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IMPACT OF FOOD AND FOLATE SUPPLEMENTATION DURING *Salmonella* TYPHI INFECTION IN *Caenorhabditis elegans*

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Abstract: *Caenorhabditis elegans* is an instructive and suitable model for studying pathogenesis of almost all human pathogens. *Salmonella* Typhi is gram-negative facultative intracellular anaerobe that causes several pathetic infections. Necessary enriched nutrient ingestion during pathological conditions may reduce the harshness of the infection. We investigated the impact of folate and food supplementation during *S.* Typhi infection on the model system, *C. elegans*. Our data indicated that folate supplementation (10 µg) increases the lifespan of *S.* Typhi infected *C. elegans* up to 20%. In combination with laboratory food source *E. coli* OP50, folate increases the infected the worm's lifespan to 40%. The wild type *C. elegans* infected by *S.* Typhi died with the LT_{50} of 60 ± 12 h. The LT_{50} of *S.* Typhi infected *folt-1* mutant strain VC959 was 96 ± 6 h. However, the folate supplemented mutant worms exhibited an extended life with LT_{50} of 120 ± 6 h. The short time exposure and pharyngeal pumping studies confirmed that *folt-1* mutant worm exhibited increased survival rate during pathogenic course at significant level when compared to wild-type. Our data revealed that *folt-1* plays a significant role in host defense system against *S.* Typhi infection and the folate supplementation in combination with food increases the host survival during *S.* Typhi infection.

***Keywords*:** *Caenorhabditis elegans*; *S.* Typhi; folate.

Introduction

Salmonella Typhi, a gram-negative facultative intracellular pathogen, is capable of infecting human being. *S.* Typhi infection persists to cause severe health vulnerability in Asia and African countries. *Salmonella* infection leads to gastroenteritis and typhoid fever. The resultant inflammation is accompanied by the infiltration of phagocytic mononuclear cells. The mechanism by which *S.* Typhi causes the proinflammatory response is not known. This may be due to the secretion of effectors by either SPI-1 or SPI-2 encoded or host PRRs (Pattern Recognition Receptor), which triggers the inflammatory response upon recognition of PAMPs (Pathogen Associated Molecular Pattern) (Tukel *et al.*, 2006). SPI-1 and SPI-2 (*Salmonella* pathogenicity islands) of *S.* Typhi are required for the invasion of non-phagocytic cells, intracellular survival and systemic pathogenesis (Lahiri *et al.*, 2010). However, although antibiotics can kill the *S.* Typhi in blood, whether they can enter into the SCV (*Salmonella* containing vacuoles) within the cells remain unclear. Continuous antibiotic treatment for typhoidal infection leads to the emergence of multi-drug resistance strains of *S.* Typhi. In this study, the impact of supplement of enriched nutrient, especially folate, during *S.* Typhi pathogenesis was explored using *C. elegans* as a host system.

C. elegans is a useful and suitable model for host-pathogen interaction studies because of the fact that the genetic and molecular tools used for its manipulation are well developed. It is measured about 1 mm in size with very transparent body and a short life cycle. It was the first multicellular organism to have its complete genome sequenced (Darby, 2005). The vitamins act as coenzyme in a series of metabolic reactions including the synthesis of precursors of DNA, RNA and the metabolism of several amino acids.

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Folate is a water-soluble vitamin that is a member of B-class, which is crucial for normal cellular function and development. *folt-1* encodes a folate transporter required for folate uptake in *C. elegans* (Balamurugan *et al.*, 2007). FOLT-1 is orthologous to human SLC19A1, SLC19A2 and SLC19A3. FOLT-1 is expressed heterologously and transports folate *in vitro*. The activity of FOLT-1 is acid-dependent but sodium-independent and inhibited by derivatives of folate like sulfasalazine, or some inhibitors such as 4,4''-diisothio-cyanatostilbene-2,2''-disulphonic acid (DIDS). The studies revealed that folt-1(ok1460) and folt-1(RNAi) *C. elegans* show significantly lowered folate uptake (Balamurugan *et al.*, 2007).

folt-1 is expressed in several tissues, including (most strongly) pharynx and posterior intestine as well as head, body wall, vulva muscles, and gonad sheath cells. During embryogenesis folate deficiency can result in a number of developmental defects (Balamurugan *et al.*, 2007). Deficiency of folate causes neural tube deformities (Daly *et al.*, 1995), cardiovascular abnormalities [Boushey *et al.*, 1995; Rimm *et al.*, 1998), anemia (Wickramasinghe, 1999), and cancer (Choi and Mason, 2000) in humans. Approximately 70% of the neural tube defects can be prevented by the supplementation of dietary folate during pregnancy (Czeizel and Dudas, 1992; Werler *et al.*, 1993).

In light of the above facts, the current study was performed to understand the impact of food source along with folate during *S.* Typhi infection in *C. elegans.*

Materials and Methods

Bacterial Strains, Nematode, Media and Culture Conditions - The wild type strain of *Salmonella* Typhi MTCC 733 was obtained from MTCC (Microbial Type Culture Collection, IMTECH, Chandigarh, India). *Escherichia coli* OP50 was provided by CGC (Caenorhabditis Genetic Centre, MN, USA). Bacterial cultures were maintained on Luria-Bertani (LB) with agar (18 g/l) at 37°C, with aeration. *C. elegans* strain N2 Bristol was provided by CGC, and maintained on Nematode Growth Medium [NGM - a minimal medium containing 3% NaCl, 3% peptone, 1.5 % agar, autoclaved (50 min), cooled, followed by addition of 0.1 ml cholesterol (5 mg/ml in 95% ethanol), 0.1 ml 1M $CaCl_2$, 0.1 ml 1M $MgSO_4$ and 2.5 ml 1M potassium phosphate buffer (pH 6.0)] agar plates containing a lawn of *E. coli* OP50 (food source under laboratory condition) at 20°C (Brenner, 1974). Folate incorporation was done by mixing 10-100 µg/ml of folate into assay buffer or NGM. All experiments were carried out using age-synchronized L4 stage worms.

C. elegans liquid killing assay - To find out the impact of folate supplementation during pathogenesis of *S.* Typhi on *C. elegans*, liquid killing assay was performed by the addition of 100 µl of the pathogen strain *S.* Typhi and *E. coli* OP50 (0.5 OD) along with 10 µg/ml of water soluble vitamin folic acid to 400 µl of M9 buffer containing ~ 20 nematodes in each well of a micro titer well plate, respectively. The negative control was the well with M9 buffer containing nematodes along with the folate and without food. Similarly, ~ 20 nematodes in M9 buffer were exposed to *S.* Typhi without the addition of folate and with addition of folate along with *E. coli* OP50. The assays were performed simultaneously. These nematodes were monitored at every 2 h intervals to record the exact time required for their death, and their survival rate was monitored.

Killing assays with folt-1 (Folic acid transporter) mutant strain, VC959 - The strain VC959 has been created by homozygous sterile deletion of *folt-1* balanced by GFP-marked translocation. *E. coli* OP50, uracil auxotroph, is commonly used laboratory food source for the maintenance of *C. elegans.* Previous host-pathogen interaction studies using *C. elegans* were carried out by simply replacing the *E. coli* OP50 with pathogenic strains. 2 ml of LB was inoculated with a single colony of the appropriate bacterial strain and grown at 37°C for 3 h. 100 µl of 3 h culture of *S.* Typhi or *E. coli* OP50 (control) was added to 400 µl of M9 buffer containing known number of mutant worms (~20 L4 stage hermaphrodites) in each well of micro titer well plate. Negative control is the well containing M9 buffer and nematodes without any bacterial culture. The worms were monitored at every 2 h interval to note the exact time required for their death on continuous exposure to pathogen. Worms were considered dead when

they did not respond to touch with a platinum wire pick. Each experimental condition was tested in triplicates.

Solid assays with C. elegans - Bacterial full-lawn was produced by swabbing 0.5 OD culture of *S.* Typhi or *E. coli* OP50 (control) on NGM plate supplemented with folate (100 µg/ml). Plates were incubated at 37°C for overnight and allowed to equilibrate at room temperature. To observe the phenotypic changes, twenty age-synchronized L4 stage worms (wild type and mutant) were placed on each plate and incubated at 20 °C.

Short-Time exposure Assays on C. elegans - The age-synchronized L4 *C. elegans* was exposed to *S.* Typhi for 24 h. After exposure, the worms were washed with M9 buffer and transferred to *E. coli* OP50 seeded plate supplemented with or without folate. The status of pathogen exposed animals was monitored at regular intervals (Sivamaruthi *et al.*, 2011).

Bacterial accumulation assay - To find out the load of *S.* Typhi within *C. elegans* during infection under different conditions, the bacterial accumulation test was carried out as described previously (Sivamaruthi *et al.*, 2011). Briefly, L4 stage hermaphrodites were collected in between the liquid assays and serially washed with M9 buffer containing 1 mM sodium azide to inhibit expulsion of bacteria from their intestine. The nematodes were treated with antibiotic (1mg/ml Gentamicin) to wash the bacteria adhered on the outer surface, lysed in phosphate-buffered saline (PBS) containing 400 mg of 1.0-mm silicon carbide particles (Himedia) and mechanically disrupted using a pestle. The worm lysates were diluted and plated on SS agar plates. The plates were incubated overnight at 37°C, colonies were quantified to calculate the number of bacteria per nematode (Sivamaruthi *et al.*, 2011).

Pharyngeal pumping assay - To determine the pumping rate, age-synchronized L4 stage worms (wild type and mutant) were placed on NGM plates seeded with *E. coli* OP50 (control) and *S.* Typhi. The pharyngeal pumping was observed using a stereomicroscope (Nikon SMZ1000, Japan) for thirty consecutive seconds (Sivamaruthi *et al.*, 2011).

Results and Discussion

Food and folate supplementation diminishes the S. Typhi infectivity

Under natural environment, the nematode may not continuously be exposed to any particular pathogen. Hence, to detect the survival rate of the nematode during *S.* Typhi infection, they were provided with the laboratory food source such as *E. coli* OP50 and time course-killing assay was performed. *S.* Typhi infected nematode have an LT_{50} of 60 ± 12 h and complete killing occurred in 102 ± 12 h. However, when the nematodes were exposed along with the food source *E. coli* OP50 and folate, the survival rate of the nematode increased with an LT_{50} of 120 ± 12 h and complete killing was observed at 146 ± 12 h. However, *E. coli* OP50 exposed nematodes survived and reproduced without any notable hindrance (Figure 1 and 6). The data suggested that the presence of water-soluble vitamin folic acid and food source during infection of *S.* Typhi diminishes the severity of infection in *C. elegans* up to 40%.

The reduction in infection was further proved by assessing the internalized bacterium (counting of live *S.* Typhi inside the infected worms). The CFU value of nourished worm was 5.6 $X10^3$/ worm and the worms without any supplementation displayed the CFU of 9.4$X10^5$/ worm (Figure 2). The results indicated that entry and multiplication of pathogen was higher during malnutrition especially during bacterial infection. The preference for the intake of non-pathogenic

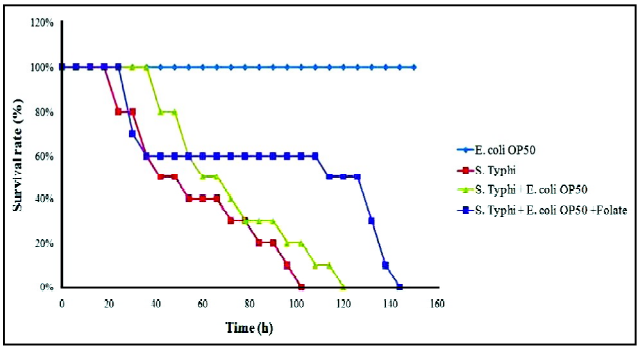
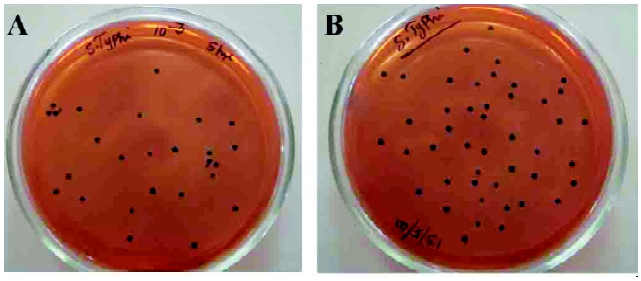


Figure 1: **Survival rate of wild type *C. elegans* in liquid assay. *S.* Typhi infected animals died with LT_{50} of 60 ± 12 h (■), In the presence of laboratory food source *E. coli* OP50 (▲), folate + *E. coli* OP50 (■) *S.* Typhi infected *C. elegans* exhibited increased survival rate. Worm exposed to *E. coli* OP50 (♦) served as control.**



CFU = 5.6×10^3 / worm CFU = 9.4×10^5/worm

Figure 2: **The bacterial accumulation (Colony Forming Unit; CFU) in *S.* Typhi infected *C. elegans*. (A) CFU of bacteria present in the worms treated with the combination of *E. coli* OP50 and *S.* Typhi. (B) Bacterial load in the worms exposed with *S.* Typhi.**

strain, by olfactory sensation, possibly is the reason for that.

In another experiment, the mortality rate of infected *C. elegans* during solid condition (LT_{50} of 250 ± 12 h) was less compared to liquid assays (Figure 3). The embryo development and egg laying ability of the infected worms were monitored and indicated that the presence of folate reduced the severity of the reproductive damages caused by the infection compared to the worms infected with *S.* Typhi without folate supplementation.

Role of folic acid during S. *Typhi infection in C. elegans*

In order to investigate the role of micronutrient, vitamin folic acid during *S.* Typhi infection, few physiological experiments with *folt-1* mutant of *C. elegans* upon *S.* Typhi infection were carried out. *S.* Typhi infected *folt-1* mutant strain died with the LT_{50} of 96 ± 6 h and the complete killing was observed at 144 ± 6 h; where as the folate supplemented mutant showed extended survival rate (LT_{50} of 120 ± 6 h) (Figure 4 and 6). In solid assay, the mutant animals fed with *S.* Typhi died completely at 314 ± 6 h. The mutant animals were exposed to *S.* Typhi for 24 h and transferred to NGM supplemented with or without folate (100 µg/ml) which causes the host mortality after 96 ± 6 h on both the plates.

The results of short time exposure assay confirmed that nearly 12 h exposure was sufficient to cause mortality of mutant worms both in the presence or absence of folate whereas

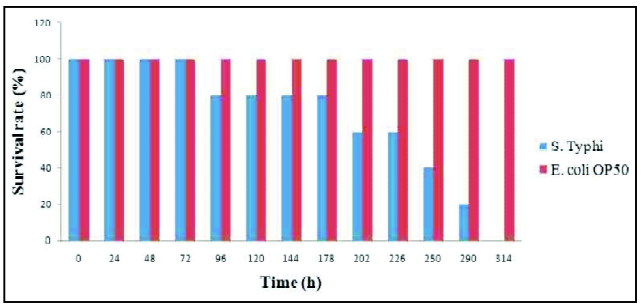


Figure 3: **The mortality rate of *C. elegans* infected with *S.* Typhi during solid assays. The animals fed with *S.* Typhi lawn died with the LT_{50} of 250 ± 12 h (■); worms exposed to *E. coli* OP50 survived without any hindrance (■).**

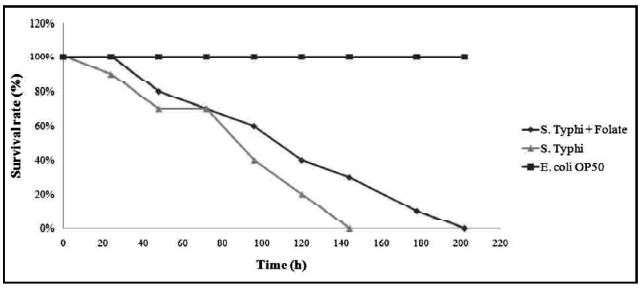


Figure 4: **Survival rate of *folt-1* mutant *C. elegans* in liquid assay. *S.* Typhi infected *folt-1* mutant strain died with the LT_{50} of 96 ± 6 h and the complete killing was observed at 144 ± 6 h (▲). The folate supplemented mutants showed an extended survival rate (LT_{50} of 120 ± 6 h) (♦).**

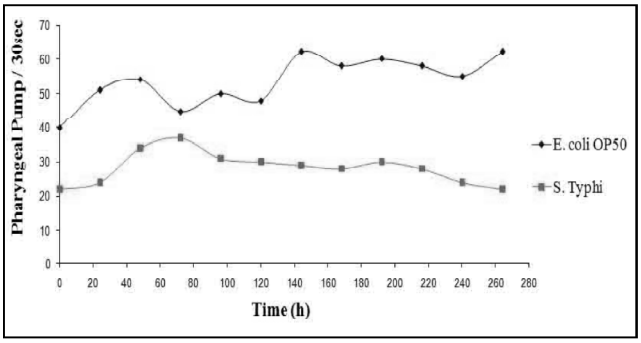


Figure 5: **The pharyngeal pumping rate of mutant infected with *S.* Typhi was not completely arrested (■) but the pumping rate has been reduced compared to control.**

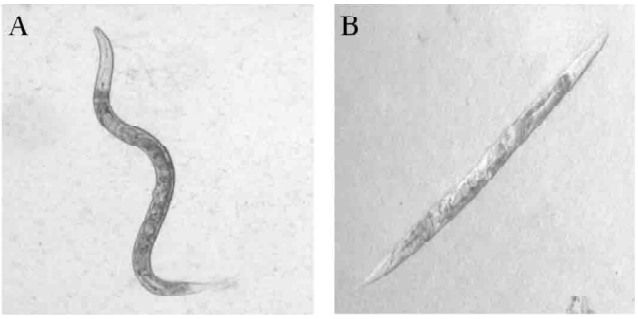


Figure 6: **The representative pictures showing live (A) and dead (B) *C. elegans* in various assays.**

the minimum infection needed for wild type was less. The silencing or knockout of *folt-1* gene leads to a substantial reduction in folate uptake compared with wild type (Balamurugan *et al.*, 2007). In this study, the results gained through assays with wild type and mutant *C. elegans* clearly enlightened that *folt-1* mutant animals were more resistant to *S.* Typhi infection compared to wild-type, suggesting that the folate receptor of the *C. elegans* might act as PRR (Pattern Recognition Receptor) of *S.* Typhi.

Pharyngeal Pumping Assay

The pharyngeal pumping rate of *S.* Typhi infected VC959 (*folt-1* mutant) was monitored under the microscope. The pumping rate of infected worm was not significantly varied over the course of infection. However, the pumping rate of the worms fed with *E. coli* OP50 lawn was higher than pathogen fed animals (Figure 5). Furthermore, the pumping rate of wild type animals infected with *S.* Typhi was completely arrested, whereas, the mutant worm exhibited reduced severity upon *S.* Typhi infection. The results indicated that *S.* Typhi infection did not completely damage the pharyngeal region of mutant animals.

Conclusion

To our knowledge this is the first study inspecting the impact of nutrient supplementation during pathogenic infection of *S.* Typhi on *C. elegans*. The results obtained through survival assays revealed that supplementation of *E. coli* OP50 (laboratory food source) and folic acid enhance the survival of *S.* Typhi infected *C. elegans* up to 40%. *S.* Typhi infected *folt-1* mutant strains exhibited increased survival compared to wild type *C. elegans* revealing that *folt-1* plays a major role in pathogen receiving (as PRR) and facilitating the firm attachment of bacterial cell to the host. Molecular based analysis may address the mechanism behind the relatively increased survival of mutant *C. elegans* against *S.* Typhi infection.

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Abbreviations

CFU, colony forming unit; CGC, *Caenorhabditis* genetics center; $LT_{50,}$ lethal time (time for half to die); NGM, nematode growth medium; PAMPs, pathogen associated molecular pattern; PBS, phosphate-buffered saline; PRRs, pattern recognition receptor; SCV, *Salmonella* containing vacuole; SPI, *Salmonella* pathogenicity islands; SS agar, *Salmonella* Shigella agar.

References

Balamurugan, K., Ashokkumar, B., Moussaif, M., Sze, J. Y and Said, H. M. (2007) Cloning and functional characterization of a folate transporter from the nematode *Caenorhabditis elegans. Am J Physiol Cell Physiol.* 293, C670-C681.

Boushey, C. J., Beresford, S. A., Omenn, G. S. and Motulsky, A. G. (1995). A quantitative assessment of plasma homocysteine as a risk factor for vascular disease - Probable benefits of increasing folic acid intakes. *J. Am. Med. Ass. 274*, 1049-1057.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans. Genetics 77*, 71–94.

Choi, S.W. and Mason, J.B. (2000). Folate and carcinogenesis: an integrated scheme. *J. Nutr. 130*, 129-132.

Czeizel, A.E. and Dudas, I. (1992). Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N. Engl. J. Med. 327*, 1832-1835.

Daly, L. E., Kirke, P. N., Molloy, A., Weir, D. G. and Scott, J. M. (1995). Folate levels and neural tube defects Implications for prevention. *J. Am. Med. Ass. 274*, 1698-1702.

Darby, C. (2005). Interactions with microbial pathogens. In Worm Book, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.21.1, pp. 1–15. *http://www.wormbook.org*.

Lahiri, A., Iyer, N., Das, P. and Chakravortty, D. (2010). Visiting the cell biology of Salmonella infection. *Microbes Infect. 12*, 809-818.

Rimm, E. B., Willett, W. C., Hu, F. B., Sampson, L., Colditz, G. A., Manson, J. E., Hennekens, C. and Stampfer, M. J. (1998). Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease among women. *J. Am. Med. Ass. 279*, 359-64.

Sivamaruthi, B. S., Ganguli, A., Kumar, M., Bhaviya, S., Pandian, S. K. and Balamurugan, K. (2011) *Caenorhabditis elegans* as a model for studying *Cronobacter sakazakii* ATCC BAA-894 pathogenesis. *J. Basic Microbiol.* 51, 540–549.

Tukel, C., Raffatellu, M., Chessa, D.,Wilson, R. P., Akcelik, M. and Baumler, A. J. (2006). Neutrophil influx during non-typhoidal salmonellosis: who is in the driver's seat? *FEMS Immunol Med Microbiol. 46*, 320-329.

Werler, M. M., Shapiro, S. and Mitchell, A. A. (1993). Periconceptional folic acid exposure and risk of occurrent neural tube defects. *J. Am. Med. Ass. 269*, 1257-1261.

Wickramasinghe, S. N. (1999). The wide spectrum and unresolved issues of megaloblastic anemia. *Semin Hematol. 36*, 3-18.