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Lactobacillus salivarius Strain FDB89 Induced Longevity in *Caenorhabditis elegans* by Dietary Restriction

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In this study, we utilized the nematode *Caenorhabditis elegans* to assess potential life-expanding effect of *Lactobacillus salivarius* strain FDB89 (FDB89) isolated from feces of centenarians in Bama County (Guangxi, China). This study showed that feeding FDB89 extended the mean life span in *C. elegans* by up to 11.9% compared to that of control nematodes. The reduced reproductive capacities, pharyngeal pumping rate, growth, and increased superoxide dismutase (SOD) activity and XTT reduction capacity were also observed in FDB89 feeding worms. To probe the anti-aging mechanism further, we incorporated a food gradient feeding assay and assayed the life span of *eat-2* mutant. The results demonstrated that the maximal life span of *C. elegans* fed on FDB89 was achieved at the concentration of 1.0 mg bacterial cells/plate, which was 10-fold greater than that of *C. elegans* fed on *E. coli* OP50 (0.1 mg bacterial cells/plate). However, feeding FDB89 could not further extend the life span of *eat-2* mutant. These results indicated that FDB89 modulated the longevity of *C. elegans* in a dietary restriction-dependent manner and expanded the understanding of anti-aging effect of probiotics.

Keywords: *Lactobacillus salivarius* FDB89, longevity, *Caenorhabditis elegans*, dietary restriction

Introduction

Probiotics have a variety of beneficial health impacts including intestinal microbiota regulation, immunomodulation and anticarcinogenic effects (Parvez *et al*., 2006). The anti-aging effects of probiotics were realized as early as 1908, when Metchnikoff reported that Bulgarian farmers, who consumed large quantities of fermented milk with lactobacilli, experienced increased longevity (Metchnikoff, 1908). However, researches on the anti-aging effects of probiotics have been limited up to the present time. Using rodents as model animals, previous studies have shown that probiotics could suppress aging-associated manifestations, such as the prevention of bone density loss, reduction in the level of serum lipid peroxidation and delaying senescence of the thymus, kidney, brain and testis (Kimoto-Nira *et al*., 2007; Zhang *et al*., 2008). However, these studies were based on senescence-accelerated or aged mice without normal physiological conditions and did not investigate the effects on life span. Thus, it is necessary to choose an optimal model for researching the anti-aging effects of probiotics.

Caenorhabditis elegans is a small, free-living soil nematode, which continues to be widely used as a model organism for research on aging, development and cell apoptosis (Salinas *et al*., 2006; Van Raamsdonk and Hekimi, 2010). In particular, for aging studies, *C. elegans* has the advantage of a short and reproducible life span and ease of cultivation. Therefore, it has been extensively used for assessing potential life-expanding effects of bio-activators. Antioxidants such as vitamin E and plant extracts have been shown to possess anti-aging effects using *C. elegans* as an experimental model system (Harrington and Harley, 1988; Brown *et al*., 2006; Saul *et al*., 2010). Lactic acid bacteria (LAB) strains have been recently reported to prolong the life span of *C. elegans* (Ikeda *et al*., 2007). However, life span extension imparted by LAB represented strain-differences and these studies did not identify possible underlying mechanisms for LAB-mediated longevity. Furthermore, previous researches have indicated that varied mechanisms of exogenous substances mediated longevity in *C. elegans*. Increased oxidative stress tolerance and dietary restriction (DR) were considered to be the major mechanisms that exogenous substances exert the anti-aging effects (Ristow and Schmeisser, 2011). Therefore, it is necessary to separately investigate the anti-aging effects of a new LAB strain on *C. elegans* and its underlying mechanism.

The *Lactobacillus salivarius* FDB89 (FDB89) strain in this study was isolated from feces of centenarians in Bama County (Guangxi, China), and was found to be a functional strain, as confirmed by our previous study (Wang *et al*., 2008). The objective of this study was to evaluate the potential life-prolonging property of FDB89 and uncover the possible underlying mechanism for FDB89-mediated longevity.

Materials and Methods

Bacterial strains and culture conditions

FDB89 strain was isolated from fecal samples of healthy cen-

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tenarians in Bama County, Guangxi, China. *E. coli* OP50 (OP50), a standard food for nematodes, was obtained from the National Institute of Biological Sciences (NIBS, China). *Clostridium perfringens* C01 (CLP) was used to exclude differences between classifications and growth conditions of FDB89 and OP50. OP50 was grown in Luria-Bertani broth (AoBoXing, China) at 37°C for 18 h while being shaken (at 220 rpm). FDB89 and CLP were cultured at 37°C in an anaerobic case with GENbox anaer (bioMérieux, France) using MRS broth (LuQiao, China) for FDB89 and BHI broth (LuQiao) for CLP for 18–24 h (Vasquez *et al.*, 2009). Bacteria were harvested by centrifugation at 4,000×g for 15 min, washed twice in sterile M9 buffer and centrifuged at 16,000×g for 15 min at 4°C to remove supernatant. Then bacteria were adjusted to a final concentration of 0.4 mg (wet weight) per µl in M9 buffer, which was used as concentrated bacteria. The densities of cells in concentrated bacteria were determined using a bacterial counting chamber (Equl, China). The concentrated bacteria were stored at -80°C until used.

Nematodes and culture conditions

C. elegans N2 (wild type) and the mutant strain *eat-2* (ad1116) were obtained from the Caenorhabditis Genetics Center (CGC, USA). *C. elegans* were maintained at 25°C on nematode growth medium (NGM) agar using standard techniques (Stiernagle, 2006), supplemented with OP50, an internationally established feed. Synchronized worms were produced by sodium hypochlorite-sodium hydroxide solution (Sulston and Hodgkin, 1988). For life span assay, mNGM plates were produced by supplementing NGM plates with filtrated sterilized carbenicillin (0.5 mM) and 5-fluoro-2′-deoxyuridine (FUdR) (50 µM) (Gruber *et al.*, 2009). Heat-killed bacterial cells (10 mg) were spread on 60 mm-diameter mNGM plates.

To detect superoxide dismutase (SOD) activity and XTT [2,3-bis-(2-methoxy-4 nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction capacity, the liquid cultivation of nematodes was established according to methods described by Houthoofd (Houthoofd *et al.*, 2002c). Briefly, fourth larval stage (L4) worms were washed off the NGM plates and suspended in S medium containing 0.5 mM carbenicillin and 50 µM FUdR, with worm densities of 1,500 to 2,000/ml (Braeckman *et al.*, 2002). Culture solution (125 ml) was transferred to 1-L Fernbach flasks, and heat-killed concentrated bacteria were added for a final concentration of 3×10^9 cells/ml. The culture was incubated at 25°C, while continuously shook at 120 oscillations/min on a gyratory shaker (Taichang, China). Turbidity at 550 nm was maintained daily and concentrated bacterial cells were added as needed to maintain a fixed supply of food.

Life span assay

L4 stage N2 or *eat-2* nematodes grown on NGM plates were transferred to mNGM plates with a platinum wire. For each life span assay, 100 worms per bacterial species were assayed in ten plates (ten worms/plate). The numbers of live and dead worms were counted using a dissecting microscope (Chong Qing Optical, China) every 24 h. A worm was considered dead when it failed to respond to a gentle touch with a platinum wire pick. Worms showing abnormal death, such as vulva explosion, hatched progeny inside the hermaphrodite or traversing the plate wall were excluded from the life-span analysis. The life span assay was conducted at least three times.

Food gradient feeding assay

Heat-killed FDB89 and OP50 bacterial suspensions were spread on mNGM plates that achieved serial concentrations ranging from 10 mg to 0.001 mg bacterial cells/plate. L4 stage N2 worms were placed on these plates. For each concentration, 100 worms were assayed over ten plates (ten worms per plate). The life span was measured as previously described. This test was conducted at least three times.

Measurements of body size, pharynx pumping rate and reproduction

L4 larvae were placed on NGM plates coated with bacterial lawns. Ten worms per bacterial species were assayed using ten plates (one worm per plate). From the first day that *C. elegans* were transferred to fresh NGM plates, the sizes of live worms were examined every 24 h. Images of adult nematodes were captured using a XSP-8CZ digital microscope (Chong Qing Optical) and the projection area of worms were analyzed as the body size using ImageJ software (National Institutes of Health, USA) (Ikeda *et al.*, 2007). To determine the pharynx pumping rate, L4 nematodes were grown on NGM plates with bacterial lawns for 30 min before counting. The pumping frequency was recorded as the number of contractions in the terminal bulb of the pharynx in a 60 sec period for an individual worm. The test was performed five times. For the reproduction assay, the animals were transferred daily to fresh NGM plates until reproduction ceased. The offspring of each animal were counted at the L2 or L3 stage. The test was performed three times.

SOD activity and XTT reduction

Nematodes in liquid cultivation were harvested at daily intervals and cleaned using Percoll and sucrose density gradients as previously described (Fabian and Johnson, 1994; Braeckman *et al.*, 2002). The cleaned live worms were suspended in PBS (pH 7.4) and homogenized using an ultrasonic cell disruptor (Scientz Biotechnology Co. Ltd., China) on ice (100 Watts, total time: 15 min, 2 sec with 2 sec intervals), and centrifuged at 1,500×g for 10 min at 4°C. The supernatant was used for assays.

SOD activity was assayed by employing a commercially available kit (Jiancheng, China) and processed according to the manufacturer's instructions. For assaying the XTT reduction capacity, 95 µl of distilled water was added to the wells of a 96-well polystyrene microtiter plate, followed by 5 µl of nematode lysate, 50 µl of a mixture containing 2 mM each of NADH and NADPH (dissolved in 0.1% $NaHCO_3$), and 100 µl of an aqueous 0.5 mg XTT/ml solution (Sigma, USA). The microtiter plate was incubated at 25°C for 1 h, then the absorbency was measured at 475 nm (Braeckman *et al.*, 2002). XTT formazan production rates are expressed as nmol per h and per mg protein using the molar extinction

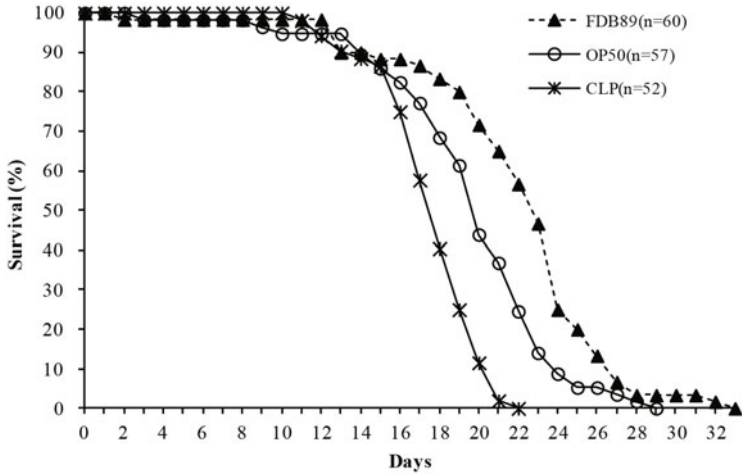

Fig. 1. Survival curves of *C. elegans* subjected to *L. salivarius* FDB89 (FDB89), *E. coli* OP50, and *C. perfringens* C01 (CLP). Day 0 refers to the first day that *C. elegans* (L4 stage) were transferred to mNGM plates.

coefficient for XTT formazan as described by Paull *et al.* (1988) ($\varepsilon_{XTT,477nm}$=16399.6 cm^{-1} M^{-1}).

The SOD activity and XTT reduction were scaled to protein content to correct for differences in biomass. The protein content of lysate was measured using a bicinchoninic acid (BCA) protein kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions.

Data interpretation and statistical analysis

The nematode survival rate was calculated by the Kaplan-Meier method, and differences in survival rates were tested for significance by use of the log rank test. Mean values were calculated for the body size assays. Statistical significance was evaluated by the repetitive measure analysis of variance (ANOVA) method with the Mauchly's sphericity test, and deemed significant at $p<0.05$. One way ANOVA with the Duncan's test was used to compare the effects of different bacteria on pharyngeal pumping rate, reproduction, SOD activity and XTT reduction. Variances were considered significant at $p<0.05$.

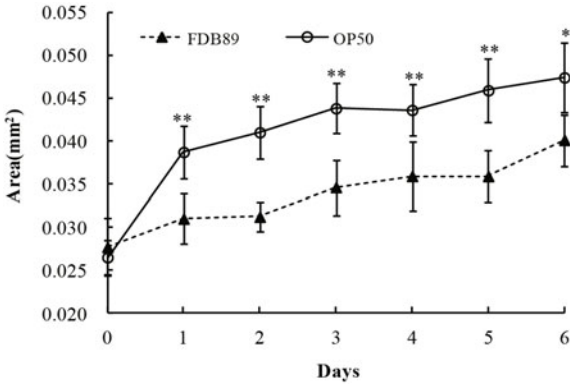

Fig. 2. Influence of FDB89 on body size of *C. elegans*. All results are presented as means±SD. Differences compared to control (OP50) were considered as significant at 0.05(*) and 0.01(**) level. Day 0 refers to the first day that *C. elegans* (L4 stage) were transferred to NGM plates.

Results

FDB89-feeding prolonged the life span of wild-type nematodes

Based on the *C. elegans* survival assay, FDB89 induced significant anti-aging effect in our study. Feeding nematodes with FDB89 increased the average life span compared to the OP50 feeding group (Fig. 1). The mean life span of worms fed FDB89 was 22.1±0.65 days, which was 11.9% greater than that of control nematodes (19.7±0.59 days). The life span of nematodes fed FDB89 was significantly extended ($p<0.05$). To exclude the differences of Gram classification and growth conditions between FDB89 and OP50, CLP was also used to feed *C. elegans*. The mean life span of worms fed CLP was 17.7±0.35 days, which was significantly shorter compared to OP50 ($p>0.05$) (Fig. 1).

FDB89 decreased reproductivity, body size and pharyngeal pumping rate of *C. elegans*

FDB89-feeding affected the reproduction rates of nematodes. No offspring were observed during the experimental period (0–10 days). The body size of *C. elegans* increased over seven consecutive days and the Mauchly's sphericity test revealed that feeding FDB89 significantly affected body size ($p<0.05$). Worms fed FDB89 were notably smaller compared to OP50 at every time point ($p<0.05$) (Fig. 2). The pharyngeal pumping rates of *C. elegans* fed FDB89 and OP50 were 186.5±5.62 and 249.2±18.99 times/min, respectively. Statistic analysis results showed that FDB89-fed nematodes exhibited a significantly decreased pumping rate compared to controls ($p<0.05$).

FDB89 increased SOD activity and XTT reduction activity of *C. elegans*

SOD activity tended to increase with the extension of cultivation time. On the second day and the fourth day, SOD-specific activities of worms fed FDB89 were notably higher than those fed OP50 ($p<0.05$), and then the activities of worms fed *C. perfringens* were increased too, but there were no significant differences compared to those fed OP50 (Fig.

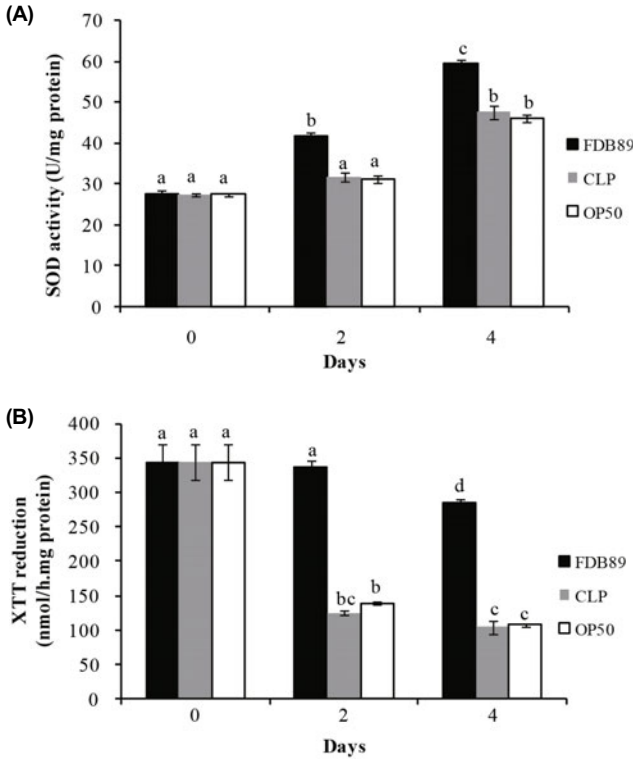

Fig. 3. Influence of FDB89 on SOD activity and XTT reduction of *C. elegans*. Error bars indicate ±SD. Different lowercase letters show that the difference arrives at 5% level with Duncan's method. Day 0 refers to the first day that *C. elegans* (L4 stage) were transferred to S medium.

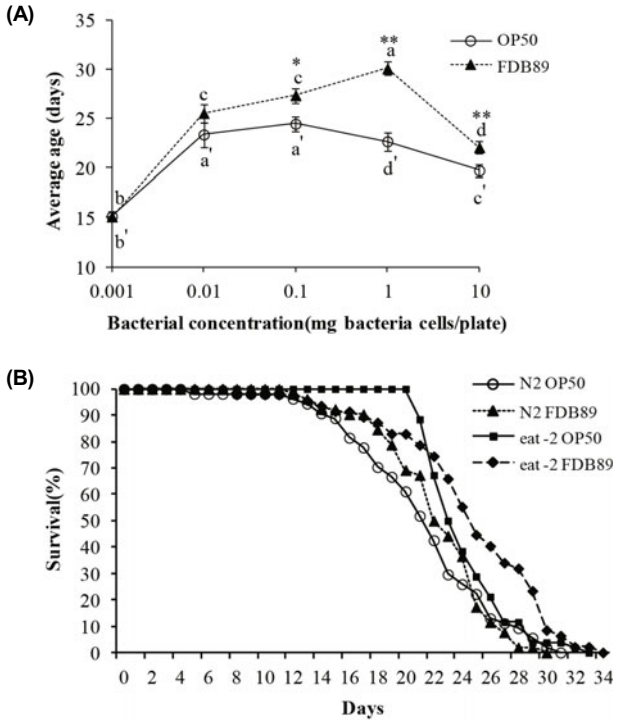

Fig. 4. (A) The life span of *C. elegans* fed by the gradient concentration bacteria. Error bars indicate±SD and differences compared to control (OP50) were considered as significant at 0.05(*) and 0.01(**) level. Different lowercase letters and lowercase letters with superscript show that the difference arrives at 5% level between inter-group. (B) The life span of N2 and *eat-2* (ad1116) mutant fed by FDB89 and OP50. Day 0 refers to the first day that *C. elegans* (L4 stage) were transferred to mNGM plates.

3A). FDB89 significantly increased the XTT reduction capability and slowed the age-dependent decrease of XTT reduction to its formazan derivative, but *C. perfringens* slightly decreased the XTT reduction capability compared to OP50 (Fig. 3B, $p<0.05$ for both species). These finds suggest that FDB89 has an effect on energy level of *C. elegans*.

FDB89 extended life span in a DR-dependent manner

Mean life span increases as food intake is reduced from *ad libitum* levels (10 mg bacterial cells/plate) until a nutrient regime that optimizes longevity is reached, past which further restriction results in reduced life span due to starvation (0.001 mg bacterial cells/plate). The mean life span of *C. elegans* fed OP50 reached a maximum at a concentration of 0.1 mg bacterial cells/plate (24.5±0.78 days), and there was no significant difference within the range of 1.0 to 0.01 mg bacterial cells/plate. Within the concentration range of 10 to 0.1 mg bacterial cells/plate, feeding FDB89 significantly increased life span compared to OP50. The maximum mean life span of *C. elegans* was reached by feeding with FDB89 at 1.0 mg bacteria cells/plate (30.1±0.70 days), and which was significantly higher than that of nematodes fed 0.1 mg bacteria cells/plate ($p<0.05$) (Fig. 4A). The mean life span of N2 fed FDB89 (22.3±0.55 days) was significantly longer than OP50 (21.3±0.70 days), which was generally consistency with the results in Fig. 1. The mean life span of *eat-2* (ad1116) mutants fed with OP50 were significantly extended compared to wild type N2 worms ($p<0.05$). However, feeding FDB89 did not further induce a statistically significant increase on *eat-2* mutant life spans ($p>0.05$). The mean life spans of N2 fed by OP50 and FDB89 were 21.3±0.70 and 22.3±0.55 days respectively. The mean life spans of *eat-2* (ad1116) mutant fed by OP50 and FDB89 were 24.3±0.40 and 24.5±0.80 days, respectively (Fig. 4B).

Discussion

In this study, we evaluated the anti-aging effect of *L. salivarius* FDB89 isolated from healthy centenarians on *C. elegans*. The results indicated that FDB89-feeding could increase the mean life span of *C. elegans*, decrease its reproductivity, body size and food intake and further alter metabolic rates in *C. elegans*. On the basis of these data, we speculated that the anti-aging effect of FDB89 was imparted by DR.

FDB89-feeding effectively increased the mean life span of *C. elegans*, and the life-prolonging rate reached a maximum of 11.9%. Similar results were obtained by Ikeda *et al.* (2007), in which feeding nematodes bifidobacteria or lactobacilli resulted in increased average life spans compared to those fed OP50 and the life-prolonging rates ranged from 17% to 33%. These results suggest that different strains exert diverse life-prolong effect, and it is necessary to separately investigate

the anti-aging effects of a new strain.

Thus far, limited publications focused in the anti-aging effect of LAB using *C. elegans* as model. Previous studies evaluated the effects of LAB on the *C. elegans* life span, but the mechanism of LAB-mediated longevity had not been clarified. The free radical theory is considered to be one of the most prominent theories to explain aging (Vina *et al*., 2007). Our results showed that FDB89-feeding caused an increase in SOD activity, which has the potential to delay aging by counteracting the impact of free radicals. However, some investigators provided evidence that oxidation resistance is not correlated with longevity in *C. elegans* (Doonan *et al*., 2008; Gems and Doonan, 2009; Pun *et al*., 2010), furthermore, our results suggested that DR was the mechanism of FDB89 extending life span. FDB89 significantly increased the XTT reduction capability of *C. elegans* which suggested that FDB89 had an effect on energy level of *C. elegans*.

DR is defined as a reduction of particular or total nutrient intake without causing malnutrition (Katewa and Kapahi, 2010). DR is known to extend life span and to retard age-related health declines in a number of different species, including rodents, worms, yeast and possibly primates (Sohal and Weindruch, 1996). There are two ways to produce DR effects: reduction of caloric intake and intake of DR mimics. Restricting nutrients without malnutrition could achieve a reduction of caloric intake (Katewa and Kapahi, 2010). DR mimics manipulate energy metabolism and induce a metabolic shift without requiring reduced food intake to obtain physiological effects, as with DR (Mehta and Roth, 2009). Our results showed that feeding *C. elegans* with FDB89 decreased pumping rate, body size and reproductivity, which indicated that FDB89 may induce longevity of *C. elegans* through reduced caloric intake. Klass reported that caloric intake is linearly related to pumping rate, which is a reliable measure of nutritional status (Klass, 1977). A decreased pumping rate may induce a reduction in caloric intake and further affect worm development (Morck and Pilon, 2006). In response to DR, the reproduction of many species was inhibited and delayed (Crawford *et al*., 2007; Martin *et al*., 2008). FDB89-feeding significantly reduced reproduction, in a similar fashion to DR. Simultaneously, our study indicated that FDB89 compounds may activate the same longevity assurance targets as DR. FDB89-feeding significantly increased life span compared to controls at 10 mg and 1 mg bacterial cells/plate, but this effect was diminished as food intake was decreased. The maximal life span of FDB89-fed *C. elegans* appeared at the concentration of 1.0 mg bacterial cells/plate, which was 10-fold greater than that of *C. elegans* fed OP50 (0.1 mg bacterial cells/plate). These results indicated that FDB89-feeding could activate DR targets at high food intake levels (Mair and Dillin, 2008). Furthermore, we used *eat-2* (ad1116) mutant as control to test the impact of DR on life span of *C. elegans*. *eat-2* mutants, which have a germline mutation in the acetylcholine receptor that reduces their pharyngeal pumping rate, likely induce a form of DR that is chronic. Therefore, if the test materials extended life span of normal nematodes in a DR-dependent manner, they could not extend the life of *eat-2* mutant anymore, which is already in the DR status. In Fig. 4B, when *eat-2* mutant was used, no significant difference was observed between OP50 and FDB89 feeding. The results suggested that FDB89 extended life span in a DR-dependent manner. We also found that FDB89-feeding led to a higher XTT reductive capacity, similar to Houthoofd's results, who reported that continued elevated XTT reductase activity was observed in dauers, which can survive for a period exceeding 8- to 10-times than that of a normal adult life span. It is therefore tempting to speculate that elevated XTT reductase activity is a good biomarker of enhanced life maintenance (Houthoofd *et al*., 2002a, 2002b, 2002c). Thus, we concluded that FDB89 mediated the longevity of *C. elegans* through a DR-dependent manner.

In conclusion, our findings have shown for the first time that *L. salivarius* FDB89 isolated from centenarians were able to prolong the life span of *C. elegans* to a maximum rate of 11.9%. Based on gradient feeding assay and life span assay of *eat-2* mutant, we concluded that the anti-aging effect of FDB89 was imparted in a DR-dependent manner. This study expands the understanding of anti-aging effect of probiotics. Further research could focus on the anti-aging effect of other bacterial strains on *C. elegans*.

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