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Effect of sunflower oil nanoemulsions on the growth and lifespan extension of the *Caenorhabditis elegans*

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Abstract

Caenorhabditis elegans has been used as a central model system for broad-spectrum research studies across diverse modern biological domains. A study was conducted to evaluate the potential of sunflower oil-based nanoemulsions for lifespan enhancement in stressed *C. elegans* worms, that had been fed on nematode growth media (NGM) seeded with two strains of pathogenic bacteria (*Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus*). Nanoemulsions were fabricated using HI-CAP® 100 modified food starch, and their stability was tested under different processing conditions (temperature, pH, ionic strength) over a period of 5 months. Lifespan assay results demonstrated that sunflower-oil-based nanoemulsions with droplet size 170.2±9.3 nm, significantly facilitated the growth of *C. elegans* under stress conditions and increased the lifespan of the worms from the previously recorded 11 days to 26 days in the current research study.

1. Introduction

Caenorhabditis elegans is a non-parasitic, small (1 mm in length), transparent, and free-living roundworm nematode with cosmopolitan distribution (Schulenburg and Félix 2017), a short life cycle of 3 days from egg stage to adulthood at 25°C, and a total lifespan of between 2-3 weeks, permitting ease of genetic manipulation, and facilitation in terms of the study of its biological attributes (Alexander *et al.,* 2014). *C. elegans* has been used as an *in vivo*, central model system across various biological disciplines (Zhang *et al.,* 2017). *C. elegans* presents significant points of interest for research in comparison with other model organisms (Morelli *et al.,* 2014), such as the convenience of use, large brood size, practicality, and ease of handling, as well as reduced costs from the perspective of research, rapid life cycle, and the ability to simulate the majority of human diseases (Zhang *et al.,* 2020). Furthermore, genomic comparison studies of *C. elegans* and humans have revealed the presence of a large number of genes associated with major human diseases, and disease pathways (Kim *et al.,* 2018). Also, the studies involving comparative proteomic analysis of close to 18,452 protein sequences from *C. elegans* have further revealed the existence of human gene analogs for approximately 83% of the worm's proteome (Kim *et al.,* 2018), and as a consequence, humans and *C. elegans* share many conserved cellular pathways, and processes in the context of human biology and disease (Poupet *et al.,* 2020).

Bacteria serve as the primary nutrition source for *C. elegans* in both natural as well as laboratory settings (Gottschling *et al.,* 2019). Various species of bacteria serve as the dietary source for the worm, in natural settings, such as *Pseudomonas medocina*, *Comomonas* sp., *Bacillus megaterium* (soil), *Acetobacter* sp., *Gluconobacter* sp. and *Enterobacter* sp. (rotting fruits) (Montalvo-Katz *et al.,* 2013). The dietary choices of *C. elegans* in the laboratory include more commonly *Escherichia coli* strain OP50, a uracil auxotroph, and to a lesser extent, the *E. coli* wild-type K12 strain, and its derivative strains, HB115 (D3), and HB101 (Zečić *et al.,* 2019). The protein-carbohydrate-fat (energy %) relationship in this bacterial diet corresponds to about 80:10:10, and therefore the diet of *C. elegans* is essentially protein-rich, albeit lipid-, and carbohydrate-poor (Gottschling *et al.,* 2019).

Until recently, aging was not regarded as an actively regulated process (Uno and Nishida, 2016). However, gene-regulated signaling pathways have been attributed to playing a significant role in the lifespan extension of

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various organisms, including *C. elegans* (Dall and Færgeman, 2019). For instance, the mutations that reduce the activity of DAF-2, the insulin/insulin-like growth factor-1 (IGF-1) homolog of *C. elegans*, almost double the lifespan of the animal (Wrigley *et al.*, 2017). Similarly, the mutations affecting the activity of AGE-1, the insulin/insulin-like growth factor signaling (IIS) downstream target, have also been associated with increasing the lifespan of *C. elegans* (Altintas *et al.*, 2016). Likewise, the inhibition of the Target of Rapamycin (TOR) signaling pathway has also been shown to increase the longevity in *C. elegans* by regulating the process of autophagy, a significant factor in the lifespan extension of the worm (Bjedov and Rallis, 2020).

Sirtuins are a family of nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase molecules with a direct association with NAD^+-aided cellular nutrient signaling, and SIR-2.1 (the *C. elegans* homolog of Sir2), by virtue of its overexpression, has been outlined as a factor in lifespan extension in *C. elegans* (Zhao *et al.*, 2020). Mediation of oxidative stress induced by reactive oxidative species (ROS) that are responsible for instigating damage in various biomolecules, for instance, DNA, lipids, and proteins, as well as promoting the deterioration of cellular structures, tissues, and, ultimately, the whole organism, is another avenue for regulation of aging-related phenomena, and consequently, lifespan extension in *C. elegans* (Hajam *et al.*, 2022). In this regard, a recent study investigated the antioxidant effects of carotenoid pigments in *C. elegans*, whereby carotenoids imparted lifespan extension characteristics through inhibition of ROS by upregulating the expression of catalase and superoxide dismutase (SOD) (Lee *et al.*, 2022). Similarly, another study demonstrated the anti-aging effects of saponin-rich extracts in C. elegans, whereby the extracts improved the lifespan of the worm, improved worm growth, enhanced stress resistance, and reduced biomarkers associated with aging (Kitisin *et al.*, 2019). Future studies are aimed at identifying the genes that provide a link between various environmental factors, such as diet, environmental temperature, as well as oxidative status, and lifespan regulation in *C. elegans* worms (Uno and Nishida, 2016).

The hydrophobicity of lipid triglycerides (TGs), however, restricts their utilization as dietary components for *C. elegans* (Watts and Ristow, 2017). Potential strategies to overcome this impediment are a mechanical scattering of TGs, partial solvency of TGs in dimethyl sulfoxide (DMSO), TGs suspended in an *E. coli* OP50 stock, and scattering of TG nanoemulsions (NEs) in media. Mechanical scattering of TGs in hydrophilic media, without the inclusion of a surfactant, may bring about creaming/sedimentation of TGs (Rodrigues, Salgueiro, Bianchini *et al.,* 2018). Furthermore, the hydrophobicity of TGs impairs their dissolvability in DMSO or other hydrophilic solvents, and the scattering of TGs in *E. coli* OP50 suspension may enzymatically alter them by way of the activity of *E. coli* layer lipases (Colmenares *et al.,* 2016) before being consumed.

Consequently, NEs are a better alternative by way of incorporation of lipids into their compositional matrix, and, therefore, are naturally more suitable for delivering TGs into the *C. elegans*. NEs have increasingly been investigated as highly capable and efficient vehicles for the delivery of water-insoluble bioactives, such as lipid-soluble vitamins, omega-6 (ω-6) oils, and β-carotenoids (Dahlawi *et al.,* 2020). Likewise, the concept of 'nanobait' has been explored, whereby *C. elegans* worms were fed nanocoated microbial cells, including *E. coli*, and the microalgae, *Chlorella pyrenoidosa*, via pharyngeal pumping, indicating an efficient delivery mechanism (Däwlätşina *et al.,* 2013).

In this research, *C. elegans* were fed on two bacterial pathogens, *S. enterica* serovar Typhimurium, and *Staphylococcus aureus*. *Caenorhabditis elegans* consumes these pathogens by way of pharyngeal pumping, whereby the rate of pumping is regulated as per the availability of food sources (Ishita *et al.,* 2020). Furthermore, *C. elegans* worms have special feeding behavior from the perspective of the water-loving nature of their preferred growth medium (Dimov and Maduro, 2019). Owing to their hydrophilic nature, bioactive components are rarely soluble in the growth media for *C. elegans*. To combat this limitation, a food-grade delivery system is vital. Food-grade NEs, therefore, could be a viable solution, allowing for the lipid component to be consumed and/or digested by the worms (Colmenares *et al.,* 2016). This also provides an excellent opportunity in the context of potential application for developing anti-aging mechanisms, by way of optimizing the delivery of bioactive substances capable of modulating molecular markers associated with the process of aging (Hernandez *et al.,* 2020).

2. Materials and methods

Sunflower oil was purchased from the local market in Lahore, Pakistan. While Hi-Cap 100 (modified starch) was donated by Ingredion (Germany GmbH). For *C. elegans* Nematode Growth Medium (NGM) was prepared in the laboratory. HI-CAP 100 is an OSA-modified straight-chain starch and is highly suitable for encapsulation applications, in particular for the encapsulation of bioactive such as vitamins, flavor compounds, spices, clouds, as well as fatty esters, at high oil loading.

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2.1 Formulation of nanoemulsions

NEs were prepared in two steps by using the high-pressure homogenizer method described by Iqbal *et al.,* 2020) with slight modifications. A coarse emulsion was prepared by combining the two phases (oil and aqueous). HI-CAP 100 5% (w/v) was dispersed with distilled water to generate the aqueous phase, while sunflower oil 10% (v/v) was utilized for the oil phase. Homogenization was achieved with the aid of a high throughput homogenizer (Troemner Talboys, Thorofare, NJ, USA) at 6000 rpm for 5 mins. The coarse emulsions were subjected to sonication (Ultra-Sonics 250W) at 25°C for 5 mins, followed by passage through a high-pressure disruption system (Constant Systems Ltd, Northamptonshire, UK) for 3 passes at 275 MPa and 25°C (Iqbal *et al.,* 2020).

2.2 Assessment of physical and chemical stability of nanoemulsions

2.2.1 Droplet size analysis

The measurement of the droplet size of the oil-in-water (O/W) NEs was achieved by way of dynamic light scattering (DLS) utilizing a Malvern Zetasizer Nano ZS90 (Malvern Instruments Inc., Worcestershire, UK). The refractive index obtained for NEs was 1.35. The NEs were subjected to 50 dilutions prior to the measurements. The readings were recorded in triplicates.

2.2.2 Measurement of zeta (ζ)-potential

A Malvern Zetasizer Nano ZS90 (Malvern Instruments Inc., Worcestershire, UK) was used for the measurement of electric charge on the surface of the NEs (25°C, 3.9V). Samples were subjected to 50 dilutions using double-distilled water (Rodrigues, Diniz, Sousa *et al.,* 2018). The readings were recorded in triplicates.

2.2.3 Effect of pH

The human digestive system experiences varying pH values (Beasley *et al.,* 2015). Accordingly, the NEs were stability tested at different pH levels (2, 5, and 7) using 1N hydrochloric acid (HCl), followed by storage at ambient temperature for 1h, before analysis was initiated (Iqbal *et al.,* 2020).

2.2.4 Effect of thermal treatments

NE samples were collected in an Eppendorf tube (1.5mL). Samples were subjected to two thermal treatments by placement in a water bath (30 mins, 63°C, and 10 mins, 95°C), followed by cooling at ambient temperature for 60 mins, and subsequent measurement for mean particle size (Iqbal *et al.,* 2020, Khalid *et al.,* 2017).

2.2.5 Effect of NaCl

The stability of NEs at varying salt concentrations was tested by collecting NEs in a beaker, NaCl concentration adjustments were made between 0.1M and 1M through the addition of NaCl solution. Samples were subjected to gentle mixing, with subsequent storage at ambient temperature for 60 mins prior to analysis (Khalid *et al.,* 2017).

2.2.6 Storage stability of nanoemulsions

The storage stability of NEs was measured by way of centrifuging NEs at 1300×*g* for 30 mins at 5°C. The NEs were later stored at ambient temperature for 5 months at pH 7. The measurement of the mean particle size of stored NEs was undertaken on a monthly basis (Iqbal *et al.,* 2020).

2.3 Application of nanoemulsions on Caenorhabditis elegans

2.3.1 Culturing of Caenorhabditis elegans with Staphylococcus aureus

C. elegans were procured from the collection maintained by the departmental laboratory. According to the method devised by Stiernagle, (2006), the NGM plates were prepared and seeded with *S. aureus* (100 µL) instead of *E. coli* OP-50 (as a food source for worms). Worms were then transferred to NGM plates for initiation of the growth of worms.

2.3.2 Culturing of Caenorhabditis elegans with Salmonella enterica serovar Typhimurium

C. elegans were procured from the collection maintained by the departmental laboratory. Per the method devised by Stiernagle (2006), the NGM plates were prepared and seeded with *S. enterica* ser. Typhimurium (100 µL) instead of *E. coli* OP-50 (as a food source for worms). The worms were once again transferred to NGM plates for propagation.

2.4 Lifespan assay of Caenorhabditis elegans with nanoemulsions

The survival lifespan of *C. elegans* worms was assayed using the protocol outlined by Sutphin and Kaeberlein (2009) with slight modifications. A total of twenty-five worms of identical size were selected and transferred to new culture plates, followed by inoculation of the NEs (100 µL). The preparation of the control plate involved the addition of worms without inoculation with NEs. For the observations related to survival rates, after every 2 days, the worms were transferred to new culture plates with identical conditions outlined above. The worms were examined under the Meiji Techno Dissecting Stereo Microscope (Model Number: EMZ-10

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+ MA502), and the results for survival analysis were recorded.

3. Results and discussion

3.1 Characterization of nanoemulsions

NEs were formulated by using 5% (w/w) HI-CAP 100 as a natural emulsifier containing 10% (w/w) sunflower oil. Figure 1 shows that O/W NEs stabilized with HI-CAP 100, exhibited slight monomodal droplet size distribution, and a mean droplet average diameter (d_{av}) of 170.2±9.3 nm. Furthermore, the NEs recorded a higher value of zeta potential -28.3±0.2 with a lower value of the polydispersity index (PDI), i.e. 0.325. It has been reported that the relatively lower value of the PDI is attributed to the higher stability of NEs during prolonged storage (Sari *et al.,* 2015).

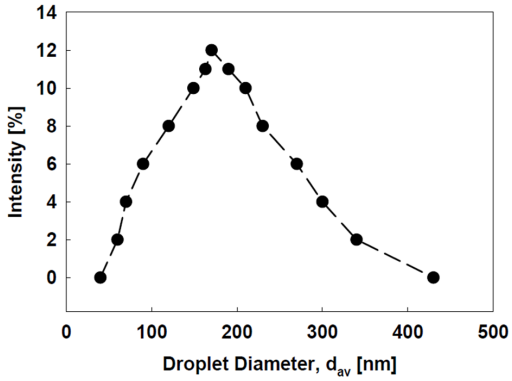

Figure 1. Particle-size distribution of oil-in-water nanoemulsions.

3.2 Physicochemical characterization

3.2.1 Effect of pH

The NEs demonstrated relative stability following changes in pH from 2 to 7, albeit with a slight increase in the d_{av}, and without any marked separation for NEs as compared to the control (Figure 2a). Also, the pH modifications had a minimal effect on the droplet size of NEs. However, in the case of ζ-potential, NEs exhibited significant changes, in particular at pH 2 (Figure 2b). As the decrease in pH consequently increases the concentration of hydrogen ions (H^+), this, in turn, induces a net positive charge on NE droplets, ultimately attributable to the negatively charged carboxylic groups of the modified starch molecules (Abbas *et al.,* 2014). In the context of food systems and the human digestive system in general, the pH value does not go below 2, as is evident from the findings associated with the stability of NEs over a pH range of 2-7 in current research.

3.2.2 Effect of temperature

In order to simulate real-world scenarios, the temperature ranges commonly employed during processing operations in the food and beverage industry

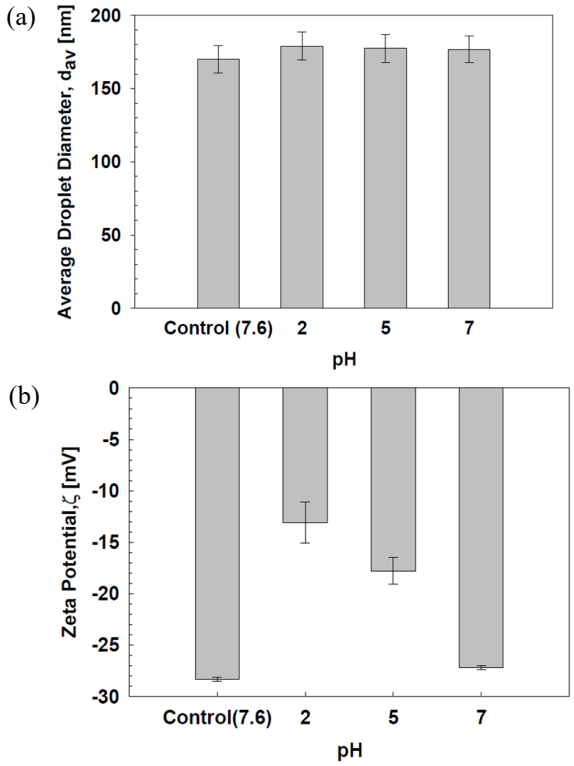

Figure 2. Effect of pH on the stability of sunflower oil NEs (a) droplet size (b) ζ –potential.

were used in current research for appraising the instability of NEs and any physicochemical changes brought on by temperature modifications. Fairly significant changes in both d_{av} and PDI of the formulated food-grade emulsions were observed when these NEs were exposed to two different temperatures, i.e., pasteurization (63°C for 30 mins), and near-boiling point (95°C for 10 mins) (Figure 3a). Regarding d_{av}, at 63°C, a small increase was recorded, with the increase much more marked at 95°C, which can be attributed to possible thinning of the emulsifier interface surrounding the droplet, and droplet aggregation as a consequence of weakening of the emulsifier interface respectively (Banasaz *et al.,* 2020). NEs formulated with synthetic emulsifiers have shown greater stability at elevated temperatures as compared to protein emulsifiers or surfactants, which tend to undergo coagulation when subjected to higher temperatures (Sari *et al.,* 2015) proteins get denatured, and consequently unfold upon exposure to elevated temperatures (Lapidus, 2017), thereby also exposing the sulfhydryl and hydrophobic groups, which, in turn, induces aggregation of proteins (Andlinger *et al.,* 2021) and ultimately results in destabilizing the NEs. In this regard, the NEs stabilized with modified starches have exhibited improved stability at elevated temperatures (Zhao *et al.,* 2019). Additionally, the ζ-potential of the formulated NEs decreased significantly at 95°C (Figure 3b), indicating a possible weakening of the emulsion interface, and subsequent release of ions from the interface.

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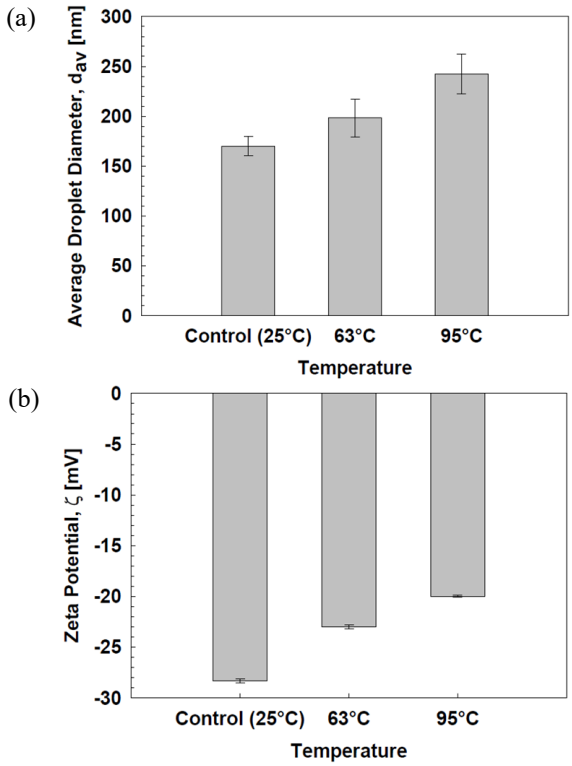

Figure 3. Effect of high temperature on (a) droplet size and (b) ζ-potential of NEs.

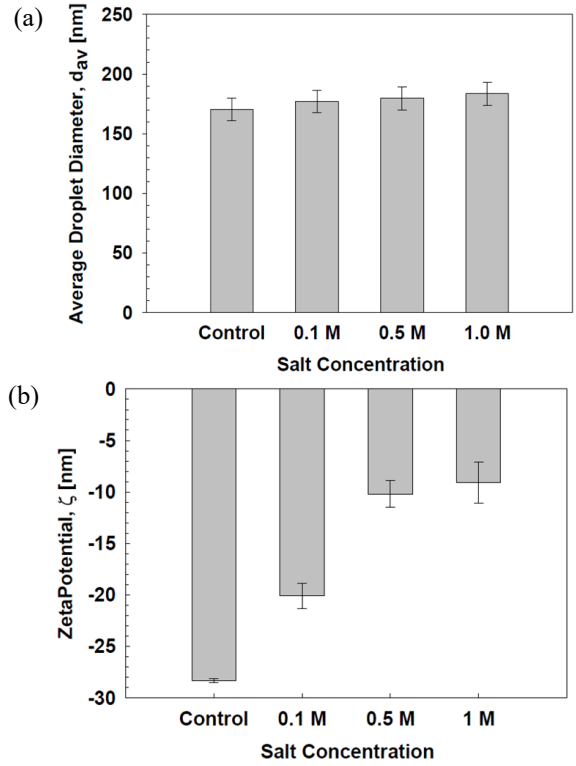

Figure 4. Effect of ionic strength on (a) droplet size and (b) ζ-potential of NEs.

3.2.3 Effect of ionic strength

Salt (sodium chloride/NaCl) and their associated ingredients are essential components of the food and beverage formulations, and therefore, have the tendency to influence both the functional performance as well as the stability of the delivery systems, pre, or post-ingestion (Aswathanarayan and Vittal 2019). Keeping this in view, the effect of various NaCl concentrations on the stability of NEs was investigated. As can be evident from Figure 4a, the effect of increasing NaCl concentrations had a nominal effect on the d_{av} of the NEs, whereby a slight increase in the d_{av} values was observed as the concentration of NaCl was increased. The increase in NaCl concentration, however, induced a decrease in the magnitude of the negative charges for the formulated NEs (Figure 4b). This decrease might be attributable to the weakening of the electrostatic interactions existing in the NEs, arguably owing to the electrostatic screening effects (McClements, 2018). Interestingly, however, despite the decrease in the ζ-potential, and consequently, the magnitude of negative charges, the NEs remained stable. This can be alluded that the magnitude of negative charges on the emulsion droplets contributed to the maintenance of the electrostatic interactions in the presence of NaCl, bringing about an overall charge stabilization effect, and, therefore, induced stability of NEs by suppressing Ostwald ripening. Another factor that could have significantly contributed toward the stability of NEs in this study is the steric hindrance induced by the modified starch (HI-Cap 100).

3.2.4 Long-term stability

One of the most common problems associated with NE formulations is their instability against various factors (de Oca-Ávalos *et al.,* 2017), particularly, concerning their application and storage for extended periods (Barkat *et al.,* 2020). Some of the major mechanisms responsible for inducing instability in NEs include gravitational separation (sedimentation or creaming), coalescence, flocculation, Ostwald ripening, and phase separation (Liu *et al.,* 2019; Sheth *et al.,* 2020). The formulated NEs in this research were found to be stable for a duration of 5 months (Figure 5) and were repeatedly characterized for centrifugation and particle-size distribution (PSD) throughout the duration of storage.

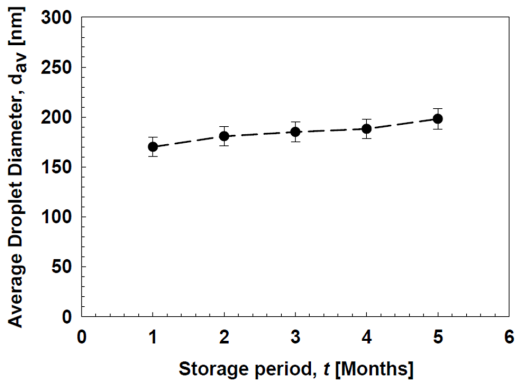

Figure 5. Effect of the storage period on the d_{av} of NEs.

3.3 Lifespan assessment

From Figure 6a, it was evident that the lifespan of *C. elegans* in the presence of *S. enterica* ser. Typhimurium

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was only limited to 11 days, as *S. enterica* ser. Typhimurium and other *S. enterica* serovars are pathogenic and ultimately, lethal for *C. elegans*, as reported in various research studies (Knodler, 2015). Furthermore, it can be noted that there was a gradual decline in the growth of *C. elegans* from a 100% survival rate on day 1 with only 20% surviving on day 10. In contrast to these results, the addition of an NE delivery system significantly increased the lifespan of *C. elegans*, with the worms' survival rate under stress showing marked improvement and increasing from the previously established 11 days (initial findings of the study) to 26 days (post-NE feeding) in current research. The presence of NEs, therefore, aided *C. elegans* to survive for an additional 15 days under stress conditions, thereby hinting at the anti-aging attributes imparted by NE formulations. This anti-aging can be attributed to the phenomenon of autophagy, a major lifespan determinant in various longevity models. Aging can be regarded as the progressive failure of cellular repair mechanisms over time, contributing to an increase in cellular and molecular damage, and accumulation of aberrant macromolecules, ultimately culminating in the loss of function (Gelino and Hansen, 2013). In contrast, autophagy has been shown to mitigate various stress-induced scenarios (nutrient deprivation, hypoxia and hypothermia) by way of recycling the damaged cellular components (the autophagic cargo) into re-usable construction materials for the synthesis of proteins, and cellular repair (Chun and Kim, 2018). The TOR signaling pathway is another major factor that affects aging, and its nutrient-dependent activation induces a pronounced metabolic shift towards cell proliferation (Bjedov and Rallis, 2020). In the case of *C. elegans*, the nutrient sensor TOR homolog, let-363, negatively regulates autophagy (Blackwell *et al.*, 2019). This regulation is induced at the transcriptional level as well, through inhibition of the nuclear translocation of transcription factors such as PHA-4/FOXA, DAF-16/FOXO, and HLH-30/TFEB, responsible for increasing the expression of genes associated with autophagy and lysosomes, factors vital for extension of lifespan in *C. elegans* (Denzel et al., 2019). Transcription factor HLH-30/TFEB also plays its role in the regulation of genes responsible for lipid metabolism, implying that under nutrient-stressed conditions, the survival of an organism is reliant on the synergistic coordination between the pathways regulating autophagy and lipid metabolism (Zhang *et al.*, 2020). This relationship could be a crucial factor in contributing toward lifespan extension in *C. elegans*, given that the food-grade delivery systems evaluated in this research study are sunflower oil-based NEs, aimed at the provision of lipids to the worm.

The NEs in this research study showed excellent results for *C. elegans* in terms of enhanced survival rates when fed on *S. aureus*. The lifespan of *C. elegans*, in this case, increased from 15 days to 28 days under stress conditions (Figure 6b). The probable reason for improved survival can be attributed to the increased solubility of lipids manifested by NE formulations administered to the worms (Banasaz *et al.,* 2020). Previous research has indicated that *C. elegans* can ingest lipid nanoparticles and researchers have confirmed that due to the small particle size of NEs, worms were able to easily ingest the NE formulations (Shen *et al.*, 2019). Lipid-based NEs can easily be metabolized by the lipase enzymes due to their large surface area (Mehanna and Mneimneh 2021) resulting in the formation of omega (ω)-6 fatty acids. These lipase-generated ω-6 fatty acids have been shown to promote autophagy, thereby contributing to an increased lifespan in *C. elegans* (Seah *et al.,* 2016). This study augments the previous research studies (Mokoena *et al.,* 2020; O'Rourke *et al.,* 2013) in this regard, and proposes that supplementation of these ω -6 fatty acids to *C. elegans* or humans can increase the lifespan of organisms.

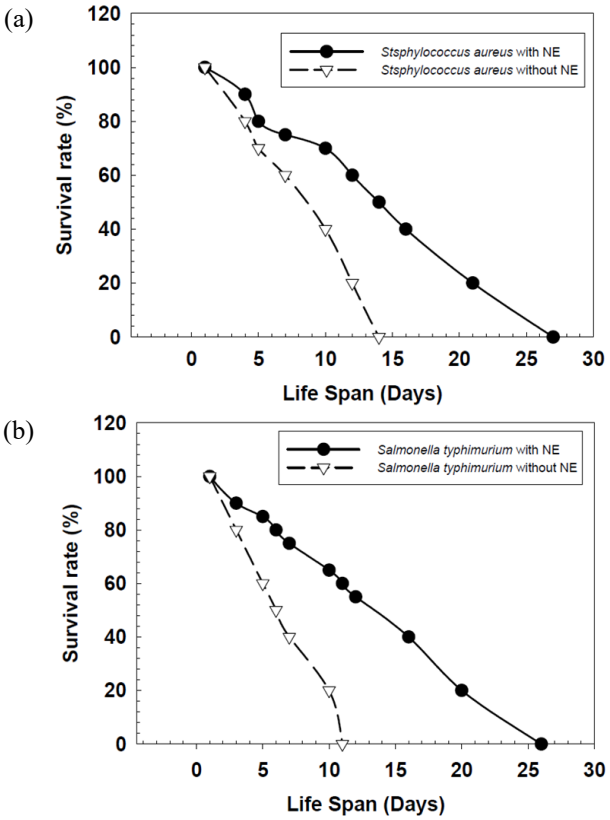

Figure 6. Survival rate and lifespan (a) *S. aureus* and (b) *S. enterica* ser. Typhimurium.

4. Conclusion

Genomic comparison studies indicate that a large number of genes from the human genome have homologs in *C. elegans*. Similarly, the presence in *C. elegans*, of the genes associated with human diseases, and their respective pathways, proves that the worm can be utilized as a key model system for studies related to

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disease simulation and genetic regulation of aging mechanisms. The current research can be viewed as a new avenue for researchers to observe the effect of lipid-based nanoemulsions in the context of the lifespan enhancement of various organisms under stress conditions. Future studies can be directed at a greater understanding of factors related to longevity, and the health of an organism, with subsequent mitigation of aging mechanisms in humans.

Conflict of interest

The authors declare no conflict of interest in writing this article.

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