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Fava bean and pea protein hydrolysates modulate stress responses in *C. elegans* through different mechanisms

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The growing global demand for protein, combined with the urgent need for effective strategies to manage conditions such as obesity and diabetes, highlights legume proteins as valuable sources of derived bioactive peptides with health-promoting properties. In this study, we employed the *Caenorhabditis elegans* model to investigate the effects of supplementation with hydrolysates derived from fava bean and pea protein on healthspan. Supplementation with fava bean and pea protein hydrolysates reduced fat accumulation and age-related lipofuscin pigment in the worms, without impairing their development. The fava bean protein hydrolysate significantly decreased total reactive oxygen species levels and enhanced stress tolerance to juglone exposure, suggesting the modulatory activity of the mitochondrial oxidative stress response. In contrast, pea protein hydrolysate improved the heat stress resistance of *C. elegans*, and gene expression and mutant analyses revealed the involvement of the endoplasmic reticulum unfolded protein response (ER-UPR) pathway in mediating its health-promoting effects. Together, our data demonstrate that fava bean and pea protein hydrolysates support healthspan in *C. elegans* by modulating distinct cellular stress response pathways and pave the way for further investigation in more complex animal models.

1. Introduction

The growing global demand for protein makes it necessary to look for environmentally friendly alternatives to animal-based sources.[1] In parallel, the alarming rise in obesity and type 2 diabetes highlights the importance of exploring protein sources as potential beneficial strategies for weight loss and obesity management by positively influencing metabolism, appetite, and body composition.[2]

Bioactive peptides are short chains of amino acids (2–15 residues) that have emerged as promising agents in combating oxidative stress and cellular aging—two key processes involved in the maintenance of metabolic health, the disruption of which significantly influences the development of diseases such as obesity and insulin resistance.[3–5] These peptides, released during protein digestion or food processing, exhibit strong antioxidant properties by scavenging free radicals and enhancing the activity of endogenous antioxidant enzymes. By reducing oxidative damage to cells and tissues, they help to preserve mitochondrial function and reduce chronic low-grade inflammation, both of which are closely linked to metabolic dysfunction.[5] Additionally, some peptides can modulate signalling pathways related to aging and metabolic regulation, potentially improving insulin sensitivity and preventing adipose tissue dysfunction.[6] Specifically, bioactive peptides have been shown to interact with cellular stress-response networks by activating antioxidant defence systems, mainly through the modulation of major oxidative protein pathways while also alleviating endoplasmic reticulum stress.[7,8] Accordingly, supplementation with sources of bioactive peptides, such as protein hydrolysates from different origins, has emerged as a valuable nutritional strategy for mitigating oxidative stress, promoting healthy aging and reducing the risk of metabolic disorders.[5] In this regard, legumes have been widely studied due to their high protein content compared to other

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plant-based foods. However, their importance lies not only in their nutritional value but also in their ability to generate, after hydrolysis, bioactive peptides with beneficial effects on metabolism.[9] In this context, pea (*Pisum sativum*) and fava bean (*Vicia faba*) proteins have gained particular attention as sources of bioactive peptides. Previous studies have shown that enzymatic hydrolysis of their proteins releases peptides with antioxidant and metabolic regulatory activities.[10–12]

In vivo models are essential for investigating the effect of bioactive peptides on the modulation of different signalling pathways and their interconnections.[13] In this context, *C. elegans* is a simple and well-established organism widely used to investigate the effect of different bioactive compounds on diverse signalling pathways, including those involved in lipid and carbohydrate metabolism, as well as cellular stress responses. Although this model has been employed to evaluate the effect of supplementation with specific peptides derived from protein hydrolysates,[14,15–17] little is known about the functional impact of legume-derived protein hydrolysates in this *in vivo* model.[18]

The objective of this work was to evaluate the potential bioactivity of fava bean and pea protein hydrolysates on lipid accumulation, oxidative stress, stress resistance and senescence using *C. elegans* as an experimental model. Gene expression and mutant analyses were performed to elucidate the possible mechanisms of action of each specific protein hydrolysate.

2. Experimental

2.1. Fava and pea protein hydrolysates

Pea and fava bean hydrolysates were obtained from the corresponding pea protein (47.8% protein) and fava bean (60.4% protein) concentrates, which were supplied by Grupo AN, S. Coop. (Navarra, Spain) and Sanygran (Navarra, Spain) and previously described by Uriz-Martínez *et al.* (2025).[19] Both concentrates were subjected to controlled enzymatic hydrolysis. The specific conditions of the method are proprietary and protected by industrial property rights.

The obtained hydrolysates were analysed for proximate composition. Protein, moisture, ash and fat were determined according to AOAC official methods[20] and carbohydrates were calculated by difference. To ensure correct hydrolysis SDS-PAGE electrophoresis (12.5% polyacrylamide) was performed.

2.2. Amino acid composition

Pea and fava bean protein hydrolysates were subjected to acid hydrolysis with 37% HCl, incubated in an oven at 105 °C for one day, and subsequently neutralized with 33% NaOH to pH 6–7. The amino acid composition was determined using a Waters™ Kairos™ Amino Acid Kit – Low Throughput. An initial protein precipitation step was carried out with 0.1% formic acid in methanol, followed by the protocol specified in the kit. The samples were then analysed by ultra-high-performance liquid chromatography (ACQUITY UPLC H-class, Waters) coupled with a quadrupole ion trap mass spectrometer (QTRAP 5500, SCIEX) (LC-MS), operating in positive electrospray ionization (ESI+) mode using MRM. Chromatographic separation was performed at 55 °C on a CORTECS C18 2.1 × 150 mm, 1.6 μm column (Waters). A gradient of water (mobile phase A) and acetonitrile (mobile phase B), both containing 0.1% formic acid, was used at a flow rate of 0.5 mL min^{-1}. Amino acid composition was expressed as a percentage of the total amino acids.

2.3. ABTS scavenging assay

ABTS scavenging activity was calculated as described by Memarpoor-Yazdi *et al.* (2012).[21] Briefly, 18 μL of protein hydrolysate samples at different concentrations (1–5 mg mL^{-1}) were mixed with 182 μL of ABTS (with absorbance previously adjusted to 0.7). After 6 minutes, the absorbance was recorded at 734 nm. A standard calibration curve was constructed using Trolox, and the results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC, μmol L^{-1} Trolox equivalents).

2.4. *C. elegans* strains

The strains used were as follows: N2 Bristol as a wild-type strain, and SJ17 zcls4 [*hsp-4*::GFP] V. *xbp-1* (zc12) and SJ30 *ire-1* (zc14) zcIs4 [*hsp-4*::GFP] V as mutant strains. All the strains were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA). *Escherichia coli* OP50 growth in LB Broth Lennox medium at 37° C was used as the standard food source.

2.5. Nematode culture and experimental design

Synchronized L1 larvae were seeded onto 6-well plates containing 4 mL of nematode growth medium (NGM) per well, with four replicates per condition. The experimental groups consisted of either control plates (NGM control, with water as a vehicle) or plates supplemented with pea or fava bean protein hydrolysates (doses: 0.5, 1.0 and 2.0 mg mL^{-1}). Doses were selected based on previous work from our group.[22] Prior to seeding the worms, 150 μL of overnight cultured *E. coli* OP50 was spread on the plates and kept at room temperature until dry. In all assays, nematodes were age-synchronized by standard hypochlorite treatment. Eggs were then hatched overnight in M9 medium at 20 °C. Approximately, 300 L1 larvae were transferred to each NGM well and grown for two days until the L4 early adult stage was reached, at which the subsequent experiments were performed.

2.6. Nile red staining

Nile red staining is a technique to quantify neutral lipids.[23] In brief, L4 worms exposed to the described treatments were collected in 1.5 mL tubes and washed with PBST (0.01% of Triton X-100 in phosphate buffered saline) twice. Subsequently, the worms were placed on ice for 15 minutes and fixed with 40% isopropanol for 3 minutes. Afterwards, the worms were stained by adding 150 μL of Nile red solution (3 μg mL^{-1}, #N3013, Sigma-Aldrich, St Louis, MO, USA), followed by incubation for 30 minutes at 20 °C in the dark. Finally, the worms were washed and mounted in 2% agarose pads for further image analysis.

2.7. Lipofuscin pigment

Lipofuscin pigment levels in worms were quantified as a biomarker of aging.[24] Briefly, L4 worms exposed to the different treatments were collected, washed and mounted in 2% agarose pads with 1% of sodium azide for further image analysis.

2.8. DHE staining

ROS levels of worms were quantified by dihydroethidium (DHE, BioReagent, ≥95% (HPCE), Sigma-Aldrich, St Louis, MO, USA) staining.[24] L4 worms were collected, washed and stained by the addition of a μmol L^{-1} DHE solution in PBST for 2 hours. Finally, the worms were washed and mounted in agarose pads (2%) with 1% sodium azide for further image analysis.

2.9. SJ30 ire-1(zc14) II; zcIs4 V. assay and SJ17 zcls4 [hsp-4::GFP] V. xbp-1 (zc12) assays

Age-synchronized L1 *ire-1* and *xbp-1* mutant worms were transferred to 6-well NGM plates previously treated with or without pea protein hydrolysates and incubated until they reached the L4 larval stage at 20 °C. Then, the worms were collected, washed, and mounted on 2% agarose pads with 1% sodium azide for further image analysis.

2.10. Egg laying

The correct development of the nematodes under the influence of different doses of fava and pea protein hydrolysates after three days of growth from the L1 larvae stage was ensured. The influence of the protein hydrolysates, both fava and pea, over the size of young adults and nematode egg laying was checked on NGM plates. The images were taken at 40× magnification using a Nikon SMZ18 stereomicroscope equipped with a Nikon DS-Fi1C high-definition colour camera.

2.11. Motility assay

Age-synchronized N2 wild type worms were transferred to 35 mm NGM plates supplemented with fava or pea protein hydrolysates and when they reached the L4 stage, their motility was studied using a WMicrotracker Smart ×8 device. The average speed and travelled distance were quantified.

2.12. Image acquisition and quantification

Images of Nile red and SJ17 mutant assays were captured at 10× magnification using a Nikon SMZ18 research stereomicroscope equipped with an *epi*-fluorescence system and a DS-FI1C refrigerated colour digital camera (Nikon Instruments Inc., Tokyo, Japan). Images were taken under the same conditions and at the integration time under a GFP filter (Ex 480–500; DM 505; and BA 535–550). Dihydroethidium (DHE)-labelled ROS formation and lipofuscin autofluorescence were detected by measuring the fluorescence intensity using a Nikon Eclipse 80i *epi*-fluorescent microscope, equipped with a TRITC filter (Ex 540–625; DM 565; and BA 605–655) and a DAPI filter (with excitation at 340–380 nm and emission at 435–485 nm), respectively (Nikon Instruments Inc., Tokyo, Japan). Image analysis was performed using ImageJ v1.53e software. The mean value, calculated as the fluorescence mean value per pixel, together with the integrated density and the volume of the worms, was determined. Approximately 30 worms were examined in four independent experiments for each condition.

2.13. Stress resistance assays

Stress resistance assays were conducted using a WMicrotracker SMART ×8 system. For the thermal stress assay, L4 stage worms were incubated at 33 °C in 35 mm plates and the average speed and travelled distance parameters were determined every 30 minutes for 6 hours. For chemical stress, L4 stage worms were incubated in 35 mm NGM agar with juglone (240 μmol L^{-1}) for 6 hours and their average speed and travelled distance were scored every 30 minutes.

2.14. ER stress assay

Worms were allowed to grow until the L4 stage and subsequently transferred to fresh 6-well agar plates with 50 μg mL^{-1} tunicamycin for six hours. Afterwards, the worms were collected and frozen in the TRIzol RNA isolation reagent (Thermo Fisher Scientific, Paisley, UK) prior to RNA extraction.

2.15. RNA extraction and quantitative PCR analysis

For gene expression analyses, approximately 800 worms were used per replicate, with 4 replicates per condition. The extraction of total RNA from the *C. elegans* N2 strain was done using the Trizol RNA isolation reagent (Thermo Fisher Scientific, Paisley, UK). The concentration and purity of RNA was determined using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). Afterwards, 1500 ng of RNA was treated with DNAse (Ambion DNase I; Thermo Fisher Scientific Inc., Waltham, MA, USA) and DNA-free RNA was reverse-transcribed into cDNA. Gene expression analyses were performed by quantitative real-time PCR (qPCR) using the TaqMan Universal PCR master mix and specific probes from Applied Biosystems Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA) and Integrated DNA Technologies Inc. (Coralville, IA, USA). The expression level of each gene was normalized compared to the expression of the housekeeping gene *pmp-3*. Gene expression differences between the control and supplemented worms were quantified using the relative quantification $2^{-\Delta\Delta Ct}$ method.

2.16. Statistical analysis

The antioxidant capacity (ABTS) was evaluated between pea and fava bean protein hydrolysates using Student's *t*-test. Body fat reduction (Nile red), together with oxidative stress and lipofuscin determination, and real-time PCR data between treatment and control groups (NGM) were evaluated using a one-way ANOVA test, followed by the Student–Newman–Keuls test or Fisher's LSD *post-hoc* test. In the motility assay, stress resistance assays and assays with mutants (SJ17 and SJ30) supplemented worms were compared to the NGM group using Student's *t*-test. All the statistical analyses were performed

using Stata/SE version 17.0 (StataCorp LLC, College Station, TX, USA), under an academic license. Experiments were performed in quadruplicate.

3. Results and discussion

3.1. Fava bean and pea protein hydrolysates exhibit differential amino acid compositions and *in vitro* antioxidant capacity

Protein hydrolysates derived from fava bean and pea protein concentrates contained 71.7% and 71.6% protein, respectively, with moisture contents of 11.1 and 9.0%, ash contents of 9.4 and 8.2%, fat contents of 1.6 and 3.7%, and carbohydrate contents of 6.2 and 7.5%.

SDS-PAGE analysis revealed comparable peptide profiles for fava bean and pea protein hydrolysates (Fig. S1). The electrophoresis showed the presence of a major band at approximately 5 kDa and the absence of bands corresponding to high molecular weight proteins. These results confirm that enzymatic hydrolysis resulted in consistent production of low molecular weight peptides in both samples. Regarding amino acid composition, a total of 18 amino acids were analysed (Table 1). The highest value of both protein hydrolysates was observed for glutamic acid (22.04% and 26.42 for fava bean and pea protein hydrolysates, respectively). The next more abundant were alanine (13.16%), lysine (8.82%), arginine (8.24%) and leucine (7.07%) in fava bean protein hydrolysate and arginine (15.17%), leucine (8.81%), lysine (6.76%) and phenylalanine (5.04%) in pea protein hydrolysate. The least abundant were tryptophan, methionine and threonine in both cases. The content of aromatic amino acids in the fava bean hydrolysate (6.32%) was practically the same as that in the pea hydrolysate (7.34%). Regarding essential amino acids, the content was similar in pea hydrolysate (29.43%) compared to fava bean hydrolysate (30.72%). Both protein sources were deficient in tryptophan, consistent with the typical amino acid profile of legumes.[25] The amino acid profiles of the fava bean hydrolysate are in line with previously reported data, particularly in terms of the high abundance of glutamic acid and arginine, although higher levels of alanine were observed, while lower levels of certain amino acids such as aspartic acid and glycine were detected compared to values described in the literature. In the case of pea protein hydrolysate, the profile also agrees with literature values, showing a similar predominance of glutamic acid together with relevant amounts of arginine and leucine, but again displaying lower levels of aspartic acid.[26,27]

Several amino acids have been recognized for their antioxidant potential, not only due to their intrinsic redox activity but also because of their ability to modulate the antioxidant signalling pathways *in vivo*. In this context, glutamic acid, which is abundant in both hydrolysates is known to modulate the NRF2 pathway and enhance the expression of antioxidant genes including superoxide dismutase (SOD). In addition, other abundant amino acids, including arginine and lysine, contribute to antioxidant activity through their intrinsic redox properties and have also been reported to influence again the NRF2 signalling pathway, suggesting that the amino acid composition of these hydrolysates could play a key role in their overall antioxidant potential.[28,29] However, the antioxidant activity of plant protein-based hydrolysates is not only determined by their amino acid composition, but also by the molecular weight distribution of the resulting peptides.[30] Accordingly, we aimed to evaluate and compare the ABTS radical scavenging activity of our fava bean and pea protein hydrolysates across a range of concentrations (Table 2). Although no ABTS scavenging activity was observed at concentrations lower than 4 mg mL^{-1}, both hydrolysates exhibited a

Table 1 Amino acid composition of fava bean and pea protein hydrolysates

AA (%)	Fava bean protein hydrolysate	AA (%)	Pea protein hydrolysate
Glutamic acid	22.04	Glutamic acid	26.42
Alanine	13.16	Arginine	15.16
Lysine	8.82	Leucine	8.81
Arginine	8.24	Alanine	8.42
Leucine	7.07	Lysine	6.76
Aspartic acid	6.58	Phenylalanine	5.04
Serine	6.30	Proline	4.74
Valine	6.10	Glycine	4.68
Glycine	4.65	Aspartic acid	3.89
Phenylalanine	4.02	Valine	3.57
Cystine	3.51	Serine	3.13
Proline	2.49	Tyrosine	2.30
Tyrosine	2.30	Isoleucine	2.01
Isoleucine	1.78	Cystine	1.83
Histidine	1.75	Histidine	1.65
Threonine	0.88	Threonine	1.19
Methionine	0.29	Methionine	0.41
Tryptophan	<LOQ	Tryptophan	0.01
EAA	30.72	EAA	29.43
HAA	37.22	HAA	35.30
PCAA	10.57	PCAA	8.40
NCAA	28.62	NCAA	30.31
AAA	6.32	AAA	7.34
BCAA	14.95	BCAA	14.39
SCAA	3.80	SCAA	2.24

Values are expressed as % of total amino acids. EAA: Essential Amino Acids; HAA: Hydrophobic Amino Acids; PCAA: Positively Charged Amino Acids; NCAA: Negatively Charged Amino Acids; AAA: Aromatic Amino Acids; BCAA: Branched Chain Amino Acids; and SCAA: Sulfur Containing Amino Acids.

Table 2 ABTS scavenging activity of fava bean and pea protein hydrolysates at different doses (1–5 mg mL^{-1})

Concentration	Fava bean protein hydrolysate	Pea protein hydrolysate	*p* value
1 mg mL^{-1}	n.d	n.d	ns
2 mg mL^{-1}	n.d	n.d	ns
3 mg mL^{-1}	n.d	n.d	ns
4 mg mL^{-1}	75.43 (12.60)	59.45 (8.46)	ns
5 mg mL^{-1}	137.02 (15.04)	73.77 (10.21)	0.007

ns: not significant. Results are expressed as Trolox Equivalent Antioxidant Capacity (TEAC, μmol L^{-1} Trolox equivalents). Mean ± SEM. Statistical analyses were performed using Student's *t*-test.

positive effect at the doses of 4 and 5 mg mL^{-1}, demonstrating their ability to neutralize reactive oxygen species (ROS). Interestingly, fava bean protein displayed a significantly higher scavenging activity mg mL^{-1} at the highest dose (5 mg mL^{-1}) as compared to the pea protein, suggesting a certain antioxidant *in vitro* effect. This is consistent with the ABTS scavenging activity of pea protein hydrolysates described by Olagunju *et al.* (2018).[31] In the case of fava bean hydrolysates, although, to the best of our knowledge, this has not been previously reported, there are several studies that report the ABTS scavenging activity of different peptides derived from fava bean protein.[32,33]

One of the reasons for the different *in vitro* antioxidant capacity could be explained by the different amino acid profile. However, the difference between the hydrolysates is not particularly pronounced, suggesting that the enhanced antioxidant capacity of fava bean protein hydrolysates might be explained by other mechanisms. Further investigation is therefore needed to explain these differences.

3.2. Fava bean and pea protein hydrolysates reduce fat accumulation and improve longevity in *C. elegans*

Peptides derived from legumes (including fava bean and pea) have been shown to influence metabolism, specifically fat accumulation and oxidative stress.[34] However, only a limited number of studies have examined their activity *in vivo*, which opens the door to explore the effects of fava bean- and pea-derived protein hydrolysates in *C. elegans*. This model allows the study of different parameters related to metabolism, such as fat accumulation, senescence, and oxidative stress.

Fat accumulation in *C. elegans* can be assessed using lipophilic dyes such as Nile red, which stain lipid droplets and therefore, they can be quantified by microscopy and image analysis. A significant reduction ($p < 0.05$) in fat accumulation was noticed in the worms supplemented with both pea and fava bean protein hydrolysates at all tested concentrations, as compared to control worms (NGM) (Fig. 1A and B). Specifically, fava bean protein hydrolysate supplementation at 2.0 mg mL^{-1} led to a 9% reduction in fat accumulation. Similarly, pea protein hydrolysate supplementation at 2.0 mg mL^{-1} led to nearly 15% reduction in fat accumulation.

One of the reasons for the reduction in lipid content induced by the hydrolysates could be lower diet intake, mimicking calorie restriction, which is known to significantly affect worm size.[35] However, worm size, assessed through body volume measurements, was not reduced or even increased (Fig. 1C and D) in the supplemented groups, suggesting that there was no lower intake in the supplemented group. Moreover, to determine if this effect was dependent on alterations in worm development, we analysed egg laying and hatching by a qualitative observation under microscopy, without observing any differences between control and supplemented groups (Fig. 1E). Only a few studies have described the lipid-lowering effects of protein hydrolysates in *C. elegans*.[18,36] There is only one previous study describing the hypolipidemic effect of legume protein hydrolysates.[18] Here, supplementation with mung bean protein hydrolysate at a dose of 100 μg mL^{-1} induced lipid lowering activity in *C. elegans*. However, when considering studies using proteins from other plant sources, lipid-lowering effects have also been reported. Specifically, Lin *et al.* (2025) showed that supplementation of spirulina protein hydrolysates in *C. elegans* at a dose comparable to that used in the present study (1 mg mL^{-1}) resulted in a lipid-lowering effect.[37] The hypolipidemic properties of protein hydrolysates could be attributed to the presence of bioactive peptides within these matrices with an anti-obesity effect, as suggested by some authors.[37,38] Specifically, a peptide (EF) present in both fava beans and peas with proven lipid-lowering activity has been previously identified.[34]

The reduced fat content induced by the hydrolysates could also be explained by an enhanced worm movement.[39] Nonetheless, we could not observe significant differences in the worm's motility between groups (Fig. S2). Thus, we decided to analyse other healthspan markers to investigate the mechanism underlying the fat-reducing activity of both hydrolysates.

Aging is a multifactorial process that is influenced by genetic, biochemical, and environmental factors. This process is characterized by the progressive loss of homeostatic balance, leading to impaired function and increased susceptibility to the development of various diseases.[40] To analyse whether supplementation with protein hydrolysates had any effect on aging, we quantified the accumulation of the age-related lipofuscin pigment, after treatment with protein hydrolysates. Supplementation with both pea and fava bean protein hydrolysates at a dose of 2.0 mg mL^{-1} led to significant reduction in the accumulation of this pigment (Fig. 2A and B). Interestingly, pea protein hydrolysate reduced the accumulation of this pigment in a dose-dependent manner. Given the observed improvement in the healthspan of worms, we analysed the gene expression of different key-signalling pathways to investigate the potential mechanisms of action. Worms supplemented with fava bean protein hydrolysate did not show any significant differences in the gene expression of lipid and glucose metabolism pathways (Fig. 2C). However, we could observe a trend toward significance in the expression of *sbp-1*, an ortholog of the human SREBP, at a dose of 2.0 mg mL^{-1}. The expression of this key transcription factor modulates lipid accumulation.[41] Interestingly, the reduction in the *sbp-1* gene was even more pronounced when worms were supplemented with pea protein hydrolysate, demonstrating the involvement of this transcription factor in the lipid-reducing effect of both supplements. Moreover, downregulation of both *daf-2* and *age-1* genes, involved in the modulation of glucose metabolism, is also observed after pea-protein supplementation (Fig. 2D). Downregulation of *daf-2* expression is associated with increased longevity in worms. Furthermore, not only *daf-2* but also its downstream target *age-1* is known to have antiaging activity. The activation of DAF-2 leads to the recruitment and activation of AGE-1/PI3K, which triggers a signalling cascade that results in the inhibition of the transcription factor DAF-16. This inhibition results in lower expression of genes

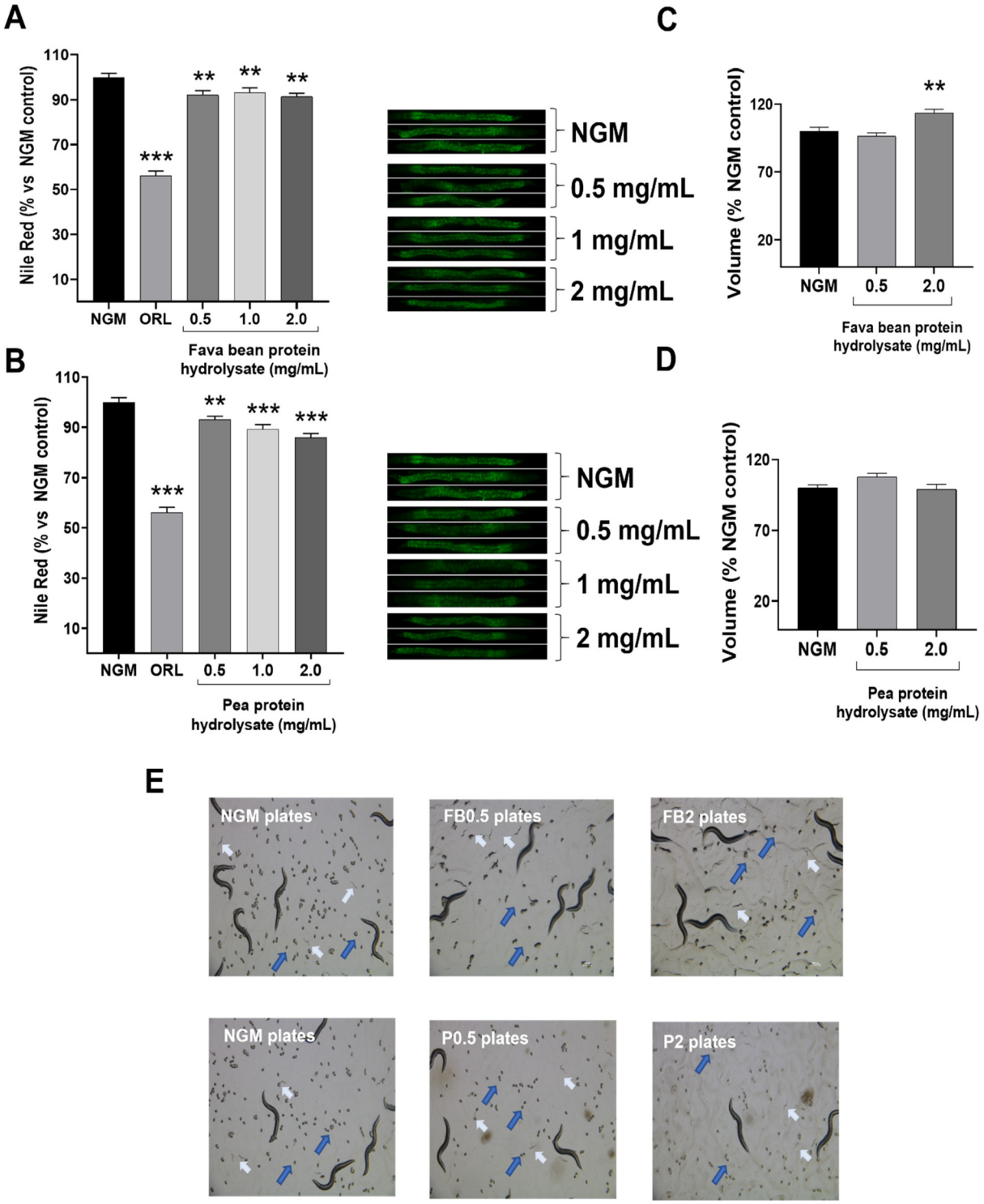

Fig. 1 Supplementation with fava bean and pea protein hydrolysates reduces fat accumulation in *C. elegans*. (A) Nile red staining of fava bean protein hydrolysate supplemented worms at doses of 0.5, 1.0 and 2.0 mg mL^{-1}. (B) Nile red staining of pea protein hydrolysate supplemented worms at doses of 0.5, 1.0 and 2.0 mg mL^{-1}. Orlistat (ORL) was used as a positive control. (C and D) Worm size of worms supplemented with (C) fava bean and (D) pea protein hydrolysates expressed as the volume. The results are expressed as mean ± SEM relative to control (NGM) worms. Statistical significance was calculated with reference to the NGM control group using one-way ANOVA and the Student–Newman–Keuls *post-hoc* test (**p < 0.01 and ***p < 0.001). (E) Supplementation with protein hydrolysates did not affect worm development and egg laying. The blue arrows indicate the eggs and the white arrows indicate the L1 larvae.

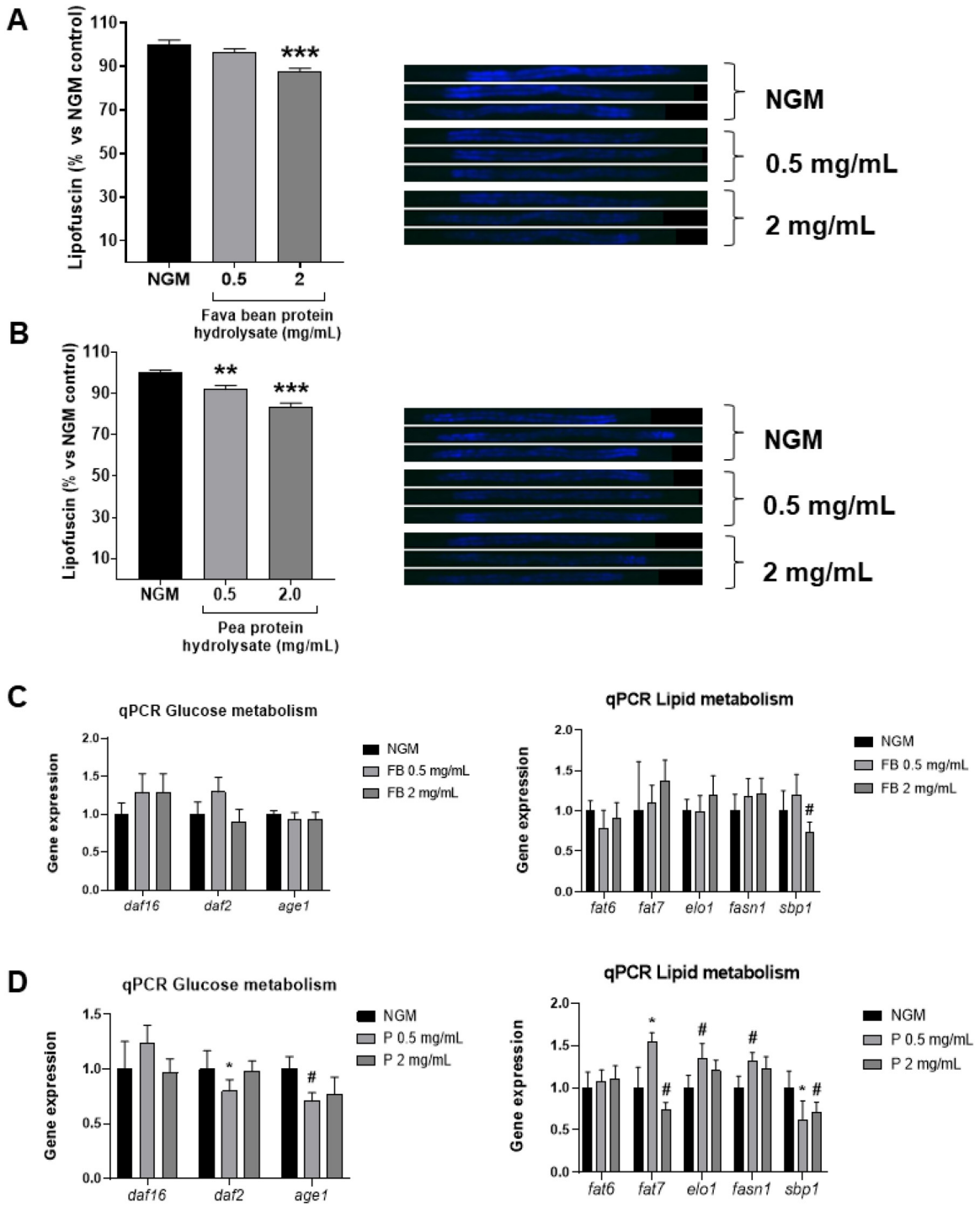

Fig. 2 Supplementation with fava bean and pea protein hydrolysate improves senescence in *C. elegans*. (A) Lipofuscin pigment quantification in worms supplemented with (A) fava bean protein hydrolysates at doses of 0.5 mg mL^{-1} and 2.0 mg mL^{-1}. (B) Lipofuscin pigment quantification in worms supplemented with pea protein hydrolysates at 0.5 mg mL^{-1} and 2.0 mg mL^{-1}. The results are expressed as mean ± SEM relative to control (NGM) worms. Statistical significance was calculated with reference to the NGM control group using one-way ANOVA and the Student–Newman–Keuls *post-hoc* test (**$p < 0.01$ and ***$p < 0.001$). (C and D) Gene expression analysis quantified by real-time PCR (qPCR) in *C. elegans* worms supplemented with (C) fava bean and (D) pea protein hydrolysates. Gene expression levels were normalized to the housekeeping gene (*pmp-3*). Data are expressed using the $2^{-\Delta\Delta Ct}$ method. Statistical significance was calculated with reference to the NGM control group using one-way ANOVA and Fisher's LSD *post hoc* test (#$p < 0.1$ and *$p < 0.05$).

related to stress resistance and longevity.[42–44] Therefore, we hypothesize that the decrease in the insulin signalling pathway could partially explain the remarkable antiaging effect of pea protein hydrolysates.

3.3. Fava bean protein hydrolysate improves the mitochondrial antioxidant response in *C. elegans*

Oxidative stress is the result of an imbalance between the production of free radicals and the body's ability to neutralize them, due to the low capacity of antioxidant mechanisms. Bioactive peptides are considered promising agents for maintaining cellular homeostasis and protecting against age-related or stress-induced damage.[45] Some bioactive peptides present in pea and fava bean protein hydrolysates are known to have *in vitro* antioxidant activity.[46,47] Thus, we aimed to investigate whether the anti-aging activity induced by the hydrolysates could be related to their potential antioxidant activity *in vivo*. Although there were no significant differences in the ROS levels when worms were supplemented with pea protein hydrolysate, a significant effect was observed when supplementing worms with fava bean protein hydrolysate at a dose of 0.5 mg mL^{-1} ($p < 0.01$) (Fig. 3A). The analysis of the expression of

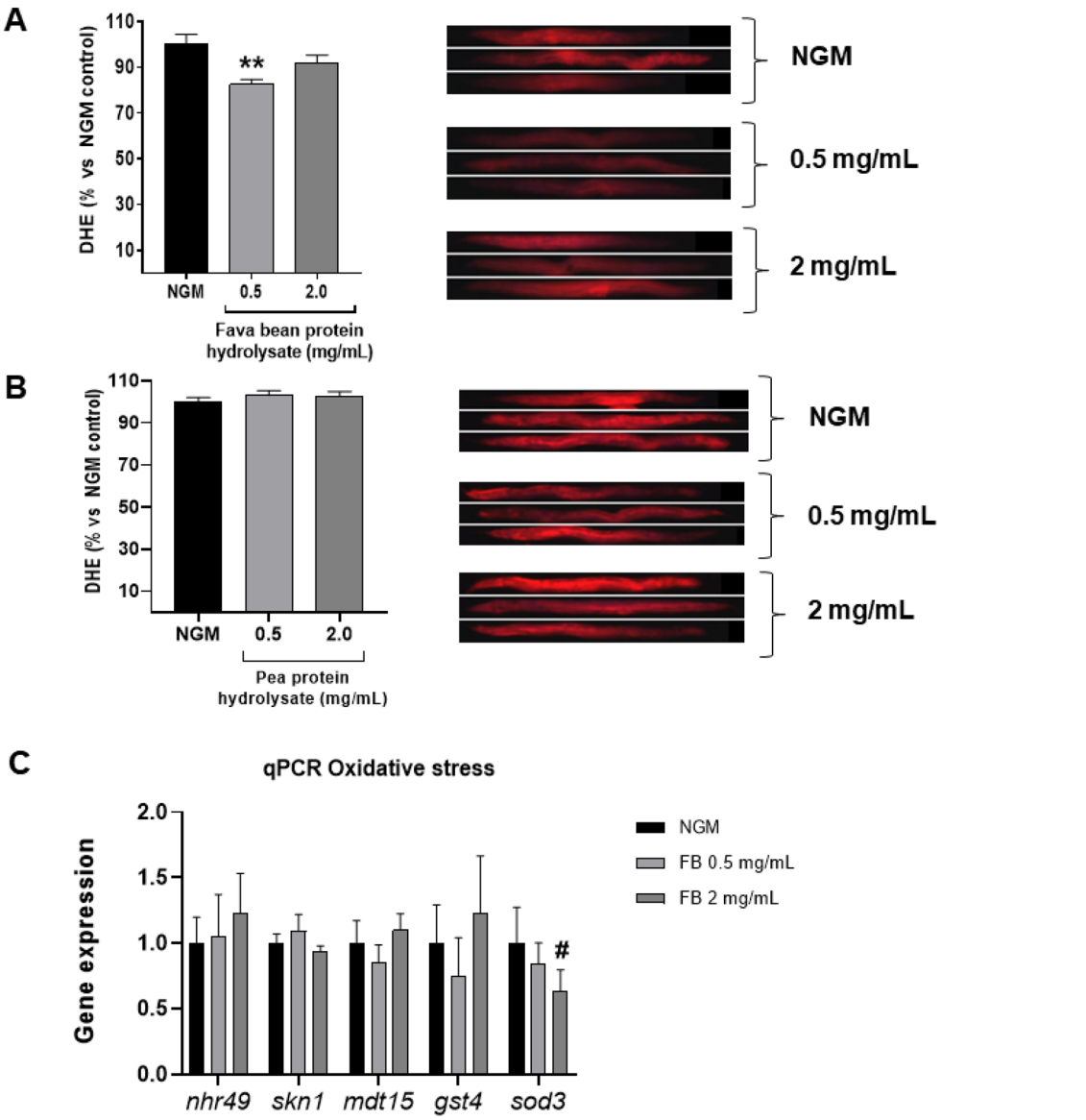

Fig. 3 Supplementation with fava bean protein hydrolysate improves oxidative stress in *C. elegans*. (A) DHE staining of fava bean protein hydrolysate supplemented worms at doses of 0.5 mg mL^{-1} and 2.0 mg mL^{-1}. (B) DHE staining of pea protein hydrolysate supplemented worms at doses of 0.5 mg mL^{-1} and 2.0 mg mL^{-1}. The results are expressed as mean ± SEM relative to control (NGM) worms. Statistical significance was calculated with reference to the NGM control group using one-way ANOVA and the Student–Newman–Keuls *post-hoc* test (**$p < 0.01$). (C) Gene expression analysis quantified by real-time PCR (qPCR) of genes related to the stress oxidative pathway in *C. elegans* worms supplemented with fava bean protein hydrolysates. Gene expression levels were normalized to the housekeeping gene (*pmp-3*). Data are expressed using the $2^{-\Delta\Delta Ct}$ method. Statistical significance was calculated with reference to the NGM control group using one-way ANOVA and Fisher's LSD *post hoc* test (#$p < 0.1$).

different oxidative stress-related genes revealed that the reduced ROS levels were accompanied by significant downregulation of *sod*-3, which showed a trend toward statistical significance after being treated with fava bean hydrolysate, in comparison with NGM control worms (Fig. 3C). SOD-3 is an extracellular enzyme that catalyses the conversion of superoxide radicals into hydrogen peroxide and oxygen molecules, playing a key role in the maintenance of the oxidative stress pathway.[48] Thus, the downregulation of this oxidative stress-related gene may reflect a lower demand for antioxidant defence induced by the fava bean protein hydrolysate.

To further investigate the antioxidant capacity of the protein hydrolysates, we investigated their sensitivity to oxidative stress by exposing worms to juglone. Juglone is a phenolic compound that induces oxidative stress and mortality in *C. elegans*.[49] Thus, worms were incubated in agar plates with juglone, and their stress resistance was studied by recording their locomotion every 30 minutes. No differences were observed in worms supplemented with pea hydrolysate (data not shown), which would be supported by the lower capacity of this hydrolysate to reduce reactive oxygen species (mitochondrial ROS) previously observed in the DHE assay. Nevertheless, worms supplemented with fava bean protein hydrolysate exhibited a trend toward significance in travelled distance parameter after exposure to this chemical stressor, suggesting better stress resistance (Fig. 4B).

This effect, together with the reduced ROS levels and the modulation of *sod-3* expression would demonstrate the mitochondrial antioxidant capacity of the fava bean protein hydrolysate, previously suggested by the *in vitro* ABTS assay. This is consistent with the results of Martineau-Côté *et al.* (2024),[33] who demonstrated that in an *in vitro* model (Nrf2-ARE reporter cell line), the principal mode of action of peptides derived from fava bean was through free radical scavenging. Several peptides identified in fava bean proteins exhibit antioxidant activity *in silico* and *in vitro*[34] but there is limited knowledge about *in vivo* activity. Therefore, this work opens a new field of research on the effect of these hydrolysates in *in vivo* models.

3.4. Pea protein hydrolysate improves thermal stress in *C. elegans* by enhancing the ER-UPR

Recent studies indicate that specific bioactive peptides—both from dietary sources or protein hydrolysates—may contribute to the maintenance of cellular homeostasis and improve stress resilience by reducing both reactive oxygen species (ROS), but also by enhancing the endoplasmic reticulum unfolded protein response (ER-UPR).[7,50] In simple organisms such as

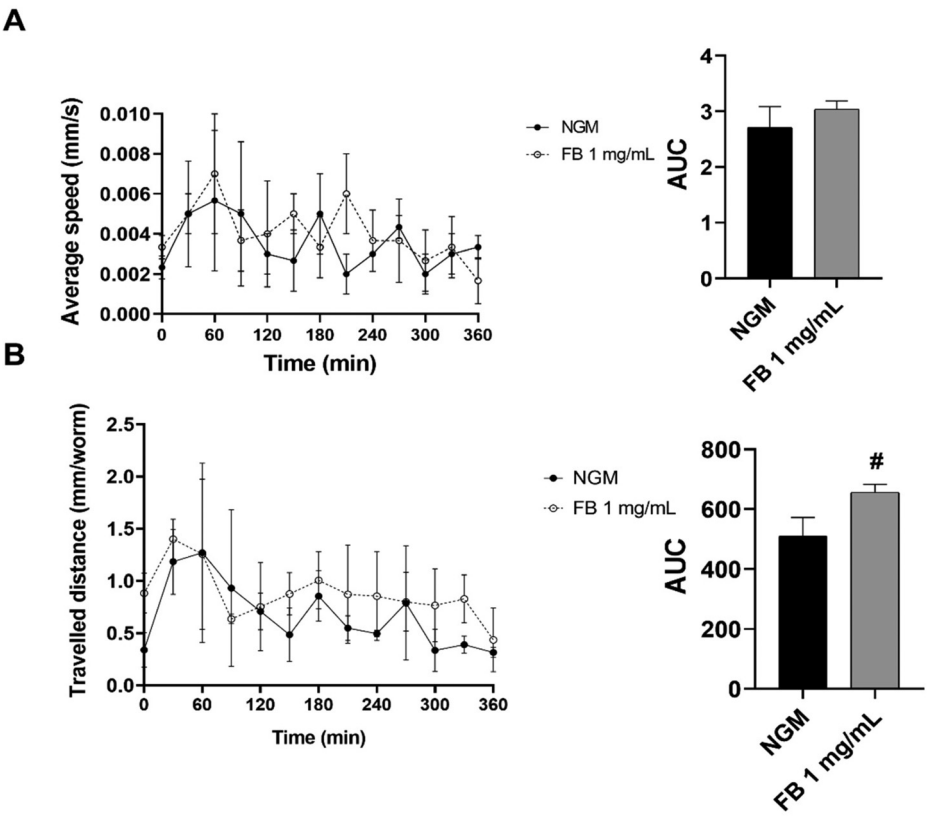

Fig. 4 Motility of *C. elegans* under chemical stress conditions. (A) Average speed and AUC of average speed for worms supplemented with fava bean protein hydrolysate (FB1), in comparison with NGM control worms. (B) Travelled distance and corresponding AUC form worms supplemented with fava bean protein hydrolysate (FB1) in comparison with NGM. Statistical significance was calculated with reference to the NGM control group using Student's-*t* test (#$p < 0.1$).

C. elegans, this is manifested by an improvement in the stress resistance and reduced senescence.

The maintenance of protein homeostasis, or proteostasis, is essential for cellular function and organismal survival. This balance is achieved through a network of mechanisms that regulate protein synthesis, folding, degradation, and recycling. During aging, the efficiency of these systems—including molecular chaperones, the ubiquitin-proteasome system, autophagy, and stress responses in the endoplasmic reticulum and mitochondria—gradually declines. As a result, misfolded and aggregated proteins accumulate, contributing to cellular dysfunction, chronic inflammation, and the development of age-related and neurodegenerative diseases.[51] Therefore, efficient proteostasis is critical for delaying functional decline and promoting healthy longevity. The unfolded protein response (UPR) is a mechanism that is activated when there is an excess of misfolded proteins in the endoplasmic reticulum. This response is mediated by three transmembrane receptors: IRE-1α, PERK and ATF6, where IRE-1α is the main sensor. Under stress conditions, IRE-1α activation occurs and results in the unconventional splicing of *xbp-1* mRNA, leading to the production of its active form, XBP-1s. This transcription factor translocates to the nucleus and activates the transcription of genes related to protein transport, folding, and degradation, leading to improved proteostasis.[52]

Thus, to understand the possible mechanism that could explain the improvement in healthspan induced by the pea protein hydrolysate, both the lipid reducing and strong anti-aging activities, the role of this supplement on the ER-UPR was explored.

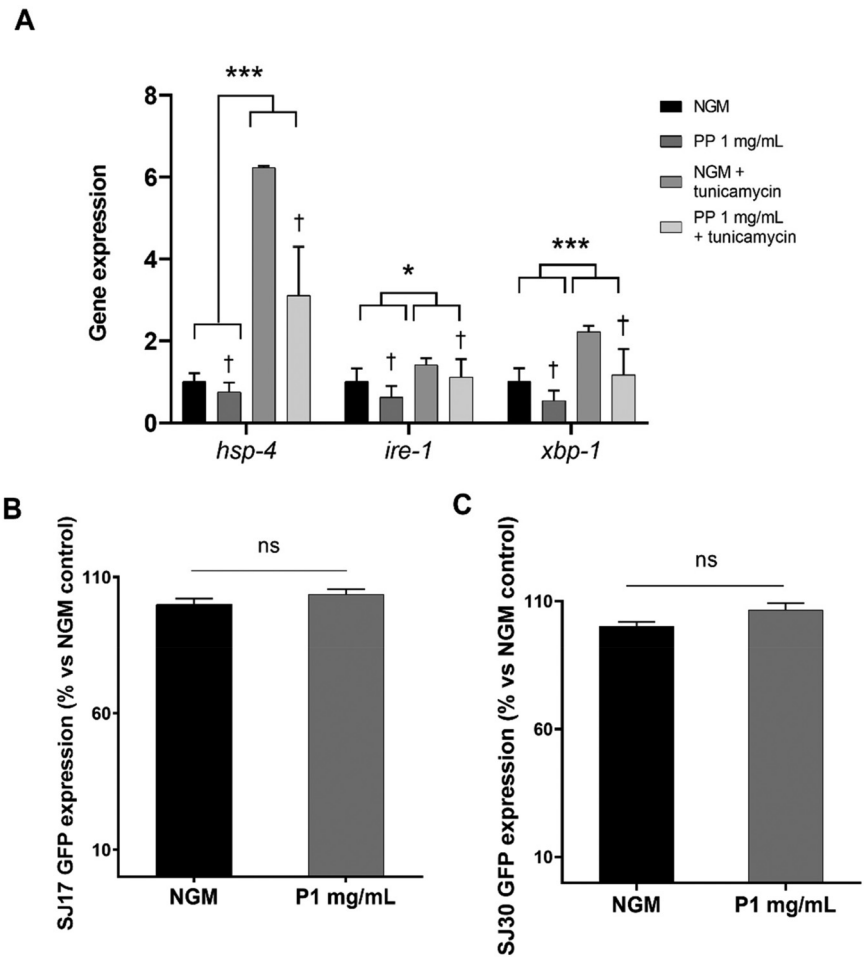

Fig. 5 (A) Gene expression analysis of key genes involved in the unfolded protein stress quantified by real-time PCR (qPCR) in *C. elegans* supplemented with pea protein hydrolysates. Gene expression levels were normalized to the housekeeping gene (*pmp-3*). Data are expressed using the $2^{-\Delta\Delta Ct}$ method. Statistical significance was calculated with reference to the NGM control group using two-way ANOVA (main effects: protein type, tunicamycin and their interaction). Two-way ANOVA results when the tunicamycin factor is significant: $*p < 0.05$ and $***p < 0.001$. Two-way ANOVA results when the protein type factor is significant: $\dagger p < 0.05$. (B) GFP quantification of *ire-1;hsp-4p*::gfp mutant supplemented with pea protein hydrolysate (1 mg mL^{-1}). The results are expressed as mean ± SEM relative to control (NGM) worms. Statistical significance was calculated with reference to the NGM control group using Student's *t* test ($^{ns}p > 0.05$). (C) GFP quantification of the *xbp-1*(zc12)*;hsp-4p*::gfp mutant supplemented with pea protein hydrolysate (1 mg mL^{-1}). The results are expressed as mean ± SEM relative to control (NGM) worms. Statistical significance was calculated with reference to the NGM control group using Student's *t*-test ($^{ns}p > 0.05$).

Gene expression analyses revealed that pea protein-supplemented worms showed lower expression of the key UPR-mediators *hsp-4*, *ire-1* and *xbp-1* under basal conditions, suggesting a reduction in the endoplasmic reticulum stress environment induced by this hydrolysate (Fig. 5A). This reducing effect was also maintained when worms were subjected to the treatment of tunicamycin for 6 hours, a well-known ER stress-inductor. Thus, the pea protein hydrolysate was able to counteract the overexpression of *hsp-4*, *ire-1* and *xbp-1* induced by tunicamycin. This effect on *xbp-1* was confirmed by SJ30 and SJ17 mutants, as the levels of HSP-4 protein remained unaffected when *ire-1* and *xbp-1* were mutated, respectively (Fig. 5B and C).

This result would suggest that pea protein hydrolysate would modulate the UPR, contributing to the maintenance of lower ER stress and improved aging.

To confirm this hypothesis, we forced the activation of the ER-UPR through thermal stress. Under hyperthermic conditions, activation of the UPR occurs,[53] which leads to a reduction in worm motility.[54] Thus, worms were incubated at 33 °C for 6 hours and their locomotion was recorded every 30 minutes using a Wmicrotracker SMART automated system. Worms supplemented with pea protein hydrolysate exhibited a significant enhancement of both average speed and travelled distance ($p < 0.05$) (Fig. 6A and B), demonstrating greater resistance to thermal stress, in comparison with untreated NGM control worms.

The better thermal resistance induced by supplementation with pea protein hydrolysate may underlie the alleviation in ER stress, which is activated under acute heat-stress conditions, as suggested by Taylor *et al.* (2013).[53] Therefore, we suggest that supplementation with pea protein hydrolysate improves ER homeostasis, reducing proteotoxic accumulation and thereby enhancing resistance to heat stress. That said, it is important to mention the existence of a crosstalk between the ER stress response pathway and other signalling pathways. In particular, interactions between the ER stress pathway and glucose and lipid metabolic pathways[55,56] could be associated with the hypolipidemic effect and the improved stress resistance previously observed in supplemented worms.

Taken together, these results indicate the involvement of multiple stress-response pathways. Despite originating from similar plant protein sources and being obtained under comparable hydrolysis conditions, fava bean and pea protein hydrolysates exerted beneficial effects in *C. elegans* through apparently different mechanisms, opening new avenues for future research aimed at identifying the specific bioactive peptides involved.

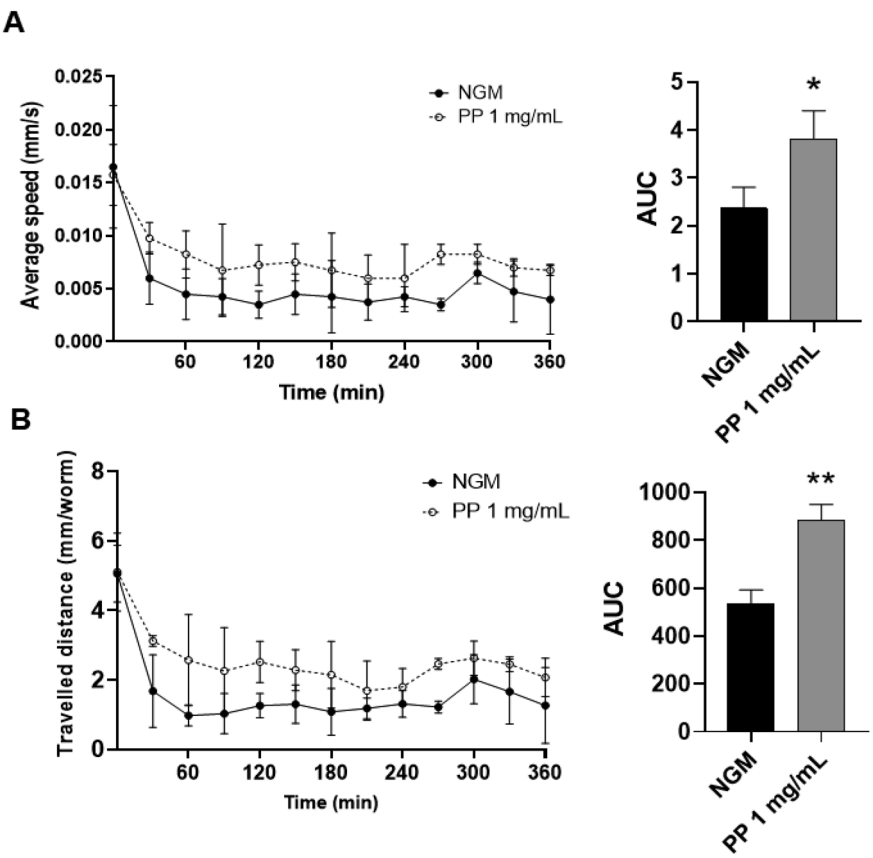

Fig. 6 Motility of *C. elegans* under thermal stress conditions. (A) Average speed and AUC of average speed for worms supplemented with pea protein hydrolysate. (B) Travelled distance and corresponding AUC form worms supplemented with pea protein hydrolysate. Statistical significance was calculated with reference to the NGM control group using Student's-*t* test (*$p < 0.05$ and **$p < 0.01$).

4. Conclusions

This study demonstrated that supplementation with fava bean and pea protein hydrolysates significantly improves healthspan in *C. elegans* by modulating different stress responses. Fava bean protein hydrolysate modulates the oxidative stress pathway, resulting in reduced DHE accumulation and enhanced resistance to chemical stress. Pea protein hydrolysate downregulates key genes of the ER-UPR stress pathway under both basal and stress conditions, thereby improving resistance to thermal stress.

The enhancement of antioxidant responses may underlie the hypolipidemic and anti-aging effects observed with supplementation of both hydrolysates. Our results serve as a starting point for further studies in more complex animal models to investigate the potential application of these hydrolysates in the prevention of metabolic syndrome related diseases.

Author contributions

Maialen Uriz-Martínez: writing – review & editing, writing – original draft, methodology, investigation (*C. elegans* experiments), formal analysis, and data curation; Diana Ansorena: writing – review & editing, writing – original draft, supervision, resources, project administration, and funding acquisition; Iciar Astiasaran: writing – review & editing, writing – original draft, and supervision; David Muñoz-Prieto: methodology, investigation (amino acid composition), and writing; Ana Isabel Yetano: methodology, investigation (protein hydrolysis and characterization), and project administration; Carolina González-Ferrero: methodology, investigation (protein hydrolysis and characterization), project administration, and funding acquisition; Paula Aranaz: writing – original draft, review & editing, resources, project administration, methodology and investigation (*C. elegans*), data curation, and funding acquisition.

Conflicts of interest

There are no conflicts to declare.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d5fo04100g.

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