Caenorhabditis Elegans Model To Study Antimicrobial Treatment On E. coli O157:H7

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CAENORHABDITIS ELEGANS MODEL TO STUDY ANTIMICROBIAL TREATMENT

ON E. COLI O157:H7

A Thesis Presented

by

PARITA R PATEL

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

MAY 2018

FOOD SCIENCE
CAENORHABDITIS ELEGANS MODEL TO STUDY ANTIMICROBIAL TREATMENT
ON E. COLI O157:H7

A Thesis Presented
by
PARITA R PATEL

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DEDICATION

I would like to dedicate to GOD for giving me the strength in all my weaknesses and for always being there for me to lead me to the path of righteousness. In addition, I would also like to thank my parents, brother, and Ajay for their endless love, support, and encouragement to achieve my goals.
ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Lynne McLandsborough for her guidance throughout my research. I am thankful for her motivation when things were going right and when things were falling apart. I also like to thank Dr. Yenohwa Park for her guidance when things weren’t working right and for showing me the right direction. Lastly, I would like to thank Ye Peng for teaching me how to handle *C. elegans* and to the staff member Cynthia Kane for her generous assistance when needing anything for conducting an experiment.
ABSTRACT

CAENORHABDITIS ELEGANS MODEL TO STUDY ANTIMICROBIAL TREATMENT ON E. COLI O157:H7

MAY 2018

PARITA R PATEL, B.S., WESTERN MICHIGAN UNIVERSITY

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Lynne A. McLandsborough

An increase in antimicrobial resistance bacteria has endangered our ability to treat infectious diseases. Lack of good in-vivo model has made it difficult to study antimicrobial resistance. In this study, we have used an inexpensive and short life span in-vivo model namely, Caenorhabditis elegans (C. elegans) to study antimicrobial treatment using pathogenic Escherichia coli O157:H7, a multidrug resistance bacterium that causes life threatening infection in humans.

We have investigated the influence of live vs. heat killed non-pathogenic E. coli OP50 (OP50) as a food source on the growth and survival of infected C. elegans mutant AU37 with E. coli O157:H7 in the presence and absence of antibiotics. This is analyzed using a liquid-based C. elegans-E. coli O157:H7 infection assay. C. elegans was synchronized and grown on a lawn of live OP50 till they reached L4-young adult stage. L4-young adults were transferred to liquid medium where the C. elegans was infected with live E. coli O157:H7 or live non-pathogenic OP50 for 24 hours. After infection, C. elegans were fed live or heat killed OP50 depending on the
experiment, and the life span and levels of E. coli O157:H7 were monitored, with and without ampicillin treatment in a 96 well transwell plate.

Our results indicate that live OP50 is an ideal food source for C. elegans growth and survival to study antimicrobial treatment. C. elegans growth rate and survival decreased in presence of heat killed OP50, which makes heat killed OP50 as a non-ideal food source for antimicrobial assay. Moreover, using live OP50 we have discovered that the ampicillin dose 8µg/ml, 16µg/ml, and 32µg/ml are effective in increasing the survival of C. elegans infected with E. coli O157:H7. However, treatment on C. elegans infected with acid stressed E. coli O157:H7 is controversial.
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CHAPTER 1

INTRODUCTION

Enterohemorrhagic *E. coli* (EHEC) is one of the groups of pathogenic *E. coli*, capable of causing hemorrhagic colitis (bloody diarrhea) and hemolytic uremic syndrome (HUS) (acute kidney failure) in humans (Lim et al., 2010). EHEC is also referred to as Shiga-toxin producing *E. coli* (STEC) because of its ability to produce Shiga-toxins. *E. coli* O157:H7 is one of the serotypes of EHEC, recognized as the leading cause of EHEC outbreaks in the United States (Ho et al., 2013). Treatment with *E. coli* O157:H7 is self-limiting, as clinical studies indicate controversial results on the use of antibiotics to increase the risk of development of HUS (Panos et al., 2006). Lack of good *in-vivo* model has limited the study of EHEC infection and treatment. In this study, we have used an in-expensive *in-vivo* model namely *Caenorhabditis Elegans* (*C. elegans*) to study *E. coli* O157:H7 infection and treatment.

Lately, *C. elegans* has gained popularity to study bacterial pathogenicity because of the simplicity of the model. *C. elegans* is inexpensive, small in size (≈1mm), have short life-span of approximately 3 weeks, and can produce a large number of offspring in short time (Corsi et al., 2015). In addition, it has 65% of the genes that are related to human diseases and has its entire genome sequenced (Shen et al., 2017). All these characteristics of *C. elegans* make them an excellent model to study bacterial pathogenicity.
The main goal of this research is to develop a *C. elegans* infection-treatment model to evaluate the sensitivity of *E. coli* cells to the ampicillin treatment in *in-vivo* model. If we will be successful in developing the *C. elegans* infection system, we can use the system to study acid stressed *E. coli* O157:H7 infection using *C. elegans* model, which is to be likely associated with EHEC infection as the cells needs to pass through our stomach acid to colonize our gut. In addition, we can use different antimicrobials to study antimicrobial resistance in *E. coli* O157:H7, as well as in other food-borne pathogens.
CHAPTER 2

LITERATURE REVIEW

2.1 Enterohemorrhagic *Escherichia coli* O157:H7

*Escherichia coli* (*E. coli*) is a facultative anaerobic gram-negative rod-shaped bacterium. Most *E. coli* strains are harmless and are present as a normal flora in the gastrointestinal tract of humans and animals (Lim et al., 2010). However, some *E. coli* strains have evolved into pathogens, and are capable of causing severe illness to humans.

Pathogenic *E. coli* has been divided into various groups based on serogroup, pathogenicity mechanisms, clinical symptoms, and/or virulence factors (Lim et al., 2010). There are six groups characterized as pathogenic *E. coli*: Enterohemorrhagic *E. coli* (*EHEC*) also referred to as Shiga toxin-producing *E. coli* (*STEC*), Enterotoxigenic *E. coli* (*ETEC*), Enteropathogenic *E. coli* (*EPEC*), Enteroaggregative *E. coli* (*EAEC*), Enteroinvasive *E. coli* (*EIEC*), and Diffusely Adherent *E. coli* (*DAEC*) (CDC, 2014). Among this groups, Enterohemorrhagic *Escherichia coli* (*EHEC*) is most commonly associated with the foodborne outbreaks (CDC, 2014). EHEC have many serotypes O26, O45, O91, O103, O111, O121, O145, and O157 (Barlow and Mellor, 2010). *Escherichia coli* serotype O157:H7 is the most common cause of EHEC outbreaks in the United States (Ho et al., 2013). EHEC is estimated to cause about 265,000 illnesses in the United States, among which *E. coli* O157:H7 accounts for about 36% of the illnesses (CDC, 2014).
One of the major pathogenic factors of \textit{E. coli} O157:H7 is the ability to produce Shiga toxin (Tarr et al., 2005). However, term EHEC is specifically referred to the serotype that produce Shiga toxins, and cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans (Lim et al., 2010). HUS is the leading cause of acute renal failure in children (Hu et al., 2013).

\textit{E. coli} O157:H7 can grow at a temperature as low as 7°C and as high as 50°C, with an optimum growth temperature at 37°C (WHO, 2018). It can grow in an acidic condition at pH as low as 4.4 (WHO, 2018). \textit{E. coli} O157:H7 activates regulatory factor RpoS which helps the bacteria survive in acidic stress condition (Jordan et al., 1999). In addition, arginine and glutamate-dependent system are also known to play an important role in the survival of \textit{E. coli} O157:H7 in acidic foods (Jordan et al., 1999).

EHEC can be destroyed through thorough cooking, as it doesn’t survive at a temperature higher than 70°C (WHO, 2018). Young children, elderly, and immunocompromised individuals are more susceptible to EHEC infection (WHO, 2018). While some individuals have asymmetric infections, others can develop severe infections associated with bloody diarrhea and abdominal cramps within three days of consuming contaminated food (Hu et al., 2013). Symptoms last an average of 3-4 days, with a maximum duration of 10 days (CDC, 2014).
2.1.1 *E. coli* O157:H7 History

*E. coli* O157:H7 was first recognized as a human disease in 1982, during undercooked hamburger outbreak, where *E. coli* O157:H7 was known as causing hemorrhagic colitis (bloody diarrhea), non-bloody diarrhea, and HUS (Kaper et al., 2004). However, it has gained public attention in the United States after Jack-in-the-Box outbreak in 1993, which has resulted in more than 700 illnesses and 4 deaths (Armstrong et al., 1996). Since then, many outbreaks associated with EHEC infection has been reported. Table 1 shows *E. coli* O157:H7 outbreaks in the United States since last five years from 2013-2017 (CDC, 2018).

<table>
<thead>
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<th>Food associated with E. coli O157:H7 outbreak</th>
<th>Number of people infected</th>
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<tr>
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<td>Romaine Lettuce</td>
<td>25</td>
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<tr>
<td></td>
<td>SoyNut Butter</td>
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<td>2016</td>
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<td>2015</td>
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</tr>
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<td>2013</td>
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2.1.2 Transmission of *E. coli* O157:H7 to humans

*E. coli* O157:H7 is a human pathogen, however, it is present as a normal flora in the intestinal tract of ruminants such as cattle, sheep, goat, and deer (Hu et al., 2013). Human exposure to *E. coli* O157:H7 is associated with ingestion of contaminated food such as undercooked ground beef and raw milk (WHO, 2018). In addition, *E. coli* O157:H7 is transmitted to humans through fecal contamination of water and food such as fruits and vegetables, cross-contamination during food preparation, and even with person-person contact (Hu et al., 2013; WHO, 2018).

2.1.3 EHEC Colonization, Diagnosis, and Treatment

In humans, EHEC infection develops when the bacteria colonize the gastrointestinal tract. The physical structure of the *E. coli* O157:H7 contains fimbriae which helps the bacteria adhering to the intestinal epithelial cells (Kaper et al., 2004).

In humans, *E. coli* O157:H7 infection can be detected by culturing stool sample on selective/differential sorbitol-MacConkey agar (Tarr et al., 2005). *E. coli* O157:H7, unlike other non-pathogenic *E. coli* strains, cannot ferment sorbitol and hence can appear colorless/beige colonies on sorbitol-MacConkey agar after overnight incubation at 37°C (Tarr et al., 2005).

In vitro studies have shown antibiotics to be effective against *E. coli* O157:H7, however, sub-lethal antibiotic concentration can increase the release of Shiga toxins and hence the development of HUS (Panos et al., 2006). In humans, hydration is the only recommended treatment for EHEC infection (CDC, 2014). Treatment with
antibiotics is controversial as some clinical studies indicate that antibiotics treatment increases the risk of development of HUS while some studies indicate antibiotics as effective in reducing the duration of the infection and may prevent the development of HUS (Panos et al., 2006). Here we proposed an inexpensive and simple in-vivo model Caenorhabditis Elegans (C. elegans) to study pathogenicity and antimicrobial treatment.

2.2 Caenorhabditis Elegans

Caenorhabditis Elegans (C. elegans) is a small free-living transparent soil nematode with about 1mm adult in length (Corsi et al., 2015). Nematodes can be observed using a dissecting microscope, which generally has 100x magnification (Corsi et al., 2015). It can easily cultivate in the laboratory with non-pathogenic E. coli OP50 as food (Riddle et al., 1997). C. elegans can reproduce in 2-4 days depending on strain and incubation temperature (Riddle et al., 1997). It has two sexes, male (rare) and a self-fertilizing hermaphrodite which can produce up to 300 offspring (Corsi et al., 2015). Their lifespan on average is up to 2-3 weeks at 25°C (Fielenbach and Antebi, 2008). Furthermore, C. elegans has a fully sequenced genome, which makes them suitable for genetic studies (Godballe et al., 2010). Research involving C. elegans does not need permission from Institutional Animal Care and Use Committees (Shen et al., 2017). Most importantly, C. elegans conserve more than 65% of the genes that are related to human diseases (Shen et al., 2017), which makes them a suitable animal model to study human bacterial infection.
2.2.1 Anatomy and Life-cycle of *C. elegans*

The body shape of *C. elegans* is unsegmented, cylindrical with an outer tube separated from an inner tube (Figure 1) (Altun and Hall, 2009; Corsi et al., 2015; Godballe et al., 2010). The outer tube of *C. elegans* contains the cuticle, hypodermis, excretory system, neurons, and muscles, where inner tube contains the pharynx, intestine, and gonad in adult (Altun and Hall, 2009; Godballe et al., 2010). The pharynx is one of the important part of the *C. elegans* as it protects worms against bacterial infection (Godballe et al., 2010). The grinder in the pharynx grinds the bacteria and prevent the whole bacteria from reaching to the intestine (Godballe et al., 2010). Besides *C. elegans* anatomy, its ability to sense different chemical signal as a nutrient or toxin helps them to stay away from the pathogens and pathogens does not have easy access to the *C. elegans* intestine (Godballe et al., 2010).

*C. elegans* has a rapid 3-5 days life-cycle (from egg to adulthood) at temperature 15°C - 20°C (Fielenbach and Antebi, 2008). Typical *C. elegans* life-cycle
consists of four larval stage from L1 to the L4 stage, and then adulthood (Figure 2). It is a good laboratory model to use for life-span study because it is easy to synchronize with the growth halt at the L1 stage in the absence of food. When food is available, worms can grow from the L1 to L4 stage after 45-50 hours at 25°C. L4 stage worms can be recognized under dissecting microscope with its unique appearance having white vulva area. In overcrowding and lack of food condition, it can go to dauer stage where it can survive up to 4 months without food and can again start to feed and reaches to the L4 stage. As the dauer can survive for months, it can be kept as a stock in a petri dish at 15°C (Corsi et al., 2015).

Figure 2 Life cycle of *C. elegans* (Altun and Hall, 2009) ([http://www.wormatlas.org](http://www.wormatlas.org)).
2.2.2 C. elegans as a model for *E. coli* O157:H7 infection and treatment

In a laboratory, *C. elegans* can easily grow on a petri dish and fed on non-pathogenic bacterial strain *E. coli* OP50. *C. elegans* can be used to study EHEC infection simply by replacing its food with pathogenic bacteria. However, it is important to understand *C. elegans* behavioral response to bacterial food as *C. elegans* can sense the difference between the nutrient and toxin (Godballe et al., 2010).

Many human bacterial pathogens kill *C. elegans* by colonizing their intestine, and the pathology is measured as a decrease in the life-span of *C. elegans* (Darby, 2005). Hence, *C. elegans* intestinal bacterial colonization can be quantified using plate count method. *E. coli* OP50 does not colonize the *C. elegans* intestine (Darby, 2005). Bacterial pathogens can kill *C. elegans* by releasing the toxin (fast-killing) or by colonizing the *C. elegans* intestine (slow killing) (Darby, 2005).

As *C. elegans* conserve about 65% of the genes related to the human diseases (Shen et al., 2017), has its full genome sequenced (Godballe et al., 2010), and because of the simplicity of the model, *C. elegans* is widely used in pathogenicity and human diseases study. Previous research on plate-liquid infection assay has shown that enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), and uropathogenic *E. coli* (UPEC) is pathogenic to *C. elegans* (Chou et al., 2013; Merkx-Jacques et al., 2013). Here, we propose a liquid-liquid infection-treatment assay as a simple experimental procedure to study the antibiotic treatment of *E. coli* O157:H7.
CHAPTER 3
OBJECTIVES

1. To determine if heat-killed *E. coli* OP50 is a good food source for *C. elegans* compared to live *E. coli* OP50 in infection-treatment assay.

2. To determine if *C. elegans* is an appropriate model to study *E. coli* O157:H7 infection and treatment *in-vivo*.

3. To determine if ampicillin treatment is effective in worms infected with acid stressed *E. coli* O157:H7 cells compared to the worms infected with non-acid stressed *E. coli* O157:H7 cells.
CHAPTER 4
MATERIALS AND METHODS

4.1 Bacterial strains, media and preservation

Bacterial strain non-pathogen *E. coli* OP50 (OP50) was used as nematode food and was obtained from Dr. Park Lab, University of Massachusetts, Amherst. Bacterial strain pathogen *E. coli* O157:H7 (laboratory number E21B) was used to infect worms and was obtained from American Type Culture Collection (ATCC) (ATCC 43895, Manassas, VA).

Concentrated *E. coli* OP50 used as nematodes food in 96 Transwell (Corning HTS Transwell 96 well pore size 0.4µm, Sigma-Aldrich, St. Louis, MO) was prepared by mixing a loop full of OP50 culture from a streaked plate into 200ml Luria-Bertani broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubated at 37°C overnight on a 125rpm shaker. Then, the mixture was centrifuged (Sorvall ST 8 small benchtop centrifuge with TX-150 swinging bucket rotor (Thermo Fisher Scientific Inc., Waltham, MA) at 2,500g for 10 minutes and the supernatant was discarded. OP50 pellet was concentrated to 100 mg/ml in a sterilized water (Solis and Petrascheck, 2011). The culture was stored at -20°C. Heat-killed OP50 was prepared by incubating concentrated OP50 in 70°C hot bath for 30 minutes (Ren et al., 2009).

Frozen bacterial stock, *E. coli* OP50 and *E. coli* O157:H7, were prepared for long-term storage. Bacterial culture was grown overnight in a Tryptic Soy Broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Sterile glycerol
(50% glycerol w/v in dH2O) was added at a proportion of 1-part glycerol:1-part TSB containing bacterial culture and mixed into a small sterile tube. The mixture was stored at -80°C. Tryptic Soy Agar (TSA) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) plate was streaked from the frozen stock each time for the new experiment. The streaked plates were discarded after a week.

### 4.2 Nematode strains, media and preservation

*Caenorhabditis elegans* (*C. elegans*) strain AU37 was purchased from Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN). Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Strain AU37 is a double mutant (*glp-4 (bn2ts); sek-1(km4)*) , where *glp-4* is a temperature-sensitive mutation that does not allow proliferation at 25°C and *sek-1* is a mutation that increases *C. elegans* susceptibility to pathogens (Zhou et al., 2011). *C. elegans* mutant AU37 were maintained at 15°C. *C. elegans* strain N2, wild-type strain, was obtained from Dr. Park Lab, University of Massachusetts, Amherst.

Nematode growth medium agar plates (NGM, US Biological Life Sciences Inc., Salem, MA, prepared with 1M Calcium Chloride, 1M Dipotassium Phosphate (Sigma-Aldrich, St. Louis, MO), and 1M Magnesium Sulfate (Thermo Fisher Scientific Inc., Waltham, MA)) were prepared according to the manufacturer’s instructions. *E. coli* OP50 was grown at 37°C in a Luria-Bertani broth for 12-14 hours on a 125rpm shaker and 1 ml of overnight culture was added to the center of NGM agar plates. The plates were incubated at room temperature for 48 hours, to allow for growth.
(considered as NGM seeded with OP50). After 48 hours, plates were sealed in a plastic bag to prevent contamination and moisture loss and moved to 4°C incubator.

Nematode working cultures were prepared by transferring a piece of an NGM agar containing *C. elegans* to a NGM plate seeded with OP50 and incubated at 15°C for 4-5 days. When the working culture plate contained many L1 stage worms (as determined by observation with a dissecting microscope), the plate was sealed with parafilm to prevent contamination and loss of moisture and stored at 15°C. The plate was kept for about two months and then discarded. New working culture nematodes were prepared using the same procedure, from an old NGM plate containing many worms.

### 4.3 Synchronization of worms

Worms were synchronized following the protocol described by Stiernagle, with some modification (2006) (wormbook.org). Synchronized worms were prepared by placing a section of NGM agar containing worms onto a new NGM plate seeded with OP50 and incubated at 15°C. When large numbers of eggs laid, eggs were collected by washing the plate with sterile water and collecting the mixture into a 15ml sterile centrifuge tube. The tube containing worms and eggs were centrifuged at 2000g for 2 minutes and the supernatant was discarded. The pellet was suspended in 10 ml bleach solution (18% household bleach (The Clorox Company, Oakland, CA) and 10% NaCl (Thermo Fisher Scientific Inc., Waltham, MA) in a sterile water) and vortexed for 2-2.5 minutes at high speed to destroy worms, then centrifuged (2000 g, 1 min), and supernatant was discarded. Pellet containing
eggs was suspended in M9 buffer (5.8g Na$_2$HPO$_4$, 2g KH$_2$PO$_4$, 0.5g NaCl, and 1g NH$_4$Cl (Thermo Fisher Scientific Inc., Waltham, MA) per 1L distilled water (Ahringer, 2006; Stiernagle, 2006)) to neutralize the reaction, centrifuged (2000g, 2 mins), and supernatant was discarded. Pellet was re-suspended into an M9 buffer, centrifuged (2000g, 2 mins), and the supernatant was discarded. Washed eggs were suspended into a 1ml M9 buffer and incubated at 25°C to hatch. After 24 hours, hatched eggs (now L1 stage worms) were transferred onto NGM plate seeded with live OP50. Worms were grown to sterile L4-young adult at 25°C which was typically achieved after a 45-50 h incubation. Mutant AU37 does not produce eggs at 25°C.

### 4.4 Determination of minimal inhibitory concentration

Minimal inhibitory concentration (MIC) *in-vitro* was performed to determine the lowest concentration of ampicillin that inhibits *E. coli* O157:H7 growth, to treat *C. elegans* infected with *E. coli* O157:H7. The antimicrobial effectiveness of ampicillin on *E. coli* O157:H7 was measured using broth microdilution method described by Reimer et al. with little modification (1981). *E. coli* O157:H7 was grown in a Mueller-Hinton Broth (MHB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) overnight and then diluted 1:10 to measure the absorbance at 625nm. 0.85% NaCl was used as a blank solution. The test was performed in a 96 well microtiter plate (Corning Incorporated, Corning, NY) in which 50µl MHB was added to each well, and a two-fold serial dilution of ampicillin was performed. Then, 50µl of an overnight *E. coli* O157:H7 culture was added to each well. The final concentrations of ampicillin
were ranging from 0.25µg/ml to 64µg/ml. The microtiter plate was incubated at 37°C for 20-24 hours, and the absorbance was measured at 625nm using ELx800 Microtiter Plate Reader (BioTek Instruments Inc., Winooski, VT). Ampicillin stock was prepared by filter sterilizing 512mg/ml ampicillin in a sterilized water using 0.22 µm filter. The stock was stored in 100µl volume at -20°C.

4.5 Bacterial colonization

Worms intestinal bacterial colonization was determined as described by Moy et al. (2006). Approximately 10 worms were collected in a small centrifuge tube and washed 3 times with approximately 250µl of 1mM sodium azide (Acros Organics, Thermo Fisher Scientific, NJ, USA). 50µl of the supernatant was plated onto Sorbitol MacConkey agar (OXOID LTD., Basingstoke, Hampshire, England) to determine the external CFU. Approximately 400mg of 1.0mm silicon carbide particles (BioSpec Inc., Bartlesville, OK, USA) was added to the tube and vortexed at high speed for approximately one minute to disrupt the worms. The suspension was then diluted and plated onto Sorbitol MacConkey agar. The plates were incubated at 37°C for 20-24 hours and the internal CFU/worm was determined.

4.6 E. coli O157:H7 growth curve

E. coli O157:H7 growth curve was performed to determine the time required for E. coli O157:H7 grown in Luria-Bertani broth with pH 6.80±0.10 and pH 5.25±0.05 to reach to the late-exponential stage. Luria-Bertani broth pH was
adjusted using 99.5% pure acetic acid (Acros Organics, Thermo Fisher Scientific, NJ, USA) (Deng et al., 1999). *E. coli* O157:H7 was grown on a Luria-Bertani broth without pH adjustment (pH 6.80±0.10) for 18-20 hours at 37°C, centrifuged at 2500g for 10 minutes, and the supernatant was discarded. M9 buffer was added to make the final *E. coli* O157:H7 concentration of 10^5 CFU/ml. *E. coli* O157:H7 concentration of 10^5 CFU/ml was then added to the pH adjusted (pH 5.25±0.05) and non-pH adjusted (pH 6.80±0.10) Luria-Bertani broth to make the final bacterial concentration of 10^4 CFU/ml. These enrichments were incubated at 37°C and the absorbance was taken at a wavelength of 625nm every hour to plot an *E. coli* O157:H7 growth curve.

### 4.7 *C. elegans*-*E. coli O157:H7* infection lifespan assay

The lifespan assay was carried out by synchronizing the worms to the L4-young adult stage as described in “synchronization of worms” section. NGM plate containing L4-young adult stage worms were washed using an M9 buffer and suspended into a sterile 2ml microcentrifuge tube, centrifuged (2000g, 2min) using accuSpin Microcentrifuge with fixed angle rotor (Fisher Scientific, Waltham, MA), and the supernatant was discarded. The worms were washed (centrifuged at 2000g, 2 min) at least three times with M9 buffer. Approximately 100 AU37 worms were added to each well in 24 well plate, containing 10^9 CFU of *E. coli* OP50 (refer to as control) and 10^9 CFU of *E. coli* O157:H7 (refer to as infected), each in 1ml M9 buffer (v/v). For N2 worms, 120μM fluorodeoxyuridine (FUDR) (Tokyo Chemical Industry
CO., LTD., Portland, OR) was added to the final concentration of 1ml to make worms infertile (Solis and Petrascheck, 2011). The plate was incubated at 25°C for 24 hours. After 24 hours, both control and infected worms were suspended into a micro-centrifuged tube and washed at least three times with M9 buffer. Approximately 10 infected worms were suspended in a 2ml micro-centrifuge tube to determine the internal bacterial colonization count. Bacterial colonization count was determined as described in section 4.5.

The treatment was performed in 96 well trans-well with varied concentration of ampicillin (8µg/ml, 16µg/ml, and 32µg/ml). The control and infected worms without ampicillin, and with ampicillin (8µg/ml, 16µg/ml, and 32µg/ml) were used to compare the results. Live or heat-killed OP50 were used as nematodes food depending on an experiment. Each treatment was performed in five duplicates. The initial number of worms in each well were counted and the plate was incubated at 25°C. The number of worms alive in each well were counted every other day till all worms died. The food in the wells was changed as needed. The experiment was repeated independently. Refer to Appendix A for detailed experimental procedure.

4.8 Heat stress assay

Heat stress assay was performed similarly as described in section 4.7., with little modification. After treatment, number of worms alive were counted in 96 well
transwell plate and the plate were incubated at 37°C. The number of worms alive was counted every 60 to 90 minutes.

4.9 Acid stress assay

Acid stress assay was performed in a similar manner as mentioned in section 4.7, with little modification. *E. coli* O157:H7 and *E. coli* OP50 cells were grown overnight on Luria-Bertani medium (pH = 5.25 ± 0.05). *E. coli* O157:H7 and *E. coli* OP50 cells were also grown in Luria-Bertani broth without pH adjustment (pH = 6.65 ± 0.05), which will refer to as infected control. *E. coli* O157:H7 and *E. coli* OP50 cells were grown to the late exponential stage, the pellet was collected, and the supernatant was discarded. The pellet was then suspended into an M9 buffer and the worms were fed with $10^6$ CFU *E. coli* O157:H7 and *E. coli* OP50 concentration per 1ml of M9 buffer (v/v).

4.10 Statistical Analysis

Statistical Analysis was performed using the Graph Pad Prism version 7.0c for Mac OS X (GraphPad Software, Inc.). Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon test were performed to analyze the significant difference in survival curves. P-value < 0.05 is considered as significantly different.
CHAPTER 5
RESULTS AND DISCUSSION

5.1 *C. elegans* mutant AU37 is preferable in infection assay compared to wild type N2

Infection assays using *E. coli* O157:H7 (E21B) were performed with *C. elegans* strains AU37 (mutant) and N2 (wild type). *C. elegans* strain AU37 is a double mutant strain, with temperature sensitivity and an increased susceptibility to pathogens (Zhou et al., 2011). Both *C. elegans* strains, AU37 and N2, were exposed to E21B or *E. coli* OP50 (OP50, non-pathogenic negative controls) for 24 hours in an M9 buffer, worms were recovered, and transferred to 96 well plate (T=0), and the worms were fed heat-killed OP50 during the duration of the experiment. E21B, an enterohemorrhagic *E. coli*, was expected to infect and shorten the life-span of the worms, while non-pathogenic OP50 was used as a negative control and was not expected to shorten the life-span.

The survival of *C. elegans* AU37 infected with *E. coli* O157:H7 had a reduced life-span compared to the AU37 infected with non-pathogenic *E. coli* OP50, however the differences were not significant (P>0.05) (Figure 3A). The survival of *C. elegans* N2 infected with E21B was not different compared to OP50 (Figure 3B). The results indicated that E21B may successfully infect AU37; however, it may fail to infect N2. We are not sure whether E21B can infect and colonize the N2 strain later in the life of worms since the assay was terminated for N2 after 19 days, when all AU37 died.
Based on the 19 days life-span study, N2 is not susceptible to E21B infection. Chou et al. has showed *E. coli* O157:H7 does colonize N2 worms (2013). However, in order to shorten the experimental duration, we concluded that the AU37 is more suitable for infection assay.

**Figure 3** Infection assay with *E. coli* O157:H7. Worms were grown to L4/ young adult stage and transferred to 24 well plate containing $10^9$ CFU/ml *E. coli* O157:H7 in M9 buffer or similar levels of *E. coli* OP50. Worms infected with *E. coli* O157:H7 and *E. coli* OP50 for 24 hours. Worms were recovered and transferred to a 96 well trans-well plate (T=0) containing heat-killed OP50 as a food source for the remainder to the experiment at 25°C and survival was counted every day for 19 days. Graph Pad Prism Log-rank (Mantel-Cox) test was used to obtain the p-value. (A) AU37 (p=0.2781) (B) N2 (p=0.5954). P-value of less than 0.05 is considering to be statistically different.
5.2 Heat-killed OP50 significantly decreases the life-span of *C. elegans* strain AU37

We originally tested the *C. elegans* AU37 infection assay using heat-killed OP50 as post-infection food in trans-well plate. This was done due to the concern that live non-pathogenic bacteria (OP50) will interfere with live pathogenic bacteria (E21B) and will mislead the intestinal colonization numbers in worms. In addition, the goal was to develop an *in-vivo* infection assay for antibiotic efficiency; we were concerned that the antibiotic will also kill OP50, which may complicate the system.

*C. elegans* AU37 fed with heat-killed OP50 had a significantly shorter the life-span compared to the worms fed with live OP50 (Figure 4). When a preliminary study was done with synchronized worms (L1 stage) fed with heat-killed OP50 vs. live OP50 on NGM lawn, we observed the worms fed with live OP50 has reached to L4/ young adult stage within 48 hours, whereas worms fed with heat-killed OP50 were significantly smaller, and we hypothesize they may possibly move to a dauer stage (Figure 5). This indicates that live OP50 is needed to provide sufficient nutrient to the *C. elegans* AU37 for its growth and survival. Live OP50 were used to feed *C. elegans* AU37 after infection in all subsequent experiments.
**Figure 4** AU37 survival assay fed with live vs. heat-killed OP50. Worms were grown to L4/young adult stage and transferred to 96 trans-well plate where worms were fed with live vs. heat-killed OP50. Survival was counted every day till all worms died. Graph Pad Prism Log-rank (Mantel-Cox) (P=0.0032) and Gehan-Breslow-Wilcoxon (P=0.0100) tests were used to obtain a p-value.

**Figure 5** Dissecting microscope image of AU37 after 48 hours. Worms were synchronized and transferred to the NGM plate containing lawn of live vs. heat-killed OP50 after 24 hours. Dissecting microscopic image was taken using a digital camera (AmScope MU1400 with FMA050 lens, AmScope, Irvine, CA) after 48 hours. Image shown are at highest magnification. (A) AU37 grown on a live OP50 lawn. (B) AU37 grown on a heat-killed OP50 lawn.
5.3 Ampicillin treatment significantly increases the life-span of infected worms

In order to determine whether *C. elegans* is an appropriate model to study *E. coli* O157:H7 infection and treatment *in-vivo*, we had fed *C. elegans* AU37 with *E. coli* O157:H7 (we will refer to these worms as infected) for 24 hours, and transferred to a transwell plate with live *E. coli* OP50 with and without treatment with ampicillin at various concentrations. The control was *C. elegans* mutant AU37 infected with *E. coli* OP50 (we will refer to as control) for 24 hours and treated with ampicillin in a 96 transwell plate. A previous study has been done testing EHEC pathogenicity on *C. elegans* (Chou et al., 2013) however, limited studies have been performed on the effectiveness of antibiotics using *C. elegans* model. Hence, minimum inhibitory concentration test *in-vitro* was performed to determine the minimum ampicillin concentration that inhibits the growth of *E. coli* O157:H7. Starting with *in-vitro* minimum inhibitory concentration (0.25-1µg/ml), we determined the minimum effective ampicillin concentration to treat *E. coli* O157:H7 infection *in-vivo* was much higher (8µg/ml).

Pathogenicity of *E. coli* O157:H7 to *C. elegans* AU37 was tested, and determined to significantly decrease in the life-span of infected worms compared to the control worms (Figure 6A). The result indicates that *E. coli* O157:H7 is pathogenic to the *C. elegans* strain AU37. Treatment with ampicillin was tested on infected worms and determined that the worms fed with ampicillin concentration 8µg/ml, 16µg/ml, and 32µg/ml after being infected for 24 hours with an initial
infection of $2.4 \times 10^2$ CFU/worm, shows significant increase in the life-span of infected worms (Figure 6 (B-D)). This indicates that ampicillin concentration $8\mu g/ml$, $16\mu g/ml$, and $32\mu g/ml$ are effective in treating E. coli O157:H7 infection in C. elegans strain AU37. Control worms were fed with ampicillin concentration $8\mu g/ml$, $16\mu g/ml$, and $32\mu g/ml$ in 96 transwell plate to ensure ampicillin concentrations was toxic to the worms (results not shown). The control results are contradictory, as ampicillin did not affect the life-span of the worms in a first trial, whereas ampicillin decreases the life-span of the worms compared to the control in a second trial. However, the treatment with ampicillin on infected worms did not appear as toxic, since the life-span of the infected worms treated with ampicillin survived significantly more than infected alone (Figure 6 (B-D)) and mimiced the survival of the control worms (not shown). Further studies on the appearance of the worms (body size and shape) and its activity (movement and pharyngeal pumping) need to study to enlighten the effect of ampicillin on C. elegans strain AU37.

Intestinal bacterial colonization assay was performed on infected and ampicillin treated worms to determine whether the ampicillin treatment reduced the bacterial colonization overtime in worms intestine. Approximately 10 worms were used to determine the intestinal bacterial colonization. Treatment with ampicillin dose $16\mu g/ml$ and $32\mu g/ml$ significantly decreased intestinal bacterial colonization in the worms (1.38 log) compared to the infected worms without ampicillin treatment (Figure 6E). The results indicates that E21B has colonized and replicated in worms intestine, which supports the findings of Chou et al. (2013);
and the treatment with ampicillin decreased the intestinal bacterial colonization in the worms. The experiment was performed twice (independently) with the significant result when infected worms were treated with ampicillin. These findings suggest *C. elegans* as an appropriate model to use as an *in-vivo* model to study bacterial infection and its treatment. However, there was variability on the life-span of the worms between both trials. This could possibly be due to the initial infection in the worms intestine (4.9 x 10^2 CFU/worm in first trial compared to 2.4 x 10^2 CFU/worm for the results shown). In both experiments, worms were fed with the same number of bacteria, however, the number of bacteria eaten by worms varies and could then influence the life-span.
**Figure 6** *C. elegans* AU37 infection with *E. coli* O157:H7 (E21B) in a liquid medium using ampicillin treatment. *C. elegans* were grown to L4/young adults and transferred to 24 well plate containing $\approx 10^9$ CFU *E. coli* O157:H7 and $\approx 10^9$ CFU *E. coli* OP50 (control) per 1ml M9 buffer (v/v). Worms were fed with E21B and OP50 for 24 hours in an M9 buffer. Worms were transferred to 96 transwell plate and fed with live *E. coli* OP50 and ampicillin (0µg/ml, 8µg/ml, 16µg/ml, and 32µg/ml). GraphPad Prism 7 was used to obtain the p-value and T50 (Table 2) (A) Life-span of control worms (OP50) and infected worms (E21B) without ampicillin treatment. (B) Life-span of infected worms with 8µg/ml ampicillin (Amp 8) and without ampicillin (E21B) treatment. (C) Life-span of infected worms with 16µg/ml ampicillin (Amp 16) and without ampicillin (E21B) treatment. (D) Life-span of infected worms with 32µg/ml ampicillin (Amp 32) and without ampicillin (E21B) treatment. (E) Intestinal colonization of infected worms at Day 0 and worms with and without ampicillin treatments (E21B, Amp 8, Amp 16, Amp 32) at Day 24, the dotted line indicated minimum detectable level ($1 \times 10^1$ CFU/worm).
Table 2 Statistical summary of *C. elegans* AU37 infection with E21B

<table>
<thead>
<tr>
<th>Infection</th>
<th>Ampicillin Treatment (µg/ml)</th>
<th>Median life-span T₅₀ (days)</th>
<th>Log-rank (Mantel-Cox) test</th>
<th>Gehan-Breslow-Wilcoxon test</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> OP50</td>
<td>0</td>
<td>20</td>
<td>0.0208</td>
<td>0.0236</td>
<td>Yes</td>
</tr>
<tr>
<td><em>E. coli</em> E21B</td>
<td>0</td>
<td>18</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>E. coli</em> E21B</td>
<td>8</td>
<td>22</td>
<td>0.0047</td>
<td>0.0075</td>
<td>Yes</td>
</tr>
<tr>
<td><em>E. coli</em> E21B</td>
<td>16</td>
<td>22</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>Yes</td>
</tr>
<tr>
<td><em>E. coli</em> E21B</td>
<td>32</td>
<td>20</td>
<td>0.0001</td>
<td>0.0002</td>
<td>Yes</td>
</tr>
</tbody>
</table>

GraphPad Prism 7 for Mac OS was used to obtain statistical summary on *C. elegans* AU37 infection with E21B in a liquid medium using ampicillin treatment. The p-value confirms the life-span study (Figure 4 (A-D)) is significant. T₅₀ indicates that the worms treated with ampicillin (8µg/ml and 16µg/ml) have significantly higher survival rate compared to the worms without treatment. A p-value less than 0.05 is significant. T₅₀ defines as a time taken when 50% of the worms died.
5.4 Heat stress is not an effective way to reduce the life span of AU37

It is known heat stress reduces life-span of the worms, hence, experimental time, imitating the results with non-heat stress worms (Shen et al., 2017). We were interested to see if we can shorten the experimental time and obtain our results faster. In heat stress assay, the *C. elegans* incubation was held at 37°C after infection. Heat stress assay shortens the time required to perform an infection-treatment experiment, from days to hours (Figure 7(A-C)). However, we concluded that heat stress is not an effective way to reduce the experimental time, as we observed inconsistent results (Table 2). Variability in results may be due to counting error in the liquid medium, since at 37°C worms stops movement and becomes straight, mimicking dying posture when still alive. In addition, the growth rate of *E. coli* is faster at 37°C compared to at 25°C (Olanya et al., 2014), thus it may be a combination of stressed *C. elegans* and increased infection rate by *E. coli* O157:H7.
Figure 7 Heat stress assay. The procedure for the infection-treatment assay was same as section 5.3, except the incubation temperature, which was 37°C. (A) Life-span of infected (E21B) and non-infected (OP50) *C. elegans* strain AU37, without ampicillin treatment. (B) Life-span of infected worms with 16µg/ml ampicillin (Amp 16) and without ampicillin (E21B) treatment. (C) Life-span of infected worms with 32µg/ml ampicillin (Amp 32) and without ampicillin (E21B) treatment.
Table 3 Heat stress assay

<table>
<thead>
<tr>
<th>Infection</th>
<th>Ampicillin Treatment (µg/ml)</th>
<th>Median life-span $T_{50}$ (Hours)</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P-value compared to E21B</td>
<td>Significant?</td>
<td>P-value compared to E21B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Log-rank (Mantel-Cox) test</td>
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<td>Log-rank (Mantel-Cox) test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gehan-Breslow-Wilcoxon test</td>
<td></td>
<td>Gehan-Breslow-Wilcoxon test</td>
</tr>
<tr>
<td>E. coli OP50</td>
<td>0</td>
<td>4</td>
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<td>5</td>
</tr>
<tr>
<td>E. coli E21B</td>
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<td>NA</td>
</tr>
<tr>
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<td>8</td>
<td>NA</td>
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<td>3</td>
</tr>
<tr>
<td>E. coli E21B</td>
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<td>1</td>
<td>0.0954</td>
<td>3</td>
</tr>
<tr>
<td>E. coli E21B</td>
<td>32</td>
<td>1</td>
<td>0.3133</td>
<td>NA</td>
</tr>
</tbody>
</table>

GraphPad Prism 7 for Mac OS was used to obtain statistical summary on heat stress assay. Non-infected worms (E. coli OP50) and infected worms with ampicillin treatment were compared with infected worms (E21B) to obtain the p-value. The p-value using Gehan-Breslow-Wilcoxon test confirms significant difference between non-infected and treated worms compared to the infected worms in experiment 1 but not significant during experiment 2. A p-value less than 0.05 is significant.
5.5 Effect of ampicillin treatment on AU37 fed with acid stressed *E. coli* O157:H7 cells

The *C. elegans* assay could be used to determine if bacterial pathogens exposed to conditions found in foods are more or less susceptible to antibiotic treatment *in-vivo*. An acid-stressed experiment was performed because of the concern that for EHEC infection the *E. coli* cells needs to pass through our stomach acid, where they may encounter acid stress, in order to colonize our gut. Growth curve was performed for *E. coli* O157:H7 grown in a Luria-Bertani broth at pH 6.90 and pH 5.25 to determine the estimate time required for the cells to reach late exponential stage (Figure 8).

To determine the effect of acid-stressed *E. coli* O157:H7 (E21B) cells on *C. elegans* infection and treatment, we had grew E21B in a Luria-Bertani broth with a pH 5.25±0.05 (referred to as acid stressed cells) for 12-14 hours at 37°C till the bacteria is in late exponential stage to fed the L4-stage worms. As a comparison, *C. elegans* AU37 were infected with E21B grown in Luria-Bertani broth with a pH of 6.65±0.05 for 6-8 hours at 37°C. Control was *C. elegans* mutant AU37 fed with *E. coli* OP50 grown in Luria-Bertani broth with a pH of 6.65±0.05 for 6-8 hours and pH of 5.25±0.05 for 16-18 hours at 37°C till the cells reach to the late exponential stage. Further experimental procedure was run in the same manner as mentioned in section 5.3.

Our results show no significant difference between infected worms fed with E21B grown at pH 6.65±0.05 and worms fed with E21B grown at pH 5.25±0.05
(Figure 9A). In addition, treatment with 8µg/ml and 32µg/ml ampicillin concentration results in no significant difference in the life-span of *C. elegans* mutant AU37 fed with E21B grown at pH 6.65±0.05 compared to the worms fed with E21B grown at pH 5.25±0.05 (Figure 9 (B, D)). However, treatment with 16µg/ml ampicillin concentration results in significantly shorter the life-span of *C. elegans* AU37 fed with E21B grown at pH 5.25±0.05 compared to the worms fed with E21B grown at pH 6.65±0.05 (p < 0.05) (Figure 9C).

Treatment with ampicillin did not increase the life-span of infected worms fed with E21B at either pH compared to the infected worms without treatment (Figure 10 (A-F)). However, it shows increased survival of the treated worms with ampicillin at the median life-span (T₅₀) compared to the infected worms with E21B at pH 6.65±0.05. These results indicate that there can be variability in this test system, since previously (Section 5.3), we found the ampicillin treatment increased the life-span of infected worms. Differences in this result may be due to higher initial infection per worm in this experiment (8.3 x 10² CFU/worm compared to 2.4 x 10² CFU/worm). Another possibility, may be due to the use of late exponential stage *E. coli* O157:H7 cells in this set of experiments, compared to the use of stationary phase *E. coli* O157:H7 cells in section 5.3. The last reason could be due to the age of our stock *C. elegans* culture due to the storage at 15°C. Despite not seeing a statistical increase in life-span, ampicillin treatment decreased the number of bacterial counts in worms intestine (Figure 9E). Figure 9E shows the bacterial colonization in worms intestine at Day 14. Initial bacterial colonization at Day 0 in
worms infected with E21B grown at pH 6.65±0.05 was 8.3 x 10^2 CFU/worm and in worms infected with E21B grown at pH 5.25±0.05 was 1.6 x 10^3 CFU/worm. At day 14, ampicillin treatment with 16µg/ml decreased the intestinal colonization of worms fed with E21B grown at pH 5.25±0.05 by approximately 1.8 log compared to the 1 log reduction in the worms fed with E21B grown at pH 6.65±0.05, however failed to increase the life-span of the infected worms. Further study is needed to understand such effect on the life-span of worms.

**Figure 8** E. coli O157:H7 growth curve at pH 6.90 vs at pH 5.25. Blue curve is the growth of *E. coli* O157:H7 in a Luria-Bertani broth pH 6.90 at 37°C. Red curve is the growth of *E. coli* O157:H7 in a Luria-Bertani broth pH 5.25 at 37°C.
Figure 9 Effect of ampicillin treatment on *C. elegans* AU37 fed with E21B grown on pH 6.65 vs. E21B grown on pH 5.25. *C. elegans* were grown to L4/ young adults and transferred to 24 well plate containing \( \approx 10^9 \) CFU E21B grown at pH 6.65±0.05 and \( \approx 10^9 \) CFU E21B grown at pH 5.25±0.05 per 1ml M9 buffer (v/v). Worms were fed with E21B for 24 hours in an M9 buffer. Worms were transferred to 96 transwell plate and fed with live *E. coli* OP50 and ampicillin (0\( \mu \)g/ml, 8\( \mu \)g/ml, 16\( \mu \)g/ml, and 32\( \mu \)g/ml). GraphPad Prism 7 was used for statistical analysis. (A) Life-span of infected worms without ampicillin treatment. (B) Life-span of infected worms with 8\( \mu \)g/ml ampicillin treatment. (C) Life-span of infected worms with 16\( \mu \)g/ml ampicillin treatment. (D) Life-span of infected worms with 32\( \mu \)g/ml ampicillin treatment. (E) Intestinal colonization of infected worms at Day 14 with and without ampicillin treatments (E21B, Amp 8, Amp 16, Amp 32), the dotted line indicates minimum detectable level (1 x \( 10^1 \) CFU/worm).
Figure 10 Effect of ampicillin treatment on C. elegans AU37 fed with E21B grown on pH 6.65 and E21B grown on pH 5.25. C. elegans were grown to L4/young adults and transferred to 24 well plate containing $10^9$ CFU E21B grown at pH 6.65 and $10^9$ CFU E21B grown at pH 5.25 per 1ml M9 buffer (v/v). Worms were fed with E21B for 24 hours in an M9 buffer. Worms were transferred to 96 transwell plate and fed with live E. coli OP50 and ampicillin (0µg/ml, 8µg/ml, 16µg/ml, and 32µg/ml). GraphPad Prism 7 was used for statistical analysis. (A) Life-span of infected worms fed with E21B grown at pH 6.65 (Infected pH 6.65) and treated with 8µg/ml ampicillin (Amp 8 pH 6.65). (B) Life-span of infected worms fed with E21B grown at pH 6.65 (Infected pH 6.65) and treated with 16µg/ml ampicillin (Amp 16 pH 6.65). (C) Life-span of infected worms fed with E21B grown at pH 6.65 (Infected pH 6.65) and treated with 32µg/ml ampicillin (Amp 32 pH 6.65). (D) Life-span of infected worms fed with E21B grown at pH 5.25 (Infected pH 5.25) and treated with 8µg/ml ampicillin (Amp 8 pH 5.25). (E) Life-span of infected worms fed with E21B grown at pH 5.25 (Infected pH 5.25) and treated with 16µg/ml ampicillin (Amp 16 pH 5.25). (F) Life-span of infected worms fed with E21B grown at pH 5.25 (Infected pH 5.25) and treated with 32µg/ml ampicillin (Amp 32 pH 5.25).
CHAPTER 6

CONCLUSIONS

In conclusion, *C. elegans* needs live *E. coli* OP50 to get enough nutrients to grow and survive. *C. elegans* strain AU37 is more suitable compared to N2 strain for the *E. coli* O157:H7 infection-treatment assay, as AU37 is more susceptible to bacterial infection compared to wild-type N2 strain.

Our results indicate that *C. elegans* is an appropriate model to use as an in-vivo study for EHEC infection and treatment as *E. coli* O157:H7 colonize the worm’s intestine and the treatment with ampicillin concentration 8µg/ml, 16µg/ml, and 32µg/ml significantly increases the life-span of the worms compared to the infected worms without ampicillin treatment.

In contrast, when ampicillin treatment was used on acid-stressed and non-acid-stressed late exponential stage *E. coli* O157:H7 cells, it failed to increase the life-span of the worms infected with acid-stressed and non-acid stressed *E. coli* O157:H7 cells. This may possibly be due to the use of late exponential stage *E. coli* O157:H7 cells compared to the use of stationary phase *E. coli* O157:H7 cells in the previous experiment. In addition, ampicillin treatment with concentration 16µg/ml on *C. elegans* infected with acid-stressed *E. coli* O157:H7 cells significantly reduces the life-span of the worms compared to the ampicillin treated (16µg/ml) non-acid stressed *E. coli* O157:H7 cells, even though ampicillin reduces the intestinal bacterial colonization to approximately 1.8 log compared to approximately 1 log for non-acid
stressed *E. coli* O157:H7. Hence, further studies need to perform to understand such effect on the life-span of the worms.
APPENDIX

EXPANDED LAB PROCEDURES

E. coli O157:H7 INFECTION-TREATMENT IN C. ELEGANS PROCEDURE

1. **Preparation of nematode growth media (NGM) plate and concentrated E. coli OP50**
   - Prepare NGM plate and concentrated E. coli OP50 a week before the start of the experiment.
   
   A. **NGM plate seeded with OP50 preparation**
      - Prepare NGM plate using NGM growth media and follow the manufacture instructions.
      - Grow *E. coli* OP50 in a Luria-Bertani broth for 12-14 hours at 37°C and pipet 1ml onto each of the 2 days old NGM plate. Let the plate sit at the room temperature for another two days. This is referred to as OP50 seeded NGM plates. Store the plates at 4°C till ready to use.
   
   B. **Concentrated *E. coli* OP50**
      - Grow *E. coli* OP50 in a Luria-Bertani broth for 12-14 hours at 37°C and centrifuge using a 50ml centrifuge tube, collect the pellet and discard the supernatant.
      - Weigh the pellet and prepare 100 mg/ml OP50 concentration using sterile water. This is referred to as concentrated OP50 for *C. elegans* food in 96 transwell plate.

2. **C. elegans stock culture preparation**
   - Chunk a piece of agar containing many AU37 worms received from CGC or from your old NGM plate containing AU37 worms and place it onto your freshly prepared NGM agar seeded with OP50. Incubate the plate at 15°C. Plate can be stored at 15°C for up to 4 months and can be used when needed.

3. **Infection-Treatment life-span assay using *C. elegans* strain AU37**
   
   **Day 1:** Chunk a piece of NGM agar
   - Chunk a piece of agar containing many AU37 worms and transfer it onto three new NGM plates seeded with OP50. Incubate the plates at 15°C for approximately 4 days for the worms to lay eggs.

   **Day 5:** Collect the eggs (synchronization of worms)
   - Wash each of the NGM plates having many eggs with approximately 3 ml sterile water for couple of time. Dispense the washed liquid containing worms into 15ml centrifuge tube.
• Centrifuge at 2000g for 2mins. Discard the supernatant.
• Prepare 10ml bleach solution (1ml 10M NaOH, 1.8ml household bleach, and 7.2ml sterile water) into a 15ml centrifuge tube (You can prepare 5ml or 10ml bleach depends on the density of worms you have).
• Add the bleach solution to the washed worms tube, and vortex at high speed for 2mins. Centrifuge at 2000g for 1min and discard the supernatant. Add approximately 10ml M9 buffer to neutralize the bleach effect and vortex little (All this process in this step should be really fast, should not exceed >3.5 mins, or the eggs won’t hatch out the next day), and then centrifuge at 2000g for 2mins, and discard the supernatant. At this stage you should have only eggs in your tube (may contains few dead worms).
• Add 10ml M9 buffer one more time and centrifuge at 2000g for another 2mins, discard the supernatant, and add 1ml of M9 buffer.
• Incubate the centrifuge tube containing eggs horizontally at 25°C for 24 hours for the eggs to hatch out. After 24 hours the worms should be in L1 stage.

Day 6: Transfer of L1 stage worms and E21B streak plate
A. Transfer the L1 stage worms using a pipet onto NGM plate seeded with OP50 and incubate the plate at 25°C for 45-50 hours. Worms should be at L4-young adult stage after 45-50 hours.
B. Streak a new Tryptic Soy Agar (TSA) plate from a frozen E21B stock and incubate at 37°C for 18-20 hours. Follow the same procedure with OP50.

Day 7: E21B and OP50 culture preparation
• Transfer 3 colonies from streaked E21B from Day 6 into two 10ml Luria-Bertani broth. Incubate the tubes at 37°C for 18-20 hours on a 125rpm shaker. Follow the same procedure with OP50.

Day 8: Infecting worms with E21B

A. Preparation of E21B and OP50 culture
• Take out E21B and OP50 culture tubes from Day 7, dilute the culture 10x, and read the absorbance at wavelength 625nm using spectrophotometer. Absorbance of 0.08 – 0.1 at wavelength 625nm is consider having approximately 10^8 CFU/ml bacteria.
• Centrifuge the original 9 ml E21B and OP50 culture containing approximately 10^9 CFU/ml bacteria at 2500g for 10 minutes and discard the supernatant.
• Add 0.9 ml M9 buffer to each tube to make the final bacterial concentration of 10^10 CFU/ml.

B. Preparation of worms
Pipet approximately 2-3ml M9 buffer into each of the Day 6 plates containing synchronized L4-young adult stage AU37 worms and collect it into a 15ml centrifuge tube. Centrifuge the worms at 2000g for 1min with M9 buffer three times to remove any OP50 residue. Discard the supernatant using pipet.

- Add 1ml of M9 buffer and transfer the mixture into 2ml micro centrifuge tube.
- Pipet five 10 µl drops on a sterile petri dish and count the number of worms. Average the worms per 10 µl and record the number.

C. Infection in 24 well plate
- Add approximately 100 worms into 24 well plate and make the final volume to 900 µl using M9 buffer.
- Add 100 µl E21B to make the final E21B concentration of 10⁹ CFU/ml. Run control on side by adding 100 µl OP50 to make the final OP50 concentration of 10⁹ CFU/ml.
- Incubate the plate at 25°C for 24 hours.

Day 9: Treatment with ampicillin

A. Preparation of bacterial food with varied ampicillin concentration
- Add concentrated OP50 to the final concentration of 3mg/ml and ampicillin to the final desired concentration using M9 buffer in a sterile tube. Mix the mixture well for 1min using vortex at high speed.
- Add 250 µl of the food mixture to the bottom of 96 transwell plate.

B. Preparing and adding worms to use in 96 transwell plate
- Transfer infected worms (E21B) from Day 8 into 15ml centrifuge tube. Follow same procedure for control (OP50) worms.
- Centrifuge the worms at 2000g for 1 minute and discard the supernatant. Add M9 buffer and repeat the procedure five times to remove most residue of E21B and OP50.
- Pipet five 10µl drops of each of the infected and control worms into a sterile petri dish to determine the number of worms per 10µl. Average and record the number.
- Transfer approximately 10 infected worms into a micro centrifuge tube to determine the bacterial colonization in worms’ intestine.
- Transfer approximately 10 worms into each of the insert well in transwell plate containing food in the bottom wells.
- Incubate the plate at 25°C and record the number of viable worms every day to every other day.

C. Bacterial colonization in the worms
• Add 1mM sodium azide to the final volume of 250µl into approximately 10 infected worms tube.
• Centrifuge the worms at 2000g for 1min and discard the supernatant using pipet. Add 1mM sodium azide to the final volume of 250µl and repeat the step 3 times.
• Add 50µl of supernatant to the Sorbitol MacConkey Agar to determine the external bacterial count.
• Add approximately 400mg of 1mm silicon carbide particles into the tube containing 10 infected worms and vortex at high speed for 1min to disrupt the worms.
• Perform serial dilution and plate onto Sorbitol MacConkey Agar to determine total bacterial count (Internal + External) per 10 worms.
• Incubate the plates at 37°C for 18-20 hours.


