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Enterococcus faecalis SI-FC-01 enhances the stress resistance and healthspan of *C. elegans* via AKT signaling pathway

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The global demographic is witnessing an unprecedented surge in aging, precipitating a dramatic rise in geriatric diseases and related health complications. Although probiotics have been extensively shown to maintain microbiome stability and confer health benefits, their potential role in decelerating the aging process remains largely unexplored. The study identified a beneficial gut microbe from human intestinal tract, *Enterococcus faecalis* SI-FC-01, which was proved to be biosafe and found to enhance the average lifespan of *C. elegans* by 33.55%. More interestingly, the *E. faecalis* SI-FC-01 also enhanced the motor ability, memory and learning ability and anti-oxidative stress ability of *C. elegans*. Moreover, it exhibited neuroprotective effects in the worm models of neurodegenerative diseases such as Parkinson's disease and Huntington's disease. Through screening various aging-associated mutants of *C. elegans*, we discovered that *E. faecalis* SI-FC-01 modulates DAF-16/FOXO signaling via the activation of AKT pathway. This activation subsequently triggers stress resistance and immune-related genes downstream of *daf-16*, thereby promoting healthspan and neuroprotection. In summary, our research indicates that *E. faecalis* SI-FC-01 holds significant potential as a dietary supplement for delaying host aging. Furthermore, it provides novel insights for potentially mitigating the progression of age-related neurodegenerative diseases.

Keywords *Enterococcus faecalis*, *Caenorhabditis elegans*, Aging, Healthspan, Neurodegenerative diseases

According to the United Nations' World Population Prospects, the proportion of people aged 65 and over is expected to grow from 9% in 2019 to 11% in 2050[1]. While this demographic shift testifies to the successes of modern medicine and public policy, it also underscores the importance of maintaining health in older age to enhance quality of life and mitigate the costs of societal aging. Aging, however, is an inevitable biological process influenced by numerous factors and is associated with a rise in age-related disorders such as cancer, cardiovascular disease, metabolic disorders, and neurodegenerative diseases[2,3]. These conditions, being the leading causes of death globally, diminish the quality of life for older adults and escalate healthcare costs, presenting both societal and economic challenges. Consequently, contemporary aging research primarily aims to enhance healthspan—the number of years a person enjoys good health—rather than merely extending lifespan[4].

Although aging is irreversible, the aging rate and the onset of age-related conditions can be delayed. Recent research has shown that selectively removing senescent cells can extend and rejuvenate the healthspan of aged animals[5,6]. Over the past two decades, the search for anti-aging interventions that reduce morbidity and increase lifespan has intensified, leading to the discovery of potential life-extending compounds such as rapamycin, metformin and nicotinamide adenine dinucleotide (NAD) precursors[7,8]. Since Metchnikoff's initial observation of a correlation between longevity and probiotics, the use of probiotics has been associated with a reduced risk of many chronic diseases. Epidemiological studies suggest that probiotics possess anti-carcinogenic, anti-cardiovascular, and anti-diabetic properties and their extracts could extend lifespan and mitigate oxidative stress, potentially preventing age-related chronic diseases[9,10]. For example, dietary supplementation with *Bifidobacteria* has been shown to enhance the healthspan in rodent models, highlighting the potential of probiotics as a viable

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strategy for delaying the aging process and its associated diseases[11]. However, research on the anti-aging effects of probiotics is limited due to a lack of suitable experimental models for determining host longevity.

C. elegans, a terrestrial, non-parasitic nematode, is a powerful genetic model for aging studies. This organism presents no ethical issues, is cost-effective to maintain, and requires less time and labor for screening, making it an ideal model for studying human diseases and screening active substances. Importantly, the interaction between diet and longevity is a crucial factor in interpreting *C. elegans* lifespan assays involving bacterial food source modifications[12]. Several studies have shown that *Lactobacilli* and *Bacillus licheniformis* can enhance host defense and extend the nematodes' lifespan[11,13]. Tests of environmental, genetic, and pharmacologic interventions have led to the discovery and characterization of numerous functional substances and endogenous pathways that can be modified to extend lifespan and healthspan.

In our high-throughput screening of 7,087 bacterial strains isolated from soil, water, and living organisms, we identified a strain of *Enterococcus faecalis* SI-FC-01, which exhibits potent anti-inflammatory and antioxidant properties, and lacks antibiotic resistance or virulence genes, making it a promising candidate for probiotic development. We found that supplemental feeding with *E. faecalis* SI-FC-01 significantly extended the healthspan and lifespan of *C. elegans*. The anti-aging effects of *E. faecalis* SI-FC-01 in worms were regulated by the DAF-16/AKT signaling pathway, which is involved in proliferation and immunity processes, and were accompanied by changes in stress-resistant genes in *C. elegans*. We also investigated the potential therapeutic effects on age-related neurodegenerative diseases. Our findings underscore a significant association between probiotics and host lifespan, offering novel perspectives on the use of probiotics. We also suggest that *E. faecalis* SI-FC-01 may have potential applications in developing nutraceuticals or pharmaceuticals, thereby contributing to the broader field of aging and healthspan research.

Results

E. faecalis SI-FC-01 extends the lifespan in *C. elegans*

It has been reported that *C. elegans* show preference when their normal food, *E. coli* OP50, is replaced with other bacteria. Therefore, in order to verify whether *E. faecalis* SI-FC-01, isolated from gut bacteria, can be fed to worms, preference and safety experiments were conducted on wild-type worm N2. For preference experiments, we performed a binary selectivity analysis. 3-day-old worms were transferred to plates containing *E. coli* OP50 or *E. coli* OP50 + *E. faecalis* SI-FC-01 bacterial lawns, and the number of worms in each of their lawns was recorded 1–2 h later. The results showed that the number of worms on each lawn correlated with what they were fed with. Worms originally fed *E. coli* OP50 would be more likely to crawl toward *E. coli* OP50 lawns, and worms originally fed *E. coli* OP50 + *E. faecalis* SI-FC-01 would be more likely to crawl toward lawns containing *E. faecalis* SI-FC-01 (Figure S1). These results indicated that the worms are not averse to *E. faecalis* SI-FC-01. In addition, the pharyngeal pump test showed that *E. faecalis* SI-FC-01 did not cause any adverse effect on the pharyngeal pumping of C. elegans (Figure S2). The results of the bacterial selectivity experiment and the pharyngeal pump experiment avoid the possibility that the subsequent results may be due to food avoidance. Furthermore, the outcomes of the hemolytic test and drug resistance test for *E. faecalis* SI-FC-01 showed that *E. faecalis* SI-FC-01 exhibited no hemolytic activity and was not drug resistant, thus indicating a certain level of safety (Figure S3; Table S2). These results suggested *E. faecalis* SI-FC-01 could serve as a dietary supplement for *C. elegans*.

To investigate the impact of *E. faecalis* SI-FC-01 on the aging process, longevity experiments were conducted by feeding *E. faecalis* SI-FC-01 bacterial solution to 3-day-old wild-type larvae (L4) and recording the daily survival rate until all worms had perished. Beyond its established use in treating diabetes, metformin has emerged as a subject of interest in anti-aging research by numerous investigations. Considering that 50 mM metformin can significantly improve the lifespan of *C. elegans*, this study compared the life-prolonging effect of the strain with the recognized drug metformin[14–17]. In these experiments, *E. coli* OP50 was used as a blank control group, while metformin supplemented with 50mM was served as a positive control group. Compared with the *E. coli* OP50 control, the mean lifespan of worms in the experimental group treated with *E. faecalis* SI-FC-01 and those in the metformin group were significantly increased by 33.55% ($P < 0.001$) and 33.33% ($P < 0.001$), respectively. In order to assess changes across the entire lifespan, this study compared the survival profiles of the three groups, showing a significant shift towards increased longevity (Fig. 1, Table S3). This data underscored the efficacy of *E. faecalis* SI-FC-01 in prolonging the lifespan of *C. elegans*.

E. faecalis SI-FC-01 improves healthspan of *C. elegans*

To assess the potential of *E. faecalis* SI-FC-01 in enhancing the health and extending the lifespan of *C. elegans*, we evaluated multiple physiological indicators throughout the life cycle (body size, gonad apoptosis, motility, learning ability, and lipofuscin accumulation). The results showed that *E. faecalis* SI-FC-01 did not affect the body size of the worms (Fig. 2a), but it was capable of reducing the number of gonadal cell apoptosis (Fig. 2b). *E. faecalis* SI-FC-01 significantly slowed down the decline in worm motility over time compared to the control group. The retardation effect became increasingly pronounced over time, with a 26.41% increase in motility observed on 12D (Fig. 2c). Subsequently, we conducted a salt aversion learning experiment with *C. elegans*, as learning is an important ability for animals to adapt to changing environments and is closely related to survival[18,19]. In the presence of a salt gradient, *C. elegans* are usually attracted to elevated salt concentrations, thus their movement towards areas of higher salt[20]. However, worms that have learned to associate starvation with high salt levels will often migrate towards regions of lower salt concentration. The Fig. 2d showed that worms nourished with *E. faecalis* SI-FC-01 had improved learning ability and displayed a greater aversion to salt than those in the *E. coli* OP50 group. Lipofuscin is an aging pigment that accumulates with age and is considered as a sign of aging[21]. In comparison to the autofluorescence observed in the *E. coli* OP50 group, the autofluorescence levels in the *E. faecalis* SI-FC-01 group at 4D, 8D and 12D was reduced by 18.02% ($p < 0.01$), 34.80% ($p < 0.001$) and 34.93%

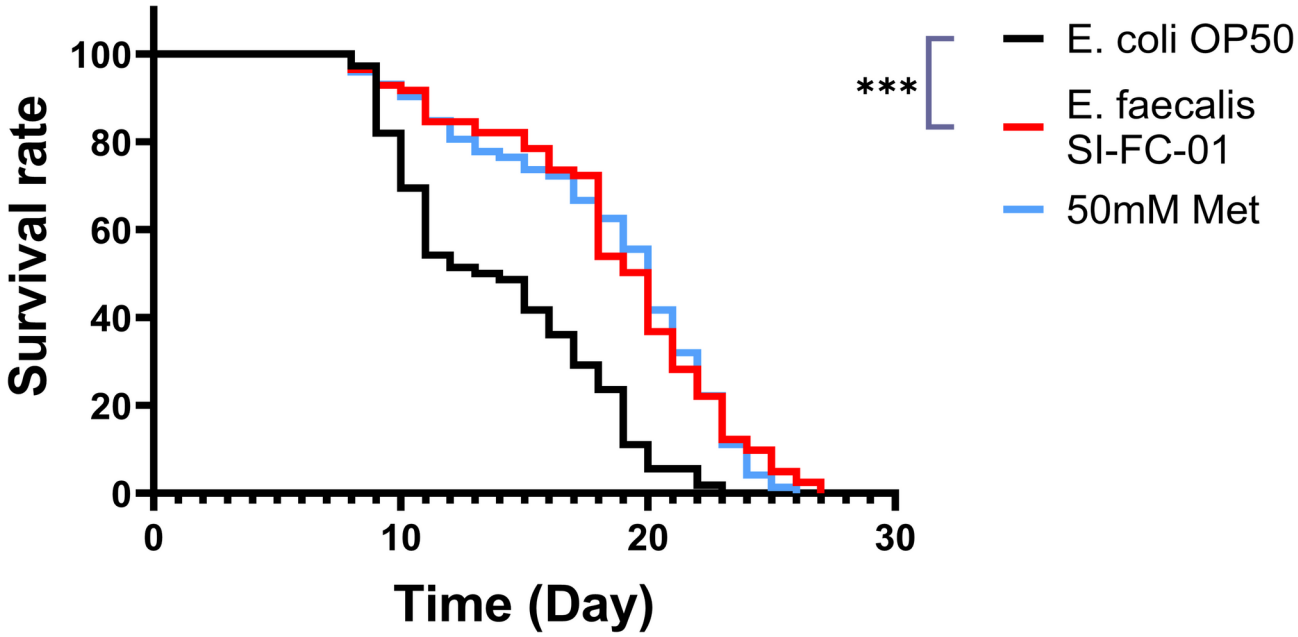

Fig. 1. *E. faecalis* SI-FC-01 prolonged the lifespan of *C. elegans*. The effect of *Enterococcus faecalis* SI-FC-01 and 50mM metformin on worm lifespan regulation was determined, and control worms were fed with *E. coli* OP50. ($N = 90$ worms, $p < 0.001$, Log rank test).

($p < 0.001$), respectively (Fig. 2e). Collectively, these results indicated that *E. faecalis* SI-FC-01 did not affect the growth and development of the worms and contributed to the preservation of superior physical capabilities.

Effects of *E. faecalis* SI-FC-01 on the transcriptome of *C. elegans*

To further explore the mechanism for the lifespan extension of *C. elegans* fed *E. faecalis* SI-FC-01, we performed an RNA-seq analysis, followed by enrichment analysis with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms. After treating 3-day-old *C. elegans* with *E. faecalis* SI-FC-01 for 5D, 47 genes were found to be upregulated and 618 genes were downregulated ($p < 0.05$, fold change $|\log FC| \geq 1$; Fig. 3a and b). Then, GO and KEGG term enrichment analysis were performed to further explore the changes in biological functions of *C. elegans* by studying the gene expression profiles of RNA-seq (Fig. 3c, Figure S5). Among them, the most highly represented GO terms altered by *E. faecalis* SI-FC-01 treatment in *C. elegans* included a range of biological process, such as transporter activity, intracellular, cellular anatomical entity, protein-containing complex, response to stimulus, multicellular organismal process, behavior, multi-organism process, biological regulation, developmental process, interspecies interaction between organisms, and metabolic process (Fig. 3c). GO enrichment analysis of the differentially expressed genes showed that the majority of the enriched pathways in worms fed with *E. faecalis* SI-FC-01 were related to immunity and defense mechanisms against external stimuli (Fig. 3d). It is notable that both GO terms and GO enrichment analyses showed that nematodes that had undergone *E. faecalis* SI-FC-01 feeding had differential genes associated with resistance. Transcriptome results on *E. faecalis* SI-FC-01 treated nematodes suggest that *E. faecalis* SI-FC-01 can affect these biological processes by regulating mRNA expression.

E. faecalis SI-FC-01 acts on the AKT signaling pathway to promote longevity

To understand the molecular mechanisms of longevity associated with *E. faecalis* SI-FC-01 feeding, we explored the AKT/DAF-16 signaling pathway, a conserved MAPK subfamily signaling pathway that plays a pivotal role in host lifespan and resistance regulation, based on KEGG enrichment derived from transcriptomic analysis. qRT-PCR results suggested that *daf-16* and *age-1*, involved in AKT/DAF-16 pathway were significantly altered (Fig. 4a). DAF-16 is a nematode homolog of the *C. elegans* FOXO transcription factor. Normally, DAF-16/FOXO remains inactive in the cytoplasm. Upon stress stimulation, it translocates to the nucleus and affects the expression of stress-responsive genes. DAF-16 is a key protein in the transfer of insulin /IGF1 signaling from the cytoplasm to the nucleus in response to stress. We used the TJ356 *daf-16(zls356)* transgenic strain to determine the nuclear localization of DAF-16 protein. The results showed that DAF-16 was mainly present in the cytoplasm of the control group (OP50), while 84.44% of the positive control group (37°C) had nuclear translocation of DAF-16. Compared with worms treated with OP50 control, the proportion of nuclear increased significantly from 10 to 62.22% after supplemental feeding with SI-FC-01 (Fig. 4b). The effect of *E. faecalis* SI-FC-01 on lifespan of *daf-16* mutant strain CF1038 *daf-16(mu86)* was further examined and we found that *daf-16* mutation almost completely suppressed the enhanced lifespan associated with *E. faecalis* SI-FC-01 feeding (Fig. 4d). In the AKT/DAF-16 signaling pathway, *akt-1* and *akt-2* are located downstream of *age-1* and upstream of *daf-16*. The qRT-PCR results showed that the expression levels of *akt-1* and *akt-2* were significantly decreased, which further

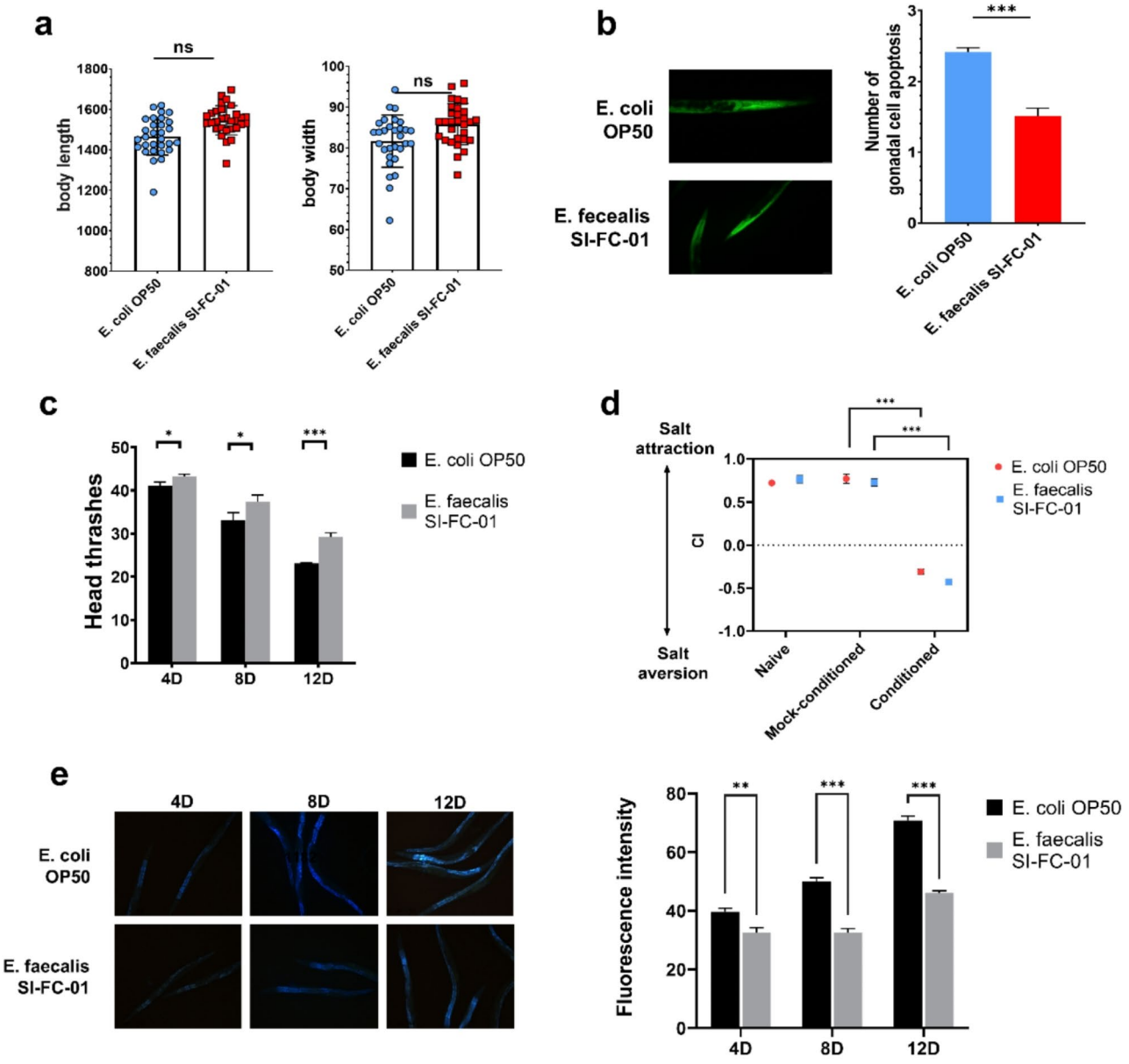

Fig. 2. *E. faecalis* SI-FC-01 can improve the health of *C. elegans*. (**a**) The body size from wild-type (N2) worms grown on *E. coli* OP50 and *E. faecalis* SI-FC-01 ($N = 30$ worms, $p > 0.05$, F-test). (**b**) Effect of feeding *E. coli* OP50 or *E. faecalis* SI-FC-01 on gonadal cell apoptosis in N2 worms ($N = 90$ worms, Student's t-test). (**c**) The head thrashes from 4D, 8D and 12D wild-type (N2) worms grown on *E. coli* OP50 and *E. faecalis* SI-FC-01 are significantly different at $p < 0.05$, $p < 0.05$ and $p < 0.001$ ($N = 60$, Student's t-test). (**d**) Altered salinity tropism after salt aversion learning in wild-type worms N2 fed *E. coli* OP50 or *E. faecalis* SI-FC-01 ($N \geq 300$ worms, $p < 0.001$ and $p < 0.001$, two-way ANOVA). **e** Changes in accumulation of lipofuscin in wild-type worms N2 after *E. faecalis* SI-FC-01 feeding in 4D,8D,12D. ($N = 60$ worms per time period, Student's t test)

confirmed that the extension of lifespan was related to AKT signaling pathway(Fig. 4c). Meanwhile, *E. faecalis* SI-FC-01 was unable to enhance the lifespan of nematode mutants TJ1052 *age-1(hx546)*, GR1310 *akt-1(MG144)* and VC204 *akt-2(ok393)* (Fig. 4e, f and g). The result means that the life-prolonging effect of *E. faecalis SI-FC-01* disappeared with the disappearance of relevant target genes (*age-1*, *akt-1*, *akt-2* and *daf-16*) in the AKT signaling. Therefore, the above results demonstrated probiotic *E. faecalis* SI-FC-01 extended the lifespan through AKT/DAF-16 signaling pathway in *C. elegans*.

E. faecalis SI-FC-01 enhances the stress resistance of *C. elegans*

With the progression of physical aging, an organism's capacity to tolerate external conditions characterized by unnatural environmental stress diminishes. To assess the effect of *E. faecalis* SI-FC-01 on the viability of *C. elegans* when subject to oxidative and thermal stress, L1 larvae were treated with *E. coli* OP50 or *E. faecalis* SI-FC-01 and subsequently exposed to temperatures of 35 °C or H_2O_2, respectively. Compared to worms cultured

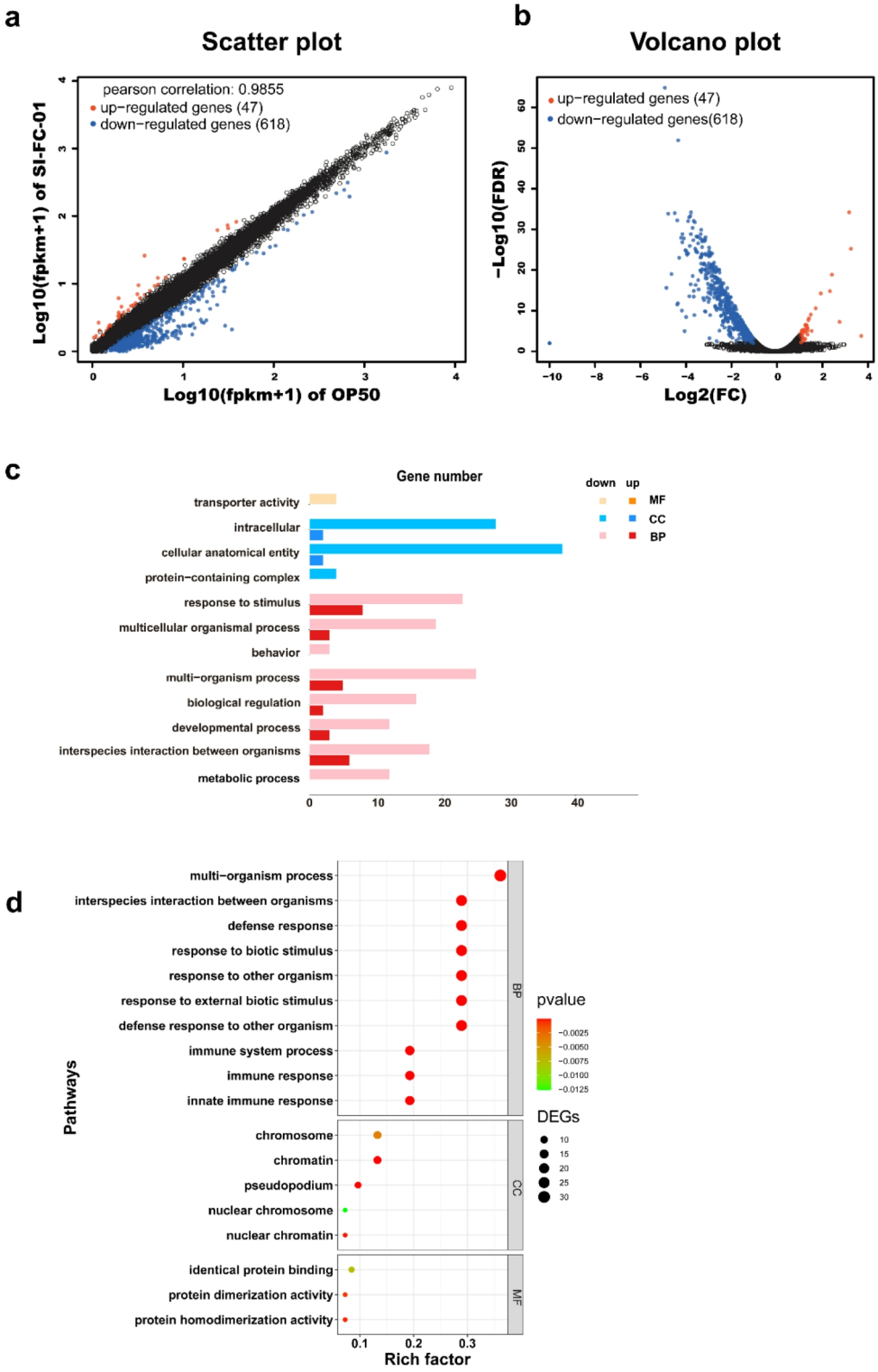

Fig. 3. The transcriptome of *C. elegans* fed with *E. coli* OP50 or *E. faecalis* SI-FC-01. Differential gene expression analysis, the red and blue dots in the figure indicate significantly up- and down-regulated genes with $|\log FC| \geq 1$ and $p < 0.05$, and black points are non-significantly different genes (**a**, **b**). (**a**) scatter plot. The x- and y-axes are the expression of the gene in the *E. coli* OP50 group and in the *E. faecalis* SI-FC-01 group. (**b**) Volcano plot. The x-axis is the value of the fold change log2(FC) in gene expression differences between *E. faecalis* SI-FC-01 treated worms and *E. coli* OP50 treated worms, and the y-axis is the negative logarithm of the p-value. (**c**) GO Enrichment pathway analysis with significant changes between *E. coli* OP50 and *E. faecalis* SI-FC-01 group. (**d**) GO enrichment analysis of genes with significant changes between *E. coli* OP50 and *E. faecalis* SI-FC-01 group.

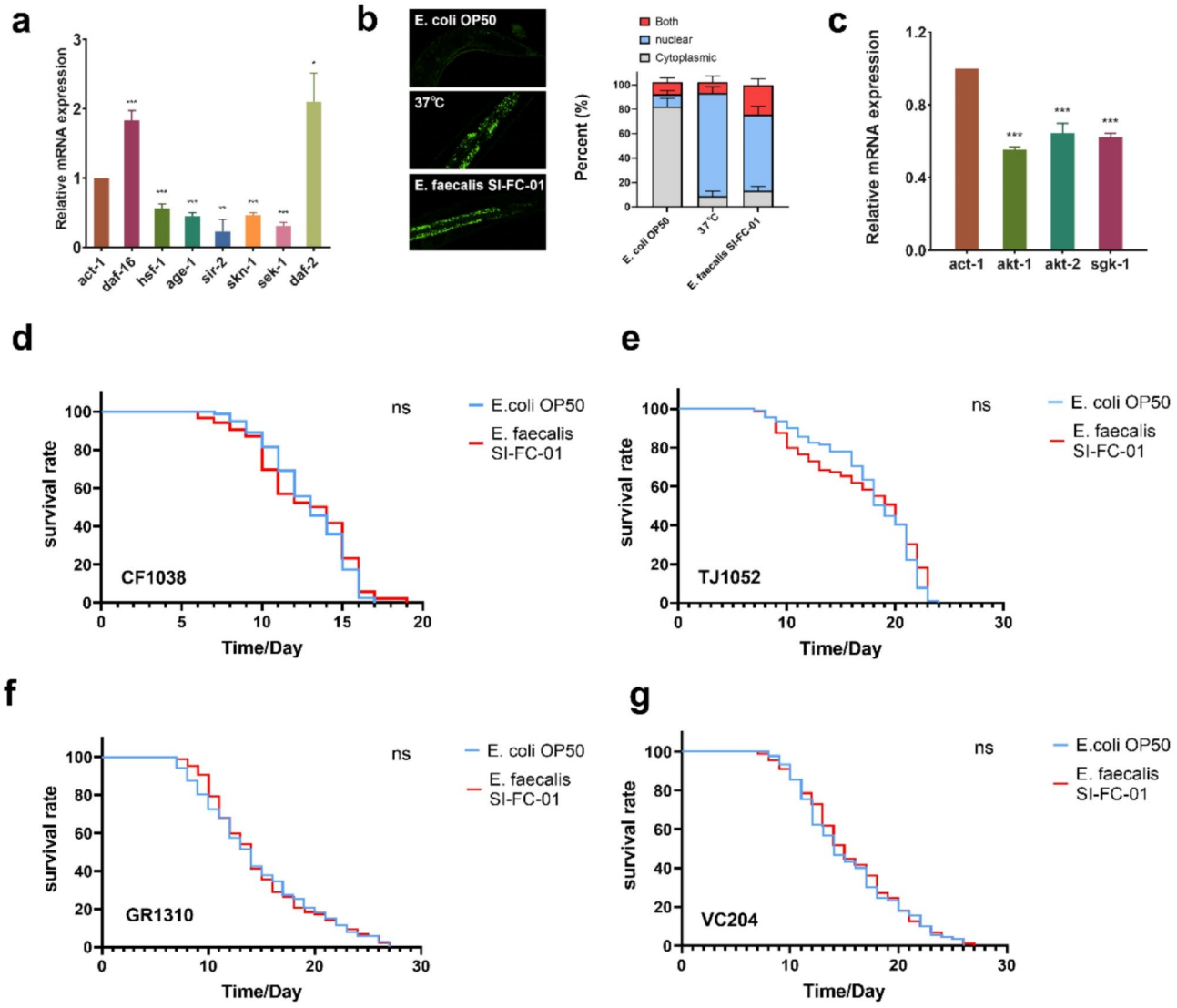

Fig. 4. *E. faecalis* SI-FC-01 acts on the AKT signaling pathway to delay aging. (**a**) Relative expression of *daf-16* upstream genes in 8-day-old worms (N2) treated with *E. faecalis* SI-FC-01 (Student's t-test). (**b**) The altered fluorescence signal of the TJ356 *daf-16(zls356)* fed with *E. faecalis* SI-FC-01 ($N = 90$ worms, $p < 0.001$, Student's t-test). (**c**) Relative expression of AKT pathway-related genes in 8-day-old worms (N2) treated with *E. faecalis* SI-FC-01 (Student's t-test). Survival curves of AKT signaling pathway mutants, CF1038 *daf-16(mu86)* (**d**), TJ1052 *age-1(hx546)* (**e**), GR1310 *akt-1(MG144)* (**f**), VC204 *akt-2(ok393)* (**g**) ($N = 90$ worms, $p > 0.05$, Log rank test).

in the *E. coli* OP50 group, those in the *E. faecalis* SI-FC-01 group had a slight resistance effect in the early stages of high-temperature treatment. However, their overall resistance to heat stress was limited (Fig. 5a). The worms cultured in the *E. faecalis* SI-FC-01 significantly increased the resistance of *C. elegans* to oxidative stress compared to worms cultured in the *E. coli* OP50 group, the survival rate of *E. faecalis* SI-FC-01 group was increased by 30% ($p < 0.001$) compared with *E. coli* OP50 group at 4 h (Fig. 5b).

Altered AGE-1/PI3K signaling has been shown to be associated with increased resistance to oxidative and heat stress[22]. Figure 5c, d, e and f showed that none of the mutant strains for *age-1*, *akt-1*, *akt-2*, or *daf-16* exhibited an enhancement in worm longevity subsequent to treatment with *E. faecalis* SI-FC-01. Moreover, qRT-PCR analysis showed that the expression of *sod-5*, *dod-17*, *dod-19*, *dod-23*, and *gst-4* was upregulated (Fig. 5g). These data suggested that *E. faecalis* SI-FC-01 enhanced *C. elegans* resistance to oxidative stress through activating the AKT signaling pathway.

E. faecalis SI-FC-01 delays the progression of age-related diseases in *C. elegans* models of degenerative disease

Neurodegenerative diseases have been consistently shown to be associated with oxidative stress[23,24]. At the same time, *E. faecalis* SI-FC-01 shows great potential in improving motor and cognitive impairment in

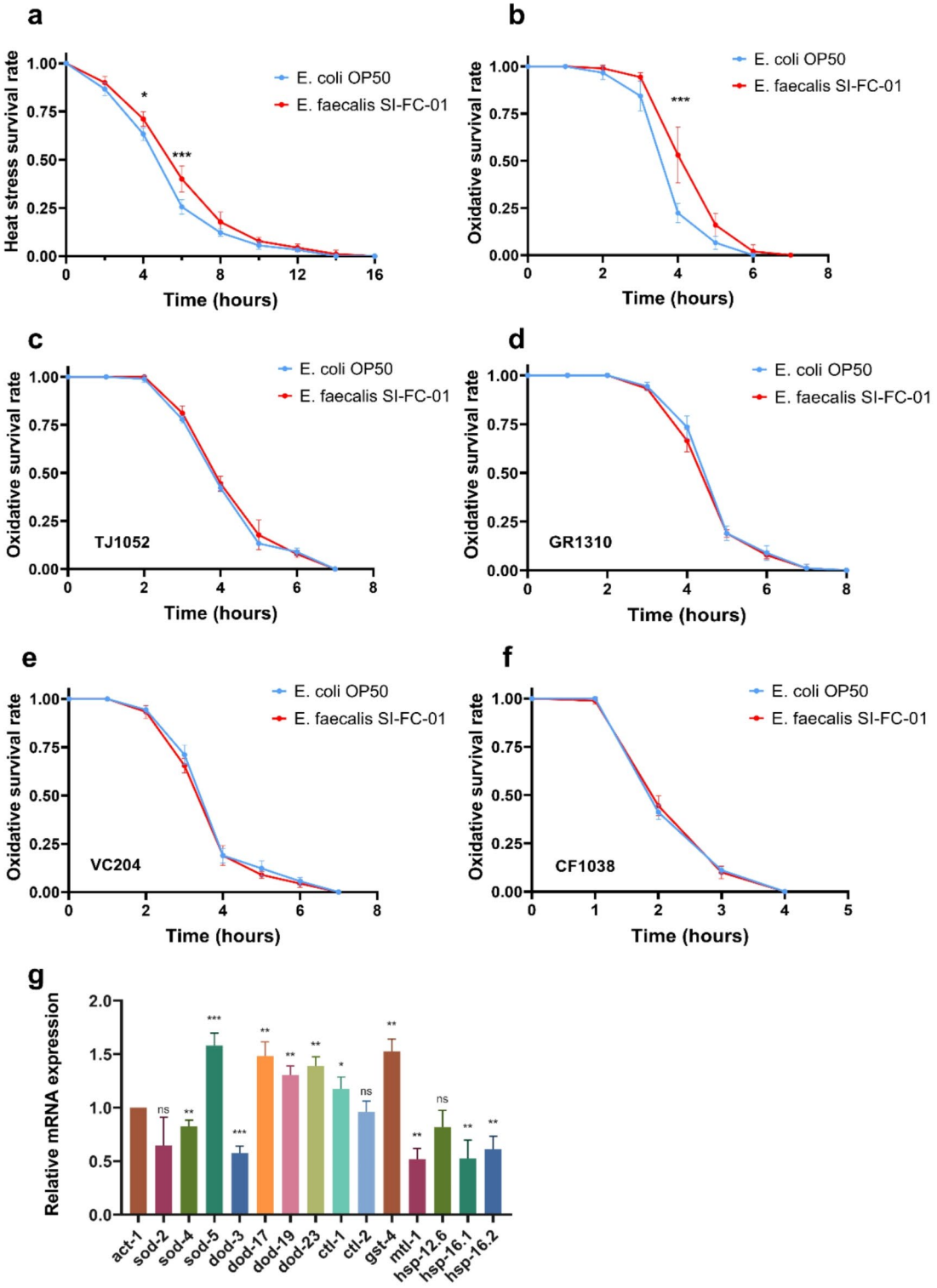

Fig. 5. *E. faecalis* SI-FC-01 can enhance the stress resistance of *C. elegans* via AKT signaling pathway. a The changes in lifespan of wild type worms N2 treated with *E. faecalis* SI-FC-01 after heat stress at 35°C ($N = 90$ worms, two-way ANOVA). The changes in lifespan of N2 (**b**), TJ1052 *age-1(hx546)* (**c**), GR1310 *akt-1(MG144)* (**d**), VC204 *akt-2(ok393)* (**e**), CF1038 *daf-16(mu86)* (**f**) treated with *E. faecalis* SI-FC-01 after H_2O_2 oxidative stress ($N = 90$ worms, two-way ANOVA). (**g**) Relative expression of *daf-16* downstream genes in 8-day-old worms (N2) treated with *E. faecalis* SI-FC-01 (Student's t-test).

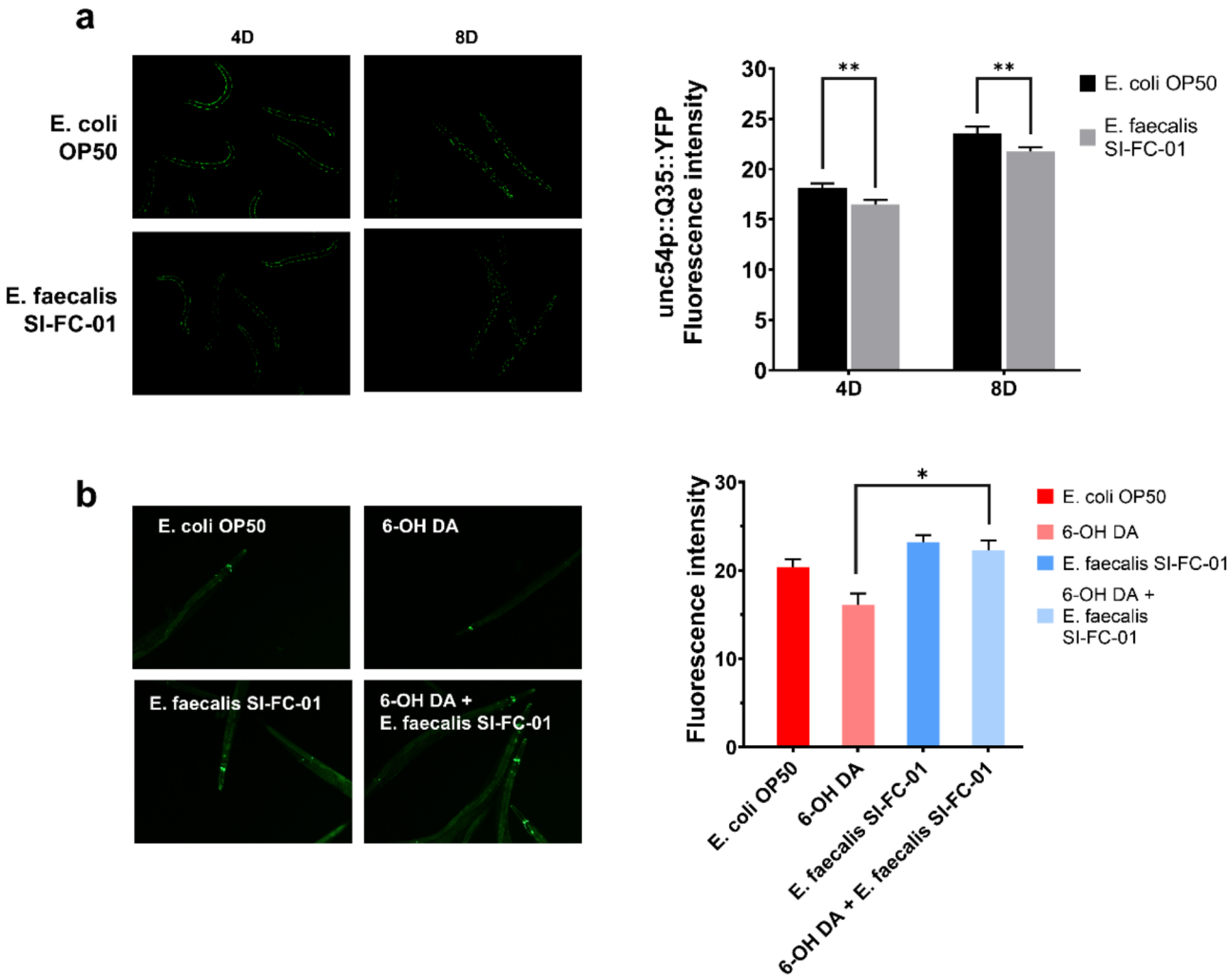

Fig. 6. *E. faecalis* SI-FC-01 can delay the progression of age-related diseases in models of degenerative disease *C. elegans*. (**a**) Effect of *E. faecalis* SI-FC-01 treatment on Poly-Q accumulation on 4D and 8D PD model worm AM140 ($N = 90$ worms, $p < 0.01$ and $p < 0.01$, Student's t-test). (**b**) Repair of dopamine neurons in 6-OH DA treated BZ555 strain by *E. faecalis* SI-FC-01 treatment ($N = 90$ worms, $p < 0.05$, Student's t-test).

neurodegenerative diseases. Therefore, we have further explored worm models for a number of neurodegenerative diseases. Huntington's disease (HD) is a neurodegenerative disorder with no current cure that develops in adulthood. AM140 is a yellow fluorescent protein (YFP)-tagged polyglutamine (PolyQ) that exhibits fluorescence from soluble to aggregated with age. *E. faecalis* SI-FC-01 significantly reduced age-related PolyQ accumulation in worm body wall muscle on days 4 and 8 (Fig. 6a). In patients with Parkinson's disease (PD), the brain exhibits progressive degeneration leading to eventual failure of dopaminergic neurons in the substantia nigra compacta (nigrostriatal pathway)[25]. BZ555 mutant worms express green fluorescent protein (GFP) in soma and axons of dopamine neurons. Treatment of B555 with 6-OH DA allows for the construction of disease models of dopamine neuron degeneration. The *E. faecalis* SI-FC-01 culture could enhance the activity of dopamine neurons and could restore 6-OH DA-induced neuronal damage (Fig. 6b). These data suggest that *E. faecalis* SI-FC-01 could improve worm expression in a model of neurodegenerative disease.

Discussion

In many developed countries, the trend of population ageing presents significant social and economic challenges. Probiotic research offers a promising avenue and compounds targeting cellular senescence shows potential to extend the health period in various research models[26,27]. Consequently, probiotics have emerged as a focal area on research into therapies designed to promote healthy aging and alleviate the socioeconomic impacts. The present study discovered that supplementation with the probiotic *E. faecalis* SI-FC-01 extended the health period and lifespan of *C. elegans*, enhanced motor and learning abilities, boosted antioxidant capacity, and delayed the onset of neurodegenerative diseases by activating the Daf-16/AKT signaling pathway.

In anti-aging research, the research focus has spread from single lifespan to the healthy lifespan[4]. Recent studies have shown that metformin and rapamycin inhibitor treatment extend the lifespan of worms and reduce

their body size and fertility[17,28]. While these drugs hold promise, they probable have side effects and urgently need the alternatives. *E. faecalis* SI-FC-01 significantly extended the lifespan of the worms by 33.55%, and reduced gonad cell apoptosis without affecting the body size and feeding rate of the worms. Dietary restriction leads to an extension of the lifespan of nematodes, it has been demonstrated that the downstream effectors of diet restriction-induced longevity contribute to the longevity extension of C. elegans[29]. No food avoidance was observed when nematodes were fed *E. faecalis* SI-FC-01, indicating that the extension of lifespan of C. elegans was not caused by dietary restriction.

Similar to the manifestations of human aging, the aging process in *C. elegans* is accompanied by decreased learning, decreased stress resistance, and accumulation of the aging pigment lipofuscin[30,31]. The increased head swing frequency, learning ability as well as the ability to resist oxidative stress after supplementation with *E. faecalis* SI-FC-01 during the worm life cycle indicated the improved health span (Fig. 2). Worms are able to sense to a number of compounds and associate a certain perception with a signal[32]. In salt-aversion learning, worms are instructed to associate salt starvation and play the opposite moving toward salt. Any alteration in neuronal activity or progression of aging reduces the learning ability of *C. elegans*, the neuroprotective effect of *E. faecalis* SI-FC-01 may improve the associative learning ability.

The molecular and cellular mechanisms governing aging are important but remain poorly elucidated. Studies on the microbiome and/or probiotics have begun to elucidate the complex interactions between animal hosts and bacteria, but the molecular mechanisms are lacking. In the Insulin/IGF-1 signaling pathway, AKT pathway is an important component, among which *age-1*, *akt-1*, *akt-2* and *daf-16* are important constructs[33]. DAF-16 is a direct homolog of the FOXO transcription factor, and its nuclear translocation is essential for regulating the expression of several age-and stress-related genes in *C. elegans*[34]. Many genes differentially regulated in aging are known or postulated to be regulated by *daf-16*[35]. In addition, *age-1* is a gene closely related to *C. elegans*, and all mutants increase its stress resistance[36]. In this study, we observed that *E. faecalis* SI-FC-01 supplementation did not improve the mean lifespan and stress resistance of *age-1*, *akt-1*, *akt-2* and *daf-16* mutants and enhanced nuclear translocation of DAF-16, suggesting a role for AKT/DAF-16 in *E. faecalis* SI-FC-01 mediated lifespan extension and stress resistance.

Parkinson's disease (PD) and Huntington's disease (HD) are age-dependent neurodegenerative disorders that are often accompanied by associated symptoms of hypokinesia and neurobehavioral abnormalities[37]. It has been confirmed that oxidative stress is one of the main pathologies of neurodegenerative diseases, and the supplementation of *E. faecalis* SI-FC-01 has been previously demonstrated to improve the stress resistance of worms[23,24]. In this study, we used an established Huntington's *C. elegans* model that accumulates Poly-Q in muscle cells, allowing us to assess the protective effects of *E. faecalis* SI-FC-01 treatment in vivo. It also examined the aggregation of neuronal damage caused by 6-OH DA in the Parkinson's model of BZ555 worms. Supplemental feeding of *E. faecalis* SI-FC-01 delayed the progression of relevant features in the PD and HD disease models of *C. elegans* by enhancing antioxidant capacity.

In conclusion, *E. faecalis* SI-FC-01 can act through the AKT pathway to target resistance genes in DAF-16/FOXO, which in turn affects the healthspan of *C. elegans* and delays the development of neurodegenerative diseases in the worm model. This study underscores the potential of probiotics as dietary supplements in delaying host aging and offers new possibilities for the screening of anti-aging natural actives.

Materials and methods

C. elegans strains and bacterial

All strains were cultured using nematode media supplemented with *E. coli* OP50, following previously described methods[38]. If not specifically labeled, strains were maintained at 20 °C. The strains used in this study included: Bristol wild type worm N2, TJ356 *daf-16(zls356)*, TJ1052 *age-1(hx546)*, GR1310 *akt-1(MG144)*, VC204 *akt-2(ok393)*, CF1038 *daf-16(mu86)*, AM140 *rmIs132[unc54p::Q35::YFP]*, BZ555 *egIs1[dat-1::GFP]*. All worm strains and *E. coli* OP50 were purchased from the Caenorhabditis Genetics Centre (CGC, University of Minnesota, Minneapolis, Minnesota, USA). *E. faecalis* SI-FC-01 used in the experimental treatment group has been stored in the Chinese Typical Culture Collection Center, with the preservation number CCTCC M 2,024,091. The *E. faecalis* SI-FC-01 treatment groups mentioned in this paper all refer to the 1:1 mixing of *E. coli* OP50 ($OD_{600}=1$) and *E. faecalis* SI-FC-01 ($OD_{600}=1$).

All the worms used in the assay were treated with synchronized hypochlorite lysate, the specific operation is as follows[39].

Bacterial selection assay and safety assessment

The assay was determined according to previous methods[40]. Worms were cultured using *E. coli* OP50 or *E. faecalis* SI-FC-01 for 3d to reach L4 stages. *E. coli* OP50 or *E. coli* OP50 + *E. faecalis* SI-FC-01 was seeded on the edges of each end of the plate, and 100 worms were placed in the center of the plate, being equidistant from *E. coli* OP50 and *E. coli* OP50 + *E. faecalis* SI-FC-01 (as shown in Figure S1). Allow the worms to move freely for 1–2 h and count the number of worms that migrated into each bacterial lawn.

Hemolysis tests were performed using Columbia blood AGAR medium[41]. The drug susceptibility test was performed by disk diffusion method. The VITEK2 Compact automatic microbial analyzer (BioMerieux, Craponne, France) paried with VITEK MS automatic rapid microbial mass spectrometry detection system (BioMerieux, Craponne, France) was used for drug susceptibility testing. Details were shown in Table S2.

Lifespan assay

Nematode lifespan was determined according to previous studies[40]. The L4 stage worms were subsequently moved to a new 30 mm NGM plate and started feeding *E. coli* OP50 with or without *E. faecalis* SI-FC-01. Nematodes were transferred to new NGM plates once every two days until all worms died. For each experiment,

bacterial strains from 90 worms were examined on three plates of 30 worms per plate. Worms that climbed walls, extricated organs, or laid eggs within their body were excluded from the statistical analysis.

Lipofuscin assay

Lipofuscin accumulation was determined with reference to previous research methods[40]. L3 worms were incubated to 4D, 8D, and 12D using NGM plates containing 50 mM of 5-fluoro-2'-deoxyuridine (5-FUDR) from Sigma-Aldrich, St. Louis, MO, and then anaesthetized by the addition of 40 mM NaN_3 after being washed three times with M9 buffer solution. Autofluorescence images of lipofuscin were captured using a fluorescence microscope (Leica, Wetzlar, Germany) with blue excitation light ranging from 405 to 488 nm. The accumulation level of lipofuscin was detected by quantifying fluorescence intensity using Image J software (National Institutes of Health, Bethesda, MD, USA). Three independent replicate experiments were performed with 20 worms in each group.

Developmental toxicity assay

Synchronized wild-type worms N2 were cultured for 72 h and then washed off the NGM plates with M9 solution. They were rinsed three times and fixed on slides using an alcohol lamp. Images of the worms were captured using a Leica microscope (Wetzlar, Germany) equipped with a digital camera and analyzed for body length and width using image J software, following previous research methods[42].

Apoptosis of gonadal cells was determined using acridine orange staining with reference to previous reports[43]. Worms cultured for 70 h were rinsed with M9 and transferred to 24-well plates. 100 µL of worm solution and 400 µL of acridine orange dye at 75 µg/mL were added to each well, and placed in the incubator at 20 °C to avoid light for staining for 50 min. Following staining, the worms were transferred from the 24-well plates to 1.5mL EP tubes, and naturally settled drops containing worms were collected onto the NGM plates coated with *E. coli* OP50 for recovery over a period of 50 min. Finally, the worms were rinsed and observed using a fluorescence microscope. A total of three independent replicate experiments were performed, each observing the fluorescence signal from 30 worms.

Feeding rates of 4-day-old, 8-day-old, and 12-day-old nematodes were compared by observing the pharyngeal pumping assays. Worms from the treated and control groups were transferred to new NGM plates, and the ball movement at the end of the pharyngeal pump of nematodes was recorded for 1 min. The assay was performed in three independent replicates with 30 nematodes each.

Motor ability assay

L3 worms were cultured for 4D, 8D, and 12D on NGM plates containing 50 mM 5-FUDR. The frequency of head swings was measured over a 20-second interval in 30 worms using a stereo microscope[38]. A total of three independent replicate experiments were performed.

Salt aversion learning assay

The learning ability of nematodes after *E. faecalis* SI-FC-01 treatment was determined as described in previous experiments[32]. As shown in Figure S4, at least 100 adult worms were titrated to the "0" point and the position to which the worms migrated after 40 min was recorded. The "learning index" was calculated by the following formula.

$$\mathrm{CI} = \frac{\text{Worms on high salt region} - \text{Worms on low salt region}}{\text{Total worms} - \text{Worms on origin region}}$$

Quantitative RT-PCR (qRT-PCR)

Approximately 500 synchronized L3 larvae were transferred to NGM plates containing 50 mM 5-FUDR with or without the addition of *E. faecalis* SI-FC-01. Following a 6-day treatment, adult nematodes were collected, and RNA was extracted using the trizol method. According to the previously described protocol, *act-1* was used as a housekeeping gene[44]. The $2^{-\Delta\Delta Ct}$ comparative threshold method was used for the relative expression of genes. Reverse transcription was performed using Transgene's TransScript One-step gDNA Removal and cDNA Synthesis SuperMix kit. The qRT-PCR was performed using the Transgene TransStart Tip Green qRT-PCR SuperMix kit. Primer sequences for *act-1* and other target genes are shown in Table S1.

Heat resistance and oxidative stress assay

The resistance experiment was based on the previous research methods[45]. In the heat resistance test, synchronized L1 larvae were laid on NGM plates transferred with or without *E. faecalis* SI-FC-01 as mentioned previously. The worms were incubated at 20 °C for 96 h, after which they were transferred to 35 °C. The survival rate was monitored by counting the number of alive and dead worms at 2-hour intervals.

For oxidative stress experiments, worm preconditioning was consistent with heat resistance experiments. Early adult worms were transferred to NGM plates containing H_2O_2 (15 µL 30% H_2O_2 per 10 mL NGM), and worm deaths were observed at hourly intervals.

Three independent replicate experiments were performed for each of the above two experiments, each involving 30 worms in each group to ensure statistical significance.

Determination of fluorescent strains

Four-day-old TJ356 worms were treated with SI-FC-01 to observe the nuclear translocation of DAF-16. Untreated TJ356 nematodes were exposed to transient heat stress at 37 °C for 20 min as a positive control for DAF-16 nuclear translocation. As previously described, micrographs of worms were viewed under confocal

fluorescence microscopy, and the results were classified as nuclear, cytoplasmic, and both[46]. The experiment was repeated three times with 30 worms each.

For the AM14 strain worms, the L3 larva worms (culture for 40 h) were transferred to experimental plates with or without *E. faecalis* SI-FC-01. AM140 worms were then collected on the fourth and eighth days. Worms were anesthetized with 40 mM of NaN_3. The aggregation of Poly-Q protein was visualized with a Leica fluorescence microscope and quantified with Image J software[45]. Three independent replicate experiments were performed, each involving 30 worms per group.

For the BZ555 strain worms, L3 larvae (culture for 40 h) were rinsed and transferred to a solution containing 10 mM ascorbic acid and 50 mM 6-hydroxydopamine (6-OH DA) in a 1:1 ratio[45]. The mixture was incubated at 20 °C with shaking every 20 min. After a one-hour incubation, the worms were washed three times with M9 buffer. The worms were placed on culture plates containing 5-FUDR, with or without *E. faecalis* SI-FC-01 for 72 h. Finally, the worms were anesthetized, photographed, and the resulting images were analyzed using image J to calculate the statistics. The worms were incubated for an additional 72 h with or without *E. faecalis* SI-FC-01. The experiment was repeated three times independently, and the number of worms in each set of experiments was 30.

RNA-seq

To collect the total RNA from the extracted samples (worm treatments were consistent with q-PCR), the Illumina TruseqTM RNA sample prep Kit method was used to construct the library. The rRNA was removed using the E.Z.N.A.®Total RNA Kit (Norcross, Georgia), thereby enriching the mRNA content. Library quality control was performed with an Agilent 2100 Bioanalyzer. Qualified libraries were subjected to double-end sequencing on Illumina novaseq 6000 platform. The edgeR software was used to analyze the significance of expression differences between samples for the expression of each transcript. Gene ontology (GO) database and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database were used to assess the functional classification and statistical overrepresentation of gene lists[47]. GO enrichment analysis and KEGG pathway enrichment analysis were performed using Goatools and KOBAS software, respectively. The experiment was repeated four times independently.

Statistics and analysis

Except for RNA-Seq, which was performed in four independent replicates, all results were derived from three independent replicate experiments. All images were created using Prism 8.0 drawing software. For the statistical analysis of the data, we employed a combination of Student's t test, F-test, Log rank test, and two-way ANOVA, utilizing multiple testing methods. In all significant analysis, the significance threshold was set at $p < 0.05$. $p<0.05$ was denoted by *, $p < 0.01$by **, and $p < 0.001$ by ***.

Data availability

The data supporting the findings of this study are available from the corresponding author mentioned above upon reasonable request.

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Author contributions

A.X., Y.L. (Ying Liu) and Y.L. (Yang Li) designed and conceived for the research, A.X., Y.L. (Ying Liu) secured the funding, Y.Y.W., W.T., Y.T.D., Y.N.Z. and C.X.Z. performed the experiments, Y.T.C and R.Y.D. analysed the data and plotted the diagram, Y.Y.W. drafted the manuscript, A.X., G.P.Z. and Y.L. (Ying Liu) checked and revised the manuscript. All authors contributed to the final manuscript file.

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Declarations

Competing interests
The authors declare no competing interests.

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