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RESEARCH ARTICLE

Coix seed oil prolongs lifespan and enhances stress resistance in *Caenorhabditis elegans*

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Abstract Coix seed oil (CSO) has many beneficial effects, but there is limited research on its influence on the processes and mechanisms related to senescence. Here, we used *Caenorhabditis elegans* as an in vivo model to investigate CSO's bioeffects on longevity. CSO (1 mg/mL) significantly extended the mean lifespan of *C. elegans* by over 22.79% and markedly improved stress resistance. Gene-specific mutant studies showed that the CSO-mediated increase in life expectancy was dependent on *mev-1*, *hsf-1* and *daf-16*, but not *daf-2*. Furthermore, CSO significantly upregulated stress-inducible genes, including *daf-16* and its downstream genes (*sod-3*, *hsp-16.2* and *gst-4*). In addition, four major fatty acids, linoleic, oleic, palmitic and stearic, played leading roles in *C. elegans*' extended lifespan. Thus, CSO increased the life expectancy of, and enhanced the stress resistance in, *C. elegans* mainly through *daf-16* and its downstream genes, but not through the insulin/insulin-like growth factor 1 signaling pathway.

Keywords Coix seed oil · *Caenorhabditis elegans* · Longevity · Stress resistance

Introduction

As a traditional Chinese medicine with important medical and nutritional values, coix seed (*Coix lacryma-jobi* L. var. *ma-yuen, adlay*) is cultivated widely in China and Japan where it is commonly used as a dietary supplement. The consumption of coix seed has human health-related benefits (Chen et al. 2011). Modern phytochemical studies have shown that oil is a main component of coix seed, and it can reduce leptin, adipose, low-density lipoprotein and cholesterol levels in rats (Huang et al. 2005), and it has blood lipid-reducing and antioxidant effects in hyperlipidemic rats (Yu et al. 2011). Thus, coix seed oil (CSO) could be used as a supplement in healthcare products and drugs for the prevention of chronic diseases. Aging is a major risk factor for several chronic diseases, including cancer, cardiovascular disease, neurodegeneration and diabetes (Niccoli and Partridge 2012). Anti-aging interventions can be used as a systemic approach to inhibit age-related diseases, and they can increase the health span and delay aging; therefore, suppressing the

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appearance of most age-related diseases (Argyropoulou et al. 2013). However, it is still unclear whether CSO consumption increases life expectancy.

Caenorhabditis elegans is a model organism with a short lifespan and strong reproductive capacity that can be easily genetically manipulated. It is a well-established model system for investigating organismal aging and exploring new pharmacological targets that are conserved across diverse species, including mammals (Honda et al. 2010).

Here, we used *C. elegans* as an in vivo model to verify the longevity-related effects of CSO. Here, 1 mg/mL CSO significantly extended the lifespan of worms, and improved stress resistance in *C. elegans* through increase the expression of genes regulating stress responses and senescence.

Materials and methods

Plant material and extract preparation

Coix seeds were sourced from Taishun County in Zhejiang Province (China). Semen Coicis (50.0 g), the dried mature seed of the perennial herbaceous plant *C. lacryma-jobi* L. was crushed into a powder using a grinder. Then, the powder was placed into a 60 °C water bath with 300 mL petroleum ether for condensation reflux. This was performed twice. The petroleum ether extract was concentrated in a rotary evaporator at 60 °C to yield a crude preparation (2.64 g) that was stored at 4 °C. The oil extract was diluted in Dimethyl Sulfoxide (DMSO, A100231, Sangon Biotech). A 10× stock solution was prepared 1 day before use in the assays, and then, the appropriate dilutions were pipetted onto nematode growth medium (NGM) plates or into 96-well plates.

Gas chromatography-mass spectrometry (GC–MS) analyses of CSO

The experiment was performed on a 7890B-5977A GC/MSD (Agilent, USA). The GC–MS conditions were as follows: inlet temperature, 250 °C; split ratio, 20:1; injection volume, 1 µL; chromatographic column, HP-5 ms; column flow, 1 mL/min; interface temperature between GC and MS, 280 °C, solvent delay, 5 min; temperature programming, 40 °C for 3 min and then rising to 260 °C at 10 °C/min for 10 min; ion source, EI, 70 eV, 230 °C; and quadrupoles temperature, 150 °C.

Caenorhabditis elegans strains and maintenance

All the strains were obtained from the Caenorhabditis Genetics Center, University of Minnesota (Minneapolis, MN, USA), including Bristol wild-type N2, CF1038(*daf-16*), CB1370(*daf-2*), CF1553[(pAD76) *sod-3*::GFP + rol6(su1006)], PS3551(*hsf-1*, *sy441*), TK22(*mev-1*, *kn1*) and CL2070(*hsp16.2*::GFP). The *C. elegans* used in this study were cultured at 20 °C (unless otherwise stated) on NGM agar plates or in 96-well plates with *Escherichia coli* OP50.

Lifespan assay

Synchronized L1 larvae (wild-type N2, *daf-16*, *daf-2*, *hsf-1* and *mev-1*) were incubated in 96-well plates (12 per well) together with S-complete, *E. coli* OP50, 50 µg/mL carbenicillin and 0.1 µg/mL amphotericin B in a total of 110 µL at 20 °C. Then, 25 µL 1.08 mM 5-fluoro-2′-deoxyuridine and 15 µL various CSO concentrations were added to each well after 48 and 72 h, respectively. The treatment day was defined as day 0 of adulthood. The survival or death of *C. elegans* was scored every 2 days until all the nematodes had died (Solis and Petrascheck 2011).

Motor ability assay

An optical microscope was used to measure the distances moved by worms (N2) treated with or without 1 mg/mL CSO at L1 larvae for 4 days, pick up the worms on NGM without *E. coli* OP50 and the distance of 10 s was measured (Brandt et al. 2009). The assay was repeated three times. Each group, which included at least 30 nematodes, was analyzed using ImageJ software.

Pharyngeal pumping assay

Nematodes (N2) treated with or without 1 mg/mL CSO at L1 stage for 4 days, and then cleaned and placed on NGM without food to count the number of the pharyngeal pumps per nematode within 10 s, with one pump being defined as one contraction of the posterior bulb/grinder (Huang et al. 2004; Papaevgeniou et al. 2019). Each group included at least 30

nematodes, and the assay was repeated independently three times.

Food intake assay

Nematodes (N2) treated with or without 1 mg/mL CSO for 4 days which were cleaned and placed on NGM without food and, then, picked into 96-well plates (10 nematodes per well), containing S-complete and *E. coli* OP50 in a 100 µL total volume per well. This was defined as day 0. The absorbance at 600 nm (OD600) of each well was measured at day 0 and day 7 (Gomez-Amaro et al. 2015). This assay was repeated independently three times.

Spawning assay

Synchronized L1 larvae (N2) were transferred to 96-well plates (12 per well) which containing 150 µL S-complete, *E. coli* OP50 and 1 mg/mL CSO mixture, incubated at 20 °C for 72 h. Young adult nematodes were collected, cleaned and picked onto new NGM plates (1 nematode per plate) containing 100 µL solution, including S-complete, *E. coli* OP50 and 1 mg/mL CSO per well (Ryu et al. 2016). Each nematode was allowed to lay eggs and was transferred to another new NGM plate every day for 3 days. The number of eggs were scored. This assay was repeated independently three times.

Reactive oxygen species (ROS) levels assay

Nematodes (N2) treated for 4 days were collected, cleaned and picked the worms into a new 96-well plate (10 nematodes per well) containing 150 µL M9 buffer. Then, 8.0 µL 1 mM 2′,7′-dichlorofluorescein diacetate, which is used to measure endogenous ROS levels, was added to the plates and nematodes were incubated at 37 °C for 2 h. A microplate reader was used to measure the fluorescence intensity at an excitation wavelength of 485 and an emission wavelength of 535 nm (Lee et al. 2010). Three independent trials of this assay were performed.

Stress assay

For the heat shock assay, nematodes (N2) treated for 4 days were transferred to new NGM plates at 37 °C, and the number of surviving nematodes were recorded once an hour until they were all dead (Strayer et al. 2003; Pietsch et al. 2009).

For the oxidative stress assay in vitro, synchronized N2 worms were treated with or without 1 mg/mL CSO for 4 days, and then picked the worms into a new 96-well plates (10 nematodes per well) which containing 200 µL M9 buffer and 300 mM paraquat (856,177, Sigma-Aldrich, St. Louis, MO, USA), and the plates were incubated at 20 °C (Possik and Pause 2015). Nematodes were observed, and their survival scored once per hour.

For the heavy metal tolerance assay, as previously described, but 300 mM paraquat was replaced with 5 mM $K_2Cr_2O_7$ (P2588, Sigma-Aldrich, St. Louis, MO, USA) (Yang et al. 2013). Nematodes were observed, and their survival scored once every 12 h. Each assay was repeated independently three times.

Fluorescence measurements in the CF1553 and CL2070 strains

Synchronized CF1553(*sod-3*::GFP) and the CL2070(*hsp-16.2*::GFP) L1 larvae (Wen et al. 2014; Ayuda-Duran et al. 2019). treated with or without 1 mg/mL CSO in 96-well plates for 4 days, collected the worms and cleaned out the drugs and OP50, subsequence, fixed with 4% paraformaldehyde for observed. A fluorescence microscope (M165FC, Leica, Wetzlar, Germany) was used to image the nematodes. The assay was repeated independently three times for each strain.

Gene expression analysis by quantitative PCR

Total RNA isolated from nematodes by Trizol (DP421, TIANGEN), simply, at least 500 nematodes cultured for 4 days as in the lifespan assay were collected and cleaned by DEPC sterile distilled water, then frozen in liquid nitrogen or − 80 °C quickly for 1 h. The RNA was isolated gently on ice according to the Trizol Kit (Sorrentino et al. 2017). The total RNA was subsequently reverse transcribed into cDNA by the All-in-One cDNA Synthesis SuperMix (B24403, Bimake.). The expression levels of genes were quantified with a SYBR Green PCR Mix (B21202, Bimake.) in a LightCycler96 (Roche, Switzerland). The actin(*act-1*) was used as housekeeping gene, and the fold changed were calculated by $2^{-\Delta\Delta Ct}$ method.

Statistical analyses

GraphPad Prism 6.0 and ImageJ were applied to conduct statistical analyses. For the lifespan assays, Kaplan–Meier survival was utilized, and *P* value was calculated using a log-rank test. Student's *t*-test and a one-way analysis of variance with Duncan's test were adopted to compare two groups and multiple groups, respectively. Differences were considered significant at $p < 0.05$.

Results

CSO extended the lifespan of wild-type *C. elegans*

To assess the pro-longevity effects of CSO, wild-type N2 nematodes were treated with different CSO concentrations (0, 0.1, 0.5, 1.0, 5.0 and 10.0 mg/mL) in 96-well plates at 20 °C. As shown in Fig. 1 and Supplementary Table S1 and S2, nematodes exposed to 1.0 mg/mL CSO had the most significant effect on prolonging mean lifespan in nematodes, which remarkably increased by 22.79% compared with control. Consequently, we used 1.0 mg/mL CSO as the treatment concentration for subsequent experiments.

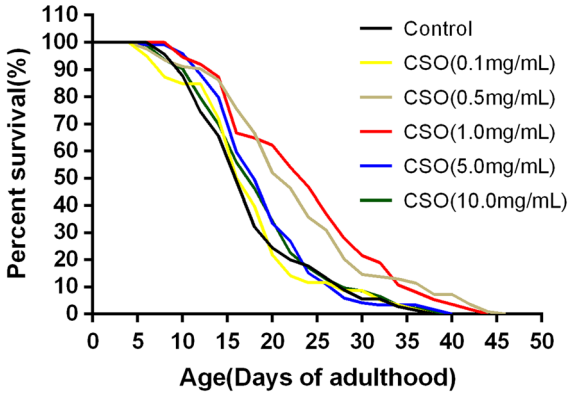

Fig. 1 CSO prolonged the lifespan of wild-type *C. elegans*. Synchronized wild-type N2 were incubated in 96-well plates (12 per well) with CSO (0.1, 0.5, 1.0, 5.0 and 10.0 mg/mL) or with 0.3% dimethylsulfoxide (control). The day nematodes were treated was defined as day 0 of adulthood. The survival or death of nematodes was scored every 2 days until all the nematodes had died. At least three independent biological replicates, containing more than 50 nematodes each, were performed. A statistical analysis was performed using Prism 6.0. Differences between the curves (treated versus untreated control) were considered statistically significant when $p < 0.05$. P values were calculated using the log-rank test

CSO had no effects on pharyngeal pumping, food intake or spawning of wild-type nematodes, but did impact on motor ability

To determine whether CSO affected the physiology of *C. elegans*, we first studied their physiological functions. N2 nematodes treated with 1.0 mg/mL CSO for 4 days showed no obvious differences in pharyngeal pumping, food intake and spawning compared with the control group (Fig. 2a–c), which suggested that its effects on lifespan might not depend on the dietary restriction (Lee et al. 2006) or reproductive signaling (Hsin and Kenyon 1999) pathway. Because systemic muscle cells gradually lose vitality, which results in a gradual decline in athletic ability and other phenotypes (Ryu et al. 2016), With aging, we measured the distances N2 nematodes treated with or without 1.0 mg/mL CSO for 4 days could move in 10 s (Fig. 2d), and 1.0 mg/mL CSO significantly enhanced the mobility of N2 nematodes, increased by 27.95% compared with the control group (Supplementary Table S3).

CSO enhanced the resistance of *C. elegans* to stress conditions

Aging and chronic disease are closely associated with increased environmental stresses (Si and Liu 2014; Lee et al. 2017). To determine whether the resistance levels of wild-type nematodes against environmental stresses were improved by CSO, we performed independent thermal stress, oxidative stress and heavy metal tolerance experiments with N2 nematodes. For the heat stress assay, nematodes treated with or without 1.0 mg/mL CSO for 4 days at 20 °C were transferred to new NGM plates and exposed to heat stress at 37 °C. We found that 1.0 mg/mL CSO significantly extended the mean lifespan of nematodes by 32.69% under thermal stress conditions compared with the control (Fig. 3a). For the oxidative stress assay, wild-type nematodes treated with or without 1.0 mg/mL CSO for 4 days were exposed to 300 mM paraquat. The mean lifespan of CSO-treated nematodes increased by 25.30% under paraquat-induced oxidative stress (Fig. 3b). For the heavy metal tolerance assay, wild-type nematodes treated with or

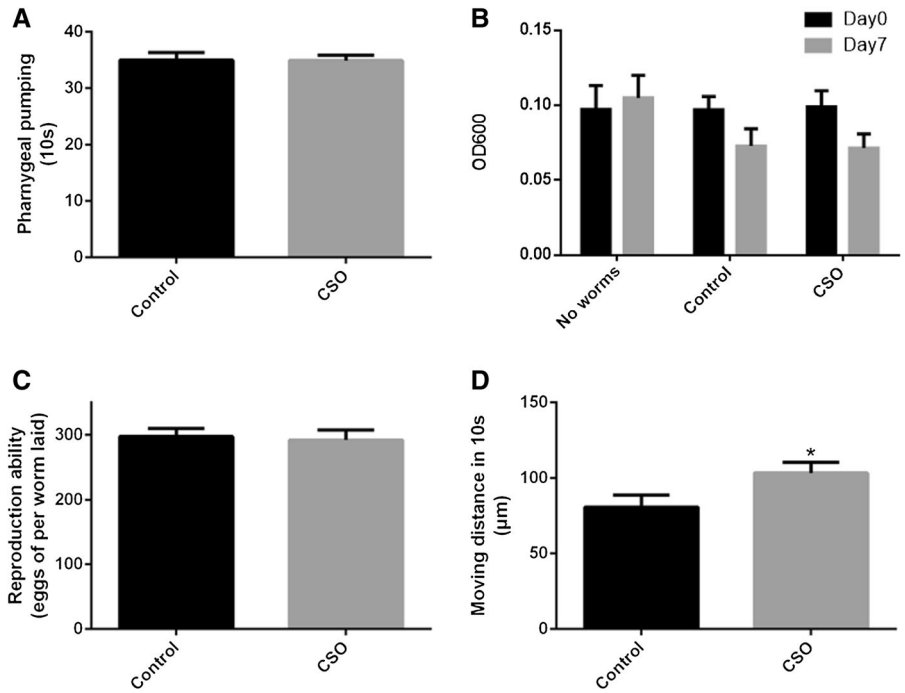

Fig. 2 Effects of CSO on the physiological functions of wild-type nematodes. **a** Numbers of pharyngeal pumps in 10 s from N2 nematodes treated with or without 1 mg/mL CSO for 4 days. **b** Nematodes (N2) treated with or without 1 mg/mL CSO for 4 days were cleaned and placed into 96-well plates (10 nematodes per well) with S-complete and *E. coli* OP50. This was designated as day 0. The absorbance at 600 nm (OD600) of each well was measured at day 0 and day 7. **c** Synchronized L1 larvae (N2) treated with or without 1 mg/mL CSO were cultured to adulthood and allowed to freely spawn. **d** An optical microscope was used to measure the distances nematodes (N2) treated with or without 1 mg/mL CSO for 4 days moved in 10 s. CSO significantly increased the motor ability of nematodes. At least three independent biological replicates were performed. Data were analyzed using Student's *t*-test with Prism 6.0. Values are presented as means ± SDs. $*p < 0.05$

without 1.0 mg/mL CSO for 4 days were exposed to 5 mM $K_2Cr_2O_7$. Similarly, the average longevity of nematodes treated with 1.0 mg/mL CSO in a heavy metal environment were significantly increased by 20.16% compared with the control group (Fig. 3c). These findings implied that CSO significantly enhanced the resistance levels of wild-type nematodes against oxidative, heavy metal and heat stresses.

CSO decreased ROS levels in *C. elegans*

Previous studies indicated that oxidative stress is important for the modulation of cell function and cell physiology (Handy and Loscalzo 2012; Bazopoulou et al. 2019), free-radical theory (Harman 1956) as a predominant mechanistic explanation for the process of aging, indicated that aging results from the accumulation of molecular damage caused by ROS. To investigate whether CSO decreased ROS levels in *C. elegans*, we measured the fluorescence intensities of treated and untreated wild type nematodes (Fig. 4). Exposure to 1.0 mg/mL CSO reduced ROS accumulation by 23.88%, suggesting that the CSO-related mechanism that increases life expectancy might involve a reduction in ROS accumulation.

CSO-related increase in the *C. elegans* lifespan is dependent on *mev-1*, *hsf-1* and *daf-16*

The deletion of *mev-1* can cause dramatic decreases in the antioxidant capacity and the expression levels of antioxidant enzymes of nematodes (Feng et al. 2015). MEV-1 affects the formation of a subunit of complex II in the electron transport in nematodes, and nematodes with *mev-1* mutation had dramatically shortened lifespans (Ishii et al. 1998). Because CSO showed a significant antioxidant activity, we performed a lifespan assay in the *C. elegans mev-1* mutant TK22. Exposure to 1 mg/mL CSO did not extend the lifespan of *mev-1* mutant nematodes (Fig. 5a), indicating that

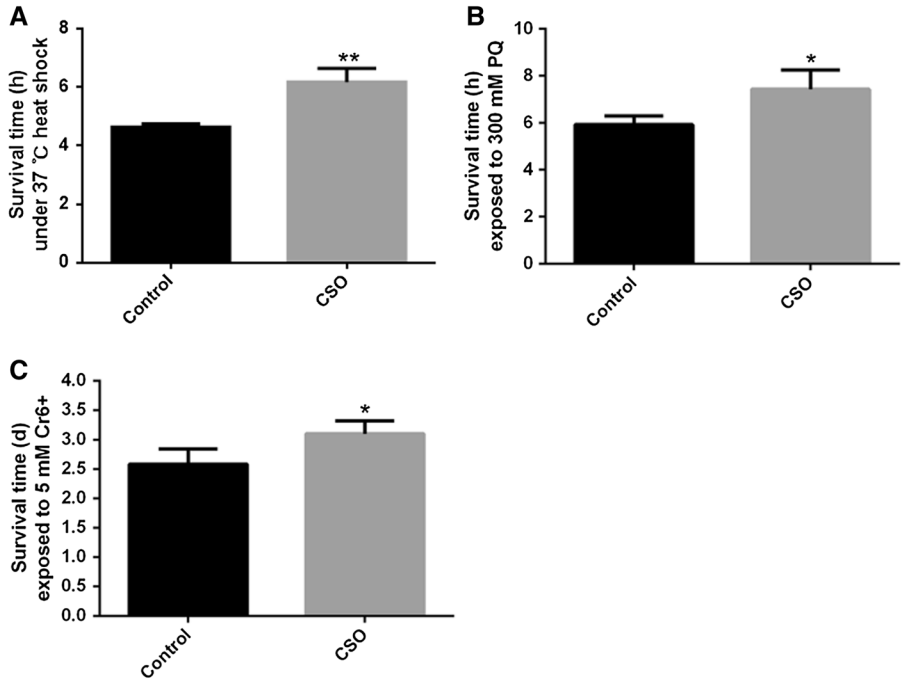

Fig. 3 CSO enhanced the resistance of wild-type nematodes against oxidative and heat stresses. **a** Wild-type nematodes treated with 1 mg/mL CSO for 4 days were transferred to new NGM plates and cultured at 37 °C. The number of surviving nematodes was recorded once an hour until they were all dead. **b** and **c** Wild-type nematodes treated with 1 mg/mL CSO for 4 days were exposed to 300 mM paraquat and 5 mM potassium dichromate, respectively, and the surviving numbers of nematodes were recorded. Each assay was repeated independently three times. Data were analyzed by Student's *t*-test using Prism 6.0. Values are presented as means ± SDs. $*p < 0.05$, $**p < 0.01$

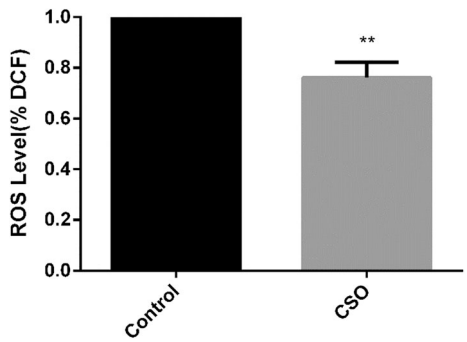

Fig. 4 CSO reduced levels of reactive oxygen species (ROS) in *C. elegans*. Nematodes (N2) treated with or without 1 mg/mL CSO for 4 days were cleaned and placed into 96-well plates (10 nematodes per well) containing M9 buffer and 10 mM 2′,7′-dichlorofluorescein diacetate. A microplate reader was used to measure the fluorescence intensity levels of excitation and emission wavelengths at 485 and 535 nm, respectively. Three independent trials were performed. Data were analyzed using Student's *t*-test with Prism 6.0. Values are presented as means ± SDs. $**p < 0.01$

mev-1 was required for the CSO-induced increase in the lifespan.

Heat Shock Factor 1 (HSF-1), a crucial longevity transcription factor, plays a key role in promoting longevity, enhancing stress resistance and maintaining protein homeostasis, which were achieved mainly by increasing the levels of molecular chaperones at the transcriptional level (Hsu et al. 2003; Cohen et al. 2006; Mohri-Shiomi and Garsin 2008; Seo et al. 2013; Nakai 2016). To test whether longevity caused by CSO in N2 nematodes required *hsf-1*, we used the *hsf-1* mutant strain PS3551 (*hsf-1*, *sy441*) in a lifespan assay (Fig. 5b). CSO failed to extend the lifespan of *hsf-1* mutant nematodes, indicating that *hsf-1* is required for the CSO-induced increase in the lifespan.

DAF-16, a homologue of mammalian FoxO transcription factor, plays a critical role in integrating different signals to modulate aging and longevity by shuttling from cytoplasm to nucleus, which involves multiple signaling pathways (Davis 2000; Greer et al. 2009; Kenyon 2010; Murphy and Hu 2013; Sun et al.

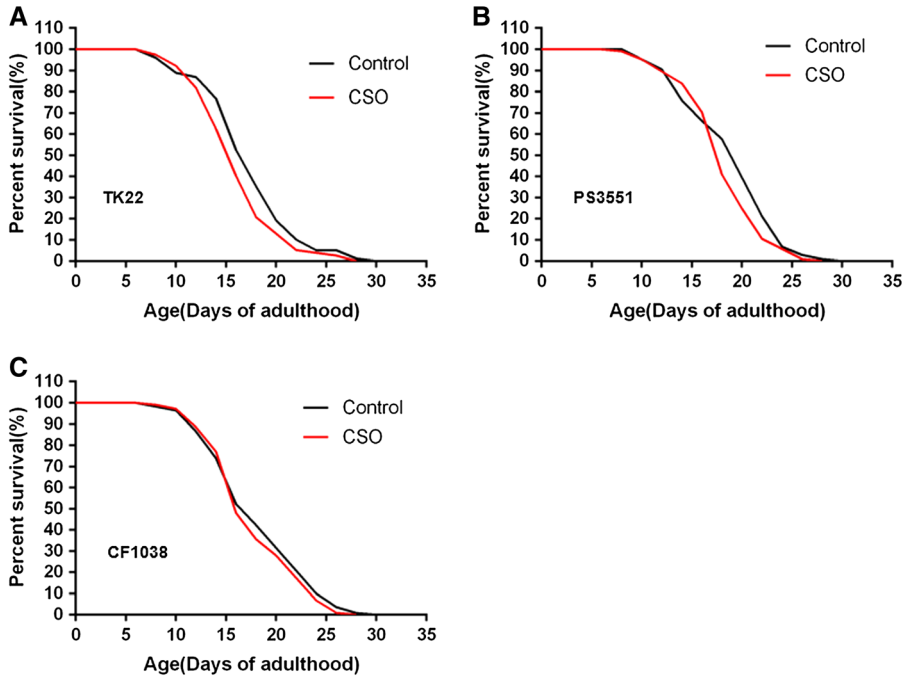

Fig. 5 CSO-related increase in the *C. elegans* lifespan is dependent on *mev-1*, *hsf-1* and *daf-16*. **a** Effects of 1 mg/mL CSO administration on the lifespan of TK22*(mev-1, kn1)* nematodes. **b** Effects of 1 mg/mL CSO administration on the lifespan of PS3551(*hsf-1, sy441*) nematodes. **c** Effects of 1 mg/mL CSO administration on the lifespan of CF1038(*daf-16*) nematodes. Three independent trials were performed for each assay

2017). In addition, many chemicals enhance stress and oxidation resistance in *C. elegans* through *daf-16* (Li et al. 2014; Shen et al. 2017). To investigate whether the CSO-mediated increase in the lifespan depended on *daf-16*, we performed a lifespan assay using CF1038 (*daf-16*) nematodes. CSO was unable to extend the longevity of *daf-16* mutant nematodes (Fig. 5c), indicating that the longevity-related effects of CSO in nematodes were dependent on the *daf-16* transcription factor.

daf-16 and its partial downstream genes were up-regulated by CSO

The *daf-16* transcription factor regulates many genes encoding stress response enzymes, including *sod-3*, *hsp-16.2* and *gst-4* (Shanmugam et al. 2017; Zhou et al. 2017). To further investigate whether the longevity-related effects of CSO were regulated by d*af-16* and its downstream target genes, we determined the mRNA levels of *daf-16* and its partial downstream genes in wild-type nematodes treated with or without 1 mg/mL CSO. The mRNA levels of *daf-16* and its partial downstream genes, *sod-3*, *hsp-16.2* and *gst-4*, were increased by CSO treatment (Fig. 6a). Moreover, we measured the protein expression levels of SOD-3 and HSP-16.2 in CF1553[(pAD76) *sod-3*::GFP + rol6(su1006)] (Fig. 6b) and CL2070(*hsp-16.2*::GFP) nematodes (Fig. 6c), and found that their expression levels were effectively increased by 18.4% and 14.8%, respectively, after being treated with 1.0 mg/mL CSO. Therefore, the CSO-related prolonged lifespan of *C. elegans* and its increased stress tolerance are dependent on *daf-16* and its downstream stress-inducible genes.

The main chemical constituents of CSO and their effects

GC–MS was used to investigate the active constituents in CSO. CSO contained at least 15 components, with linoleic acid, oleic acid, palmitic acid and stearic acid being the major constituents (Supplementary Table S7 and Fig. S1), and these four main components (12:9:4:1 linoleic acid: oleic acid: palmitic acid:

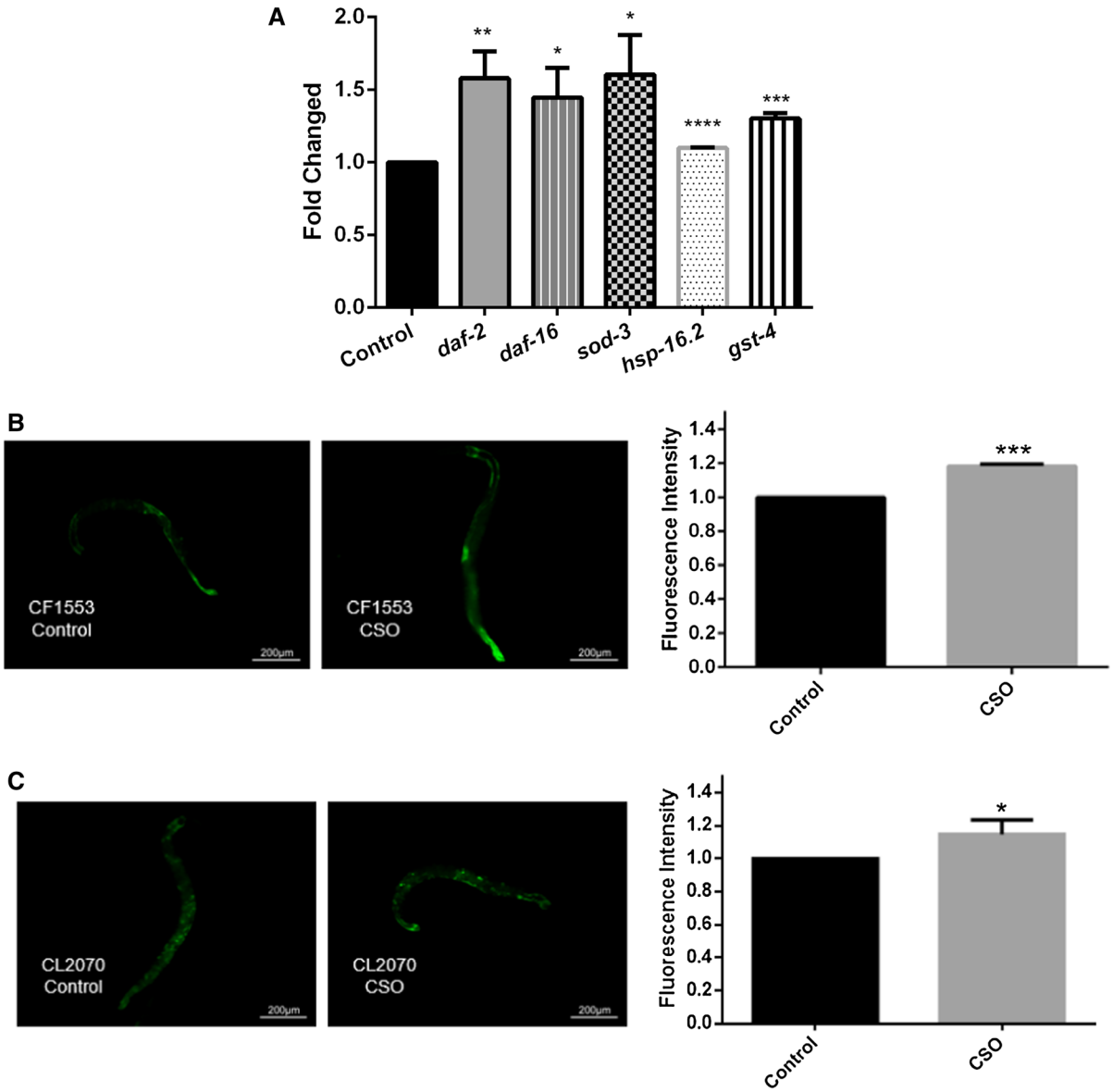

Fig. 6 *Daf-16* and its partial downstream genes were up-regulated by CSO. **a** The transcript levels of *daf-16* and its partial downstream genes in wild-type nematodes treated with 1 mg/mL CSO for 4 days. **b** The expression of SOD-3 in CF1553 nematodes treated with 1 mg/mL CSO for 4 days was up-regulated significantly compared with the control. **c** CSO enhanced the expression of HSP16.2 in CL2070 nematodes. Three independent trials were performed per assay. Data were analyzed using Student's *t*-test with Prism 6.0. Values are presented as means ± SDs.*$p < 0.05$, ***$p < 0.001$

stearic acid according to their content ratio) accounted for ∼ 0.26 mg/mL of 1 mg/mL CSO. To evaluate whether these four main fatty acids play leading roles in increasing the lifespan of *C. elegans*, we used a 0.26 mg/mL mixture in lifespan and ROS level assays and found that this mixture could effectively increase the life expectancy of nematodes by 21.8% (Fig. 7a), which is equivalent to the activity of 1 mg/mL CSO. Moreover, the 0.26 mg/mL mixture significantly reduced ROS levels by 24.3% (Fig. 7b). Thus, these four fatty acids appear to be the main functional components of CSO.

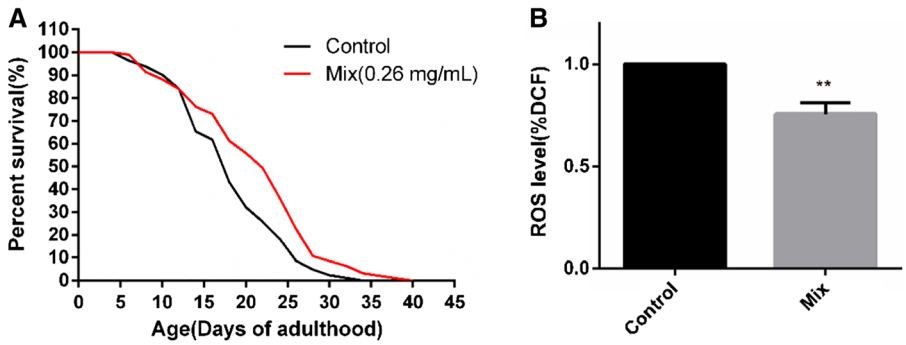

Fig. 7 Four fatty acids of CSO play a leading role in the increased *C. elegans* lifespan. **a** The mixture of four fatty acids at a 0.26 mg/mL concentration (including 0.12 mg/mL linoleic, 0.09 mg/mL oleic, 0.04 mg/mL palmitic and 0.01 mg/mL stearic acids) had significant effects on prolonging the average life span of nematodes by 21.8% ($p < 0.01$). **b** The 0.26 mg/mL mixture of four fatty acids significantly reduced the ROS level by 24.3% in wild-type nematodes. Three independent trials were performed per assay. Data were analyzed using Student's *t*-test with Prism 6.0. Values are presented as means ± SDs. $^{**}p < 0.01$

Discussion

Aging, as a complex biological process, still remains incompletely understood. Coix seed as a traditional medicinal and food homologous plant has high medicinal value. CSO has reported beneficial effects on human health (Huang et al. 2005; Yu et al. 2011). However, the influence of CSO on organism lifespan has not been previously reported.

It was showed that the main components of CSO are oleic acid, linoleic acid, palmitic acid and stearic acid by GC–MS Analysis (Supplementary Fig. S1). Oleic acid, a monounsaturated fatty acids, is the most abundant in CSO and could improve insulin sensitivity, enhance stress resistance, and inhibit cancer cell proliferation (Guzman et al. 2016; Palomer et al. 2018). Linoleic acid, the secondary abundant fatty acid in CSO, widely reported it could anti-obesogenic, antidiabetic, anticarcinogenic and reduce lipid accumulation in vivo (Zhai et al. 2010; Naughton et al. 2016; den Hartigh 2019). In this study, we showed that 1.0 mg/mL CSO, which mainly included linoleic, oleic, palmitic and stearic acids, significantly extended the lifespan of wild-type nematodes (Fig. 1, Table S1). In addition, a mixture made up of these four acids in the same proportion and concentration as in CSO also prolonged the lifespan, which indicated that the four main components of CSO might be the major functional substances. Interestingly, 1.0 mg/mL CSO had no obvious effects on the physiological functions of nematodes, including pharyngeal pumping, food intake and spawning (Fig. 2), which suggested that the increase in the lifespan caused by CSO exposure might not depend on the diet restriction or reproductive signaling pathways (Hsin and Kenyon 1999; Lee et al. 2006).

There is a correlation between stress resistance and longevity in nematodes(Si and Liu 2014; Lee et al. 2017). Our work indicated that 1.0 mg/mL CSO significantly enhanced the resistance of wild-type nematodes against oxidative and heat stress (Fig. 3 and Tables S4–S6), and remarkably reduced ROS accumulation level (Fig. 4). Moreover, the use of mutant nematodes has determined that CSO-induced prolonged life and increased resistance to stress depend on the regulation of *mev-1*, *hsf-1*, and *daf-16*, which are key genes for enhanced resistance and longevity (Hsu et al. 2003; Cohen et al. 2006; Li et al. 2014; Feng et al. 2015; Shen et al. 2017). Furthermore, mRNA levels of *daf-16* and its partial downstream genes, *sod-3*, *hsp-16.2* and *gst-4*, were evidently upregulated by CSO (Fig. 6a), suggesting that the CSO-related prolonged lifespan of *C. elegans* and its increased stress tolerance are dependent on *daf-16* and its downstream stress-inducible genes.

In this study, we confirmed that *daf-16* plays a vital role in the longevity of nematodes, which led us to hypothesize that CSO may extend the lifespan of nematodes through the insulin/IGF-1 signaling (IIS) pathway, which exerts longevity effects mainly through *daf-16*. However, the increase in life expectancy caused by CSO did not depend on *daf-2* (Fig. S2A). Moreover, the mRNA level of *daf-2* in nematodes treated with 1.0 mg/mL CSO was

surprisingly up-regulated (Fig. S2B) compared with the control. Thus, CSO prolonged the lifespan of wild-type nematodes through *daf-16* and its related genes, not through the IIS signaling pathway. The JNK family, as a subgroup of the mitogen-activated protein kinase superfamily, is a molecular sensor for stresses, such as ultraviolet radiation, ROS, DNA damage, heat and inflammatory cytokines (Davis 2000). The JNK signaling pathway is parallel to the IIS signaling pathway prior to acting on the *daf-16* gene. This could ultimately prolong the lifespan of nematodes and enhance the resistance to heavy metal toxicity by promoting *daf-16* expression in the nucleus. However, unlike the IIS signaling pathway, the JNK signaling pathway promotes the entry of the *daf-16* gene product into the nucleus through phosphorylation, indicating antagonism between the two signaling pathways (Mizuno et al. 2004; Oh et al. 2005). In this study, CSO promoted the expression of the *daf-2* gene and antagonized the IIS signaling pathway, but the expression of *daf-16* and its downstream target genes increased significantly. Therefore, we speculated that CSO plays a key role in prolonging the life expectancy of, and enhancing stress resistance in, nematodes, which might be related to the JNK signaling pathway.

Our findings further confirm the beneficial effects of CSO and may contribute to the further usage of CSO to promote healthy aging and treat age-related diseases in humans.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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