing, especially involving cutting (49).

Nematodes are roundworms that occur worldwide in almost every soil environment. *Caenorhabditis elegans* is a free-living, microbivorous nematode common to soil environments globally. It feeds on bacteria and has a life span of 2-3 weeks, given optimal conditions. *C. elegans* consist of an outer tube having a cuticle, hypodermis, neurons, and muscles surrounding a pseudocloelmic space that contains the intestine and gonad. (57) *C. elegans* are filter feeders, meaning they take in liquid with suspended particles containing bacteria and spitting out the

liquid while retaining the particles (6). They have limited temperature range (12-26 degrees C) in which they remain viable and fertile. *C. elegans* have a rapid 3-day life cycle and are 1-1.5mm in length (47). *C. elegans* possess several advantages as a test model, which includes rapid growth and its genetic molecular tools being well defined (35). The adult male has 1031 somatic nuclei, where as the adult hermaphrodite has only 959 (47).

Recently, research has centered on *C. elegans* as a test model for pathogenic bacteria contamination in soil to pre-harvest crops (16). Previous studies also show *C. elegans* to be attracted to and survive on several strains of pathogenic bacteria (2). Past studies involved the exposure of *C. elegans* to pathogenic bacteria and cantaloupe juice and determining their attraction to one or the other. The study concluded a preferred attraction of the nematode to *Salmonella* Poona rather than cantaloupe juice. Further studies demonstrated *C. elegans* to serve as a safeguard, protecting ingested pathogenic bacteria against sanitizers. (17) The relevance of this study and similar studies is to see what factors besides anthropogenic sources are contributing to contamination of fruits and vegetables.

Due to *C. elegans* not being commonly found in agricultural soils, attention has turned to other nematode species. (15) *Diploscapter* is a small soil dwelling, bacterial feeding nematode common in compost and sewage (3). Several species have been found around plant roots, and unusual places such as: the pharyngeal glands of the Argentine ant, thermal waters, root galls, and occasionally the human urinary tract (20). Reports on numerous species of the genus *Diploscapter* have shown this particular nematode is commonly found in an array of agricultural habitats (48).

From the order Rhabditida, *Diploscapter* is distinctly different from its sister genus *C. elegans* (10). Species of *Diploscapter* are approximately one third the size of *C. elegans* and

consist of females with 0.27 - 0.35mm long bodies, clearly striated cuticles, anterior labial hooks (3μm), stoma 17.5-20 x 2.0-2.2 μm, poorly muscular corpus, excretory pore opposite anterior half of oesophangeal bulb, vulva at 56 - 58 % of body length, and tail regularly tapering to a filiform tip and measuring 4 - 5 anal body widths long (48). Reported *Diploscapter* species reproduce parthenogenetically, which is the development of an unfertilized egg (50). They have a 4-6 day life cycle and 14-day life span. Although *Diploscapter* does not survive well on biocontrol isolates of *Bacillus cepacia*, as do other bacterial-feeding nematodes (18), almost nothing is known about its ability to feed on various bacteria.

Compared to *C. elegans*, *Diploscapter* have a strikingly higher thermal tolerance and have been found in composted manure (37) , but nearly nothing is known about ability to survive in compost. *Diploscapter* has been successfully cultured on non-pathogenic *E.coli* OP50 at 30°C.

Research performed in this thesis is centered on three strains of pathogenic bacteria and one species of nematode, *Diploscpater*. Of the several bacterial feeding species of nematodes, this one was selected due to its ubiquity in agriculture soils of the United States. Studies with *Diploscapter* assessed their survival and reproduction in the presence of pathogenic bacteria, both on agar and in compost. Research was also performed to determine if *Diploscapter* was attracted to pathogenic bacteria, and to determine if they disperse the pathogenic bacteria in a manner that increases the potential of pre-harvest produce contamination.

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CHAPTER 3

POTENTIAL ROLE OF *DIPLOSCPATER*, A FREE-LIVING BACTERIVOROUS NEMATODE, AS A VECTOR OF FOODBORNE PATHOGENIC BACTERIA TO PRE-HARVEST FRUITS AND VEGETABLES [1]

[1] Potential Role of *Diploscapter*, A Free-living Bacterivorous Nematode, As A Vector of Foodborne Pathogenic Bacteria to Pre-harvest Fruits and Vegetables, Daunte' S. Gibbs, Gary L. Anderson, Larry R. Beuchat, Lynn K. Carta, and Phillip L. Williams. To be submitted to *Applied Environmental Microbiology*.

ABSTRACT

Diploscapter, a free-living soil nematode commonly found in compost, sewage and agricultural soil in the United States, was studied to determine its potential role as a vehicle of *Salmonella enterica* serotype Poona, enterohemorrhagic *Escherichia coli* O157:H7, and *Listeria monocytogenes* in contaminating pre-harvest fruits and vegetables. The ability of *Diploscapter* to survive on agar media, in cow manure, in composted turkey manure, and to be attracted to and ingest foodborne pathogens inoculated into soil or a mixture of soil and composted turkey manure was investigated. Worms survived and reproduced in lawns of *S. enterica* serotype Poona, *E. coli* O157:H7, and *L. monocytogenes* on agar media, or in cow manure and composted turkey manure. Attraction of *Diploscapter* to colonies of pathogenic bacteria on tryptic soy agar within 10, 20, 30, and 60 min and 24 h was determined. At least 60% of the worms initially placed 0.5 – 1 cm away from bacterial colonies migrated to the colonies within 1 h. Within 24 h, ≥ 90% of the worms were embedded in colonies. The potential of *Diploscapter* to disperse pathogenic bacteria after exposure of to bacteria inoculated into soil or a mixture of soil and composted turkey manure was investigated. Results indicate that *Diploscapter* can disperse pathogenic bacteria after exposure to pathogens in these milieus. These studies demonstrate the potential of *Diploscapter* to serve as a vector of foodborne pathogenic bacteria in soil, with or without amendment with compost, to the surface of pre-harvest fruits and vegetables in contact with soil.

INTRODUCTION

Associations between minimally processed fruits and vegetables and improved health, including the prevention of certain cancers, have stimulated an increase in per capita consumption of fresh produce (8). In the U.S., outbreaks of human bacterial infections associated with consumption of produce have also increased in the past decade (16). These events have prompted scientists to identify factors that may contribute to contamination of pre-harvest and post-harvest fruits and vegetables (4). Agronomic practices that may facilitate to contamination of pre-harvest produce are of concern and microbiota in soil microenvironments have been studied to determine their potential roles (9). A lack of appropriate hygienic practices in growing, harvesting, processing, distributing, and preparing fruits and vegetables eaten raw can have a significant impact in contributing to increased risk of human microbial infections (5).

Numerous types of microorganisms inhibit the soil environment, but meso-fauna of particular interest with regard to their role as potential vectors of foodborne pathogens are free-living nematodes that feed on bacteria. Although these nematodes are universal in agricultural soils, very little is known about their potential role as vectors of human pathogens that may be present as a result of application of manure, improperly treated irrigation water, or runoff water from nearby livestock operations. Free-living, bacterivorous nematodes are attracted to areas of high organic activity in soil, largely in the top 5 cm of soils to which animal manure is applied, where they ingest bacteria as a nutrient source (9).

Studies have shown that *Caenorhabtitis elegans* is attracted to and ingests foodborne pathogens (1, 9), but a more commonly found free-living nematode in agricultural soils is *Diploscapter* sp. Several species of the genus *Diploscapter* are reported to be present in a range of agricultural habitats (19). In comparison to *C. elegans*, *Diploscapter* has a markedly higher

thermal tolerance (15). Agricultural practices involving the use of compost, manure, and poorly treated irrigation water result in increased numbers of nematodes (2, 10), but the behavior of *Diploscapter* in soil environments and its ability to feed on foodborne pathogens have not been described. The feeding behavior and reproductive cycles of *Diploscapter* and other free-living nematodes render them as potential vectors for dispersing pathogenic bacteria in agricultural soil environments.

The primary objectives of this study were: 1) to determine survival and reproduction characteristics of *Diploscapter* fed on foodborne pathogenic bacteria; 2) to determine if *Diploscapter* is attracted to pathogenic bacteria; and 3) to determine if pathogenic bacteria ingested by *Diploscapter* and adhere to worm in soil are subsequently dispersed. Information gained in this study will be of value in assessing the potential role of *Diploscapter* as a vector of pathogenic bacteria to pre-harvest fruits and vegetables.

MATERIALS AND METHODS

Procedure for culturing *Diploscapter*. A *Diploscapter* sp. obtained from the nematode stock collection at the Nematology Laboratory, USDA-ARS, Beltsville, Md. was used in all experiments. The worms were maintained on Nematode Growth Medium (NGM) agar on which a lawn of *Escherichia coli* strain OP50 had formed by incubating surface inoculated plates for 24 h at (37°C) (7). *Diploscapter* used in experiments was harvested from plates incubated at 30°C for 7 days.

Bacterial strains. Three foodborne pathogenic bacteria were used: *Salmonella enterica* serotype Poona (strain 01A4754, from patient with salmonellosis associated with consuming cantaloupe), enterohemorrhagic *Escherichia coli* O157:H7 (strain SEA-13B88, from patient infection associated with consuming apple cider), and *Listeria monocytogenes* (strain G1091,

from coleslaw). Non-pathogenic *E. coli* OP50 was used as a control. The three pathogens and *E. coli* OP50 were grown in tryptic soy broth (TSB, pH 7.3) (Difco / BBL, Sparks, Md.) supplemented with nalidixic acid (50 µg/ml) (TSBN) at 21°C, with transfers using loop inoculum (ca. 10 µl) at 24-h intervals. Cells from these cultures were used in all experiments. Stock cultures were stored at 4°C.

Copper lethality test. The sensitivity of *Diploscapter* to copper was determined. *Diploscapter* grown on NGM agar for 7 days at 30°C collected in 5 ml of sterile K medium (pH 4.8), which contains, per liter of deionized water: 2.36 g of potassium chloride and 3.0 g of sodium chloride (21). Worms (ca. 100 – 200) were deposited in a sterile 5-ml glass tube and placed into a 15-ml centrifuge tube. The suspension was centrifuged (500 x *g*, 2 min, 21°C), the supernatant was removed, and 1 ml of fresh K medium was added to the small tube. Worms were resuspended using a sterile glass pipette. These steps were repeated twice more and the supernatant was removed; fresh K medium was added, and worms were deposited in the center of a small (60 mm diameter x 15 mm deep) petri plate. A sufficient amount of K medium was deposited on the worms to facilitate separation and counting. This technique was developed to enable precision counting of *Diploscapter*, which is approximately one-third the length of *C. elegans*.

A stock solution containing $CuCl_2$ at 1200 µg/ml of deionized water was diluted to give solutions containing 0.5, 1.0, 1.5, 2, 3.5, and 4.0 µg/ml. *Diploscapter* (10 – 15 worms) were immersed in 1 ml of solution at each concentration for 24 h at 30°C. Viability was determined by probing each worm with a platinum wire while viewing through a dissecting microscope (60x magnification). Worms were judged to be alive if moving or dead if no movement was

observed. LC_{50} values for copper were generated by Probit from log-transformed data in Toxstat® 3.4 after passing normality (20).

Survival and growth of *Diploscapter* on agar media inoculated with bacteria. Aliquots (0.1 ml) of 24-h cultures of *S.* Poona, *E. coli* O157:H7, *L. monocytogenes*, and *E. coli* OP50 grown in TSBN were surface spread on NGM agar and tryptic soy agar (TSA, pH 7.3) (BBL/Difco) supplemented with nalidixic acid (50 μg/ml) (TSAN) and incubated at 21°C for 24 h, allowing bacterial lawns to develop. In the center of each 24-h bacterial lawn, 10 μl of K medium containing 40-50 *Diploscapter* was deposited. Worms were incubated at 30°C and examined for viability at 3 and 10 days.

Survival of *Diploscapter* in cow manure and composted turkey manure. Fresh cow manure was obtained from the College of Veterinary Medicine, University of Georgia, Athens, GA. Samples (3 g) were placed in petri plates (35 mm diameter x 10 mm deep) along with 5 μl of K medium containing 20 – 25 *Diploscapter* and incubated at 30°C for 24 or 48 h. Worms were recovered from manure using a centrifugation / flotation technique in a colloidal silica suspension (Ludox extraction) and examined for viability using the ASTM method (3) approved for *C. elegans*. Composted turkey manure was obtained from Dr. P. Millner at the United States Department of Agriculture, Agricultural Research Service, Beltsville, Md. Samples (3 g) were placed in small (35 mm diameter x 10 mm deep) petri dishes and inoculated with 20 –25 worms. Worms were separated from the compost after incubating the mixture for 24 or 48 h at 30°C using the Ludox extraction method (3) and analyzed for viability. In another series of experiments, composted turkey manure was amended into soil at a ratio of 2.33 g of soil to 1 g of compost, inoculated with 20 – 25 *Diploscapter*, incubated for 24 and 48 h at 30°C, and analyzed for numbers of viable worms. Tifton soil (94% sand, 4% silt, and 2% clay), obtained from the

University of Georgia Soil Testing Laboratory, was used. All experiments were repeated at least three times.

Attraction assays. Two 10-µl aliquots of TSBN culture containing *E. coli* O157:H7, *S.* Poona*, L. monocytogenes*, or *E. coli* OP50 were deposited 1 cm apart on the surface of TSAN in petri dishes (60 mm diameter x 15 mm deep), allowed to dry 10 - 15 min, and incubated at 21°C for 24 h. A suspension (5 µl) of *Diploscapter* (25 - 50 worms) in K medium was placed between the sites of inoculation. The location of the worms on the surface of TSAN incubated at 21°C for 10, 20, 30, and 60 min and 24 h was monitored using a computer-captured image technique (1).

Attraction assays using three test pathogens inoculated on the same TSAN plate were also conducted. Suspensions (10 µl) of 24-h TSBN cultures were deposited on the surface of TSAN at locations 2 cm apart. *Diploscapter* (25 – 50 worms in 5 µl of K medium) was deposited at a point on the plate equidistant (1.5 cm) from the three inoculated sites and monitored for location up to 24 h as described above.

Dispersal of pathogenic bacteria. *Diploscapter* (20 – 25 worms / 2.33 g of soil) that were washed by suspending worms in K medium and bubbling (x 10), allowed to settle into a pellet, supernatant was suctioned off, worms were re-suspended and steps were repeated twice more. Pathogenic bacteria (5.8 – 6.2 $\log_{10}$ CFU/ 2.33g of soil) was then inoculated into soil amended with composted turkey manure (1 g of compost per 2.33 g of soil), as well as unamended soil, and incubated at 30°C for 24 or 48 h. Worms were extracted as described above. Using a platinum wire, two worms were placed on the surface of TSA plates with and without nalidixic acid (50 µg/ml) for 2 h. Worms were removed and the plates were incubated at 37°C for 24 h to allow for bacterial colony formation. The number of colony-forming units of

each bacterium shed by the two worms was determined by counting the number of colonies formed on TSAN.

Bacterial vectoring from soil to carrot. Raw carrots (*Daucus carota* L) were obtained from a local market. Two types of samples were prepared. The first type consisted of pieces (1 cm x 2 cm x 1 cm deep) cut from the surface of carrots and the second type consisted of disks (ca. 1.5 cm diameter x 1 cm thick) prepared by cutting the carrot transversely. The first series of experiments was done using the first type of sample. A 24-h TSBN culture (1 ml) of test pathogen or *E. coli* OP50 was deposited in a 35 x 10 mm petri dish and agitated to cover the entire bottom surface. Tifton soil (autoclaved at 121°C fro 30 min) not inoculated or inoculated with *Diploscapter* (20 – 25 worms / 2.33 g of soil) was placed on top of the culture at a depth of 1 cm. K-medium (0.5 ml – 1.0 ml) was pipetted onto the soil for the purpose of maintaining a moist environment. Plates were sealed and incubated at 30º C for 24 h to enable equilibration. A piece of carrot was placed external surface down firmly on the surface of soil containing or not containing *Diploscapter*, incubated at 30º C for 24 h, and analyzed for population of test bacteria.

The second series of experiments involved placing a carrot disk within the surface of moistened soil (1 cm deep), then inoculating the surface with 1 ml of a 24-h TSBN culture 1-1.5 cm away from the disk. To minimize the capillary movement of water that may draw the bacteria to the carrot surface, water was added to soil samples prior to applying the carrot disk. Worms (25 - 50) were placed on top of the soil at a point 0.5 - 0.75 cm away from the disk and 0.5 – 0.75 cm away from the site of inoculation with *S.* Poona, *E. coli* O157:H7, or *L. monocytogenes*. After incubating for 3 days at 30°C, disks were removed and analyzed for populations of test pathogens.

Carrot pieces and disk were removed from the surface of soil using a sterile forceps, placed into 0.5 ml of sterile 0.1 % Bacto peptone solution, and vortexed for 2 – 3 min. The peptone wash solution was serially diluted and surface plated (0.1 ml) on TSAN. Plates were incubated at 37°C for 24 h before colonies were counted. Mean values were analyzed using MS Excel Data Analysis of Variance (ANOVA) statistical analysis. All experiments were replicated three times. Statistically significant differences in mean values were determined.

RESULTS AND DISCUSSION

Copper lethality test. Copper is naturally found in the environment and is required by most organisms as an essential element, given proper dose. For many years, Cu has been commonly used as a reference toxicant for environmental toxicological studies using a variety of organisms. Since there is no known sensitivity of *Diploscapter* to Cu concentrations, its sensitivity was investigated for comparison to published data from other nematode species. Copper is commonly used as a reference toxicant for *C. elegans*, (12) and it has also been used with *Pristionchus pacificus* and *Panagrellus redivivus*. For this reason, the LC_{50} of Cu to *Diploscapter* was determined in hopes to compare to data generated for other nematode species. An LC_{50} value of 0.81 mg Cu/l was determined for *Diploscapter*. Studies involving other free-living nematodes have reported a range of LC_{50} values: *P. redivivus* (160 mg Cu/l), *C. elegans* (85.4 mg Cu/l), and *P. pacificus* (18.9 mg Cu/l) (6). In comparison to these nematodes, *Diploscapter* is the most sensitive to copper. The reason for these differences is not known, but it is interesting to note that the most sensitive species, *Diploscapter* (0.27 - 0.35 mm in length) is smaller than the other species (*P. redivivus* at 0.9 – 1.8 mm in length, *P. pacificus* at 0.75 – 1.2 mm in length and *C. elegans* at 1.0 – 1.5 mm in length). Size, as well as morphological and physiological characteristics, may contribute to differences in sensitivity to copper. The wider-

range in worm age due to non-synchronized populations may have also contributed to the lower LC_{50} value (i.e., exposed population may include juveniles and may also account for the increased variation in the data.

Survival of *Diploscapter* as affected by medium and bacterium. *Diploscapter* survived and reproduced on NGM agar inoculated with pathogenic bacteria or *E. coli* OP50 for up to 10 days (Figure 1a). The increase in number of *Diploscapter* was unaffected by the type of test bacterium. Populations were too numerous to count, i.e. > 900 worms, at 10 days and remained at those populations for up to 30 days, regardless of the bacterium used as a nutrient source. Several life cycles occurred within this 30-day period. Given that *Diploscapter* has a 4 - 6 day life cycle and 14-day life span (19), compared to its behavior on NGM, *Diploscapter* did not increase in population or survive well on bacterial lawns grown on TSAN, a nutrient rich agar medium (Figure 1b). Previous studies using *C. elegans* indicated that worms grown on rich agar media live for significantly shorter times than those cultivated on NGM agar (11). Presumably, rich media and virulence factors associated with gram-positive bacteria are inhibitory or lethal to worms (13). Bacterial lawns on NGM were consistently less dense than on TSAN, allowing for better observation and visibility of *Diploscapter*. Another explanation for differences in growth and survival on the two media may be related to pH. Potassium phosphate is used to buffer NGM agar, which kept the medium at a pH of 6.0 - 6.3, even after bacterial lawns are established. The initial pH of TSAN was 7.3 and after bacterial lawns were established, the pH increased to above 8. *C. elegans* is tolerant to pH ranging from 3.2 – 11.8 for at least 96 h (14), but the sensitivity of *Diploscapter* to pH is unknown.

Survival in cow manure and composted turkey manure. Manure-amended soils are often used to grow vegetables and fruits intended to be eaten raw. Since application of manure

and compost is believed to stimulate microbial activity and affect nematode populations (4), the ability of *Diploscapter* to survive in cow manure and composted turkey manure was investigated. Changes in populations of *Diploscapter* inoculated into raw cow manure and composted turkey manure were determined 24 and 48 h after inoculation. More than 90% of the worms remained viable in cow manure after 48 h (Figure 2a). The optimum temperature for growth of *Diploscapter* is 30°C, compared to 20°C for *C. elegans*. The recognized tolerance of *Diploscapter* to elevated temperature (15) and its presence in compost (2) prompted an investigation to determine its ability to survive in composted turkey manure. *Diploscapter* was inoculated into composted turkey manure and held for up to 48 h at 30°C before analyzing for changes in the number of viable worms. More than 80% of the population survived (Figure 2a).

Survival of *Diploscapter* in soil amended with composted turkey manure and inoculated with pathogens or *E. coli* OP50 was examined. The presence of test bacteria did not markedly alter survival of *Diploscapter* in compost-amended soil, although higher survival of the worm was observed in amended soil containing *E. coli* OP50, compared to survival in the presence of pathogens (Figure 2b). The lowest percentage of *Diploscapter* that survived occurred in amended soil inoculated with *L. monocytogenes*.

Attraction of *Diploscapter* to pathogenic bacteria. Once the ability of *Diploscapter* to survive and grow using foodborne pathogens as nutrient sources was demonstrated, its attraction to the same pathogens was evaluated. Worms were attracted to *E. coli* O157:H7*, S.* Poona*,* and *L. monocytogenes* as well as to non-pathogenic *E. coli* OP50 (Figure 3). More than 50% of the worms initially 1 cm from the sites of inoculation migrated to all bacteria within 60 min. At 24 h, ≥ 95% of the worms migrated to colonies formed at sites of inoculation. Worms were more slowly attracted to pathogens than to *E. coli* OP50, which could be due to worms being

conditioned for this food source, and markedly less attracted to *L. monocytogenes* than to *E. coli* O157:H7 or *S.* Poona during the first 60 min of incubation. Studies have shown free-living nematodes such as *C. elegans* are attracted to bacteria over distances of several centimeters (9). Since most free-living nematode activity occurs within the top 5 cm of soil, attraction of *Diploscapter* to pathogenic bacteria (at a distance > 1 cm) that may enter the soil from sources such as manure, runoff water, or irrigation water would seem plausible. We attempted to assess attraction over greater lengths by placing the worms at a distance of approx. 5 cm from each strain of bacteria over 60 min followed by a 24 h observation. With these studies only 1 to 3 worms (out of 20 – 25) were found in the seeded bacteria with the majority remaining closer to the initial area of placement. Compared to *C. elegans*, which is approximately 3 times greater in length, *Diploscapter* was found to be more restricted in movement.

Dispersal of pathogenic bacteria. Dispersal of bacteria that were in and on *Diploscapter* that had fed on foodborne pathogens or *E. coli* OP50 for 24 h in soil amended with composted turkey manure was investigated. Worms dispersed all four bacteria on TSAN during a subsequent 24-h incubation period (Figure 4). There were no significant differences in the number of each test bacterium shed on the TSAN.

Vectoring of bacteria by *Diploscapter* from soil to carrot. Experiments were conducted to determine if *Diploscapter* would transport pathogenic bacteria through soil. Results showed that the number of pathogenic bacteria on the surface of carrot pieces or disk was unaffected ($P > 0.05$) by the presence of *Diploscapter* in or on the soil. Additional studies are needed to better understand the potential role of *Diploscapter* as a vector in contaminating pre-harvest produce in soil.

In summary**,** compared to *C. elegans*, *Diploscapter* is more sensitive to copper and migrates more slowly to sites at which pathogenic and non-pathogenic bacteria was inoculated. Results also show that *Diploscapter* may lack the ability to sense the presence of bacteria at distances greater than 1 cm. Environmental vectors are among the primary factors known to cause contamination of produce with foodborne pathogens (4, 5), and the potential for pre-harvest contamination by bacterivorous nematodes may be among these potential factors. Results suggest that *Diploscapter* ingests and passes or releases adhering foodborne pathogenic bacteria. We were not able to demonstrate that *Diploscapter* serves as a vector in soil to transport pathogens to carrots. Our observations serve, however, as foundation for more extensive research to determine the role of *Diploscapter* as one of the several free-living nematodes that may impact the level of microbiological safety of pre-harvest fresh fruits and vegetables.

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LEGENDS FOR FIGURES

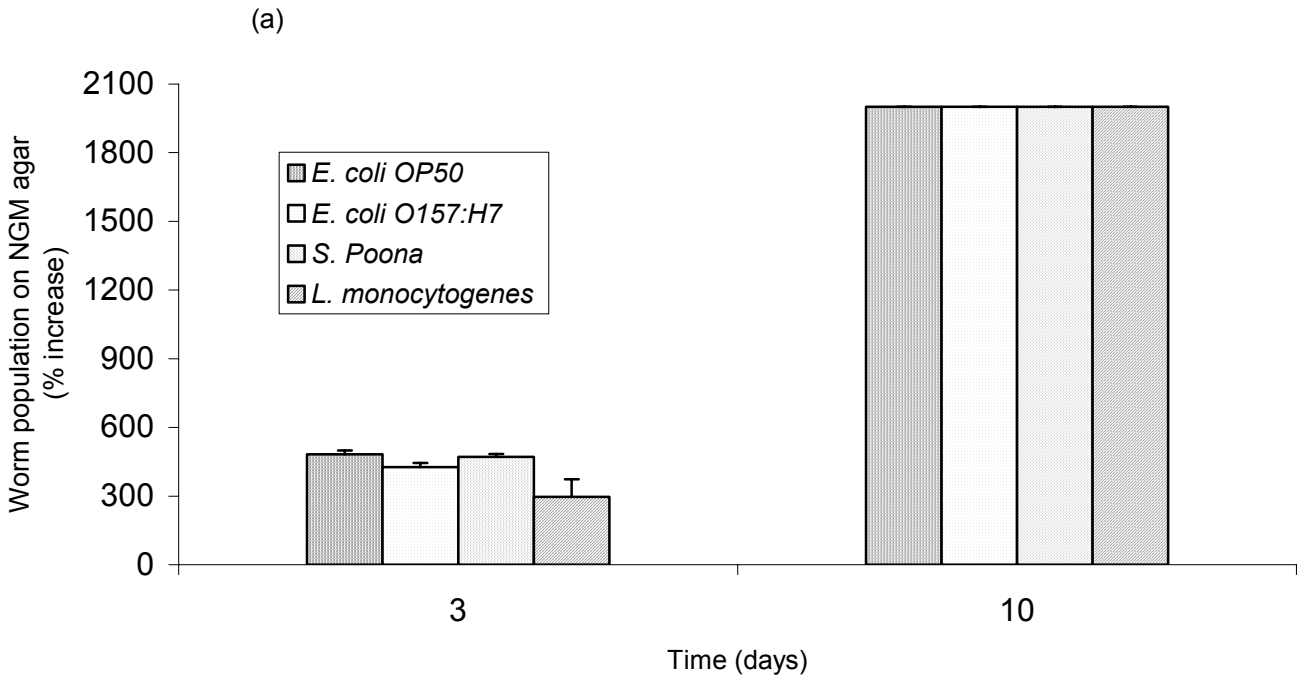
Figures 1. Percent increase in number of *Diploscapter* deposited on NGM agar (a) and TSAN (b) inoculated with *E. coli* OP50 and pathogenic bacteria and incubated for 3 or 10 days at 30°C.

Figures 2. Survival of *Diploscapter* in cow manure and composted turkey manure (a) and in soil amended with composted turkey manure inoculated with *E. coli* OP50, *E. coli* O157:H7, *S.* Poona, or *L. monocytogenes* (b).

Figure 3. Attraction of *Diploscapter* to *E. coli* OP50, *E. coli* O157:H7, *S.* Poona, and *L. monocytogenes* on TSAN.

Figure 4. Dispersal of pathogenic bacteria and *E. coli* OP50 by *Diploscapter* on TSAN within 24 h after exposure to bacteria inoculated into soil amended with composted turkey manure. Values ($\log_{10}$ CFU/worm) are means of the number of colonies formed on TSAN by bacteria shed by *Diploscapter* during the 24-h incubation period, after removing from inoculated compost-amended soil.

Figure 1


(a)
2100
1800
1500
1200
900
600
300
0
Worm population on NGM agar (% increase)
E. coli OP50
E. coli O157:H7
S. Poona
L. monocytogenes
3
10
Time (days)

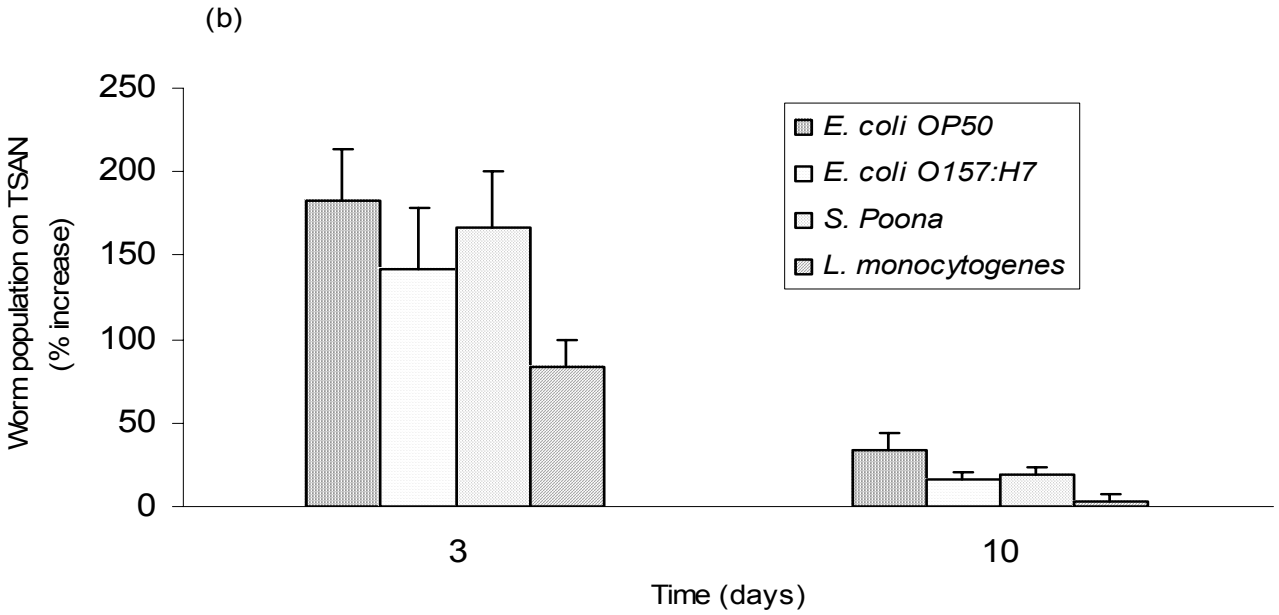
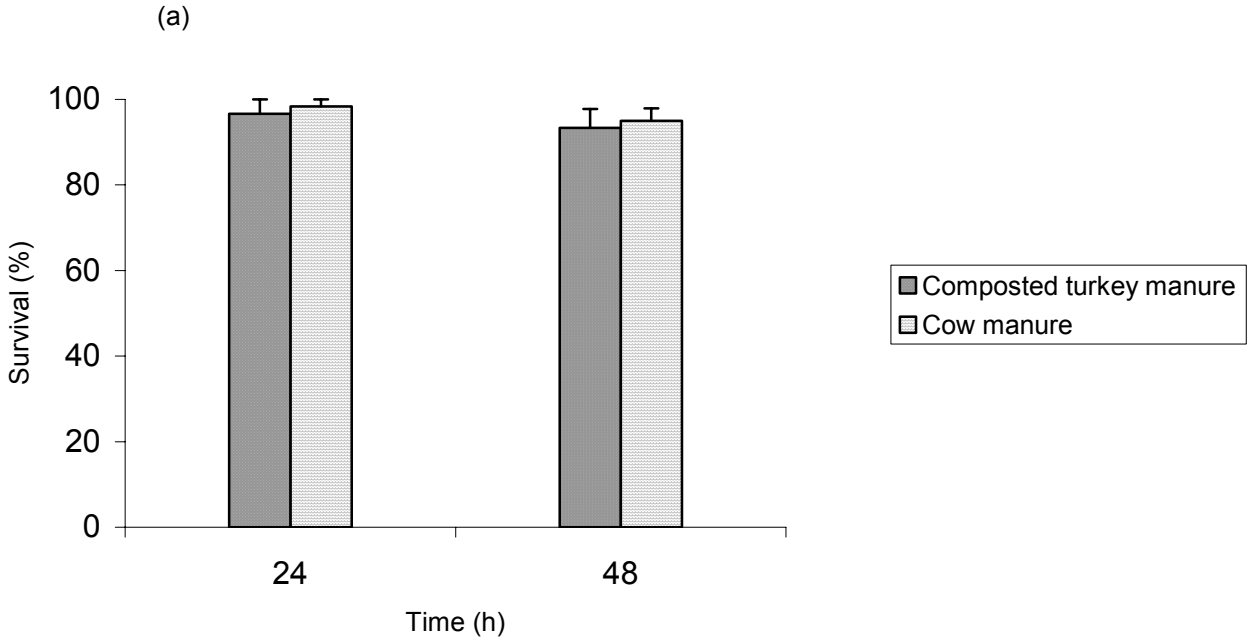

(b)
250
200
150
100
50
0
Worm population on TSAN
(% increase)
E. coli OP50
E. coli O157:H7
S. Poona
L. monocytogenes
3
10
Time (days)

Figure 2


(a)
100
80
60
40
20
0
Survival (%)
24
48
Time (h)
Composted turkey manure
Cow manure

(b)

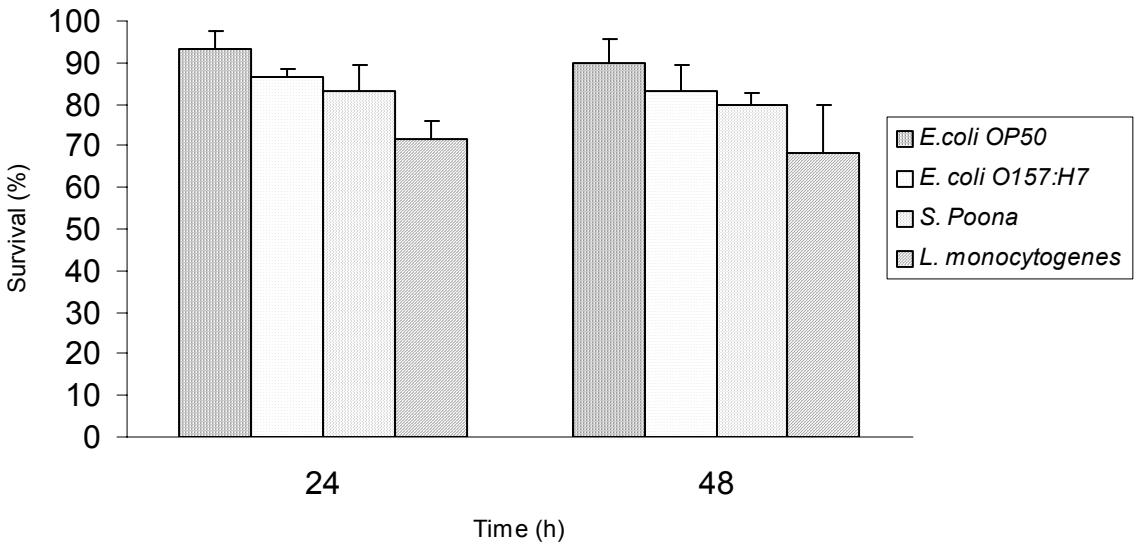

100
90
80
70
60
50
40
30
20
10
0
Survival (%)
24
48
Time (h)
E.coli OP50
E. coli O157:H7
S. Poona
L. monocytogenes

Figure 3

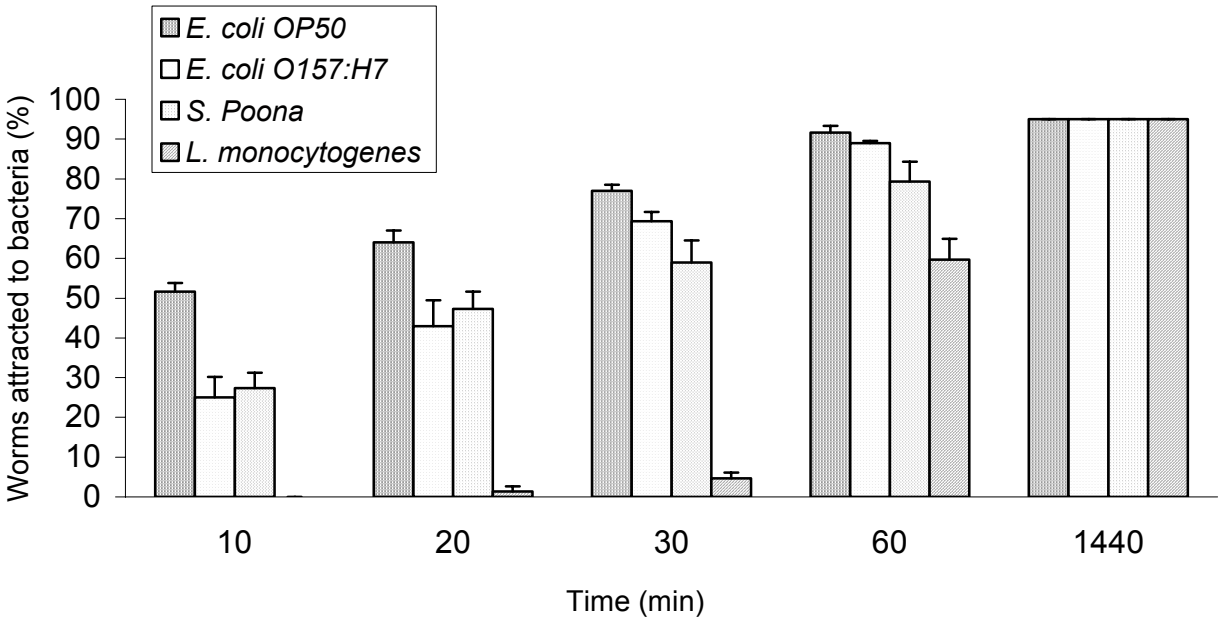
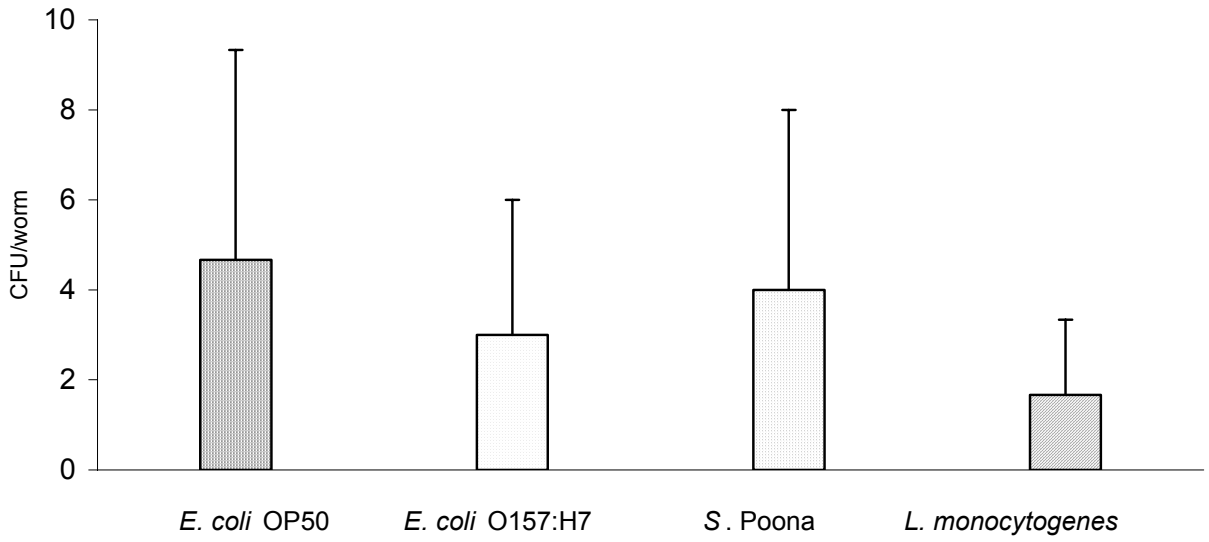

E. coli OP50
E. coli O157:H7
S. Poona
L. monocytogenes
Worms attracted to bacteria (%)
100
90
80
70
60
50
40
30
20
10
0
10
20
30
60
1440
Time (min)

Figure 4


10
8
6
4
2
0
CFU/worm
E. coli OP50
E. coli O157:H7
S. Poona
L. monocytogenes

CHAPTER 4

SUMMARY AND CONCLUSIONS

Anthropogenic influence may be the primary sources of fruit and vegetable contamination, but bacterial feeding microorganisms may play a significant role in vectoring these pathogens from the soil to the plant in pre-harvest contamination. As a result, bacterial-feeding nematodes may largely influence pathogenic bacterial contamination of pre-harvest produce. Thus, increasing the risk of human bacterial infections.

The objectives of this thesis were: 1) to determine survival and reproduction characteristics of *Diploscapter* fed on foodborne pathogenic bacteria; 2) to determine if *Diploscapter* is attracted to pathogenic bacteria; and 3) to determine if pathogenic bacteria ingested by *Diploscapter* and adhere to worm in soil are subsequently dispersed.

The results from this study were expected to be similar to those of previous investigations involving *C. elegans*. Compared to *C. elegans*, *Diploscapter* were more sensitive to copper, which was noted by a much lower LC50 value. *Diploscapter* demonstrated an attraction to pathogenic bacteria but did so in a much slower manner than *C. elegans*, which was expected, given *Diploscapter*'s smaller size and associated mobility. Attraction of *Diploscapter* to pathogenic bacteria at distances greater than 1 cm was not clearly demonstrated. We found that even with surface washing, it was not possible to transfer *Diploscapter* without associated surface bacteria. It was concluded that the presence of additional bacterial growth could contribute to the absence of strong attraction to test colonies at distances greater than 1 cm as well as minimizing preference among different bacterial strains.

Findings from this study support the potential for pre-harvest contamination by bacterial feeding nematodes. Results indicate *Diploscapter* has the potential to ingest and pass viable pathogenic bacteria capable of causing human infection.

Our study does not provide definitive evidence to support the hypothesis that *Diploscapter* do serve as vectors of pathogenic bacteria to pre-harvest produce, but it does support the potential role of *Diploscapter* as a contributor. It is hoped this research can serve as the general foundation for further extensive studies involving *Diploscapter*. Such research should examine a number of issues including the effectiveness of sanitizers and additional work on the organism attraction to actual fruits and vegetables as well as include produce contamination assays. Also, more information is needed on basic aspects of this nematodes biology including pH tolerance and methods for establishing synchronized cultures.