

**ENVIRONMENTAL FACTORS REGULATE DEVELOPMENTAL RATE IN**  
***C. ELEGANS***

MSc Thesis – Sabih Rashid

McMaster University – Biochemistry and Biomedical Sciences

**INVESTIGATING THE IMPACT OF BACTERIAL DIET ON  
DEVELOPMENTAL RATE IN THE MODEL ORGANISM *CAENORHABDITIS*  
*ELEGANS***

**By**

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the  
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MSc Thesis – Sabih Rashid

McMaster University – Biochemistry and Biomedical Sciences

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### ABSTRACT

Environmental factors, such as diet, can have a significant impact on the health of animals, influencing lifespan, development, and disease progression. The model organism *Caenorhabditis elegans* is a bacterivore whose development is characterized by an invariant pattern of cell division. This study investigated how *C. elegans* developmental rate is altered in response to 48 different bacterial diets. The bacterial species studied had a wide range of effects on developmental progression, with some bacteria dramatically decreasing developmental rate, while others caused developmental arrest in early larval stages. From these analyses, *Staphylococcus* species that caused very slow development of animals in the L1 stage were selected for further characterization. The slow developmental rate observed in these animals was rescued by supplementation with essential amino acids. Genetic analysis revealed that these effects were mediated through TOR signaling and were independent of insulin signaling. Loss of *daf-15(raptor)*, a central component of TOR complex 1, inhibited the rescuing effects of the amino acid supplementation on developmental rate, suggesting that the slow development induced by *Staphylococcus* could be rescued by activating the TORC1 pathway. Autophagy, negatively regulated by TOR, is increased in worms fed *Staphylococcus* species, suggesting TOR activity is reduced, and supplementation through amino acids reduced autophagy. These data suggest that TOR signaling is reduced in *C. elegans* in response to certain bacterial diets, resulting in decreased developmental rate, and that this effect may be due to amino acid deficiency.

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## TABLE OF CONTENTS

Abstract	iii
Acknowledgements	iv
Table of contents	v
List of figures	viii
List of abbreviations	xi
Declaration of academic achievement	xii
Chapter 1 – Introduction	1
1.1 – General introduction	2
1.1.1 - Environmental regulation of health and disease	2
1.1.2 – Introduction to <i>C. elegans</i>	3
1.2 – Development of <i>C. elegans</i>	5
1.2.1 - Introduction to <i>C. elegans</i> development	5
1.2.2 - The dauer stage	7
1.2.3 - Factors regulating developmental rate in <i>C. elegans</i>	8
1.3 – Signaling pathways that regulate <i>C. elegans</i> development	9
1.3.1 - Insulin/insulin-like growth factor signaling in <i>C. elegans</i>	8
1.3.2 - TOR signaling in <i>C. elegans</i>	12
1.4 - <i>C. elegans</i> metabolism, nutrition, and diet	15
1.4.1 - <i>C. elegans</i> diet and development	15
1.4.2 - <i>C. elegans</i> essential nutrients	15
1.4.3. - Bacterial metabolism	18
1.5 - <i>C. elegans</i> innate immunity and pathogen response	19
1.6 – Project Goals	23
Chapter 2 – Materials and Methods	25

2.1 – Bacteria and nematode strains	26
2.2 – Nematode synchronization and general maintenance	28
2.3 – Monitoring <i>C. elegans</i> development	28
2.3.1 - More precise determination of developmental stage using <i>hlh-8::gfp</i> worms	29
2.3.2 - Analysis of <i>mlt-10::gfp-pest</i> worms	29
2.3.3. - Measuring developmental rate of worms at different larval stages on the test bacteria	30
2.3.4 – Testing whether metabolite supplementation increases <i>C. elegans</i> developmental rate on certain bacteria	31
2.4 - Determining whether signaling pathways play a role in slowing development on the test bacteria	34
2.4.1 - Observing <i>cki-1::gfp</i> expression in L1 worms	34
2.4.2 – Measuring autophagy using <i>LGG-1::gfp</i>	34
2.4.3 – Measuring developmental rate of TOR and insulin/IGF-1 signaling pathway mutants on the test bacteria	34
2.5 - Testing survival of worms	35
2.5.1 - Survival of adult worms	35
2.5.2 - Survival of L1 worms	36
2.5.3 - Survival of worms following starvation	36
2.6 – Statistical analysis	37
Chapter 3 - Results	40
3.1 - <i>C. elegans</i> developmental rate is influenced bacterial diet	41
3.1.2 - Timing of <i>C. elegans</i> molting is altered in response to bacterial diet	45
3.1.3 – Earlier larval stages are more heavily slowed on some bacterial diets	48
3.2 - Loss of <i>daf-16</i> does not rescue development of worms on slow growth/arrest causing bacteria	56
3.3 - Nutrient supplementation increases developmental rate of worms on test bacteria	60

3.3.1 - Heme supplementation rescues arrested development of worms	60
3.3.2 - Amino acids, but not vitamins, appears to increase developmental rate of worms on <i>Staphylococcus</i> species	64
3.3.3 – Supplementation with different amino acids rescues development on different <i>Staphylococcus</i> species	69
3.3.4 - Addition of glucose does not increase developmental rate in worms fed <i>Staphylococcus</i> species	75
3.4 - TOR signaling is required for amino acid-mediated developmental rescue on <i>Staphylococcus</i> species	77
3.5 - Some bacterial species are pathogenic to <i>C. elegans</i>	84
3.5.1 - Survival of adult worms is decreased on some bacterial diets	84
3.5.2 - Survival of L1 worms is reduced on the <i>Staphylococcus</i> species	88
Chapter 4 – Discussion and conclusion	91
4.1 - <i>C. elegans</i> development is heavy slowed by some bacterial species	92
4.2 – Loss of <i>daf-16</i> does not rescue slow development on tested bacteria	94
4.3 – Supplementation with specific nutrients can accelerate developmental rate in response to poor bacterial diets	95
4.4 – TOR signaling regulates slow development on <i>Staphylococcus</i> species	98
4.5 – Pathogenic effects from tested bacteria may impact development	100
4.6 - Conclusions and future directions	102
Chapter 5 – Supplementary Figures	107
References	123



**LIST OF FIGURES**

Figure 1.1 – The developmental cycle of <i>C. elegans</i> grown at 25 °C on <i>Escherichia coli</i>	6
Figure 1.2 - The insulin/insulin-like growth factor 1 pathway in <i>C. elegans</i>	11
Figure 1.3 - The Target of Rapamycin (TOR) signaling pathway in <i>C. elegans</i>	14
Figure 1.4 – The MAPK immune response pathway in <i>C. elegans</i>	22
Table 2.1 – List of <i>C. elegans</i> strains used	26
Table 2.2 – List of bacterial strains used	27
Table 2.3 - Concentration of vitamins in mix	32
Table 2.4 - Concentration of amino acids in mix	33
Table 2.5 - Concentration of non-essential amino acids in mix	33
Figure 2.1 – Protocol for <i>E. coli</i> OP50 to test bacteria transfer experiment	38
Figure 2.2 – Protocol for survival assay	39
Figure 3.01 – Bacterial diet influences developmental rate in <i>C. elegans</i>	42
Table 3.1 - Categorized bacterial impact on <i>C. elegans</i> developmental rate	43
Figure 3.02 - Timing of <i>C. elegans mlt-10::gfp-pest</i> activation is severely delayed on certain bacterial diets	47
Figure 3.03 – <i>hlh-8::gfp</i> expression changes throughout <i>C. elegans</i> development	50
Figure 3.04 – <i>hlh-8::gfp</i> transgenic worm strains allow for precise determination of <i>C. elegans</i> larval stages	51
Figure 3.05 - <i>C. elegans</i> developmental rate is adjusted in response to bacterial diet	52
Figure 3.06 – Developmental slowing in <i>C. elegans</i> induced by bacterial diets occurs primarily in the L1 stage	54
Figure 3.07 – Developmental slowing in <i>C. elegans</i> induced by <i>Staphylococcus</i> species occurs primarily in the L1 stage	55
Figure 3.08 – <i>cki-1::gfp</i> is expressed in seam cells of <i>C. elegans</i> for several days when fed bacteria that induce slow development	57
Figure 3.09 – <i>daf-16</i> mutant development is not increased compared to wild type worms on test bacteria	59

Figure 3.10- Heme supplementation rescues developmental arrest induced by certain bacteria	62
Figure 3.11 – Proportion of fluorescent <i>mlt-10::gfp-pest</i> worms over time when grown on <i>E. coli</i> OP50 plates supplemented with heme.	63
Figure 3.12 - Casamino acid supplementation, but not vitamins, increases developmental rate of <i>C. elegans</i> grown on certain microbial bacterial species	65
Figure 3.13 - Amino acid supplementation increases developmental rate of worms fed some bacteria	67
Figure 3.14– Amino acid supplementation increases developmental rate of worms fed <i>Staphylococcus</i> species primarily in the L1 stage	68
Figure 3.15 – Different amino acid mixes rescue <i>C. elegans</i> development on different bacteria	71
Figure 3.16 - Amino acid induced changes in developmental rate depend on bacterial diet	72
Figure 3.17 - D-amino acid supplementation does not affect <i>C. elegans</i> developmental rate	74
Figure 3.18 - Non-essential amino acids, but not glucose, increase developmental rate of worms on some <i>Staphylococcus</i> species	76
Figure 3.19 - <i>rsk-1</i> mutations inhibits amino acid-mediated rescue of development of worms fed <i>Staphylococcus</i> species	80
Figure 3.20 - Developmental rate of <i>daf-15</i> mutant worms is not increased after amino acid supplementation on test bacteria	81
Figure 3.21– LGG-1:: <i>gfp</i> puncta count is increased on test bacteria, and reduced following amino acid supplementation	83
Figure 3.22 – Colonization of <i>C. elegans</i> intestine by <i>Staphylococcus warneri</i>	86
Figure 3.23 – <i>C. elegans</i> survival is decreased on several human respiratory microbiota.	87
Figure 3.24 - Survival of L1 worms is lower on <i>Staphylococcus</i> species and following starvation	90
Figure 4.1 - Regulation of <i>C. elegans</i> developmental rate by different bacterial species	105

Table S3.1 - Timing of <i>C. elegans mlt-10::gfp-pest</i> activation is severely delayed on certain bacterial diets	108
Figure S3.1 - <i>C. elegans</i> developmental rate is adjusted in response to bacterial diet	111
Figure S3.2 - Amino acid supplementation increases developmental rate of worms fed <i>Staphylococcus</i> species	117
Figure S3.3 - Amino acid supplementation increases developmental rate of worms fed <i>Staphylococcus</i> species primarily in the L1 stage	118
Figure S3.4 – Different amino acids rescue <i>C. elegans</i> development on different bacteria	120
Table S3.2 – <i>C. elegans</i> survival is decreased on different bacterial species	121
Table S3.3 - Early survival of <i>C. elegans</i> is decreased on <i>Staphylococcus</i> species	122

## **LIST OF ABBREVIATIONS**

- ANOVA – Analysis of variance  
ATP – Adenosine triphosphate  
BCAA – Branched-chain amino acid  
BHI – Brain Heart Infusion  
CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats  
dsRNA – Double-stranded RNA  
GFP– Green fluorescent protein  
L1 – Larval stage 1  
L2 – Larval stage 2  
L3 – Larval stage 3  
L4 – Larval stage 4  
mTOR – Mechanistic Target of Rapamycin  
NGM – Nematode Growth Medium  
RNAi – RNA interference  
TOR – Target of Rapamycin  
TORC1 – TOR complex 1  
TORC2 – TOR complex 2

**DECLARATION OF ACADEMIC ACHIEVEMENT**

This study was primarily designed and by the author, Sabih Rashid, under the supervision of Dr. Lesley MacNeil. Dr. MacNeil contributed significantly to the design of the experiments, as well as interpretation of the data. All experiments were carried out by the author. The thesis was written by Sabih Rashid, with significant input from Dr. MacNeil.

**CHAPTER 1 – INTRODUCTION**

## **1.1 - General Introduction**

### 1.1.1 – Environmental regulation of health and disease

The health and physiology of organisms is determined not only by genetics, but by environmental factors, as well. Environmental factors such as diet, pathogen exposure, and even exposure to different non-pathogenic bacterial species can impact an organism's metabolism, development, immune system, and lifespan (Rogol et al., 2000; Wolowczuk et al., 2008; Ege et al., 2011; English and Uller, 2016). Gene-environment interactions also play a substantial role in regulating development and disease, adding a further layer of complexity to the regulation of health and physiology (Edwards and Cooper, 2006; Kunes and Zicha; 2009, Baye et al., 2011; Eberhart et al., 2017). Diet can have a large impact on many human diseases, such as obesity and cardiovascular disease (Hruby et al., 2016; Anand et al., 2016). This environmental factor also provides a therapeutic opportunity, as control of diet and nutrition can minimize disease risk (Champagne et al., 2011; Segal and Opie, 2015). Understanding how environmental factors affect health and disease is invaluable to developing strategies to manipulate and control exposure to these factors, thus improving quality of life. In addition to diet, the microbiota, the collection of microorganisms that inhabits every individual, also influences human health (McFall-Ngai et al., 2013; Ursell et al., 2012). Changes in the composition of the microbiome have been associated with several diseases in humans (Clemente et al., 2012; Qing et al., 2017; Hou et al., 2017). The microbiota presents another therapeutic opportunity and attempts to manipulate the microbiota to improve disease outcomes have already shown some

success (Kang et al., 2017). Studying environmental regulation of human physiology presents many opportunities to further the understanding of health and disease progression, but the complexity of the human system, as well as ethical concerns, pose a challenge. The use of model organisms to study the impact of environmental factors on health may provide a solution. Understanding how factors such as diet and bacteria impact the health and development of a model organism with conserved genes and signaling pathways can provide insight into how those same factors impact human systems.

#### 1.1.2 – Introduction to *C. elegans*

*Caenorhabditis elegans* are found in soil throughout many parts of the world (Leung et al., 2008; Frezal and Felix, 2015). They exist primarily as self-fertilizing hermaphrodites, with 0.1-0.2% of the population being males that arise through nondisjunction of sex chromosomes during meiosis (Ward and Carrel, 1979; Chasnov and Chow, 2002). *C. elegans* are simple organisms, composed of 959 somatic cells in the hermaphrodite and 1031 in the males (Sulston et al., 1983). Although sex-specific differences affect most tissues, the basic body plan is similar for both males and hermaphrodites, and 650 cells are identical in both sexes (Reviewed in Herman, 2006). The entire neural connectome of *C. elegans* has also been mapped, and 302 neurons identified in nematode hermaphrodites (White et al., 1986). *C. elegans* are transparent animals, allowing for easy observation of their anatomy under a microscope. Their ease of maintenance, short development cycle, and large brood size ensures that a sizeable



population of genetically identical animals can be grown very quickly and easily for laboratory use.

*C. elegans* is a powerful genetic model that is amenable to genetic manipulation. Generation of mutant and transgenic animals is relatively simple in *C. elegans*, and powerful genetic techniques, such as CRISPR, have been established for use in the worm (Friedland et al., 2013; Dickinson and Goldstein, 2016). A large number of *C. elegans* mutants is readily available due to the collaborative efforts of the *C. elegans* research community (Thompson et al., 2013; The *C. elegans* Deletion Mutant Consortium, 2012). *C. elegans* are also sensitive to RNA interference, allowing for genes to be knocked down through simple feeding of dsRNA-producing bacteria (Timmons et al., 2003).

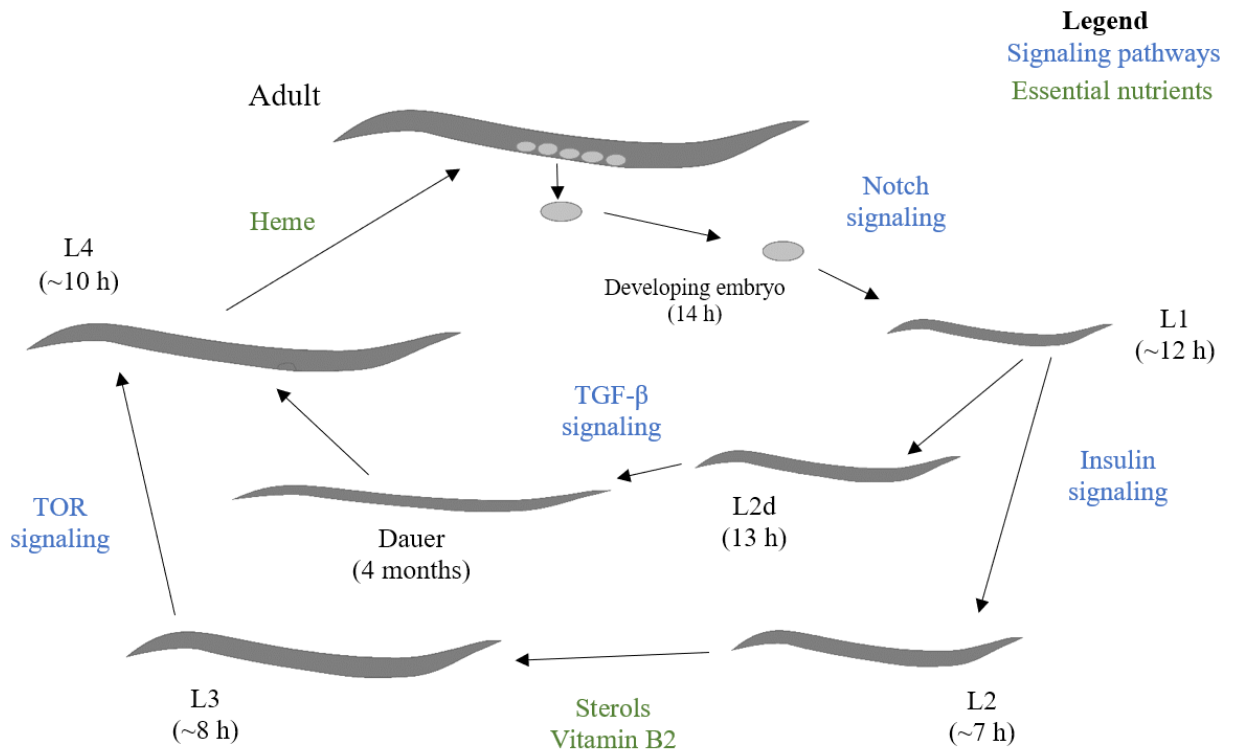
*C. elegans* were the first multicellular organism whose genome was fully sequenced (*C. elegans* Sequencing Consortium, 1998). The current genome annotation contains 20,359 predicted protein-coding genes (Sugi, 2016). *C. elegans* share a number of conserved genes and pathways with higher organisms, including humans, and orthologs of hundreds of human genes have been identified, including 533 disease-related genes (Kaletta and Hengartner, 2006; O'Brien et al., 2004). Many significant findings in the field of biology, including the discovery of the first microRNA and the identification of apoptosis, were first reported in *C. elegans* (Lee et al., 1993; Sulston and Horvitz, 1977). In addition to being used as a model for genetics and neurobiology, *C. elegans* have been commonly used as model organisms in the fields of microbiology and ecology, highlighting their versatility (Leung et al., 2008; Issi et al., 2017; Zhang et al., 2017).

## **1.2 - Development of *C. elegans***

### **1.2.1 - Introduction to *C. elegans* development**

*C. elegans* development is separated into an embryonic stage, four distinct larval stages (denoted L1 to L4), and the adult stage (Figure 1.1). *C. elegans* develop from embryos to adults in approximately three days at 20 °C on a diet of *E. coli* OP50, a uracil auxotroph mutant strain used for growth in laboratories, but developmental rate can be altered by temperature and diet (Gilbert, 2000; Palgunow et al., 2012; MacNeil et al., 2013; Gómez-Orte et al., 2018). Without sufficient food, *C. elegans* undergo larval arrest, during which they suspend development until sufficient nutrition is provided (Baugh, 2013).

*C. elegans* can produce approximately 300 eggs over the course of their lifespan, the majority of which are laid during the first few days of adulthood (Muschiol et al., 2009). Embryogenesis occurs partially in-utero, and the embryo continues to develop inside the egg after it is laid by the parent. Worms hatch from eggs after approximately 10 hours of development. Following this, worms will begin their development if environmental conditions are optimal, growing into gravid adults after approximately 60 hours (Gilbert, 2000). Each larval stage is separated by a molt, during which time worms shed their older cuticle and form a new, larger cuticle to accommodate their growing size. Each larval stage is divided into three substages: lethargus, in which animals decrease their activity; apolysis, where the old cuticle is loosened; and ecdysis, where the worm releases the old cuticle (Page and Johnstone, 2007).



**Figure 1.1 - The developmental cycle of *C. elegans* grown at 25 °C on *Escherichia coli***  
*C. elegans* hatch from embryos and progress through four distinct larval stages before becoming fertile adults. At 25 °C, worms take approximately 51 hours to grow from eggs to adults. During times of stress or starvations, worms are able to enter an alternate larval stage, the dauer, where they can survive for many months. Several signaling pathways regulating development shown in blue, positioned where they have important regulatory roles e.g. TGF-β signaling regulates dauer development, while loss of TOR signaling causes developmental arrest at L3. Some essential nutrients are also shown in green, positioned where they are needed for developmental progression e.g. heme is required for worms to develop from L4 to adults. Adapted from Strange, 2003.

### 1.2.2 - The dauer stage

*C. elegans* enter an alternate L3 larval stage in response to poor environmental conditions, known as the dauer stage (Golden and Riddle, 1984). Dauer worms effectively remain in stasis, not moving or feeding, and develop a tough cuticle inside which they can survive for months (Fielenbach and Antebi, 2008). During the first larval stage, *C. elegans* receive chemical signals from the environment, relaying information regarding nutrient availability, the density of the population, and temperature (Hu et al., 2007). If cues indicate an environment that is not optimal for growth, worms will enter a pre-dauer L2 stage. If these signals persist, worms will not proceed to the L3 stage and instead enter the dauer state. When favorable environmental conditions are restored, worms exit the dauer state and enter the L4 stage, developing normally into adulthood (Riddle et al., 1997).

During the dauer stage, worms metabolize fat stores to produce energy. Worms are able to survive on stored energy reserves for up to four months as dauers because many energy-consuming metabolic activities are suspended (Braeckman et al., 2009). Several genes and pathways regulating the dauer phenomenon have been identified (Wang and Kim, 2003). The insulin signaling pathway plays an important role in regulation of dauer entry, as loss of the insulin receptor *daf-2* results in constitutive dauer formation (Vowels and Thomas, 1992; Kimura et al., 1997). Previously known as dauer pheromone, several ascarosides regulate entry into the dauer state (Golden and Riddle, 1984; Kim et al., 2009). Loss of the G-protein subunits GPA-2 and GPA-3 results in a dauer defective phenotype, suggesting that these G-protein coupled receptors may

function to sense ascarosides and regulate dauer formation (Golden and Riddle, 1985, Zwaal et al. 1997). The TGF- $\beta$  pathway also regulates dauer formation, comprising a separate branch of the dauer regulatory pathway (Estevez et al, 1993; Inoue and Thomas, 2000; Shaw et al, 2007). The chemosensory amphid neuron ASI secretes the TGF- $\beta$  ligand DAF-7 in response to favorable environmental conditions, binding to *daf-1* and *daf-4* receptors to regulate dauer formation (Georgi et al, 1990; Estevez et al, 1993; Inoue and Thomas, 2000). Chemosensory neurons thus function to regulate dauer entry in *C. elegans* in response to environmental cues.

### **1.3 – Signaling pathways that regulate *C. elegans* development**

#### 1.3.1 - Insulin/insulin-like growth factor signaling in *C. elegans*

A complex network of signaling pathways exist in *C. elegans* that coordinate and interact to regulate cellular processes and ensure proper development. Insulin, TOR, and Notch signaling regulate development in response to nutrition (Riddle et al., 1981; Greenwald et al., 1983; Long et al., 2002; Jia et al., 2004; Ruaud et al., 2011). IGF/insulin signaling and TOR signaling are perhaps the most important regulators of early larval development in the worms, and both control development and metabolism in response to nutrient levels (Murphy and Hu, 2013).

Insulin/insulin-like growth factor signaling regulates developmental progression and longevity in *C. elegans*. The first long-lived mutant was identified in *C. elegans*, with a loss-of-function mutation in the insulin receptor DAF-2 leading to worms living twice as long as their wild-type counterparts (Kenyon et al., 1993; Kenyon, 2011). Insulin

signaling in *C. elegans* is comprised of one insulin receptor, DAF-2, which signals to a number of downstream targets, including the transcription factor DAF-16 (Figure 1.2). Forty insulin-like peptides (ILPs) are present, although the specific role of each ILP in development has not been fully elucidated (Hua et al., 2003). Many of these activate DAF-2, although some ILPs function as antagonists to the pathway. Activation of DAF-2 results in a kinase cascade that phosphorylates DAF-16 and prevents its entry into the nucleus. Only when DAF-16 is present in the nucleus can it regulate its downstream targets. (Ogg and Ruvkun, 1998; Lin et al., 2001; Fukuyama et al., 2006; Murphy et al., 2013). Control of *daf-16* nuclear translocation is one way by which *C. elegans* development is regulated (Lin et al., 2001; Ruaud et al., 2011). Many components of the insulin signaling pathway, including the PI3K/Akt kinase cascade, are conserved between *C. elegans* and humans, making *C. elegans* a useful tool for the study of the pathway (Murphy and Hu, 2006; Bulger et al., 2017).

Loss-of-function mutations of insulin signaling components result in changes to developmental rate and lifespan in *C. elegans* (Kenyon et al., 1993). *Daf-2* mutants are long-lived and dauer constitutive, entering the dauer state even when sufficient food is present. Conversely, *daf-16* mutants have short lifespans, cannot form dauers, and are able to bypass starvation-induced L1 arrest, as well as developmental arrest induced by starvation at later developmental stages (Vowels and Thomas, 1992; Ogg et al 1997; Fielenbach and Antebi, 2008; Gami and Wolkow, 2006; Kaplan and Baugh, 2016; Schindler et al., 2014). DAF-16 mutant animals continue development despite starvation, and some early cellular divisions, including those of the P and M cell lineages, occur

despite the lack of available nutrients (Kasuga et al., 2013). This may be due to a decrease in expression of the cell cycle inhibitor *cki-1* (Buck et al., 2009). Without *cki-1*, worms undergo additional larval cell divisions (Kostić et al., 2003). In wildtype worms, *cki-1* is upregulated in seam cells during starvation, and knockdown of *daf-16* diminishes the effect (Baugh and Sternberg, 2006), suggesting *daf-16* mutants have a reduced capability to inhibit cell division.

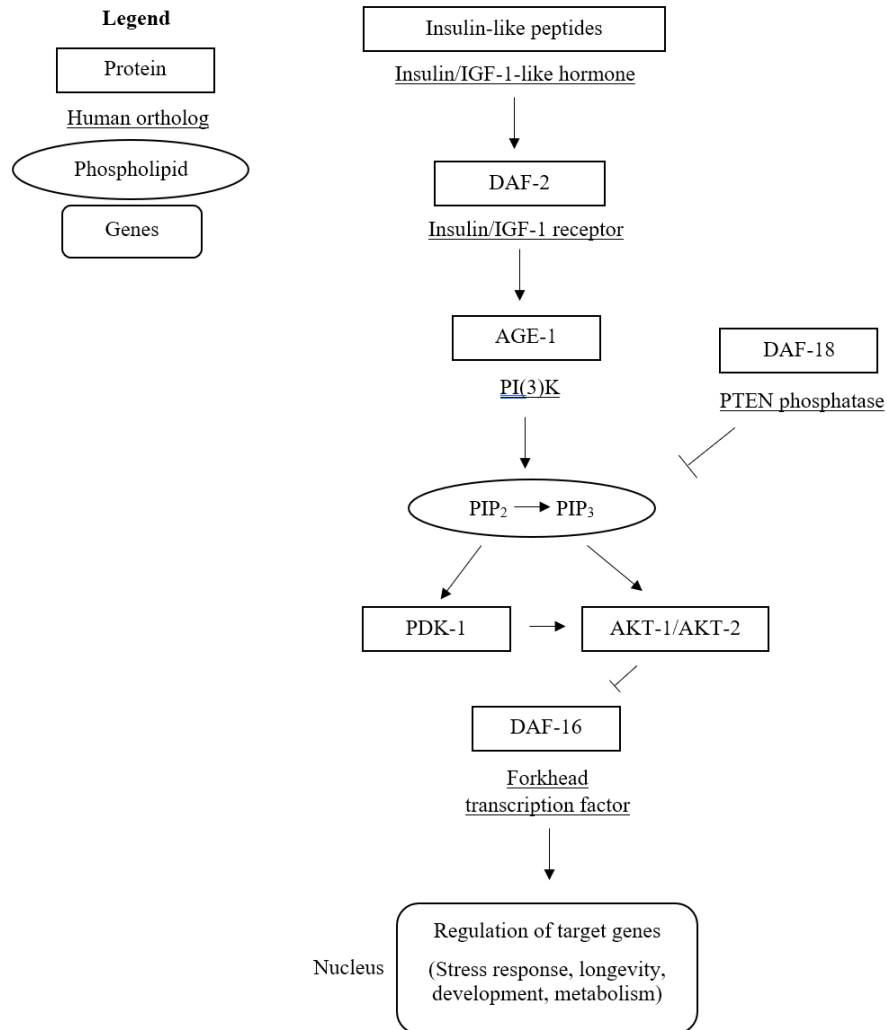


Figure 1.2 - The insulin/insulin-like growth factor 1 pathway in *C. elegans*

Activation of DAF-2 by agonistic insulin-like peptides results in the recruitment of the AGE-1 kinase which converts PIP<sub>2</sub> to PIP<sub>3</sub>. PIP<sub>3</sub> activates PDK-1 that in turn phosphorylates and activates ATK-1 and ATK-2, which phosphorylate DAF-16, excluding it from the nucleus and preventing its regulation of downstream targets. Conversely, binding of DAF-2 by antagonistic ILPs inhibits its activity, preventing the downstream kinase cascade and resulting in DAF-16 translocating to the nucleus to regulate its target genes, leading to increased resistance, longevity, upregulation of immunity genes etc. DAF-18 dephosphorylates PIP<sub>3</sub>, thus inhibiting activation of PDK-1 and AKT-1/2. Figure adapted from Guarente and Kenyon, 2000.



### 1.3.2 - TOR signaling in *C. elegans*

TOR signaling is an important regulator of *C. elegans* development. The mechanistic target of rapamycin, or mTOR, is the central protein of the conserved TOR signaling pathway, and is found in many organisms, including humans and *C. elegans*. First discovered in *Saccharomyces cerevisiae*, TOR is responsible for integrating nutrient and metabolic signals to promote growth and development (Heitman et al., 1991; Wullschlegel et al., 2006). TOR/LET-363 is a serine/threonine kinase that forms two functionally distinct multi-protein complexes, TORC1 and TORC2. These complexes share several subunits but are defined by one of two adaptor proteins, Raptor/DAF-15 (TORC1) or Rictor/RICT-1 (TORC2). Both complexes are conserved in *C. elegans* and promote developmental progression (Long et al., 2002; Soukas et al., 2009).

In *C. elegans*, loss of the TOR kinase *let-363* or the Raptor ortholog *daf-15* causes animals to enter an irreversible dauer-like state in L3. The mechanisms regulating the arrest specifically at the L3 stage are not fully understood, but are likely mediated through regulation of protein synthesis, as mutations in regulators of protein translation, such as eIF-4G, phenocopy many of the defects seen in *let-363* mutants (Long et al., 2002). Knockdown of *daf-15* increases longevity similar to *daf-2* (Jia et al., 2004). *Daf-15* mutants alter metabolism, accumulating increased fat relative to wild type animals (Jia et al., 2004). *daf-15* expression is regulated by DAF-16, suggesting the insulin and TORC1 signaling pathways interact to regulate growth and development in response to nutrition (Jia et al., 2004). Thus, effects on development through one of the pathways may require activity of the other.

One crucial role of TORC1 is to mediate an appropriate response to amino acid availability. The H<sup>+</sup>-adenosine triphosphatase ATPase (V-ATPase) is one example of an amino acid sensor that signals to the TOR complex, interacting with the Ragulator scaffolding complex in response to the presence of amino acids (Zoncu et al., 2011). The Ragulator anchors RAG GTPases to the lysosome, which recruits TOR to the lysosome and facilitates its binding with the GTP-binding protein Rheb, activating the TORC1 complex (Sancak et al., 2008; Sancak et al., 2010). *C. elegans* orthologs of Rheb and the Rag GTPases, as well as V-ATPase (Honjoh et al., 2009, Schreiber et al., 2010, Oka et al., 1998), suggesting these components may perform similar regulatory roles in *C. elegans*. However, the mechanisms by which worms sense amino acids and relay nutrient signals to TOR remain unclear. The MGL-1/2 metabotropic glutamate receptors, expressed in amphid neurons, regulate the starvation response in *C. elegans* in response to amino acids, suggesting sensing of amino acids through neurons may relay the signal (Kang and Avery, 2006).

The TORC1 complex activates several downstream targets, including the S6 Kinase, and the translation initiation factor eIF-4E BP1, regulating growth and longevity through control of protein synthesis (Brown et al., 1995; Hara et al., 1997). In *C. elegans*, both RSKS-1/S6K and IFE-2/eIF-4E BP1 are downstream targets of TOR and may function in a similar manner to regulate development (Pan et al., 2007; Hansen et al., 2007).

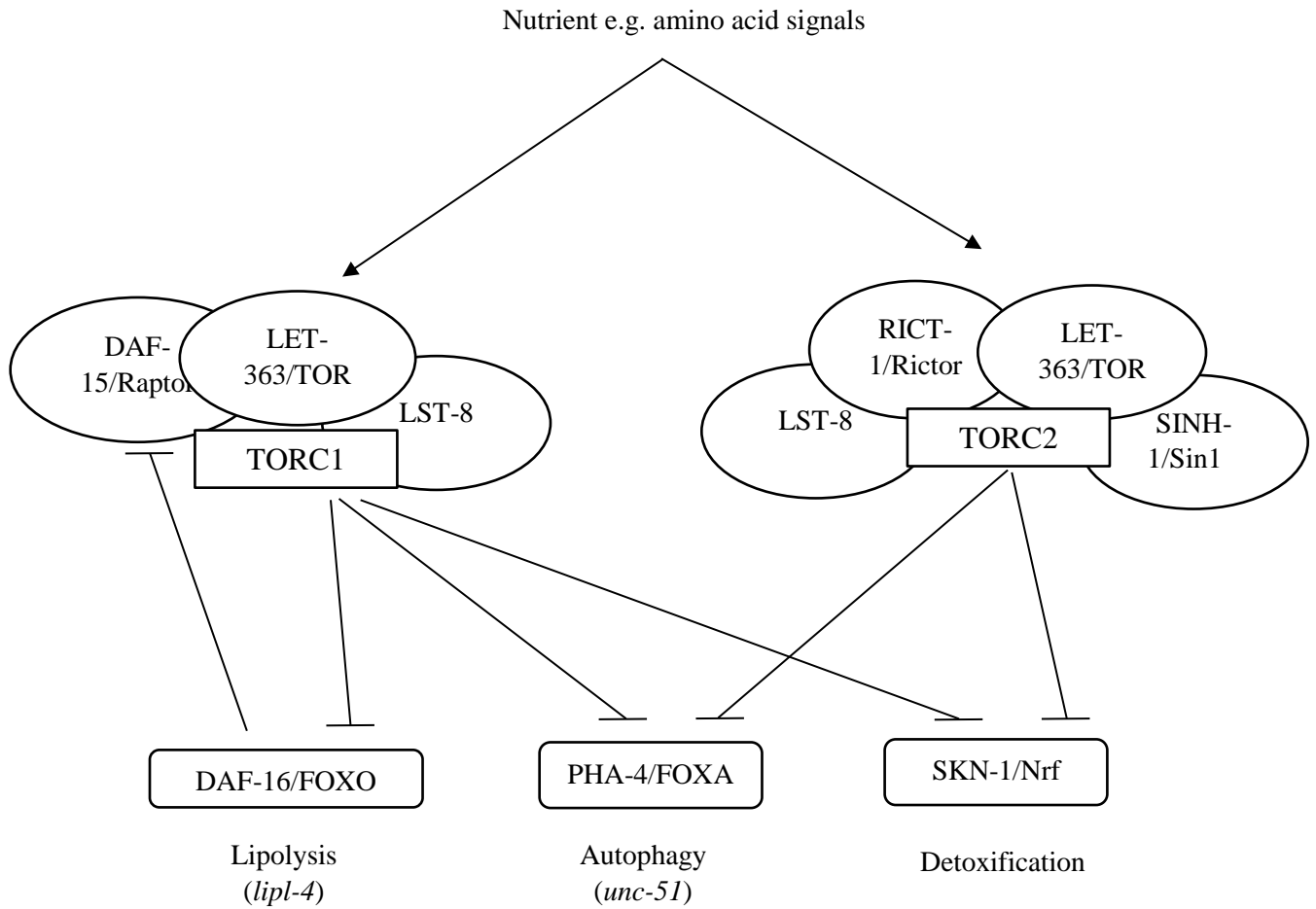


Figure 1.3 - The Target of Rapamycin (TOR) signaling pathway in *C. elegans*  
Signals from nutrients such as amino acids leads to the recruitment and activation of LET-363, the TOR kinase, which acts in two distinct complexes to modulate downstream transcription factors, including DAF-16, PHA-4, and SKN-1, regulating metabolism, growth, and longevity. Adapted from Lapierre et al., 2012.

## **1.4 - *C. elegans* metabolism, nutrition, and diet**

### 1.4.1 - *C. elegans* diet and development

*C. elegans* are able to survive and thrive feeding on a number of different bacterial species (Samuel et al., 2016). They can also be propagated in axenic media, although they develop at a very slow rate (Szewczyk et al., 2003). Different bacterial diets can influence a number of traits, including developmental rates, longevity, and fertility (Leroy et al., 2012; MacNeil et al., 2013; Sánchez-Blanco et al., 2016). For example, *Comamonas aquatica*, accelerates the developmental rate of *C. elegans* relative to *E. coli* OP50 (MacNeil et al., 2013). Changes in gene expression accompany this effect, with several genes associated with molting and metabolic enzymes regulated in response to diet. Additionally, the effect on developmental rate was independent of both TOR and insulin signaling, potentially acting through nuclear hormone receptors. Incidentally, while developmental rate was increased, the brood size of worms was decreased, suggesting that different bacterial diets may offer both advantages and disadvantages to the worm (MacNeil et al., 2013).

### 1.4.2 *C. elegans* essential nutrients

*C. elegans* have a number of essential nutrients that they require in their diet for optimal growth. In order to regulate development and, if needed, prevent larval progression in the absence of sufficient nutrients, *C. elegans* uses several developmental checkpoints where they will arrest their development until the necessary nutrients are supplied (Lee et al., 2009; Schindler et al 2014). Although animals can remain

developmentally arrested for several days on low-nutrient or inedible diet, survival in this arrested state decreases over time (Schindler et al., 2014; Szewczyk et al., 2006).

Heme is one nutrient that worms require in their diet. While worms are heme auxotrophs, they have heme-trafficking proteins that are homologs of several proteins found in vertebrates (Sinclair and Hamza, 2015). The heme responsive *hrg-4* is responsible for importing heme in the intestine, where it is stored or transferred to other tissues such as hypodermal cells and oocytes. In addition to obtaining heme from their diet, worms also receive some heme from the parent during embryogenesis. This process is mediated by the heme responsive gene *hrg-3* (Chen et al., 2011). *C. elegans* use heme both as a cofactor, and as a source of iron (Rao et al., 2005). Without sufficient heme, development is arrested at the L4 stage; conversely, excess heme, in concentrations of 800  $\mu\text{m}$  or higher, causes arrest at the L2 or L3 stage (Severance et al., 2010; Chen et al., 2011).

In addition to heme, several vitamins and amino acids are also required for proper development in *C. elegans*. Vitamins and micronutrients act as cofactors and donor molecules to fuel metabolic reactions. Depletion of these vitamins can induce developmental slowing or developmental arrest. However, the mechanisms that tie these arrests to the developmental timer are largely unknown. Vitamins such as riboflavin, thiamin, and Vitamin B12 are all required for development (Dougherty et al., 1959). The role of these vitamins in worms is similar to those in other organisms, with many acting as required cofactors and thus playing a role in metabolism (Bito et al., 2013). Vitamins, or lack thereof, can also indirectly alter *C. elegans* behavior. Lack of vitamin B2, for

example, results in a decrease of TOR activity and protease secretion in the intestine, allowing worms to sense and avoid low quality food (Qi et al., 2017). Vitamins thus play an essential role in *C. elegans* metabolism and development.

Vitamin B12 as well as Vitamin B9, or folate, act in the methionine/SAM cycle (Yilmaz and Walhout, 2014). Reducing folate intake in worms results in reproductive defects, as well as a shorter lifespan (Austin et al., 2010). Addition of low doses of folic acid can increase lifespan and delay aging through inhibition of both TOR and insulin signaling, suggesting it may affect *C. elegans* developmental rate as well (Rathor et al., 2015). Vitamin D can also affect *C. elegans* health, increasing longevity by activating stress response genes including *skn-1*, which regulates oxidative stress responses, and *ire-1*, required for the unfolded protein response (Mark et al., 2016). Micronutrients may thus have a major impact on the development and growth of an organism, and it is possible that *C. elegans* health on different bacterial diets may be affected by the presence, or absence, of such nutrients.

Like humans, *C. elegans* require several amino acids in their diet. The essential amino acids in *C. elegans* include the same nine amino acids essential in humans (histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine and valine), as well as arginine (Braeckman et al., 2009). In addition to being required for protein synthesis, these amino acids are required for the synthesis of several metabolites and can also serve as energy sources in low nutrient conditions (Kühnl et al., 2005). For example, mutations in the branched-chain  $\alpha$ -ketoacid dehydrogenase, which is required in the branched-chain amino acid (BCAA) catabolic pathway, induces developmental

slowing where worms remain in the L1 and L2 stage for several days (Jia et al., 2016). Three of the essential *C. elegans* amino acids (leucine, isoleucine, and valine) are BCAAs and are used in this pathway, suggesting lack of any or all three may induce similar developmental slowing. Many amino acids increase longevity when supplemented into the *C. elegans* diet (Edwards et al., 2015). These effects required activity of DAF-16, except in the case of tryptophan, which appears to increase lifespan by activating ER and mitochondrial stress response pathways. Amino acids play a significant role in the regulation of metabolism, longevity, and development, and are thus an important component of diet for *C. elegans*. Given that both worms and humans require the same amino acids in their diet, studying how amino acids regulate growth in *C. elegans* could provide insight into effects of the metabolites on human health.

#### 1.4.3. - Bacterial metabolism

Bacteria, even of the same genera or species, exhibit vast diversity in the metabolites that they produce (Saccenti et al., 2015). Metabolism of bacteria impacts their own survival and proliferation and can also affect other organisms that consume or harbor the bacteria. One example of this is the host-associated microbiota. This relationship is exemplified by the interaction between humans and their gut microbiota. Bacteria in the human gut, and elsewhere, are capable of metabolizing nutrients from the human diet, including fatty acids, amino acids, and some carbohydrates (Krishnan et al., 2015). This metabolism not only sustains the bacteria, but can also provide their hosts with essential nutrients, such as vitamins and amino acids, which are not synthesized by humans but are amply provided by bacteria living within the human gut (Metges, 2000). Bacterial

metabolic products can directly impact host growth, such as in *Drosophila*, where acetic acid produced by *Acetobacter pomorum* regulates development of the organism through modulation of insulin signaling (Shin et al., 2011). Conversely, bacterial metabolites can have negative effects on the host. Differences in gut microbiota composition may be one factor contributing to diseases such as obesity (Riva et al., 2016).

Bacteria that *C. elegans* regularly encounter in their natural habitat have been identified, and the effects these bacteria have on *C. elegans* survival and growth have been examined, identifying a large number of bacteria that can sustain *C. elegans* growth, and many that inhibit growth (Samuel et al., 2016). Given the numerous ways in which human gut microbiota is known to impact human health and metabolism, it is likely that similar interactions exist with *C. elegans* and the bacteria it encounters in its natural habitat, making it an excellent model for studying the effects of the microbiome (Zhang et al., 2017). Discovering bacterial effects on *C. elegans* metabolism, development, or immunity can help identify possible effects that similar bacteria could have in humans.

### **1.5 - *C. elegans* innate immunity and pathogen response**

One aspect of *C. elegans* physiology that is often overlooked in the context of development is the impact of pathogens and the worms' immune system. Organisms may reallocate resources from growth to defense and survival during infection (reviewed in Rauw, 2012; Leroy et al., 2012; Marsh and May, 2012). Several pathogens, including *M. nematophilum* and *P. aeruginosa* slow *C. elegans* development (Marsh and May, 2012;



Fasseas et al., 2013). Numerous signaling pathways involved in the *C. elegans* immune response have been identified, including the p38 MAPK pathway, which is involved in the immune response to *D. coniospora* (Couillault et al., 2004), and the insulin signaling pathway, which also regulates development (Kurz and Tan, 2004). Infection by *Pseudomonas* causes the secretion of the insulin-like peptide INS-7, an agonist of DAF-2, activating the insulin signaling pathway and reducing the expression of immunity-related genes (Kawli and Tan, 2008). Similarly, loss of *daf-2* increases survival of worms on pathogenic bacterial strains (Garsin et al., 2003). Activation of insulin signaling through DAF-2 is necessary for developmental progression, but also appears to reduce pathogen resistance. The MAPK pathway (Figure 1.4) may also regulate developmental rate in worms in response to infection, as the pathway inhibits the activity of the *let-7* miRNA, which regulates developmental timing in worms (Ren and Ambros, 2015). A pathogenic infection may thus regulate developmental rate in *C. elegans* by modulating signaling pathways such as insulin and MAPK.

Many different bacterial species can infect *C. elegans* (Couillault and Ewbank, 2002), and this infection may occur through a number of methods, including penetration of the cuticle, or via colonization of the gut after ingestion. Both bacteria and fungi are capable of penetrating the cuticle, triggering a wound-healing response and activating the innate immune response (Chisholm and Xu, 2012). Infection by the fungus *Drechmeria coniospora*, for example, causes transcriptional upregulation of antimicrobial peptides in the epidermis to fight off the infection (Pujol et al., 2008). In addition to the immune response, *C. elegans* can protect against infection by avoiding pathogenic bacteria. *C.*

*C. elegans* use chemosensation to sense specific compounds produced by pathogenic bacteria, such as serrawettin W2, a surfactant of *Serratia marcescens* (Pradel et al 2007). This response is mediated by G-protein coupled receptors on olfactory neurons (Engelman and Pujol, 2010). Avoidance can also be learned; this type of avoidance is mediated by an increase in serotonin signalling in the case of learned aversion to *Pseudomonas aeruginosa* (Zhang et al., 2005). *C. elegans* also use a physical barrier, the cuticle, as the first line of defense against pathogens. Some pathogens, such as *Staphylococcus aureus*, infect both *C. elegans* and humans (Sifri et al., 2003). Thus, it is important to understand the effects these pathogens can have on worms, as those findings may have implications for human health, as well.

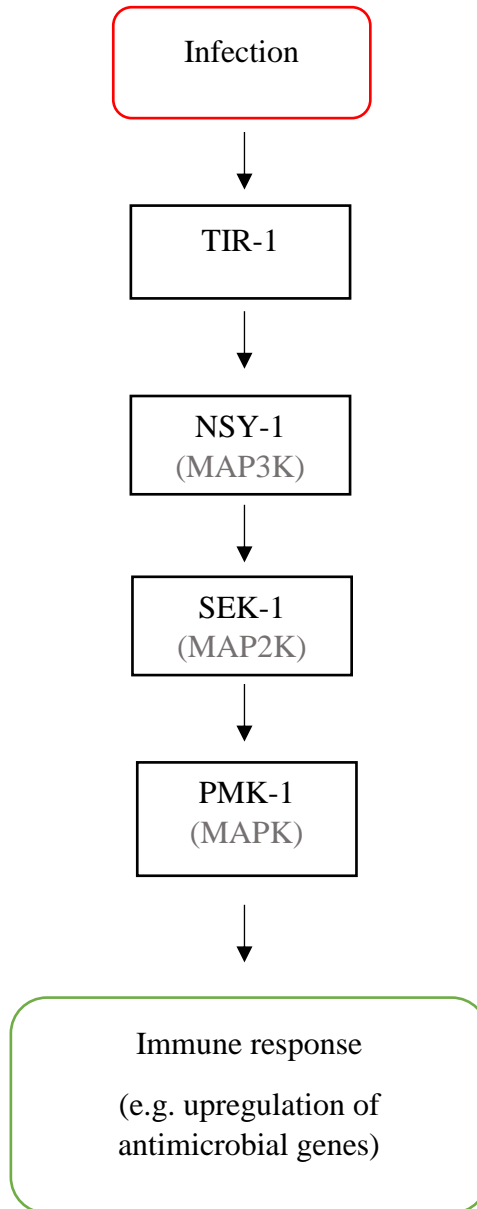


Figure 1.4 – The MAPK immune response pathway in *C. elegans*

Infection by a pathogen activates the toll-interleukin 1 receptor (TIR-1), which in turn leads to the activation of the MAP kinase pathway, comprised of NSY-1 (MAP3K), SEK-1 (MAP2K), and PMK-1 (MAPK). Phosphorylation of PMK-1 results in upregulation of antimicrobial peptides, such as ShK toxins, which act as an immune response. Figure adapted from Kim and Ausubel, 2005.

## **1.6 - Project Goals**

### **Rationale**

Development is a complex, tightly regulated process, with pathways such as insulin signaling and TOR interacting to determine the optimal rate of growth given environmental and metabolic constraints. Diet plays a major role in development, as sufficient nutrition and availability of essential metabolic compounds are required for growth (Rogol et al., 2000). Developmental rate in *C. elegans* is influenced by several factors and can be used as a broad measure of health effects. The impact that bacteria might have on human physiology is a challenge to study given how complex human systems are and the difficulty of performing experiments. As such, *C. elegans* models are a useful tool for studying host-microbe interactions, particularly as it relates to microbiota, not only due to their ease of use, but also because of the number of genes and pathways that are conserved between *C. elegans* and humans, including insulin and TOR signaling, both of which regulate development in response to nutrition (Hashmi et al., 2013). As *C. elegans* display diverse growth rates on different bacterial species, determining how developmental rate of worms changes in response to a single bacterial diet can be an effective method to study the effect that environmental factors can have on signaling pathways. Learning how signaling pathways in *C. elegans* are able to respond to different environmental factors can further understanding of the function of these same pathways in human systems.

### Hypothesis

Many questions remain regarding how environmental factors, such as diet and nutrition, regulate development. To better understand the link between environmental factors and development, *C. elegans* were used as a model organism to identify mechanisms by which different bacterial diets alter developmental rate. We hypothesize that *C. elegans* slow or arrest developmental progress when fed certain bacterial diets due to a lack of nutrients, and that these changes in development are regulated by nutrient sensing pathways such as insulin or TOR signaling.

### Aims

The primary aims of this study were to:

- 1) Determine developmental rate of worms fed different bacterial species
- 2) Determine whether changes in developmental rate of worms fed these bacteria were caused by missing metabolites, regulation by signaling pathways, and/or pathogenic effects
- 3) Rescue development of worms fed these bacteria through metabolite supplementation or knockout of relevant genes

**CHAPTER 2 – MATERIALS AND METHODS**

## 2.1 - Bacteria and nematode strains

*Caenorhabditis elegans* were maintained on nematode growth medium (NGM) (Stiernagle et al., 2006) agar seeded with *Escherichia coli* OP50, and stored at 20 °C or 15 °C, depending on the strain.

For most experiments, bacteria were grown on ¼ Brain Heart Infusion (BHI) agar plates. BHI agar is composed of 14.5 g/L Casein Peptone, 10.0 g/L Brain Heart Infusion from Solids, 5.0 g/L Animal Tissue peptone, 5.0 g/L Sodium Chloride, 2.5 g/L Disodium Phosphate, and 2.0 g/L Dextrose. 5 mg/L cholesterol was also added to the ¼ BHI agar.

Table 2.1 – List of *C. elegans* strains used

Strain name	Genotype	Relevant human ortholog	Reference
N2	Wild type	N/A	N/A
PD4666	<i>ayIs6[hlh-8::gfp+dpy-20(+)]</i>	N/A	Harfe et al., 1998
GR1395	<i>mgIs49 [mlt-10::gfp-pest; ttx-1::gfp]</i>	N/A	Hayes et al., 2006
CF1038	<i>daf-16(mu86)</i>	FOXO3	Lin et al., 1997
GG60	<i>glp-1(g60)</i>	Notch	Chang et al., 2003
VC1027	<i>daf-15(ok1412)/nT1; +/nT1</i>	Raptor	The <i>C. elegans</i> Deletion Mutant Consortium, 2012
MH5197	<i>npri-3(ku540)</i>	NPRL3	Zhu et al., 2013
RB1206	<i>rsk-1(ok1255)</i>	RPS6KB1	The <i>C. elegans</i> Deletion Mutant Consortium, 2012
MAH236	<i>sqIs13 [lgg-1p::GFP::lgg-1 + odr-1p::RFP]</i>	MAP1LC3B	Lapierre et al, 2013

*E. coli* OP50 was obtained from the Caenorhabditis Genomic Center (University of Minnesota). A collection of bacteria isolated from human respiratory clinical samples were used in this study (Table 2.2) and were a generous gift from Dr. Michael Surette (McMaster University).

Table 2.2 – List of bacterial strains used

Bacterial strain	
<i>Escherichia coli</i> OP50	<i>Kocuria rhizophila</i>
<i>Abiotrophia defectiva</i>	<i>Kocuria rhizophila</i>
<i>Actinomyces naeslundii</i>	<i>Neisseria flavescens</i>
<i>Actinomyces naeslundii</i>	<i>Neisseria mucosa</i>
<i>Actinomyces radidentis</i>	<i>Rothia dentocariosa</i>
<i>Actinomyces radidentis</i>	<i>Selenomonas sputigena</i>
<i>Actinomyces sp.</i>	<i>Staphylococcus aureus</i> S1
<i>Actinomyces sp.</i>	<i>Staphylococcus aureus</i> S2
<i>Actinomyces sp.</i>	<i>Staphylococcus caprae</i>
<i>Actinomyces sp.</i>	<i>Staphylococcus epidermidis</i>
<i>Actinomyces sp.</i>	<i>Staphylococcus warneri</i>
<i>Afipia broomeae</i>	<i>Streptococcus gordonii</i>
<i>Corynebacterium diphtheriae</i>	<i>Streptococcus gordonii</i>
<i>Corynebacterium durum</i>	<i>Streptococcus intermedius</i>
<i>Corynebacterium durum</i>	<i>Streptococcus massiliensis</i>
<i>Corynebacterium mucifaciens</i>	<i>Streptococcus mutans</i>
<i>Corynebacterium mucifaciens</i>	<i>Streptococcus oralis</i>
<i>Corynebacterium pseudodiphtheriticum</i>	<i>Streptococcus parasanguinis</i>
<i>Corynebacterium striatum</i>	<i>Streptococcus sanguinis</i>
<i>Corynebacterium urealyticum</i>	<i>Streptococcus sanguinis</i>
<i>Dietzia sp.</i>	<i>Streptococcus sinensis</i>
<i>Eikenella corrodens</i>	<i>Streptococcus sp.</i>
<i>Gemella sanguinis</i>	<i>Streptococcus sp.</i>
<i>Granulicatella adiacens</i>	<i>Streptococcus vestibularis</i>



## 2.2 - Nematode synchronization and general maintenance

*C. elegans* were synchronized via bleaching using standard protocols (Porta-de-la-Riva et al., 2012). Worms grown on *E. coli* OP50 NGM agar plates were washed with M9 buffer and eggs and animals were transferred to 15ml conical tubes. Eggs were pelleted by centrifugation and the supernatant removed. For the bleaching process, 800 µl of 6% hypochlorite solution (bleach) and 400 µl of 5M sodium hydroxide were added to 2 mls of M9, and tubes were lightly shaken until the bodies of worms were disintegrated, leaving only eggs. Eggs were washed four times with M9 buffer to remove the bleaching solution. The final solution containing the eggs, resuspended in M9, was incubated, with rocking, at 20 °C for approximately 24 hours, at which point most eggs had hatched and the larvae arrested in the L1 stage. For growth of worms, 3.5 cm 1/4 BHI agar plates were prepared and seeded with the test bacteria, with the bacteria grown for approximately 18 hours at 37 °C. Approximately fifty L1 worms were dispensed onto each 3.5 cm 1/4 BHI agar plate containing test bacteria. All plates were maintained at 20 °C, unless otherwise noted.

## 2.3 - Monitoring *C. elegans* development

Animals were observed under a dissecting microscope daily for up to ten days, and the developmental stage, were noted. For initial characterization, the developmental stage was determined qualitatively, via observation of body morphology. Specifically, the morphology of the vulva was used to distinguish between the L3 and L4 stages. For a

quantitative measure of developmental rate on different bacterial diets, the time taken for 50% of the worm population to reach adulthood was used.

### 2.3.1 – More precise determination of developmental stage using *hlh-8::gfp* worms

To more accurately determine the larval stages of worms during development, the PD4666 strain (*ayIs6 [hlh-8::gfp fusion + dpy-20(+)]*) was used (Harfe et al., 1998). *hlh-8::gfp* is expressed in the M cell lineage that eventually differentiates into vulval and body wall muscle cells. Expression of *hlh-8* changes over the course of development, allowing for the precise identification of developmental stage of the worms via examination of fluorescent cell counts and positioning of fluorescent cells within the worm.

Synchronized L1 worms were set up onto bacteria, as described. A collection of 48 bacterial species derived from the human respiratory microbiota, as well as an *E. coli* OP50 control, were tested (Table 2.2). Worms were observed daily for 10 days. For each bacterial plate, 20 worms were counted, and the number of fluorescent cells in each worm recorded. This assay was repeated twice for a total of three biological replicates.

### 2.3.2 - Analysis of *mlt-10::gfp-pest* worms

For thorough analysis of *C. elegans* development, molting frequency was observed using the strain GR1395, which contains a *mlt-10::gfp-pest* fusion reporter (Hayes et al., 2006), which contains a fluorescent reporter that turns on transiently before molting occurs at the end of each larval stage, before being rapidly degraded (Meli et al., 2010).

To cover a full 24 hours of *mlt-10* expression in the worms, one set of worms were synchronized in L1 and put onto bacteria as described above, while a second set, bleached separately was plated 10 hours later. Both sets were observed every two hours, up to a total duration of ten hours, under a fluorescence-equipped microscope, with the stage of the worms noted and the ratio of fluorescing to non-fluorescing worms determined. Combined, the two sets provided data for worms throughout the day. All worms were grown at 20 °C, with population sizes ranging from 80 to 500 individuals.

### 2.3.3. - Measuring developmental rate of worms at different larval stages on the test bacteria

Synchronized L1 worms were plated onto *E. coli* OP50 ¼ BHI plates until they reached the desired larval stage (Figure 2.1). Worms were grown from synchronized L1s for 15 hours for the L2 stage, 24 hours for the L3 stage, and 34 hours for the L4 stage. When worms reached the desired stage, plates were washed with 7 mls of M9 solution, and the solution of worms was washed a second time, after which 7 mls of 50 µg/ml Kanamycin solution was added. Worms were incubated in the kanamycin solution for 4 hours, then washed once with M9 plated onto the test bacteria. For the L1 stage, two different treatments were carried out. One set of worms was plated directly onto the test bacteria. The second set of worms was put on *E. coli* OP50, then washed immediately and transferred, to ensure that the kanamycin treatment was sufficient to kill off the bacteria, in which case the results from both treatments should be identical.

After being placed onto the test bacteria, worms were monitored daily. The total number of adult worms on each plate, which ranged from 50-150, was counted, and the day on which 50% of the plate population was adults was recorded.

#### 2.3.4 – Testing whether metabolite supplementation increases *C. elegans* developmental rate on certain bacteria

For testing heme, hemin chloride powder was dissolved in ultrapure water to create a stock with a concentration of 10 mM, and filter-sterilized, and added to ¼ BHI agar medium. Concentrations of 2, 10, 20, 100, and 200 µM were tested.

For vitamins, MEM Vitamin Solution (100x) (Thermofisher, Catalog # 11120052) (Table 2.3) was added to ¼ BHI agar at 1x concentration.

For testing casamino acids, Bacto Casamino Acids (acid-hydrolyzed casein) powder was added to ¼ BHI mix prior to autoclaving in different quantities depending on the desired final concentration. Concentrations of 0.5, 1, 2, and 5% were used. Test bacteria were grown on the supplemented plates as described.

For testing amino acids, each individual essential amino acid was mixed together to form different amino acid mixes, with the concentrations dictated by the requirements for the *C. elegans* Maintenance Medium, or CeMM (Szewczyk et al., 2003, measurements provided by protocol from the Conley lab, Table 2.4). For initial experiments, amino acids were added to molten agar. When testing different amino acid supplements, solutions were filter-sterilized and added on top of the bacterial plates and left to dry.

For testing non-essential amino acids, 1x solutions were prepared from MEM Non-Essential Amino Acids Solution (100x), which contained some of the non-essential amino acids, though tyrosine, cysteine, and glutamine were not included (Thermofisher, Catalog # 11140050, Table 2.5). A solution of the mix added on top of bacterial plates, as described above.

Table 2.3 – Concentration of vitamins in mix

Vitamin	Concentration (mM)
Choline chloride	0.714
Calcium D-pantothenate	0.210
Folic Acid	0.227
Nicotinamide	0.820
Pyridoxal hydrochloride	0.490
Riboflavin	0.0266
Thiamin hydrochloride	0.297
i-Inositol	1.11
Sodium chloride	146.0

Table 2.4 – Concentration of essential amino acids in mix

Amino acid	Concentration (mM)
Arginine	5.60
Histidine	1.82
Lysine.HCl	7.02
Tryptophan	0.901
Methionine	2.61
Threonine	6.02
Leucine	11.0
Isoleucine	6.56
Valine	8.71
Phenylalanine	3.77

Table 2.5 – Concentration of non-essential amino acids in mix

Amino acid	Concentration (mM)
Glycine	10
L-Alanine	10
L-Asparagine	10
L-Aspartic acid	10
L-Glutamic acid	10
L-Proline	10
L-Serine	10

## 2.4 - Determining whether signaling pathways play a role in slowing development on the test bacteria

### 2.4.1 - Observing *cki-1::gfp* expression in L1 worms

To determine whether *cki-1* expression was increased in worms that were fed bacteria that slowed or arrested development, worm strains carrying a *cki-1::gfp* fluorescent reporter were synchronized and grown on the test bacteria, and observed over several days. The fluorescence intensity was noted qualitatively via observation under a fluorescence-equipped stereo microscope. Worms were also mounted onto slides on days 1, 3, and 5 and observed under a fluorescence-equipped compound microscope. The number of fluorescent seam cells was counted. Cells in 30 worms per bacteria were counted.

### 2.4.2 – Measuring autophagy using LGG-1::gfp

MAH236 animals, containing an LGG-1::gfp reporter, were synchronized in L1 before exposure to test bacteria. Worms were mounted onto slides 10 hours after being put on the bacteria, and observed under a fluorescence-equipped compound microscope. The number of GFP-positive puncta within the seam cells was counted. Five seam cells were counted in 30 worms.

### 2.4.3 – Measuring developmental rate of TOR and insulin/IGF-1 signaling pathway mutants on the test bacteria

The MH5197 and RB1206 strains, containing loss-of-function mutations of *npri-3* and *rsk-1*, respectively, were synchronized and plated onto ¼ BHI plates growing the

test bacteria, with and without amino acids, and their development noted over several days. The CF1038 strain, containing a *daf-16* mutation, was bleached and the eggs immediately put on the bacteria, rather than as synchronized L1s, as the *daf-16* mutation prevents worms from entering L1 arrest and thus would continue to grow even without food.

The VC1027 strain, containing a *daf-15* mutation, was maintained and propagated at 15 °C, eggs were collected and added to ¼ BHI plates growing the test bacteria, with and without amino acids, development was monitored over several days. On days three to six, older (L3) worms were transferred from amino-acid supplemented plates onto *E. coli* OP50 NGM plates, while younger (L1-L2) worms were transferred on days five and six. All transferred worms were then observed for several days, and the number of worms that developed into adults were noted as heterozygotes, while that remained arrested in L3 were noted as *daf-15* mutant homozygotes.

## 2.5 - Testing survival of worms

To determine whether the test bacteria were inducing pathogenic effects in the worms, several different assays looking at survival rates of worms was performed.

### 2.5.1 - Survival of adult worms

Survival of worms was measured using a slow-killing assay, as described in Tan et al., 1999. The GG60 strain of *C. elegans*, a *glp-1* mutant, was used as the strain has temperature-dependent sterility, which eliminated the need to clear plates of eggs. *glp-1*



worms were grown at 15 °C, the permissive temperature, to allow for propagation (Figure 2.2). Worms were then synchronized and the eggs were plated onto ¼ BHI *E. coli* OP50 plates. Animals were not synchronized in L1 because of the decreased survival of *glp-1* mutant worms when left in M9 buffer. For survival assays, animals were grown at 25 °C to induce sterility. Once worms reached the L4 stage, approximately 2 days later, they were washed twice in M9 buffer, and 7 mls of 50 µg/ml Kanamycin was added to kill any *E. coli* OP50. Worms were incubated in the Kanamycin solution for 4 hours before being washed once with M9 and plated on the test bacteria. Worms were observed every day, including the day they were put onto plates, and the number of live and dead worms counted. The assay continued until all worms were dead.

#### 2.5.2 - Survival of L1 worms

PD4666 worms with a *hlh-8::gfp* reporter were synchronized and plated onto ¼ BHI plates growing the test bacteria to provide better visibility of worms. The number of visible worms on all plates was counted every day for the first six days, and any missing worms noted as ‘dead’.

#### 2.5.3 - Survival of worms following starvation

Wild-type worms were bleach synchronized and kept rocking in 5 mls of M9 buffer solution for up to six days. Each day, an aliquot of worms in M9 was plated onto *E. coli* OP50 grown on NGM plates. The number of live worms on each plate was counted two days after plating.

## 2.6 – Statistical analysis

The differences in LGG-1::*gfp* puncta count were analyzed using two-way ANOVA with multiple comparisons using the program Graphpad Prism. Bacterial species and amino acid supplementation status were the two factors tested, with Bonferroni post-hoc analyses, using  $p \leq 0.5$  as the threshold for statistical significance.

Comparison of adult survival data was performed using the Wilcoxon log-rank test in Graphpad Prism. L1 survival data was analyzed using one-way ANOVA.

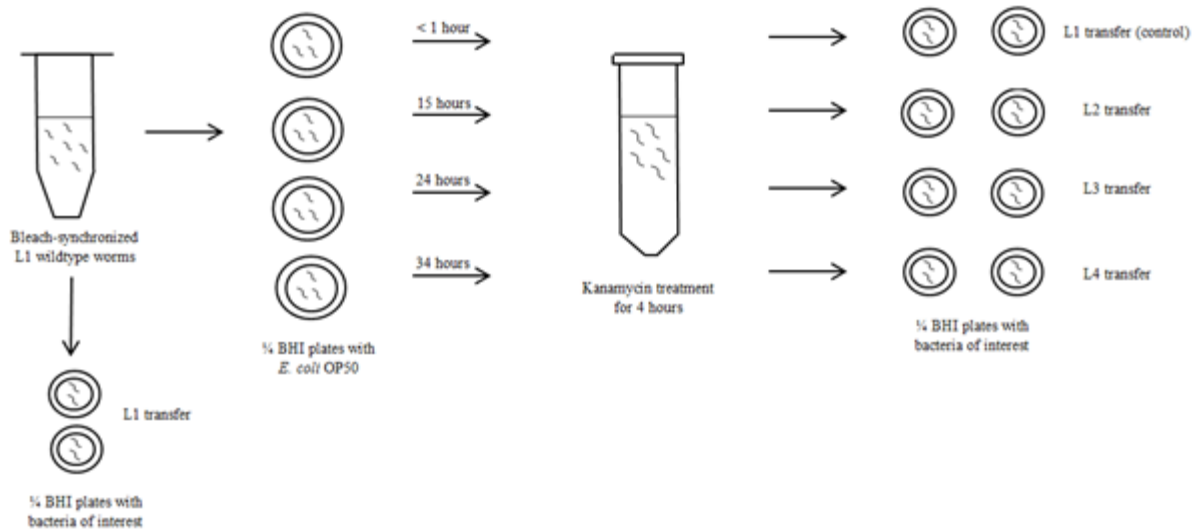


Figure 2.1 – Protocol for *E. coli* OP50 to test bacteria transfer

Worms were synchronized and added to ¼ BHI plates seeded with *E. coli* OP50. One set of plates was set aside as a control, while worms on the other plates were washed, separately, at the indicated timepoints. Worms from each set of plates were washed twice with M9 buffer, then placed into tubes containing 7 mls of M9, supplemented with 50 µg/mls of Kanamycin to kill any remaining bacteria. Worms were incubated in the Kanamycin solution for 4 hours, after which they were put onto ¼ BHI plates growing the test bacteria. Worms from each set of plates ('L1 transfer plates' etc.) were washed, treated, and plated separately, resulting in four sets of bacterial plates, each having worms at a different larval stage.

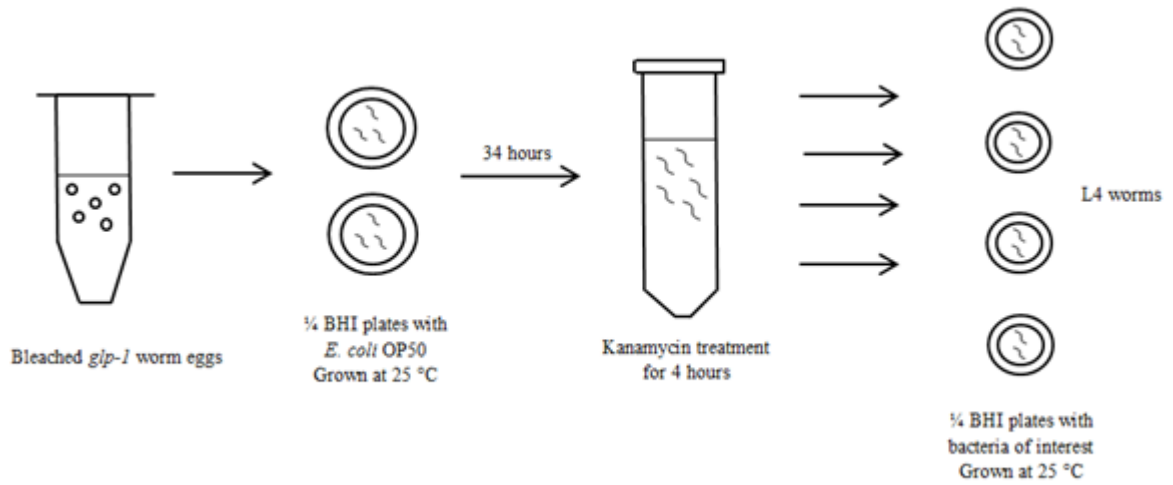


Figure 2.2 – Protocol for survival assay

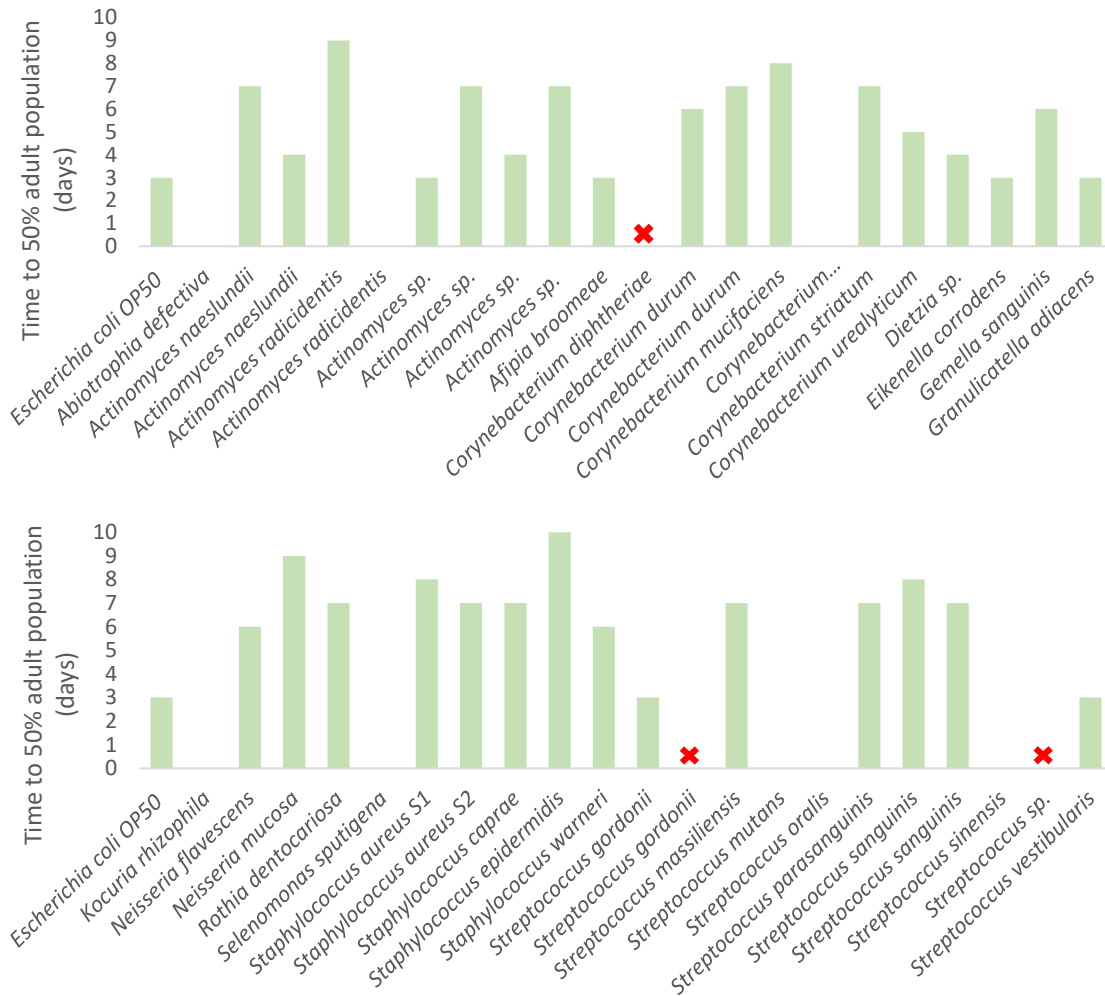
*glp-1* worms were grown at the permissive temperature of 15 °C to ensure reproduction and propagation. Worms were then bleached and put onto 1/4 BHI plates growing *E. coli* OP50. Worms were maintained at the restrictive temperature of 25 °C until they reached the L4 stage, approximately 34 hours later. Worms were then washed off with M9 to remove as much bacteria as possible, then incubated in 7 ml of M9 with 50 µg/mls of Kanamycin to kill off any remaining bacteria. After 4 hours of incubation in the solution, worms were plated onto 1/4 BHI plates growing the test bacteria, then maintained at 25 °C, the restrictive temperature, to ensure sterility. Worm survival was then measured over the next few weeks, until worms on all plates had died.

## **CHAPTER 3 – RESULTS**

*C. elegans* alter growth and developmental rate in response to environmental factors, such as nutrition, and these changes are regulated by conserved signaling pathways. A *C. elegans* model was used to study the effects of different bacterial diets on developmental rate, and to determine how these changes in developmental rate were regulated.

### 3.1 - *C. elegans* developmental rate is influenced by bacterial diet

*C. elegans* fed the control *E. coli* OP50 diet developed into adults within three days (Figure 3.01, Table 3.1). Of 42 bacterial strains tested, six others were able to fully support development and enabled worms to develop into adults in three days. 26 bacterial strains increased the time it took worms to develop into adults. A third subset of 11 bacterial strains prevented worms from developing into adults. This was due in some cases to worms dying before developing into adults (e.g. on *Corynebacterium diphtheriae*) or because worms arrested their development at an early larval stage (e.g. on *Actinomyces radidentis*).



**Figure 3.01 – Bacterial diet influences developmental rate in *C. elegans***

Time taken for *C. elegans* to develop into adults when fed different bacterial diets. The day on which at least 50% of the worm population on each plate was adults is depicted. On bacteria where time to adult is depicted as 0, development was arrested at an earlier larval stage. Red crosses denote bacteria that did not support growth and instead caused worm death. N > 50 for each condition.

Table 3.1 – Categorized bacterial impact on *C. elegans* developmental rate

<u>Fast growth (7)</u>	<u>Slow growth (26)</u>	<u>Arrested development (8)</u>	<u>Dead (3)</u>
<i>Escherichia coli</i> <b>OP50 (control)</b>	<i>Actinomyces naeslundii</i>	<i>Abiotrophia defectiva</i>	<i>Corynebacterium diphtheriae</i>
<i>Actinomyces sp.</i>	<i>Actinomyces naeslundii</i>	<i>Actinomyces radidentis</i>	<i>Streptococcus gordonii</i>
<i>Afipia broomeae</i>	<i>Actinomyces radidentis</i>	<i>Corynebacterium pseudodiphtheriticum</i>	<i>Streptococcus sp.</i>
<i>Eikenella corrodens</i>	<i>Actinomyces sp.</i>	<i>Kocuria rhizophila</i>	
<i>Granulicatella adiacens</i>	<i>Actinomyces sp.</i>	<i>Selenomonas sputigena</i>	
<i>Streptococcus gordonii</i>	<i>Actinomyces sp.</i>	<i>Streptococcus mutans</i>	
<i>Streptococcus vestibularis</i>	<i>Corynebacterium durum</i>	<i>Streptococcus oralis</i>	
	<i>Corynebacterium durum</i>	<i>Streptococcus sinensis</i>	
	<i>Corynebacterium mucifaciens</i>		
	<i>Corynebacterium striatum</i>		
	<i>Corynebacterium urealyticum</i>		
	<i>Dietzia sp.</i>		
	<i>Gemella sanguinis</i>		
	<i>Neisseria flavescens</i>		
	<i>Neisseria mucosa</i>		
	<i>Rothia dentocariosa</i>		



MSc Thesis – Sabih Rashid  
McMaster University – Biochemistry and Biomedical Sciences

<u>Fast growth (7)</u>	<u>Slow growth (26)</u>	<u>Arrested development (8)</u>	<u>Dead (3)</u>
	<i>Staphylococcus aureus S1</i>		
	<i>Staphylococcus aureus S2</i>		
	<i>Staphylococcus caprae</i>		
	<i>Staphylococcus epidermidis</i>		
	<i>Staphylococcus warneri</i>		
	<i>Streptococcus massiliensis</i>		
	<i>Streptococcus parasanguinis</i>		
	<i>Streptococcus sanguinis</i>		
	<i>Streptococcus sanguinis</i>		

### 3.1.2 - Timing of *C. elegans* molting is altered in response to bacterial diet

While many of the bacterial species tested caused slow developmental progression or arrest, it was not clear how, or when, this effect was occurring. To gain a better understanding of when development of the worms was being slowed, the *mlt-10::gfp-pest* reporter strain was used (Hayes et al., 2006). This fluorescent reporter turns on and off during every larval stage, allowing for more precise measurement of developmental timing. Wild-type *C. elegans* grown on *E. coli* OP50 display oscillatory *mlt-10* expression, with the majority of worms fluorescing shortly before each of the four molts (Frand et al., 2005). The *mlt-10::gfp-pest* expression in a population of animals over time can thus be used to monitor developmental progression.

For worms grown on *E. coli* OP50, the majority of worms (96-100% of the plate population) fluoresce at specific timepoints throughout the course of the experiment, where worms are developing into the next larval stage, as expected (Figure 3.02). Due to data being collected from two separate populations, slight differences in developmental timing led to the appearance of an additional peak in the data from the first set of plates (light green in Figure 3.02). For worms fed *E. coli*, there is a peak in the number of worms that are fluorescing during the L1 to L2 molt, at around 18 hours. However, on bacteria that induce slow worm growth, such as *Staphylococcus epidermidis*, no more than 24% of the worms on the plate are fluorescent at any time, and the first timepoint where worms begin to fluoresce is much later than worms on *E. coli* OP50, suggesting development is slowed before the first molt. Additionally, the percent of fluorescent

worms does not peak and then quickly subside as expected. Instead, there is a prolonged period where a portion of the population is expressing *mlt-10::gfp-pest*. It is unclear whether this is due to asynchronous development, leading to worms fluorescing briefly at different timepoints, or due to *mlt-10* being expressed for extended periods of time in only a portion of the population.

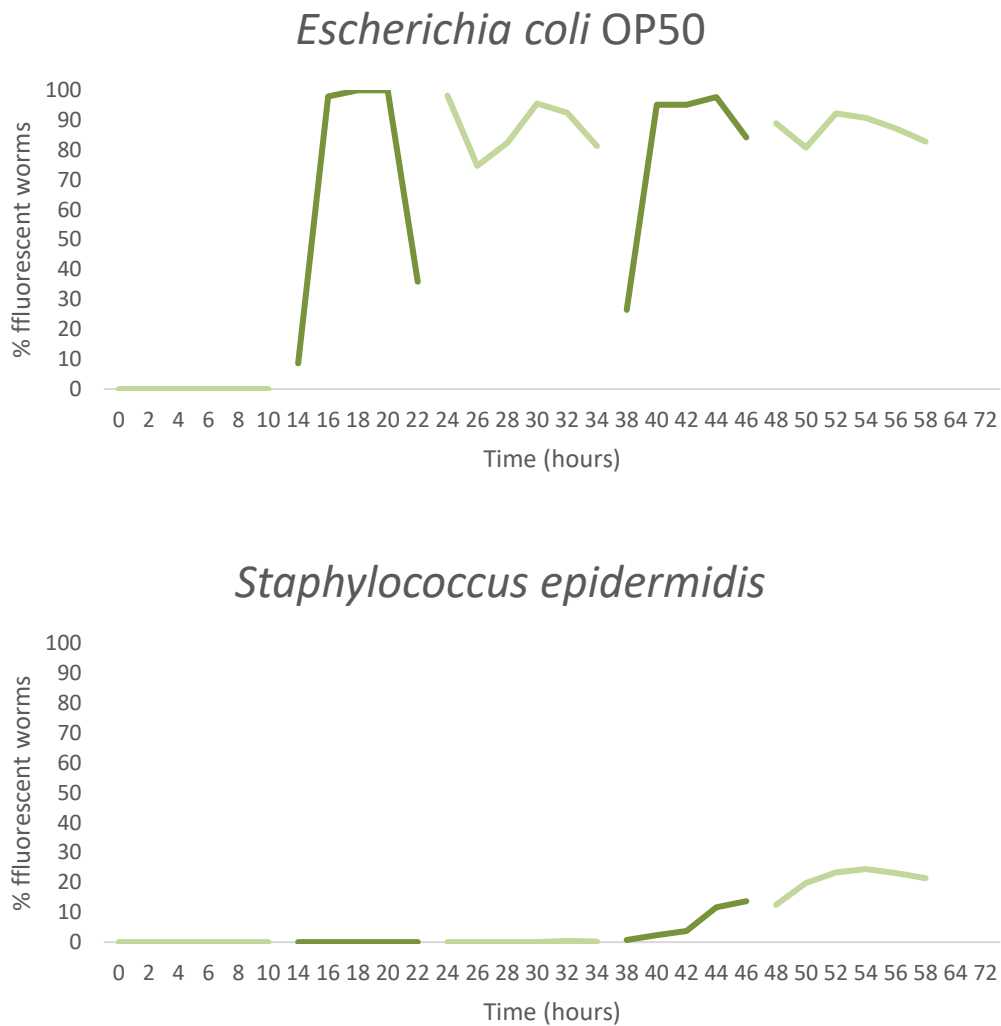


Figure 3.02 - Timing of *C. elegans mlt-10::gfp-pest* activation is severely delayed on certain bacterial diets

*mlt-10::gfp-pest* worms were synchronized in L1 and grown on test bacteria. Two sets of plates were used, with worms being put onto the second set of plates 10 hours after the first. Plates were screened every 2 hours for 10 hours per day, for up to 3 days. The proportion of fluorescent worms in the population was recorded, and data from both sets of plates combined to display the *mlt-10::gfp-pest* fluorescence activation pattern over the entire day, the second set displayed in dark green. Data for additional bacteria can be found in Supplementary Table S3.1.

### 3.1.3 – Earlier larval stages are more heavily slowed on some bacterial diets

Accurately identifying the developmental stage of worms is essential to determining the timing of developmental slowing. The developmental stage of worms in the early larval stages is often difficult to determine based on their appearance under a stereomicroscope, particularly on bacteria that obscure a clear view of worms. To this end, the PD4666 strain, containing an *hlh-8* translational GFP reporter, was used. HLH-8 is a transcription factor expressed exclusively in the M lineage sex mesoblast and subsequent differentiated cells (Philogene et al., 2012). The pattern of *hlh-8::gfp* within the cells of the worm changes throughout the development of the worm (Figure 3.03). Initially, only one fluorescent cell is present in the early L1 stage (Figure 3.04 A), dividing into two cells later in the L1 stage (Figure 3.04 B) and eventually becoming 16 in the L2 stage (Figure 3.04 C). Thus, the reporter strain can be used to identify larval stages of worms.

The developmental timing of worms exposed to 48 different bacterial strains was measured using *hlh-8::gfp* (Figure 3.04 and Supplementary Figure S3.1). The number of GFP-positive cells were counted each day for 10 days. On *E. coli* OP50 worms were in the L3 stage after 1 day, while they were L4 after two days and adults after three days (Figure 3.05). Conversely, developmental rate was severely slowed in animals exposed to several other bacterial species, including *Staphylococcus aureus* S1. For the first seven days worms were in the L1 stage, as only 1 or 2 fluorescent cells were visible. From days eight to ten, more developed worms began to appear, and worms eventually developed to the adult stage. The *hlh-8::gfp* experiment revealed that bacteria that induce very slow

development primarily affect the L1 stage (Figure 3.05). Additionally, development on some of these bacteria was also more asynchronous than on *E. coli* OP50, with some worms developing to later larval stages faster than others.

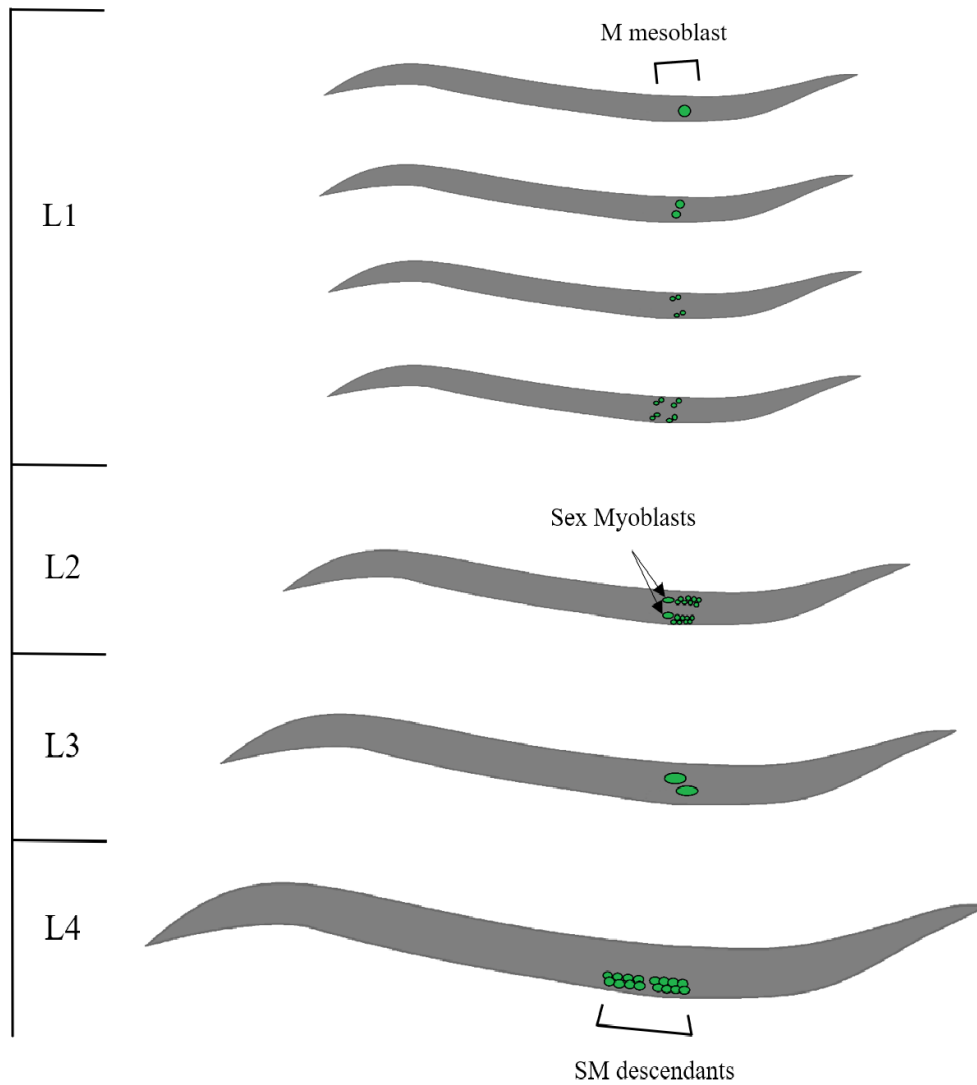


Figure 3.03 – *hlh-8::gfp* expression pattern changes throughout development  
Drawings of *C. elegans* in various larval stages, not to scale. Green circles depict nuclei of cells where *hlh-8::gfp* is expressed in M mesoblast descendants.

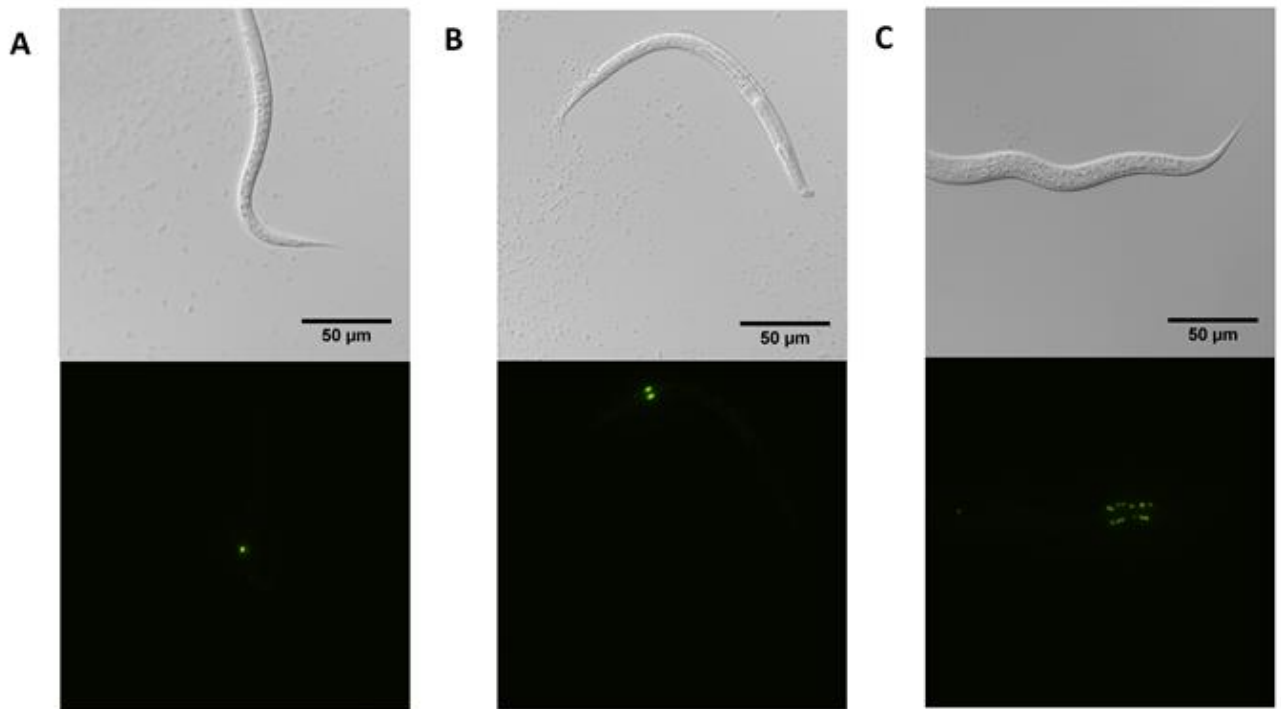
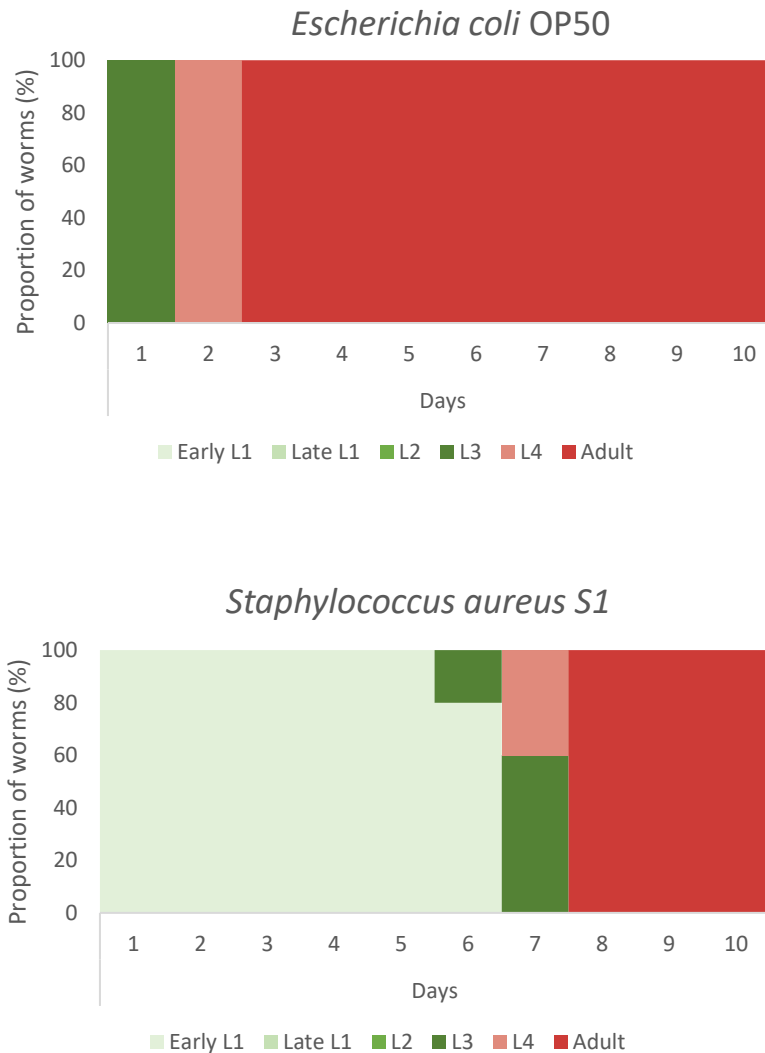


Figure 3.04 – *hlh-8::gfp* transgenic animals allow for precise determination of *C. elegans* larval stages

Images of *hlh-8::gfp* worms at different stages, taken under both Nomarski (top) and fluorescent illumination (bottom). A) An early L1 worm with one visible M-cell. B) A late L1 worm with two visible M-cells. C) An L2 worm with 16 visible M-cells.



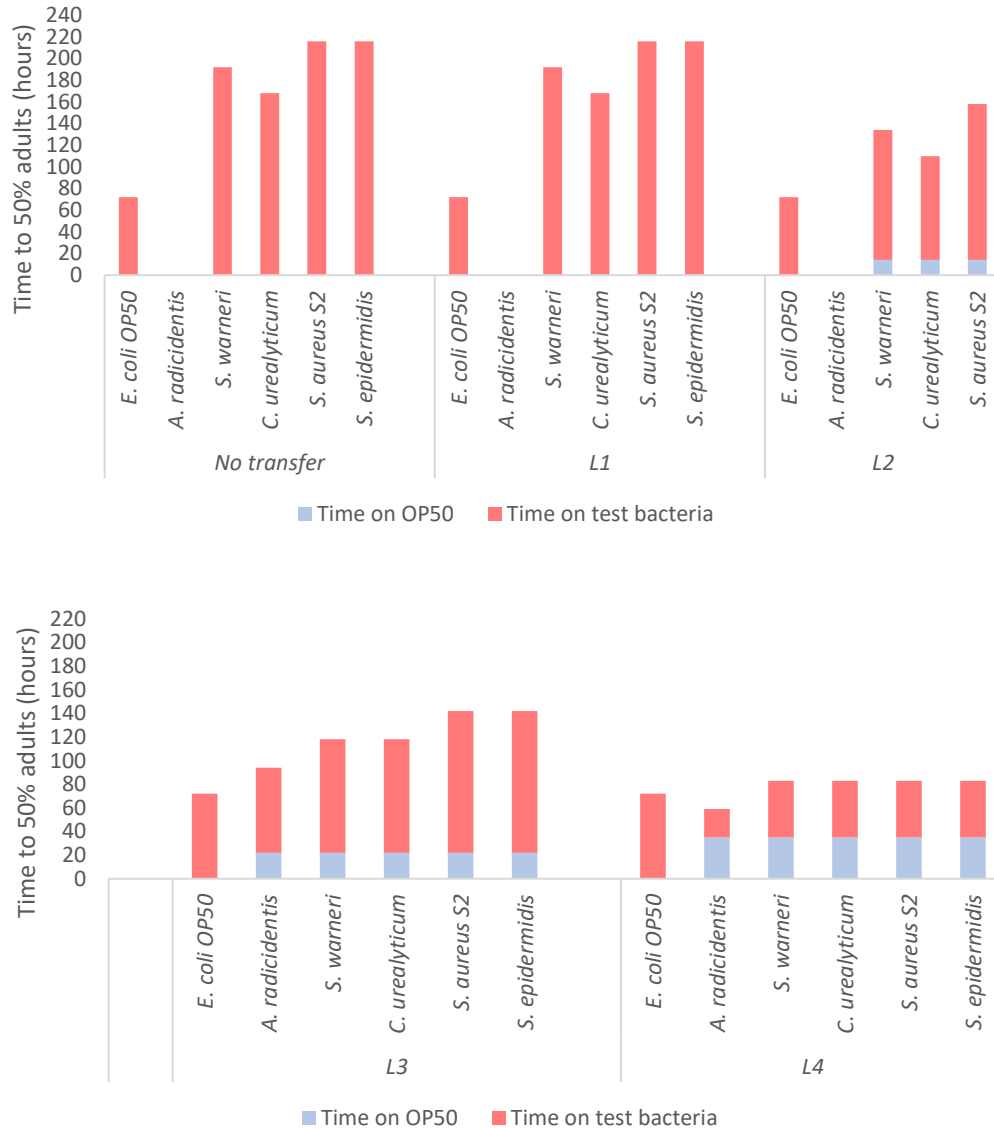


**Figure 3.05 - *C. elegans* developmental rate is adjusted in response to bacterial diet**  
Synchronized L1 *hlh-8::gfp* worms were plated onto ¼ BHI plates seeded with the indicated bacterial species. Animals were observed daily, and 20 worms from each plate examined under a fluorescent stereomicroscope and the number of fluorescent cells in each worm counted. For each bacterial diet, the percent of worms on each plate with a given number of fluorescent cells is displayed over 10 days. Data for other bacteria found in Supplementary Figure S3.1.

Previous results show that certain bacterial species slowed development particularly in the L1 stage. To determine whether slowing or arrest were restricted to specific larval stages, worms were initially grown on *E. coli* OP50, then transferred to test bacteria at indicated larval stages.

Worms developed slowly when put directly onto the test bacteria, with most bacteria causing slow development and one strain, *Actinomyces radidentis*, causing developmental arrest (Figure 3.06), consistent with the previous experiments. Worms transferred to the test bacteria in the L1 stage grew slowly, with worms on *Staphylococcus warneri* taking 192 hours to develop into adults. Generally, worms transferred in L1 grew at the same rate as animals grown only on the test bacteria.

Worms transferred in the L2 stage developed more slowly than those grown on *E. coli* OP50 alone, taking up to 150 hours to develop into adults on a bacteria such as *Staphylococcus aureus* S2. This demonstrates that the impacts of *Staphylococcus* species, *A. radidentis* and *C. urealyticum* on developmental rate are not confined to the first larval stage. However, when the transfer experiment was repeated with data recorded at multiple timepoints per day (Figure 3.07), it was determined that the L1 stage is greatly extended, as worms transferred in that stage remain in L1 for 148 hours, while worms transferred at the later larval stages only remain in those stages for 44 hours before developing to the next stage. The slowing of developmental rate induced by the *Staphylococcus* species appears to be greatest in the L1 stage, but other larval stages are also slowed to an extent.



**Figure 3.06 – Developmental slowing in *C. elegans* induced by bacterial diets occurs primarily in the L1 stage**

Time taken for *C. elegans* to develop into adults when transferred to the test bacteria at different larval stages. At specific time points after plating, corresponding to entry into different developmental stages, worms were washed off and transferred to the test bacteria. As a control, worms were also plated directly onto the test bacteria.

Development was monitored daily by counting the percent of adult worms in the population until 50% of the population of each plate reached the adult stage.  $N \geq 50$  for each plate.

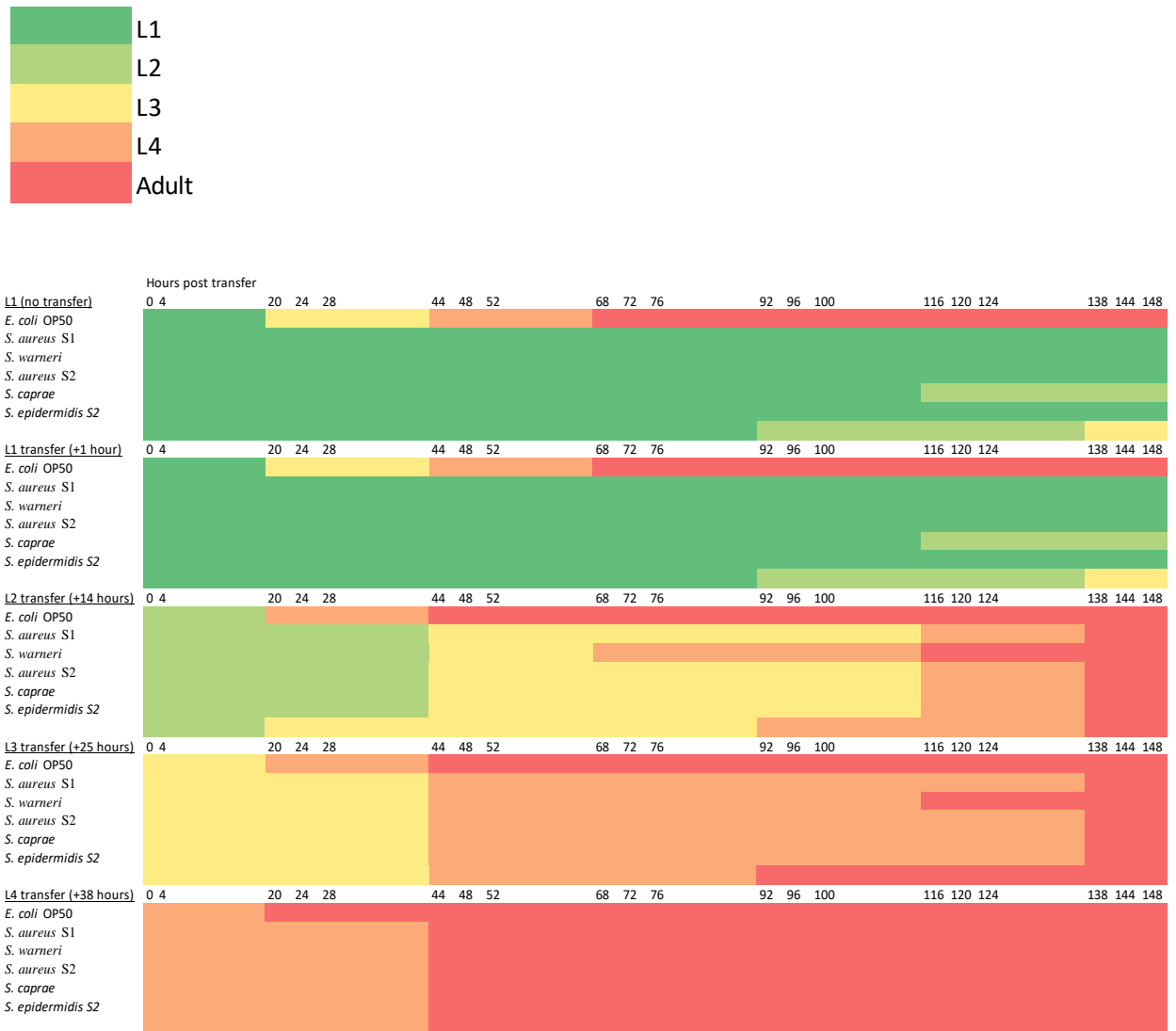


Figure 3.07 – Developmental slowing in *C. elegans* induced by *Staphylococcus* species occurs primarily in the L1 stage

Time taken for *C. elegans* to develop into adults when transferred to the test bacteria at different larval stages. At specific time points after plating, corresponding to entry into different developmental stages, worms were washed off and transferred to the test bacteria. As a control, worms were also plated directly onto the test bacteria. Development was monitored at the indicated time-points. Colors indicate developmental stages.

### 3.2 - Loss of *daf-16* does not rescue development of worms on slow growth/arrest causing bacteria

One cause of slow development is poor nutrition. If the bacterial diet was deficient in critical nutrients or if the bacteria was not recognized as food, a starvation response would likely be triggered, and development would arrest. This may be mediated by *daf-16*, the insulin signaling effector, which controls entry of worms into L1 diapause. *daf-16* mutants are able to overcome starvation-induced developmental arrest in the L1 stage. We asked whether *daf-16* mutants could also override developmental arrest or slowing in response to specific bacterial diets.

The cyclin-dependent kinase inhibitor 1 (*cki-1*) is an inhibitor of cell-cycle entry in *C. elegans* and other organisms (Buck et al., 2009). *cki-1* expression is increased in the seam cells of starved animals, and is regulated by DAF-16. Thus, if worms fed bacteria that cause slow development are experiencing starvation, and this effect is regulated by insulin signaling, then *cki-1* expression would be increased.

During L1 arrest, *cki-1::gfp* is expressed. L1 worms fed *E. coli* OP50 show *cki-1* expression for 8 hours (Figure 3.08). In worms fed the *Staphylococcus* species, *cki-1::gfp* is expressed for several days, and is consistent with the fact that worms remain in the L1 stage for several days when fed these bacteria. This may implicate *cki-1*, and its regulator *daf-16*, as a mediator of the slow L1 development seen in these worms.

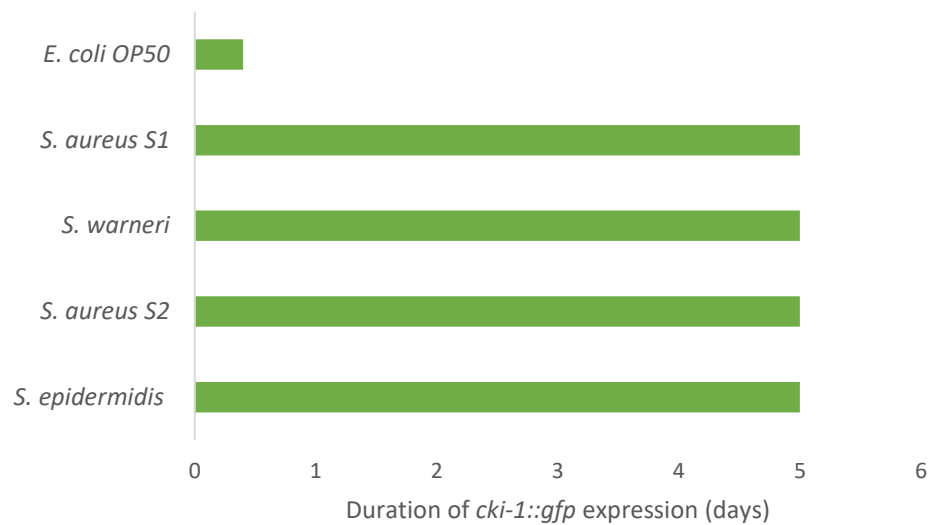


Figure 3.08 – *cki-1::gfp* is expressed in seam cells of *C. elegans* for several days when fed bacteria that induce slow development

The duration of *cki-1::GFP* expression in animals fed the indicated bacteria. Worms were mounted on agar slides and viewed under a fluorescent compound microscope to confirm that *cki-1::gfp* was expressed in the seam cells. 30-50 worms were scored from each bacterial plate every day, for six days. Bars represent the number of days on which 50% of the worms from each bacterial plate had visible fluorescence in the seam cells.

To test whether DAF-16 is mediating developmental slowing, developmental rate of wild-type and *daf-16(mu86)* mutant animals on the test bacteria were compared. Worms were put onto bacteria as eggs rather than arrested L1s because *daf-16* mutants bypass the L1 arrest, and thus hatching worms in M9, as in the other developmental assays, would not guarantee that the population would remain synchronized. For all bacterial species that were tested, the developmental rate of the *daf-16* mutants was the same as that of the wild-type worms (Figure 3.09). This suggests that *daf-16* is not controlling developmental rate, and that other regulatory mechanisms are responsible for the slow development that is observed.

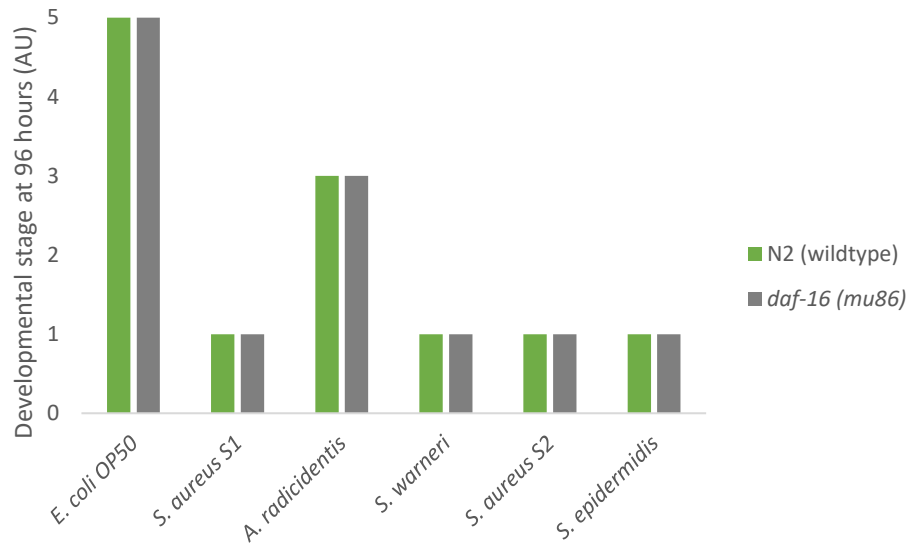


Figure 3.09 – *daf-16* mutant developmental rate is not increased compared to wild type worms on test bacteria

N2 (wild type) and *daf-16(mu86)* worms were fed bacteria that slow or arrest development. Development was monitored over several days via observation under a stereo microscope. Bars depict developmental stage of worms at 96 hours, with 1 referring to the L1 stage, 2 to the L2 stage, 3 to the L3 stage, 4 to the L4 stage, and 5 to the adult stage.  $N \geq 50$  for each treatment.



### 3.3 – Nutrient supplementation increases developmental rate of worms on test bacteria

Although several different bacterial diets induced very slow development, several species induced similar rates of development, suggest a related mechanism may be mediating the changes in developmental rate. *C. elegans* have a number of dietary requirements to maintain growth and development, including sterols, heme, and certain vitamins and amino acids (reviewed in Braeckman et al., 2009). Thus, if some bacterial diets have a deficiency of certain essential nutrients, development may be slowed or even arrested. To test this hypothesis, different nutrients were supplemented into bacterial plates in an attempt to rescue development.

#### 3.3.1 - Heme supplementation rescues arrested development of worms

When lacking heme, worms arrest at the L4 stage (Rao et al., 2005). Of the bacteria that caused developmental arrest, many were in the *Streptococcus* genus. Some Streptococci are capable of hemolysis, meaning they have the ability to break down red blood cells to obtain heme (Patterson, 1996). Streptococci are able to obtain heme from their environment, or from hosts that they infect (Francis et al., 1985, Eichenbaum et al., 1996). To test whether heme deficiency was causing developmental arrest or slowing in the worms, heme was supplemented and developmental rate was measured. Worms grown on *E. coli* OP50 developed as expected, developing to adults in three days, regardless of the amount of heme present (Figure 3.10). The slowed development when fed *Staphylococcus warneri*, *Corynebacterium urealyticum*, *Staphylococcus aureus* S2, and *Staphylococcus epidermidis* was not rescued by heme supplementation. Conversely,

heme supplementation increased the developmental rate of worms fed *Streptococcus salivarius*, *Streptococcus agalactiae*, *Actinomyces radidentis*, and *Streptococcus sinensis* and rescued the developmental arrest induced by these bacteria.

It is possible that heme has an innate ability to accelerate *C. elegans* development. To control for this possibility, *E. coli* OP50 was supplemented with different concentrations of heme and developmental progression was assessed using the *mlt-10::gfp-pest* fluorescent reporter, which fluoresces periodically as the worm molts throughout development (Frand et al., 2005). Although there were some differences in *mlt-10::gfp-pest* expression between the plates with different heme concentrations, higher concentrations of heme did not appear to accelerate developmental rate relative to when animals were fed *E. coli* OP50 without heme, suggesting that heme did not accelerate developmental rate in animals that were not heme-deficient (Figure 3.11).

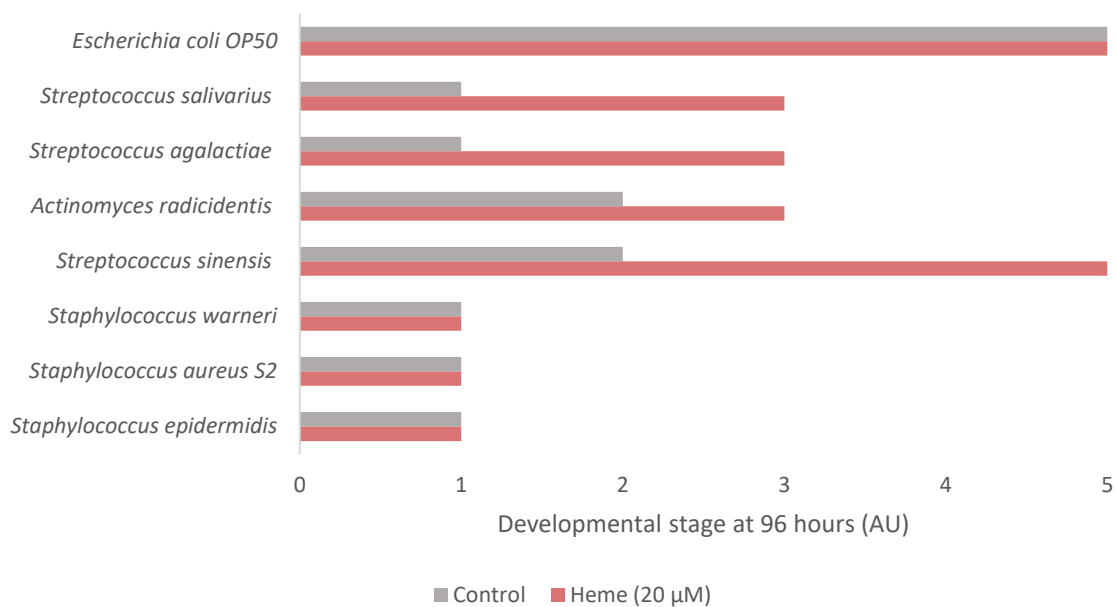


Figure 3.10 - Heme supplementation rescues developmental arrest induced by certain bacteria

*C. elegans* were fed bacteria that slow or arrest development, on plates with and without heme supplementation. Developmental stage of worms was recorded over six days. Bars depict developmental stage of worms at 96 hours, with 1 referring to the L1 stage, 2 to the L2 stage, 3 to the L3 stage, 4 to the L4 stage, and 5 to the adult stage.  $N \geq 50$  for each treatment.

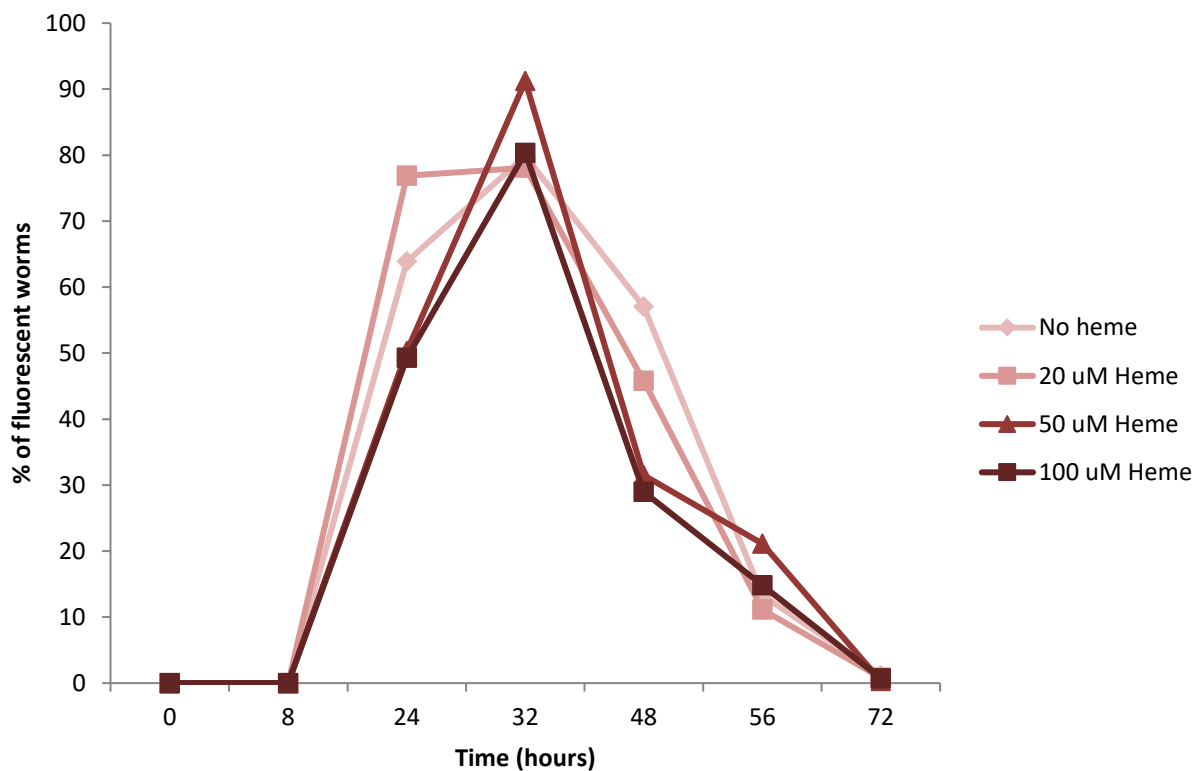


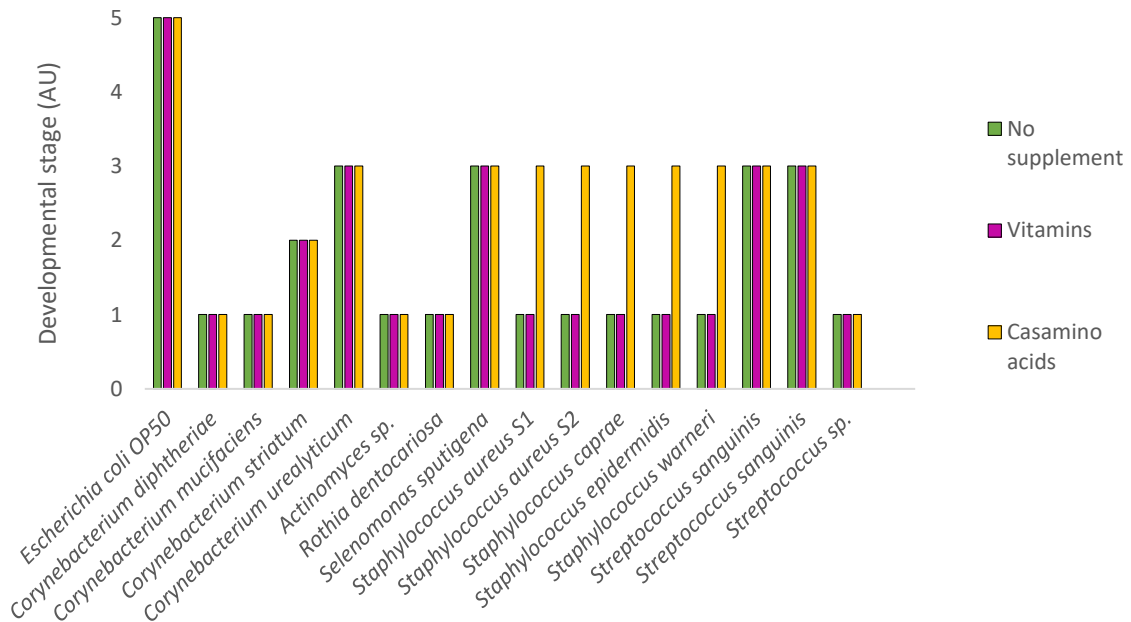
Figure 3.11 – Proportion of fluorescent *mlt-10::gfp-pest* worms over time when grown on *E. coli* OP50 supplemented with heme

Synchronized *mlt-10::gfp-pest* worms were added to  $\frac{1}{4}$  BHI plates with *E. coli* OP50, supplemented with the indicated amounts of hemin chloride. *mlt-10::GFP-pest* worms on each plate were monitored twice a day, 8 hours apart, for 3 days, and the number of fluorescent worms in the population recorded.

3.3.2 - Amino acids, but not vitamins, appears to increase developmental rate of worms on *Staphylococcus* species

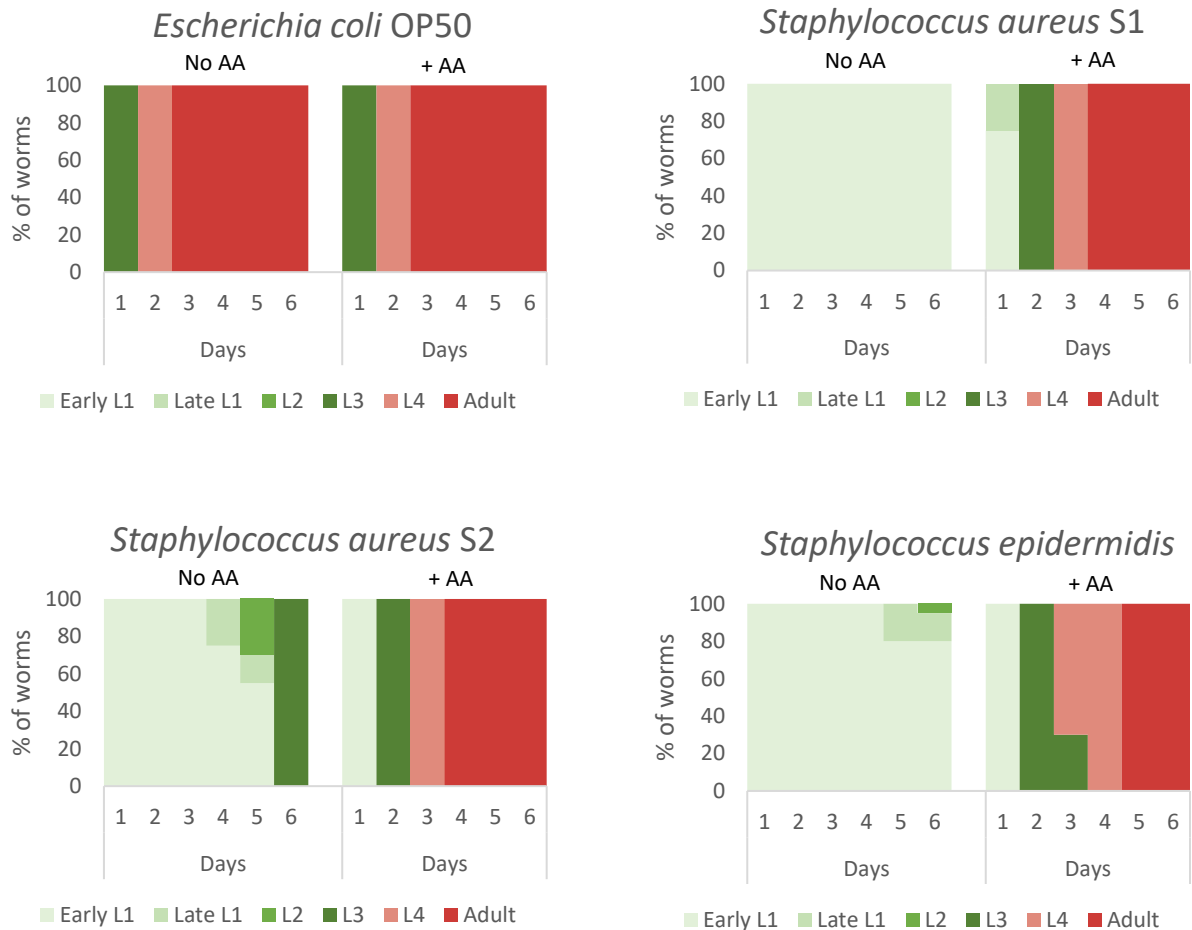
*C. elegans* require certain vitamins and amino acids in their diet for proper growth and development (Braeckmann et al., 2009). To test whether a vitamin deficiency was inducing slow development in worms grown on the test bacteria, a commercial vitamin mix (See Materials and Methods, Table 2.2) was used to supplement the media on which bacteria and worms were grown. No difference in developmental rate was observed between the control and vitamin supplemented treatments for any of the bacterial species tested (Figure 3.12).

To test if an amino acid deficiency was slowing development, a 2% casamino acid mix was supplemented into the ¼ BHI media. The casamino supplementation had no effect on development for 11 of the 16 bacteria tested, including the *E. coli* OP50 control (Figure 3.12). Developmental rate of animals fed five of the bacterial strains, the *Staphylococcus* species, was increased by casamino acid supplemented bacteria.



**Figure 3.12 - Casamino acid supplementation, but not vitamins, increases developmental rate of *C. elegans* grown on certain microbiotal bacterial species**  
*C. elegans* were grown on the listed bacterial plates with no supplementation, 1x vitamin mix supplementation, or 2% casamino acid supplementation. Worms were grown at 20 °C and observed daily under a stereomicroscope, with the larval stage of worms recorded. Bars depict developmental stage of worms at 120 hours, with 1 referring to the L1 stage, 2 to the L2 stage, 3 to the L3 stage, 4 to the L4 stage, and 5 to the adult stage.  $N \geq 50$  for each treatment.

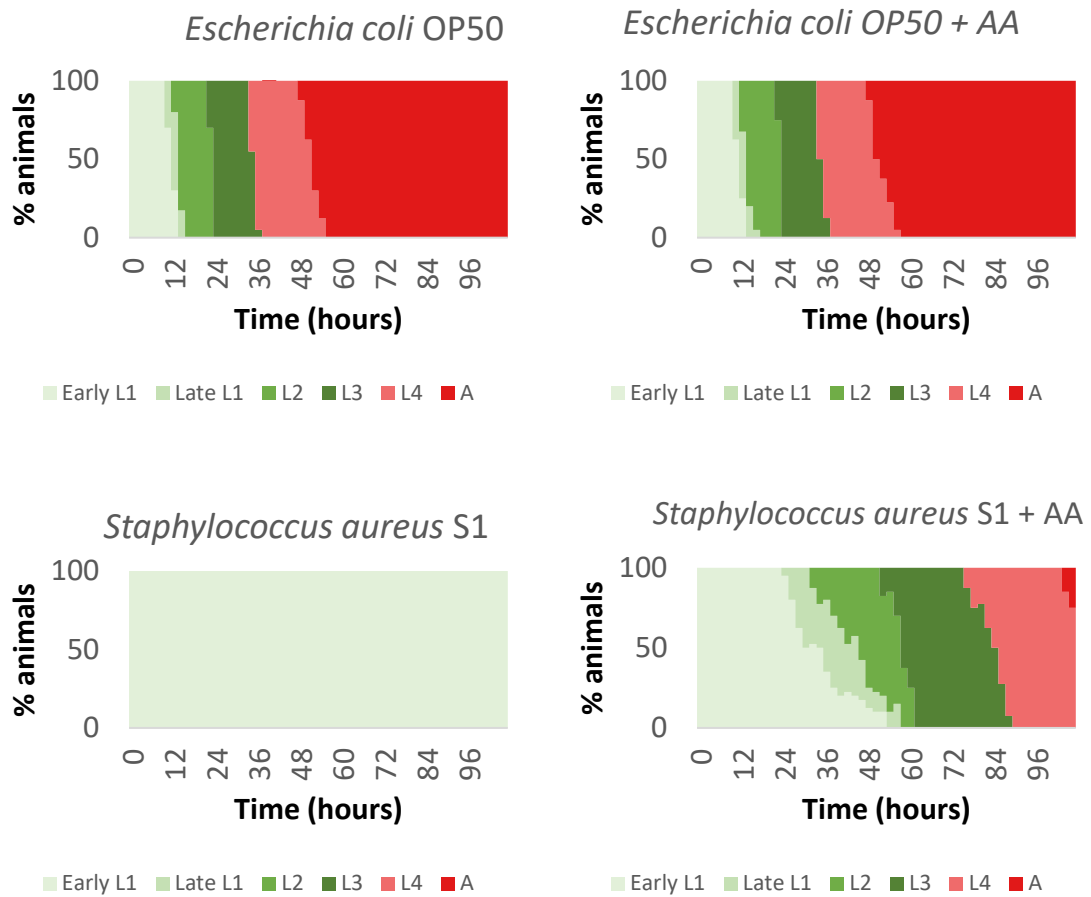
To determine which specific amino acids were required to increase developmental rate, a *hlh-8::gfp* fluorescent reporter was used to measure the developmental age of worms with and without supplementation of ten specific essential amino acids, added directly into the agar (see Materials and Methods, Table 2.2). Similar to the casamino acid supplementation, worms developed more rapidly on *Staphylococcus* species when essential amino acids were added (Figure 3.13, Supplementary Figure S3.2). Worms remained in the L1 stage for the first six days on *S. aureus* S1 without supplementation, but developed out of L1 on day 2 and into adults by day 4 when amino acids were supplemented. A more thorough analysis of the amino-acid supplementation effect was performed next in an effort to determine the approximate length of larval stages following the supplementation, with worm populations observed every two hours on two separate plates, grown 12 hours apart, for several days (Figure 3.14, Supplementary Figure S3.3). As with the previous *hlh-8::gfp* experiment, developmental rate of worms on amino acid supplemented *Staphylococcus* was much faster than on plates without supplementation, with worms developing out of the L1 stage after approximately 36 hours on bacteria such as *S. aureus* S1. However, even with supplementation, developmental rate of animals grown on the *Staphylococcus* species was slower than for those grown on *E. coli* OP50.



**Figure 3.13 - Amino acid supplementation increases developmental rate of worms fed some bacteria**

*C. elegans* carrying an *hlh-8::gfp* reporter were grown on the listed bacteria with and without essential amino acid supplementation and observed daily, with the number of visible fluorescent cells and/or larval stage of worms recorded. Data for 40 worms were collected per plate. Colors indicate number of visible M cells observed in the worm or its larval stage. Additional data in Supplementary Figure S3.2.





**Figure 3.14– Amino acid supplementation increases developmental rate of worms fed *Staphylococcus* species primarily in the L1 stage**

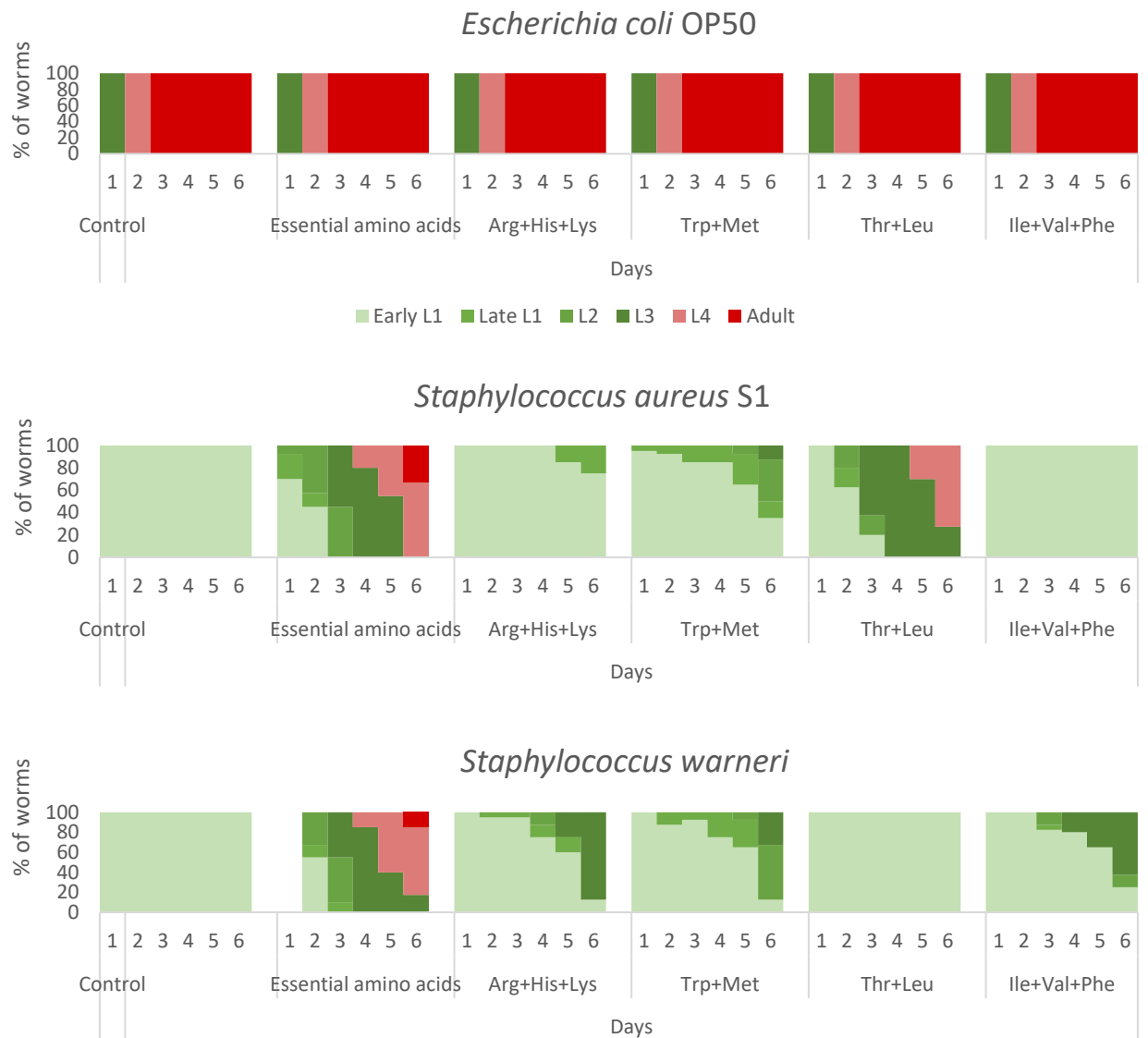
*C. elegans* carrying an *hlh-8::gfp* reporter were grown on the listed bacteria with and without essential amino acid supplementation. Two sets of plates were used for the experiment, with worms put onto the plates 12 hours apart. Worms were observed every 2 hours for 10 hours a day, under a fluorescence-equipped microscope, with the number of visible fluorescent cells and/or larval stage of worms recorded. Data for 40 worms were collected per plate. Colors indicate number of visible M cells observed in the worm or its larval stage. Additional data in Supplemental Figure S3.3.

3.3.3 – Supplementation with different amino acids rescues development on different *Staphylococcus* species

To determine which specific amino acids accelerated developmental rate, mixtures of 2-3 amino acids were supplemented on top of bacterial plates. (Figure 3.15, Supplementary Figure S3.4). Different amino acid mixtures rescued development to different degrees. On *S. aureus* S1, worms remained in the L1 stage for the first six days of development with no supplementation, but supplementation with the threonine/leucine mix greatly increased their developmental rate, with worms reaching the L4 stage after five days. Addition of tryptophan and methionine also increased developmental rate, with some worms reaching the L3 stage on day six. Conversely, the isoleucine/valine/phenylalanine mix did not increase developmental rate. For worms fed *Staphylococcus warneri*, the threonine/leucine mix had no impact on development, while supplementation with the other three mixes increased developmental rate. None of the mixes increased developmental rate to the same extent as the mixture of all ten essential amino acids.

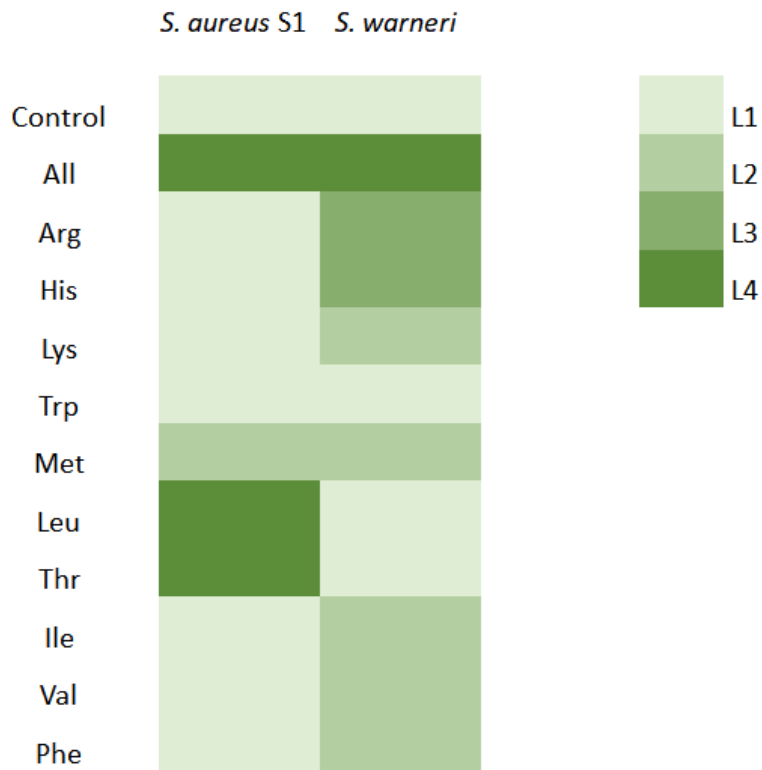
After testing the amino acid mixes, individual amino acids were supplemented into bacteria. Both threonine and leucine greatly increased developmental rate of worms fed *S. aureus* S1 (Figure 3.16). Conversely, while the tryptophan/methionine mixture increased developmental rate (Figure 3.15), tryptophan alone had no effect, although methionine was able to slightly increase developmental rate. For worms fed *S. warneri*, all eight supplemented amino acids increased developmental rate, with arginine and histidine being the most effective. Worms on the control plate were in the L1 stage after

120 hours, and arginine/histidine supplementation accelerated development to L3. Taken together, these data suggest that the type of amino acids required to increase developmental rate is dependent on the type of bacteria that are used as a diet.



**Figure 3.15 – Different amino acid mixes rescue *C. elegans* development on different bacteria**

*C. elegans* carrying an *hlh-8::gfp* reporter were grown on the listed bacteria with no supplementation, supplementation with all 10 essential amino acids, or supplementation with fewer amino acid mixes, and observed daily, with the number of visible fluorescent cells and/or larval stage of worms recorded. Data for 40 worms were collected per plate. Colors indicate larval stage of worm. Additional data in Supplementary Figure S3.4.



**Figure 3.16 – Amino acid induced changes in developmental rate depend on bacterial diet**  
*C. elegans* carrying an *hlh-8::gfp* fusion gene were grown on the listed bacteria with no supplementation or supplementation with a single amino acid. Worms were observed once a day, with the larval stage of worms recorded. Shade of green indicates larval stages of worms at 120 hours, with darker shades indicating later stages.  $N \geq 50$  for each treatment.

To determine whether non-essential amino acids could also increase developmental rate of worms fed the *Staphylococcus* species, a commercial mix containing some non-essential amino acids was added to the *Staphylococcus* species and developmental rate was measured (see Materials and Methods, Table 2.4). Developmental rate was tracked on supplemented and un-supplemented plates over several days. Worms developed at the same rate on supplemented plates compared to the controls fed *E. coli* OP50, *S. warneri*, *S. aureus* S2, and *S. caprae* (Figure 3.18). However, when grown on *S. aureus* S1 and *S. epidermidis*, worms developed faster on bacteria supplemented with non-essential amino acids compared to the control. While the increase in developmental rate was smaller than the increase observed with essential amino acid supplementation, this may suggest that some non-essential amino acids can increase developmental rate of worms on these bacterial species.

D-amino acids are produced by many species of bacteria, being essential components of peptidoglycan and other compounds (reviewed in Radkov and Moe, 2014). While *C. elegans* make use of L-amino acids from their diet, it is possible that a large quantity of D-amino acids may interfere with amino acid metabolism in the worm by competing for amino acid transporters with L-amino acids. To determine if D-amino acids could slow development of *C. elegans*, worms were grown on *E. coli* OP50 supplemented with D-amino acids. No discernible difference in developmental timing was observed between the control *E. coli* OP50 fed animals, and animals supplemented with D-amino acids (Figure 3.17). This suggests that D-amino acid production by the *Staphylococcus* species is not the cause of development slowing.

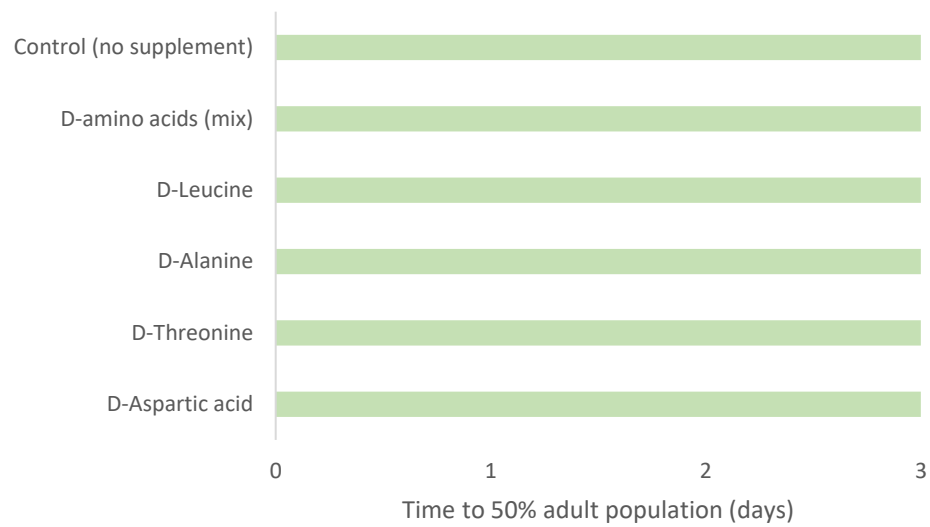


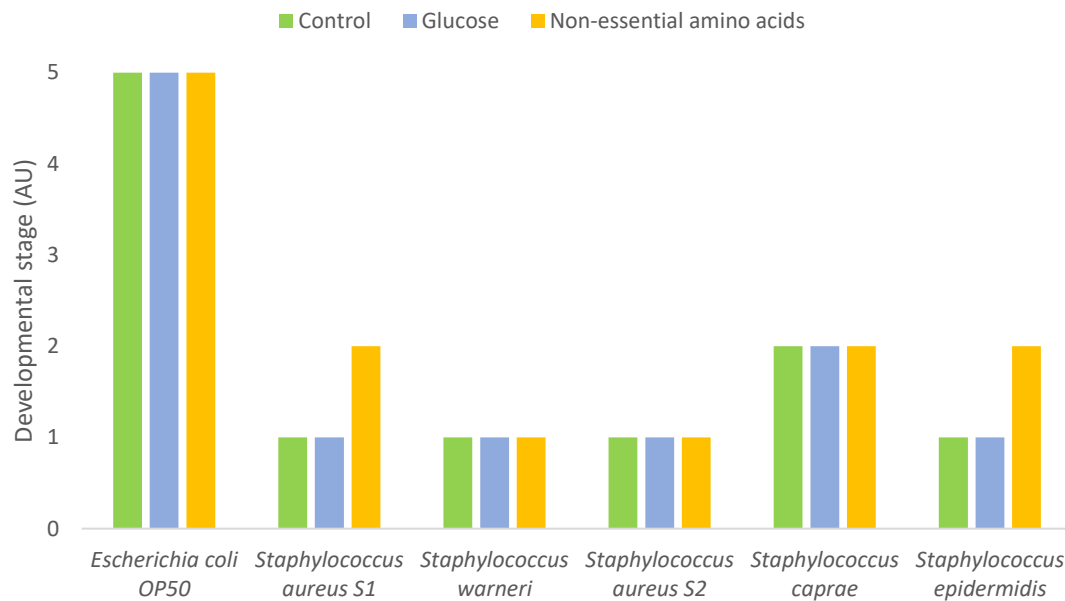
Figure 3.17- D-amino acid supplementation does not affect *C. elegans* developmental rate  
*C. elegans* carrying an *hlh-8::gfp* fusion gene were fed *E. coli* OP50 supplemented with single D-amino acids, a mix of four D-amino acids, or a control with no supplementation. Developmental rate of worms was determined once a day. Bars depict the time taken for at least 50% of the worm population on each plate to reach adulthood.

### 3.3.4 – Addition of glucose does not increase developmental rate in worms fed

#### *Staphylococcus* species

An alternate explanation for why amino acid supplementation resulted in increased development in the worms may be that worms are simply obtaining more energy. Amino acids may provide the worms with a source of energy, as amino acids can serve as energy sources in low-nutrient conditions (reviewed in Hayamizu, 2017). If additional energy is the reason worms are able to develop faster on the test bacteria following amino acid supplementation, then other sources of energy may also have the potential to increase their developmental rate. Developmental rate of worms on the *Staphylococcus* species was measured following glucose supplementation. Two percent glucose was tested, as this concentration is sufficient to decrease lifespan in *C. elegans*, suggesting it is sufficient to induce physiological changes in worms (Lee et al., 2009). Development of worms on glucose-supplemented bacteria did not differ from development on control (Figure 3.18). This suggests that the ability of amino acids to rescue developmental rate on worms fed the *Staphylococcus* species was not due to additional of energy, as an alternate energy source in the form of glucose did not increase developmental rate. Alternatively, the amount of added glucose may not have provided sufficient energy to the worms. Measuring levels of ATP in the worms fed different diets could help determine how much energy each diet provided to the worms.





**Figure 3.18 - Non-essential amino acids, but not glucose, increase developmental rate of *C. elegans* on some *Staphylococcus* species**

*C. elegans* carrying an *hh-8::gfp* fusion gene were fed bacteria that induce slow development, as well as an *Escherichia coli* OP50 control. Worms were grown with no supplementation, supplementation with 2% glucose, or supplementation with non-essential amino acids. Worms were observed once a day, with the number of visible fluorescent cells and/or larval stage of worms recorded. Bars depict developmental stage of worms at 96 hours, with 1 referring to the L1 stage, 2 to the L2 stage, 3 to the L3 stage, 4 to the L4 stage, and 5 to the adult stage.  $N \geq 50$  for each treatment.

3.4 - TOR signaling is required for amino acid-mediated developmental rescue in *Staphylococcus* species

Amino acid supplementation was sufficient to increase the developmental rate of worms grown on the *Staphylococcus* species. The mechanism of developmental slowing induced by these bacteria is likely tied to amino acid sensing or metabolism, as supplementation with both essential and non-essential amino acids, but not other metabolites or energy sources, led to an increased developmental rate in the worms fed these bacteria. The target of rapamycin (TOR) signaling pathway is a highly conserved pathway that ties nutrient sensing to growth, and is one of the most important regulators of amino acid metabolism and sensing in *C. elegans*. Thus, it is likely that reduced TOR signaling plays a role in the slow development of worms on certain bacteria if amino acid deficiency is the cause. Reducing or eliminating TOR in *C. elegans* can lead to slow development and developmental arrest (Long et al., 2002, Sheaffer et al., 2008).

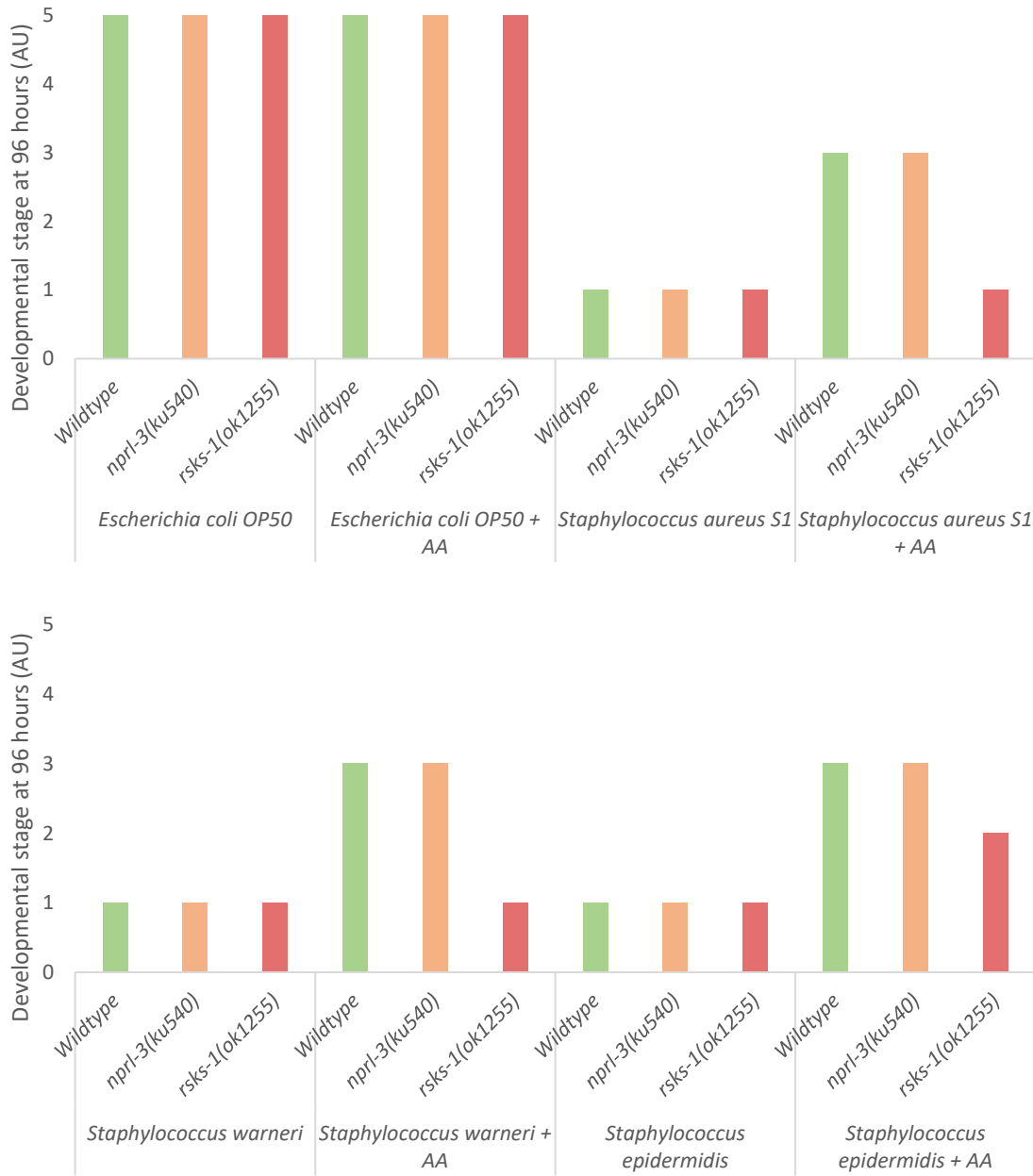
To test whether TOR signaling was required for the amino-acid mediated rescue of developmental rate, *rsk-1*, and *daf-1* mutants (TOR pathway components) were grown on the test bacteria. The gene *rsk-1/S6* kinase is a downstream effector of TORC1 that regulates lifespan and development (Sheaffer et al., 2008). The *rsk-1* mutants developed more slowly compared to wild type animals on amino acid supplemented *Staphylococcus*, with worms growing at the same rate as those without amino acid supplementation for the first few days (Figure 3.19) Developmental rate of the *rsk-1* mutant worms did increase after 5 days on the amino-acid supplemented bacteria, but it was nevertheless slower than the increase observed with wild-type worms. This suggests that *rsk-1*, a kinase

downstream of TORC1, is one factor that is needed for amino acid induced increase in developmental rate.

If *Staphylococcus* slows development by reducing TOR signaling, increased activation of the TOR pathway may suppress developmental slowing in response to these bacterial diets. *nprl-3* forms part of a complex that negatively regulates TOR in worms, and loss of function of *nprl-3* is able to rescue developmental arrest induced by a lack of monomethyl branched-chain amino acids (Zhu et al., 2013). However, developmental rate was similar in wild type worms and *nprl-3* mutants fed the *Staphylococcus* species (Figure 3.19), suggesting the mutation was not able to sufficiently increase TOR activity and increase developmental rate.

DAF-15 is the *C. elegans* homolog of Raptor, a component of TOR complex I (Jia et al., 2004). Loss-of-function *daf-15* mutants enter a terminal dauer-like larval arrest at the L3 stage. Since these animals do not produce offspring, a mutant strain balanced by translocation was used, segregating wild type heterozygotes and mutant homozygotes (The *C. elegans* Deletion Mutant Consortium, 2012). Because no visible markers distinguished the homozygous *daf-15* mutants from the wild type heterozygotes at the early larval stages, worms were transferred onto *E. coli* OP50 plates to confirm their genotype. Wild type worms that were picked onto *E. coli* could be identified as heterozygotes because they would develop into adults, while *daf-15* mutants would remain in the L3 stage. If TOR signaling is required for amino acid-mediated rescue of development on the test bacteria, amino acid supplementation should increase the developmental rate of wild type worms, but not the *daf-15* mutants. This would lead to a

mix of older wild type and younger mutant worms on the amino acid supplemented plates. The *daf-15* mutant worms were grown on *Staphylococcus aureus* S1 and *Staphylococcus warneri* with amino acid supplementation. To verify the suppression of the amino acid rescue, L3 and L4 worms were transferred from the *Staphylococcus* onto *E. coli* OP50 plates from days three to six to confirm that they were heterozygotes, which would be determined if worms developed into adults. Additionally, younger worms were transferred on days five and six to confirm that these animals were homozygous *daf-15* mutants that had not benefited from the amino acid supplementation. The older worms picked on days three and four on amino acid-supplemented *S. aureus* S1 and *S. warneri* all developed into adults, and were therefore wild type heterozygotes (Figure 3.20). Older worms picked on days five and six were also mostly heterozygotes, with the exception of *S. aureus* S1, where approximately 14% older worms picked on Day 6 were homozygous mutants. Conversely, younger worms picked on days five and six were primarily homozygous *daf-15* mutants, as those worms arrested in the L3 stage. Taken together, this suggests that the *daf-15* mutant worms were not rescued by amino acid supplementation, and that TORC1 signaling was required for the accelerated developmental rate induced by amino-acid addition.



**Figure 3.19 - *rsk-1* mutations inhibits amino acid-mediated rescue of development of worms fed *Staphylococcus* species**

Wildtype, *nprl-3*, and *rsk-1* mutant worms were grown on *Staphylococcus* species with and without essential amino acid supplementation. Development was tracked once a day for several days. Bars depict developmental stage of worms at 96 hours, with 1 referring to the L1 stage, 2 to the L2 stage, 3 to the L3 stage, 4 to the L4 stage, and 5 to the adult stage.  $N \geq 50$  for each treatment.



Figure 3.20 - Developmental rate of *daf-15* mutant worms is not increased after amino acid supplementation on test bacteria

Ratio of wildtype and mutant *daf-15* worms grown on amino acid supplemented *Staphylococcus* species. Worms that developed faster on amino acid supplemented plates were picked onto *Escherichia coli* OP50 plates and their subsequent development observed. Slow-growing worms were also picked and transferred Days 5 and 6. Worms that developed into adults on *E. coli* OP50 plates were identified as wild type and worms that arrested at the L3 stage were identified as *daf-15* mutants.

One consequence of decreased TOR signaling is an increase in autophagy. To determine if autophagy was increased in animals fed *Staphylococcus*, a fluorescent reporter, LGG-1::*gfp*, was used. LGG-1 is a vacuolar protein that is recruited to the autophagosome and localized in cells with increased autophagic vesicles (reviewed in Zhang et al., 2015). A GFP-tagged LGG-1 thus allows for identification of cells with increased autophagy, as these cells will have more LGG-1::*gfp* puncta. The number of LGG-1 positive puncta increases when TOR activity is reduced in *C. elegans*, consistent with TOR inhibition of autophagy (Hansen et al., 2008). To determine levels of autophagy in worms fed different bacterial diets, LGG-1::*gfp* worms were grown on the test bacteria, with and without amino acid supplementation. No GFP-positive puncta were observed in worms fed *E. coli* OP50, with or without amino acid supplementation (Figure 3.21). However, the number of puncta was significantly increased in worms fed *Staphylococcus* species, suggesting an increase in autophagy. Conversely, worms fed *Staphylococcus* supplemented with amino acids had a decreased number of puncta compared to worms on bacteria without supplementation, suggesting the addition of amino acids was sufficient to reduce autophagy. Because autophagy is negatively regulated by TOR, it is possible that the high levels of autophagy observed in worms fed the *Staphylococcus* species are a result of decreased TOR activity, while TOR activity is increased following amino acid supplementation, thus leading to reduced autophagy.

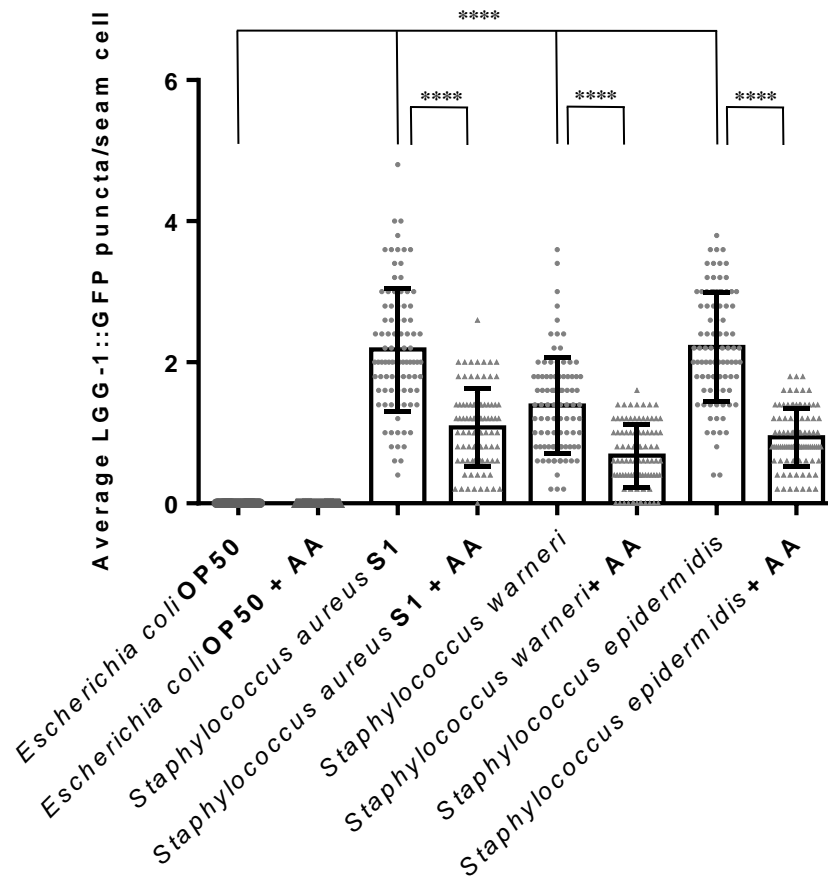


Figure 3.21– LGG-1::*gfp* puncta count is increased on test bacteria, and reduced following amino acid supplementation

LGG-1::*gfp* worms were grown on test bacteria as well as an *E. coli* OP50 control. The number of puncta in five seam cells from each worm was counted, with points on the graph representing the average number of puncta per seam cell per worm. Data from three separate biological replicates is depicted, with N = 90 per treatment. One-way ANOVA was performed to compare data for the three test bacteria to the *E. coli* OP50 control, as well as between the supplemented and un-supplemented bacteria. With the exception of the *E. coli* OP50 supplemented vs. un-supplemented comparison, all comparisons were statistically significant, with p-values < 0.0001



### 3.5 - Some bacterial species are pathogenic to *C. elegans*

One possible mechanism behind the slowed development caused by certain bacteria could be pathogenesis. If the bacteria caused infections in the worms, it would compromise their immune system or induce stress, and, in doing so, impact their ability to develop (Hodgkin et al., 2000; Leroy et al., 2012). There was some evidence to suggest that the bacteria may have been infecting the worms, such as colonization of the worm intestine (Figure 3.22). Previous attempts to test pathogenesis using killed bacteria as diet, which would not be as pathogenic as live bacteria, were unsuccessful, as killing the bacteria using UV radiation proved ineffective. Additionally, heat-killing bacteria was not a viable option as the heat destroys the essential nutrient Vitamin B2 (Qi et al., 2017), and attempts to supplement B2 into the dead bacteria was unsuccessful. Instead, the effect of the bacteria on survival was examined. Establishing which bacteria caused pathogenic effects would help distinguish between other bacteria that might affect development through alternate mechanisms, such as nutrition. If the test bacteria are pathogenic, then survival of worms on those bacteria should be decreased relative to animals grown on *E. coli* OP50.

#### 3.5.1 - Survival of adult worms is decreased on some bacterial diets

The longevity of *C. elegans* on the different test bacteria was measured using a standard survival assay. The difficulty of scoring survival in L1 worms, as well as the asynchronous development of worms on many of the bacteria, meant that determining the lifespan of worms starting from development would be difficult. Thus, the survival assay

was started with a synchronized population of worms in the L4 stage. For convenience, *glp-1* worms were used due to their temperature-sensitive sterility, allowing for worms to be grown on the same plates without a need for transfer to be rid of larvae. Late L4 worms were transferred to the test bacteria and monitored daily, with counts made of both living and dead worms each day until all worms were dead (see Materials and Methods, Figure 2.2).

Worms fed *E. coli* OP50 had one of the highest survival rates (Figure 3.23). *Actinomyces radidentis* had a survival rate comparable to the *E. coli* OP50 control. The only other bacterium tested that led to survival rates comparable to *E. coli* OP50 was *Staphylococcus warneri*. Worms fed *Corynebacterium urealyticum*, *Actinomyces sp.*, and the other *Staphylococcus* species, which all slow development, had lower survival rates compared to the *E. coli* control. Overall, the tested bacteria that induced slow development of worms also resulted in decreased survival.

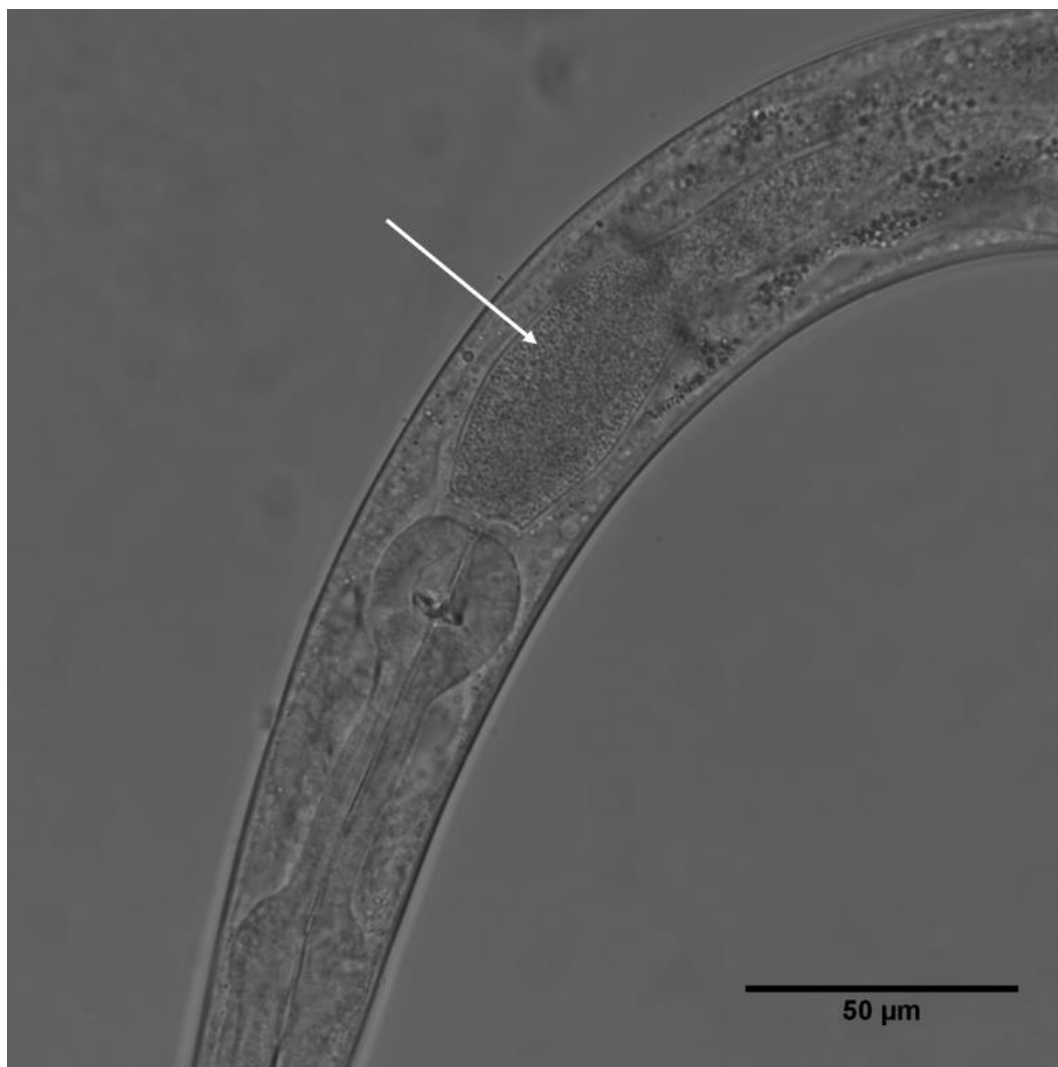


Figure 3.22 – Colonization of *C. elegans* intestine by *Staphylococcus warneri*  
Worms fed bacteria that induce slow development were mounted onto agar slides as adults and viewed under a compound microscope under high magnification, and colonization of their intestines was confirmed by the presence of bacteria in the gut, as shown by the arrow.

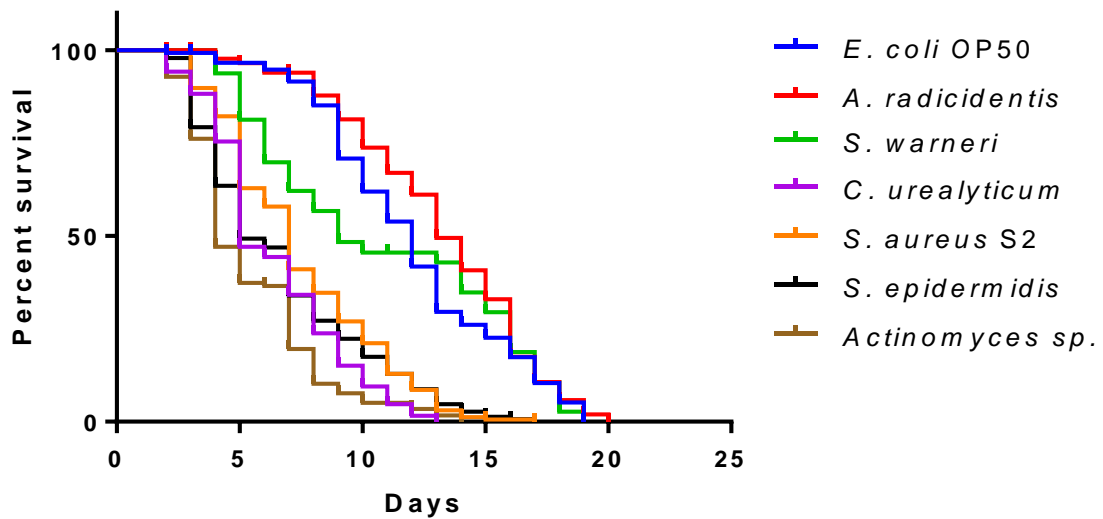


Figure 3.23 – *C. elegans* survival is decreased on several bacterial species  
Survival rate of worms grown on bacteria that cause slow development or arrest. *glp-1* worms were grown on *E. coli* OP50 until the late L4 stage, then transferred to the indicated bacteria and grown at 25 °C. Number of live and dead worms were counted each day until all worms on plate were dead. Lost worms were censored. Data for additional trials in Supplementary Table S3.2.

3.5.2 - Survival of L1 worms is reduced on the *Staphylococcus* species

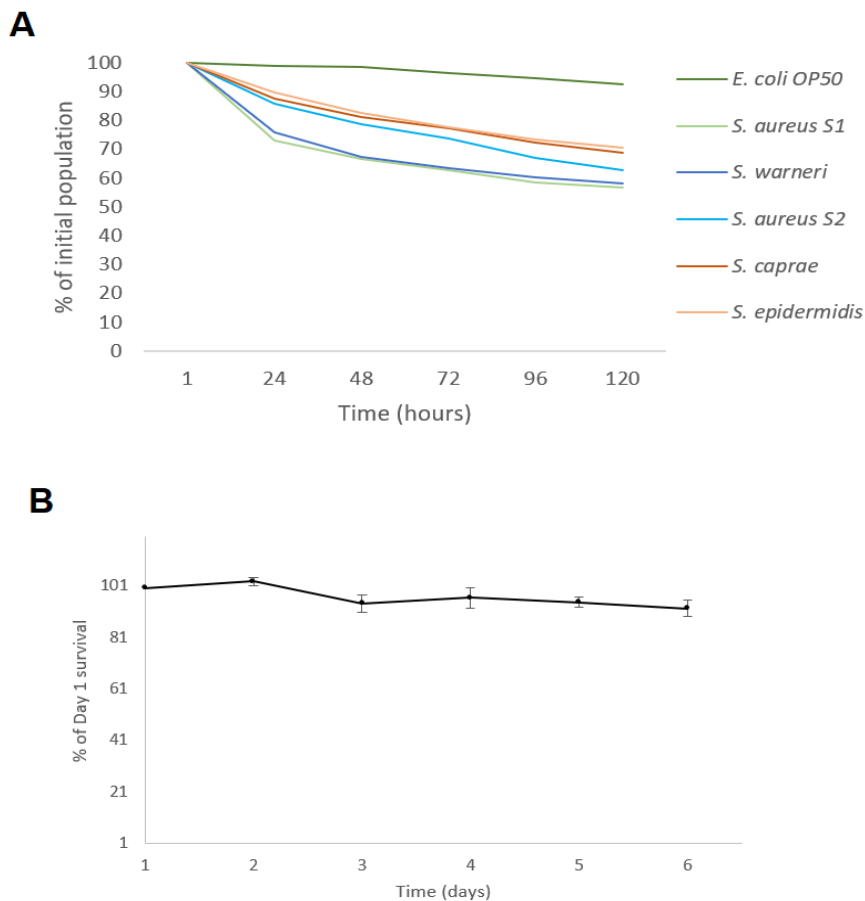
It is clear that survival is compromised on many of the bacteria, suggesting a pathogenic effect from the bacteria. Thus far, however, only adult worms have been examined. Many of these bacteria induce developmental slowing primarily in the L1 stage, thus any pathogenic effects induced by the bacteria that are affecting development may primarily impact worms in the L1 stage. To test this hypothesis, survival in the early larval stages was examined by plating L1 worms onto the *Staphylococcus* species. The number of live worms was counted every day for the first six days, which is the approximate duration of the L1 stage of worms on the test bacteria.

Worms grown on *E. coli* OP50 had a high rate of survival, as expected (Figure 3.24 A). After the first six days, 90% of the worm population was present. Conversely, the survival rate for worms was much lower on the other bacteria tested, with survival ranging from 50 to 70% at this time point. Across multiple trials *S. aureus* S1 and *S. warneri* had the lowest survival, while *S. epidermidis* had the highest. Within Day 4 survival of worms on all of the test bacteria was significantly lower than the *E. coli* OP50 control.

An alternate explanation for the slow development of worms fed certain bacteria may be starvation. If worms do not have enough food to grow, their development will be slowed or even arrested. Worms are able to survive for long periods in the L1 stage when starved; however, their survival decreases with time (Lee et al., 2012). If worms are starving on the test bacteria, that could be one explanation for their lower survival rates noted on the bacteria. To test whether starvation was causing low survivability, an L1

starvation survival assay was performed. Worms were kept in a solution of M9 following synchronization and left to starve. Worms from this solution were put onto plates every day for six days, and a count made of the surviving worms.

Worms did not appear to have a low survival rate throughout the course of the survival assay, with the survival rate of worms on Day 6 being approximately 10% lower than worms on Day 1 (Figure 3.24 B). The loss of worms due to starvation is comparable to the loss of worms noted in the L1 survival assay with *E. coli* OP50 but is much lower than the worms fed the *Staphylococcus* species. Thus, the low survival of worms in the L1 stage fed the *Staphylococcus* species is likely due to pathogenic effects, not starvation. Given that both adult and L1 worms had greatly reduced survival on these bacteria, it is quite probable that the worms are suffering from some pathogenic effects, and this may in turn be impacting their development.



**Figure 3.24 – Survival of L1 worms is decreased on *Staphylococcus* species and following starvation**

A) L1 worms have reduced survival on *Staphylococcus* species. *C. elegans* carrying an *hlh-8::gfp* fusion gene were grown on different *Staphylococcus* species. Worms were observed 1 hour after plating and then daily under a dissecting microscope, with the number of live worms counted per plate, and then observed again once a day for the next five days.  $N \geq 150$  for each bacteria. Lines represent percentage of the total initial population of worms grown on each bacteria over time. Data for additional trials in Supplementary Table S3.3.

B) Starvation in M9 slightly reduces survival L1 survival. *C. elegans* were synchronized and arrested in L1 in an M9 solution. Worms were plated onto separate *E. coli* OP50 NGM plates every day for 6 days. The number of surviving worms on each plate was counted two days after worms had been put onto the plate. All plates done in triplicate, 90-150 worms counted per plate. The line represents the number of surviving worms on the plate on each day as a percentage of the number of worms that survived on the first day. Data shown is an average of three separate biological replicates, error bars are standard deviation.

**CHAPTER 4 – DISCUSSION AND CONCLUSION**



Environmental factors, such as diet, can have huge impacts on health and disease, and understanding how organisms respond to the environment can provide insight into how human health is impacted by the environment. The model organism *Caenorhabditis elegans* was used to study the effects of a single environmental factor, their bacterial diet, and how this factor altered the developmental rate of the organism. In particular, the study aimed to investigate how *C. elegans* development is regulated in response to different bacterial diets.

Development of *C. elegans* is severely slowed in response to a number of bacterial species in the *Staphylococcus* genera, with the majority of developmental slowing happening in the first larval stage. Furthermore, the study determined that supplementation with amino acids increased developmental rate of worms grown on these bacteria, although the specific amino acids required differed between bacterial species. Activation of TOR signaling was required for this amino-acid mediated developmental rescue, suggesting the slow development on the tested bacteria was being regulated, at least in part, through TOR signalling. This regulation of development by TOR highlights the importance of the pathway in responding to different nutritional cues, such as a deficiency of an essential amino acid, by mediating changes to the organism's developmental rate.

#### 4.1 *C. elegans* development is heavy slowed by some bacterial species

The developmental rate of *C. elegans* exposed to 42 bacterial species, isolated from the human respiratory microbiota, was determined, and several bacterial species that

severely slow or arrest development were identified. Of the 42 species tested, 26 species increased the time taken for worms to develop into adults, while eleven species prevented worms from developing into adults. The varied developmental rates of worms exposed to different bacterial diets highlights the adaptability of the species, as worms were able to regulate their development in response to specific bacterial diets.

Tracking developmental progression of worms exposed to different bacteria revealed that many bacterial species slowed *C. elegans* development primarily in the L1 stage. Because several different bacterial species induced similar effects on developmental rate, a similar mechanism may be the cause. Activation of *mlt-10::gfp-pest* was delayed in worms fed slow growth-inducing bacteria, suggesting the slowing is happening before the new cuticle is formed. Additionally, the increase in the number of fluorescent animals of the *mlt-10::gfp-pest* worms does not occur as a peak in time, but instead is prolonged, as observed with *mlt-10::gfp-pest* expression on *Staphylococcus epidermidis*. This may indicate asynchrony in the developmental rate of individual animals within the population, causing some worms to turn on *mlt-10* earlier than others, leading to a more extended period of fluorescence observed in the population. This asynchrony was also observed using the *hlh-8::gfp* reporter, where previously synchronized populations observed after a few days were comprised of worms in multiple larval stages. One possible explanation for the asynchronous growth of a population is that some worms may not be feeding on the bacteria. *C. elegans* have the ability to distinguish between high- and low-quality food and may avoid consuming food that is low-quality (Shtonda and Avery, 2006; Qi et al., 2017). Thus, some of the worms

exposed to these bacteria may be feeding less and thus developing more slowly. This stochastic variation within a population may be one mechanism by which worms are able to adapt to challenging environmental conditions, ensuring that a portion of their population is able to survive.

Exposure to the bacteria in later larval stages also slowed development, suggesting that the effect of these bacteria is not exclusive to the L1 stage. However, the slowing effect was most pronounced in the L1 stage. It is possible that in the L1 stage *C. elegans* are more susceptible to the bacterial factors that are inducing slow development. The L1 stage may be a critical developmental checkpoint where worms slow their development in response to challenging environmental factors such as nutrient deficiencies or pathogens. Conversely, the increased developmental rate of worms when transferred at later larval stages may be due to worms storing nutrients while feeding the *E. coli* OP50 diet.

#### 4.2 – Loss of *daf-16* does not rescue slow development on tested bacteria

When *C. elegans* were fed *Staphylococcus* species, an increase in the expression of cell cycle inhibitor *cki-1* was noted, suggesting seam cell division was halted and animals were undergoing developmental arrest. DAF-16 is one regulator of *cki-1* in the L1 stage (Baugh and Sternberg, 2006). It thus seemed likely that L1 arrest or developmental slowing may be mediated by DAF-16. However, *daf-16* mutants grown on the test bacteria did not develop more rapidly than wild type animals, suggesting *daf-16* was not playing a role in the L1 extension. As loss of *daf-16*, the primary insulin signaling effector, does not appear to increase the slow developmental rate of worms

induced by the tested bacteria, insulin signaling may not be the main regulator of this developmental adjustment.

#### 4.3 – Supplementation with specific nutrients can accelerate developmental rate in response to poor bacterial diets

Supplementation with different nutrients increased developmental rate of worms on some bacterial species, though worms did not grow as well as on the *E. coli* OP50 control in any treatment. Heme was able to rescue developmental arrest of worms grown on *A. radidentis*, *S. sinensis*, *S. salivarius* and *S. agalactiae*, suggesting the cause of developmental arrest induced by those bacteria, and potentially others, is a lack of heme. Heme is an essential nutrient for *C. elegans*, and worms arrest in the L4 stage when heme is missing from their diet (Rao et al., 2005). These bacteria may not be producing enough heme, leaving worms with a deficiency. Based on KEGG data, certain isolates of *A. radidentis* and *S. salivarius* can produce heme, while *S. agalactiae* cannot (Kanehisa and Goto, 2000; Kanehisa et al., 2016; Kanehisa et al., 2017). However, even if these bacteria are producing heme, it is uncertain whether the amount produced is sufficient for the worms. It is not clear why developmental arrest induced by these bacteria happens in the earlier larval stages, rather than in L4, as documented by other studies (Rao et al., 2005). Heme is deposited into the *C. elegans* oocyte from the parents to support early development (Chen et al., 2011). Some bacterial species may have the potential to acquire heme from the worms themselves, thus leaving them with a deficiency and inducing arrest at an earlier larval stage.

Although certain vitamins are essential nutrients for worms, supplementation of a commercial vitamin mix to bacteria did not rescue the slow developmental rate of worms induced by the tested bacteria. It is possible that vitamins are not the limiting factor in regulating growth of *C. elegans* on these bacteria. Alternatively, the bacteria themselves may be obtaining and metabolizing the vitamins, preventing the worms from accessing them. If this were the case, adding vitamins to the bacteria would not rescue development. Instead, a method would need to be used where the bacteria could not compete for the available vitamins. Feeding worms bacteria that has been killed may potentially address this issue.

Amino acid supplementation increased developmental rate of *C. elegans* fed strains of *Staphylococcus aureus*, *S. warneri*, *S. caprae*, and *S. epidermidis*, which all slow development in the L1 stage, suggesting an amino acid deficiency was causing developmental slowing on the tested bacteria. The specific amino acids required to increase developmental rate varied between bacterial strains, although threonine and leucine were the most effective at improving development on all tested bacteria save *S. warneri*. Why an amino acid deficiency causes development to only be slowed, and not arrested, is unclear. It is possible that worms are able to accrue small amounts of the needed amino acids from the bacteria or the plates, and over time this is sufficient to allow them to develop into adults.

Supplementation with non-essential amino acids increased developmental rate of worms fed *S. aureus* S1 and *S. epidermidis*. This may suggest amino acids in general are

able to increase developmental rate. Amino acid supplementation may be providing more energy to the worms, leading to improved developmental rate. However, supplementation with glucose failed to increase the developmental rate of worms fed the *Staphylococcus* species, suggesting addition of energy sources was not sufficient to rescue development. Alternatively, the glucose supplementation may not have provided enough energy to the worms. A more effective method of measuring whether changes in energy levels can increase developmental rate may be to measure ATP production in the worms following supplementation of different nutrients (Palikaras and Tavernarakis, 2016).

Amino acid deficiency is not sufficient to fully explain the decreased developmental rate in response to the *Staphylococcus* species, as even with supplementation animals developed more slowly than those fed *E. coli* OP50. However, an amino acid deficiency likely contributed to the slow development of worms on these bacteria. This adaptability in developmental rate in response to a lack of nutrition demonstrates a method by which organisms are able to adapt to challenging environmental conditions. In this case, *C. elegans* may be regulating their growth so they can make optimal use of their limited metabolic resources. Determining the mechanism by which worms are able to sense this nutritional deficiency and subsequently slow their development may shed further light on how organisms integrate nutrient sensing with regulation of metabolism and growth.

#### 4.4 – TOR signaling regulates slow development on *Staphylococcus* species

TOR signaling is a regulator of development in *C. elegans* (Jia et al., 2004), and many TOR components are activated by amino acids in several species (reviewed in Kim and Guan, 2011). Mutation of the TOR kinase *let-363* slows developmental rate, eventually leading to developmental arrest at the L3 stage (Long et al., 2002). It is possible that a decrease in TOR activity due to a lack of nutrients could similarly slow development in the early larval stages, while supplementation with amino acids rescues this effect. If this were the case, hyperactivating TOR may improve developmental rate on these bacteria, while knocking out TOR components should suppress the effects of amino acid supplementation.

Loss of *daf-15*/Raptor, a central TORC1 component, suppressed the effect of amino acid supplementation in worms fed *Staphylococcus* diets. Similarly, loss of *rsks-1*, a downstream mediator of TORC1, partially suppressed the effects of amino acid supplementation. This finding suggests that the S6 kinase may be one mediator of the TOR signaling pathway that is required for the developmental rescue. Given that *rsks-1* did not completely suppress the effects of amino acid supplementation as worms did show an increase in developmental rate after several days, it is likely that *rsks-1* is not the only factor regulating the effect of amino acid supplementation, and that other factors downstream of TORC1 are also required for the effects of amino acid supplementation on the *Staphylococcus* species. TOR signaling regulates both DAF-16 and SKN-1 to regulate development (Jia et al., 2004; Robida-Stubbs et al; 2013, Ruf et al., 2014), and thus other factors may be regulating development in response to an amino acid deficiency.

It was hypothesized that the *nprl-3* mutant could potentially increase developmental rate of worms on the bacteria, as it is a negative regulator of TOR, and loss-of-function rescues developmental arrest in worms lacking leucine (Zhu et al., 2013). However, the *nprl-3* mutants did not develop any faster than the wild type worms when fed a *Staphylococcus* diet. It is possible that another regulator of TOR, or perhaps an effector downstream of TOR, remained inhibited due to a lack of amino acids, resulting in developmental rate not being increased. Indeed, the study by Zhu et al., 2013 suggests effects of *nprl-3* mutants on rescuing developmental rate or arrest require TOR activity to be increased. Thus, TOR if being suppressed through some other mechanism, then the *nprl-3* mutation may not have any effect.

In this study, the observed increase in LGG-1::*gfp* positive puncta, a marker of autophagy, in worms fed bacteria that slow development may suggest that TOR activity is reduced in those worms, as TOR is a negative regulator of autophagy (Hansen et al., 2008). Additionally, amino acid supplementation decreases the number of LGG-1::*gfp* positive puncta in worms fed the *Staphylococcus* species. Taken together, this demonstrates that worms may have reduced TOR activity when fed the *Staphylococcus* species, leading to slow development, and this is partially rescued by the supplementation of amino acids,

As the TOR signaling pathway integrates nutrient signals to regulate development, a nutrient deficiency could lead to TOR inactivation to prevent developmental from progressing in the absence of essential nutrients. To further elucidate the mechanism by which inactivation of TOR signalling slows development in *C. elegans* when worms are



fed the *Staphylococcus* species, other components in the TOR signalling pathway, perhaps those downstream of *rsks-1*, should be studied. Additionally, determining whether *C. elegans* are sensing a deficiency in amino acids could also explain how TOR is regulated. As the TOR signaling pathway is conserved in humans, understanding the mechanisms of how the pathway integrates nutritional cues to regulate development and metabolism in worms can shed light on how human systems do the same.

#### 4.5 – Pathogenic effects from tested bacteria may impact development

In addition to metabolic regulation of development, pathogenic effects can also impact worm growth (Hodgkin et al., 2000; Fasseas et al., 2013; Ren and Ambros, 2014). Even with metabolite supplementation, developmental rate of worms on the test bacteria were slower than worms fed *E. coli* OP50, suggesting other mechanisms may also be slowing development. Many of the bacteria that cause very slow development are of the *Staphylococcus* genus, some of which are known to be pathogenic to worms (Sifri et al., 2003; Irazoqui et al., 2010). Further, many of the bacterial species examined colonized the worm intestine. It is likely that some of the bacteria tested are infecting the worms, and this may be one way in which development is being slowed.

The *Staphylococcus* strains that slowed development also reduced survival, suggesting they are pathogenic. How this pathogenic effect slows developmental rate is unclear. Previous studies have shown that transcription factors can regulate development in response to infections, and balance the worms' immune response with developmental timing (Ren and Ambros, 2014), and this may be one mechanism by which infection by

the *Staphylococcus* species is slowing development. Data from the survival assay may have been confounded by the fact that bacterial survival was not accounted for. Given that the survival assay lasted for multiple weeks, bacteria may have begun to die as well, reducing their impact on worm survival. Future survival assays should attempt to determine how long each bacterial species is able to survive to ensure their effects on worms are not changing over the course of the assay.

Survival of worms in the L1 stage was also reduced on the *Staphylococcus* species. If worms are allocating resources to fighting a pathogenic species and attempting to survive, then growth may be slowed. Survival can also be decreased as a result of prolonged starvation (Lee et al., 2012). A study by Lee et al in 2012 suggests that the LT<sub>50</sub> (50% of lethality time) for starved worms at 20 °C is 16 days. However, a lower lethality rate was observed for worms starved in M9 compared to worms grown on the *Staphylococcus* species. This suggests that starvation does not reduce worm survival to the same extent as growth on the *Staphylococcus* species. The conditions used to test starvation survival involved starving worms in an M9 solution. This is not equivalent to how worms naturally experience survival, where larvae may obtain some nutrients from their eggs, and this may have confounded the results from the starvation assay.

Given the results of the survival assays, there is evidence that the bacteria used in this study have pathogenic effects. Worms have reduced survival when fed the bacteria in the adult stage, after development has already completed. Additionally, low survival in L1 suggests infection is happening as early as the first larval stage, which may indicate that this infection may be causing the slow L1 development. Tests of pathogenesis should be

continued to distinguish pathogenic effects from nutritional ones. Ideally, growing worms on killed bacteria could provide more evidence of this, as worms would not have to deal with infection from dead bacteria and may be able to develop faster. However, thus far attempts to grow worms on killed bacteria have been unsuccessful, as the most effective method of killing bacteria, through heat, also causes worms to arrest development. Future studies examining slowing of development through pathogenesis should attempt to identify a way to kill bacteria without compromising development.

#### 4.6 - Conclusions and future directions

This study identified several bacterial species that severely slow the developmental rate of *C. elegans*, particularly in the L1 stage, as well as some of the factors that are regulating these changes in development (Figure 4.1). In the case of the *Staphylococcus* species, supplementation with amino acids partially rescued developmental rate. *Staphylococcus* are known pathogens of *C. elegans*, and all of the *Staphylococcus* species used in this study colonize the worm intestine and reduce the survival rate of worms in both the adult and L1 stage, suggesting worms are suffering from pathogenic effects. Infection by bacteria can slow growth (Hodgkin et al., 2000; Fasseas et al., 2013), with one study suggesting the *let-7* miRNA, which regulates developmental timing, is inhibited by p38 MAPK pathway, which activates innate immunity in response to the pathogenic bacteria *Pseudomonas aeruginosa* (Ren and Ambros, 2014). The *Staphylococcus* species may be activating the p38 MAPK cascade in a similar manner and in turn negatively regulating miRNAs like *let-7* that control

developmental timing, thus slowing development in the worms. A future study should aim to determine activity of the p38 MAPK pathway in worms fed the *Staphylococcus* species as well as determine the expression of *let-7* family miRNAs.

While pathogenic effects may be one explanation for the slow development observed in worms fed the *Staphylococcus* species, this study suggests an alternate mechanism. The increase in developmental rate with amino acid supplementation on these bacteria was mediated through TORC1 signaling. There is evidence to suggest that some *Staphylococcus* species can reduce the activity of TORC1 in mammalian systems, and that downregulation of TOR activity is important for mounting an immune response (Magryś et al., 2018). The subsequent increase in autophagy, also noted in the present study, helps with clearing of infected cells (Ivanov and Roy, 2013). Alternatively, decreased availability of amino acids may result in low TOR activity, thus slowing development. TORC1 signaling is increased in response to amino acid availability (Sancak et al., 2008), which may explain why addition of even non-essential amino acids resulted in slightly increased developmental rate.

This study identified a potential mechanism by which *C. elegans* regulate their developmental rate in response to a challenging bacterial diet. When exposed to a *Staphylococcus* diet, worms may be experiencing both a nutrient deficiency and a pathogenic effect. Through regulation by the TOR signaling pathway, worms are able to slow their developmental rate until they are provided with additional amino acids. However, while the study found that amino acid supplementation requires TOR activity to increase developmental rate of worms on the *Staphylococcus* species, questions remain

regarding the mechanism of how development is slowed initially. TOR activity appears to be decreased, but it is unclear whether this occurs in response to amino acid sensing, or whether TOR is inhibited through a pathogen response. Additionally, this study identified the S6K branch of TORC1 signaling as one regulator of amino-acid mediated developmental rescue, but loss of *rsks-1/S6K* did not fully suppress the effects of amino acid supplementation, suggesting other factors play a role. Additionally, the mechanism by which S6K regulates development is also unclear, although previous studies have suggested that TOR and S6K may be repressing *pha-4/FoxA* to regulate development (Sheaffer et al., 2009). Future experiments should focus on discovering the genetic pathways that control development through regulation by TOR. Understanding how conserved signaling pathways, such as the TOR pathway, regulate metabolism and growth in response to diet can provide more information regarding how environmental signals are integrated by organisms and used to regulate physiological processes. This may have implications for human health, as it could shed light on how environmental factors influence human physiology to regulate health and development.

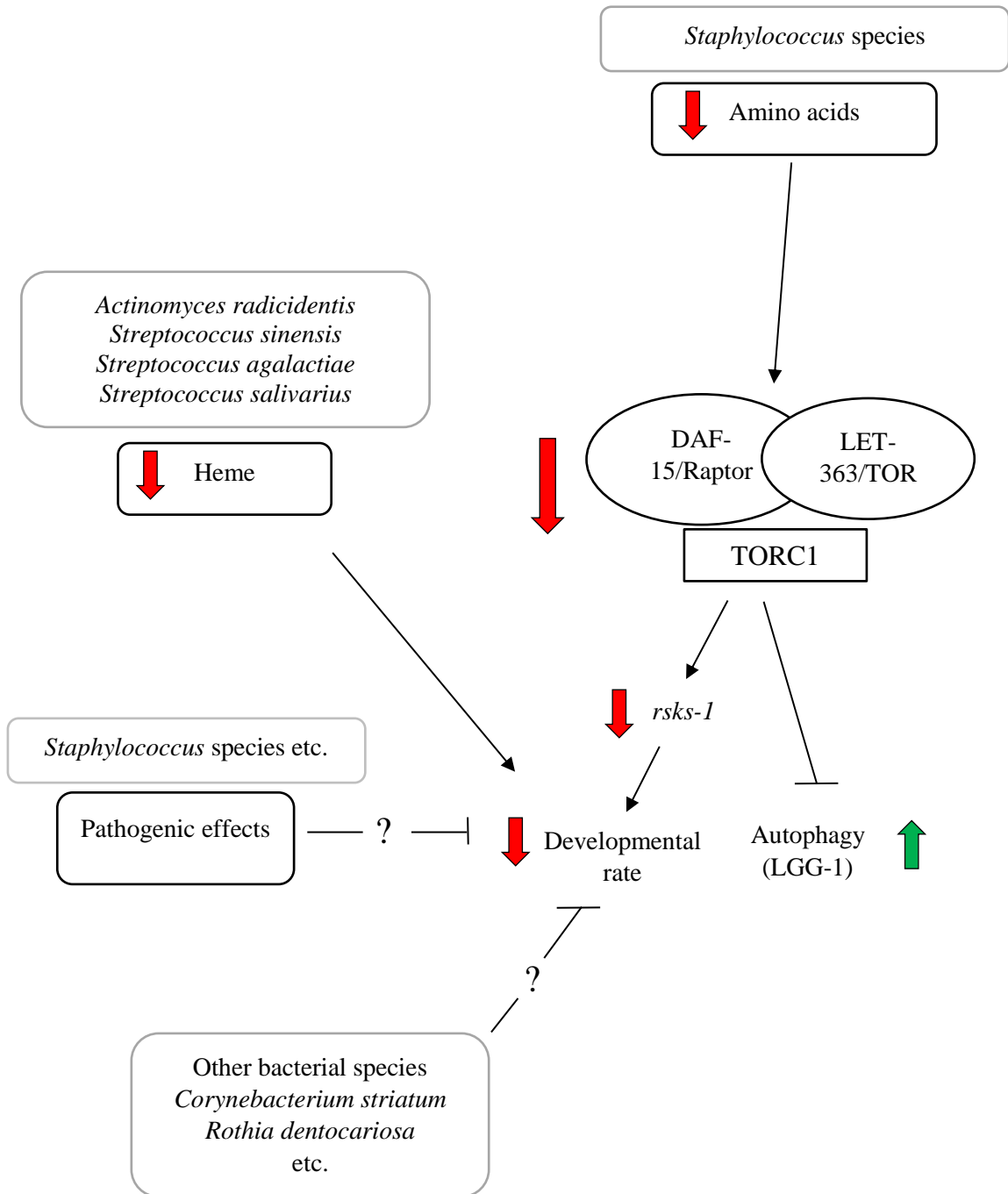


Figure 4.1 – Regulation of *C. elegans* developmental rate by different bacterial species

Working model demonstrating how different bacterial species arrest or slow developmental rate through different methods. Feeding on *Staphylococcus* species results in an amino acid deficiency in worms, reducing TOR activity and slowing developmental rate through downstream effectors such as *rsk-1*. Autophagy is also increased as a result. Some species, such as *Actinomyces radidentis* and *Streptococcus sinensis*, cause developmental arrest through a heme deficiency. Several species, including the *Staphylococcus* species, may be slowing development through pathogenic effects. A set of bacteria, including *Corynebacterium striatum* and *Rothia dentocariosa*, slowed development, but the mechanisms behind this effect remain unknown.

**CHAPTER 5 – SUPPLEMENTARY FIGURES**



Trial 1

% of fluorescent worms

<b>Time (hours)</b>	0	2	4	6	8	10	14	16	18	20	22	24	26	28	30
<i>E. co-li</i> OP50	0	0	0	0	0	0	9	98	100	100	36	98	75	82	96
<i>S. warneri</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>C. urealyticum</i>	0	0	0	0	0	0	0	0	0	0	0	0	3	1	10
<i>S. aureus</i> S1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. epidermidis</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

% of fluorescent worms

<b>Time (hours)</b>	32	34	38	40	42	44	46	48	50	52	54	56	58	64	72
<i>E. coli</i> OP50	93	81	26	95	95	98	84	89	81	92	91	87	83	0	0
<i>S. warneri</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>C. urealyticum</i>	15	15	15	11	8	8	17	14	38	59	31	29	24	13	72
<i>S. aureus</i> S1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	34
<i>S. epidermidis</i>	0	0	1	2	4	12	14	13	20	23	24	23	21	23	2

Trial 2

% of fluorescent worms

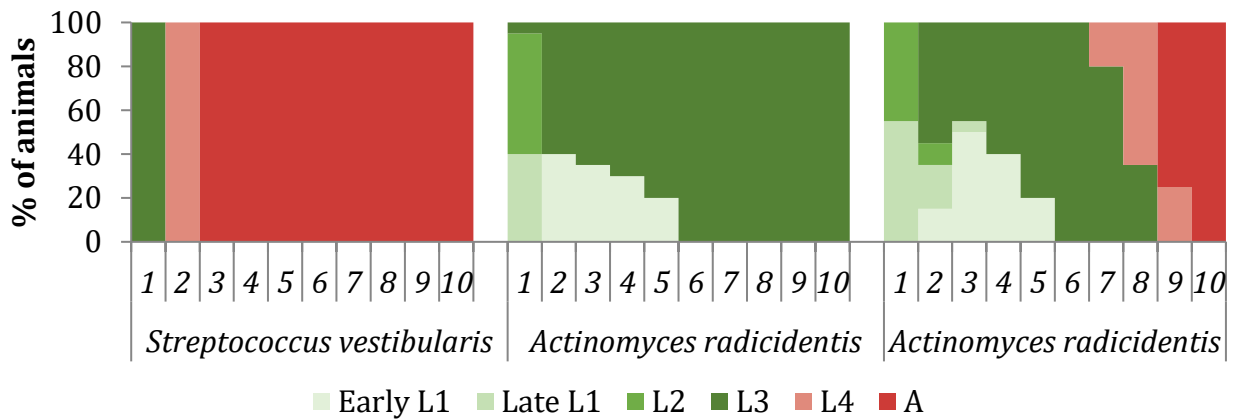
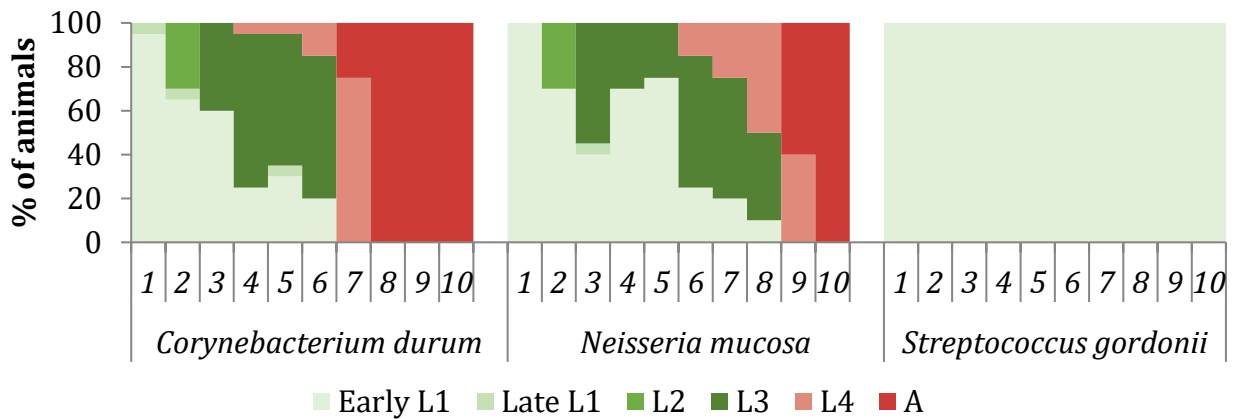
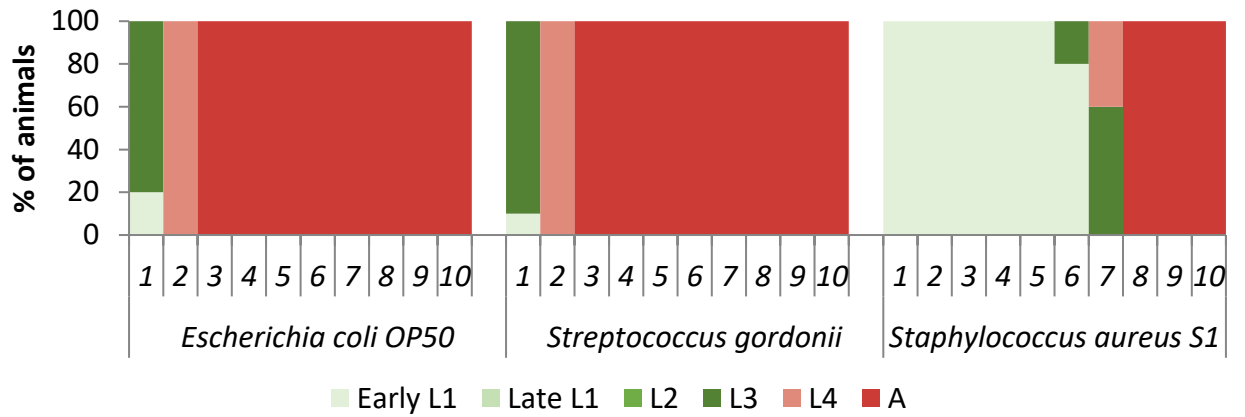
<b>Time (hours)</b>	0	2	4	6	8	10	14	16	18	20	22	24	26	28	30
<i>E. coli</i> OP50	0	0	0	0	0	0	57	68	100	89	50	20	11	47	70
<i>S. warneri</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>C. urealyticum</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. aureus</i> S1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	6
<i>S. epidermidis</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0

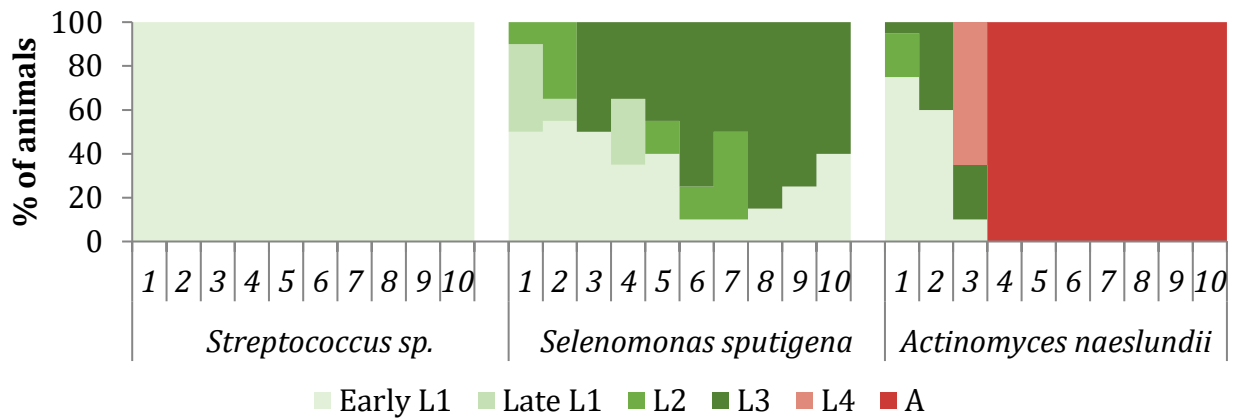
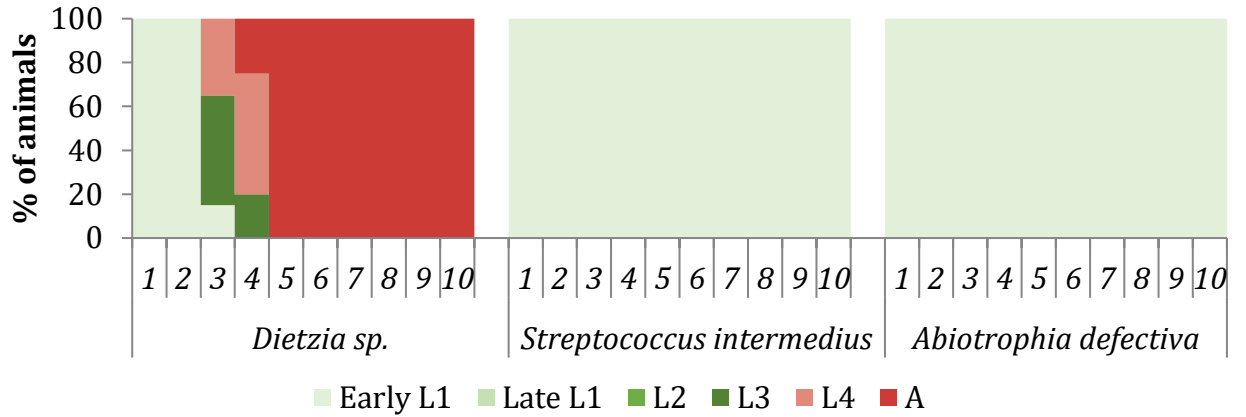
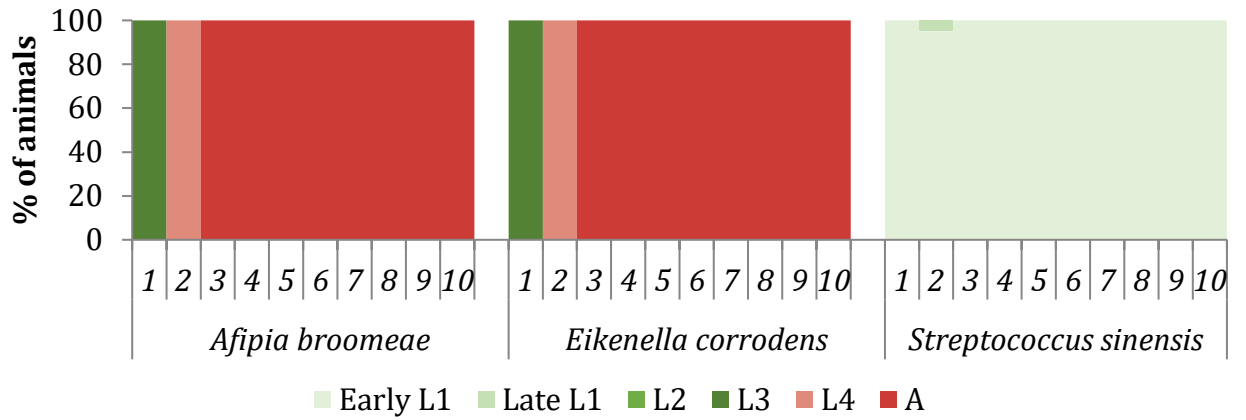
% of fluorescent worms

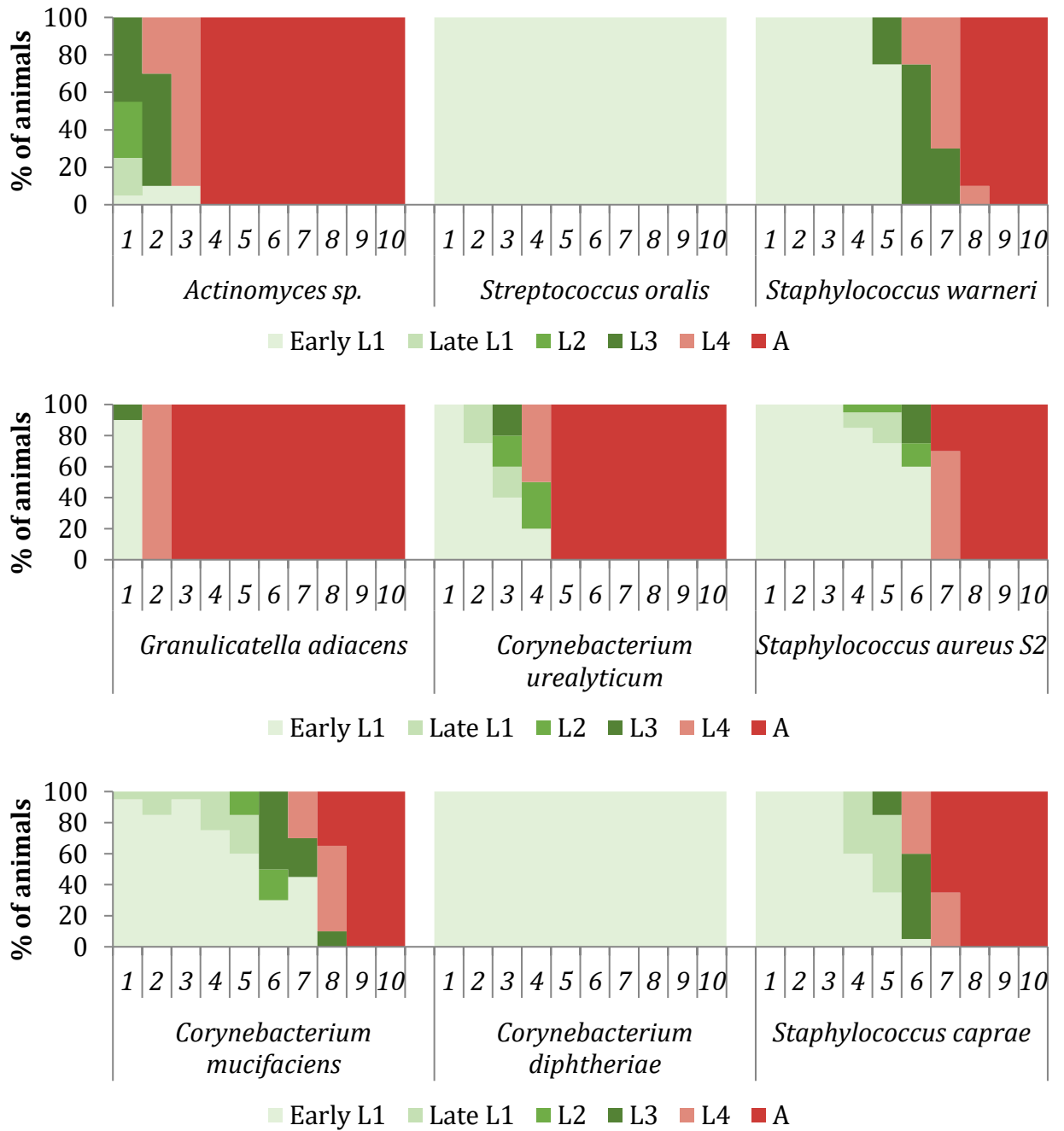
<b>Time (hours)</b>	32	34	38	40	42	44	46	48	50	52	54	56	58	64	72
<i>E. coli</i> OP50	45	13	65	80	85	88	96	23	72	96	67	50	18	2	0
<i>S. warneri</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>C. urealyticum</i>	0	0	11	15	15	12	20	25	35	60	42	25	28	5	50
<i>S. aureus</i> S1	6	0	7	7	6	5	5	5	6	6	1	6	2	23	32
<i>S. epidermidis</i>	0	0	0	0	0	0	0	41	56	49	38	30	19	35	2

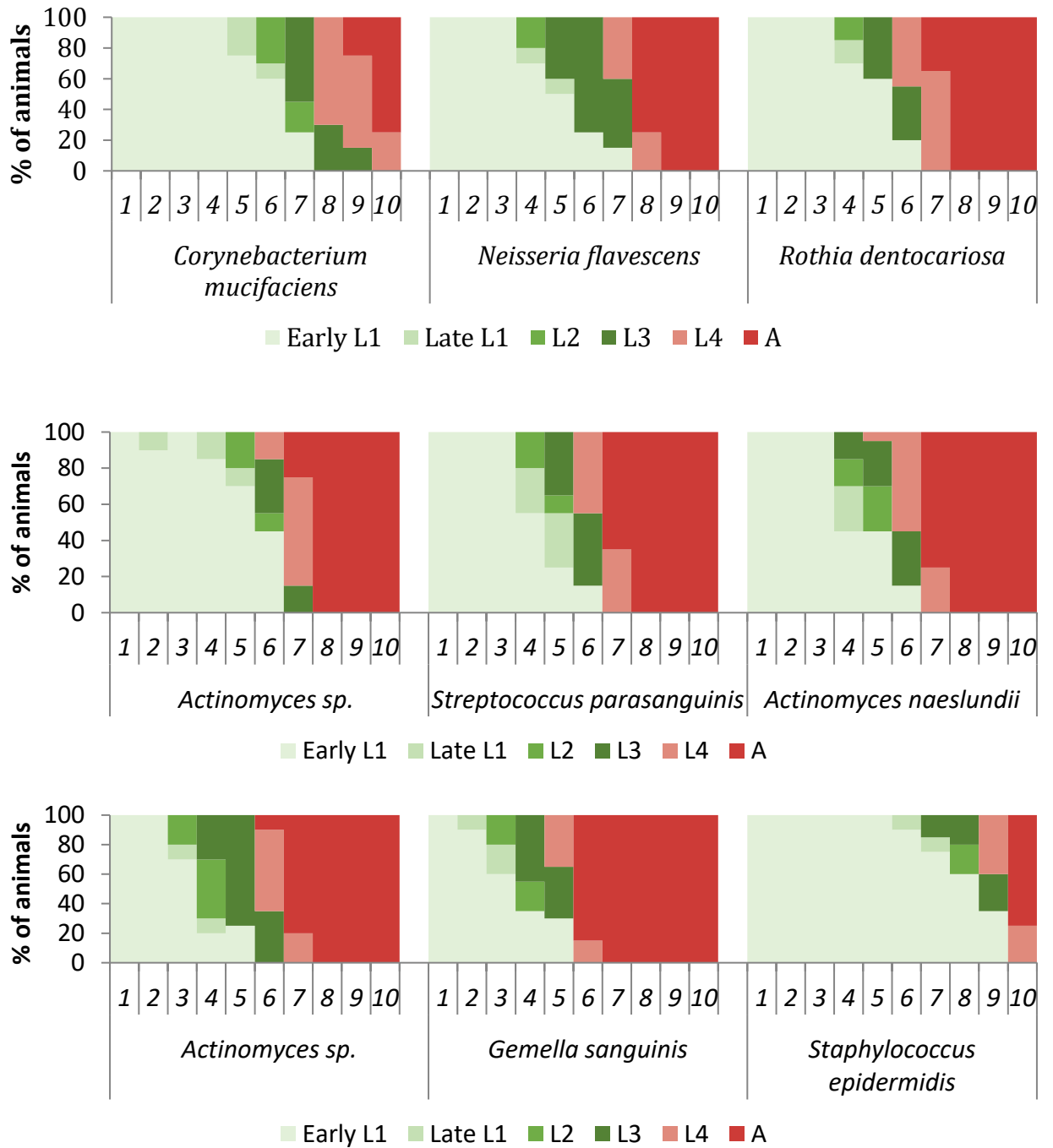
Table S3.1 - Timing of *C. elegans mlt-10::gfp-pest* activation is severely delayed on certain bacterial diets

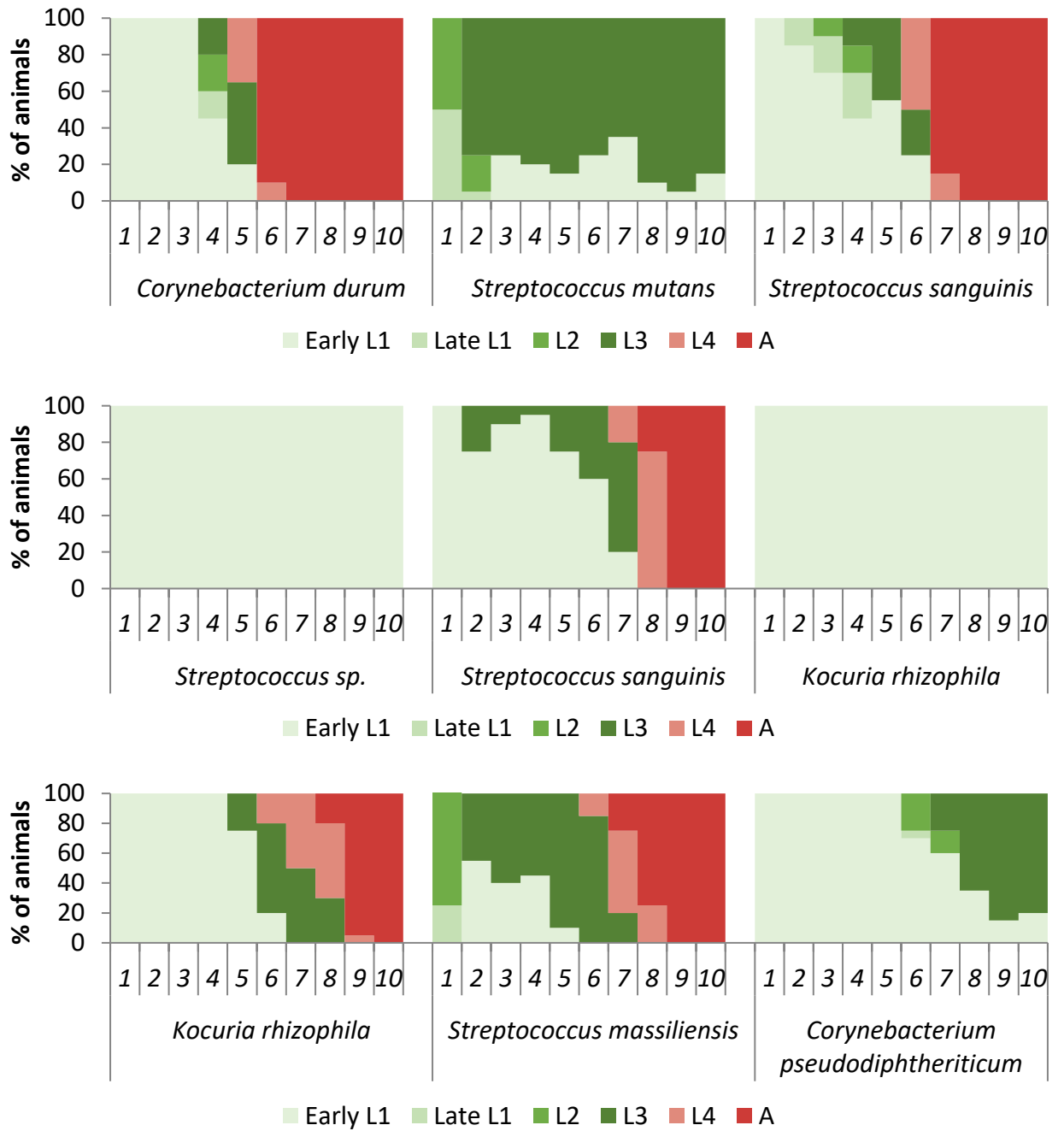
*mlt-10::gfp-pest* worms were synchronized and grown on test bacteria. Two sets of plates were used, with worms being put onto the second set of plates 10 hours after the first. Animals were screened using a fluorescence equipped stereomicroscope every 2 hours for 10 hours per day, for up to 3 days. The proportion of fluorescent worms on the plate was recorded, and data from both sets of plates combined to display the *mlt-10::gfp-pest* fluorescence activation pattern over the entire course of the experiment. The intensity of green shading represents the proportion of fluorescent worms on each plate. Data for the second set of plates is displayed under bolded timepoints. N >50-150 for each plate.













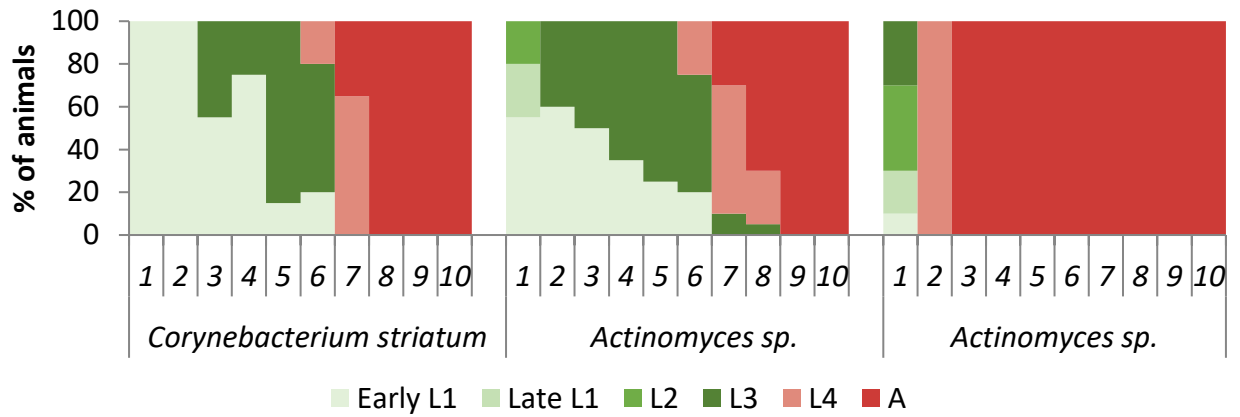
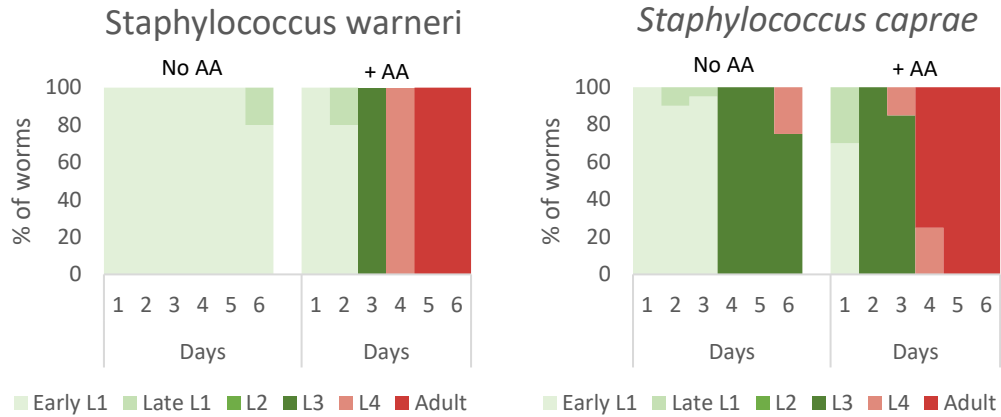


Figure S3.1 - *C. elegans* developmental rate is adjusted in response to bacterial diet  
 Synchronized L1 *hlh-8::gfp* worms were plated onto ¼ BHI plates seeded with the indicated bacterial species. Plates were observed daily, and 20 worms from each plate examined under a fluorescent stereomicroscope and the number of fluorescent cells in each worm counted. For each bacteria, the proportion of worms with a given number of fluorescent cells is displayed over 10 days.



**Figure S3.2 - Amino acid supplementation increases developmental rate of worms fed *Staphylococcus* species**

*C. elegans* carrying an *hlh-8::gfp* fusion gene were synchronized and arrested in L1 for 18 hours before being plated onto different human respiratory microbiota species, as well as an *Escherichia coli* OP50 control, grown on 1/4 BHI plates with and without essential amino acid supplementation. Worms were grown at 20 °C and observed daily using a fluorescence-equipped microscope, with the number of visible fluorescent cells and/or larval stage of worms recorded. Data for 20 worms were collected per plate. Colors indicate number of visible M cells observed in the worm or its larval stage.

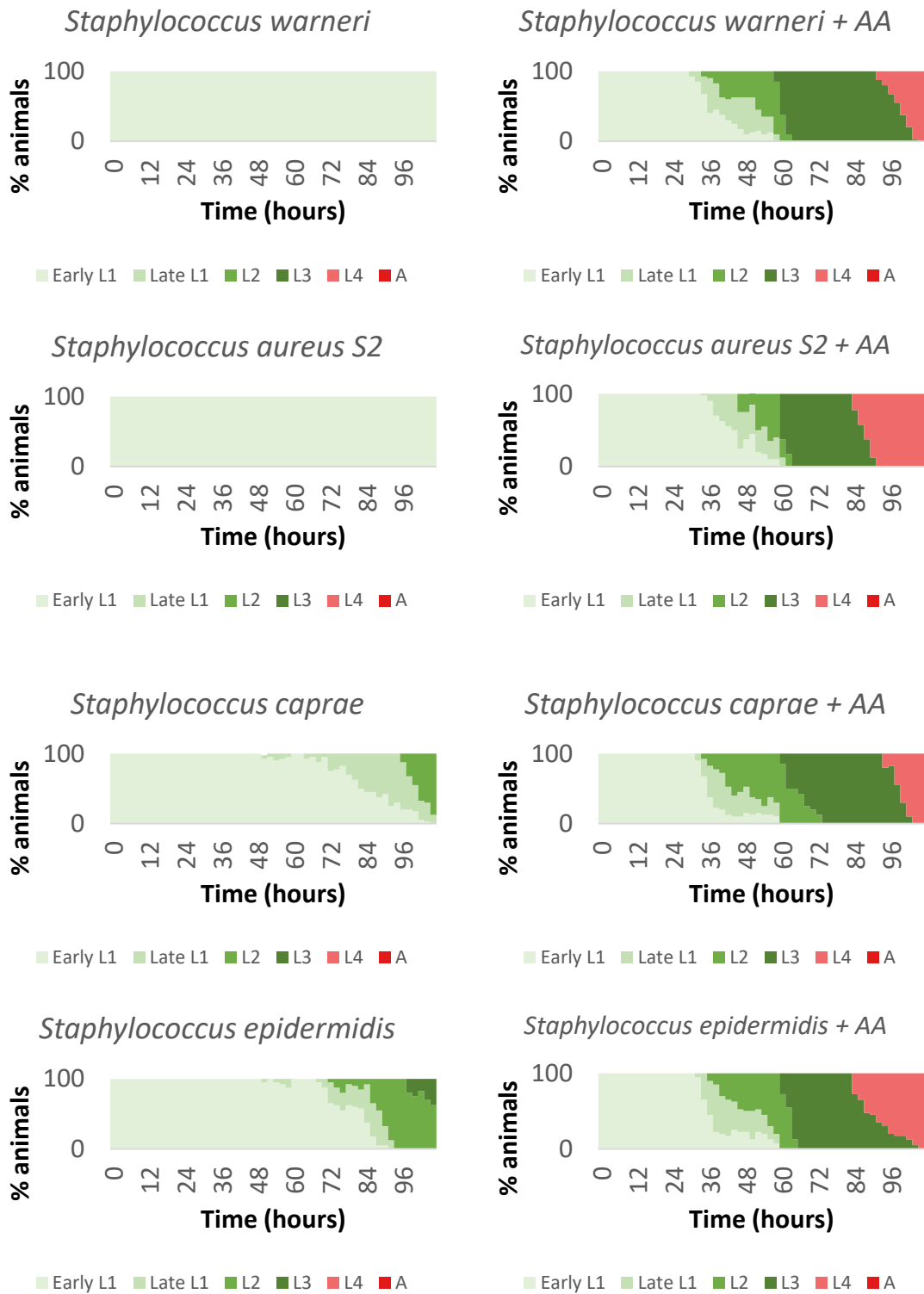
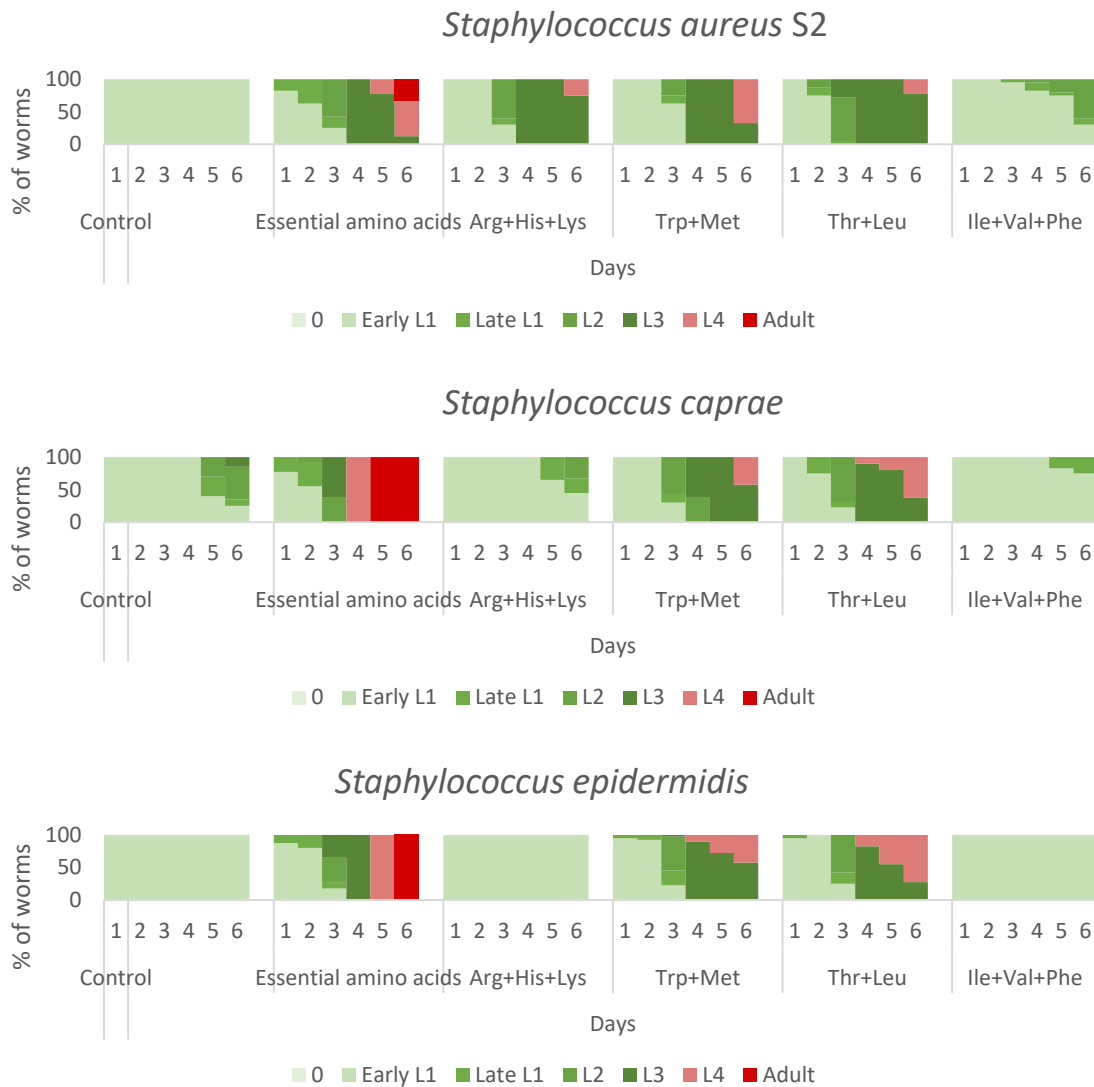


Figure S3.3 -Amino acid supplementation increases developmental rate of worms fed *Staphylococcus* species primarily in the L1 stage

*C. elegans* carrying an *hlh-8::gfp* fusion gene were synchronized and arrested in L1 for 18 hours before being plated onto different human respiratory microbiota species, as well as an *Escherichia coli* OP50 control, grown on 1/4 BHI plates with and without essential amino acid supplementation. Two sets of plates were used for the experiment, with worms put onto the plates 12 hours apart. Worms were grown at 20 °C and observed every 2 hours for 10 hours a day, under a fluorescence-equipped microscope, with the number of visible fluorescent cells and/or larval stage of worms recorded. Data for 40 worms were collected per plate. Colors indicate number of visible M cells observed in the worm or its larval stage.



**Figure S3.4 – Different amino acids rescue *C. elegans* development on different bacteria**  
*C. elegans* carrying an *hlh-8::gfp* fusion gene were fed bacteria that induce slow development, as well as an *Escherichia coli* OP50 control. Worms were grown on plates with no supplementation, supplementation with all 10 essential amino acids, or supplementation with fewer amino acid mixes, as indicated. Worms were observed once a day, with the number of visible fluorescent cells and/or larval stage of worms recorded. Data for 40 worms were collected per plate. Colors indicate larval stage of worm.

	Trial #	Population size	Median survival (days)	Significance
<i>E. coli</i> OP50	1	126	12	n/a
	2	142	13	n/a
<i>A. radicidentis</i>	1	179	13	ns
	2	165	12	ns
<i>S. warneri</i>	1	110	9	ns
	2	173	10	ns
<i>C. urealyticum</i>	1	171	5	p < 0.0001
	2	144	6	p < 0.0001
<i>S. aureus</i> S2	1	206	7	p < 0.0001
	2	127	7	p < 0.0001
<i>S. epidermidis</i>	1	150	5	p < 0.0001
	2	138	5	p < 0.0001
<i>Actinomyces</i> sp.	1	248	4	p < 0.0001
	2	130	5	p < 0.0001

Table S3.2 – *C. elegans* survival is decreased on certain slow-growth inducing bacterial species

*glp-1* worms were bleached and plated onto *E. coli* OP50 plates as eggs, and allowed to grow at 25 °C to induce sterility. Worms were washed off plates with M9 after reaching the L4 stage and placed in a Kanamycin solution for 4 hours. After Kanamycin treatment, worms were put onto the bacterial and grown at 25 °C. Number of live and dead worms were counted each day until all worms on plate were dead. Lost worms were censored. Table depicts total initial worm population size for each bacteria, median survival, and significance based on log-rank test comparing survival to the *E. coli* OP50 control.

	Trial #	Population size	% Survival (Day 6)	Significance
<i>E. coli</i> OP50	1	189	92.4	n/a
	2	296	88.5	n/a
<i>S. aureus</i> S1	1	212	56.6	p < 0.0001
	2	283	59.7	p < 0.0001
<i>S. warneri</i>	1	189	58.2	p < 0.001
	2	267	61.4	p < 0.0001
<i>S. aureus</i> S2	1	196	62.8	p < 0.001
	2	266	67.3	p < 0.01
<i>S. caprae</i>	1	192	68.8	p < 0.01
	2	279	64.5	p < 0.01
<i>S. epidermidis</i>	1	202	70.3	p < 0.01
	2	262	66.8	p < 0.001

Table S3.3 - Early survival of *C. elegans* is decreased on *Staphylococcus* species  
*C. elegans* carrying an *hlh-8::gfp* fusion gene were grown on the indicated *Staphylococcus* species. Worms were observed 1 hour after plating and then daily using a dissecting microscope, with the number of live worms counted per plate, and then observed again once a day for the next five days. All tests done in triplicate, with a total of 190-200 worms screened per bacteria. Table depicts initial worm population size for each bacteria, percent survival of worms on day 6 compared to day 1, and significance based on one-way ANOVA comparing survival on day 6 with the *E. coli* OP50 control.

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