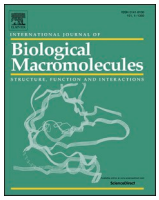
International Journal of Biological Macromolecules 278 (2024) 135098

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Synergistic anti-aging effect of *Dendrobium officinale* polysaccharide and spermidine: A metabolomics analysis focusing on the regulation of lipid, nucleotide and energy metabolism

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ARTICLE INFO

Keywords:
Dendrobium officinale polysaccharide
Spermidine
Synergistic anti-aging

ABSTRACT

The importance of synergy has been underscored in recent medical research for augmenting the efficacy of therapeutic interventions, targeting multiple biological pathways simultaneously. Our prior research elucidated that *Dendrobium officinale* polysaccharide (DOP) has the potential to prolong the lifespan of *Caenorhabditis elegans* (*C. elegans*) via regulating gut microbiota. Concurrently, spermidine (Spd), as a mimicking caloric restriction, facilitates autophagy and exerts a pronounced anti-aging effect. To enhance the anti-aging capabilities of DOP, we conducted a comprehensive study examining the combined effects of DOP and Spd in *C. elegans*, incorporating metabolomics analysis to investigate the underlying mechanisms. A combination of 250 mg/L DOP and 29.0 mg/L Spd yielded the most favorable outcomes in lifespan extension, evidencing a synergistic effect with a combination index (CI) of 0.65. In oxidative and heat stress tolerance assays, the observed CIs were 0.50 and 0.33, respectively. Metabolomic analysis highlighted significant alterations in metabolites related to lipid, nucleotide and energy metabolism, notably regulating glycerol 3-phosphate, linoleoyl glycerol, docosapentaenoic acid and β-nicotinamide mononucleotide, nicotinamide adenine dinucleotide. The effects of DS on lipid metabolism were further validated using Oil Red O staining and triglyceride level in *C. elegans*. The results indicated that DS may primarily be via modulating lipid metabolism. To further confirm these findings, a high-fat diet-induced mouse model was employed. Consequently, it can be inferred that the synergistic anti-aging impact of DOP and Spd is likely mediated primarily through alterations in lipid metabolic processes.

1. Introduction

In an era where the demographic shift towards an aging society is pronounced, there is a compelling necessity to explore the efficacious strategies that mitigate the aging process, avert age-associated pathologies, and enhance life quality [1]. Dietary interventions are increasingly recognized for their safety, efficacy, broad-spectrum utility, and cost-effectiveness, offering the potential to prolong lifespan or sustain health through the modulation of nutritional signaling, gut microbiota composition, and energy metabolism [2–4]. The longstanding and successful application of herbal drug combinations in traditional Chinese medicine has catalyzed our investigation into the synergistic potential of phytochemicals endowed with health-promoting properties [5]. In contemporary medical research, synergy assessment is pivotal for augmenting treatment efficacy and impacting multiple biological targets concurrently [6]. A prior review also posited that an individual dietary supplement typically exerts anti-aging effects via one or two distinct pathways. Hence, the synergistic application of multiple supplements may result in additive therapeutic benefits [7]. For instance, concurrent administration of resveratrol and spermidine (Spd) has demonstrated potential for synergistic enhancement of autophagy both in vitro and in vivo [8]. Resveratrol can enhance sirtuins (SIRT) deacetylase activity,

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https://doi.org/10.1016/j.ijbiomac.2024.135098
Received 30 April 2024; Received in revised form 23 August 2024; Accepted 24 August 2024
Available online 26 August 2024

while Spd acts as an acetyltransferase inhibitor. The combination index (CI) serves as an efficacious metric for analyzing the synergistic potential of multi-component combinations at fixed ratios, with synergism inferred when the CI is <1, and a lower CI value denotes stronger synergy.

Dendrobium officinale (DO) is a traditional functional food in China and has obtained increasing attention. DO polysaccharide (DOP) is its major active component and constitutes up to 36.30 %, with a yield exceeding 20 % in water-alcohol extraction [9]. Accumulating evidence underscores the profound antioxidant, immunomodulatory, anti-obesity, anti-diabetes, and anti-tumor capacities of DOP [10–12]. The underlying mechanisms of these beneficial effects include regulation of the nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), cytokines and host metabolism [12]. These functions and mechanisms are also related to the aging process. Recently, the potential anti-aging effects of DOP have also received attention [13,14]. For example, DOP has been shown to regulate age-related lineage commitment between osteogenic and adipogenic differentiation in bone marrow mesenchymal stem cells and aged mice [13]. Moreover, our preliminary experiments have shown promising results, demonstrating that DOP can extend the lifespan and improve the motor abilities of *C. elegans*.

Caloric restriction (CR) has been demonstrated to effectively extend a healthy lifespan. Caloric restriction mimics (CRMs) are compounds that activate the salutary pathways associated with CR, circumventing the subjective discomfort typically linked with CR or intermittent fasting. CRMs could promote autophagy via facilitating protein deacetylation, thus exerting anti-aging effects [7]. Four CRMs, including Spd, caffeic acid, epigallocatechin gallate, and hydroxy citric acid, were selected to evaluate their synergistic anti-aging effects with DOP in *C. elegans*. The results showed that Spd had the most pronounced synergistic effect with DOP on the extended lifespan of *C. elegans*. Spd, abundantly present in whole-grain products, vegetables, and legumes, is attributed with various health benefits, encompassing lifespan extension, anti-aging, and anti-inflammatory actions, as well as enhancements in cardiovascular, neurological, renal, and muscular health [15,16]. For example, Yang et al. found that Spd could inhibit neurodegeneration and delay aging via the PINK1-PDR1-dependent mitophagy pathway in *C. elegans* [17].

Previous research has extensively explored the anti-aging activities and mechanisms of dietary polysaccharides or CRMs. Polysaccharides are known to manifest anti-aging effects predominantly through immunomodulation and the regulation of gut microbiota, while CRMs induce autophagy and modulate nutrient-sensing pathways, including insulin/IGF-1 signaling (IIS) pathway, forkhead box O (FOXO) pathway and MAPK pathway. However, research on their synergistic impacts, particularly from a metabolomics perspective, is notably limited. Recent research has highlighted the important role of metabolism in regulating aging and longevity. The host organism generates numerous metabolites through various metabolic processes, and specific metabolites have been found to act as signaling molecules that regulate longevity [18]. Therefore, our study aims to determine the co-administration of DOP with Spd can synergistically decelerate the aging process, with a specific focus on elucidating the underlying mechanisms through metabolomic analysis. There is a growing market demand for natural and effective anti-aging products, providing a significant commercial opportunity for successful findings. This study proposes a novel synergistic anti-aging approach, but further animal and clinical trials are necessary to confirm the efficacy and safety of this approach.

2. Material and methods

2.1. Extraction of DOP

The extraction of DOP was conducted in accordance with a previous study [19]. Specifically, dried DO powder (60 mesh, Table S1) was defatted and removed small molecular impurities by employing hexane and 95 % ethanol (National Pharmaceutical Group Chemical Reagent Co., Ltd., Beijing, China) in a 1:10 ratio, respectively. Subsequently, the processed powder was exposed to a hot water bath at 95 °C for 2 h, using a volume of deionized water that was 30 times the weight of the powder. The resultant supernatant was then collected, concentrated, and subjected to precipitation using 75 % ethanol. The precipitate was resuspended and then destarched with thermostable α-amylase, followed by two freeze-thaw-centrifugation cycles and dialysis of the supernatant for 72 h (molecular weight cutoff: 3500 Da). Thereafter, the supernatant was precipitated with 75 % ethanol, and the resulting precipitate was collected, freeze-dried, and yielding the final DOP.

2.2. Determination of food clearance of C. elegans

Synchronized *C. elegans* were cultured at 20 °C to achieve the L4 larval stage. Subsequently, a food clearance assay was performed using a 96-well plate (Fig. 1A) [20]. A solution containing *Escherichia coli* (*E. coli*) OP50 and M9 buffer, supplemented with 5-fluoro-2-deoxyuridine (50 μM), was prepared in each well. Spd were added to the wells to achieve the final concentrations of 14.5, 29.0, 58.0, 116.0 and 232.0 mg/L, respectively. One hundred worms at the L4 stage were then introduced into each well containing the test solution and incubated at 20 °C. The optical density at 600 nm (OD_{600}) was recorded at 12 h intervals over a total duration of 96 h.

2.3. Determination of lifespan and stress tolerance of C. elegans

Based on the outcomes of the food clearance assay, concentrations of 14.5, 29.0 and 58.0 mg/L of Spd were selected for the lifespan assay (Fig. 1C). Specifically, synchronized L4 stage wild-type N2 *C. elegans* were placed onto nematode growth medium (NGM) supplemented with *E. coli* OP50 as a food source, along with varied concentrations of DOP and Spd, for a duration of 72 h [20]. To prevent egg laying and subsequent the generation of progeny that could affect lifespan metrics, 5-fluoro-2′-deoxyuridine was incorporated into the NGM. At 48 h intervals, the nematodes were relocated to fresh NGM plates. The survival of the *C. elegans* was monitored by counting the number of live worms at each interval. An individual worm was classified as deceased or was censored in the analysis if it exhibited no movement in response to a gentle mechanical stimulus applied with a platinum wire.

The oxidative and heat stress tolerance of *C. elegans* was assessed following methodologies described in the previous study, with minor modification [21]. Synchronized L4 stage wild-type N2 *C. elegans* were transferred to fresh NGM plates for a 72-h intervention period with DOP, Spd and combined DOP and Spd (Fig. 1C). Subsequent to this intervention, the worms from each treatment group were transferred onto new NGM plates containing 0.1 % hydrogen peroxide (H_2O_2) and incubated at 20 °C to conduct the acute oxidative stress tolerance assay. For the assessment of heat stress tolerance, the worms from each group were relocated to an incubator maintained at 37 °C. The survival of these worms was hourly documented for a duration of 12 h.

2.4. Determination of locomotion behavior of C. elegans

The motility of the worms was classified into three categories: Class A, B, or C. Class A comprised worms capable of spontaneous sinusoidal movement. In contrast, Class B included worms that, upon stimulation, exhibited movement but not in a sinusoidal manner. Class C was assigned to worms that could only exhibit movement in either the head or tail upon stimulation [22,23]. Additionally, the locomotive behavior was also quantified by measuring the frequency of body bends and head swings of worms [20]. On day 10 of life, body bending frequency was analyzed by counting the number of sinusoidal movements executed by the worms within 60 s. Similarly, head swing frequency was determined by the number of times the worm's head oscillated from one side to the

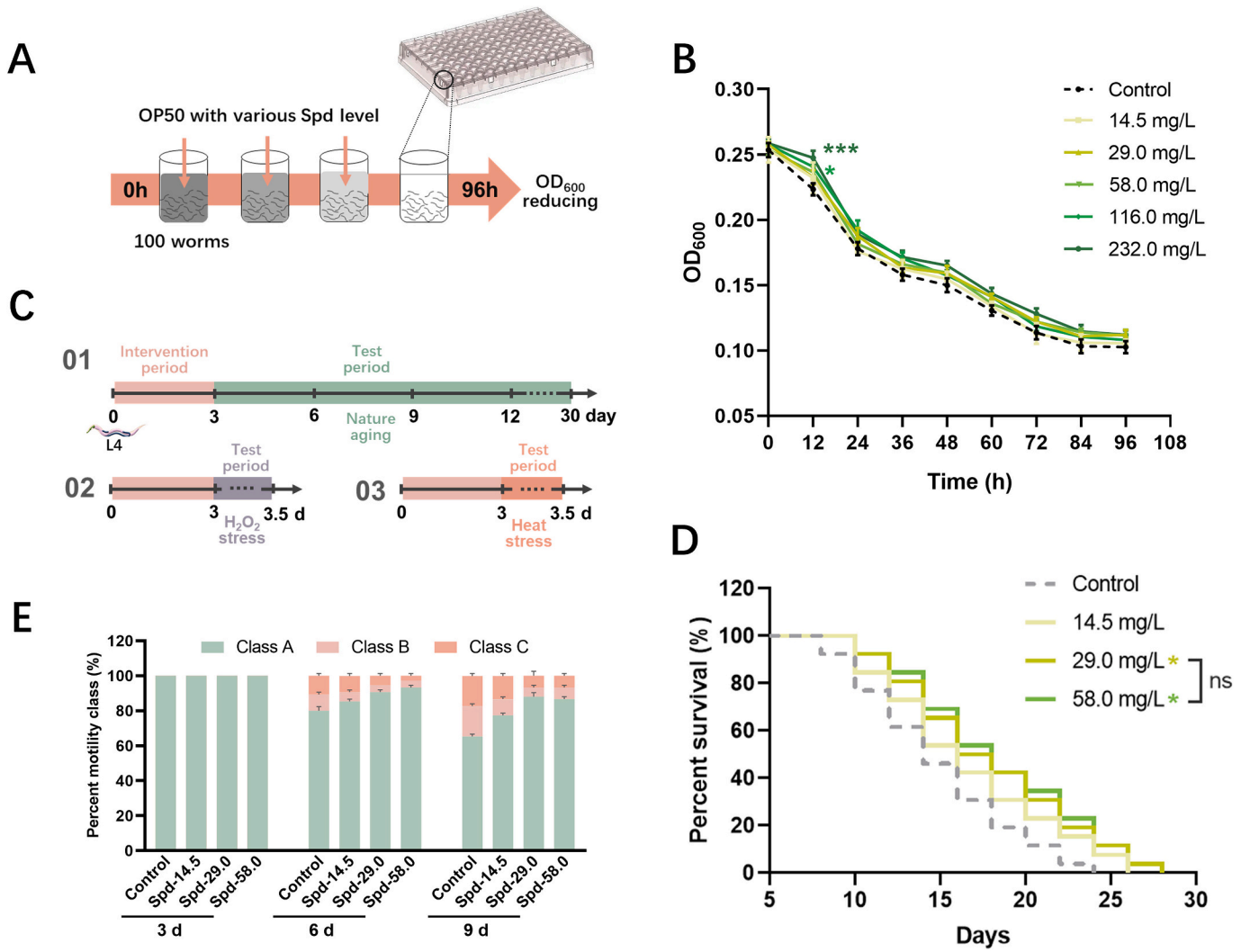

Fig. 1. Effects of Spd on food intake (A, B), lifespan (C, D) and motility capacity (E) of *C. elegans*.
Statistical annotations indicate significant differences: *, $p<0.05$; **, $p<0.01$, relative to the control group.

other within the same time frame.

2.5. qPCR analysis

The extraction of total RNA and its reverse transcription were performed using Trizol Reagent and the RevertAid First Strand cDNA Synthesis Kit (Vazyme Biotech, Nanjing, China), respectively. Subsequently, quantitative PCR (qPCR) was conducted employing the RT SuperMix Perfect (Vazyme Biotech, Nanjing, China). The specific primer sequences used for RT-qPCR are detailed in Table S2, with *act-1* serving as the reference gene [26,27]. The relative mRNA expression levels of the target genes were normalized to the internal reference gene and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Untargeted metabolomics analysis

The sample pretreatment method and the subsequent liquid chromatography-mass spectrometry (LC-MS) analyses were conducted according to previous studies [24]. The raw LC-MS datasets were transformed into visual outputs utilizing Compound Discoverer 3.3 software (ThermoFisher Scientific, US). For further analysis, these data were subjected to examination through the online platform Wekemo Bioincloud (accessible at https://bioincloud.tech/task-meta) [25].

2.7. Oil Red O staining and determination of TG level and antioxidant enzyme activities

Oil Red O staining was conducted according to a previous study with minor modifications [21]. Following a three-day treatment with DOP and Spd at 20 °C, the worms were harvested and washed thrice in M9 buffer, and then fixed by adding 600 μL of 40 % isopropanol to the worm pellet and gently rocking at room temperature for 3 min. Subsequently, the worms were stained with the Oil Red O working solution (Aladdin, Shanghai, China) for 2 h at room temperature, with continuous rotation at 30 rpm. After staining, the worms were washed three times in M9 buffer. Imaging was performed using an inverted fluorescence microscope (Nikon, T1-SAM), and ImageJ was employed to quantify the mean intensity of Oil Red O staining. The determination of triglyceride (TG) level and activity of SOD and CAT were conducted using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.8. Mice experimental design

Male C57BL/6J mice, aged six weeks, were procured from Gempharmatech Co., Ltd. (Nanjing, China), and acclimatized in a specified pathogen-free (SPF) facility maintained at a constant temperature of 24 ± 1 °C and relative humidity of 50 ± 10 %, under a 12-h light-dark cycle, for a two-week adaptation phase. Subsequently, forty mice were

randomly allocated into five groups ($n = 8$): control, model, DOP, Spd and combined DOP and Spd (DS) (Fig. 6A). The control group of mice was maintained on a normal diet, while the experimental groups administered a high-fat diet (60 % kcal from fat) provided from Nantong Telofei Biotechnology Co., LTD (Nantong, China) for a duration of 12 weeks. Detailed administration doses for DOP, Spd, and DS treatments were illustrated in Fig. 6A. The body weight measurements were conducted weekly. Following a 12 h fasting period, the mice were euthanized, and blood samples were collected for subsequent analyses. All experimental procedures were conducted in accordance with the guidelines for the Administration of Experimental Animals established by Jiangnan University, and the protocols received approval from the Committee of Experimental Animal Welfare Ethics at Jiangnan University (JN.No20230915c0960111[409]).

2.9. Determination of serum biochemical indices

A volume of 70 μL serum samples was diluted with 0.9 % saline to a total volume of 210 μL. Levels of plasma glucose (Glu), triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL—C) were quantified using an automatic biochemistry analyzer (Mindray BS-480, Shenzhen, China).

2.10. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) and were analyzed using GraphPad Prism version 8.3. Statistical assessment was performed via one-way analysis of variance (ANOVA) with subsequent post hoc analysis using Tukey's multiple comparison test. Survival curves were evaluated using the Mantel-Cox test. A p-value of <0.05 was deemed to denote statistical significance.

3. Results

3.1. Effect on the food clearance of C. elegans

Food intake markedly influences the lifespan of *C. elegans*; for instance, dietary restriction has been shown to prolong lifespan in various animal models [28]. Therefore, prior to conducting lifespan assays, it is imperative to assess food clearance to ascertain the appropriate concentration range for Spd administration (Fig. 1A). The absorbance of *E. coli* OP50 serves as an indicator of worm food concentrations. Specifically, at Spd concentrations of 116.0 and 232.0 mg/L, a slower food clearance rate was noted at 12 h (Fig. 1B, $p < 0.05$), whereas at lower concentrations of Spd, the rate of food consumption remained consistent with the control group throughout the 96 h duration ($p > 0.05$). Hence, Spd concentrations of 14.5, 29.0, and 58.0 mg/L were selected for further investigation in the lifespan assay.

3.2. Effect on the lifespan and locomotor capacity of C. elegans

The effects of Spd on the lifespan of *C. elegans* were investigated (Fig. 1C). Compared to the control group, the survival curves for the 14.5 mg/L Spd group exhibited a marginal rightward shift, although this alteration did not reach statistical significance ($p > 0.05$, Fig. 1D). Conversely, at concentrations of 29.0 and 58.0 mg/L, Spd treatment resulted in a noticeable rightward shift in the survival curves compared to the control ($p < 0.05$), yet no significant difference was detected between these two concentrations.

To assess the potential physiological effects of Spd treatment on the *C. elegans*, the locomotor capacity was monitored. Motility is an indicator of muscle function and directly correlates with aging [22,23]. Initially, until day 3, motility for all nematodes was categorized as Class A. Subsequently, from day 4 to day 6, there was a noticeable trend of motility transitioning towards Classes B and C. Nonetheless, nematodes in all Spd treatment groups demonstrated a decelerated progression to these classes compared to the control group. Specifically, by day 9, only 65.3 % of nematodes in the control group maintained Class A status. In contrast, 66.7 %, 77.3 %, and 86.7 % of nematodes treated with three different concentrations of Spd remained in Class A, respectively ($p < 0.05$), with no significant differences observed between the 29.0 and 58.0 mg/L Spd groups ($p > 0.05$).

In summary, Spd treatment not only prolonged lifespan but also enhanced the health status of the nematodes, indicating that doses of 14.5, 29.0, and 58.0 mg/L Spd are effective in extending lifespan and preserving a relatively high level of locomotor function. However, no significant differences were found between the 29.0 and 58.0 mg/L Spd groups. Therefore, in the following synergistic effect assay, the lower Spd levels (14.5 and 29.0 mg/L) were chosen to acquire a better synergy effect.

3.3. Synergistic effect of DOP and Spd on the lifespan and stress resistance of C. elegans

In our preliminary assays, it was observed that DOP at concentrations of 125 and 250 mg/L exhibited anti-aging effects without significantly affecting food intake in *C. elegans* (data not presented). To augment its anti-aging effects, we sought to determine the synergistic potential of DOP when combined with Spd. Accordingly, the lower efficacious concentrations of 125 and 250 mg/L for DOP (referred to as DOP-L and DOP-H, respectively) and 14.5 and 29.0 mg/L for Spd (referred to as Spd-L and Spd—H, respectively) were selected for synergistic evaluation.

The impacts of each dosage of DOP and Spd, as well as their combined administration, on the lifespan and stress resistance of *C. elegans* were investigated (Fig. 2A-F). The group receiving the combination of higher DOP and Spd (DS-HH) exhibited the most pronounced improvement in lifespan, stress tolerance, and locomotor behavior ($p < 0.05$). Specifically, the median survival times for the control, DOP-L, DS-LL, and DS-LH groups were 15, 17, 20, and 20 days, respectively, while for the DOP-H, DS-HL, and DS-HH groups, the median survival times were 18, 20, and 21 days, respectively (Fig. 2A-B). Similar patterns were observed in the stress tolerance assays, particularly in the oxidative stress assessment (Fig. 2C-F). In the oxidative stress assay demonstrating the most significant effects, the control group exhibited a median survival of 4.5 h, whereas the DOP-L, DOP-H, DS-LL, DS-HL, DS-LH, and DS-HH groups exhibited median survival times of 5.0, 6.0, 6.5, 6.5, 8.0, and 8.0 h, respectively.

In the A-F, statistical significance is denoted as follows: * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, **** for $p<0.0001$, each compared to the control group; In the G and H, significance is evaluated by comparing the DOP and Spd only group with the control group, and the DS group with DOP groups. * for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, **** for $p<0.0001$.

CompuSyn software was employed to calculate the synergy indices for DOP and Spd (Table 1). The specific time point for determining the combination index (CI) was selected based on the 50 % survival rate of the worms in the control group. A CI value of ≤ 0.3 denotes strong synergistic effects, >0.3 to ≤ 0.7 indicates synergistic effects, and > 0.7 to ≤ 0.9 suggests weak synergistic effects. Conversely, values >1.1 to ≤ 1.45 are indicative of weak antagonistic effects, >1.45 to ≤ 3.3 suggest antagonistic effects, and > 3.3 denotes strong antagonistic effects. In this context, the combination of 250 mg/L DOP and 29.0 mg/L Spd (DS-HH group) exhibited the most favorable outcomes in enhancing lifespan, manifesting synergistic effects in lifespan and two stress resistance assays with CIs of 0.65, 0.50, and 0.33, respectively. Notably, this synergistic effect was even more pronounced in the stress resistance experiment.

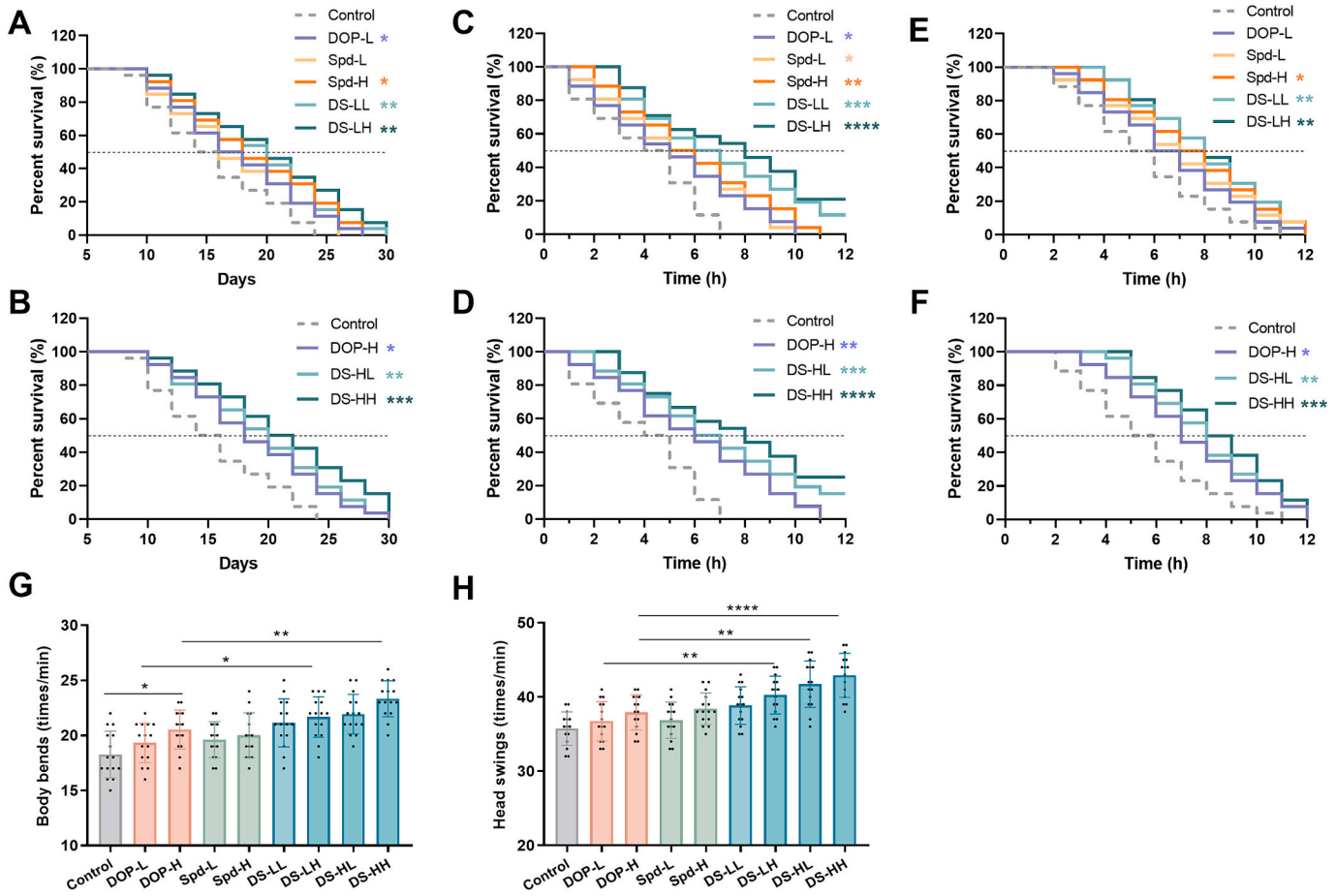

Fig. 2. Synergistic effect of DOP and Spd on the lifespan (A, B), oxidative stress tolerance (C, D), heat stress tolerance (E, F) and locomotion behavior (G, H) of *C. elegans*.

Table 1
Combination index (CI) of anti-aging function of DOP and Spd.

Group	Lifespan		H_2O_2 stress		Heat stress	
	Survival (%)	CI	Survival (%)	CI	Survival (%)	CI
Control	50.00	–	50.00	–	50.00	–
DOP-L	61.54	–	53.85	–	65.38	–
DOP-H	73.08	–	61.54	–	73.08	–
Spd-L	65.38	–	57.70	–	69.23	–
Spd-H	69.23	–	65.38	–	73.08	–
DS-LL	69.23	1.14	69.23	0.58	76.92	0.57
DS-LH	73.08	0.98	73.08	0.62	80.77	0.42
DS-HL	73.08	1.24	73.08	0.54	80.77	0.53
DS-HH	80.77	0.65	76.92	0.50	84.62	0.33

DS-LL, combined low DOP (125 mg/L) and low Spd (14.5 mg/L) treatment group; DS-LH, combined low DOP (125 mg/L) and high Spd (29.0 mg/L) treatment group; DS-HL, combined high DOP (250 mg/L) and low Spd (14.5 mg/L) treatment group; DS-HH, combined high DOP (250 mg/L) and high Spd (29.0 mg/L) treatment group.

3.4. Synergistic effect of DOP and Spd on the locomotion behavior of *C. elegans*

Upon comparing the four groups treated with DOP and Spd alone to the control group, significant changes were observed only in the number of body bends in the DOP-H group ($p < 0.05$, Fig. 2G). While a slight increase in the number of head swings was noted in the groups treated with DOP and Spd alone, this increment was not statistically significant ($p > 0.05$, Fig. 2H). Overall, treatment with either DOP or Spd alone effectively extended the lifespan of *C. elegans*, albeit with little impact on their locomotion behavior. Contrastingly, DS-HH group demonstrated the most significant improvements in both the number of body bends and head swinging, with these effects being statistically different from those observed in the DOP alone group ($p < 0.05$), indicating a synergistic enhancement of locomotor behavior.

3.5. Synergistic effect of DOP and Spd on the relative mRNA expression of aging-related genes and antioxidant enzyme activities of *C. elegans*

The qPCR was used to assess the effects of DOP, Spd and DS on the mRNA expression of genes associated with aging in *C. elegans* (Fig. S1). Cotreatment with DOP and Spd resulted in a significant upregulation of *sir-2.1* and *daf-16*, while a significant decrease in the expression of *daf-2* was observed ($p < 0.01$). Notably, the relative mRNA expression of *daf-16* exhibited the highest increase, with a 4.05-fold rise in DS group, compared to the control group. As shown in Fig. S1D and S1E, the activities of SOD and CAT were also dramatically increased in the DS group ($p < 0.01$).

3.6. Synergistic effect of DOP and Spd on the metabolic changes of *C. elegans*

The DS-HH group exhibited optimal synergistic effects in lifespan extension, achieving the lowest CI, and hence, this combination concentration was selected for mechanistic investigation via metabolomic analysis (Fig. 3). Metabolomic screening and integration in both positive and negative ion modes yielded a total of 401 metabolites. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) demonstrated that the quality control (QC) group displayed tight clustering, indicating

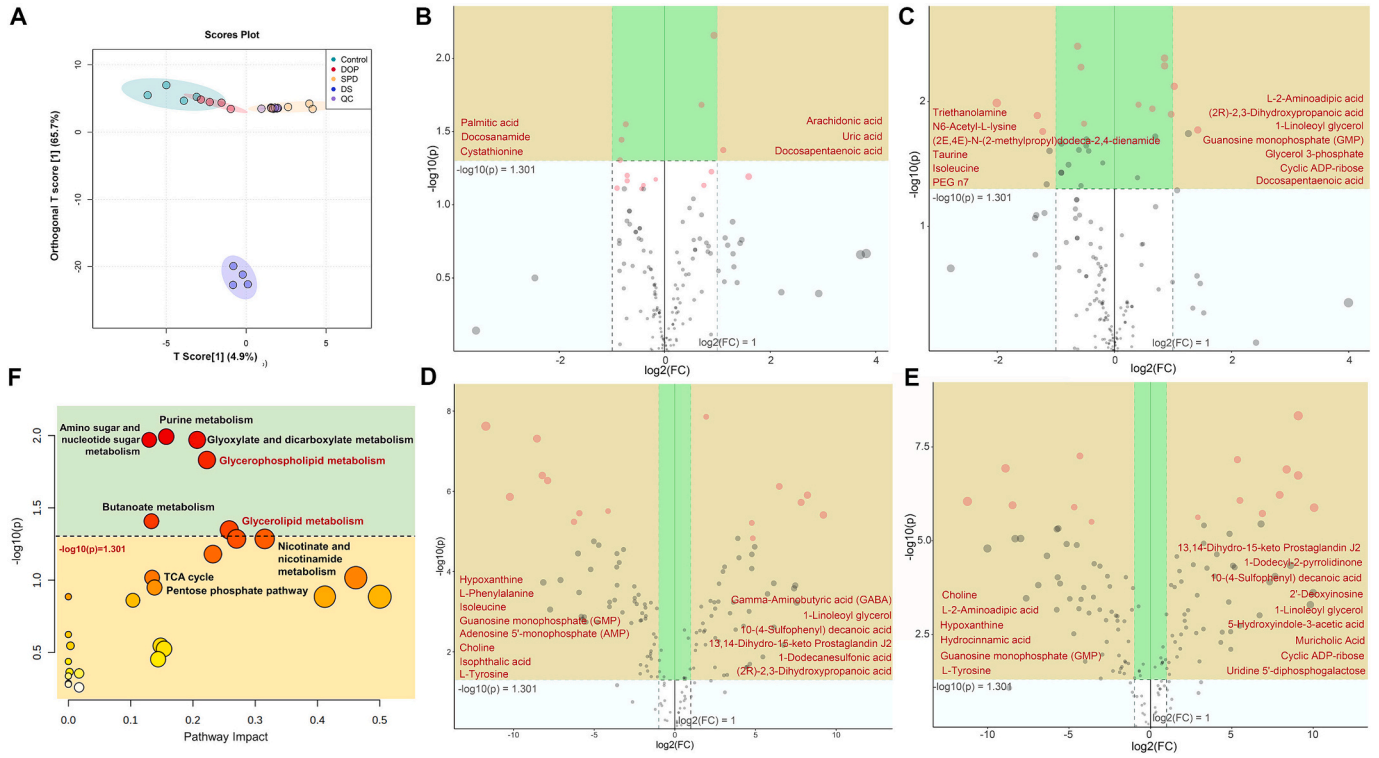

Fig. 3. Synergistic anti-aging effect of DOP and Spd in *C. elegans* based on the metabolomic analysis.
(A) Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) score plot from the metabolome datasets; (B)-(E) The top 15 different metabolites between control and DOP-treated groups, control and Spd-treated groups, DOP and combined DOP-Spd (DS) treatment groups, and Spd and DS treatment groups, respectively; (F) Pathway analysis plots of the differential metabolites.

consistent instrument performance and minimal data variability. A pronounced segregation was observed between the control, DOP, Spd, and DS groups, particularly between the DS group and the other three, highlighting substantial differences in metabolite profiles across the groups, with the DS group showing distinct variations (Fig. 3A).

Further analysis entailed a clustering assessment combined with a heatmap, delineating the distribution pattern of the top 20 metabolites ($p < 0.05$, Fig. S2). These metabolites were classified according to metabolic pathways, encompassing carbohydrate metabolism, lipid metabolism, nucleotide and energy metabolism, amino acids and other metabolic processes. Among these, 6 metabolites were associated with lipid metabolism, and another 6 were linked to nucleotide and energy metabolism, illustrating the metabolic changes influenced by the three treatments.

As delineated in Fig. 3B-E, the regions shaded in yellow represent metabolites fulfilling the criteria of $p < 0.05$ and a fold change (FC) > 2.0 or FC < 0.5, indicating statistically significant alterations between the control and the DOP, Spd groups, as well as between the DS and the DOP, Spd groups, respectively. Metabolites represented by red dots are the top 15 identified in terms of significance or abundance changes. The DS group exhibited a greater number of metabolite fluctuations compared to the groups treated solely with DOP or Spd. Intriguingly, relative to the control, DOP administration resulted in increased levels of docosapentaenoic acid, arachidonic acid, and uric acid, while reducing cystathionine, docosanamide, and palmitic acid levels, suggesting that DOP modulates both fatty acid and nucleotide metabolism ($p < 0.05$, Fig. 3B). Moreover, Spd treatment led to an upregulation of 1-Linoleoyl glycerol, cyclic ADP-ribose, docosapentaenoic acid, glycerol 3-phosphate, guanosine monophosphate (GMP), L-2-Aminoadipic acid and adenosine ($p < 0.05$, Fig. 3C), highlighting its influence on fatty acid and nucleotide metabolism, albeit with a distinct metabolite profile compared to DOP. The concurrent administration of DOP and Spd was observed to further enhance fatty acid and nucleotide metabolism, thereby generating a more pronounced synergistic effect (Fig. 3D-E). This synergy likely results from the complementary actions of DOP and Spd on the metabolic pathways, underscoring their potential combined impact on the metabolic changes in *C. elegans*.

The Variable Importance in Projection (VIP) score, obtained from OPLS-DA, was utilized to quantify the significance of metabolite changes, with VIP values exceeding 1.0, indicating metabolites substantially contribute to the differentiation observed in the model. These metabolites demonstrated changes with *p*-values <0.05 and an FC >1.5 or <0.75. Ultimately, a total of 36 metabolites were identified as significantly associated with lipid, carbohydrate, nucleotide, and energy metabolism pathways (Table 2). Notably, the levels of citric acid, linoleoyl glycerol, β-nicotinamide mononucleotide (NMN), 5-hydroxyindole-3-acetic acid and taurine exhibited an increase of >10-fold ($p < 0.05$). The pathway analysis and interaction network of the differential metabolites in *C. elegans* were analyzed. The results indicated that DOP and Spd had a greater impact on glycerophospholipid metabolism and glycerolipid metabolism than other pathways (Fig. 3F). Additionally, glycerol-3-phosphate played a crucial role in the interaction network of the differential metabolites, indicating that the anti-aging effect of DS may primarily mediate through alterations in lipid metabolism (Fig. S3).

3.7. Synergistic effect of DOP and Spd on lipid level of C. elegans

Oil Red O staining, a method for highlighting lipid droplets microscopically, combined with TG quantification, serves as a reliable measure for in vivo lipid levels. As illustrated in Fig. 4A-D, a significant reduction in lipid accumulation was observed in *C. elegans* treated with DOP+Spd, whereas the DOP and Spd treatments alone resulted in a decrease that was not statistically significant. This reduction was visually evident as a substantial decrease in the intensity of stained lipid droplets in worms treated with DOP+Spd compared to the control group ($p < 0.001$, Fig. 4E). Additionally, a decrease in TG level indicates

Table 2
The significantly changed metabolites after DOP and Spd treatment.

No.	*m/z*	Compound name	Chemical Formula	Fold change			
				DOP vs. Con	Spd vs. Con	DS vs. DOP	DS vs. Spd
Carbohydrate metabolism							
1	209.02906	Citric acid	$C_6H_8O_7$	1.73	1.69	27.31	28.00
2	289.03161	D-Sedoheptulose 7-phosphate	$C_7H_{15}O_{10}P$	0.93	0.72	0.36	0.46
3	229.03151	D-Xylulose 5-phosphate	$C_5H_{11}O_8P$	0.93	0.72	0.23	0.30
4	133.01355	Malic acid	$C_4H_6O_5$	1.08	1.02	0.08	0.09
5	260.0285	D-Mannose 6-phosphate	$C_6H_{13}O_9P$	0.88	0.90	0.06	0.06
Lipid metabolism							
6	337.27233	Linoleoyl glycerol	$C_{21}H_{38}O_4$	1.67	2.03	29.75	25.00
7	329.2469	Docosapentaenoic acid	$C_{22}H_{34}O_2$	2.16	2.68	3.48	2.80
8	279.2320	Linoleic acid	$C_{18}H_{32}O_2$	1.16	0.87	1.49	1.98
9	303.2316	Arachidonic acid	$C_{20}H_{32}O_2$	1.91	1.63	1.36	1.59
10	255.2317	Palmitic acid	$C_{16}H_{32}O_2$	0.60	0.54	0.82	0.92
11	140.0112	O-Phosphorylethanolamine	$C_2H_8NO_4P$	1.63	2.10	0.65	0.51
12	104.10693	Choline	C5H13NO	1.15	1.31	0.06	0.05
13	454.29151	Glycerophospho-N-palmitoyl ethanolamine	$C_{21}H_{44}NO_7P$	2.75	1.16	0.02	0.05
14	171.0057	Glycerol 3-phosphate	$C_3H_9O_6P$	1.12	1.57	0.06	0.04
Nucleotide and energy metabolism							
15	335.06277	β-Nicotinamide mononucleotide (NMN)	$C_{11}H_{15}N_2O_8P$	0.83	0.85	15.72	15.29
16	540.05155	Cyclic ADP-ribose	$C_{15}H_{21}N_5O_{13}P_2$	1.78	1.97	10.70	9.65
17	565.0454	Uridine 5′-diphosphogalactose	$C_{15}H_{24}N_2O_{17}P_2$	1.11	0.99	6.66	7.52
18	151.02534	Xanthine	$C5H4N4O_2$	1.16	0.97	3.10	3.72
19	302.5323	UDP-*N*-acetylglucosamine	$C_{17}H_{27}N_3O_{17}P_2$	1.18	1.04	1.93	2.19
20	268.1032	Adenosine	$C_{10}H_{13}N_5O_4$	2.44	2.40	2.02	2.05
21	332.56074	Nicotinamide adenine dinucleotide (NAD+)	$C_{21}H_{27}N_7O_{14}P_2$	1.40	1.62	2.33	2.01
22	167.0203	Uric acid	$C_5H_4N_4O_3$	1.62	1.40	1.65	1.90
23	136.06134	Adenine	$C_5H_5N_5$	1.10	0.80	1.17	1.61
24	346.05421	Adenosine 5′-monophosphate	$C_{10}H_{14}N_5O_7P$	0.95	0.92	0.44	0.45
25	300.0475	N-Acetyl-α-D-glucosamine 1-phosphate	$C_8H_{16}NO_9P$	0.63	0.64	0.35	0.34
26	362.04905	Guanosine monophosphate (GMP)	$C_{10}H_{14}N_5O_8P$	0.76	1.09	0.03	0.02
Amino acid and the other metabolism							
27	190.05011	5-Hydroxyindole-3-acetic acid	$C_{10}H_9NO_3$	3.01	0.91	13.61	45.08
28	124.00667	Taurine	$C_2H_7NO_3S$	2.59	0.40	5.60	36.03
29	102.05553	ϒ-Aminobutyric acid (GABA)	$C_4H_9NO_2$	0.98	0.88	3.84	4.30
30	399.14315	S-Adenosyl-methionine	$C_5H_9NO_4$	1.02	0.94	0.57	0.62
31	221.05909	Cystathionine	$C_7H_{14}N_2O_4S$	0.61	0.84	0.36	0.26
32	356.10185	5′-S-Methyl-5′-thioadenosine	$C_{11}H_{15}N_5O_3S$	1.15	1.12	0.24	0.25
33	173.10361	DL-Arginine	$C_6H_{14}N_4O_2$	0.87	0.88	0.15	0.15
34	74.0244	Glycine	$C_2H_5NO_2$	0.55	0.39	0.09	0.13
35	188.1751	N8-Acetylspermidine	$C_9H_{21}N_3O$	2.05	2.76	0.16	0.12
36	148.05994	L-Glutamic acid	$C_5H_9NO_4$	2.29	1.99	0.007	0.008

diminished lipid synthesis, potentially leading to lower lipid accumulation [29]. Compared to the control group, DOP+Spd treatment led to TG reductions of 37.68 % in *C. elegans*, highlighting its lipid-lowering efficacy (Fig. 4F).

3.8. The synergistic anti-aging effect of DOP and Spd in mice

Based on the metabolomic analyses conducted on *C. elegans*, the synergistic anti-aging effects of DOP and Spd appear to be linked to alterations in lipid metabolism (Fig. 5). The impact of a high-fat diet (HFD) on lipid and energy metabolism is well-documented, with established associations with obesity and metabolic disorders [30]. Recent studies have also explored the relationship between HFD and aging, notably finding the correlations between HFD, increased body weight, and accelerated epigenetic aging [31]. Consequently, metabolically compromised mice, induced by an HFD, were utilized to further investigate the synergistic anti-aging potential of DOP and Spd (Fig. 6A).

As shown in Fig. 6B, HFD led to a significant increase in body weight relative to the control group. Conversely, treatments with DOP only and the combined DOP and Spd (DS) significantly mitigated this weight gain, with the DS treatment demonstrating a more pronounced effect ($p < 0.05$). Regarding the blood biochemical parameters, compared to the model group, all three treatment groups notably decreased glucose and TG levels. However, reductions in low-density lipoprotein cholesterol (LDL-C) were observed only with DOP and DS treatments, and a decrease in total cholesterol (TC) levels was exclusive to the DS group ($p < 0.05$, Fig. 6C-G). These findings indicate that the DS combination manifested the most substantial improvements in glucose and lipid metabolism, surpassing the effects of treatments with either DOP or Spd alone.

4. Discussion

4.1. The synergistic effects of DOP and Spd on lifespan, oxidative and heat stress tolerance

The concept of synergy has been extensively investigated in the context of anticancer drug combination screening [6,32]. Jaaks et al. assessed the potency and efficacy of 2025 clinically relevant two-drug combinations, compiling a comprehensive dataset for 125 molecularly characterized breast, colorectal, and pancreatic cancer cell lines [6]. The scale and breadth of the study provide insights into combination response, revealing that synergistic outcomes are most probable when targeted agents are employed within molecularly defined patient

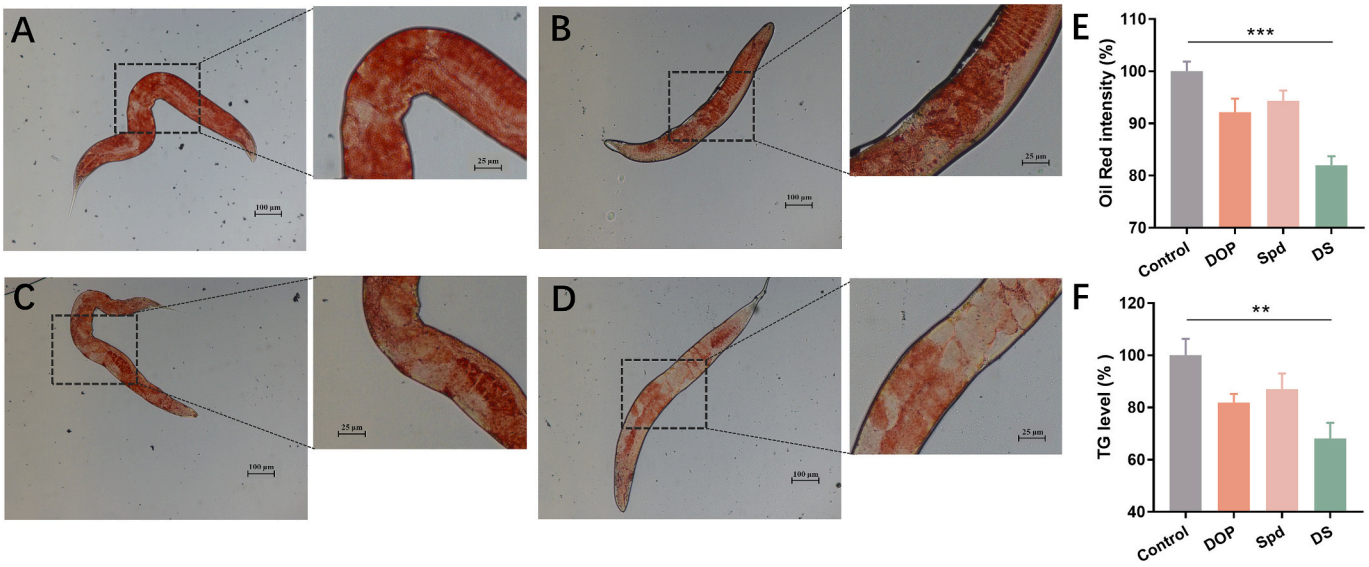

Fig. 4. Synergistic effect of DOP and Spd on the lipid level in *C. elegans*.
(A-D) Representative images of Oil Red O staining of worms in the control, DOP, Spd and DS groups, respectively; (E) Relative Oil Red O intensity; (F) TG levels. Statistical annotations indicate significant differences: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ relative to the model group.

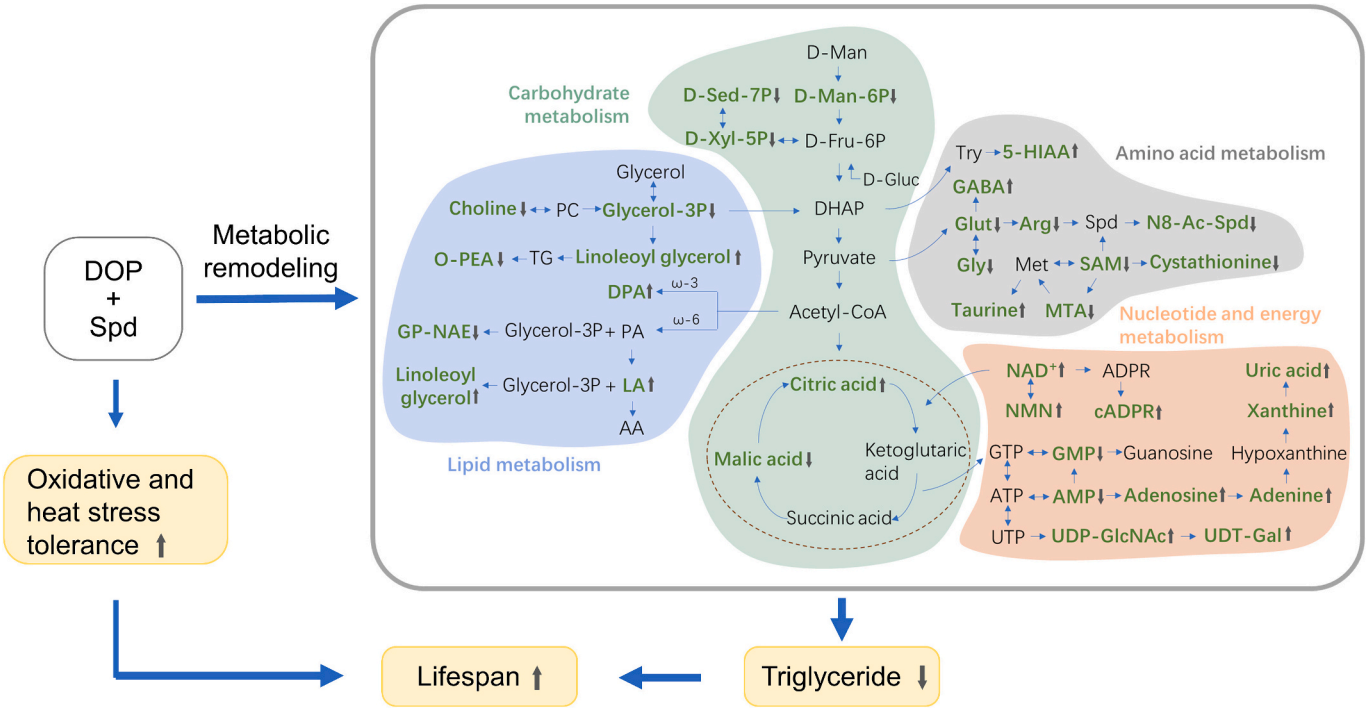

Fig. 5. The possible synergistic anti-aging mechanism of DOP and Spd.
GP-NAE, Glycerophospho-N-palmitoyl ethanolamine; UDP-GlcNAc, UDP-*N*-acetylglucosamine; O-PEA, O-Phosphorylethanolamine; SAM, S-Adenosylmethionine; MTA, 5′-S-Methyl-5′-thioadenosine; PC, Phosphatidylcholine; TG, Triglyceride; 5-HIAA, 5-Hydroxyindole-3-acetic acid; Sac, Saccharopine; DPA, Docosapentaenoic acid; PA, Palmitic acid; LA, Linoleic acid; AA, Arachidonic acid; DHAP, Dihydroxyacetone phosphate.

populations. Interestingly, drugs with weak or modest efficacy as single agents or those distanced by one or two nodes within a protein-protein interaction network frequently manifest synergistic interactions [6]. For example, it has been demonstrated that low doses of two CRMs, Spd and resveratrol, can synergistically induce autophagy through distinct mechanisms in *Saccharomyces cerevisiae*, *C. elegans* and mice [33].

Based on these findings, the investigation into the synergistic anti-aging effects of DOP and CRMs was proposed in *C. elegans*. After several screening experiments, Spd was selected for further synergistic anti-aging study. *C. elegans* has a short life cycle and is easy to culture, making it an ideal model organism for studying aging and anti-aging mechanisms. In our study, the CI values for lifespan extension, oxidative stress tolerance and heat stress tolerance were 0.65, 0.50 and 0.33, respectively. These values indicate varying degrees of synergy between DOP and Spd in these different assays. According to the criteria for CI, where a value <1.0 suggests synergy, the lower CI in oxidative and heat stress tolerance assays indicates a stronger synergistic effect compared to anti-aging assays.

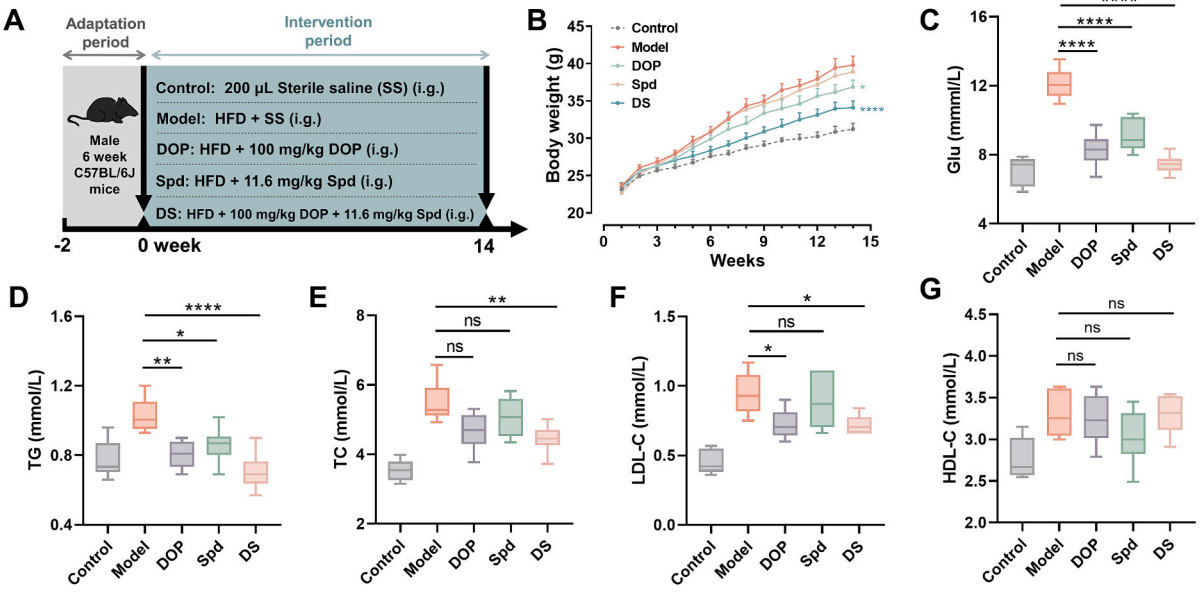

Fig. 6. The synergistic anti-aging effect of DOP and Spd in mice.
(A) The experimental design scheme for the mice study; (B) The body weight changes throughout the experiment; (C-G) The levels of Glu, TG, TC, LDL-C and HDL—C, respectively.
Statistical annotations indicate significant differences: *, $p<0.05$; **, $p<0.01$, relative to the model group.

The molecular mechanisms and signaling pathways involved in different stress responses are diverse. DS treatment might have a stronger activation effect on certain stress response pathways. Heat shock proteins (HSPs) play a key role in the heat stress response, and the DS treatment may significantly increase the expression of HSPs, thereby protecting cells from heat-induced damage more effectively. Antioxidant stress responses involve enzymes that clear reactive oxygen species (ROS), such as superoxide dismutase, catalase, and glutathione peroxidase. Previous studies have reported that DOP could extend the lifespan and combat obesity in *C. elegans* by enhancing the activities of these antioxidant enzymes [29,34]. DS administration may increase the activity of these antioxidant defense systems, but not as significantly as it enhances the activation of HSPs. Moreover, lifespan extension depends on multiple physiological processes, including metabolic regulation, gene expression, cellular repair and autophagy, rather than being driven by a single stress response [35]. DS treatment may have strong effects on specific pathways, such as increasing the expression of HSPs and antioxidant enzymes, but their overall impact on lifespan is moderated by the complexity of these processes.

4.2. *The synergistic effects of DOP and Spd on lipid metabolism*

The exploration of these anti-aging effects has seldom focused on metabolomic analysis. Therefore, it is imperative to decipher the molecular mechanisms through which DOP and Spd extend lifespan, particularly from a metabolomic perspective. Metabolome analysis during aging in *C. elegans* has revealed the top 10 altered pathways, mainly including the biosynthesis of amino acids, purine metabolism, carbon metabolism, cysteine and methionine metabolism, fructose and mannose metabolism, glycerolipid metabolism, and glycerophospholipid metabolism [36]. Our comprehensive metabolomic data analysis suggests that the synergistic anti-aging effect of DOP and Spd in *C. elegans* is associated with several metabolic pathways, including glycerolipid metabolism, glycerophospholipid metabolism, glyoxylate and dicarboxylate metabolism, purine metabolism, butanoate metabolism, and amino sugar and nucleotide sugar metabolism, which are primarily involved in the regulation of lipid, nucleotide, energy and carbohydrate metabolism. These findings are congruent with prior research, underscoring the multifaceted nature of their anti-aging mechanisms and highlighting the necessity for further investigation into their combined effects.

In our study, levels of glycerol-3-phosphate (glycerol-3P), linoleoyl glycerol, choline, and special fatty acids showed significant changes in *C. elegans* in the DS group compared to the groups treated solely with DOP or Spd. These results suggest that coadministration of DOP and Spd has synergistic effects on lipid metabolism. Linoleoyl glycerol, a molecule combining linoleic acid with glycerol, exhibits significant positive effects, especially in lipid metabolism [37]. The decrease of choline and glycerol-3P levels may confer advantageous effects within specific physiological contexts, including in relation to aging and metabolic health. Diminished glycerol-3P levels could reduce substrate utilization for TG synthesis, promoting lipid homeostasis and bolstering metabolic wellness [38], thus potentially extending lifespan and attenuating age-related metabolic dysfunctions. In contrast, diets enriched in choline have been implicated in the promotion of atherosclerosis in murine models [39]. Additionally, the results of the POUNDS Lost Trial indicated that choline reduction was significantly predictive of reductions in body fat composition, fat distribution, and resting energy expenditure among overweight and obese participants [40]. Hence, a lower choline level may contribute to healthier aging, effective weight management, and improved cardiovascular health.

Fatty acids, encompassing both saturated and unsaturated types, are pivotal for human health. Saturated fatty acids, predominantly comprising palmitic, stearic, and arachidic acids, when excessively accumulated, are implicated in the pathogenesis of atherosclerosis and cardiovascular diseases [41]. In contrast, unsaturated fatty acids play beneficial roles in metabolic health. Specifically, linoleic acid, an ω-6 double unsaturated fatty acid (DUFA), has been reported to lower total, LDL, and VLDL cholesterol levels, alongside serum TG, while elevating HDL cholesterol levels [42]. Docosapentaenoic acid (DPA) is an ω-3 polyunsaturated fatty acid (PUFA). Studies have shown that in older adults, with an average age of 74.4 years, elevated levels of DPA in circulation were linked to the increased likelihood of healthy aging [43], and higher DPA levels correlated with a reduced risk of premature mortality [44]. Sun et al. demonstrated that the anti-aging effects of polysaccharides extracted from ginsenoside residues (GRP) are closely

associated with fatty acid metabolism in *C. elegans*, as revealed by microbiome and transcriptomic analyses [20]. Post-GRP treatment, an enhancement in fatty acid degradation was observed in *C. elegans*, leading to the preferential biosynthesis of beneficial fatty acids, such as ω-3 PUFAs.

The TG content showed significant changes in *C. elegans* between the control and DS groups when measured directly. However, no significant change was found in the metabolome analysis. Several possible reasons could explain this discrepancy: 1) direct biochemical assays for TG are often more sensitive and specific than global metabolomics approaches, which may detect a broad range of metabolites but with lower sensitivity for specific compounds; 2) the efficiency of lipid extraction in metabolomics protocols might not be optimal for triglycerides, leading to an underestimation of their levels.

4.3. The synergistic effects of DOP and Spd on energy, nucleotide and other metabolism

D-Mannose-6-phosphate (D-Man-6P) is the intermediate in the mannose metabolic pathway, while D-Sedoheptulose 7-phosphate (D-Sedoheptulose-7P) and D-Xylulose-5-phosphate (D-Xyl-5P) are intermediate in the pentose phosphate pathway and can be further linked to the glycolytic pathway through its conversion to D-Fructose-6-phosphate (D-Fru-6P) and then metabolized to pyruvate. Pyruvate is a central metabolite in metabolism that can enter the citric acid cycle via acetyl-CoA formation, which is a vital link between glycolysis and the TCA cycle. In our study, relative to groups treated solely with DOP or Spd, levels of D-Man-6P, D-Sedoheptulose-7P, D-Xyl-5P, and D-Fru-6P were notably reduced in the DS group. These observations suggest that concurrent administration of DOP and Spd enhances the flux through both the pentose phosphate pathway and glycolysis, subsequently facilitating increased flux into the TCA cycle, and resulting in augmented production of citric acid, highlighting the synergistic effect of DOP and Spd on central carbon metabolism.

Nucleotide metabolism plays a pivotal role in the aging process, influencing both cellular energy homeostasis and genomic stability. The availability of nucleotides is essential for optimal mitochondrial function, which is central to energy production and is intricately linked with the aging process. Additionally, the role of nucleotide synthesis pathways in extending lifespan has been highlighted, demonstrating that enhancing nucleotide metabolism and digestion can ameliorate age-related phenotypes and promote longevity [45,46]. Uric acid, as the end metabolite in purine degradation, manifests dichotomous health impacts. Elevated concentrations of uric acid can precipitate conditions such as gout, nephrolithiasis, and cardiovascular pathologies. Conversely, uric acid inherently functions as an antioxidant, mitigating oxidative stress. Thus, a slight elevation in uric acid levels has been shown to diminish H_2O_2-induced oxidative damage in the gastrointestinal tract via the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [47].

N-acylspermidines are conserved metabolites downstream of mitochondrial SIRT that facilitate annotation of SIRT enzymatic activities in vivo and may contribute to SIRT-dependent phenotypes [48]. After exogenous Spd treatment, N8-acylspermidine was detected, but with no significant changes in *C. elegans* embryos [49]. However, in our study, N8-acylspermidine was significantly increased in *C. elegans* in Spd and DOP alone group, compared with those of the control group, indicating that Spd and DOP would promote the production of N8-acylspermidine. S-adenosyl-L-methionine (SAM) is the universal methyl donor in eukaryotic cells, and it is capable of acting as a cofactor in the transfer of methyl groups to DNA, RNA, proteins, and lipids [50]. DNA methylation is closely linked to the aging process and has emerged as a valuable tool for age prediction with an absolute median error of 2.77 years [51]. Thus, the observed decrease in SAM levels among worms in the DS group suggests a potentially positive impact on slowing down the aging process.

4.4. The synergistic effects of DOP and Spd on the signaling pathway related to aging

Because longevity-associated signaling pathways are highly conserved, similarity can be observed between these pathways in *C. elegans* and higher mammals, such as rodents and humans [26]. About 83 % of the proteins in the *C. elegans* proteome have homologs in humans [52]. It has been reported that multiple signaling pathways, such as the mitochondrial signaling pathway, IIS pathway, heat shock transcription factor, MAPK pathway, dietary restriction pathways, and mitochondrial pathways, play important roles in regulating aging and stress resistance in *C. elegans* [52]. Numerous genetic and molecular mechanisms underpinning the anti-aging effect of DOP or Spd have been documented. Notably, previous studies have identified that 29.0 mg/L of Spd promotes anti-aging effects primarily through the induction of autophagy [33], while DOP primarily exerts its anti-aging influence by modulating the antioxidant enzyme and NRF2 antioxidant signaling pathway [13,34]. The distinct mechanisms of action for DOP and Spd suggest the potential for synergistic anti-aging effects, which, to date, remain underexplored.

The IIS pathway is an evolutionarily conserved mechanism involved in the regulation of longevity and metabolism across different species [26]. In *C. elegans*, several genes are involved in the IIS pathway: *daf-2* encodes a receptor tyrosine kinase homolog, and *daf-16* is an ortholog of human FOXO1, which encodes DAF-16 [53–55]. DAF-16 further activates the expression of numerous anti-stress and longevity genes, such as *sod-3*, and *ctl-1*, *hsp-16.2*, which eventually enhance the anti-aging ability of *C. elegans* [26]. The anti-aging effect of various polysaccharides has been linked to these pathways. For example, *Lonicera japonica* polysaccharide (LJP) has been shown to prolong the lifespan and healthspan of *C. elegans* via DAF-16. LJP also upregulates the expression of *daf-16* and its downstream target genes, including *sod-3*, *gst-4* and *hsp-16.2* [56]. Moreover, *Polygonati Rhizoma* polysaccharide (PRP) may prolong the lifespan of *C. elegans* by down-regulating *daf-2* and activating *daf-16* and *sod-3* [57]. Transgenic nematode experiments corroborate these findings, suggesting that the age-delaying effects of PRP are related to the *daf-2*, *daf-16*, and *sod-3* genes within the insulin signaling pathway.

SIRTs, notably SIRT1, have been extensively studied for their role in extending lifespan, preventing aging-related diseases and maintaining metabolic homeostasis [58]. SIRT1-mediated deacetylation could activate or inhibit FOXO, and selectively directing FOXO to specific targets [58]. The *sir2.1* gene, an ortholog of human SIRT1, plays a crucial role in regulating longevity-related genes in *C. elegans*, including the activation of DAF-16 [26]. Eisenberg et al. found that the ability of Spd to extend lifespan was not abrogated in *C. elegans* following the deletion of *sir2* or other SIRT genes, indicating that the lifespan extension effect of Spd is independent of *sir2* [59]. However, in our study, the DS treatment was identified to activate *sir2.1*, suggesting a potential interaction with this pathway. The mRNA expression of *sir2.1* and *daf-16* was upregulated in *C. elegans* in the DOP and DS groups, while the mRNA expression of *daf-2* and *hsp-16.2* showed significant changes in the Spd group. These results suggest that the synergistic anti-aging effects of DOP and Spd may be due to their anti-aging effects being mediated through different pathways.

4.5. The relationship between the aging signaling pathway and the metabolic changes

The relationship between the signaling pathway related to aging and the metabolic changes has been reported. For example, Gao et al. conducted comprehensive metabolomic analyses on extended-lifespan nematode strains, notably *daf-2* mutants characterized by attenuated IIS, and *eat-2* mutants serving as models of caloric restriction, in comparison to the wild-type N2 strain. The findings highlighted a pronounced enhancement in purine metabolism within these long-lived

strains, evidenced by elevated concentrations of adenine, xanthine, and adenosine monophosphate (AMP) [46].

SIRT1 was associated with fat and energy metabolism and insulin sensitivity [58]. For example, in differentiated adipocyte cell lines, SIRT1 inhibits adipogenesis and enhances fat mobilization through lipolysis by suppressing the activity of PPARγ [60]. Moreover, Feige et al. found that SIRT1 activation protected mice from diet-induced obesity by increasing the rate of fatty acid oxidation [61]. Nicotinamide adenine dinucleotide (NAD^+) is a coenzyme for SIRT protein deacetylases. Synthesis of NAD^+ decreases during aging, which is thought to limit the activity of enzymes that require it for their catalytic activity [62]. Replenishment of cellular NAD^+ has been shown to exert positive effects on aging and related diseases. For example, NAD^+ administration has been reported to ameliorate aging-induced damage to the blood-brain barrier through the CX43-PARP1 axis [63] and to restore female reproductive capacity in the context of reproductive aging [64]. NMN, a precursor in NAD^+ biosynthesis, has been recognized for its ability to boost cellular NAD^+ synthesis, thereby addressing age-related conditions linked to reduced NAD^+ concentrations in tissues [45]. NMN supplementation has been observed to reverse intestinal aging attributed to mitochondrial DNA mutations in aged male mice [65], highlighting the potential of increased NAD^+ levels in activating SIRT-mediated longevity pathways. Additionally, cyclic ADP ribose has been shown to activate SIRT1 and the mechanistic target of rapamycin complex 1 (mTORC1) signaling in intestinal stem cells [66].

4.6. The future commercial applications

As the global market for anti-aging products continues to expand rapidly, translating the findings of anti-aging research into commercially viable products is of paramount importance. A market research report from Exactitude Consultancy showed that the global market for anti-aging products was valued at USD 48.6 billion in 2022 and is projected to grow at a compound annual growth rate (CAGR) of approximately 7.2 % from 2023 to 2030 (https://exactitudeconsultancy.com/reports/40921/anti-aging-supplements-market). The most direct application of DOP and Spd is in dietary supplements and functional foods. However, before these applications can be realized, future research must focus on conducting comprehensive animal and human clinical trials to validate the efficacy and safety of these supplements.

In addition to the biological efficacy, the economic aspect of the two ingredients is also an important consideration. The results showed that the effect of 250 mg/L DOP and 29.0 mg/L Spd on extending lifespan under oxidative and heat stress conditions was similar to that of 750 mg/L DOP and 72.5 mg/L Spd alone in *C. elegans* (data not shown). This combination approach results in a cost reduction of 66.7 % compared to DOP alone and 60 % compared to Spd alone. Therefore, the synergistic anti-aging effects of DOP and Spd not only enhance biological efficacy but also offer economic advantages. This dual benefit underscores the potential of combining these compounds for the development of cost-effective, highly effective anti-aging supplements. Moreover, climate change is a universal issue, and numerous recent studies have adopted sustainable and green industrial practices to reduce greenhouse gas emissions [67–75]. Considering the industrial production, it is necessary to make the DOP and Spd extraction processes more efficient. This includes reducing energy consumption and minimizing greenhouse gas emissions through novel technologies or methods.

5. Conclusion

The combination of 250 mg/L DOP and 29.0 mg/L Spd had a synergistic impact on extending the lifespan in *C. elegans* with a CI of 0.65. Subsequent metabolomic analyses revealed significant changes in metabolites associated with lipid, nucleotide and energy metabolism, specifically glycerol 3-phosphate, linoleoyl glycerol, β-nicotinamide mononucleotide, nicotinamide adenine dinucleotide and citric acid, between the group receiving the combined DOP and Spd treatment and the groups treated with each compound separately. The effects of DS on lipid metabolism were further validated in *C. elegans* and high-fat diet-induced mouse model. Consequently, it can be concluded that the combination of DOP and Spd presents a novel and synergistic approach to anti-aging, with the synergistic effects likely being mediated primarily through alterations in lipid metabolic pathways. The potential of combining these two compounds would promote the development of cost-effective, highly effective anti-aging supplements. However, these results are preliminary and require further validation through clinical trials in humans.

Longevity is a complex, multifactorial phenomenon influenced by metabolite signals. Many questions remain unanswered, such as the efficient and rapid screening of synergistic anti-aging substance combinations, and the expansion of the repertoire of pro-longevity signaling metabolites, alongside an understanding of their regulatory mechanisms. Moreover, translating these natural chemical processes to benefit human health opens an exciting new frontier for research. The discovery of additional aging- and longevity-regulatory metabolites through advancements in metabolomics, supported by AI-driven chemical annotation, holds significant promise. Innovative approaches in chemical proteomics and imaging with functional genetics and genomics will provide robust platforms for detailed mechanistic studies. Future studies in this rapidly evolving field will be crucial for developing next-generation phytochemicals and metabolites, or their combinations, aimed at combating aging and age-related diseases, as well as formulating nutraceutical strategies to promote healthy aging.

CRediT authorship contribution statement

Hui Duan: Writing – original draft, Methodology, Data curation. **Qun Yu:** Investigation, Funding acquisition. **Yang Ni:** Funding acquisition, Data curation. **Jinwei Li:** Writing – review & editing, Supervision. **Leilei Yu:** Writing – review & editing, Supervision. **Xiaowei Yan:** Supervision, Funding acquisition. **Liuping Fan:** Supervision, Funding acquisition.

Declaration of competing 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.

Acknowledgments

This work was supported by the key research and development program of Xinjiang Autonomous Region (2022B02026-5), the Chongzuo Science and Technology Project (2021021479), Jiangsu Province Excellent Postdoctoral Program (2023ZB662), the National Natural Science Foundation of China (32202026, 32302117) and China Postdoctoral Science Foundation (2024M751157).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.135098.

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