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Simple Evaluation of *Listeria monocytogenes* Pathogenesis Using *Caenorhabditis elegans* Animal Model

Kyoung Hee Yang^{1,†}, Bohyun Yun^{2,†}, Hye Jin Choi², Sangdon Ryu², Woong Ji Lee², Mi-Hwa Oh³, Min-Ho Song⁴, Jong Nam Kim⁵, Sangnam Oh⁶, Younghoon Kim², and Young Jun Kim^{1,*}

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*Corresponding author : Young Jun Kim
Department of Food and Biotechnology,
Korea University, Sejong 30019, Korea
Tel: +82-44-860-1435
Fax: +82-44-865-0220
E-mail: yk46@korea.ac.kr

*ORCID

Kyoung Hee Yang
<https://orcid.org/0000-0003-2430-3818>
Bohyun Yun
<https://orcid.org/0000-0001-6723-5849>
Hye Jin Choi
<https://orcid.org/0000-0002-5977-2780>
Sangdon Ryu
<https://orcid.org/0000-0001-5338-8385>
Woong Ji Lee
<https://orcid.org/0000-0001-7243-0702>
Mi-Hwa Oh
<https://orcid.org/0000-0001-7838-5260>
Min-Ho Song
<https://orcid.org/0000-0002-4515-5212>
Jong Nam Kim
<https://orcid.org/0000-0002-8034-7156>
Sangnam Oh
<https://orcid.org/0000-0002-2428-412X>
Younghoon Kim
<https://orcid.org/0000-0001-6769-0657>
Young Jun Kim
<https://orcid.org/0000-0003-1447-7857>

† These authors contributed equally to this study

¹Department of Food and Biotechnology, Korea University, Sejong 30019, Korea

²Department of Animal Science and Institute of Milk Genomics, Chonbuk National University, Jeonju 54896, Korea

³Animal Products Research and Development Division, National Institute of Animal Science, Rural Development Administration, Wanju 55365, Korea

⁴Division of Animal and Dairy Science, Chungnam National University, Daejeon 34134, Korea

⁵Department of Beef Science, Korean National College of Agriculture and Fisheries, Jeonju 54874, Korea

⁶Department of Functional Food and Biotechnology, Jeonju University, Jeonju 55069, Korea

Abstract *Listeria monocytogenes* is a major cause of serious foodborne illness in the dairy foods. Although *Caenorhabditis elegans* model is well established as a virulence model of pathogenic bacteria, its application on *L. monocytogenes* is critically unclear. The objective of this study was to carry out an evaluation of *L. monocytogenes* toxicity using *C. elegans* nematode as a simple host model. We found that *C. elegans* nematodes have high susceptibility to *L. monocytogenes* infection, as a consequence of accumulation of bacteria in the worms' intestine. However, *L. innocua*, which is known to be non-toxic, is not accumulate in the intestine of worms and is not toxic similarly to *Escherichia coli* OP50 known as the normal feed source of *C. elegans*. Importantly, immune-associated genes of *C. elegans* were intensely upregulated more than 3.0-fold when they exposed to *L. monocytogenes*. In conclusion, we established that *C. elegans* is an effective model for studying the toxicity of *L. monocytogenes* and we anticipate that this system will result in the discovery of many potential anti-listeria agents for dairy foods.

Keywords *Listeria monocytogenes*, *Caenorhabditis elegans*, toxicity, immune response

Introduction

Human listeriosis is one of the most common foodborne diseases in European Union (EU) and USA [European food safety authority (EFSA), 2010; Scallan et al., 2011].

In 2014, a total of 2,161 cases of listeriosis were recorded in the EU (EFSA, 2016). Also, the Center for Disease Control and Prevention (CDC) estimates that 1,600 illnesses and 260 deaths caused by listeriosis, occur annually in the United States (US) (CDC, 2016). According to a recent study, the genus *Listeria* consists of 17 species with validly published names. Of these, *Listeria monocytogenes* and *L. ivanovii* are considered pathogenic (Weller et al., 2015) and only *L. monocytogenes* are regarded as foodborne pathogens and pose a threat to public health in human (Escolar et al., 2017). In general, *L. monocytogenes* virulence assessment has been performed using laboratory animals such mice, guinea pigs and monkeys (Cabanes et al., 2008). However, the use of such models often involved ethical and financial burdens. Furthermore, the breeding cycle of mammals and the number of animals required became challenging factors in animal-related research. Due to these limitations, various surrogate animal models such as invertebrates including *Galleria mellonella*, *Drosophila melanogaster*, and Zebra fish (*Danio rerio*) have been used to evaluate host-pathogen interactions with *L. monocytogenes* (Chambers et al., 2012; Martinez et al., 2017; Shan et al., 2015).

Among these surrogate animal models, *Caenorhabditis elegans* has number of practical advantages as a model system for screening; the process is easy to perform, rapid, low-cost, can be scaled-up, and ethical acceptability (Park et al., 2014). Furthermore, the body of this nematode is transparent, allowing clear observation of all cells in mature and developing animals. Also, they have intestinal cells that are similar in structure to human intestinal cells (Irazoqui et al., 2010). By confirming that the majority of human disease genes and disease pathways are commonly present in *C. elegans*, the use of this nematode has emerged as promising model for the assessment of virulence of numerous human pathogens (Kaletta and Hengartner, 2006).

The main objective of this study was to perform a risk assessment of *L. monocytogenes* associated with the major foodborne outbreaks using *C. elegans* model. Furthermore, we aimed to demonstrate the importance of *L. monocytogenes* virulence factor by confirming the regulation of *C. elegans* immune genes against *L. monocytogenes*.

Materials and Methods

Nematode and bacteria

C. elegans CF512 *fer-15(b26) II; fem-1(hc17) IV (fer-15; fem-1* worms) strains were used in this study. *C. elegans* strains were routinely maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 using standard procedures (Kim and Mylonakis, 2012). *L. monocytogenes* ScottA (serotype 4b) (Olier et al., 2002), *L. monocytogenes* strains 17, 18, 70, 106, 303, 3982, 3990, V7, Brie-1, LCDC (Yun et al., 2012), and *L. innocua* KU011 (obtained from Lab. of Food Microbiology in Korea University) were cultured on brain-heart infusion medium (BHI; BD Science, San Jose, CA, USA) at 37°C.

C. elegans solid killing assays

The *C. elegans* solid killing assay was performed as described previously, but with slight modifications (Kim and Mylonakis, 2012). Briefly, we prepared listeria pre-conditioning plates by spreading 20 µL of overnight cultured *Listeria* strains (approximately $ca\ 2.0 \times 10^9$ CFU/mL) on NGM agar plates. Worms were synchronized through hypochlorite bleaching, hatched overnight and were subsequently cultured on NGM plates with *E. coli* OP50. Synchronized L1 larvae were transferred to NGM plates with *E. coli* OP50, and worms were allowed to develop to L4 larvae. On day 3, we used 30 L4/young adult hermaphrodites per plate and also transferred the worms to listeria pre-conditioning, incubated at 25°C.

Worms were transferred to a new plate after every 24 h to determine the survival rates of the worms. The worms were considered dead when they did not respond to touching with a platinum wire pick. Each experimental condition was carry out with triplicate.

Measurement of the *L. monocytogenes* colonization in the *C. elegans* gut environments

To measure the colonization of *C. elegans*, the numbers of bacterial cells in worm intestines were measured (Kim and Mylonakis, 2012). Synchronization of worm cultures was achieved by hypochlorite treatment. The synchronized L4 larvae were transferred to NGM plates with *Listeria* strains. After exposing *C. elegans* to listeria pre-conditioning plates for 24 h, 10 worms were picked randomly, washed twice in M9 buffer, and placed on BHI plates containing 100 µg/mL kanamycin (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg/mL streptomycin (Sigma-Aldrich). These plates were put in 5 µL of 25 µg/mL gentamicin solution (Sigma-Aldrich) for 5 min, and results were compared with those of *E. coli* OP50 as a control. Afterward, worms were washed 5 times with M9 buffer, transferred into a 1.5-mL tube containing M9 buffer with 1% Triton X-100 (Sigma-Aldrich), and mechanically disrupted using a pestle (Kontes Glass Inc., Vineland, NJ, USA). These diluted worm lysates were plated and incubated at 37°C in modified LB agar for 18 h or PALCAM agar for 48 h (Oxoid Ltd., Basingstoke, UK). Each experimental condition was carried out with triplicate.

RNA isolation and qRT-PCR

All the RNA from worms was quickly isolated following the protocol of the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified using the RNeasy mini kit (Qiagen, Valencia, CA, USA) including an on-column DNase digestion with RNase-free DNase (Qiagen). After RNA isolation, 50 ng of all the RNA was used for a quantitative real-time PCR (qRT-PCR) using the qPCRBIO SyGreen 1-Step Hi-ROX (PCR Bio-systems, London, UK). qRT-PCR was performed using the StepOnePlus™ instrument (Applied Biosystems, Foster City, CA, USA). Primers were designed using Primer3Input Software (v0.4.0) and were listed as shown in Table 1. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ threshold cycle method (Livak and Schmittgen, 2001). The control gene *snb-1* was used to normalize the genes' expression data (Kim and Mylonakis, 2012).

Table 1. Oligonucleotides used in this study

Genes	Sequence
<i>C15C8.3</i>	f: 5'-ATCGGAGCACCAAAGAGTGT-3'
	r: 5'-AAGAATGCACCCGTAAGTGG-3'
<i>cpr-5</i>	f: 5'-GACAACGGAACCCCATACTG-3'
	r: 5'-CTCGATTCCACACTCGTTGA-3'
<i>lys-5</i>	f: 5'-TCCCAGAATTTATCATTTCATCG-3'
	r: 5'-TGGCATTCTTGACATTTTGC-3'
<i>clcc-60</i>	f: 5'-ACGGGCAAGTTATTGGAGAG-3'
	r: 5'-ACACGGTATTGAATCCACGA-3'
<i>snb-1</i>	f: 5'-CCGGATAAGACCATCTTGACG-3'
	r: 5'-GACGACTTCATCAACCTGAGC-3'

f, represent forward primer; r, represents reverse primer.

Statistical analysis

C. elegans survival was analyzed using the Kaplan-Meier method, and the differences were determined using the log-rank test (STATA6; STATA, College Station, TX, USA). The differences in the bacterial numbers from CFU counting were determined using the Student *t* test. Each experiment was performed with duplicates. A p-value of 0.05 in all replicate experiments was considered statistically significant.

Results and Discussion

L. monocytogenes* kills *C. elegans

It has been well-established that *C. elegans* is a suitable organism for virulence models of pathogenic bacteria because they are simple and have a short lifespan as economic invertebrate animal model (Darby, 2005; Merckx-Jacques et al., 2013). In several previous studies, a relationship has been established between nematode-pathogenic bacteria and bacteria that are capable of killing mice (Sifri et al., 2002). In this study, we studied the feasibility of *C. elegans* for the virulence study of *L. monocytogenes*. Initially, we examined whether *L. monocytogenes* Scott A and *L. monocytogenes* EGD-e cause food poisoning and influences the lifespan of *C. elegans*. As same manner, we employed the *L. innocua* KU011, which is nonpathogenic listeria strain as control. The nematodes was highly susceptible when exposed to *L. monocytogenes*. All *L. monocytogenes* strains used in this study showed that *C. elegans* leads to faster death than *E. coli* OP50; however, *L. innocua* KU011 showed similar death period to *E. coli* OP50 (Fig. 1). The killing effect of *C. elegans* infected with *L. monocytogenes* were briefly reported in the previous studies (Thomsen et al., 2006). We found that *C. elegans* exposed to *L. innocua* KU011 was not significantly different from *C. elegans* exposed to non-pathogenic *E. coli* OP50 used as a standard normal feed source for worms in killing assays with *fer-15; fem-1* worms ($p=0.2651$); hence, we concluded that both of them are not harmful to *C. elegans*. In addition, we found that *L. monocytogenes* strains also killed *C. elegans* much faster as compared to the avirulent *E. coli* OP50 and *L. innocua* KU011 ($p<0.0001$). As a result, *L. monocytogenes* is highly toxic to *C. elegans* host compared to worms feeding on *E. coli* OP50 and *L. innocua* KU011.

L. monocytogenes* accumulates on intestinal tract of *C. elegans

Since some pathogenic bacteria caused a persistent lethal infection in intestinal tract, we evaluated the possibility that *L. monocytogenes* cells colonize the *C. elegans* intestine, thereby killing the *C. elegans*. *E. coli* OP50 and *L. innocua* KU011 showed similar nematode survival rates and intestinal bacterial load. As a result, no bacterial cells could be detected in the nematode intestine 24 h after feeding on *E. coli* OP50 and *L. innocua* KU011. On the other hand, *L. monocytogenes* strains colonize the *C. elegans* intestine more than *E. coli* OP50 or *L. innocua* KU011. The strains of *L. monocytogenes* that were harmful to *C. elegans* showed high colonization of 1.10 ± 0.17 – 3.69 ± 0.10 CFU/mL in nematode intestinal tract after 24 h of exposure (Fig. 2; $p<0.05$ for all strains of *L. monocytogenes*, compared with worms feeding on *E. coli* OP50 or *L. innocua* KU011). In particular, the most toxic *L. monocytogenes* Scott A showed a high colony counts of 3.69 ± 0.10 CFU/mL in the nematode intestinal tract. After being digested by the nematode, burden of Gram-positive pathogens including *Staphylococcus aureus* and *Enterococcus faecalis* in the nematode intestine are prerequisite for virulence of pathogens (Garsin et al., 2001). Thus, we concluded that *L. monocytogenes* strains may be pathogenic by colonizing the intestinal tract of *C. elegans*.

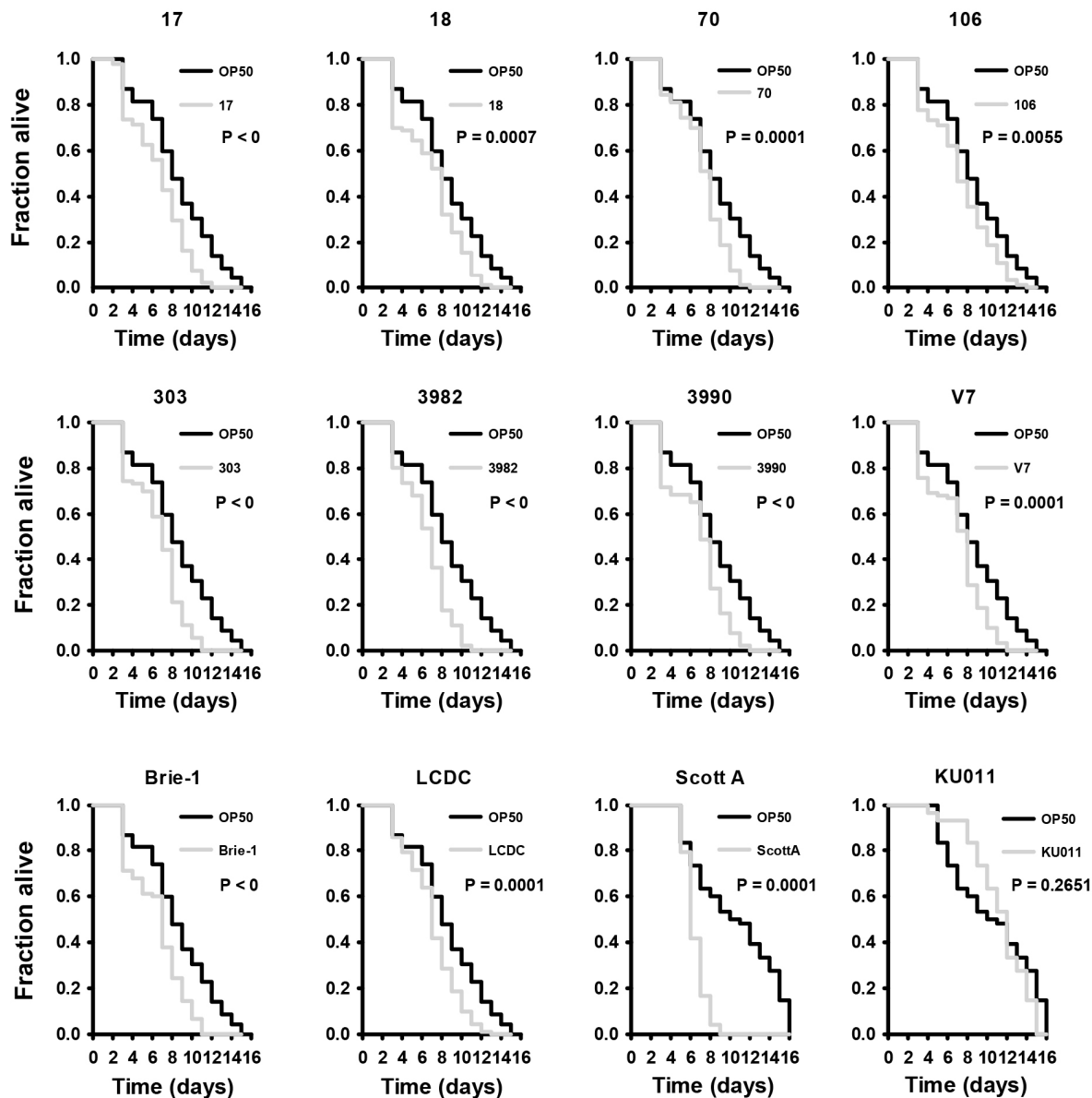


Fig. 1. Solid killing assays of *Caenorhabditis elegans* worms infected with the *Listeria monocytogenes* strains, or *Listeria innocua* KU011. Survival curves were compared using the log rank test and considered statistically different ($p < 0.05$). $p < 0$ means that the p -value is less than 0.0001.

L. monocytogenes regulates the immune related genes of *C. elegans*

Having established that *C. elegans* nematodes are kill by *L. monocytogenes* strains infection. We aimed to determine the immune mechanism associated with infection of *C. elegans* with *L. monocytogenes*. Four strains with the highest toxicity among *L. monocytogenes* strains were tested. We selected 4 immunity-related target genes that critically increased by Gram-positive pathogens such as *E. faecalis* and *S. aureus* as follows; C15C8.3 (encodes aspartyl proteases), *cpr-5* (encode cysteine proteases), *lys-5* (encodes a lysozyme), and *clec-60* (encode C-type lectins). The transcription of the nematode immune gene to *L. monocytogenes* strains exposure was analyzed in comparison with *E. coli* OP50, the standard normal feed source for *C. elegans*. With reference to our previous reports (Kim and Mylonakis, 2012), all of the immune associated genes were significantly upregulated by the exposure of *L. monocytogenes* strains ($p < 0.05$ for all transcriptions of immune related genes

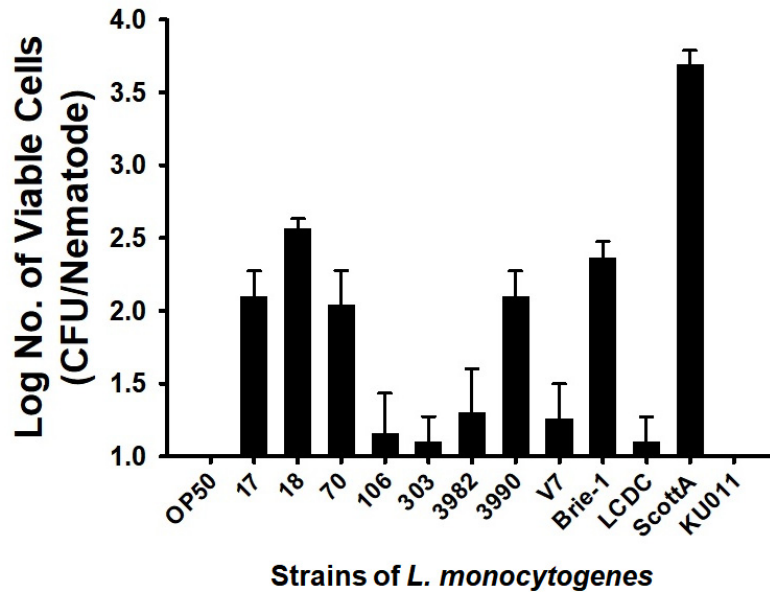


Fig. 2. Evaluation of colonization (CFU/nematode) of *Listeria monocytogenes* in the nematode intestine. Error bars show standard deviations ($p < 0.05$ for all strains of *L. monocytogenes*, compared with worms feeding on *E. coli* OP50 or *L. innocua* KU011).

in *C. elegans* with infection of *L. monocytogenes* strains, comparing with *E. coli* OP50). More specifically, our qRT-PCR results indicated that infection with *L. monocytogenes* ScottA intensely induced the transcription of C15C8.3 (6.8 ± 0.1 -fold), *cpr-5* (3.3 ± 0.4 -fold), *lys-5* (10.0 ± 1.6 -fold), and *clec-60* (4.1 ± 0.2 -fold) (Fig. 3). *L. monocytogenes* strain 17, induced the transcription of C15C8.3 (11.7 ± 1.5 -fold), *cpr-5* (1.6 ± 0.7 -fold), *lys-5* (19.5 ± 6.7 -fold), and *clec-60* (18.0 ± 5.9 -fold). For *L.*

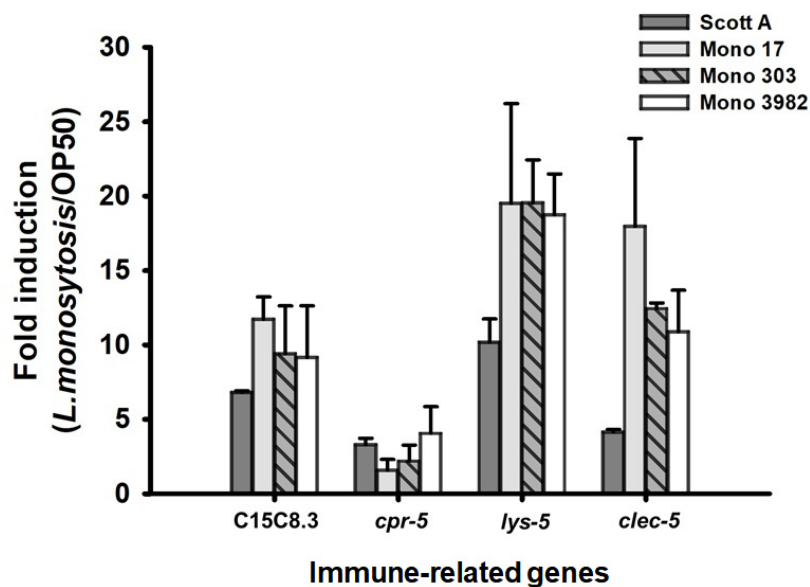


Fig. 3. Regulation of transcription of immune genes in *Caenorhabditis elegans* by *Listeria monocytogenes*. qRT-PCR analysis evaluating the impact of *L. monocytogenes* strains ScottA, 17, 303, and 3,982 infections on genes associated with nematode immune responses (*E. coli* OP50 were used for comparison). Transcript levels were measured in young adult *fer-15*; *fem-1* worms infected with *L. monocytogenes* strains for 24 h. ($p < 0.05$ for all transcriptions of immune related genes in *C. elegans* with infection of *L. monocytogenes* strains, comparing with *E. coli* OP50).

monocytogenes strain 303, induced the transcription of C15C8.3 (9.4±3.2-fold), *cpr-5* (2.2±1.1-fold), *lys-5* (19.5±3.0-fold), and *clec-60* (12.4±0.4-fold). *L. monocytogenes* strain 3982, induced the transcription of C15C8.3 (9.2±3.5-fold), *cpr-5* (4.1±1.8-fold), *lys-5* (18.7±2.8-fold), and *clec-60* (10.9±2.8-fold) (Fig. 3).

C15C8.3 encodes aspartyl proteases while *cpr-5* encodes cysteine proteases. Its primary function is lumen hydrolysis of bacterial proteins (McGhee et al., 2007). Transcriptional upregulation of these genes was observed in previous studies in response to Gram-positive bacteria (Kim and Mylonakis, 2012). The gene encoding proteases were also up-regulated in gram-positive bacteria, *L. monocytogenes* infection. Lysozyme plays an important role in animal immunity by catalyzing the cleavage of peptidoglycan, a major component of the bacterial cell wall (O'Rourke et al., 2006). The destruction of bacterial cells is believed to play an important role both in defending against infectious bacteria and in digesting bacteria as a nutrient source (Schulenburg and Boehnisch, 2008). The lysozymes quantified in this study showed changes similar to qPCR results of a previous studies (Boehnisch et al., 2011; Treitz et al., 2015), with *lys-5* increased. C-type lectin-like domain containing proteins (CTLN) have been described to play a role in binding of specific pathogen-derived molecules (Ghazarian et al., 2011). Transcriptional upregulation of genes coding for C-type lectins has been observed in previous studies in response to gram-positive bacteria (O'Rourke et al., 2006; Treitz et al., 2015). The transcription of *clec-60* was also observed to have significantly increased in response to the *L. monocytogenes* in our study. Taken together, the induction of these genes was strongly associated with pathogenesis of *L. monocytogenes*. Interestingly, C15C8.3, *lys-5*, and *clec-5* were highly expressed in the remaining strains than *L. monocytogenes* ScottA, which has the highest toxicity with the fastest killing of *C. elegans*. This results suggests that the death of *C. elegans* is associated with a high colony for the intestines.

In conclusion, this study established that *L. monocytogenes* are not digested by the nematode and they can successfully colonize in intestinal tract of nematodes. This persistence resulted in toxic condition for *C. elegans* host. In addition, these results indicate that conditioning with the *L. monocytogenes* specifically stimulated the transcription of genes associated with nematode immune response to Gram-positive pathogens.

Conflicts of Interest

The authors declare no potential conflict of interest.

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Author Contributions

Conceptualization: Oh S, Kim Y, Kim YJ. Data curation: Oh S, Kim Y, Kim YJ. Formal analysis: Yang KH, Yun B, Oh S, Kim Y, Kim YJ. Methodology: Yang KH, Yun B, Choi HJ, Ryu S, Lee WJ, Oh MW, Song MH, Kim JN, Oh S, Kim Y, Kim YJ. Software: Yang KH, Yun B, Oh S, Kim Y, Kim YJ. Validation: Yang KH, Yun B, Choi HJ, Ryu S, Lee WJ, Oh MW,

Song MH, Kim JN, Oh S, Kim Y, Kim YJ. Investigation: Oh S, Kim Y, Kim YJ. Writing - original draft: Yang KH, Yun B, Choi HJ, Ryu S, Lee WJ, Oh MW, Song MH, Kim JN, Oh S, Kim Y, Kim YJ. Writing - review & editing: Yang KH, Yun B, Choi HJ, Ryu S, Lee WJ, Oh MW, Song MH, Kim JN, Oh S, Kim Y, Kim YJ.

Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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