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Fat Lowering Effects of Piperine in Caenorhabditis elegans

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FAT LOWERING EFFECTS OF PIPERINE IN *CAENORHABDITIS ELEGANS*

A Thesis Presented

by

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ABSTRACT

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Piperine is an alkaloid found in pepper plant, which exhibits many health benefits including anti-aging, anti-cancer, anti-diabetic, and anti-inflammatory effects. However, the anti-obesity research of piperine is limited. Therefore, this study was done to determine the effects of piperine on fat accumulation, using in *vivo* model, *Caenorhabditis elegans*. Treatment with 100μM piperine reduce fat content of wild-type *C. elegans* by 17% over control. Pumping rate was not affected by piperine, suggesting piperine has no effects on food intake of worms. Mutant strains were tested to determine the fat reducing mechanisms. *daf-2*, *daf-12*, and *daf-12* mutant strains failed to decrease the fat content by piperine, suggesting piperine may regulates fat accumulation via DAF-2, DAF-12, and DAF-16.

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CHAPTER 1

INTRODUCTION

The global prevalence of overweight and obesity continues to rise significantly[1]. In the year 2000, the World Health Organization (WHO) officially recognized obesity—defined as having a Body Mass Index (BMI) equal to or greater than 30 kg/m^2—as a distinct disease[2]. Individuals who are overweight or obese are at a heightened risk of developing chronic diseases, including cardiovascular diseases, diabetes[3], and various types of cancer[4]. According to estimates from the National Health and Nutrition Examination Survey (NHANES), the prevalence of obesity among teenagers and adults in the United States stands at 19.7% and 41.9%, respectively[5].

Certain plant-based foods contain bioactive compounds that have been demonstrated by researchers to possess anti-obesity properties. Examples of such compounds include resveratrol[6], curcumin[7], deacyl gymnemic acid[8], and pectolinarin[9]. Piperine (1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]-(E,E)-piperidine 1-piperonylpiperidine) is an alkaloid extracted from *Piper nigrum* (black pepper) and *Piper longum* (long pepper)[10]. Extensive research has been conducted on piperine, revealing its anti-obesity[11], anti-inflammatory[12], anti-cancer[13], antimicrobial[14], cardio-protective[15], anti-diabetic[16], anti-aging[17], and anti-allergic effects[17]. Nevertheless, the specific mechanisms underlying the anti-obesity effects of piperine remain relatively underexplored.

The nematode *Caenorhabditis elegans*, a soil-dwelling organism, offers numerous advantages as a model organism compared to traditional animal models. Its small body size, short lifespan, and large brood size make *C. elegans* a particularly useful model for obesity research[18]. Additionally, *C. elegans* shares more than 65% of its genes with disease-associated genes in humans[19], which facilitates the identification of genes associated with fat accumulation and obesity.

CHAPTER 2

LITERATURE REVIEW

2.1 *Caenorhabditis elegans* background

2.1.1 Introduction

Caenorhabditis elegans, is a small, transparent, and non-parasitic organism that inhabits temperate soil environments. First found by Sydney Brenner in 1963, *C. elegans* has been a strong animal model in research areas, such as aging, Alzheimer's, Parkinson's, and obesity[20-24]. Compared with traditional animal models, *C. elegans* shows benefits in research due to its small body size (0.25mm-1mm), short life-span and reproductive cycle, wide living temperature (16-25°C), and large brood size[18]. In addition, using *C. elegans* as the research model does not require approval from the Institutional Animal Care and Use Committee.

2.1.2 Life Cycle

C. elegans is a nematode species in which more than 99% of individuals are hermaphrodites. Hermaphrodites possess a dual reproductive system, capable of producing both sperm and oocytes[25]. Moreover, due to the limited availability of sperm, the self-fertilization of hermaphrodites results in a brood size of around 300 progeny[25]. In contrast, the remaining approximately 1% of the *C. elegans* population consists of males. These males mate with hermaphrodites, leading to the large brood size to around 1200-1400 progeny[25].

The life cycle of *C. elegans* consists of six stages, starting from the embryo, followed by four larval stages (L1-L4), and finally, the adult stage[26]. The appropriate culturing temperature for *C. elegans* is around 16°C to 25°C, with higher temperatures accelerating the worms' growth. At 25°C, their growth rate is 2.1 times faster than at 16°C and 1.3 times faster than at 20°C[27].

In laboratory culture conditions at 20°C, *C. elegans* takes approximately 16 hours to transit from the embryo to the L1 stage. Each stage lasts around 12 hours, except for the L1 stage, which lasts 16 hours[27]. After each stage, there is a period called lethargus during which the worms produce a new cuticle. The lethargus ends with molting, shedding the old cuticle[28].

At the L4 stage, approximately 12 hours after molting, *C. elegans* reaches adulthood and starts producing progeny, a process that lasts for about 5-6 days. However, when L2 larvae experience adverse conditions, such as lack of food or crowding, they can enter an alternative life cycle called the "dauer" stage[29]. In this stage, dauer larvae exhibit enhanced resistance to environmental stresses and can survive for several months. If provided with enough food, dauer larvae can resume development and molt into slightly different L4 larvae, bypassing the L3 stage[30].

2.1.3 Compound Intake Behavior

Caenorhabditis elegans employs two distinct mechanisms to intake compounds, both reliant on sensory cilia for nutrient detection[31]. One method involves diffusion

through the skin, which is covered by a resilient, yet pliable exoskeleton known as the cuticle. The cuticle is replaced at the end of each larval stage, ensuring continued efficacy in nutrient intake[32]. The adult worms' cuticle exhibits a thickness of approximately 0.5 mm and is primarily composed of cross-linked collagens. Additionally, it comprises a diverse array of insoluble proteins termed cuticlins, along with associated glycoproteins and lipids. This intricate cuticle composition imparts strength, flexibility, and protective attributes, all of which are vital for the worms' survival and adaptation to their environment[18,32].

The other intake mechanism involves injection through the pharynx, enabling the capture of foods with a diameter ranging from 0.5 to 1μm[33]. This alternative method complements the diffusion-based intake and further diversifies the nutrient acquisition strategies of *C. elegans*.

2.1.4 Application of *C. elegans* in Modern Research

With more than 65% of genes related to human diseases genes, *C. elegans* become a great model to study aging and obesity (Summarized in Table 2.1). In summary, *Caenorhabditis elegans* has proven to be an excellent model for obesity research, particularly in elucidating target gene in response to specific treatments.

Table 2.1 Genes related to obesity in *C. elegans*.

Genes name	Function	Reference
aak-1, aak-2	AMP-Activated Kinase	[34]
cebp-2	CCAAT-enhancer-binding proteins	[35]
daf-2	Insulin/insulin-like growth factor 1 receptor	[36]
daf-12	Nuclear receptor of farnesoid X acid	[37]
daf-16	FOXO transcription factor	[36]
eat-2	EATing: abnormal pharyngeal pumping	[38]
fard-1	Fatty Acyl-CoA ReDuctase	[39]
fasn-1	Fatty Acid Synthase	[40]
fat-5, fat-6, fat-7	FATty acid desaturase	[41]
nhr-49	Hepatocyte nuclear factor 4 (HNF4) and peroxisome proliferator-activated receptor α (PPARα)	[42]
sbp-1	Sterol regulatory element Binding Protein	[43]
sir-2.1	Mammalian sirtuin	[44]
tub-1	TUBby-related	[45]

2.2 Piperine Background

2.2.1 Introduction

Piperine, an alkaloid found in several Piper species, including *Piper nigrum*[46], *Piper longum*[47], *Piper chaba*[48], *Piper guineense*[49], and *Piper sarmentosum*[50], offers a multitude of health benefits, making it a compound of significant interest. It has been extensively studied and has demonstrated various beneficial properties, including anti-aging[51], anti-cancer[52], anti-diabetic[53], anti-inflammatory[54], and anti-tumor effects[55]. These diverse properties highlight the potential of piperine as a promising natural compound for various health applications.

Despite the extensive research on its various benefits, there is still a limited number of reports regarding its potential role in anti-obesity effects. While some studies

have indicated its anti-obesity effects, much more research is required to fully understand the mechanisms behind its impact on lipid metabolism[7,8,11,53,56].

2.2.2 Anti-Cancer Effects of Piperine

Piperine has emerged as a promising anti-cancer agent, showing efficacy against various types of cancer, including breast[57], colorectal[58], lung[59], ovarian[55], and prostate cancer[13], through diverse mechanisms.

Numerous research suggested that piperine can be effective in preventing breast cancer[52,57,60-62]. For instance, piperine was tested in 4 cell lines of triple-negative breast cancer cells (TNBC), which was considered to be lacking estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER 2)[62], including MDA-MB-231, MCF-7, T-47D, and MDA-MB-468. The data showed that piperine reduced proliferation of those cells through inhibiting the survival-promoting protein kinase B (Akt) activation and causing caspase-dependent apoptosis[52]. Additionally, piperine impedes the cellular cycle of the aforementioned cell lines by augmenting expression of $p21^{Waf1/cip1}$, an inhibitor that disrupts the progression of the cell cycle. This inhibitory effect by piperine was linked with the S phase, G1 phase, and G2 phase of the cell cycle, resulting in an elevated proportion of cells existing within the G2/M phase[52,63]. Nonetheless, piperine was suggested to suppress TNBC by targeting the up-regulator epidermal growth factor receptor (EGFR)[60]. There were more evidences have shown that piperine can inhibit breast cancer cell line, such as 4T1[64],

MCF-7[65-68], MDA-MB-231[69].

In addition, other studies reported the effect of piperine treatment on colorectal cancer cells: SW480, HCT-116, and DLD-1 over periods ranging from 24 to 72 hours[58,70]. Observations indicated that the epithelial-to-mesenchymal transition (EMT) process - a mechanism enabling cancer cells to diverge from the original site, permeate into adjacent tissues, and ultimately migrate to distant organs[71] - was inhibited by piperine due to the downregulation of the EMT regulator Snail, and the transducers and activators of transcription 3 (STAT3)[58]. Moreover, piperine impeded the Wnt/β-catenin pathway, suppressing proliferation and migration in colorectal cancer cells[70]. Piperine has also shown anti-cancer effects in other types of cancers such as lung, ovarian and prostate (summarized in Table 2.2)

Table 2.2 Summary of anti-cancer effects of piperine

Cancer type	Experimental model	Concentration/dose	Duration	Effects
Lung [59,72,73]	Swiss Albino mice (*in vivo*)	100 mg/kg	4 weeks	↓ oxygen species ↑ free radical scavenging enzymes
	Swiss Albino mice (*in vivo*)	50 mg/kg	16 weeks	↓ oxygen species ↑ free radical scavenging enzymes
	A549 cells	88-1400µM	48 hr	↑ caspase activity ↑ apoptosis cell cycle arrest at G2/M phase
Ovarian [55,74]	W1	88-350µM	72 hr	↓ cell survival
	A2780	70µM	48 hr	↓ cell viability ↑ apoptosis
Prostate [13,75-78]	LNCaP, PC-3 and DU145	40-160µM	48 hr	↓ cell proliferation ↑ autophage cell cycle arrest at G0/G1-phase
	LNCaP, DU-145, 22RV1, and PC-3	50-200µM	24, 48, 72 hr	↓ cell proliferation
	LNCaP, and PC-3	0.1-100µM	24 hr	↑ apoptosis cell cycle arrest at G1 phase
	PC-3 and LNCaP	0.1-100µM	24 hr	cell cycle arrest at G0/G1 phase
	DU145	40-320µM	48 hr	↓ cell proliferation ↓ migration

2.2.3 Anti-Inflammatory Effects of Piperine

Piperine has shown significant potential as an anti-inflammatory agent based on extensive research. It effectively down-regulates inflammatory markers, thus suppressing inflammation in various conditions. Piperine suppressed the production of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-2, IL-6, IL-8, and increase the expression of IL-10, transforming growth factor-β1 (TGF-β1), suggesting its potential use in managing sciatica, epilepsy and arthritis[12,54,79-82].

Furthermore, some revealed intricate mechanisms governing the regulation of inflammatory mediators by piperine. A study conducted by Yu et al. uncovered that the expression of mRNA-520a (miR-520a) in sciatic nerve tissue was augmented in male Sprague Dawley rats administered with piperine for 15 days. This up-regulation led to the suppression of p65 expression, consequently resulting in the downregulation of IL-1β & TNF-α, and the up-regulation of IL-10 & TGF-β1[79,83,84].

Others reported that piperine inhibited two IL-6 expression pathways: the P38 mitogen-activated protein kinase (MAPK) pathway and signal transducer and activator of transcription-3 (STAT3) activation in gastric cancer cells, which suggest down-regulation of IL-6 by piperine[85]. Moreover, multiple studies reported that piperine suppresses the expression of IL-2 and IL-8 by inhibiting pathways including Janus kinase (JAK)/STAT, MAPK, phosphoinositide 3-kinases (PI3K)/Akt, and Src/epidermal growth factor receptor (EGFR)[86,87]. Overall, the evidence from these

studies suggests that piperine is an anti-inflammatory agent, with potential applications in various inflammatory conditions and diseases.

2.2.4 Anti-Obesity Effects of Piperine

Numerous *in vivo* and *in vitro* investigations have reported the anti-obesity properties of piperine (*in vivo* studies are summarized in Table 2.3)[56,88]. Researchers reported that piperine diminish the fat content of 3T3-L1 adipocytes[88,89], an established *in vitro* model for obesity research[90]. After a 48-hour treatment with 20μM of piperine, a notable reduction in lipid content was observed in the 3T3-L1 adipocytes[88]. Concurrently, there was a marked decrease in the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), fatty acid synthetase (FAS), sterol regulatory element-binding protein-1c (SREBP-1c), CCAAT-enhancer binding protein alpha (C/EBP-α), and fatty acid binding proteins-4 (FABP-4), whereas uncoupling protein-1 (UCP-1) was significantly increased[88]. These data aligns with other *in vitro* studies involving HER2-overexpressing breast cancer cells[61], and HepG2 hepatocytes[91], as well as *in vivo* experiments utilizing C57BL/6 mice[92-95], Sprague-Dawley rats[96,97], and Wistar rats[98,99]. However, others reported that piperine enhanced the expression of PPAR-γ, which was contradictory with results from 3T3-L1 adipocytes[100,101].

Wang et al.[102] revealed that piperine mitigated fat accumulation via inhibition of fat absorption. In an experiment involving male Sprague Dawley rats administered with a piperine dosage of 27mg/kg for eight weeks, the fecal fat content was significantly

elevated compared to control, suggesting a decreased absorption of fat.

In summary, piperine reduces fat accumulation through inhibition of fat absorption, as well as affecting the expression of PPAR-γ, FAS, SREBP1-c, C/EBP-α, FABP-4, and UCP-1.

Table 2.3 Summary of *in vivo* studies on piperine & obesity

Animal model	Concentration/dose of piperine	Duration	Expression	Reference
male Wistar rats	36 mg/kg	15 days	↑ PPAR-γ	[98]
male Wistar rats	100 mg/kg	30 days	↓ FAS	[99]
Sprague-Dawley rats	27 mg/kg	8 wk	↑ PPAR-γ	[96]
Sprague-Dawley rats	27 mg/kg	8 wk	↓ FAS	[96]
C57BL/6 mice	50 mg/kg	3 wk	↑ PPAR-γ	[92]
C57BL/6N mice	50 mg/kg	10 wk	↓C/EBP-α	[95]
C57BL/6 mice	40 mg/kg	10 wk	↓C/EBP-α	[11]
C57BL/6 mice	40 mg/kg	10 wk	↓ SREBP1-c	[11]
C57BL/6 mice	30 mg/kg	12 wk	↓ FAS	[93]
C57BL/6N mice	50 mg/kg	10 wk	↓ FAS	[95]

2.2.5 Studies of Piperine in *Caenorhabditis elegans*

Previously, there was a study of piperine on *Caenorhabditis elegans*[103]. *C. elegans* was treated with 50μM and 100μM of piperine. It was found that the activity of superoxide dismutase (SOD) and catalase significantly increased, suggesting that piperine enhances the antioxidant capacity of *C. elegans*. This study provides the potential health benefits of piperine to modulate antioxidant defenses in *C. elegans*.

2.3 Conclusion

Piperine, an alkaloid derived from pepper plants, has been demonstrated to possess a wide range of beneficial effects, including anti-cancer, anti-inflammation, anti-oxidant, and anti-obesity properties. These properties suggest that piperine could serve as a therapeutic agent for various diseases. With limited research on anti-obesity effect of piperine, we conducted experiments to determine the effects of piperine on lipid metabolism using *Caenorhabditis elegans* as a model organism. *C. elegans* is widely used in obesity research due to its genetic simplicity and similarities to higher organisms in many biological processes, including lipid metabolism.

CHAPTER 3

METHODS AND MATERIALS

3.1 Materials:

Piperine (>97.5%) and 5-fluoro-2'-deoxyuridine (FUDR) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ampicillin and carbenicillin were purchased from Fisher Bioreagents (Pittsburgh, PA, USA). Infinity™ Triglycerides Liquid Stable Reagent was purchased from Fisher Diagnostics (Middletown, VA, USA). Pierce™ BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Middletown, VA, USA). *C. elegans* wild-type (N2), *C. elegans* mutants: *aak-2*(*ok524*), *aak-1*(*tm1944*)*;aak-2*(*ok524*), *daf-2*(*e1370*), *daf-12*(*rh61rh411*), *daf-16*(*mgDf50*), *fat-5*(*tm420*), *fat-6*(*tm331*), *fat-7*(*wa36*), *fat-5*(*tm420*)*;fat-6*(*tm331*), *fat-5*(*tm420*)*;fat-7*(*wa36*), *nhr-49*(*tm1011*), *sbp-1*(*ep79*), *tub-1*(*ok1972*), and *E. coli* OP50 were purchased from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). *aak-1*(*tm1944*) was purchased from National BioResource Project (Osaka, Japan).

3.2 Methods:

3.2.1 Maintenance:

The worms were bred on NGM plates supplemented with *E. coli* OP50. The respective formulations for both NGM and *E.coli* OP50 were adopted from a previous study[104]. To synchronize the worms to the L1 stage, gravid worms and their eggs were gathered and subjected to a bleach solution, followed by two rounds of washing with

an M9 solution before being transferred to an S-complete solution (recipes for the bleaching solution, M9, and S-complete are as described in the Wormbook)[27,105,106].

Upon hatching from eggs, the L1 stage worms were nourished with *E. coli* OP50 and included ampicillin (100μg/ml) and carbenicillin (50μg/ml). After a period of 40 hours at 20°C, the L4 stage worms were relocated to a 12-well plate at a density of 800-1,000 worms per well. If the worms were fed with dead *E. coli* OP50 (heating in 65°C waterbath for 30 min)[107], the L4 stage worms should be washed twice first with M9 solution, and then dissolved in S-complete solution. Each well was further supplemented with more S-complete solution, ampicillin (100μg/ml), carbenicillin (50μg/ml), and FUDR (12μM) to achieve a final volume of 1ml per well. The inclusion of FUDR served to prevent the hatching of eggs.[104] Subsequently, the worms were administered with 1μL of piperine derived from a 100mM stock in dimethyl sulfoxide (DMSO, ⩾99.9%) to reach 100μM of piperine, which acted as the vehicle for piperine.

3.2.2 Pumping Rate:

The rate of pumping was determined by quantifying the pharyngeal contractions of randomly chosen nematodes over a span of 30 seconds.

3.2.3 Triglyceride and Protein Quantification:

Post a 48-hour treatment, nematodes were relocated to centrifuge tubes. Following five times washing with water to remove *E. coli* OP50, the resultant worm pellet was resuspended with 100-150µL of 0.05% Tween 20[108]. Then, the worms were subjected to ultrasonication for a maximum duration of three minutes to obtain homogeneous solution. Quantification of triglycerides (TG) and protein was accomplished utilizing Infinity™ Triglycerides Reagent and BCA Protein Assay Kit, respectively. The standard solution for TG quantification was glycerol ranging from 0 to 100µg/ml, whereas the standard solution for protein quantification used bovine serum albumin from 0 to 2000µg/ml. Absorbance readings were obtained using a SpectraMax i3 Microplate Reader (Molecular Devices LLC, Sunnyvale, CA, USA) with SoftMax Pro 6 (version 6.5) software. The TG sample underwent a 5-minute incubation at 37°C prior to measurement at 510nm, while the protein sample was incubated at 37°C for 30 minutes and then measured at 562nm[104].

3.2.4 Real-time Polymerase Chain Reaction

Real-time PCR was executed utilizing TRIzol® (Thermo Fisher Scientific, Inc., Middletown, VA) to extract total mRNA, which was then subject to reverse transcription using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Inc., Middletown, VA) and a thermal cycler (Bio-Rad Laboratories Inc.,

Hercules, CA)[104,109]. The gene expression was then quantified with the aid of the StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA).

The tested genes (FAM): *atgl-1*(Ce02406730_g1), *fard-1*(Ce02493083_m1), *fasn-1*(Ce02411648_g1), *sir-2.1*(Ce02459017_g1). The housekeeping genes (VIC): ama-1(Ce02462726_m1). The TaqMan gene expression assays were used (Thermo Fisher Scientific, Inc., Middletown, VA).

3.2.5 Statistical Analysis

Statistical evaluations were conducted by the Student t-test via GraphPad Prism software (version 10, San Diego, CA). A *p*-value less than 0.05 was determined as a significant difference.

CHAPTER 4

RESULTS

4.1 Piperine Reduced Fat Content in Wild-type *C. elegans*

After reaching the adult stage, wild-type *C. elegans* were treated with 100µM piperine for 48 hours. Piperine at 100µM reduced triglyceride levels by 17% over the control (p=0.0002) (Fig. 4.1A). We then tested piperine with dead *E. coli* OP50 to determine if live *E. coli* OP50 contributed to any of observed effects on piperine[107]. We observed significant reduction of triglyceride levels by piperine over the control even with dead *E. coli* OP50 (Fig. 4.1B) (p=0.0408), which suggests that piperine itself is responsible for the reduction of fat accumulation.

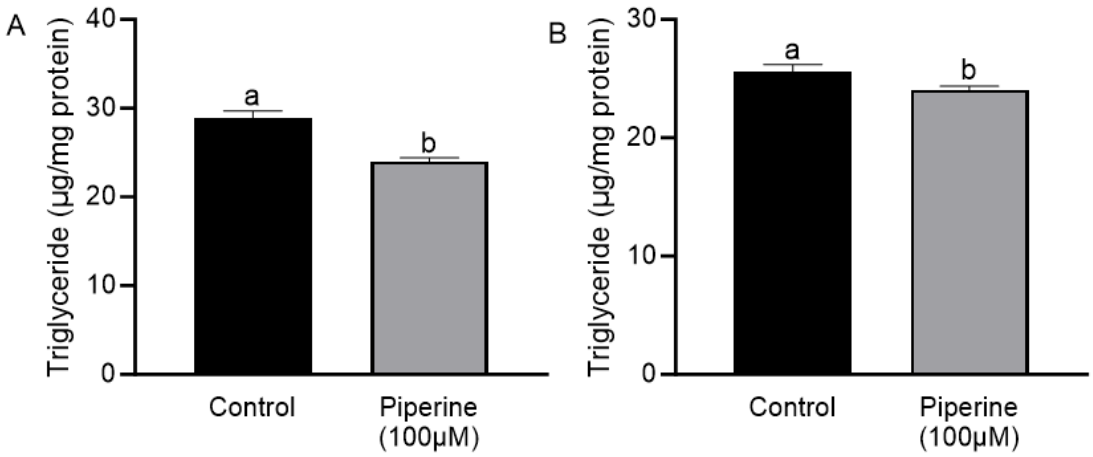

Figure 4.1 Effects of piperine on triglyceride content in *C. elegans*. Wild-type adult worms (N2 Bristol) were subjected to a 48-hour treatment with 100µM piperine, feeding with (A) live *E. coli* OP50 and (B) dead *E. coli* OP50. Triglyceride levels were standardized by protein content. Data are means ± S.E (A: n=8, taken from two independent experiments; B: n=6, taken from one experiment). Values denoted by distinct letters indicate a significant difference at $p < 0.05$.

4.2 The Effects of Piperine on Pumping Rate

Wild-type worms were treated with piperine for 48 hours, and the pumping rate was determined by counting pharyngeal contractions over 30 seconds. There was no significant difference in pumping rate between the control and piperine groups, suggesting that piperine did not influence food consumption. (Fig. 4.2)

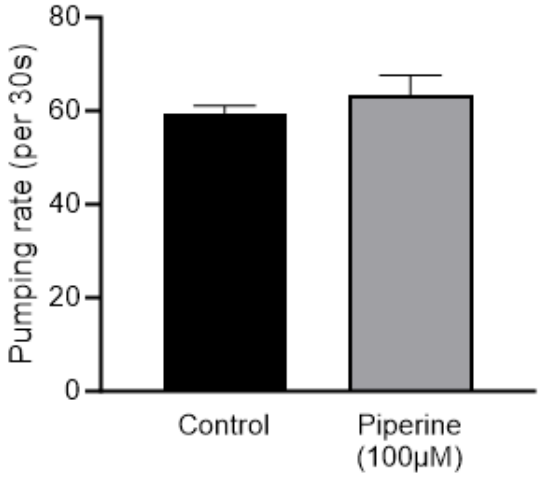

Figure 4.2 Effects of piperine on *C. elegans* pumping rate. Wild-type adult worms (N2 Bristol) were subjected to a 48-hour treatment with 100μM piperine. The pharyngeal contrations were counted for 30 seconds of randomly selected worms. Data are means ± S.E (n=12).

4.3 Piperine's Effects on Fat Accumulation in Mutants

AAK-1 and AAK-2 are homologs of the catalytic alpha subunit of the mammalian 5-AMP-activated protein kinase (AMPK), an intracellular ATP/AMP ratio sensor.[110] AMPK regulates energy homeostasis by modulating various down stream target, including ACC1, FAS, and SCD1[111,112]. The results showed that fat accumulation was reduced by piperine in both *aak-2* (19%, p=0.0039) and *aak-1*;*aak-2* (16%, p=0.0008) mutants over the control. No significant difference of TG was observed by piperine in the *aak-1* mutants. Even though *aak-1* may play a role in

piperine's effect, based on significant effects of piperine in *aak-1*;*aak-2* double mutant, these results suggest that the *aak-1* and *aak-2* might not be involved in piperine's fat-reducing effects (Fig 4.3A).

In *C. elegans*, the genes *fat-6* and *fat-7* are responsible for encoding Stearoyl-CoA Desaturases (SCDs), while a closely related gene, *fat-5*, encodes a palmitoyl-CoA desaturase[113]. Specifically, FAT-5 transforms palmitic acid (16:0) into palmitoleic acid (16:1), whereas FAT-6 and FAT-7 convert stearic acid (18:0) into oleic acid (18:1Δ9)[114]. The data indicated that piperine decreased fat accumulation in *fat-5*, *fat-6*, and *fat-5*;*fat-7* mutants by 16% (p=0.0197), 11% (p=0.0017), and 17% (p=0.0026) compared to the control, respectively. There was a lack of statistically significant difference between the control and piperine groups in *fat-7* mutants, suggeting *fat-7* might play a role in piperine's effects. However, given the results from *fat-5*;*fat-7* double mutant, which showed a significant decrease of piperine group over control, our results suggest that *fat-7* may not be critical for piperine's fat-lowering effects (Fig 4.3B). Overall, the fatty acid desaturases genes, *fat-5*, *fat-6*, and *fat-7*, may not play important roles in the fat-reducing effects of piperine.

NHR-49, a homolog of peroxisome proliferator-activated receptor α (PPAR α), plays key roles in fat metabolisms of *C. elegans* through regulation of fatty acid β-oxidation and expression of *fat-7*[115,116]. Piperine reduced fat accumulation by 13% (p<0.0001) compared to control group in *nhr-49* mutant, which suggested *nhr-49* is independent of piperine's fat decrease effects (Fig 4.3C).

In *C. elegans*, *sbp-1* serves as the homolog to the sterol regulatory element-binding protein-1c (SREBP-1c). SREBP regulates lipid metabolism by controlling the expression of genes like *elo-2*, *fat-2*, and *fat-5*[117]. Piperine significantly reduced fat accumulation in *sbp-1* mutants by 27% (p=0.0033) compared to the control, suggesting *sbp-1* might not involve in piperine's fat lowering effects.

TUB-1, which is the homolog of human tubby proteins, also known to regulate fat storage[118]. Piperine decrease of fat by 21% (p=0.0003) compared to the control in *tub-1* mutant, suggesting it is not involved in the process of fat reduction (Fig 4.3C).

DAF-16, homolog to the class O of mammalian Forkhead (FOXO) transcription factors, functions downstream of *daf-2*, the nematode counterpart of the insulin/insulin-like growth factor receptor (IIS). Both DAF-2 and DAF-16 are integral to maintaining energy balance in the organism[119]. Compared with control, piperine had no significant effect on fat accumulation in *daf-2* and *daf-16* mutants, which suggests *daf-2* and *daf-16* play important role on fat reduction effects of piperine (Fig 4.3D).

DAF-12, a homolog to human farnesoid receptors, regulates dauer diapause and lipid metabolisms in *C. elegans*[120]. Piperine had no effects on TG in *daf-12* mutant, suggesting *daf-12* involved in the fat reduction effects of *C. elegans* (Fig 4.3D).

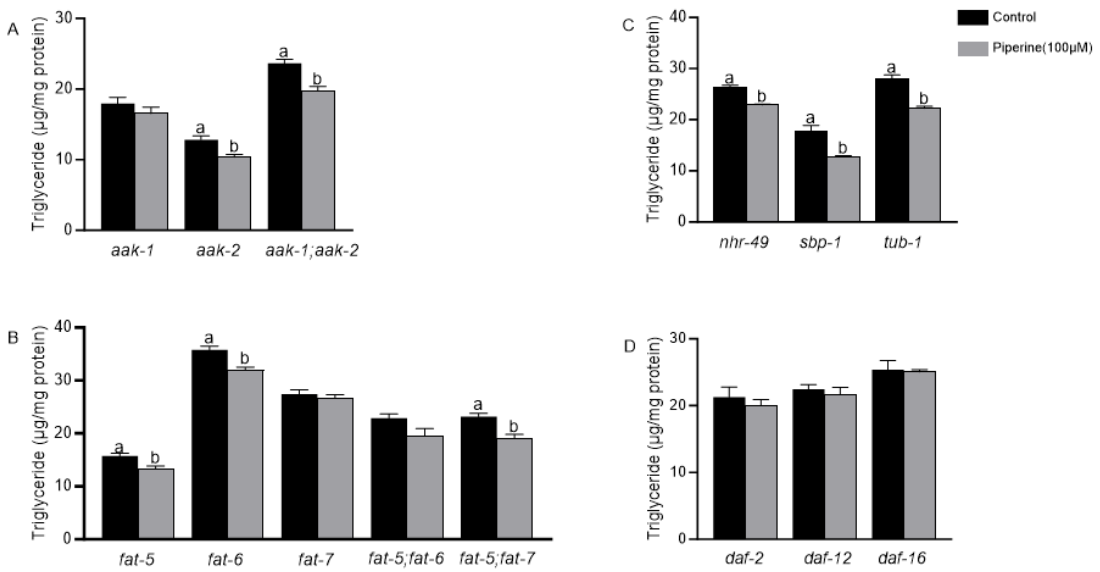

Fig 4.3 Effects on fat accumulation of piperine in *C. elegans* mutants. Mutants were subjected to a 48-hour treatment with 100μM piperine after adult stage. Triglyceride levels were standardized by protein content. Data are means ± S.E (n=4-8). Values with difference letters indicate a significant difference at each strain at of $p < 0.05$.

4.4 Piperine's Effects on Relative Gene Expressions

ATGL-1, FARD-1, FASN-1, and SIR-2.1, are homologs of human adipose triglyceride lipase[121], fatty acyl-CoA reductase[122], fatty acid synthase[123], and sirtuin 1[124], respectively. Relative gene expressions of *atgl-1*, *fard-1*, *fasn-1*, and *sir-2.1* showed no significant difference between control and 100μM piperine, suggesting those genes were not involved in the piperine's fat reduction effects.

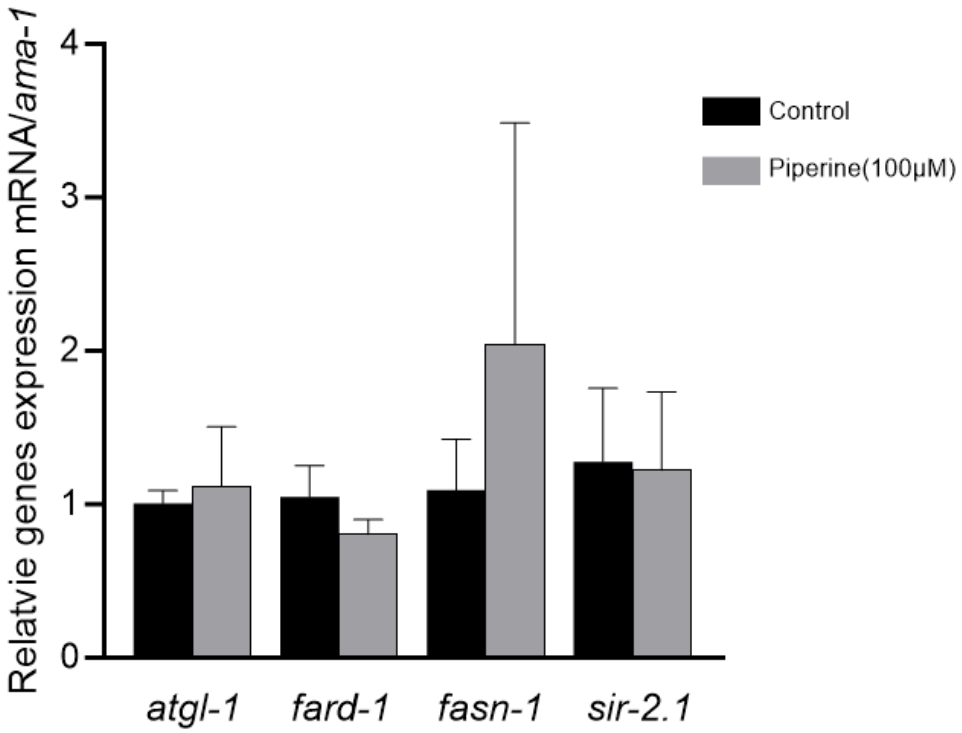

Figure 4.4 Effects of piperine on relateive gene expressions in *C. elegans* Mutants were subjected to a 48-hour treatment with 100μM piperine. Gene expressions were measured by real-time PCR, subsequently normalized by *ama-1* expression. Data are means ± S.E (n=3).

CHAPTER 5

DISCUSSION

Piperine, an alkaloid derived from pepper plants, is known for its diverse health advantages[46-50]. While numerous studies have documented its impacts on aging[51], cancer[52], diabetes[53], and inflammation[54], research on piperine's anti-obesity properties remains relatively limited. Hence, this study focused on piperine's potential in reducing fat accumulation, utilizing *C. elegans* as the chosen invertebrate model. Our findings indicate that piperine reduces fat accumulation in *C. elegans*, without altering in their food intake rate. Piperine's lipid-lowering effects are modulated through the insulin/insulin-like growth factors (IGF) signaling (IIS) pathway and DAF-12.

Contrary to previous research indicating that piperine may modulate fat accumulation through down-regulation of SREBP-1c, C/EBP-α, FAS, FABP-1 and up-regulation of UCP-1[88], our data found no involvement of *aak-1*, *aak-2*, *nhr-49*, *sbp-1*, and *tub-1* in piperine's fat-reducing effects. Additionally, the study observed inconsistent findings concerning PPAR-γ expression; piperine up-regulated PPAR-γ in rodent models[92,98] but down-regulated in 3T3-L1 adipocytes[88], suggesting that piperine's effect may vary depending on models tested.

Our investigation suggests that *daf-2*, *daf-12*, and *daf-16* might have a significant role in piperine's fat-reducing effects. DAF-2, a homolog of the IIS receptor, exerts influence on fat metabolism, independent of feeding behavior[125,126]. DAF-16, a transcription factor downstream of *daf-2*, is implicated in fat metabolism regulation via

its influence on fatty acid dehydrogenases *fat-7*, alcohol dehydrogenases *sodh-1*/*dod-11*, *sodh-2*/*dod-14*, short-chain acyl-CoA dehydrogenases *acdh-1*/*dod-12*, acyl CoA synthetase *dod-9*, and sterol signaling ligands *daf-9*[127,128]. Our results suggest that *fat-7* was not involved in piperine's effects, while other downstream targets of DAF-16 may be involved. Additional studies, including assessing *daf-16* expression in *daf-2* mutants, are warranted to clarify whether piperine acts directly on DAF-16, or whether its effects are mediated via DAF-2. Furthermore, upstream regulators of DAF-16, such as *age-1*, *pdk-1*, *akt-1*, and *akt-2*, may also be implicated in piperine's effects, which need to be determined[129].

Previous studies found the significance of DAF-12 in regulating energy metabolism, particularly its role in promoting aerobic fatty acid utilization[130]. DAF-12 activation by Δ7-dafachronic acid has been demonstrated to reduce triglyceride levels through enhanced lipolysis and fatty acid β-oxidation in *C. elegans*[131]. In addition, DAF-12 modulates fat accumulation via its downstream genes *fard-1*[132], and via PPARα[133]. However, our findings suggest that piperine's effects on fat metabolism are independent from its actions on *fard-1* and *nhr-49*. Other reported that the DAF-12 nuclear receptor remains unliganded in the absence of dafachronic acid[127]. And, DAF-12 operates its action via the Insulin/Insulin-like Growth Factor Signaling (IIS) pathway[134]. This was further supported by the fact that certain DAF-16/FOXO target genes depend on DAF-12, suggesting that DAF-16/FOXO and DAF-12 may function in parallel[135]. Given these findings, additional research is warranted to determine the

relationship between DAF-12 and the IIS pathway following treatment with piperine. In summary, this study provides insights into the potential effects of piperine on reducing fat accumulation via IIS pathway and DAF-12.

CHAPTER 6

FUTURE STUDY

The current study demonstrates the fat-reducing effects of piperine in *C. elegans* without altering food intake. Based on mutant experiments and RT-PCR analysis, it has been observed that the IIS pathway and DAF-12 are implicated in the fat-reducing effects of piperine. However, much remains to be further investigated.

Piperine elicits its effects via the IIS pathway, *daf-16* and *daf-2* (which is an upstream regulator of *daf-16*). However, it remains uncertain whether the piperine directly target *daf-16*, or indirectly through regulation of its upstream gene, *daf-2*. To elucidate this, testing piperine on DAF-16 expression using RT-PCR in *daf-2* mutants could be completed.

Additionally, there are other genes involved in the IIS pathway, which act upstream of *daf-16*—including *age-1*, *pdk-1*, *akt-1*, and *akt-2*—that may be regulated by piperine[125]. Furthermore, certain signaling pathways, such as the Target of Rapamycin (TOR) pathway, the JNK signaling pathway, and germline signaling pathways, should be further tested for their potential role in fat metabolism regulation by piperine[129]. Moreover, the relationship between DAF-12 and the IIS pathway remains to be determined.

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