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Comparison of Three Fermentation Methods for Enhancing Antioxidant and Anti-Aging Properties in *Dendrobium officinale* Polysaccharides

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ABSTRACT

Excessive free radicals accelerate aging, while antioxidant supplementation mitigates oxidative damage and promote lifespan extension. *Dendrobium officinale* polysaccharides (DOPs) exhibit strong antioxidant properties, which can be enhanced through fermentation. In this study, we compared three fermentation methods, including *Lactobacillus plantarum* (LP), *Saccharomyces cerevisiae* (SC), and a combination of *Lactobacillus plantarum* & *Lactobacillus bulgaricus* (CL), based on previous research. Our results showed that LP fermentation significantly reduced the molecular weight of DOPs, and produced the most favorable outcomes, both *in vitro* and *in vivo*. Specifically, LP-fermented DOPs effectively reduced all three commonly used oxidative free radicals OH, DPPH and ABTS *in vitro*. It also reduced the production of reactive oxygen species (ROS) and increased the expression of antioxidant enzymes, including glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) in *Caenorhabditis elegans*. This led to improved motility, increased heat stress resistance, and extended lifespan of the nematode. These findings underscore LP-fermented DOPs as the most effective fermentation method, offering antioxidant benefits to combat aging and oxidative stress-related diseases.

Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CAT, catalase; CL, DOPs fermented by combination of *Lactobacillus plantarum* & *Lactobacillus delbrueckii subsp. Bulgaricus*; CTR, control; DCF, 2',7'-dichlorofluorescein; DOPs, *Dendrobium officinale* polysaccharides; GR, glutathione reductase; LP, DOPs fermented by *Lactobacillus plantarum*; ROS, reactive oxygen species; SC, DOPs fermented by *Saccharomyces cerevisiae*; SOD, superoxide dismutase; UNF, unfermented DOPs.

Jian-Peng Lei and Yun-Shu Li contributed equally to this study.

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Summary

- Fermentation reduces the molecular weight and enhances the anti-oxidation activity of *Dendrobium officinale* polysaccharides.
- Fermentation using *Lactobacillus plantarum* most effectively enhances the antioxidant properties of *Dendrobium officinale* polysaccharides.
- *Dendrobium officinale* polysaccharides fermented with *Lactobacillus plantarum* markedly extend the lifespan of *Caenorhabditis elegans*.
- *Dendrobium officinale* polysaccharides fermented with *Lactobacillus plantarum* significantly promote healthy aging in *Caenorhabditis elegans*.

1 | Introduction

Aging is a universal and inevitable biological process. While various hypotheses have been proposed to explain the cellular and molecular mechanisms of aging, recent research suggests it primarily results from the accumulation of molecular damage (Gladyshev et al. 2021; Schumacher et al. 2021). Among these theories, the free radical theory of aging is one of the most prominent describing how free radicals damage organisms during aging (Viña 2019; Melov et al. 2000). The mitochondrial electron transport chain generates reactive oxygen species (ROS) during aerobic metabolism (Nolfi-Donagan et al. 2020; Okoye et al. 2023). Excess free radicals, which contain unpaired and highly reactive electrons, induce oxidative stress and disrupt redox balance. This leads to protein and lipid peroxidation, which in turn damage mitochondria and DNA. These effects impair cellular and tissue functions, contribute to various pathological conditions, and accelerate aging (Leyane et al. 2022). Antioxidants neutralize free radicals, stabilize their activity, and prevent further oxidative damage (Hajam et al. 2022; Imelda et al. 2022). They also activate intracellular enzymes that regulate inflammation and immune responses (Morris et al. 2022), while enhancing DNA repair by modulating signaling pathways and supporting cellular self-repair mechanisms (S. Jiang et al. 2022).

Humans and other mammals have a natural antioxidant system consisting of both enzymatic and non-enzymatic components that help maintain a balance of free radicals (L. He et al. 2017; Mendonça et al. 2022). However, this system alone is insufficient to fully counteract oxidative damage. Supplementation with antioxidants can help mitigate aging-related oxidative stress and potentially extend lifespan (S. Li et al. 2018). While natural antioxidants provide protective effects, synthetic ones, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have shown to disrupt endocrine function, impair reproduction, and even increase cancer risk with prolonged use (Adefegha et al. 2021; Yang et al. 2018). Therefore, identifying a safe and effective natural antioxidant is crucial for delaying aging and protecting human health.

Dendrobium officinale (*D. officinale*, Tiepi Shihu) is a traditional Chinese herbal medicine and a valuable functional food known for its medicinal and nutritional benefits. It was recognized as the “foremost of the nine sacred medicinal herbs of China” in

the Taoist classic *Dao Zang* during the Kaiyuan era of the Tang Dynasty (Wei et al. 2016). The primary bioactive compounds in *D. officinale* include polysaccharides and alkaloids (Lee et al. 2018). Polysaccharides, consisting of sugar chains linked by glycosidic bonds, widely present in plants, animals, fungi, and microorganisms. They play key roles in physiological processes, including cell differentiation, proliferation, and signal transduction (Qiu et al. 2025; Yu et al. 2018; A. Zhang et al. 2024). Research has shown that polysaccharides, including those from *D. officinale*, exhibit a range of biological activities such as antidiabetic, anticoagulation, antitumor, immune regulation, antioxidant, and gut microbiota regulation (Liang et al. 2025). Notably, the hemiacetal hydroxyl groups in natural polysaccharides contribute to their strong free radical scavenging ability, which enhances their antioxidant activity.

Studies have shown that microbial fermentation can enhance the physicochemical properties and bioactivity of DOPs (Chen et al. 2022; R. Li et al. 2022). However, there has been no comprehensive study that compares which and how specific fermentation method maximizes the biological activity of these polysaccharides. This study compares three well-established fermentation methods using *Saccharomyces cerevisiae* (*S. cerevisiae*), *Lactobacillus plantarum* (*L. plantarum*), and a 1:1 mixture of *L. plantarum* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (*L. bulgaricus*) under their optimal fermentation conditions. The physicochemical changes were characterized and their impact on antioxidant activity has been assessed both *in vitro* and in *Caenorhabditis elegans* (*C. elegans*) models. The results offer valuable insights into optimizing fermentation techniques for enhancing the antioxidant potential of DOPs, with implications for their application in the development of antioxidant and anti-aging health supplements, skincare products, and pharmaceuticals.

2 | Materials and Methods

2.1 | Materials

Fresh stems of *Dendrobium officinale* (*D. officinale*, wild *Dendrobium* from Huoshan) were purchased from Hainan Xinwang Biotechnology Co. Ltd. The *D. officinale* fermentation broth with *Lactobacillus plantarum* (*L. plantarum*) was provided by the Laboratory Center of the College of Food Science and Engineering, Hainan University. *L. plantarum* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (*L. bulgaricus*) were obtained from Beina Chuanglian Biotechnology Co. Ltd. *Saccharomyces cerevisiae* (*S. cerevisiae*) was purchased from Angel Yeast Co. Ltd. Wide type *Caenorhabditis elegans* (*C. elegans*) strains were provided by Dr. Dawei Shi of the International Center for Aging and Cancer, Hainan Medical University.

2.2 | Isolation and Purification of DOPs

Fresh *D. officinale* stems were dried, powdered, and extracted with distilled water (1:30, w/v) at 90°C for 2 h. The supernatant was autoclaved, adjusted to pH6, and fermented with *L. plantarum* (LP), *S. cerevisiae* (SC), or a probiotic mixture (1:1 *L. plantarum*: *L. bulgaricus*) (CL) as described (Chen et al. 2022; X. Wang et al. 2022; Xing et al. 2021). The fermentation conditions

for each group were carefully standardized for this comparative study. For LP fermentation, the medium consisted of 10% skim milk, inoculated at 2% (v/v), and fermented at 42°C for 48 h under anaerobic conditions in air-tight jars with anaerobic gas-generating sachets. The initial pH was set to 6, and no agitation was applied. For SC fermentation, the medium consisted of a 2% glucose solution, inoculated at 2% (v/v), and incubated at 37°C for 48 h with a shaking speed of 150 r/min. The initial pH was adjusted to 6. For the CL group, the fermentation conditions were identical to those for LP, using a 10% skim milk medium, inoculated at 2% (v/v), and fermented under anaerobic conditions at 42°C for 48 h. The inoculum size of 2% (v/v) was chosen based on prior optimization experiments to ensure efficient fermentation, and all bacterial strains were activated according to the manufacturer's instructions before inoculation. The proportion of *D. officinale* juice in the fermentation medium was 20% (v/v) for all groups. The isolation and purification of DOPs was performed as previously described (X. Wang et al. 2022). Briefly, post-fermentation supernatants were alcohol-precipitated (4°C, 48 h), freeze-dried, and dissolved for protein removal via Sevag method. The aqueous phase was dialyzed (3,500 Da, 72 h), lyophilized, and further purified sequentially by DEAE-52 ion-exchange and Sephadex G-100 chromatography to obtain refined polysaccharides.

2.3 | Monosaccharide Composition Analysis of DOPs

The monosaccharide composition was determined according to the method described by Corrado et al. with minor modifications (Sarzanini et al. 2000). Briefly, an accurately weighed sample was placed into a 10 mL ampoule bottle, followed by the addition of 2.0 mL of 2 mol/L trifluoroacetic acid (TFA). After purging with nitrogen gas, the ampoule was sealed and hydrolyzed at 120°C for 10 h. Subsequently, 1.0 mL of the hydrolysate was transferred and dried under a nitrogen stream with methanol to remove residual TFA. The residue was redissolved in 1 mL of water to obtain the sample solution. Then, 250 µL of this solution was precisely mixed with 250 µL of 0.6 mol/L NaOH and 500 µL of 0.4 mol/L 1-phenyl-3-methyl-5-pyrazolone (PMP) methanol solution. The mixture was reacted at 70°C for 1 h, cooled in cold water for 10 min, and neutralized with 500 µL of 0.3 mol/L HCl. The resulting solution was extracted three times with 1 mL of chloroform by vortexing for 1 min and centrifuging at 3000 rpm for 10 min each time. The upper aqueous layer was carefully collected for analysis. The PMP derivatives (20 µL injection volume) were analyzed using a high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Japan) equipped with an Xtimate C18 column (4.6 × 200 mm, 5 µm; Welch Materials, China) maintained at 30°C. The mobile phase, consisting of 0.05 M potassium dihydrogen phosphate solution (pH adjusted to 6.70 with NaOH) and acetonitrile (83:17, v/v), was delivered at a flow rate of 1.0 mL/min. Detection was performed at a wavelength of 250 nm.

2.4 | Molecular Weight Determination of DOPs

The molecular weight and distribution of the DOPs were determined by gel permeation chromatography (GPC) using an

LC20 HPLC system equipped with a RID-20 refractive index detector (Shimadzu Corporation, Japan). The system was fitted with a TSKgel GMPWXL aqueous gel column (TOSOH, Japan) and a Rheodyne 7725i manual injector (Rheodyne, USA) with a 20 µL sample loop. The sample was dissolved in the specified solvent at a concentration of 3–10 mg/mL and filtered through a 0.45 µm membrane prior to injection. The eluent was a 0.1 N NaNO₃ aqueous solution containing 0.06% NaN₃, delivered at a flow rate of 0.6 mL/min. The column temperature was maintained at 35°C. A calibration curve was established using a set of narrow-distribution polyethylene glycol (PEG) standards (TOSOH, Japan).

2.5 | FTIR Spectroscopy of DOPs

Fourier-transform infrared (FTIR) spectroscopy was performed on a Thermo Scientific iS50 spectrometer (Thermo Fisher Scientific, USA). The sample was thoroughly ground with approximately 100 mg of potassium bromide (KBr) and pressed into a transparent pellet under a pressure of 20 MPa. The FTIR spectrum was recorded in the transmission mode with a resolution of 4 cm⁻¹, accumulating 32 scans, and a data interval of 0.482 cm⁻¹.

2.6 | Determination of Free Radical Scavenging Activity of Fermented *D. officinale* Crude Polysaccharides

2.6.1 | Determination of Hydroxyl Radical Scavenging Activity

Determined by mixing FeSO₄, salicylic acid, and polysaccharide solutions (sample group, A_m) or distilled water (control, A_n; blank, A₀). H₂O₂ was added to A_m and A_n, while the blank received water. After incubation (37°C, 1 h) and centrifugation, absorbance was measured at 510 nm.

2.6.2 | Determination of DPPH Radical Scavenging Activity

Assessed by combining polysaccharide solutions (A_m), DPPH/ethanol (blank, A₀), or ethanol (control, A_n). After incubation (RT, 20 min) and centrifugation, absorbance was measured at 517 nm.

2.6.3 | Determination of ABTS Radical Scavenging Activity

Preparing ABTS⁺ standard solution (A₀) by reacting ABTS with potassium persulfate (dark, 4°C, 12 h), followed by ethanol dilution to adjust absorbance (734 nm). Polysaccharide solutions (A_m) or water (control, A_n) were mixed with ABTS⁺, incubated (30 min), and absorbance measured at 735 nm.

For all assays, scavenging rates were calculated as:

$$\text{Scavenging Rate (E, \%)} = [1 - (A_m - A_n)/A_n] \times 100\%$$

2.7 | Determination of GR, CAT, and SOD Levels in *C. elegans*

Synchronized *C. elegans* were cultured for 5 days on standard NGM plates or plates supplemented with 200 µg/mL of

unfermented DOPs or polysaccharides fermented by three distinct microbial strains. Nematodes were harvested using sterile M9 buffer, washed twice via centrifugation, and resuspended in M9 buffer containing PMSF and protease inhibitors. Homogenization was performed using a high-throughput tissue grinder (SCIENITZ-48, Scientz Biotechnology, China) with grinding beads, followed by three cycles of grinding and cooling on ice. Homogenates were centrifuged, and supernatants were collected for protein quantification using a BCA assay kit (NewCell & Molecular, China). The activities of superoxide dismutase (SOD), glutathione reductase (GR), and catalase (CAT) were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) following manufacturer protocols.

2.8 | Determination of ROS Levels in *C. elegans* Using H₂DCFDA Fluorescent Probe

Synchronize *C. elegans* and cultivate them on NGM plates with *Escherichia coli* OP50 until the L4 stage. Transfer 30 L4 worms per group to NGM dishes with different components: standard medium (CTR), unfermented DOPs (UNF), or DOPs fermented by *L. plantarum* (LP), *S. cerevisiae* (SC), or a mixture of *L. plantarum* and *L. bulgaricus* (CL). All plates include 5-fluorouracil to inhibit reproduction and are incubated at 20°C for 5 days. Subsequently, wash the worms with M9 buffer and centrifuge. After washing, the worms resuspended in M9 buffer were transferred to a black 96-well microplate. Each well was then mixed with H₂DCFDA (MCE, America) fluorescent probe (100 µM, 1:1 volume ratio). The microplate was incubated at 30°C with shaking (30 rpm) for 2 h to induce oxidative stress. Fluorescence intensity (excitation: 485 nm, emission: 535 nm) was measured using a multifunctional microplate reader immediately after incubation and periodically over 4 h at 30°C. Data were analyzed using SPSS software to evaluate intergroup differences.

2.9 | Measurement of Intracellular ROS Levels in *C. elegans* Using a Multifunctional Microplate Reader

C. elegans were cultured on NGM plates and exposed to the H₂DCFDA fluorescent probe to assess ROS production. Heat-induced oxidative stress was applied at 30°C for 2 h. For imaging, a 2% agarose gel was prepared using a dry block thermostat, flattened on a microscope slide, and supplemented with anesthetic. Worms were mounted on the gel, covered with a coverslip, and visualized via fluorescence microscopy (Olympus IX51, Japan). The imaging was performed with a 10× objective lens, and exposure time was set at 600 ms to capture ROS fluorescence. Fluorescence intensity quantification was performed using ImageJ software. Experiments were conducted in triplicate.

2.10 | Effects of DOPs on the Locomotor Activity of *C. elegans*

Worms in both the control and experimental groups, with comparable physiological states, were transferred onto 2% agarose gel pads. The worms were allowed to acclimate and move freely for 2 min. Subsequently, under a stereomicroscope, the

number of head swings - defined as a single sinusoidal bending motion of the head - was recorded over a 30-second observation period. This count served as an indicator of locomotor activity. A total of 20 worms were observed per group.

2.11 | Heat Stress Resistance Assay in *C. elegans*

C. elegans were cultured and grouped as previously described, with 60 worms assigned to each group. After 5 days of normal cultivation, the worms were transferred to a 35°C constant-temperature incubator to induce heat stress. Worm viability was assessed under a stereomicroscope. A worm was considered dead if it failed to exhibit head or body movement in response to gentle stimulation with a picker. The number of worms killed in each group was recorded every 2 h. Based on the collected data, survival curves were plotted to evaluate worm viability under heat stress conditions at 35°C.

2.12 | Effects of DOPs on the Lifespan of *C. elegans*

Synchronized *C. elegans* were randomly selected and evenly distributed into five groups: CTR, UNF, LP, SC, and CL. The experimental groups were treated with DOPs at a concentration of 200 µg/mL. Each group consisted of 20 worms, with three replicates per group. Worms were cultured at 20°C in a constant-temperature and humidity incubator, L4 considered the starting point of the lifespan assay. The worms were transferred daily to fresh NGM plates seeded with *E. coli* OP50. Worm viability was monitored daily under a microscope. A worm was considered dead if it showed no head or body movement when gently touched with a picker. Starting from day 7, the number of dead worms on each plate was recorded in detail, and survival rates were calculated. Worms that burrowed into the medium or died from non-experimental causes were excluded from the analysis. Survival curves were plotted with survival rate (%) on the y-axis and lifespan (days) on the x-axis. The lifespan of the last surviving worm in each group was considered the maximum lifespan.

2.13 | Statistical Analysis

One-way ANOVA (analysis of variance) was used to compare data with a single factor for groups, while two-way ANOVA was used for independent measurements with a factor for group and a factor for time. The Tukey's post-test was performed with a significance level of 5% ($p \leq 0.05$) when there was a difference between the variables. The analyses were performed using the GraphPad Prism 9.5 statistical package (GraphPad Software Inc. La Jolla - free trial, CA, USA). $p < 0.05$ was considered statistically significant.

3 | Results

3.1 | Characterization of the Fermented *D. officinale* Polysaccharides

Fermentation can potentially influence the molecular weight, monosaccharide composition, glycosidic linkages, and other

characteristics of *Dendrobium officinale* polysaccharides (DOPs). To investigate these effects, three well-established fermentation methods were compared: *Lactobacillus plantarum* (LP), *Saccharomyces cerevisiae* (SC), and a 1:1 mixture of *Lactobacillus plantarum* and *Lactobacillus bulgaricus* (CL). First, we compared the yields of fermented DOPs from the three fermentation methods. As shown in Figure 1A, the results indicated a 20–40% loss of DOPs in all cases after fermentation, though no significant differences in yield were observed

between the methods. The relative molecular weights of the DOPs before and after fermentation were determined by gel permeation chromatography (GPC) (Figure 1B). The average molecular weight of unfermented DOPs (UNF) was calculated to be 99,652 Da, while those of DOPs fermented with LP, SC, and CL decreased to 4546 Da, 46,631 Da, and 8673 Da, respectively (Figure 1C). Notably, DOPs fermented with LP and CL exhibited a reduction in molecular weight by approximately 10–20 times.

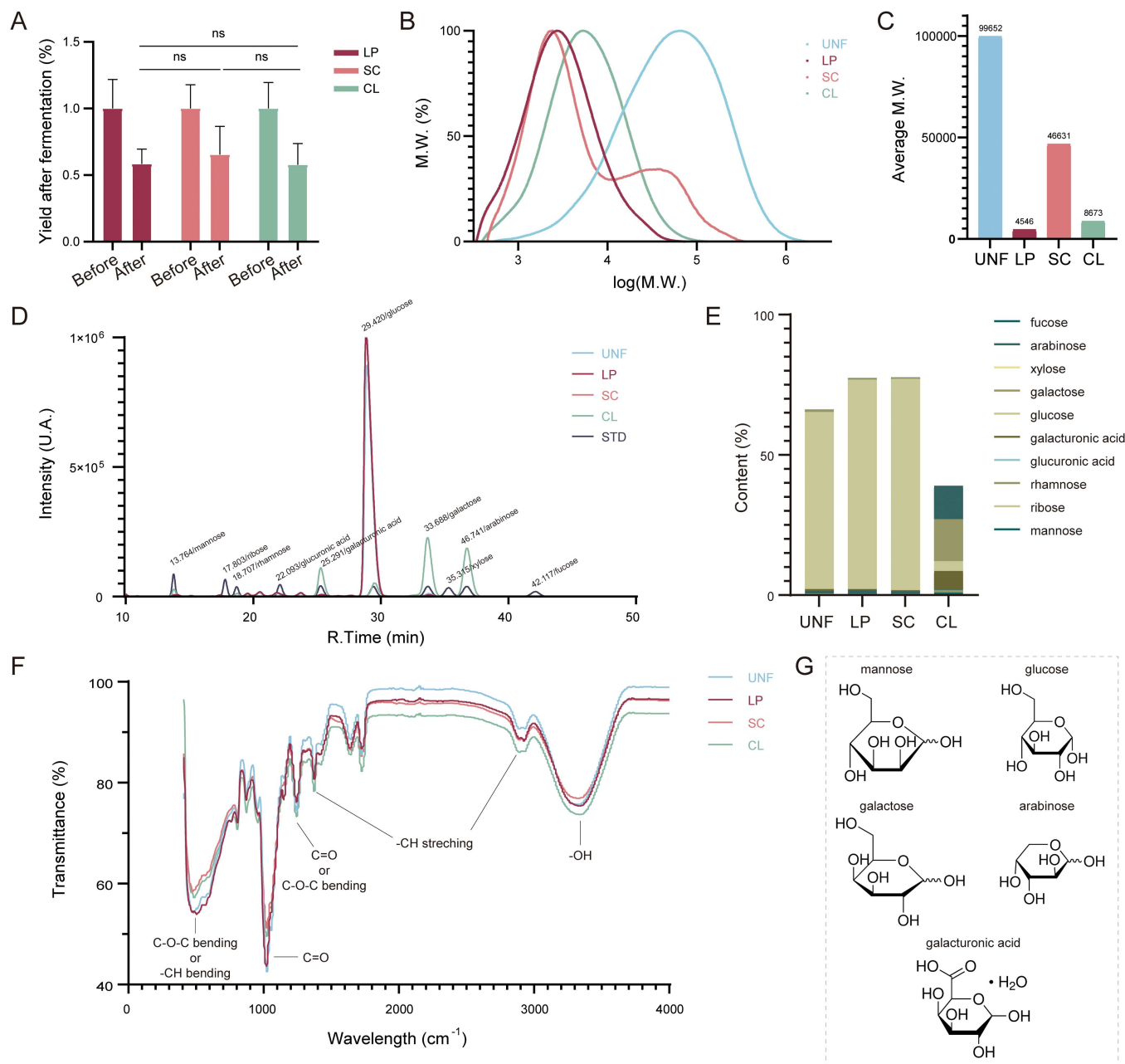


FIGURE 1 | Characterization of the *D. officinale* Polysaccharides (DOPs) before and after fermentation. (A) Yield of DOPs after fermentation. No significant differences in the yield of DOPs were observed. Student *t*-test was used for statistical analysis. (B) Molecular weight distribution of unfermented and fermented DOPs detected by GPC. (C) Comparison of average molecular weight calculated from panel B. The average molecular weight is labeled on the bar chart. (D) Monosaccharide composition of unfermented and fermented DOPs detected by FPLC. (E) Comparison of monosaccharide content between unfermented and fermented DOPs based on the data from panel D. (F) FTIR spectra of unfermented and fermented DOPs. Characteristic functional groups are labeled. (G) Chemical structures of several monosaccharides (obtained from <https://www.sigmaaldrich.com>). UNF: unfermented DOPs; LP: DOPs fermented by *L. plantarum*; SC: DOPs fermented by *S. cerevisiae*; CL: DOPs fermented by combination of *L. plantarum* & *L. bulgaricus*. STD: standard monosaccharide solution.

Further analysis of the monosaccharide composition of DOPs before and after fermentation was performed by FPLC following alkaline hydrolysis (Figure 1D). The results showed that the monosaccharide composition of DOPs fermented by LP and SC remained largely unchanged, with glucose remaining the predominant monosaccharide in both fermented DOPs (Figure 1E). However, fermentation with CL resulted in a significant alteration in the monosaccharide profile: the proportion of glucose significantly decreased, while the proportions of galactose, arabinose, and galacturonic acid increased considerably (Figure 1E).

Subsequently, the functional group changes in DOPs before and after fermentation were analyzed by fourier-transform infrared spectroscopy (FTIR). The absorption peaks at 1024 cm^{-1} and 3330 cm^{-1} , characteristic of polysaccharides, correspond to C—O and O—H stretching vibrations, respectively. The peaks at 2890 cm^{-1} and 1372 cm^{-1} correspond to C—H stretching vibrations in sugar molecules. As shown in Figure 1F, there were no significant changes in the functional groups of DOPs after fermentation.

We were particularly interested in understanding why the monosaccharide composition of DOPs after CL fermentation changed so drastically, while the FTIR spectra did not show any significant alteration. To address this, we examined the chemical structures of monosaccharides that showed notable changes (Figure 1G). Our analysis revealed that mannose, galactose, arabinose, and glucose share very similar structures, with the only major difference being that galacturonic acid contains an additional C—O group, a characteristic functional group of polysaccharides. This explains why the significant change in monosaccharide composition did not result in detectable changes in the FTIR spectra.

3.2 | Free Radical Scavenging Capacity of Fermented DOPs

In order to evaluate whether the free radical scavenging capacity of DOPs is enhanced after fermentation, three commonly used oxidative free radicals: OH, DPPH and ABTS were tested. As shown in Figure 2, compared to UNF, LP fermentation significantly enhanced the scavenging capacity of all three free radicals tested at a relatively low concentration of 2.5 mg/mL . SC fermentation increased OH scavenging at 7.5 mg/mL and DPPH scavenging at 10 mg/mL , but have no effect on ABTS scavenging. Furthermore, CL fermentation failed to improve the free radical scavenging capacity of the DOPs. Thus, LP fermented DOPs exhibit the best *in vitro* antioxidant capacity.

3.3 | Antioxidant Activity of Fermented DOPs in *C. elegans*

Reactive oxygen species (ROS) are major contributors to oxidative damage and play a central role in the aging process across various species, including humans (Leyane et al. 2022). The overproduction of ROS and the subsequent oxidative stress are closely associated with aging (Hajam et al. 2022). Prior to evaluating the *in vivo* antioxidant effects of DOPs, we tested their biocompatibility as a prerequisite to rule out any potential

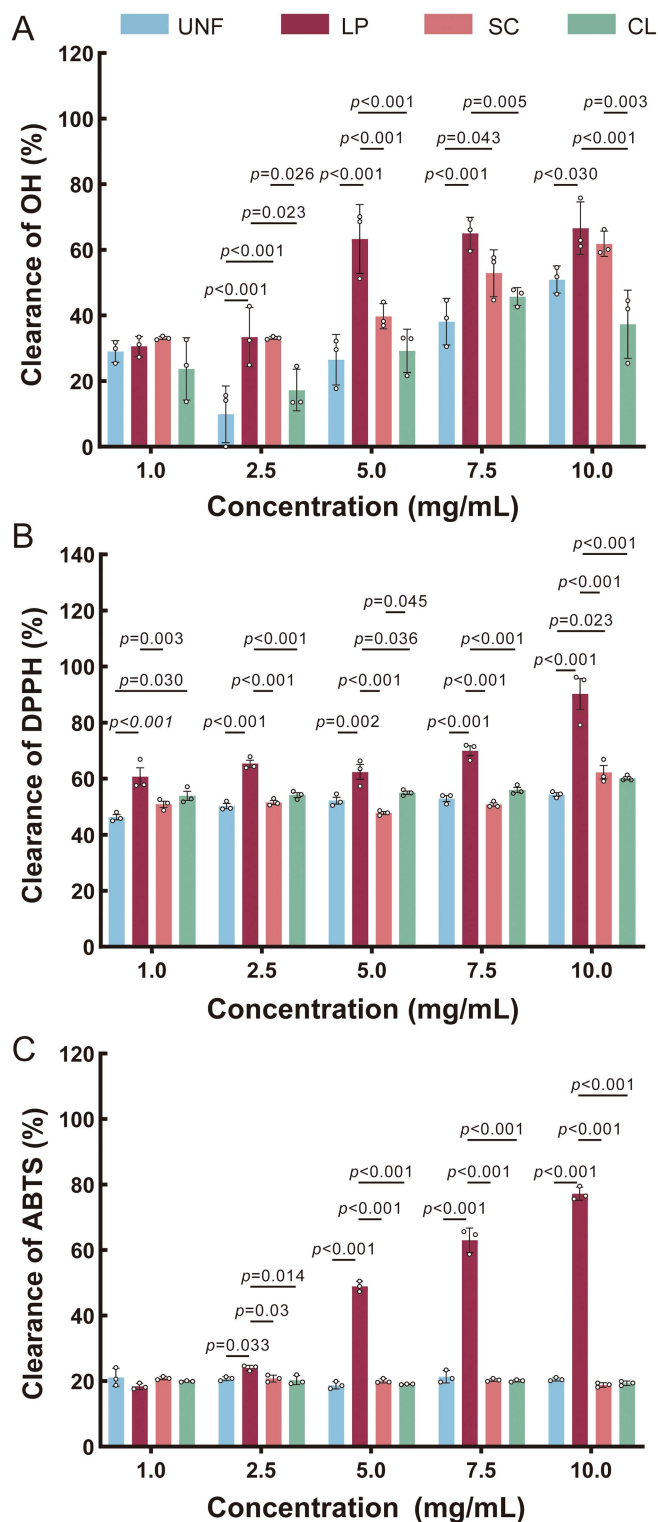


FIGURE 2 | Comparison of *in vitro* free radical scavenging capacities of fermented and unfermented DOPs. Increasing concentrations of polysaccharides were tested for: (A) OH radical scavenging capacity. (B) DPPH radical scavenging capacity. (C) ABTS radical scavenging capacity. UNF: unfermented DOPs; LP: DOPs fermented by *L. plantarum*; SC: DOPs fermented by *S. cerevisiae*; CL: DOPs fermented by combination of *L. plantarum* & *L. bulgaricus*. One-way ANOVA followed by Tukey's post-hoc tests was used for statistical analysis, $p < 0.05$ was considered statistically significant, and all significant P values are indicated.

cytotoxicity (Figure S1). *C. elegans* was selected as a model organism which was known for its simple structure, short lifespan, ease of manipulation and convenient observation. *C. elegans* were exposed to high temperature (30°C) for 2 h to induce oxidative stress and elevate ROS levels. Intracellular ROS levels were then measured using the fluorescent probe H₂DCFDA, a cell-permeable, non-fluorescent compound that, upon entering the cell, is oxidized by ROS to produce the highly fluorescent 2',7'-dichlorofluorescein (DCF). As shown in Figures 3A and 3B, the UNF group exhibited no significant difference compared to the control group (CTR), while all three fermented DOPs demonstrated significantly reduced green fluorescence, indicating that the fermented DOPs effectively reduced intracellular ROS accumulation in *C. elegans*. No significant differences were observed between the fermented groups (Figure 3B). The fluorescent images represent a single time point, to further confirm our findings, ROS levels were also measured over time using a multifunctional microplate reader within live worms, yielding consistent results (Figure 3C).

Antioxidant enzymes such as glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) are essential for combating oxidative stress, maintaining oxidative homeostasis, and protecting the body from disease caused by oxidative damage (Vilchis-Landeros et al. 2024). As shown in Figure 3D-F, the enzymatic activity of GR, SOD and CAT in *C. elegans* were evaluated after treatment with fermented DOPs at a concentration of 200 µg/mL for 5 days. The results indicated that UNF only increased CAT activity, but did not affect the activities of GR and SOD. SC and CL fermentation did not have an impact on the expression profile of antioxidant enzymes of UNF. Interestingly, LP fermentation not only significantly increased CAT activity, but also significantly enhanced the activities of GR and SOD. Notably, LP fermentation led to an eightfold increase in GR activity and 1.5-fold increase in SOD activity comparing with UNF. Collectively, these findings demonstrate that LP fermentation significantly strengthens the antioxidant defense system in *C. elegans*, effectively alleviating oxidative stress and providing the strongest protective effect against oxidative stress.

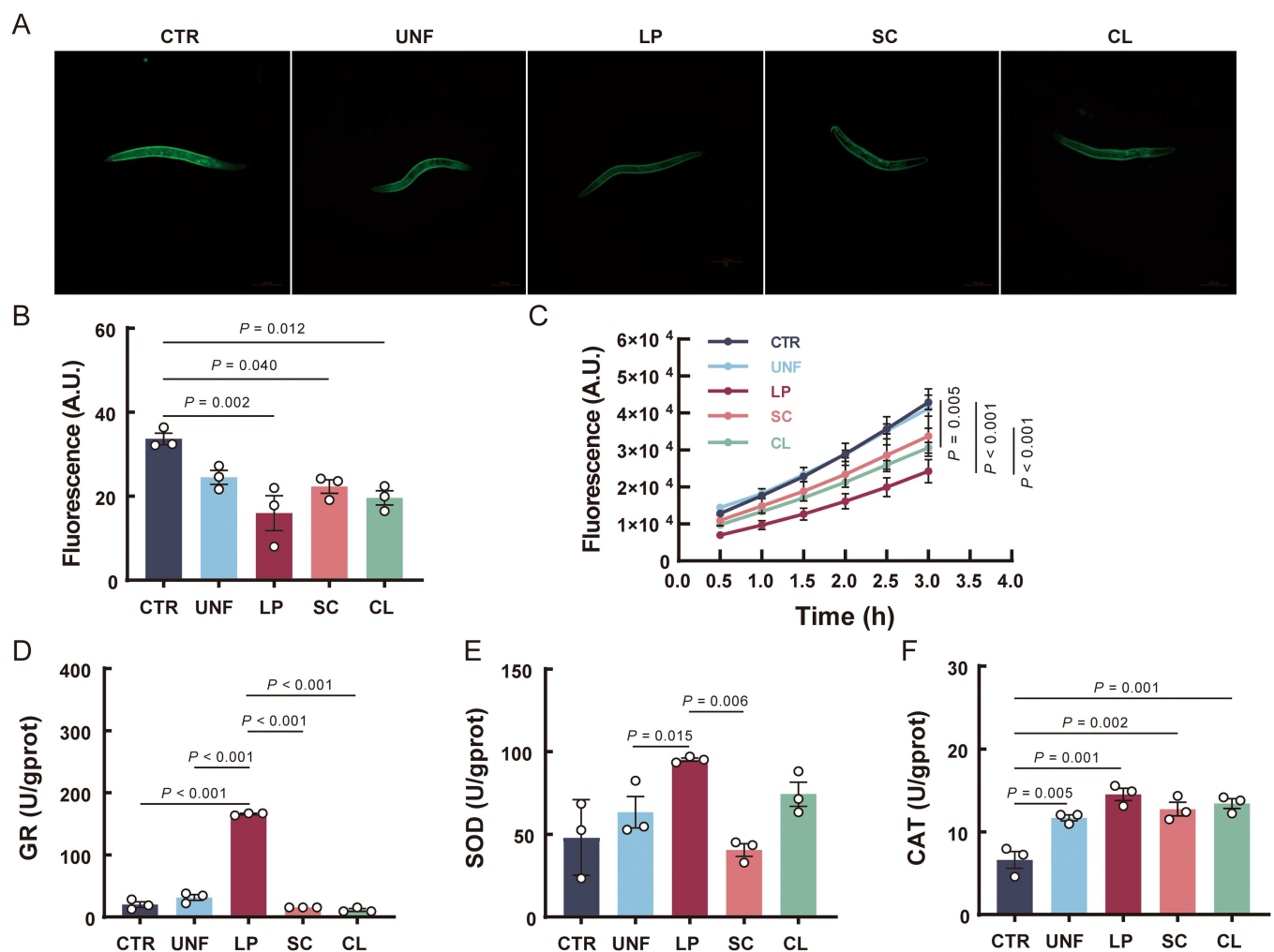


FIGURE 3 | Antioxidant Activity of Fermented DOPs in *C. elegans*. (A) Representative fluorescent images of *C. elegans* fed with or without fermented or unfermented DOPs. (B) Statistical analysis of (A) from three independent experiments. (C) Intracellular ROS levels in living *C. elegans* fed with or without fermented or unfermented DOPs. (D-F) Enzymatic activities of GR (D), SOD (E) and CAT (F) in *C. elegans* fed with or without fermented or unfermented DOPs. CTR: control; UNF: unfermented DOPs; LP: DOPs fermented by *L. plantarum*; SC: DOPs fermented by *S. cerevisiae*; CL: DOPs fermented by combination of *L. plantarum* & *L. bulgaricus*. One-way ANOVA followed by Tukey's post-hoc tests was used for statistical analysis, $p < 0.05$ was considered statistically significant, and all significant P values are indicated.

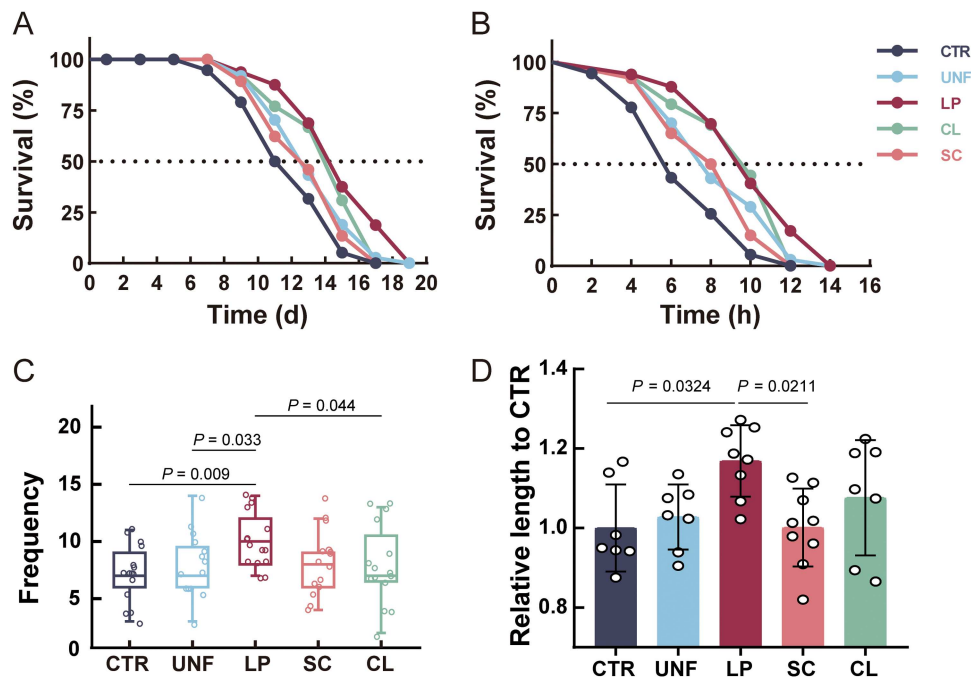


FIGURE 4 | Fermented DOPs enhance vitality and extend lifespan in *C. elegans*. (A) Normal lifespan of *C. elegans* fed with or without fermented or unfermented DOPs. The number of censored worm counts are 38, 36, 37, 37, and 40 for CTR, UNF, LP, SC and CL groups, respectively. (B) Heat stress lifespan of *C. elegans* fed with or without fermented or unfermented DOPs. The number of censored worm counts are 90, 99, 100, 100 and 97 for CTR, UNF, LP, SC and CL groups, respectively. Statistical analysis of (A) and (B) was performed using two-way ANOVA. (C) Locomotor activity of *C. elegans* fed with or without fermented or unfermented DOPs. (D) Length of *C. elegans* fed with or without fermented or unfermented DOPs. Statistical analysis of (C) and (D) was performed using One-way ANOVA followed by Tukey's post-hoc tests, $p < 0.05$ was considered statistically significant, and all significant P values are indicated. CTR: control; UNF: unfermented DOPs; LP: DOPs fermented by *L. plantarum*; SC: DOPs fermented by *S. cerevisiae*; CL: DOPs fermented by combination of *L. plantarum* & *L. bulgaricus*.

3.4 | Fermented DOPs Enhance Vitality and Extend Lifespan in *C. elegans*

Lifespan extension is widely recognized as one of the most direct and reliable biological indicators of the ability to slow down the aging process (Shen et al. 2018). To assess this, the overall lifespan of *C. elegans* cultured on NGM plates supplemented with 200 $\mu\text{g/mL}$ of either unfermented or fermented DOPs was compared. Survival curves revealed a significant rightward shift in all treated groups (Figure 4A), indicating that both fermented and unfermented DOPs can extend the lifespan of the nematodes. It is important to note that only LP fermented DOPs significantly extended lifespan compared to unfermented UNF ($p = 0.0405$), indicating the lifespan extension effects of LP fermented DOPs. Since lifespan extension in *C. elegans* is often correlated with enhanced stress resistance, the worms' thermotolerance by exposing them to heat stress at 35°C was evaluated. In line with the lifespan results, the survival curves also displayed a notable rightward shift in all treated groups, exhibiting similar patterns (Figure 4B). Both LP and CL fermentation significantly enhanced the heat stress resistance effects of DOPs compared to UNF ($p = 0.0003$ and $p = 0.0202$, respectively). Taken together, LP fermented DOPs not only prolong lifespan but also improve the heat stress resistance of *C. elegans*.

Aging has been shown to have a detrimental impact on the locomotor activity, with movement capacity serving as a key

indicator of health, and reflecting the state of health aging. As shown in Figure 4C, only the LP-fermented DOPs significantly increased the locomotor activity of the worms compared to the UNF group. Additionally, the size of the worms serves as another indicator of health. Consistent with the aforementioned results, while UNF group has no effect on the size of the worms, only LP-fermented DOPs significantly increased the length of *C. elegans* compared to the CTR group (Figure 4D). These results suggest that while both unfermented and fermented DOPs extended the lifespan of *C. elegans*, only the LP-fermented DOPs effectively promoted healthy aging in the worms.

4 | Discussion

4.1 | Antioxidant Effects of Fermented DOPs

Our research demonstrated that fermented DOPs significantly enhanced the antioxidant activity both *in vitro* and *in vivo*, leading to improvements in lifespan and stress resistance. Among the different fermentation methods, LP-fermented DOPs exhibited the most notable antioxidant effects. In particular, LP-fermented DOPs extended the lifespan of *C. elegans* under both normal and heat-induced oxidative stress (35°C) conditions, and also improved locomotor activity. These findings support the potential of LP-fermented DOPs in promoting healthy aging by enhancing stress resistance.

4.2 | Mechanistic Insights into Antioxidant Activity

The antioxidant mechanisms of DOPs are closely linked to the activation of crucial enzymes such as GR, CAT, and SOD, which work synergistically to neutralize ROS and protect cells from oxidative damage. GR maintains reduced glutathione (GSH), an essential antioxidant, while CAT decomposes hydrogen peroxide into water and oxygen, reducing cytotoxicity (Dutta et al. 2022). SOD helps convert superoxide radicals into less harmful molecules. Together, these enzymes contribute to maintaining cellular redox balance, which is critical for overall physiological health.

4.3 | Fermentation Impact on Molecular Properties

Our results showed that fermentation, particularly LP and CL fermentation, significantly reduced the molecular weight of DOPs by 10 to 20 times. The reduced molecular weight of DOPs may significantly enhance their solubility, increase their total surface area, and improve their hydrogen-donating capacity (R. Li et al. 2022), which could further contribute to their enhanced antioxidant activity. Interestingly, CL fermentation also led to an enriched monosaccharide composition. However, this change in monosaccharide composition does not appear to be the primary factor responsible for the enhanced antioxidant activity. Existing research suggests a positive correlation between the presence of arabinose and mannose in polysaccharides and their antioxidant activities (H. Wang et al. 2023). However, these polysaccharides typically have high molecular weights. Our results indicate that reducing the molecular weight may play a more significant role in enhancing antioxidant activity. Further studies into other structural modifications (Lin et al. 2024), such as glycosidic bonds and chemical modifications, will be necessary to fully understand these effects.

4.4 | Fermented DOPs Exhibited Better Overall Health Benefits

Various studies have shown that unfermented DOPs possess notable bioactivities, including antioxidant, anti-inflammatory, and immune-regulatory effects (Liang et al. 2025). Consistent with previous studies, our results demonstrated that fermentation significantly enhanced the *in vivo* and *in vitro* antioxidant capabilities of DOPs, potentially improving their bioavailability and overall health benefits. While unfermented DOPs were effective in extending lifespan in our study, the LP-fermented DOPs exhibited superior effects across all tested assays.

4.5 | Broader Applications of Fermented DOPs

Beyond aging research, the remarkable antioxidant properties of DOPs offer broad potential across various industries (Chu et al. 2022; Duan et al. 2024). The LP-fermentation process significantly enhances the antioxidant potential of DOPs, making it valuable for industrial and therapeutic applications. In the cosmetics industry, fermented DOPs are being explored as a potent ingredient for anti-aging, whitening, and

moisturizing skincare products, especially when combined with other active compounds like niacinamide (Duan et al. 2024). In the pharmaceutical sector, DOPs' structural advantages, including higher acetylation and increased uronic acid content (Gu et al. 2020), make it a promising candidate for therapies aimed at oxidative stress management, immune regulation, and anti-aging. Additionally, in the food industry, DOPs serve as an excellent natural additive that enhances both nutritional value and functional benefits of food products (Y. He et al. 2022). As research advances and purification technologies improve, the scope of DOPs' applications is expected to expand, offering substantial benefits for human health and quality of life.

5 | Conclusion

This study compared three well-established fermentation methods for DOPs, and found that LP fermentation markedly reduced the molecular weight of DOPs, resulting in significantly enhanced antioxidant and anti-aging activities compared to unfermented, SC-fermented and CL-fermented DOPs. However, we have only studied the antioxidant activity and anti-aging effects of DOPs in *C. elegans*. Further studies in other animal models and human trials are necessary to validate these activities. Additionally, there may be other more effective fermentation strains and methods yet to be explored, making it essential to establish standardized comparative methods for future research.

Author Contributions

Jian-Peng Lei: writing – original draft, methodology, formal analysis, data curation. **Yun-Shu Li:** methodology, formal analysis, data curation. **Hao-Yu Wang:** methodology, data curation. **Xin-Jian Wu:** data curation, methodology. **Jin-Qi Liu:** methodology, data curation. **Zhen-Yuan Xu:** methodology, data curation. **Yu-Hang Jiang:** methodology, data curation. **Ting Wang:** methodology, data curation. **Meihong Fu:** project administration, supervision. **Jian Dai:** supervision. **Fei-Fei Gao:** conceptualization, writing – review and editing. **Xian-Ying Cao:** conceptualization, supervision, writing – review and editing. **Kai Wang:** conceptualization, supervision, funding acquisition, methodology, writing – review and editing.

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Conflicts 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.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Figure S1: Biocompatibility of DOPs. Cell viability was measured by CCK-8 assay. Increasing concentrations of UNF (A), LP (B), CL (C), and SC (D) were applied to fibroblasts (human renal proximal tubular epithelial cell line HK2). One-way ANOVA followed by Tukey's post-hoc tests was used for statistical analysis, $P < 0.05$ was considered statistically significant, and all comparisons are non-significant (ns).