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Original Article

Curcumin-mediated alleviation of dextran-induced leaky gut in *Drosophila melanogaster*

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Abstract

Aging is commonly characterized by a decline in the physiological functioning of the body organs, with one hallmark being the impairment of intestinal function, leading to increased intestinal permeability known as leaky gut. The aim of this study was to investigate the potential of curcumin to prevent the development of leaky gut in *Drosophila melanogaster* utilizing the smurf fly method. In this study, flies aged 3–5 days underwent a 10-day dextran sulfate sodium (DSS) treatment to induce intestinal permeability, followed by a smurf assay using brilliant blue dye and locomotor testing the next day. Flies displaying the smurf phenotype were divided into four groups: untreated control and curcumin-treated (10 µM, 50 µM, and 250 µM). After 21 days of treatment, flies were reassessed for the smurf phenotype and underwent locomotor testing. On day 23, flies were subjected to RT-qPCR analysis. By inducing increased intestinal permeability through the administration of DSS, a higher proportion of flies exhibiting the smurf phenotype and a reduced survival rate in the DSS-treated group were observed. Such phenotypes were reversed, decreased number of flies displaying the smurf phenotype and improved fly survival, upon the incorporation of curcumin in the fly food at concentrations of 10, 50, and 250 µM. Subsequent molecular analysis revealed upregulated expression of *sod1*, *cat*, and *pepck* genes, while no significant changes were observed in the expression of *sod2*, *indy*, and *srl* genes following treatment with curcumin at high concentration. Overall, our findings provide insight into the potential effect of curcumin to alleviate the phenotypical features associated with DSS-induced leaky gut, possibly via the selective regulation of aging-related genes.

Keywords: Fruit fly, leaky gut, aging, intestine, curcumin

Introduction

Leaky gut syndrome is a condition characterized by impaired intestinal epithelial barrier function, leading to increased intestinal permeability [1]. The compromised barrier allows various substances from the intestinal lumen to enter the systemic circulation, resulting in an elevated risk of infection, inflammation, and gastrointestinal disorders [2,3]. Several factors, including psychological stress, intestinal inflammation, diet, and alcohol intake, contribute to the heightened intestinal permeability [4]. In addition to those factors, several exogenous chemical substances, one of the examples is dextran sulfate sodium (DSS), can also induce intestinal epithelial damage and simulate the pathophysiology of intestinal inflammation. DSS reduces protein expression at tight junctions, thereby increasing intestinal permeability [5,6]. DSS also

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triggers the production of reactive oxygen species (ROS), which further exacerbates intestinal permeability and leaky gut syndrome. Elevated levels of ROS contribute to inflammatory processes within the intestinal milieu, culminating in the apoptosis of the mucosal epithelial cells. This cascade of events compromises the structural integrity of the intestinal epithelial barrier, consequently amplifying intestinal permeability [7,8].

Curcumin, a hydrophobic phenolic compound found in turmeric (*Curcuma longa* L), possesses notable antioxidant and anti-inflammatory properties [9]. These attributes make curcumin as a potential candidate for preventing age-related diseases [10]. Previous *in vitro* studies have demonstrated curcumin's ability to restore barrier function and reduce paracellular permeability in human intestinal epithelial cell lines [11,12]. In addition to its antioxidant effect, curcumin has been reported to exhibit immunomodulatory, antimicrobial, and anti-inflammatory properties. The antimicrobial activity has been shown to be effective against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Helicobacter pylori*. The combination of *Moringa oleifera* Lam. and *C. longa* is predicted to have antiviral and anti-inflammatory activity through a dual inhibitory mechanism involving cryptochlorogenic acid and curcumin [13-16]. One study reported that curcumin may mitigate damage to the intestinal barrier by inhibiting inflammatory responses and attenuating stress injury [17]. However, mechanisms of curcumin-mediated protection in the alleviation of leaky gut remains unclear.

To investigate the pathological consequences of leaky gut in metazoan species, researchers have employed the smurf fly method utilizing *Drosophila melanogaster* as a model organism. This technique involves administering standard fly food containing a non-absorbable blue dye. The presence of a blue color change on the body of *D. melanogaster* indicates increased intestinal permeability [18, 19]. The primary objective of this study was to examine the effect of curcumin on the survival, locomotor, and expression of aging-related genes (*indy*, *srl*, and *pepck*) and endogenous antioxidant genes (*sod1*, *sod2*, and *cat*) in relation to the curcumin-mediated protection against leaky gut phenotype. The *indy* gene is associated with caloric restriction, while the *srl* gene is related to mitochondrial activity [20,21]. Considering the known association between leaky gut syndrome and elevated ROS levels, investigating endogenous antioxidant genes (*sod1*, *sod2*, and *cat*) becomes crucial to understand the mechanisms involved in leaky gut repair and regulation of aging systems and/or oxidative stress [8]. The results of this study aim to provide valuable insights into the mechanisms through which curcumin may exhibit therapeutic potential for treating leaky gut in the smurf *D. melanogaster* model.

Methods

Fly stocks

The genotype of the fly line used in this study was *Oregon-R* (3–5 days old), kindly provided by the Host Defense and Responses Laboratory at Kanazawa University, Japan. Flies were maintained in fly culture vials with standard cornmeal-agar food under customary conditions (25°C, 12-hour light: 12-hour dark cycle).

Fly food and curcumin preparation

The standard fly food was prepared by mixing all required ingredients as specified in the laboratory recipe. The mixture was stirred and heated until thickened, and the final mixture was transferred into vials and allowed to solidify for 24 hours. To create the Smurf food, FD&C #1 dye (Brilliant blue) (Harapan Indowarna Lestari, Tangerang, Indonesia) was added to the standard fly food at a concentration of 2.5%. The Smurf fly assay was utilized to detect the smurf phenotype, which is associated with a leaky gut [19].

Curcumin was dissolved in 96% ethanol to prepare a stock concentration of 50 mM. Subsequently, this stock solution underwent dilution using 96% ethanol to produce concentrations of 10 mM and 2 mM. Each dilution was added to the standard fly food to achieve varying curcumin concentrations of 250 µM, 50 µM, and 10 µM.

Experimental design and study groups

In this study, all flies underwent DSS treatment before undergoing additional treatments in the presence or absence of curcumin. For the DSS treatment, flies aged between 3 to 5 days post-eclosion were carefully separated under CO_2 anesthesia and transferred to a vial containing filter paper immersed in a solution comprising 5% DSS and 5% sucrose for a duration of ten days. The filter paper was replaced every 12 hours. Administration of DSS induced inflammation associated with increased intestinal permeability, commonly known as leaky gut [22].

Following a 10-day DSS treatment, flies were tested for smurf assay and locomotor test. Flies exhibiting the smurf phenotype were then allocated into four groups, each comprising five vials with ten flies per vial. Group 1, the untreated control, received standard food. Group 2 was administered curcumin at a concentration of 10 µM, group 3 at 50 µM, and group 4 at 250 µM. The curcumin solution was introduced into vials containing fly feed at the specified concentration for testing and then thoroughly mixed until homogeneous.

After 21 days of treatment in each group, re-tested for smurf fly assay and locomotor test. The gene expression analysis was conducted afterward on day 23 for all groups. This methodology facilitated the comparison of the number of smurf flies before and after curcumin treatment. The experimental design and workflow are visually depicted in **Figure 1**.

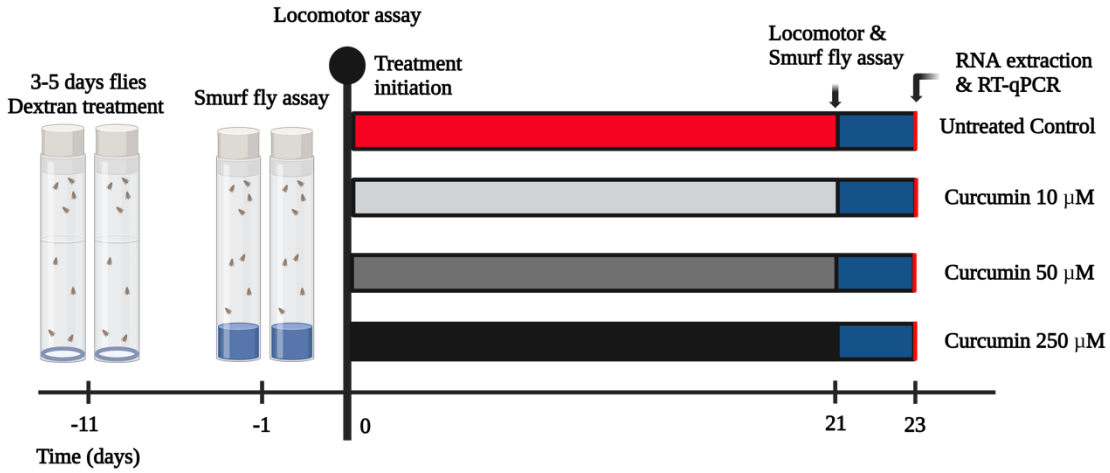

Figure 1. Experimental design to induce leaky gut in *Drosophila melanogaster*. Flies aged 3–5 days were exposed to dextran sulfate sodium (DSS) treatment for a period of ten days. Following the DSS treatment, on day 4 prior to curcumin treatment initiation, flies were maintained on standard food supplemented with 2.5% FD&C #1 blue dye (for the smurf fly assay). Subsequently, flies exhibiting the smurf phenotype were subjected to the following treatments: standard food (untreated control), curcumin 10 µM, curcumin 50 µM, and curcumin 250 µM. RNA extraction and RT-qPCR were performed 23 days after the initiation of treatment in each group.

Smurf fly assay

Following a 10-day DSS treatment, flies were placed on their standard food supplemented with FD&C #1 dye or Brilliant blue (Harapan Indowarna Lestari, Tangerang, Indonesia) at a concentration of 2.5% to conduct the smurf fly assay. The observation of a blue coloration in the fly body (smurf) after four days served as a robust indicator of leaky gut, correlated with increased intestinal permeability and mortality [19].

Locomotor test

For locomotor testing, empty vials were prepared for each treatment group (one vial per treatment group), and a circular line was drawn at the top of each vial, approximately 5 cm from the top edge, serving as the finish line. Then, 10–15 test flies were transferred into locomotor treatment vials, which were categorized based on treatment type. The locomotor treatment vials were tapped three times onto the testing table to ensure the flies settled at the bottom of the vial. Subsequently, the number of flies that crossed the finish line within 15 seconds was counted, and the percentage of negative geotaxis was calculated for each treatment group.

Gene expression analysis

RNA isolation in each treatment group involved the collection of five live flies per group, which were subsequently transferred to microtubes. The flies were then homogenized using a micropestle, and RNA isolation was carried out using the PureLink RNA Mini Kit (Invitrogen, Thermo Fisher Scientific) following the manufacturer's protocol. The RNA samples, utilized for gene expression level analysis, were quantified using a nano spectrophotometer (BioDrop, Biochrom) and processed using the reverse transcriptase quantitative PCR (RT-qPCR) approach. The expression levels of *sod1, sod2, cat, pepck, srl*, and *indy* genes were individually examined in each treatment group utilizing real-time qPCR (RT-qPCR) with the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit with ROX (Invitrogen).

Real-time qPCR was carried out using sets of specific primers for each gene (sequence of primers are listed in **Table 1**) in a reaction volume of 10 µl, following a series of cycles: 37°C for 15 min, 95°C for 10 min, 40 cycles of amplification with each cycle consisting of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The final step involved a melting curve analysis spanning from 60°C to 95°C. The expression level of an internal control gene, *rp49* (ribosomal protein), was used to normalize gene expression levels. The expression level of all genes was further analyzed using Rotor Gene Q software (Qiagen, Hilden, Germany).

Table 1. List of RT-qPCR primers used in this study

Gene	Primer sequence	
	Forward	Reverse
sod1	5'–AGGTCAACATCACCGACTCC–3'	5'–GTTGACTTGCTCAGCTCGTG–3'
sod2	5'–TGGCCACATCAACCACAC–3'	5'–TTCCACTGCGACTCGATG–3'
cat	5'–TTCCTGGATGAGATGTCGCACT–3'	5'–TTCTGGGTGTGAATGAAGCTGG–3'
pepck	5'–CCGCCGAGAACCTTATTGTG–3'	5'–AGAATCAACATGTGCTCGGC–3'
srl	5'–CTCTTGGAGTCCGAGATCCGCAA–3'	5'–GGGACCGCGAGCTGATGGTT–3'
indy	5'–CTGCCCAACTCTGTCCTCTTACTG–3'	5'–CAGGATCAGGTACAGAGGATGGAT–3'
rp49	5'–GACGCTTCAAGGGACAGTATC TG–3'	5'–AAACGCGGTTCTGCATGAG–3'

Statistical analysis

All datasets obtained from both the survival and gene expression studies were statically analyzed. The survival test data were subjected to analysis using the Kaplan-Meier test method, specifically employing log rank test. For the gene expression data, which were presented in Ct values, processing was carried out using the Rotor Gene Q software. Subsequent statistical analysis involved the application of one-way ANOVA with GraphPad Prism 9 software (GraphPad Software Inc., California, USA). In all analyses, a significance level of $p<0.05$ was deemed statistically significant.

Results

Curcumin-mediated protection of flies from DSS-induced leaky gut

In this study, we investigated whether curcumin could confer protection to the integrity of the gut in *D. melanogaster*. To achieve this, a smurf fly assay was carried out. In this assay, *D. melanogaster* underwent treatment with DSS to induce the leaky gut phenotype.

Flies treated with DSS and subjected to the smurf fly assay (staining with brilliant blue) exhibited a blue coloration throughout their entire body, indicative of the smurf phenotype, thereby suggesting impaired intestinal permeability associated with leaky gut (**Figure 2A**). Upon treatment with curcumin, a notable reduction in the smurf phenotype was observed. Each concentration of curcumin led to a diminished number of smurf flies compared to the untreated control group (**Figure 2B**). These results indicated that curcumin treatment possessed the potential to ameliorate leaky gut conditions, as evidenced by the decreased occurrence of the smurf phenotype post-treatment.

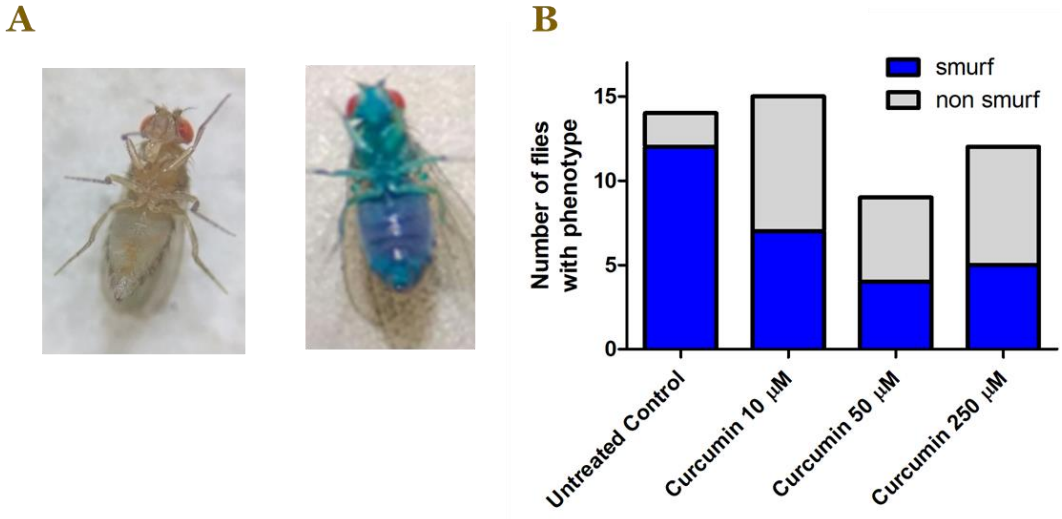

Figure 2. Reduction of smurf fly phenotype following curcumin treatment. The smurf fly assay, utilized to detect leaky gut, manifested as a blue discoloration on the abdomen that extends throughout the body (A). The number of flies exhibiting the smurf phenotype was reduced in flies receiving curcumin treatment (B). All flies underwent dextran sulfate sodium (DSS) treatment to induce the leaky gut phenotype and subsequently received curcumin treatment. One group, designated as the untreated control, received DSS treatment without curcumin treatment, and was compared to all groups receiving curcumin treatment.

Improvement of survival of DSS-treated flies in the presence of curcumin

We hypothesized that curcumin treatment may alleviate the reduction of flies' survival upon DSS treatment. To test this, a longevity assay was conducted to assess the lifespan of *D. melanogaster* following DSS treatment in the presence or absence of curcumin. The results demonstrated that the DSS-treated group exhibited a shorter lifespan in the absence of curcumin (**Figure 3A**). Additionally, in conjunction with the longevity assay, a locomotor assay was performed. Curcumin treatment at all concentrations did not yield any significant differences in locomotor activity compared to the untreated control group (**Figure 3B**).

Upregulation of *sod1* and *cat* expressions in the DSS-treated flies upon curcumin treatment

We hypothesized that the observed enhancement in fly survival subsequent to curcumin treatment could potentially be associated with the upregulation of endogenous antioxidant genes, such as *sod1*, *sod2*, and *cat*. To investigate this hypothesis, RT-qPCR analysis was conducted to assess the pharmacological impact of curcumin on the expression levels of *sod1*, *sod2*, and *cat* in DSS-treated flies. Data indicated that curcumin treatment led to a notable increase in the expression of *sod1* and *cat* at a concentration of 250 µM, in comparison to the untreated control (**Figure 4**). However, there was no significant change observed in the expression of *sod2* (**Figure 4B**).

Curcumin effect on the expression of aging-related genes

In our previous study, we observed that curcumin treatment could modulate the expression of several metabolism-related and aging-related genes, including *pepck, srl,* and *indy* in the autoinflammatory model of *D. melanogaster*. Here, we investigated whether a similar molecular profile in the expression of these genes occurred in DSS-treated flies. The expression of *pepck* exhibited a significant increase in *D. melanogaster* following treatment with curcumin at a concentration of 250 µM (**Figure 5A**). However, the expression of *srl* (**Figure 5B**) and *indy* (**Figure 5C**) remained unchanged in the DSS-treated flies in the presence of curcumin.

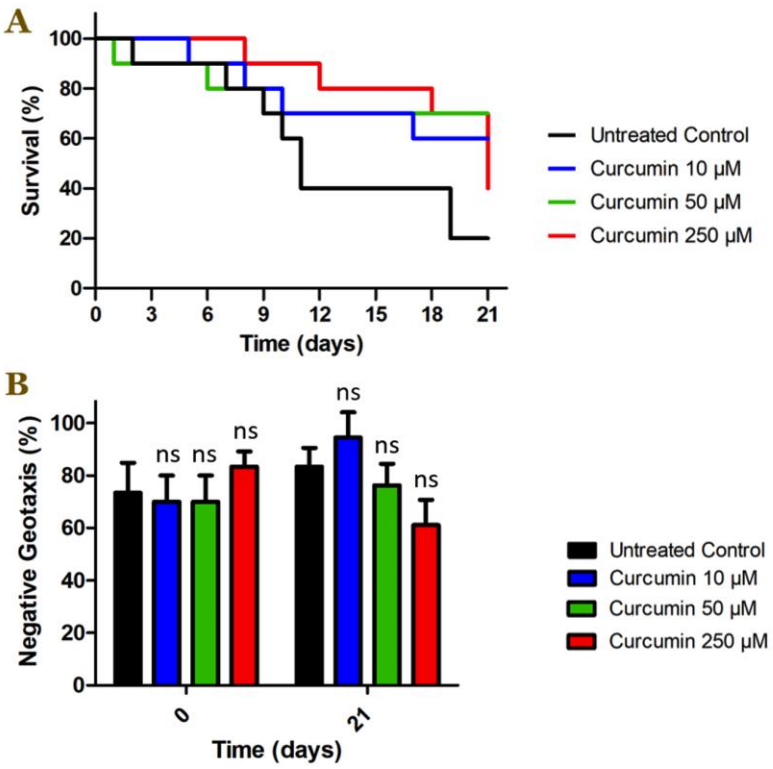

Figure 3. Curcumin improved the lifespan (A) but not the locomotor (B) of dextran sulfate sodium (DSS)-treated flies. All flies were subjected to DSS treatment to induce the leaky gut phenotype, followed by curcumin treatment. A separate group, designated as the untreated control, underwent DSS treatment without curcumin treatment and served as the comparative reference against all groups receiving curcumin treatment. Ns: non-significant.

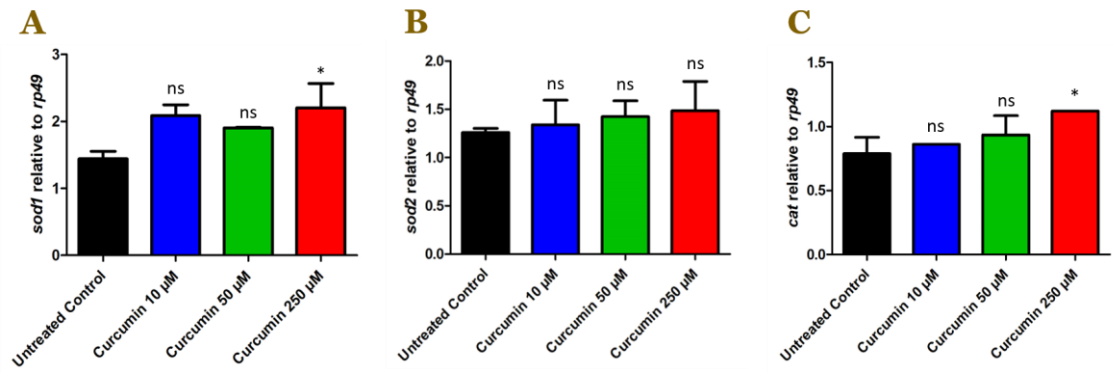

Figure 4. Upregulated expression of endogenous antioxidant genes in the presence of curcumin. The expression levels of three endogenous antioxidant genes, *sod1* (A), *sod2* (B), and *cat* (C) were analyzed relative to the expression of an internal control *ribosomal protein 49* (*rp49*). Ns: non-significant; * indicates statistically significant at $p<0.05$.

Discussion

The findings of this study demonstrated that curcumin administration at each concentration resulted in a reduced number of DSS-treated smurf flies. These results suggest that curcumin has the potential to alleviate leaky gut, as indicated by the reduction in smurf flies following DSS treatment. Furthermore, the survival of DSS-treated flies was improved in the presence of curcumin. This is similar to the previous observation; curcumin administration could extend the lifespan of autoinflammatory model of *D. melanogaster* [23]. Improvement in the survival of flies following treatment with certain substances has been demonstrated to correlate with the

upregulation of endogenous antioxidant genes, such as *sod1, sod2,* and *cat* [23, 24]. However, treatment of flies with curcumin at all concentrations did not result in significant differences in locomotor activity compared to the untreated control group, suggesting that curcumin does not affect the locomotor activity of *D. melanogaster*.

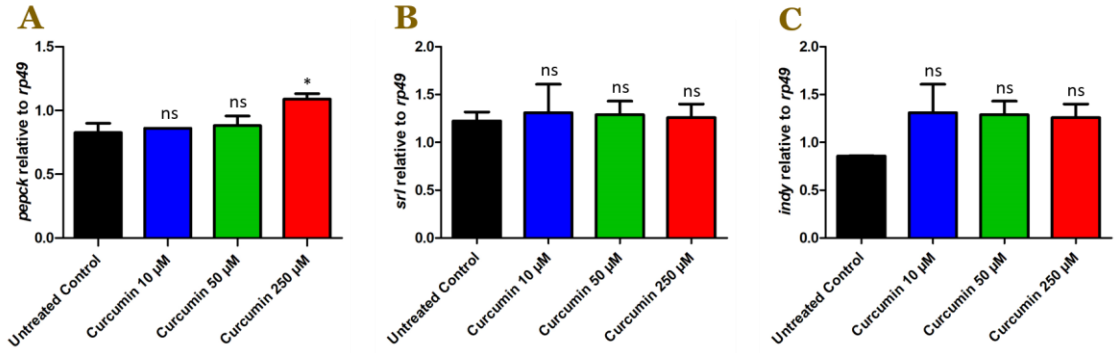

Figure 5. Expression of aging-related and metabolism-related genes in the presence of curcumin. The expression levels of *pepck* (A), *srl* (B), and *indy* (C) were analyzed relative to the expression of an internal control ribosomal protein 49 (rp49). Ns: non-significant; * indicates statistically significant at $p<0.05$.

We were wondering how curcumin can reduce the smurf phenotype and improve the survival of DSS-treated flies. Previously, a study underscored the potent antioxidant properties of curcumin, which can prevent or ameliorate changes in epithelial permeability, counter lipid peroxidation, and address key factors associated with aging [25]. We also observed that curcumin can improve the survival of autoinflammatory model of *D. melanogaster* via the upregulation of genes related to endogenous antioxidant activity, metabolism, and aging [23]. To further investigate this, RT-qPCR analysis of several genes related to endogenous antioxidant activity, metabolism, and aging was carried out.

The analysis of endogenous antioxidant gene expression in *D. melanogaster* following curcumin administration revealed a significant increase in the expression of the *sod1* and *cat* genes at a concentration of 250 µM compared to the untreated control group. The upregulation of these endogenous genes indicates an antioxidant effect of curcumin compounds, as they enhance and modulate endogenous antioxidants *sod1* and *cat*. These results are consistent with the ability of curcumin to enhance endogenous antioxidant activity. Curcumin stabilizes nuclear factor-erythroid 2-related factor 2 (Nrf2) and upregulates heme oxygenase-1 (HO-1), which triggers the Nrf2 pathway and plays a crucial role in activating antioxidant enzymes [25]. Conversely, the impact of curcumin on the expression of *sod2* does not yield a notable increase. This may be attributed to the lesser role of SOD2 compared to SOD1 as an endogenous antioxidant, accounting for 90% of the total roles of SOD [26].

As one of the endogenous antioxidants, catalase (expressed by *cat* gene) plays a vital role in reducing the levels of free radical molecules. The catalase converts hydrogen peroxide (H_2O_2) into water molecules (H_2O) and oxygen (O_2). Increasing the expression of the *cat* gene leads to a higher breakdown of hydrogen peroxide (H_2O_2), resulting in a reduction in ROS accumulation [27,28].

The *pepck* gene encodes the enzyme phosphoenolpyruvate carboxykinase, which is involved in the process of gluconeogenesis. In *D. melanogaster*, the *pepck* gene is homologous to *PCK2* (in humans [29]. The phosphoenolpyruvate carboxykinase catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, a precursor that regulates pyruvate homeostasis. Increased *pepck* gene expression promotes gluconeogenesis, which can stimulate autophagy and contribute to lifespan extension [11, 29].

The Peroxisome Proliferator-Activated Receptor-Gamma Coactivator (PGC-1), homologous to the spargel (encoded by *srl*), is involved in regulating mitochondrial biogenesis and activity. It is also associated with cell differentiation and the maintenance of cell function during the aging process [30]. Moreover, spargel is involved in the autophagy mechanism associated with aging

[31]. When pathological conditions arise, the protein spargel (PGC-1) can activate autophagy [30, 31]. Upregulation of this gene may serve as a marker for increased mitochondrial activity.

The Drosophila I'm Not Dead Yet (*Indy*) gene is responsible for encoding INDY, a plasma membrane transporter that exhibits the highest affinity for citrate among tricarboxylic acid (TCA) cycle intermediates. *Indy* is homologous to the *SLC13A5* gene that encodes a sodium-coupled citrate transporter in humans [32]. INDY influences metabolism by affecting the function of dicarboxylic transporters in the TCA cycle. The role of INDY has also been implicated in caloric restriction. Previous studies have shown that calorie restriction can activate autophagy as part of its anti-aging effects [33,34].

The absence of an increase in the expression of *srl* and *indy* genes indicates that curcumin does not affect mitochondrial activity. Consequently, the gut repair effect of curcumin does not occur through the mitochondrial pathway but rather through the upregulation of antioxidant genes. Therefore, based on this study, curcumin emerges as a promising candidate for addressing leaky gut associated with increased oxidants and inflammation by enhancing endogenous antioxidant activity.

It is important to acknowledge that our study, while shedding light on curcumin's potential protective effect on leaky gut, is subject to certain limitations. Our findings are based solely on phenotypic observations and gene upregulation analyses, which may offer a somewhat narrow perspective on the actual extent of physiological damage occurring within the *Drosophila* intestines. Additionally, the absence of quantification regarding the amount of curcumin ingested by *Drosophila* to achieve this effect further limits the comprehensiveness of our findings. Nevertheless, despite these constraints, it's important to recognize that such limitations can serve as catalysts for future research endeavors, potentially leading to broader and more nuanced insights into the mechanisms underlying curcumin's protective properties against intestinal permeability.

Conclusions

Curcumin treatment increased the survival rate of fruit flies without impacting their locomotor activity. To elucidate the underlying mechanisms driving this phenotype, the expression of several genes linked to antioxidants, metabolism, and aging was determined. Our findings demonstrated elevated expression of *sod1*, *cat*, and *pepck* upon treatment with 250 µM curcumin, while no significant alterations were observed in *sod2*, *srl*, and *indy* expression.

Ethics approval

Not required.

Acknowledgments

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Conflict of interest

All the authors declare that there are no conflicts of interest.

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Underlying data

All data underlying the results are available from the corresponding author upon reasonable request.

How to cite

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