Coffee Bioactives Regulate Lipid Metabolism in Caenorhabditis elegans

Renalison Farias Pereira

University of Massachusetts Amherst

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COFFEE BIOACTIVES REGULATE LIPID METABOLISM IN Caenorhabditis elegans

A Dissertation Presented

by

RENALISON FARIAS PEREIRA

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2020

Department of Food Science
COFFEE BIOACTIVES REGULATE LIPID METABOLISM
IN Caenorhabditis elegans

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RENALISON FARIAS PEREIRA

Approved as to style and content by:

Yeonhwa Park, Chair

David A. Sela, Member

John Gibbons, Member

Daeyoung Kim, Member

Erick A. Decker, Department Head
Department of Food Science
ACKNOWLEDGMENTS

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Coffee consumption is associated with reduced risk of metabolic syndrome, obesity and diabetes. Although the mechanisms of action are not yet elucidated, the effects of coffee and its bioactive components on lipid metabolism may account for the overall coffee effects on human health. Therefore, this study investigated the molecular mechanisms of coffee and its bioactive components on lipid metabolism using *Caenorhabditis elegans* as a model system. Green coffee bean extract (GCBE), the chlorogenic acid 5-O-caffeyolquinic acid (5-CQA), and the coffee diterpenes cafestol and kahweol reduced fat accumulation via distinct lipid metabolism pathways and/or behavior changes in *C. elegans*. The fat-lowering effects of GCBE and 5-CQA were similarly dependent on sterol regulatory element binding protein (*shb-1*) and forkhead box O (*daf-16*), both involved in lipogenesis, while cafestol reduced fat accumulation dependent on the FXR homolog, *daf-12*, in *C. elegans*. Moreover, cafestol increased worm’s moving speed, an
indicator of energy expenditure. The effects of cafestol on energy expenditure was also dependent on *tub-1* (homolog of the human TUBBY proteins), which it is likely to be a downstream target for DAF-12 in *C. elegans*. The other coffee diterpene, kahweol, also reduced fat accumulation, but instead kahweol’s fat-lowering effects were related to a reduced food intake in *C. elegans*. The effects of kahweol on lipid metabolism involved the downregulation of the insulin-like receptor *daf-2*, known to be regulated by food intake. Therefore, the suggested mechanisms of the effects of coffee bioactives on lipid metabolism may be applied for human health corroborating with the fact that consumption of coffee is associated with lower risk of obesity.
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CHAPTER 1

INTRODUCTION

Coffee consumption is associated with reduced risk of metabolic syndrome, obesity and diabetes, which may be related to the effects of coffee and its bioactive components on lipid metabolism [1, 2]. Green coffee bean extract (GCBE) is commercially available as dietary supplement for weight loss even though its claims are not yet approved by FDA. GCBE’s fat-lowering effects are associated with chlorogenic acids, however other coffee components may influence the effects of GCBE on lipid metabolism [3]. In addition, coffee contains diterpenes (cafestol and kahweol) and caffeine, which it is the most studied and a known neuromodulator as adenosine receptor antagonist [4].

There are evidences that lipid metabolism-related transcription factors (e.g. peroxisome proliferator-activated receptors and sterol regulatory element binding proteins) and enzymes (e.g. AMP-activated protein kinase) are regulated by coffee and/or its bioactive compounds, which suggest that coffee regulates lipogenesis, lipid uptake, transport, fatty acid β-oxidation and lipolysis [2]. However, there are still inconsistent reports about the effects of coffee and/or its compounds on lipid metabolism [2, 3], then identifying the underlying mechanisms of actions of coffee and its compounds can be helpful to apply these on human health.
CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Coffee is an ancient drink that is increasingly popular around the world. People who drink coffee are not only attracted to its flavor, but also to its potential health benefits, including lower risk of metabolic syndrome, obesity and diabetes [1, 2, 4]. As altered lipid metabolism is common to these conditions, the effects of coffee bioactives on lipid metabolism have been suggested as underlying mechanisms of the health benefits of coffee [1, 2]. Therefore, this review primarily discusses the current knowledge of coffee and its bioactive components on lipid metabolism.

2.2 Coffee composition

The composition of regular coffee varies mostly according to type of beans, roasting and brewing methods [5, 6]. The most popular coffee beans are from Coffea arabica (Arabica) or C. canephora (Robusta) with significant differences in their composition, including caffeine content; e.g. drinks from Robusta beans had higher caffeine levels than Arabica [5]. Roasting coffee beans degrades heat unstable compounds (e.g. phenolic acids and trigonelline) and changes their sensory profile [4]. For instance, light or medium roast coffee beans are used to make coffee drinks with more chlorogenic acids (CGA) than dark roast coffee beans [5]. Brewing methods influence the coffee drink composition as well; Turkish-style coffee drink had higher concentrations of diterpenes (cafestol and kahweol) than filtered coffee drink [7]. These
differences in composition have shown to influence the potential biological properties of coffee [6].

The most common coffee extraction is performed by hot water from beans, but other plant parts or solvents are used to develop other coffee products. Water extraction from the coffee fruit (pulp) or silver skin (bean testa), usually discarded in the regular coffee production, retained some of the coffee bioactive compounds, containing about 1% CGA and 1-3% caffeine [8–10]. Different solvent extraction methods change the coffee extract composition. Decaffeinated coffee, which has 2-15 mg caffeine per serving, can be produced by organic solvents or supercritical CO₂ methods [4, 11]. Ethanol extraction is used to make the commercially available green coffee bean extracts (GCBE), which contain 27-50% CGA and 2-10% caffeine [12–14]. Taken together, coffee processing methods have a great impact on its composition and related biological properties.

2.3 Coffee bioactive compounds

Caffeine (Fig. 2.1), an alkaloid that has a variety of potential biological effects, is found at concentration between 50-380 mg/100 mL in regular coffee drink [1, 4]. Caffeine is an adenosine receptor antagonist, related to its mostly known function as a neuromodulator, that boosts energy expenditure [15, 16]. Although caffeine has some benefits, some people need to control caffeine intake due to potential adverse effects, such as increased blood pressure [1].

There are different CGA esters in coffee and their concentrations combined range from 35-500 mg/100 mL in the regular coffee drink [4]. CGA esters are formed between cinnamic acids (caffeic acid, ferulic acid, p-coumaric acid) and quinic acid; here, any
CGA ester is mentioned as CGA [17]. Among them, 5-O-caffeyolquinic acid (Fig. 2.1) is the most studied CGA ester and is linked to the GCBE’s effects [3]. In addition, the CGA precursors or its degraded products were related to antioxidant properties [18–20].

![Diagram showing effects of coffee on lipid metabolism](image)

**Figure 2.1** Illustrative summary of the overall effects of coffee and its bioactive compounds on lipid metabolism. Molecular structures of chlorogenic acids (phenolic acids) representative: 5-O-caffeoylquinic acid; alkaloids: caffeine and trigonelline; diterpenes: cafestol and kahweol. ↓, decrease; ?, inconclusive; ↑, increase.

Trigonelline (Fig. 2.1), an alkaloid derivative of niacin (vitamin B3), is present at 40-50 mg/100 mL in regular coffee drink [4]. Although limited, trigonelline has shown potential biological effects, such as antioxidant and anti-inflammatory effects [21].
Additionally, trigonelline has shown to be potential anti-diabetes and anti-obesity agent, which may also be linked to niacin’s effects on lipid metabolism [22–24].

Cafestol (Fig. 2.1) is one of the coffee diterpenes found at 0.25-0.3 mg/100 mL in the regular coffee drink, and up to 4 mg/100 mL in unfiltered coffee drink [7]. High amounts of cafestol intake increased blood cholesterol levels; daily intake of 60 mg cafestol increased about 30 mg/dL total cholesterol levels in humans after 28 days [25]. Cafestol is an agonist of farnesoid X receptors (FXR), partially responsible for the increase of blood cholesterol levels by inhibiting bile acid synthesis [26, 27]. On the other hand, cafestol has shown beneficial biological effects, such as anti-obesity, anti-diabetes, anticancer and anti-inflammatory properties [28–32].

Kahweol (Fig. 2.1), present at range of 0.14-0.2 mg/100 mL in the regular coffee drink, is another diterpene mostly found in Arabica coffee beans [4, 7]. In vitro studies have shown that kahweol is a potential antioxidant, anti-obesity and anticancer agent [33–35]. Although kahweol and cafestol are structurally similar, their effects on lipid metabolism have been shown to be different; cafestol was more effective as cholesterol-raising factor, while kahweol was more effective as an adipogenesis inhibitor [25, 33].

2.4 Coffee regulates lipid metabolism

2.4.1 Coffee and human health

Human studies have shown that moderate consumption of coffee (2-3 cups/day) is associated with reduced risk of metabolic syndrome, obesity and type 2 diabetes [1, 2]. Daily consumption of coffee (510 mg CGA and 120 mg caffeine) or GCBE (372 mg CGA and 14.48 mg caffeine) ameliorated some parameters for metabolic syndrome after
8 weeks, including reduced body fat and insulin resistance [36, 37]. Consistently, daily intake of 600 mg CGA increased fat oxidation in healthy male subjects after 5 days [38].

The effects of coffee are influenced by genetic differences in the population; i.e., rate of caffeine metabolism contributed significantly to physiological responses to coffee [39, 40]. Daily intake of coffee (174.4 mg CGA and 175.2 mg caffeine) reduced postprandial glucose levels in people who metabolizes caffeine slowly, but increased postprandial glucose levels in people who metabolize caffeine quickly after 12 weeks [39]. However, a follow-up study reported that hypertensive patients who metabolize caffeine slowly had higher risk of impaired fasting glucose, compared to whom metabolize caffeine quickly or non-coffee drinkers [40].

A systematic review of clinical trials has discussed inconsistent results of different types of coffee on glucose metabolism and suggested that CGA and other compounds than caffeine within coffee contribute to the coffee’s effects on human health [41]. For instance, a cross-over study showed that decaffeinated coffee (equivalent to 17-24 mg caffeine or 0.24-0.33 mg caffeine/kg body weight), but not caffeinated coffee (equivalent to 101-144 mg caffeine or 1.4-2.0 mg caffeine/kg body weight), improved insulin sensitivity in healthy men [42]. Therefore, many of the inconsistent effects of coffee on human health may be due to variation of coffee composition.

Although some epidemiological studies show that moderate coffee consumption is associated with reduced risk of cardiovascular diseases, whether coffee has adverse or beneficial effects on blood lipids profile and its mechanisms is still being investigated [43–45]. A meta-analysis showed that coffee consumption (2.4-8 cups/day) increased total cholesterol, low-density lipoproteins (LDL) and triglycerides levels after 2-11 weeks
Others have shown that coffee has a null or beneficial effect on lipid profile; coffee or GCBE did not have an impact on lipid profile in healthy subjects [37, 39], while coffee reduced blood triglycerides levels in subjects with high cholesterol levels after 8 weeks [36]. In addition, unfiltered coffee was strongly associated with the undesirable changes in lipid profile, probably due to inhibitory effects of cafestol on bile acid synthesis [25, 44]. Overall, these human trials provide limited evidence that coffee and its bioactive compounds regulate lipid metabolism. This review will summarize the current proposed mechanisms of action of coffee and its bioactive compounds on lipid metabolism (Tables 2.1 and 2.2).
<table>
<thead>
<tr>
<th>Material</th>
<th>Doses(^a)</th>
<th>Time (day)</th>
<th>Model</th>
<th>Effects(^b)</th>
<th>Targets(^c)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green Coffee Bean Extracts</td>
<td>5 mg/mL (50% CGA and 2% caffeine)</td>
<td>3</td>
<td><em>Caenorhabditis elegans</em></td>
<td>↓TG</td>
<td>↓ACC; ↑ACS2; ↑ECH4; ↑FOXO; ↓FAR4; ↑HSL; ↓SREBP</td>
<td>[3]</td>
</tr>
<tr>
<td></td>
<td>1% diet (27% CGA and 10% caffeine)</td>
<td>14</td>
<td>ddY mice (♂)</td>
<td>↓b.w.; ↓TG</td>
<td>↑CPT</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td>330 mg/kg b.w. (p.o.) (28% CGA and 9% caffeine)</td>
<td>70</td>
<td>High fat diet-fed ICR and C57BL/6 mice (♂)</td>
<td>↓b.w.; ↓Chol; ↓HDL; ↓Leptin; ↓TG</td>
<td>↓ACRP30; ↓PPARγ</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>200 mg/kg b.w. (p.o.) (50% CGA)</td>
<td>42</td>
<td>High fat diet-fed C57BL/6J mice (♂)</td>
<td>↑Adiponectin; ↓b.w.; ↓Chol; ↓FFA; ↓Glucose; ↓LDL; ↓Leptin; ↓TG</td>
<td>↑AMPK; ↑ATGL; ↓C/EBPα; ↑CPT1; ↓FAS; ↑HSL; ↑PPARα; ↓PPARγ; ↓SREBP1c; ↓SREBP2</td>
<td>[14]</td>
</tr>
<tr>
<td>Water extracts</td>
<td>1% diet (77% CGA)</td>
<td>14-105</td>
<td>High fat diet-fed C57BL/6J mice (♂)</td>
<td>↓b.w.; ↓Chol; ↑Energy expenditure; ↓Glucose; ↓Inflammation; ↓Insulin; ↓Leptin; ↓TG</td>
<td>↓ACC1; ↓ACC2; ↓FAS; ↑miR-122; ↓SREBP1c; ↓SCD1; ↑UCP2</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>0.5% water</td>
<td>70</td>
<td>High fat diet-fed Swiss mice (♂)</td>
<td>↑Adiponectin; ↓Glucose; ↓Inflammation; ↓Insulin; ↑Leptin</td>
<td>↑PKB</td>
<td>[47]</td>
</tr>
<tr>
<td></td>
<td>2% diet</td>
<td>63</td>
<td>High fat diet-fed C57BL/6J mice (♂)</td>
<td>↓b.w.; ↓Inflammation; ↓Insulin; ↓TG</td>
<td>↓SCD1</td>
<td>[48]</td>
</tr>
<tr>
<td>0.1% water</td>
<td>119 Aged C57BL/6 NCr mice (♂)</td>
<td>↑ATP; ↓FFA; ↑Locomotor activity; ↓TG</td>
<td>↓MTOR; ↑PPARα</td>
<td>[49]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 g/kg b.w. (p.o.) (1.2% CGA and 0.4% caffeine)</td>
<td>84 High fat diet-fed Wistar rats (♂)</td>
<td>↓b.w.; ↓Chol; ↓Insulin; ↓LDL; ↓TG</td>
<td>↑LXRα; ↓NPC1L1; ↑PPARα; ↓PPARγ</td>
<td>[8, 50]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* b.w., body weight; p.o., per os.

*b* ↓, decrease; ↑, increase; b.w., body weight; Chol, cholesterol; FFA, free fatty acids; TG, triglycerides.

*c* ACC, acetyl-CoA carboxylase; ACRP, adipocyte complement-related protein; AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; C/EBP, CCAAT/enhancer binding protein; CPT, carnitine palmitoyl transferase; ECH, enoyl-CoA hydratase; FAR, fatty acid- and retinoid-binding protein; FAS, fatty acid synthase; FOXO, forkhead box O; HSL, hormone sensitive lipase; LXR, liver X receptor; miR, microRNA; mTOR, mammalian target of rapamycin; NPC1L1, NPC1-like intracellular cholesterol transporter 1; PKB, protein kinase B; PPAR, peroxisome proliferator activated receptor; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein; UCP, uncoupling protein.
Table 2.2. Summary of mechanistic studies of coffee bioactive compounds on lipid metabolism.

<table>
<thead>
<tr>
<th>Material</th>
<th>Doses&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Time (day)</th>
<th>Model</th>
<th>Effects&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Targets&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Cafestol</td>
<td>60 µM</td>
<td>2</td>
<td><em>Caenorhabditis elegans</em></td>
<td>↑Energy expenditure; ↓TG</td>
<td>↑FXR; ↑HADHA; ↑TUBBY</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>0.05% diet</td>
<td>56</td>
<td>High fat diet-fed C57BL6/J mice (♂)</td>
<td>↓Bile acids; ↓b.w.; ↑FFA ↑Glycerol; ↓Inflammation; ↓Insulin; ↓Leptin; ↓TG</td>
<td>↑ATGL; ↑CPT1 ↑FAS; ↑HSL; ↑SREBP1c; ↑UCP1</td>
<td>[32]</td>
</tr>
<tr>
<td>Caffeine</td>
<td>5%</td>
<td>20</td>
<td>Zebrafish</td>
<td>↓b.w.; ↓TG</td>
<td>↓ACC1; ↑ACOX; ↓ATG12; ↓BECN1; ↓CD36; ↓SREBP1; ↓UCP2</td>
<td>[52]</td>
</tr>
<tr>
<td></td>
<td>60 mg/kg b.w.</td>
<td>4 h</td>
<td>Obese yellow KK mice (♀)</td>
<td>↑Adrenaline; ↑FFA</td>
<td>↑UCP1; ↑UCP2; ↑UCP3</td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td>(s.c.) 30 mg/kg b.w.</td>
<td>3-28</td>
<td>C57BL/6 mice (♂)</td>
<td>↓b.w.; ↓TG</td>
<td>↑ACC; ↓CPT1α; ↑LC3; ↓MTOR; ↓SQSTM1</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>(i.p.); 0.05%</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>water</td>
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<tr>
<td></td>
<td>20 mg/kg b.w.</td>
<td>70</td>
<td>High energy diet-fed C57BL/6 mice (♂)</td>
<td>↓b.w.; ↓Chol ↓FFA; ↓TG</td>
<td>↓ACC; ↑AMPK; ↑cAMP</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>(p.o.) 20 mg/kg b.w.</td>
<td>42</td>
<td>High fat diet-fed Sprague-Dawley rats (♂)</td>
<td>↓b.w.; ↓TG</td>
<td>↓ACC; ↓FAS; ↑IRS1; ↑PPARα; ↓SREBP1c</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td>(p.o.) 60 mg/kg b.w.</td>
<td>11-14</td>
<td>High fat diet-fed C57BL/6; ob/ob mice (♂)</td>
<td>↓b.w.; ↓Glucose; ↓TG</td>
<td>↓A1R; ↑UCP1</td>
<td>[15]</td>
</tr>
<tr>
<td>Chlorogenic acids</td>
<td>2.65 mg/mL</td>
<td>3</td>
<td><em>Caenorhabditis elegans</em></td>
<td>↓TG</td>
<td>↓ACC; ↓ACS2; ↑ECH4; ↓FAR4; ↑FOXO; ↓C/EBP; ↓SREBP</td>
<td>[3]</td>
</tr>
<tr>
<td>Dose</td>
<td>Species</td>
<td>Diet/Fed Model</td>
<td>Changes</td>
<td>References</td>
<td></td>
<td></td>
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<tr>
<td>80 mg/kg b.w. (i.p.)</td>
<td>High fat diet-fed ICR hamsters (♂)</td>
<td>↓b.w.; ↓Chol; ↓FFA; ↓Glucose; ↓HDL; ↓Insulin; ↓LDL; ↓TG</td>
<td>↑HL; ↓LPL; ↑PPARα</td>
<td>[57]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02% diet</td>
<td>High fat diet-fed ICR mice (♂)</td>
<td>↑Adiponectin; ↓b.w.; ↓Chol; ↓FFA; ↓Insulin; ↓Leptin; ↓TG</td>
<td>↓ACAT; ↓FAS; ↓HMGR; ↑PPARα</td>
<td>[58]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 mg/kg b.w. (i.p)</td>
<td>Lepr&lt;sup&gt;db/db&lt;/sup&gt; mice (♂)</td>
<td>↑Adiponectin; ↓b.w.; ↓Chol; ↓FFA; ↓Glucose; ↓Insulin</td>
<td>↓ACC; ↑AMPK; ↑CaMKK</td>
<td>[59]</td>
<td></td>
<td></td>
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<tr>
<td>90 mg/kg b.w. (p.o.)</td>
<td>High fat diet-fed Sprague-Dawley rats (♂)</td>
<td>↓b.w.; ↓Chol; ↓FFA; ↓Glucose; ↓Insulin; ↓HDL; ↓LDL; ↓TG</td>
<td>↓ACC; ↑CD36; ↑CPT2; ↓FABP4; ↓FAS; ↓LPL; ↓LXRα; ↑PPARα; ↑RXRα</td>
<td>[60]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mg/kg b.w. (i.p.)</td>
<td>High fat diet-fed C57BL/6J mice (♂)</td>
<td>↓b.w.; ↓Chol; ↓FFA; ↓Glucose; ↓Insulin; ↓TG</td>
<td>↑ACOX1; ↓CD36; ↑CPT1; ↓FABP4; ↓MGAT; ↑PPARα; ↑PPARγ</td>
<td>[61]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mg/kg b.w. (p.o.)</td>
<td>High fat diet-fed Sprague-Dawley rats (♂)</td>
<td>↓b.w.; ↓Chol; ↓FFA; ↓TG</td>
<td>↓ACC; ↑AMPK; ↑CPT1</td>
<td>[62]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mg/kg b.w. (p.o.)</td>
<td>High fat diet-fed ICR mice (♂)</td>
<td>↑Adiponectin; ↓b.w.; ↓Chol; ↑HDL; ↓LDL; ↓TG</td>
<td>↓C/EBPα; ↓FABP4; ↓FAS; ↓LPL; ↓SREBP1c; ↑PPARα; ↑PPARγ</td>
<td>[63]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trigonelline</td>
<td>Goko rats (♂)</td>
<td>↓Bile acid; ↓Chol; ↓FFA; ↓Glucose; ↓Insulin; ↓TG</td>
<td>↑FAS</td>
<td>[23]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mg/kg b.w. (p.o.)</td>
<td>Alloxan-induced diabetes Wistar rats (♂)</td>
<td>↑b.w.; ↓Chol; ↓Glucose; ↑HDL; ↓LDL; ↓TG</td>
<td>↓Intestinal Lipase</td>
<td>[64]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mg/kg b.w. (p.o.)</td>
<td>High fat (or cholesterol) diet-fed C57BL/6J mice (♂)</td>
<td>↓b.w.; ↓Chol; ↓Glucose; ↓Insulin; ↓TG</td>
<td>↑AMPK; ↑BECN1; ↓CD36; ↑MTOR; ↑PPARγ; ↑SREBP1</td>
<td>[22]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> b.w., body weight; i.p, intraperitoneal; p.o, per os; s.c., subcutaneous.

<sup>b</sup> ↓, decrease; ↑, increase; b.w., body weight; Chol, cholesterol; FFA, free fatty acids; TG, triglycerides.
A1R, adenosine 1 receptor; ACAT, acyl-CoA cholesterol acyltransferase; ACC, acetyl-CoA carboxylase; ACOX, acyl-CoA oxidase; AMPK, AMP-activated protein kinase; ATG, autophagy related; ATGL, adipose triglyceride lipase; BECN, beclin; C/EBP, CCAAT/enhancer binding protein; cAMP, cyclic AMP; CaMKK, calcium/calmodulin-dependent protein kinase kinase; CD, cluster of differentiation; CPT, carnitine palmitoyl transferase; ECH, enoyl-CoA hydratase; FABP, fatty acid-binding protein; FAR, fatty acid- and retinoid-binding protein; FAS, fatty acid synthase; FOXO, forkhead box O; FXR, farnesoid X receptor; HADHA, hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha; HL, hepatic lipase; HMGR, HMG-CoA reductase; HSL, hormone sensitive lipase; IRS, insulin receptor substrate; LC, microtubule-associated protein 1A/1B-light chain; LPL, lipoprotein lipase; LXR, liver X receptor; MGAT, monoacylglycerol acyltransferase; MTOR, mammalian target of rapamycin; PPAR, peroxisome proliferator activated receptor; RXR, retinoid X receptor; SREBP, sterol regulatory element-binding protein; UCP, uncoupling protein.
2.4.2 Coffee reduces lipogenesis

Coffee has shown to have fat-lowering effects in humans, which was associated with reduced lipogenesis [2]. Coffee extracts [3, 14, 46, 48], CGA [3, 58–60, 62, 65], caffeine [52, 54, 56, 65], trigonelline [23] and cafestol [32] have shown to reduce activity of key enzymes for lipogenesis: acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and/or stearoyl-CoA desaturase (SCD). ACC and FAS are responsible for the first two steps of de novo lipogenesis, while SCD for the synthesis of monounsaturated fatty acids for fat storage [66]. The enzymatic inhibitory effects of coffee and/or its bioactive compounds were in part via regulation of upstream transcription factors for lipogenesis: CCAAT/enhancer-binding proteins (C/EBP), peroxisome proliferator-activated receptors (PPAR, especially PPARγ), and/or sterol regulatory element-binding proteins (SREBP) [3, 13, 14, 22, 32, 46, 50, 52, 56, 61, 63, 65]. These transcription factors are well-known to regulate adipogenesis, including lipogenesis [66, 67].

In addition, coffee reduces lipogenesis by regulating another metabolic pathway, AMP-activated protein kinase (AMPK), which inhibits ACC and FAS [59, 66]. In fact, coffee, CGA, caffeine and trigonelline were able to activate AMPK [22, 55, 59, 62, 68–70]. Many factors regulate AMPK activity, including the second messenger cyclic AMP (cAMP), which is increased by caffeine [55]. Along with increased cAMP, caffeine and CGA activated Ca²⁺/calmodulin-dependent protein kinase (CaMK), which can subsequently regulate the AMPK activation [59, 69, 70].

Coffee can also activate the forkhead box O (FOXO), involved in the insulin-signaling pathway known to regulate lipogenesis. GCBE and CGA reduced body fat dependent to increased FOXO nuclear translocation, leading to an decreased lipogenesis
in Caenorhabditis elegans [3]. These suggest that the fat-lowering effects of coffee by inhibition of lipogenesis are potentially from its effects on insulin-mediated pathway via FOXO.

Epigenetic modifications by coffee might contribute to its effects on lipogenesis as well; coffee and CGA upregulated miR-122, a microRNA abundant in the liver with the inhibition of SREBP, ACC and FAS in murine hepatocytes [46]. Similarly, others reported that coffee increased miR-96, a microRNA involved in SREBP expression in human intestinal epithelial Caco-2 cells [71, 72]. Therefore, coffee and its bioactive compounds may inhibit lipogenesis via epigenetic changes.

### 2.4.3 Coffee compounds regulate lipid uptake and transport

Coffee can regulate the fatty acid translocase (FAT/CD36/SR-B2), a key transmembrane protein for lipid uptake and transport [73]. Caffeine, CGA and trigonelline have shown to decrease the diet-induced hepatic CD36 overexpression [22, 52, 60, 61]. CD36 is not only important for the uptake of dietary fatty acids, but also able to bind lipoproteins in the liver [74, 75]. Thus, the decreased expression of CD36 by coffee compounds is probably related to changes in blood lipid profile, including reduced triglycerides, cholesterol and LDL levels [22, 52, 60, 61]. It was further suggested that caffeine, CGA and trigonelline regulated CD36 via AMPK- and PPARγ -dependent pathways [22, 60, 61, 73, 76].

Other lipid-binding proteins involved in lipid uptake and transport, such as fatty acid-binding proteins (FABP) and fatty acid transporters proteins (FATP), were regulated by coffee bioactive components [3, 33, 77, 78]. It is suggested that the decreased lipid uptake and transport is related to lipogenesis inhibition; FABP4 (also called aP2), a target
for PPARγ, was downregulated by caffeine and kahweol in adipocytes [33, 78].

Consistently, a fatty acid- and retinoid-binding protein, FAR-4, was required for GCBE and CGA to reduce fat accumulation in *C. elegans* [3]. However, coffee compounds can increase lipid uptake and transport in the muscle driven by fatty acid β-oxidation; caffeine increased lipid uptake and transport in muscle tissue by regulating FABP, FATP1 and FATP4, partially dependent on mitochondrial CD36 [77]. Taken together, coffee bioactive compounds regulate tissue-specific lipid uptake and transport via CD36 and other lipid-binding proteins.

### 2.4.4 Coffee increases fatty acid β-oxidation

There are many reports of coffee and its bioactive compounds on regulating fatty acid β-oxidation [3, 12, 14, 32, 52, 54, 56–58, 60–63, 65]. Coffee, CGA, caffeine and cafestol have shown to increase the rate-limiting enzyme for mitochondrial fatty acid β-oxidation, carnitine palmitoyl transferase (CPT), which transports acyl-CoA from cytosol into mitochondria [12, 14, 32, 54, 60–62]. In addition, peroxisomal fatty acid β-oxidation was increased by CGA and/or caffeine via regulation of acyl-CoA oxidases (ACOX), the first step of the peroxisomal fatty acid β-oxidation [52, 61, 65, 79].

It is suggested that coffee regulates fatty acid β-oxidation enzymes by activating PPARα in the liver and adipose tissues [14, 50, 56–58, 60, 61, 63]. Moreover, PPARβ/δ, involved in the fatty acid β-oxidation in muscle tissue, may play a role in the coffee’s effects; caffeine upregulated PPARβ/δ in muscle cells [67, 80]. However, it was reported that a coffee extract and CGA did not act as PPAR agonists in kidney CV-1 cells [46]. Therefore, the mechanism in which coffee and its bioactive compounds activate PPAR is yet to be clear.
Other nuclear hormone receptors are reported to be involved in the coffee bioactive components’ effects on fatty acid β-oxidation. For instance, CGA has shown to increase expression of retinoid X receptor (RXR) and decrease liver X receptor (LXR) [60], which share similarities with PPARα [81]. There is evidence that cafestol acts as a FXR agonist [26]; FXR is not only involved in cholesterol metabolism, but involved in fatty acid β-oxidation [82, 83]. Thus, it is possible that cafestol regulates fatty acid β-oxidation via FXR [51]. Therefore, it can be considered that coffee has pleiotropic effects by regulating transcription factors that potentially impact fatty acid β-oxidation.

2.4.5 Coffee regulates lipolysis

Coffee and caffeine consumption increased lipolysis, measured by free fatty acids and/or glycerol, peaking after 2-4 hours in humans [84–86]. It was suggested that caffeine increases lipolysis in adipose tissue by inhibiting adenosine receptor and increasing catecholamine levels via the sympathetic nervous system [15, 53, 87]. The lipolytic effects of caffeine are mediated by the increased cAMP levels that activate enzymes for lipolysis, especially hormone-sensitive lipases (HSL) [55, 66, 87]. Consistently, GCBE, CGA and cafestol upregulated HSL and adipose triglyceride lipases (ATGL), both responsible for lipolysis in adipose tissue [14, 32, 88]. However, GCBE, not CGA, upregulated HSL expression in C. elegans [3]. Since post-transcriptional regulation of these enzymes is important [89], the effects of coffee and its compounds on lipases activities will need to be determined to confirm the activities of coffee on lipolysis.

The lipolytic effects of coffee compounds are probably regulated by an additional pathway, the mammalian target of rapamycin (mTOR) [90]. mTOR was inhibited by
coffee, caffeine, trigonelline and kahweol in vivo or in vitro [22, 34, 49, 54].

Consistently, the lipolytic effects of caffeine were related to autophagy-lysosomal pathway dependent on AMPK and CaMK, known to cross-talk with mTOR [54, 70]. Therefore, the effects of coffee and its compounds on the nutrient-sensing pathways mTOR, AMPK and CaMK may contribute to the effects of coffee on lipolysis.

2.4.6 Coffee reduces lipid digestion

Coffee and its bioactive compounds may reduce dietary lipid digestion, partially due to inhibition of digestive lipase [8, 91, 92]. GCBE inhibits pancreatic lipase activity, in which half-maximal inhibitory concentration (IC_{50}) was estimated to be 1.98 mg/mL in in vitro digestive simulation [92, 93]. The lipase inhibitory effects of coffee is more likely due to CGA than caffeine; IC_{50} for CGA was 13-287 µM and IC_{50} for caffeine was > 500 µM [92]. Trigonelline was also found to inhibit lipase and other digestive enzymes in rats [64]. GCBE inhibited pancreatic lipase by decreasing surface area of lipid emulsion and increasing lipid droplet size in vitro [93].

Coffee bioactive compounds can also affect lipid digestion, reducing the function or synthesis of bile acids, emulsifying agents that enhance lipid digestion [8, 27]. CGA was able to bind bile acids in vitro, suggesting that it reduces the bile acid function for lipid digestion [8]. Moreover, cafestol was found to inhibit bile acid synthesis in rodents, which potentially changes lipid digestion [26, 27]. Therefore, the inhibition of bile acid synthesis and lipase activity by coffee and its bioactive compounds may reduce dietary lipid digestion.
2.5 Conclusion

In conclusion, coffee and its bioactive components have shown to regulate lipid metabolism. Although there is more evidence for coffee extracts, especially GCBE, CGA and caffeine, other, less studied compounds (trigonelline, cafestol and kahweol) have shown potential to act on lipid metabolism in vivo and/or in vitro studies. Many questions about their mechanisms on lipid metabolism remain to be answered, and perhaps with the use of ‘omics’ technologies in humans, we will be able to understand and validate the effects of coffee on human health in future.
CHAPTER 3

OBJECTIVES OF THE PROJECT

The long-term goal of this project was to facilitate the development of coffee-related products that may improve human health. The objective was to investigate the role of coffee and its bioactive components on lipid metabolism in Caenorhabditis elegans. The central hypothesis was that coffee and/or its bioactive compounds modulate lipid metabolism in C. elegans, a microscopic transparent worm that has been used for obesity studies and to elucidate pathways on lipid metabolism due to its similarities with humans. The rationale of this project was that by understanding the effects of coffee and its bioactive components on lipid metabolism in C. elegans, we will be able to propose mechanisms to be applied in humans.

Specific aims:

- Determine the effects of green coffee bean extract (GCBE) and chlorogenic acids (CGA) on fat accumulation in C. elegans. This aim was designed to test the hypothesis that CGA are responsible for the anti-obesity effects of GCBE and their mechanisms on lipid metabolism are similar in C. elegans.

- Determine the underlying mechanisms of action of cafestol on lipid metabolism in C. elegans. The working hypothesis of this aim was that cafestol as an farnesoid X receptor (FXR) agonist regulates lipid metabolism in C. elegans.

- Investigate the potential anti-obesity effects of kahweol in C. elegans. The aim was to test the hypothesis that kahweol reduces fat accumulation by modulating feeding behavior in C. elegans.
CHAPTER 4

GREEN COFFEE BEAN EXTRACT AND 5-O-CAFFEYOYLQUINIC ACID
REGULATE FAT METABOLISM IN Caenorhabditis elegans

4.1 Introduction

Coffee consumption has been associated with reduced weight gain and body fat [36, 94]. This is known to be attributed to coffee polyphenols, such as chlorogenic acids found in high concentrations in unroasted coffee, also known as green coffee beans. Green coffee bean extract (GCBE) usually consists of 50% chlorogenic acids, which are various esters formed between cinnamic acids and quinic acid [95]. Among them, 5-O-caffeoylquinic acid (5-CQA) is one of the most studied chlorogenic acid esters associated with effect of GCBE [95–98].

GCBE and its main chlorogenic acids have shown potential as natural anti-obesity agents. In in vitro studies, GCBE and chlorogenic acids inhibit lipase activity, suggesting potential reduction of fat absorption [93]. Others reported that GCBE and chlorogenic acids attenuate obesity and insulin resistance in rodent models [61, 99–101]. Human studies showed that GCBE reduces body weight and improves blood lipid profile [102, 103]. However, others reported inconsistent effects of GCBE (with ~70% of chlorogenic acids) on fat accumulation or insulin resistance in mice [104]. Although limited, reports have suggested that GCBE and chlorogenic acids act on AMP-activated protein kinase (AMPK) and lipogenesis pathway [14, 46, 59]. Therefore, identifying the mechanism of actions for GCBE and chlorogenic acids would be helpful to apply these as functional foods.
*Caenorhabditis elegans* is a multi-organ, microscopic and transparent roundworm, mostly known for its use in aging studies, but it is also used for obesity research [105]. *C. elegans* has conserved fat and energy regulatory pathways, and its known-whole genome sequence makes this model useful for determining mechanisms by using knockout mutants and standard molecular biology techniques [105, 106]. Previously, GCBE and 5-CQA extended the lifespan of *C. elegans* [107, 108], however, there is no report determining GCBE and 5-CQA’s effects on fat metabolism in this model. Thus, the goal of the current study was to investigate the mechanisms of action of GCBE and chlorogenic acids on fat metabolism using *C. elegans* as a model system. GCBE used in the current study consists of about 50% chlorogenic acids; thus, 5-CQA was used as a representative of chlorogenic acids [98, 109].

### 4.2 Materials and methods

#### 4.2.1 Materials

GCBE powder, which contained about 50% chlorogenic acids and 2% caffeine, was purchased from NuSci Institute & Corp. (Batch No. 201511005, Walnut, CA, USA). 5-*O*-caffeoylquinic acid (5-CQA, IUPAC numbering, CAS 327-97-9; named previously as chlorogenic acid or 3-*O*-caffeoylquinic acid, pre-IUPAC numbering; [110, 111]) was purchased from Sigma-Aldrich Co. (purity ≥ 95%, St. Louis, MO, USA) or Cayman Chemical (purity ≥ 95%, Ann Arbor, MI, USA). The amounts of triglyceride and protein were quantified using Infinity™ Triglycerides Reagent and Pierce™ Coomassie Plus (Bradford) protein reagent from Fisher Diagnostics and Thermo Fisher Scientific (Middletown, VA, USA), respectively. TRIzol and gene expression probes were
purchased from Thermo Fisher Scientific (Middletown, VA, USA). 2’-Deoxy-5-fluorouridine (FUDR) was from TCI America (Portland, OR, USA), carbenicillin was from Fisher Bioreagents (Pittsburgh, PA, USA), ampicillin was from Sigma-Aldrich Co. (St. Louis, MO, USA), and other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA). *Escherichia coli* OP50 and *C. elegans* strains were obtained from *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA): N2, Bristol (wild-type); CE541 [*sbp-1(ep79)* III.]; CE548 [*sbp-1(ep79)* III.; epEx141]; GR1307 [*daf-16(mgDf50)* I.]; RB1515 [*far-6(ok1811)* IV.]; RB1600 [*tub-1(ok1972)* II.]; RB1667 [*tax-6(ok2065)* IV.]; RB1716 [*nhr-49(ok2165)* I.]; RB754 [*aak-2(ok524)* X.]; TJ356 [*daf-16p::daf-16a/b::GFP + rol-6(su1006)*].

### 4.2.2 *C. elegans* culture

Worms were cultured according to established protocols [105, 112]. Worms were cultured on nematode growth media (NGM) plates (1.7% agar, 2.5 g/L peptone, 51 mM NaCl, 25 mM KPO₄ buffer pH 6.0, 5 μg/L cholesterol, 1 mM CaCl₂, 1 mM MgSO₄) and incubated at 25°C, except *sbp-1* mutants (20°C), on an incubator (model DT2-MP-47, TriTech Research Inc., Los Angeles, CA). To obtain synchronized population of worms, eggs were collected using bleach solution [112]. M9 buffer (41 mM Na₂HPO₄, 15 mM KH₂PO₄, 8.6 mM NaCl, 19 mM NH₄Cl) were used to wash the eggs, and then the eggs were incubated overnight with S-complete (100 mM NaCl, 5.7 mM K₂HPO₄, 44 mM KH₂PO₄, 5 μg/L cholesterol, 0.1 M C₆H₅K₃O₇ pH 6.0, 3 mM CaCl₂, 3 mM MgSO₄, 50 μM disodium EDTA, 2.5 μM FeSO₄ •7 H₂O, 1 μM MnCl₂•4 H₂O, 1 μM ZnSO₄ •7 H₂O, 0.1 μM CuSO₄ •5 H₂O). In liquid media, L1 stage worms (~1000 worms/mL) were cultured in S-complete with ampicillin (100 μg/mL), carbenicillin (50 μg/mL) and dead
\textit{E. coli} OP50 to prevent degradation of extract and compound by live bacteria. After two days, L4 stage/young adult worms were treated with FUDR (0.12 mM) to prevent eggs from hatching. Green coffee bean extract (GCBE, 10 or 50 mg/mL) and 5-\textit{O}-caffeoylquinic acid (5-CQA, 5.33 or 26.5 mg/mL) were freshly prepared with S-complete prior to each experiment, then, sterilized with 0.22 \(\mu\)m syringe filter (Fisherbrand, Pittsburgh, PA, USA) prior to use. Finally, GCBE (1 or 5 mg/mL) or 5-CQA (0.53 or 2.65 mg/mL) was added to media and incubated for 3 days.

4.2.3 Triglyceride quantification

First, worms were collected and washed thrice with water, then samples were homogenized by sonication with 0.05\% Tween 20 for 3 min [113]. Infinity™ Triglycerides Reagent was used to quantify triglyceride with glycerol as standard. Protein levels was determined with Pierce™ Coomassie Plus (Bradford) Protein Reagent with bovine serum albumin as standard. Absorbances were determined by SpectraMax i3 microplate reader and SoftMax Pro 6 (version 6.5) from Molecular Devices (Sunnyvale, CA, USA).

4.2.4 Food intake, worms size and locomotive behavior

Worms were transferred to low-peptone NGM plates with thin layer of live \textit{E. coli} OP50 as previously described [113]. For food intake, pharynx contractions (pumping rate) per 30 s of worms were counted using a stereomicroscope. For size and locomotive behavior, worms were recorded for 1 min and then average speed [\(\mu\)m/s; (forward distance + reverse distance)/time], width (\(\mu\)m), and length (\(\mu\)m) were measured by
WormLab tracking system (model MSCOP-002, MBF Bioscience, MicroBrightField Inc., Williston, VT, USA) and WormLab software (3.1.0 64-bit, MBF Bioscience, Williston, VT, USA).

4.2.5 Real-time PCR

Total RNA was isolated using TRIzol following the manufacturer’s protocol (Thermo Fisher Scientific, Inc., Middletown, VA). cDNA was generated using high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Inc., Middletown, VA) and standard thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA). Relative quantitative RT-PCR was carried out using StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA). Results were analyzed using the comparative threshold cycle (Ct) method and expressed as fold change of gene expression \(2^{-\Delta\Delta Ct}\) [114]. TaqMan gene expression assays (Thermo Fisher Scientific, Inc., Middletown, VA) used were aak-1 (Ce02406989_g1), aak-2 (Ce02404250_m1), acs-2 (Ce02486193_g1), acs-11 (Ce02431951_m1), atgl-1 (Ce02406730_g1), cebp-2 (Ce02421574_g1), daf-16 (Ce02422838_m1), daf-2 (Ce02444336_m1), ech-4 (Ce02438697_g1), far-6 (Ce02466862_g1), fasn-1 (Ce02411648_g1), hosl-1 (Ce02494529_m1), let-363 (Ce02417512_m1), mdt-15 (Ce02406575_g1), nhr-49 (Ce02412667_m1), pod-2 (Ce02427721_g1), and ama-1 (Ce02462726_m1, internal control).

4.2.6 Fluorescence imaging and quantification

Measurement of SBP-1 linked to green fluorescence protein (GFP) expression or localization of DAF-16::GFP were performed following protocols previously described
Worms were mounted on a thin layer of half dried 3% agarose in water on microscopic glass slides with 10 mM sodium azide, then covered with cover slip. Pictures were taken using Nikon Eclipse Ti-U (Nikon Instruments Inc., Melville, NY) and fluorescence was measured using NIS-Elements Basic Research software (version 4.20.01, Nikon Instruments Inc., Melville, NY). Whole body of adult worms were selected, and intensity was measured subtracting the intensity of background for SBP-1::GFP (strain CE548). Worms with DAF-16::GFP (strain TJ356) were visually defined as nuclear, intermediate or cytoplasmic depending on localization of transcription factor DAF-16 [116].

4.2.7 Proteomic analysis

Worms samples for proteomic analysis were prepared according to previously described method [117]. Samples were enzymatically digested with sequence grade Lys-C/Trypsin (Promega) using the Barocycler NEP2320 (Nest Group). LC-MS/MS analysis was conducted using the nano Eksigent 425 HPLC system (Nano cHiPLC 200 µm × 0.5 mm ChromXP C18-CL 3 µm 120 Å trap column and Nano cHiPLC 75 µm × 15 cm ChromXP C18-CL 5 µm 120 Å analytical column) coupled with the Triple TOF 5600 plus (Sciex, Framingham, MA). Data was acquired by monitoring 50 precursor ions at 250 ms/scan. The subsequent output was analyzed using MaxQuant computational proteomics platform version 1.5.3.30. MaxQuant settings were as follows: initial precursor mass: 0.07 Da; fragment mass tolerance: 0.02; amino-acid minimum peptide length: 7; data analysis method: ‘label-free quantification’ (LFQ) checked and ‘match between runs’ interval set to one minute; randomized fasta databases; protein
FDR: 1%; enzymes: Trypsin/P and LysC; maximum missed cleavages: 2; maximum modifications per peptide: 3; fixed modifications: iodoethanol (C); and variable modifications: acetyl (Protein N-term) and Oxidation (M). The resulting output was compared both against a common contaminants database and the *C. elegans* sequence database from Universal Protein Database (UniProt) (retrieved 03-01-2018) [118]. An in-house script was used to determine LFQ intensity. LFQ values were transformed using a log2(x) function. To interpolate missing values, a “zero-fill” value of the lowest intensity value (16.3705) was used when a protein was absent (i.e., detected in 0/3 biological replicates), and the average treatment value was used when a protein was detected in 2/3 biological replicates. Proteins detected in 1/3 replicates were not used for subsequent analysis.

4.2.8 Statistical Analysis

Data was analyzed by one-way (Fig. 4.3, 4.4A, 4.7B and Table 4.1) or two-way (Fig. 4.1, 4.2 and 4.5D) analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test with SAS Software (version 9.4, SAS Institute, Cary, NC). Localization of DAF-16::GFP (Fig. 4.4C) was analyzed using Chi-Square test. Groups were considered statistically different when $P < 0.05$ compared to the control. For proteomic data (Fig. 4.6, 4.7A, 4.7B and Table 4.3), statistical analysis was conducted using R environment (www.cran.r-project.org). Proteins with $P < 0.05$ compared to the control (ANOVA followed by Tukey test) were categorized in Table 4.2 by molecular function or biological process gene ontology terms, ranked by protein enrichment, using
4.3 Results

Wild-type *C. elegans* were treated with different concentrations of GCBE (1 or 5 mg/mL) and 5-CQA (0.53 or 2.65 mg/mL) for 3 days from L4 stage/young adults to determine whether GCBE and 5-CQA reduce fat accumulation. GCBE at 5 mg/mL and 5-CQA at 2.65 mg/mL decreased triglyceride content by 29% (*P*=0.0001) and 23% (*P*=0.0001) compared to the control, respectively (Figure 4.1).

![Figure 4.1](image)

**Figure 4.1** Green coffee bean extract (GCBE) and 5-O-caffeoylquinic acid (5-CQA) reduced fat accumulation in *C. elegans*. L4 stage/young adult worms (wild-type) were treated with GCBE (1 or 5 mg/mL) or 5-CQA (0.53 or 2.65 mg/mL) for 3 days. Triglyceride levels were normalized by protein content. Data are means ± S.E. (n = 9-15, collected from three independent experiments). Means with different letters are significantly different at *P* < 0.05.
Next, we determined GCBE and 5-CQA’s effects on worms feeding and locomotor behavior. Pharynx pumping rate was measured for feeding behavior, and our results showed no difference on pumping rates between the treated worms and the control group (Table 4.1). Treatments of GCBE and 5-CQA at the tested doses did not change locomotor behavior of *C. elegans* (Table 4.1). GCBE at 1 mg/mL and 5-CQA at 2.65 mg/mL decreased worm width 11% (*P*=0.0128) and 8% (*P*=0.0030) compared to the control, respectively, while no difference in worm length was observed after treatment of GCBE and 5-CQA (Table 4.1). Decreased width may be reflected in reduced body fat, seen in Fig. 4.1, and consistent to previous report [113]. Taken together, these results suggest GCBE and 5-CQA’s fat-lowering effects are unlikely to be associated with changes on food intake or energy expenditure.

**Table 4.1.** Effect of green coffee bean extract (GCBE) and 5-O-caffeoylquinic acid (5-CQA) on pharynx pumping rate, locomotive behavior and body size of wild-type *Caenorhabditis elegans*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mg/mL</th>
<th>Pumping rate (contractions/30 s)</th>
<th>Speed (µm/s)</th>
<th>Length (µm)</th>
<th>Width (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>71 ± 2</td>
<td>41.9 ± 4.1</td>
<td>626.0 ± 10.7</td>
<td>42.5 ± 0.6a</td>
</tr>
<tr>
<td>GCBE</td>
<td>1</td>
<td>69 ± 4</td>
<td>42.2 ± 9.2</td>
<td>629.6 ± 24.0</td>
<td>37.7 ± 1.3b</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>63 ± 2</td>
<td>39.4 ± 7.9</td>
<td>609.2 ± 20.7</td>
<td>41.4 ± 1.1a</td>
</tr>
<tr>
<td>5-CQA</td>
<td>0.53</td>
<td>65 ± 4</td>
<td>44.5 ± 5.2</td>
<td>618.9 ± 13.7</td>
<td>40.7 ± 0.8a</td>
</tr>
<tr>
<td></td>
<td>2.65</td>
<td>65 ± 2</td>
<td>44.6 ± 5.2</td>
<td>618.0 ± 13.6</td>
<td>39.2 ± 0.8b</td>
</tr>
</tbody>
</table>

L4 stage/young adult worms were treated with GCBE or 5-CQA for three days. Worms were transferred to a low-peptone NGM plates with *E. coli* OP50. Number of pharyngeal contractions of randomly selected worms was counted for 30 s (n = 10-24). Speed, length and width were measured on automatic tracking system (n = 30-116). Data are mean ± S.E. Means with different letters at each variable are significantly different at *P* < 0.05.
Next, selected *C. elegans* mutants were used to determine whether certain genes are required for GCBE and 5-CQA’s fat-lowering effects. Sterol regulatory element-binding protein (SREBP) in mammals is one of major regulators for lipogenesis that is conserved in *C. elegans* as the ortholog, SBP-1 [106]. Our results showed the treatments of GCBE and 5-CQA had no effects on fat accumulation in *sbp-1* knockdown nematodes (Figure 4.2). These results suggested GCBE and 5-CQA’s effects on fat reduction requires SBP-1.

DAF-16, an ortholog of mammalian Forkhead box O (FOXO) transcription factor, is part of insulin/insulin-like growth factor receptor signaling (IIS) pathway, which regulates energy homeostasis in *C. elegans* [106]. Although 5-CQA’s fat-lowering effect was abolished, GCBE increased fat accumulation by 26% (*P*=0.0003) compared to the control in *daf-16* mutants (Fig. 4.2), both of which were different from those in the wild-type (Fig. 4.1). These results suggest that fat-lowering effects of GCBE and 5-CQA may depend on DAF-16 as well.

NHR-49 in *C. elegans* is functional ortholog to the mammalian peroxisome proliferator-activated receptor α (PPARα), which regulates fatty acid β-oxidation [106]. GCBE and 5-CQA’s fat-lowering effects remained in *nhr-49* mutant (*P*=0.0003 and *P*<0.0001, respectively, Fig. 4.2), suggesting that GCBE and 5-CQA’s fat-lowering effects are independent of NHR-49.

AMP-activated protein kinase (AMPK) is a key player in energy homeostasis, acting as a nutrient sensor and also regulating fat metabolism [106]. GCBE and 5-CQA’s fat-lowering effects remained in *aak-2* mutant (*P*=0.0154 and *P*=0.0333, respectively,)
Fig. 4.2), suggesting that GCBE and 5-CQA’s fat-lowering effects are independent of aak-2.

*tub-1*, an ortholog of mammalian TUBBY, is known to affect fat accumulation and act synergistically with intestinal enzymes responsible for fatty acid β-oxidation [106]. Treatment of GCBE and 5-CQA significantly reduced fat accumulation in *tub-1* mutant (*P*=0.0013 and *P*=0.0408, respectively, Fig. 4.2). These results suggest GCBE and 5-CQA’s fat-lowering effects are independent of *tub-1*.

**Figure 4.2.** Effects of green coffee bean extract (GCBE) and 5-*O*-caffeoylquinic (5-CQA) on fat accumulation in mutant *C. elegans*. L4 stage/young adult worms were treated with GCBE (5 mg/mL) or 5-CQA (2.65 mg/mL) for 3 days. Triglyceride levels were normalized by protein content. Data are means ± S.E. (n = 3-13, collected from 1-4 independent experiments). Means with different letters at each variable are significantly different at *P* < 0.05.
Based on results in Fig. 4.2 that GCBE and 5-CQA’s fat-lowering effects are dependent on SBP-1, we further determined the effect of GCBE and 5-CQA on the expression levels of sbp-1, and its target genes; acetyl-CoA carboxylase (ACC/pod-2) and fatty acid synthase (FAS/fasn-1), which are key enzymes of de novo fatty acid synthesis [105]. GCBE and 5-CQA reduced expression of SBP-1::GFP by 14% compared to the control (P=0.0149 and P=0.0133, respectively, Fig. 4.3A). GCBE and 5-CQA reduced transcript levels of pod-2 significantly by 12% and 24% compared to the control (P=0.0053 and P=0.0002, respectively), without any effect on fasn-1 (Fig. 4.3C).

![Figure 4.3](image)

**Figure 4.3.** Effects of green coffee bean extract (GCBE) and 5-O-caffeoylquinic (5-CQA) on sbp-1, pod-2 and fasn-1 expression in *C. elegans*. Worms were treated with GCBE (5 mg/mL) or 5-CQA (2.65 mg/mL) for 3 days. A – SBP-1::GFP expression was measured using fluorescence microscope (n = 30-40). B – Representative images of SBP-1::GFP expression. C – Gene expression of pod-2 and fasn-1 using real time-PCR (n = 3). Data are means ± S.E. Means with different letters at each variable are significantly different at P < 0.05.
Based on results in Fig. 4.2 that GCBE and 5-CQA’s fat-lowering effects are dependent on DAF-16, we then determined the effect of GCBE and 5-CQA on the expression levels of IIS pathway: daf-2 (an ortholog of insulin/insulin-like growth factor receptor and an upstream regulator of DAF-16) and daf-16. Although GCBE significantly increased transcript levels of daf-2 by 11% ($P=0.0158$) compared to the control, GCBE and 5-CQA did not have any effect on daf-16 (Fig. 4.4A). Since it is known that DAF-16 can be regulated post-transcriptionally by different environmental conditions, such as dietary restriction, effects of GCBE and 5-CQA on nuclear translocation of DAF-16 were determined [105, 116]. Our results showed that GCBE and 5-CQA induced nuclear translocation of DAF-16::GFP compared to the control ($P=0.046$ and $P=0.024$, respectively, Fig. 4.4C). These results suggest that effects of GCBE and 5-CQA are mediated by post-transcriptional regulation of DAF-16, independent of DAF-2 in C. elegans.

Effects of GCBE and 5-CQA on genes expression that were associated with fat/energy metabolism were determined to further evaluate their role in the lipid metabolism. cebp-2 (an homolog of CCAAT/enhancer-binding proteins) plays a significant role in lipid metabolism in C. elegans [105]. Treatment of 5-CQA, but not GCBE, significantly downregulated cebp-2 by 22% ($P=0.0052$) compared to the control (Fig. 4.5).

Consistent to the observation in Fig. 4.2, GCBE and 5-CQA had no effects on AMPK, both aak-1 and aak-2 (Fig. 4.5). let-363, a TOR (target of rapamycin) homolog in C. elegans, act as nutrient sensor to regulate growth and metabolism with extensive cross talk between AMPK and IIS pathway [106] and also known to be regulated by
DAF-16 activity [119]. The results showed that GCBE and 5-CQA did not change *let-363* transcript levels (Fig. 4.5).

**Figure 4.4.** Effects of green coffee bean extract (GCBE) and 5-*O*-caffeoylquinic (5-CQA) on markers of insulin-signaling pathway in *C. elegans*. L4 stage/young adult worms were treated with GCBE (5 mg/mL) or 5-CQA (2.65 mg/mL) for 3 days. A – Gene expression of *daf-2* (insulin/insulin-like growth factor receptor ortholog) and *daf-16* (DAF-16/FOXO transcription factor) using real time-PCR (*n* = 3). B – Representative images of DAF-16::GFP in worms scored as cytoplasmic (control) and nuclear (GCBE and 5-CQA). C – Distribution of DAF-16::GFP (%) scored as cytoplasmic, intermediate or nuclear (*n* = 60-80). Data are means ± S.E. Means with different letters at each variable are significantly different at *P* < 0.05.

There are two lipases that are important for lipolysis in *C. elegans*: *atgl-1* (a homolog of adipose triglyceride lipase) and *hosl-1* (a homolog of hormone-sensitive lipase) [105]. Treatments of GCBE and 5-CQA did not affect *atgl-1* expression, while
GCBE, but not 5-CQA, significantly upregulated *hosl-1* by 13% compared to the control ($P=0.0116$) (Fig. 4.5). These suggest GCBE increased lipolysis by HOSL-1, which may be independent of effects of chlorogenic acids.

Mitochondrial acyl-CoA synthetase gene, *acs-2*, encodes a protein for fatty acid β-oxidation, which it is known to be regulated by changes of diet in *C. elegans* [106, 120]. GCBE and 5-CQA had distinct effect on *acs-2* levels; GCBE upregulated *acs-2* by 49% ($P=0.0079$), while 5-CQA downregulated *acs-2* by 39% ($P=0.0209$) compared to the control (Fig. 4.5), suggesting that GCBE and 5-CQA regulate ACS-2 differently.

![Figure 4.5. Effects of green coffee bean extract (GCBE) and 5-O-caffeoylquinic (5-CQA) on fat/energy metabolism-related genes expression. L4 stage/young adult worms were treated with GCBE (5 mg/mL) or 5-CQA (2.65 mg/mL) for 3 days. Data are means ± S.E. (n = 3). Means with different letters at each variable are significantly different at $P < 0.05$.](image)

Nuclear hormone receptor, NHR-49, interacts with mediator subunit MDT-15 regulating fat metabolism genes, including *acs-2* [106]. Treatments of GCBE and 5-CQA did not change *nhr-49* or *mdt-15* expression compared to the control (Fig. 4.5),
suggesting that GCBE and 5-CQA act independently of NHR-49 and MDT-15. This is consistent with the result in Fig. 4.2 that GCBE and 5-CQA’s fat-lowering effects are independent of NHR-49.

Next, we attempted to identify GCBE and 5-CQA-regulated proteins levels *in vivo* using a shotgun proteomic approach. A total of 63 proteins were identified as differentially regulated by GCBE or 5-CQA compared to the control (Table 4.3). Proteins were further clustered by molecular function and biological process terms, as listed in Table 4.3, which include oxidoreductase activity, aldehyde dehydrogenase, protein homeostasis, biosynthesis of amino acids, membrane, metal ion binding, ion transport ATP-binding, reproduction and development, ranked by protein enrichment score (Fig 4.6).
Figure 4.6. Clusters of proteins identified as differentially expressed in GCBE or 5-CQA-treated wild-type worms compared to the control. L4 stage/young adult worms (wild-type) were treated with GCBE (5 mg/mL) or 5-CQA (2.65 mg/mL) for 3 days, then proteins identified by shotgun proteomic approach ($P < 0.05$, $n = 3$). A – Clusters of proteins with common molecular function or biological process terms using Database for Annotation, Visualization and Integrated Discovery (DAVID) ranked by protein enrichment. B - Number of non-redundant proteins by clusters.
### Table 4.2. Protein functional classification and term annotation of proteins differently expressed in green coffee bean extract (GCBE) or 5-**O**-caffeoylquinic acid (5-CQA)-treated *C. elegans* compared to control.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Terms</th>
<th>Number of non-redundant proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidoreductase activity</td>
<td>GO:0055114 - Oxidation-reduction process</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>GO:0016491 - Oxidoreductase activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UP Keyword - Oxidoreductase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cel01130 - Biosynthesis of antibiotics</td>
<td></td>
</tr>
<tr>
<td>Aldehyde dehydrogenase</td>
<td>cel00330 - Arginine and proline metabolism</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IPR016162 - Aldehyde dehydrogenase, N-terminal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IPR016161 - Aldehyde/histidinol dehydrogenase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IPR015590 - Aldehyde dehydrogenase domain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IPR016163 - Aldehyde dehydrogenase, C-terminal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GO:0016620 - Oxidoreductase activity, acting on the aldehyde or oxo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>group of donors, NAD or NADP as acceptor</td>
<td></td>
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<tr>
<td>Protein homeostasis</td>
<td>GO:0005839 - Proteasome core complex</td>
<td>9</td>
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<tr>
<td></td>
<td>IPR001353 - Proteasome, subunit alpha/beta</td>
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<tr>
<td></td>
<td>GO:0004298 - Threonine-type endopeptidase activity</td>
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<td>UP Keyword - Threonine protease</td>
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<td></td>
<td>GO:0051603 - Proteolysis involved in cellular protein catabolic</td>
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<td>GO:0000502 - Proteasome complex</td>
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<td></td>
<td>GO:0006508 - Proteolysis</td>
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<td></td>
<td>GO:0008233 - Peptidase activity</td>
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<td>cel03050 - Proteasome</td>
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<tr>
<td>Biosynthesis of amino acids</td>
<td>cel00280 - Valine, leucine and isoleucine degradation</td>
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<td></td>
<td>cel00410 - beta-Alanine metabolism</td>
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<td></td>
<td>cel00071 - Fatty acid degradation</td>
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</tr>
<tr>
<td></td>
<td>cel01130 - Biosynthesis of antibiotics</td>
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<tr>
<td>Membrane</td>
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<td>GO:0016021 - Integral component of membrane</td>
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<td>UP Keyword - Transmembrane helix</td>
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<td>GO:0046872 - Metal ion binding</td>
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<td></td>
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<td>GO:0000166 - Nucleotide binding</td>
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<td>Reproduction &amp; Development</td>
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<td>8</td>
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<tr>
<td></td>
<td>GO:0002119 - Nematode larval development</td>
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</tr>
<tr>
<td></td>
<td>GO:0009792 - Embryo development ending in birth or egg hatching</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>GO:0000003 - Reproduction</td>
<td>15</td>
</tr>
</tbody>
</table>

Gene Ontology Consortium (GO terms), KEGG pathway (cel terms), InterPro (IPR terms) and UniProt (UP keyword) used to cluster different proteins by DAVID.
Table 4.3. Proteins identified as differentially expressed in green coffee bean extract (GCBE) or 5-O-caffeoylquinic acid (5-CQA)-treated *C. elegans* compared to the control.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein names</th>
<th>Category</th>
<th>GCBE Log2-fold change (P-value)</th>
<th>5-CQA Log2-fold change (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2FLJ1</td>
<td>STomatin-Like</td>
<td>Membrane</td>
<td>0 (1)</td>
<td>5.3587 (0.0001606)</td>
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<tr>
<td>O61217</td>
<td>Purine nucleoside phosphorylase</td>
<td>Other</td>
<td>0 (1)</td>
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<tr>
<td>U4PAM5</td>
<td>C-type LECTin</td>
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<td>O16294</td>
<td>GMP reductase</td>
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<td>V6CLQ2</td>
<td>Heat Shock 16.2 kDa Protein</td>
<td>Other</td>
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<td>6.2387 (0)</td>
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<td>NADH dehydrogenase [ubiquinone] flavoprotein 1</td>
<td>Oxidoreductase activity</td>
<td>0 (1)</td>
<td>6.4542 (0.000001)</td>
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<td>Probable NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 2</td>
<td>Oxidoreductase activity</td>
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<tr>
<td>Q9TZC4</td>
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<td>Membrane</td>
<td>6.6991 (0)</td>
<td>6.5993 (0)</td>
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<td>P54889</td>
<td>Probable delta-1-pyrroline-5-carboxylate synthase</td>
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<td>-4.919 (0)</td>
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<td>Q9TYP9</td>
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<td>Q9N538</td>
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GCBE increased 25 protein levels and decreased 20 protein levels, while 5-CQA increased 20 proteins levels and decreased 8 protein levels, compared to the control (Fig. 4.6B). Among those proteins, the ones that are potentially associated with fat metabolism are enoyl-CoA hydratase (ECH-4, Q09603), fatty acid-CoA synthetase family protein (ACS-11, Q20264), fatty acid/retinol binding protein (FAR-6, Q9XUB7) and serine/threonine-protein phosphatase 2B catalytic subunit (TAX-6, Q0G819) (Fig. 4.7).

**Figure 4.7.** Effects of green coffee bean extract (GCBE) and 5-O-caffeoylquinic (5-CQA) on fat metabolism-associated proteins and genes. L4 stage/young adult worms were treated with GCBE (5 mg/mL) or 5-CQA (2.65 mg/mL) for 3 days. A - Fat metabolism-associated proteins levels identified as differentially expressed in GCBE or 5-CQA-treated wild-type worms compared to the control by shotgun proteomic approach. Data are fold change compared to the control (n = 3, *P < 0.05, **P < 0.001). B - Effects of GCBE and 5-CQA on target proteins-encoding genes expression in wild-type worms. Data are means ± S.E. (n = 3). C - Effects of GCBE and 5-CQA on fat accumulation in mutant C. elegans of identified proteins. Triglyceride levels were normalized by protein content. Data are means ± S.E. (n = 4-8, collected from 1-2 independent experiments). Means with different letters at each variable are significantly different at P<0.05.
Enoyl-CoA hydratase (ECH-4) and fatty acid-CoA synthetase (ACS-11) are enzymes involved in fatty acid β-oxidation [120]. GCBE and 5-CQA increased levels of enoyl-CoA hydratase (ECH-4) by 56% and 48%, respectively, compared to the control \((P=0.0234\) and 0.0398, respectively) (Fig. 4.7A). GCBE, but not 5-CQA, increased fatty acid-CoA synthetase (ACS-11) by 79% compared to the control \((P=0.0117)\) (Fig. 4.7A). At transcript levels, GCBE increased \(ech-4\) expression by 9% \((P=0.0405)\), but neither GCBE or 5-CQA affects \(acs-11\) expression (Fig. 4.7B). Taken together our proteomics and gene expression results suggest GCBE and 5-CQA regulate fatty acid β-oxidation through different enzymes, such as ECH-4 and ACS-11.

Lipid-binding proteins, known as intracellular lipid chaperones, are suggested as a target for metabolic diseases treatment [121, 122]. \(C.\ elegans\) contains different types of lipid binding proteins; LBPs, homolog of mammals fatty-acid binding proteins (FABPs), and FARs, fatty acid and retinoid-binding proteins, which it is structurally unique in \(C.\ elegans\) [121]. GCBE decreased levels of FAR-6 by 99% \((P<0.0001)\) (Fig. 4.7A), which was confirmed at transcript levels; GCBE decreased \(far-6\) expression by 37% \((P=0.0002)\) (Fig. 4.7B). Consistently, our results showed that GCBE’s fat-lowering effect was abolished in \(far-6\) mutants (Fig. 4.7C). Furthermore, our results showed that 5-CQA’s fat-lowering effects were also abolished in \(far-6\) mutant (Fig. 4.7C), even though 5-CQA did not change FAR-6 at translational or transcript levels (Fig. 4.7A & B). These results suggest GCBE and 5-CQA require FAR-mediated lipid uptake and transport in \(C.\ elegans\) for their effects on reducing fat accumulation, which may be independent of transcriptional and translational levels.
Calcineurin, a serine/threonine phosphatase, has multiple biological functions, including fat metabolism [123, 124]. Our proteomics results showed GCBE increased calcineurin catalytic subunit (TAX-6) by 19% compared to the control (P=0.0153, Fig. 4.7A) and 5-CQA decreased TAX-6 levels by 99% compared to the control (Fig. 4.7A). However, GCBE and 5-CQA significantly reduced fat accumulation in tax-6 mutant (P=0.0044 and P=0.0179, respectively, Fig. 4.7C), which suggests that TAX-6 is not a requirement for their effects on fat accumulation in C. elegans.

4.4. Discussion

Coffee is one of the most consumed beverages in the world and is rich in polyphenols [36, 94, 95]. Previous reports suggest that consumption of GCBE, which consists of ~50% of chlorogenic acids, could reduce body weight, improve lipid profile and insulin sensitivity [59, 93, 96, 99, 101–103]. However, there is limited knowledge regarding how GCBE regulates fat accumulation. In the current study, we found that GCBE and 5-CQA, one of the main chlorogenic acids, reduce fat accumulation in C. elegans dependent on sbp-1 and daf-16, which are involved in lipogenesis and insulin-signaling pathway, respectively. We identified lipid-binding protein, FAR-6, involved in lipid uptake and transport, was identified as a possible target for GCBE and 5-CQA. These are consistent to previous reports that chlorogenic acids reduce fat accumulation by regulating lipogenesis-related molecules, such as SREBP and ACC [46], and induce nuclear translocation of DAF-16 [108]. Therefore, chlorogenic acids are likely to be responsible for GCBE effects on fat metabolism. Moreover, our study is the first to show GCBE and 5-CQA influence on fatty acid β-oxidation and lipid transport pathways.
Similar to the GCBE and 5-CQA’s effects on fat accumulation in *C. elegans*, hesperidin, piceatannol and cranberry phenolic extract reduce fat accumulation via SBP-1 pathway, regulating its downstream target genes [113, 115, 125]. Also, GCBE and 5-CQA act via IIS pathway modulating the FOXO transcription factor, DAF-16, similarly to other bioactive compounds, such as piceatannol [126]. Although there are similarities on mechanism of actions between different compounds, the effective doses of GCBE (5 mg/mL) and 5-CQA (2.65 mg/mL or 7.5 mM) on fat reduction in *C. elegans* were higher compared to other phenolics compounds and plant extracts [105, 113, 115, 127]. It is not currently possible to extrapolate doses from *C. elegans* to other mammals, therefore the significance of the doses used in the study may need to be carefully interpreted for other animal models [105].

The effects of GCBE on fat metabolism are known to be associated with chlorogenic acids, but in the current study there were some inconsistent results between GCBE and 5-CQA. Chlorogenic acids vary in concentrations and forms within GCBE [95, 97, 98]. Thus, the mixture of chlorogenic acids within GCBE may act in a different way to the singular one, 5-CQA, on fat metabolism pathways in *C. elegans*. In addition to chlorogenic acids, GCBE contains caffeine, trigonelline, sucrose, and amino acids [97, 98], which may contribute to differences between GCBE and 5-CQA. Therefore, it is reasonable to speculate that inconsistent results between GCBE and 5-CQA may derive from the different composition of the treatments used.

The current results showed that GCBE and 5-CQA potentiated activity of DAF-16 via nuclear translocation independent to their effects on daf-2 or calcineurin catalytic subunit (TAX-6). IIS pathway includes DAF-2, an ortholog of the mammalian
insulin/insulin-like growth factor receptor and DAF-16 as a downstream key regulator for its effects on fat metabolism [106]. DAF-2 act to inhibit DAF-16, however, there are other independent pathways that can target DAF-16 activity [128]. Thus, the current results suggest that GCBE and 5-CQA may change DAF-16 post-transcriptionally via different pathways; calcineurin signaling pathway, which inhibits DAF-16 [123], AMPK system and TOR signaling pathway [128]. Alternatively, the current results suggest that the reason GCBE was able to activate DAF-16, independent of daf-2 and TAX-6, might be due to sucrose present in GCBE [124]. Others reported that GCBE and 5-CQA activate DAF-16 via AMPK system [59, 108], although the current results do not show that GCBE and 5-CQA act on AMPK system. However, we cannot exclude the possibility that GCBE and 5-CQA’s fat-lowering effects are dependent on daf-16 by post-transcriptional regulation of AMPK.

GCBE and 5-CQA reduced fat accumulation in wild-type worms (Fig. 4.1), but we observed that GCBE significantly promoted fat accumulation on daf-16 mutants, while 5-CQA alone did not (Fig. 4.2). This may be due to the difference of sugar contents between two treatments; GCBE contains about 10% of sucrose [97, 98]. Excess dietary sugar leads to increased fat accumulation, which may be independent to DAF-16 [106, 129]. Thus, sugar within GCBE may be the responsible for the promotion of fat accumulation on daf-16 mutant. These results suggest that with activation of DAF-16, GCBE and 5-CQA were able to reduce fat accumulation regardless to the presence of sugar (~10%).

GCBE and 5-CQA had different effects on acs-2 expression, which encodes an enzyme involved in fatty acid β-oxidation, downstream of NHR-49 [106]. The results in
Figures 4.2 & 4.4 suggest that effects of GCBE and 5-CQA on fat accumulation are independent of *nhr-49*, thus it is unlikely that GCBE and 5-CQA modulate *acs-2* via NHR-49-dependent pathway, rather potentially via SBP-1-dependent pathway [127, 130]. In addition, decreased *acs-2* expression by 5-CQA should result in reduced fatty acid β-oxidation, which is not consistent to 5-CQA’s fat-lowering effect. Consistently, it was previously reported that proanthocyanins reduce fat accumulation in *C. elegans* also downregulating *acs-2*, which was suggested to be by compensatory transcriptional responses occurred due to fat depletion [127]. In addition to *acs-2*, other enzymes related to fatty acid β-oxidation were modulated by GCBE and/or 5-CQA; enoyl-CoA hydratase (ECH-4) and fatty acid-CoA synthetase (ACS-11). Inconsistent effects on fatty acid β-oxidation by GCBE and 5-CQA may derive from the additional components, such as caffeine, present in GCBE, which also needs to be further tested.

Fatty acid and retinoid-binding proteins (FARs), structurally unique in *C. elegans*, are one type of lipid binding proteins responsible for lipid uptake and transport [121]. Our results showed GCBE decreased *far-6/FAR-6* levels and its fat-lowering effects was abolished on *far-6* mutant, while 5-CQA’s fat-lowering effects was also abolished without changes on *far-6/FAR-6* levels. A different type of lipid binding protein is a target for metabolic diseases treatment in humans, which may be functionally similar to FARs [121, 122]. The importance of FARs on fat metabolism in *C. elegans* needs to be further studied. Taken together, our results suggest that *far-6* is important for the effects of GCBE and 5-CQA on fat metabolism, which may involve different mechanism on this target.
GCBE and 5-CQA changed proteins levels associated with several biological processes, particularly oxidoreductase activity, aldehyde dehydrogenases and protein homeostasis in *C. elegans*. The changes on protein levels may be associated with other biological effects, such as GCBE and 5-CQA’s antioxidant activities, which may be associated with oxidoreductase proteins [96, 107, 131]. Additional experiments will be necessary to confirm GCBE and 5-CQA’s effects on these protein levels *in vivo*, and also to explain the difference on responses between GCBE and 5-CQA treatments.

**4.5. Conclusion**

In summary, GCBE and 5-CQA reduce fat accumulation in *C. elegans* dependent on SBP-1 (involved in lipogenesis), DAF-16 (involved in insulin signaling pathway) and FAR-6 (involved in lipid uptake and transport). Chlorogenic acids are most likely responsible for GCBE’s fat-lowering effects. Furthermore, *C. elegans* has been proven as a model system for mechanistic studies with respect to fat metabolism.
CHAPTER 5

CAFESTOL INCREASES FAT OXIDATION AND ENERGY EXPENDITURE IN

*Caenorhabditis elegans* VIA DAF-12-DEPENDENT PATHWAY

5.1 Introduction

Coffee is one of the most consumed beverages in the world and is known to have many health benefits including reduced risk of obesity [132]. The anti-obesity effects of coffee are mostly associated with its major bioactive compounds, such as caffeine and chlorogenic acids [3, 132]. However, coffee diterpenes, such as cafestol, are also reported to contribute to the coffee’s fat-lowering effects, which may be independent of its effects on circulating lipid levels [26, 30, 32, 33, 133]. In addition to its effects on lipid metabolism, cafestol has a potential use for human health due to its anticancer, anti-inflammatory and anti-diabetes properties [132].

It is known that cafestol is an agonist of farnesoid X receptor (FXR), bile acid receptor that regulates cholesterol homeostasis and other lipid metabolic pathways, including fatty acid metabolism [26, 82]. However, little is known about the mechanism of action of cafestol on fatty acid metabolism. *Caenorhabditis elegans*, a multi-organ millimetric roundworm, is an animal model used for mechanistic studies of bioactive compounds on fatty acid metabolism with the use of various knockout gene mutants along with standard biology techniques [105]. *C. elegans* has fatty acid metabolic pathways similar to humans, including the nuclear hormone factor DAF-12 as the homolog of FXR [134, 135]. Thus, we hypothesized that cafestol reduces fat accumulation through DAF-12/FXR pathway and/or complementary mechanisms in *C. elegans*. 

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5.2 Materials and methods

5.2.1 Materials

Cafestol was obtained from Chengdu Biopurify Phytochemicals Ltd (purity ≥ 98%, lot PRF8042041, CAS 469-83-0, PubChem CID 108052, Chengdu, China). Chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA), but the following were obtained from elsewhere: 2’-Deoxy-5-fluorouridine (FUDR, TCI America, Portland, OR, USA), carbenicillin (Fisher Bioreagents, Pittsburgh, PA, USA) and ampicillin (Sigma-Aldrich Co., St. Louis, MO, USA). Reagents and kits for photocolorimetric assays and PCR were purchased from manufacturers, as stated in their respective methods section. Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA) provided Escherichia coli OP50 and C. elegans strains: N2, Bristol (wild-type); AA86 [daf-12(rh61rh411) X.]; AA120 [daf-12a::GFP + lin-15(+)]; CB1370 [daf-2(e1370) III.]; CE541 [sbp-1(ep79) III]; GR1307 [daf-16(mgDf50) I.]; RB1600 [tub-1(ok1972) II.]; RB1716 [nhr-49(ok2165) I]; RB754 [aak-2(ok524) X].

5.2.2 C. elegans culture

Different C. elegans strains were cultured on nematode growth media (NGM) plates at 20°C or 15°C (daf-2 mutant) on an incubator (model DT2-MP-47, Tritech Research Inc., Los Angeles, CA) as previously described [3, 105]. Adult worms were dissolved using bleach solution followed by washing several times with M9 buffer and then S-complete to obtain eggs for a synchronized population [105]. L1 stage worms (~1000 worms/mL) were fed with live E. coli OP50 in S-complete containing ampicillin (100 μg/mL) and carbenicillin (50 μg/mL). We had to include 0.12 mM FUDR to prevent
eggs from hatching after worms had reached L4 stage/young adult stage in both control and treated-worms, even though FUDR is known to affect worm metabolism [105, 115]. Cafestol was diluted in dimethyl sulfoxide (DMSO) at 30 mM stock solution. Adults worms were treated with cafestol (30 or 60 µM) or 0.2% DMSO (control), then incubated for 2 days.

5.2.3 Triglyceride quantification

Worms were transferred to centrifuge tubes, then washed thrice with water [115]. After sonication in 0.05% Tween® 20, triglyceride quantification was determined using Infinity™ Triglycerides Reagent (Fisher Diagnostics, Middletown, VA, USA) with glycerol as standard. Quantity of protein from the same sample was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Middletown, VA, USA) with bovine serum albumin as standard. Absorbances were measured by using SpectraMax i3 microplate reader and SoftMax Pro 6 (version 6.5) from Molecular Devices (Sunnyvale, CA, USA).

5.2.4 Behavior and body size

Low-peptone NGM plates were used to assess worm behavior and size as previously described [117]. Briefly, feeding behavior was measured by pharynx contractions (pumping rate). An automatic tracking system (WormLab, model MSCOP-002, MBF Bioscience, MicroBrightField Inc., Willinston, VT, USA) was used to record 1 min video of the multiple worms; then width, length, maximum amplitude and speed
[(forward distance + reverse distance)/time] were calculated by WormLab software (3.1.0 64-bit, MBF Bioscience, Williston, VT, USA).

5.2.5 Reproduction

Brood size, progeny and hatchability of C. elegans was measured by counting the number of eggs and larvae on a bench microscope [117]. Briefly, L4/young adult worms were individually transferred to an NGM 12-well plate with E. coli and 60 µM cafestol or 0.2% DMSO for 2 days. Worms were transferred daily to a new well until no eggs and larvae were observed. Hatchability is the ratio of total laid eggs (brood size) to offspring larvae (progeny) [117].

5.2.6 Real-time PCR

Two-step RT-PCR was performed by using TRIZol® (Thermo Fisher Scientific, Inc., Middletown, VA) to extract total RNA from worms samples; then high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Inc., Middletown, VA) and standard thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA) were used to produce cDNA templates following the respective manufacturer’s protocols. Gene expression was quantified by using StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA) and TaqMan® gene probes (Thermo Fisher Scientific, Inc., Middletown, VA): aak-1 (Ce02406989_g1), aak-2 (Ce02404250_m1), acs-2 (Ce02486193_g1), acs-11 (Ce02431951_m1), atgl-1 (Ce02406730_g1), cebp-2 (Ce02421574_g1), cpt-5 (Ce02419317_g1), daf-12 (Ce02500122_g1), daf-16 (Ce02422838_m1), daf-2 (Ce02444336_m1), ech-1.1 (Ce02485968_g1), ech-4 (Ce02438697_g1), far-6
(Ce02466862_g1), *fard-1* (Ce02493083_m1), *fasn-1* (Ce02411648_g1), *fat-6*
(Ce02465318_g1), *fat-7* (Ce02477066_g1), *hosl-1* (Ce02494529_m1), *kat-1*
(Ce02434540_g1), *let-363* (Ce02417512_m1), *lrp-1* (Ce02409772_m1), *mdt-15*
(Ce02406575_g1), *nhr-13* (Ce02471923_m1), *nhr-49* (Ce02412667_m1), *nhr-80*
(Ce02421189_g1), *pod-2* (Ce02427721_g1), *sir-2.1* (Ce02459017_g1), *tub-1*
(Ce02435686_m1) and an internal control *ama-1* (Ce02462726_m1). Comparative
threshold cycle (Ct) method was used and results were expressed as fold change of gene
expression ($2^{-\Delta\Delta Ct}$).

### 5.2.7 DAF-12 expression

DAF-12 linked to green fluorescence protein (DAF-12::GFP) expression was
measured by a fluorescence imaging technique using the strain AA120 as previously
described [3]. Paralyzed worms by 10 mM sodium azide were put on microscopic glass
slides with a 3% agarose pad. A fluorescence microscope, Nikon Eclipse Ti-U, and NIS-
Elements Basic Research software version 4.20.01 (Nikon Instruments Inc., Melville,
NY) were used to take the pictures and collect data.

### 5.2.6 Statistical Analysis

SAS Software (version 9.4, SAS Institute, Cary, NC) was used to analyze data by one-way
(Fig. 5.2, 5.3, 5.6 and 5.7) or two-way (Fig. 5.1, 5.4 and 5.5) analysis of variance
(ANOVA) followed by Tukey-Kramer multiple comparison test. Significance of
differences was defined as $P < 0.05$. 

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5.3. Results

After 2 days of treatment, cafestol at 30 and 60 µM decreased triglyceride content by 12% \((P=0.0001)\) and 20% \((P<0.0001)\) compared to the control, respectively (Fig. 5.1), in adult wild-type \textit{C. elegans}.

![Graph showing triglycerides (µg/mg protein) for different concentrations of cafestol (0, 30, and 60 µM).](image)

**Figure 5.1.** Cafestol reduced fat accumulation in \textit{C. elegans}. Adult worms (wild-type) were treated with cafestol (30 or 60 µM) for 2 days. Triglyceride levels were normalized by protein content. Data are means ± S.E. \((n = 11-12, \text{ collected from three independent experiments})\). Means with different letters are significantly different at \(P < 0.05\).

Furthermore, 60 µM cafestol decreased body size of worms: width by 5% \((P=0.0004, \text{ Fig. 5.2A})\) and length by 7% \((P<0.0001, \text{ Fig. 5.2B})\) compared to the control, which may be reflected by reduced body fat, observed in Fig. 5.1 [113]. Next, to assess whether cafestol’s fat-lowering effects were due to changes in feeding behavior, we evaluated pharynx pumping rate, as an indicator of food intake, after treatment with cafestol [117]. Our results showed the cafestol did not change feeding behavior (Fig. 5.2C), suggesting that cafestol’s fat-lowering effects are independent of food intake.
Figure 5.2. Cafestol decreased body size but did not change feeding behavior of wild-type *C. elegans*. Adult worms were treated with cafestol (30 or 60 µM) for 2 days, then transferred to a low-peptone NGM plates with live *E. coli* OP50. Body size was measured by an automatic tracking system (n = 127-177): A – Width; B – Length; C - Number of pharyngeal contractions of randomly selected worms was counted for 30 s. Data are mean ± S.E. (n = 10-24). Means with different letters at each variable are significantly different at $P < 0.05$.

Since fat reduction may be linked to changes in reproduction, we further determined whether cafestol changes the reproductive ability of the worms [136].

Cafestol delayed the *C. elegans* reproduction, during the 2 days of cafestol treatment, but
the total number of laid eggs, offspring larvae and hatchability were not changed by cafestol compared to the control (Fig. 5.3).

**Figure 5.3.** The effects of cafestol on the reproduction of *C. elegans*. A – number of laid eggs per worm (brood size). B – number of offspring larvae (progeny). C – hatchability. Data are means ± S.E (n = 3–4). Means with different letters at each variable are significantly different at *P* < 0.05.
Next, we used *C. elegans* knockout mutants, involved in lipid and energy metabolism, to determine the genetic requirement for cafestol’s fat-lowering effects. As cafestol is a known agonist of FXR, we tested whether the fat-lowering effects of cafestol are dependent on the FXR homolog in *C. elegans*, DAF-12 [26, 134, 135]. Cafestol’s fat-lowering effects were abolished in *daf-12* mutant (Fig. 5.4). We further determined the effect of cafestol on the expression levels of *daf-12* and a DAF-12-target gene, *fard-1* (homolog of the human fatty acyl-CoA reductase-1, involved in biosynthesis of ether lipids) [137]. Cafestol at 60 µM upregulated *fard-1* (*P*=0.0069, compared to the control), without changes on *daf-12* expression (Fig. 5.5A) nor at protein levels, by DAF-12::GFP expression (Fig. 5.6). These suggest that the fat-lowering effects of cafestol are mediated by post-translational regulation of DAF-12/FXR in *C. elegans*.

**Figure 5.4.** Effects of cafestol on fat accumulation in *C. elegans* knockout mutants. Adult worms were treated with cafestol (30 or 60 µM) for 2 days. Triglyceride levels were normalized by protein content. Data are means ± S.E. (*n* = 4-12, from 1-3 independent experiments). Means with different letters at each variable are significantly different at *P* < 0.05.
Figure 5.5. Effects of cafestol on lipid metabolism-related genes expression on wild-type worms measured by real time-PCR (A-C). Data are means ± S.E. (n=3-11, from 1-3 independent experiments). Means with different letters at each variable are significantly different at $P < 0.05$. 
Figure 5.6. The effects of cafestol on DAF-12::GFP expression. Worms were treated with cafestol (60 µM) or control (0.2% DMSO) for 2 days, then transferred to microscope agarose pad. A – DAF-12::GFP expression was measured using fluorescence microscope. Fluorescence intensity unit was collected from the images of the whole worm body. Data are means ± S.E (n = 14-22). Means with different letters at each variable are significantly different at $P < 0.05$. B – Illustrative images of DAF-12::GFP expressed in cafestol-treated and control worms.

There are several key players that regulate energy homeostasis important for the overall fat accumulation [106]. Insulin/insulin-like growth factor signaling (IIS) pathway regulates energy homeostasis and its major players in C. elegans are DAF-2, a homolog of insulin/insulin-like growth factor receptor, and its downstream target DAF-16, an ortholog of mammalian Forkhead box O transcription factor. Cafestol reduced fat accumulation in daf-2 and daf-16 mutants by 9% and 11% at 60 µM, respectively, compared to their respective control ($P=0.0325$ and $P=0.0003$, respectively, Fig. 5.4). Consistently, cafestol did not change daf-2 nor daf-16 transcript levels (Fig. 5.5A). These
results suggest that fat-lowering effects of cafestol are independent of the IIS pathway regulators, 

In *C. elegans*, adenosine monophosphate-activated protein kinase (AMPK), which its subunits are encoded by *aak-1* and *aak-2*, regulates cellular energy along with SIR-2.1 (ortholog of sirtuin 1, a nicotinamide adenine dinucleotide-dependent deacetylase) [106, 117]. In addition, the target of rapamycin (TOR), encoded by *let-363* in *C. elegans*, has extensive cross-talk with AMPK and IIS pathways to regulate energy homeostasis [106]. Both 30 and 60 µM cafestol reduced fat accumulation by 9% in *aak-2* mutant (*P* = 0.0132 and *P* = 0.0178, respectively, Fig. 5.4), suggesting that cafestol’s fat-lowering effects are independent of *aak-2*, the subunit responsible for the kinase activity of AMPK [117]. Also, cafestol did not change *aak-1*, *aak-2*, *sirt-2.1* nor *let-363* transcript levels (Fig. 5.5A). These results suggest that cafestol reduces fat accumulation independent of AMPK, SIR-2.1 or LET-363 pathways.

TUBBY-like proteins are involved in the development of obesity via neurologic control of energy expenditure [138, 139]. The underlying mechanisms of TUBBY pathway on lipid metabolism are not yet elucidated, but *C. elegans* TUB-1, the ortholog of mammalian TUBBY, is known to act on fatty β-oxidation synergistically with the intestinal 3-ketoacyl-CoA thiolase (KAT-1) [106]. Cafestol did not reduce fat accumulation in *tub-1* mutant (Fig. 5.4), suggesting that cafestol fat-lowering effects are dependent on TUB-1 pathway. Thus, we further determined the effect of cafestol on the *tub-1* and *kat-1* transcripts levels. 60 µM cafestol upregulated expression of *tub-1* by 89% compared to the control (*P* = 0.0096, Fig. 5.5A). However, cafestol did not change *kat-1*
transcription (Fig. 5.4A). Overall, these suggest that cafestol’s fat-lowering effects are via TUB-1-dependent pathway, however, independent to its effect on kat-1 in C. elegans.

CCAAT/enhancer-binding proteins (C/EBPs) are a family of transcription factors that regulate adipocyte differentiation [105]. Even though C. elegans lacks adipocytes and its fat accumulates mostly in the intestine as body fat, the C. elegans homolog of C/EBP, CEBP-2, regulates body fat and lipogenesis [106, 140]. Our results showed that cafestol did not change the expression of cebp-2 on wild-type worms (Fig. 5.5B), which suggest that cafestol’s fat-lowering effects are independent to cebp-2.

Lipogenesis is regulated by several key players in C. elegans, including transcription factors and enzymes for the conversion of fatty acids into triglycerides [105, 106]. Sterol regulatory element-binding protein (SREBP) is a natural target for anti-obesity agent due to its importance for lipogenesis [3, 113, 115]. In C. elegans, SBP-1, the SREBP ortholog, has as downstream targets: pod-2 (encodes acetyl-CoA carboxylase), fasn-1 (encodes fatty acid synthase homolog) and fatty acid stearoyl-CoA desaturases (fat-6 and fat-7) [106]. Additionally, the nuclear hormone receptors, NHR-80 and NHR-13, also control fatty acid desaturation [141]. Our results showed 60 µM cafestol reduced fat accumulation in sbp-1 mutants by 20% ($P=0.0342$, Fig. 5.4), suggesting that cafestol’s fat-lowering effects are independent of sbp-1. Moreover, cafestol did not change the expression of pod-2, fasn-1, fat-6, fat-7, nhr-80 nor nhr-13 on wild-type worms (Fig. 5.5B). These results suggest that cafestol’s fat-lowering effects may be independent to its effect on lipogenesis.

In C. elegans, the nuclear hormone NHR-49 is the functional homolog to the mammalian peroxisome proliferator-activated receptor α (PPARα), one of the regulators
for fatty acid β-oxidation [106, 120, 141]. Other co-factors and several enzymes are involved in the fatty acid β-oxidation, such as *mdt-15* (encodes a mediator complex that interacts with NHR-49 protein), fatty acid-CoA synthetases (*acs-2* and *acs-11*), enoyl-CoA hydratases (*ech-4* and *ech-1.1*) and carnitine palmitoyl transferase (*cpt-5/CPT*) [106, 120]. Our results showed that 60 µM cafestol reduced fat accumulation in *nhr-49* mutant by 21% (*P*<0.0001, Fig. 5.4), suggesting that cafestol’s fat-lowering effects are independent of *nhr-49*. Consistently, the expression of *nhr-49*, *mdt-15*, *acs-2*, *acs-11*, *ech-4* and *cpt-5* were not influenced by cafestol (Fig. 5.5C). However, 60 µM cafestol upregulated *ech-1.1* expression by 2-fold relative to the control (*P*=0.0293, Fig. 5.5C). Taken together, our results suggest that cafestol regulates fatty acid β-oxidation via *ech-1.1*.

Lipolysis, lipid uptake and transport are also important factors for the overall fat accumulation [3, 105]. Several lipases responsible for the triglycerides breakdown are found in *C. elegans*, such as *atgl-1* (a homolog of adipose triglyceride lipase) and *hosl-1* (a homolog of hormone-sensitive lipase) [105]. However, cafestol did not influence *atgl-1* or *hosl-1* expression (Fig. 5.5B). In *C. elegans*, lipid-binding proteins, such as fatty acid and retinoid-binding proteins (FAR) and low-density lipoproteins (LDL), are involved in lipid uptake and transport [3, 142]. Cafestol did not change expressions of *far-4* nor *lrp-1* (an LDL receptor-related gene) (Fig. 5.5C). Overall, these results suggest that cafestol does not regulate lipolysis or lipid uptake and transport in *C. elegans*.

Since the cafestol’s fat-lowering effects were abolished in *daf-12* and *tub-1* mutants (Fig. 5.3), we determined whether cafestol regulates the expressions of *tub-1*, *ech-1.1* and *fard-1* dependent on *daf-12* and/or *tub-1*. As expected, the upregulation of
fard-1 by cafestol was abolished in daf-12 mutant. In addition, the effects of cafestol on tub-1 and ech-1.1 expressions were abolished in daf-12 mutant (Fig. 5.7A), suggesting that cafestol activated tub-1 and ech-1.1 via daf-12. Next, we determined whether tub-1 is required for cafestol’s effect on daf-12, fard-1 and ech-1.1 using tub-1 mutant. Although no change of daf-12 expression by cafestol was observed, cafestol downregulated fard-1 expression by 13% (P=0.0321, compared to the control, Fig. 5.7A) without changes on ech-1.1 expression in tub-1 mutant. Overall, these results suggest DAF-12 is an upstream regulator of tub-1 to regulate ech-1.1 for the cafestol’s fat-lowering effects.

Locomotor behavior can be assessed as indicative of energy expenditure for the overall fat accumulation in C. elegans [117]. We further determined whether cafestol’s fat-lowering effects were due to changes on locomotor behavior. Cafestol increased speed on wild-type worms by 38% compared to the control (P= 0.0072, Fig. 5.7B). Since TUB-1 is located in ciliated sensory neurons, involved in the sensory and locomotor behavior in C. elegans [143–145], we determined the effects of cafestol on locomotor behavior in the mutants of tub-1 as well as daf-12 since results in Fig. 5.7A showed that daf-12 regulates tub-1 expression. The increased effects of cafestol on speed were abolished in tub-1 and daf-12 mutant (Fig. 5.7B). Taken together, these suggest that cafestol increases energy expenditure via DAF-12 and TUB-1-dependent pathways in C. elegans.

5.4. Discussion

Coffee is one of the most consumed beverages in the world and is known to be associated with reduced risk of obesity [132]. Phenolic acids, due to their abundance in coffee, are mostly suggested to contribute to the fat-lowering effects of coffee [3].
However, diterpenes found in coffee beans, such as cafestol, also were previously reported to regulate fatty acid metabolism [26, 32, 33, 133]. In the current study, we found that cafestol reduced fat accumulation dependent on daf-12, FXR homolog, and tub-1, the ortholog of mammalian TUBBY in C. elegans. Both DAF-12 and TUB-1 were found to be involved in the increased energy expenditure and ech-1.1 upregulation by cafestol. These results support the potential use of cafestol for metabolic disorders [28, 30].

![Graph A](image1.png)

**Figure 5.7.** Cafestol’s fat-lowering effects are dependent on daf-12 and tub-1. A – Effects of cafestol on the target genes expression on daf-12 and tub-1 mutants measured by real time-PCR (n=3). B – Effect of cafestol on locomotor behavior of C. elegans. Cafestol (60 µM) increased energy expenditure (speed) on wild-type C. elegans (n = 127-177), but its effects were abolished on tub-1 mutant (n = 157-170) and daf-12 mutant (n = 78-80). Data are means ± S.E. Means with different letters at each variable are significantly different at $P < 0.05$. 
There are reports that cafestol does not change circulating lipids, including blood cholesterol levels [28, 30, 146]. Consistently, our study shows that cafestol did not change \textit{lrp-1} expression, the LDL receptor-related gene involved in the cholesterol transport in \textit{C. elegans} [147]. However, others reported the increased blood cholesterol and/or triglycerides after cafestol treatment in humans and rodents [26, 133]. It was suggested that cafestol increased blood cholesterol levels via not only FXR, but also another nuclear hormone factor, pregnane X receptor, which is involved in detoxifying pathways in a hyperlipidemic mice model [26]. However, the effects of cafestol on cholesterol metabolism may be absent in this model since \textit{C. elegans} requires regular cholesterol intake due to lack of cholesterol synthesis and has typically low levels of cholesterol [105]. Thus, the current observation along with the fact that \textit{C. elegans} does not synthesize cholesterol, contributed to the discrepancy of cafestol’s effects on cholesterol homeostasis between mammals and \textit{C. elegans} [105, 135]. Moreover, the delayed reproduction by cafestol may be related to cholesterol and DAF-12’s effects on the germline signaling pathway in \textit{C. elegans} [134, 136]. The potential effects of cafestol on blood lipids and reproduction needs further study.

In addition to its role in cholesterol metabolism, it is also known FXR agonists have potential use for preventing obesity and fatty liver disease by regulating lipid metabolism, including lipogenesis, lipolysis and fatty acid β-oxidation [82, 83]. In fact, known FXR agonists, ivermectin and epigallocatechin-3-gallate, showed anti-obesity effects in HepG2 cells and \textit{C. elegans} [83, 89, 148]. Consistently, DAF-12/FXR activation by the steroid-derived hormone, Δ7-dafachronic acid, reduces triglyceride levels by increasing lipolysis and fatty acid β-oxidation in \textit{C. elegans} [134]. However, in the current study we only
observed cafestol increases fatty acid β-oxidation, particularly via ech-1.1, without any effects on lipogenesis or lipolysis, even though the cafestol’s fat-lowering effects was via DAF-12-dependent pathway.

It has been previously reported that FXR potentiates fatty acid β-oxidation via PPARα [82]. However, our results failed to support that cafestol activates ech-1.1 dependent on nhr-49/PPARα. In addition to nhr-49/PPARα, CEBP-2 and TUB-1 are also known to modulate fatty acid β-oxidation [106, 140, 149]. The current results show that cafestol had no effect on cebp-2, but upregulated tub-1, suggesting that cafestol activates daf-12, then tub-1 subsequently improves fatty acid β-oxidation by ech-1.1. However, there is no previous knowledge if daf-12 regulates tub-1 followed by ech-1.1. These need to be further validated along with the relevance of these observations in humans. In addition, we cannot exclude the possibility that cafestol influences additional targets of lipid homeostasis, independent of transcriptional levels, to reduce overall fat accumulation.

The current results showed that cafestol downregulated fard-1 in tub-1 mutant, but its expression was increased due to daf-12 in the wild-type worm. It is known that ether lipids, in which FARD-1/FAR-1 is responsible for their synthesis, regulates fatty acid profile by modulating de novo fatty acid synthesis-related genes, including pod-2, fat-6 and fat-7 [150]. However, their role on overall fat accumulation is still unclear [150]; no changes on body fat or triglycerides levels in C. elegans were observed with the reduced FARD-1 activity [137, 150], while mulberry leaf polyphenols reduce fat accumulation with upregulation of fard-1 via DAF-12 in C. elegans [136]. Based on our results, we infer that cafestol regulates the synthesis of ether lipids via FARD-1 in part tub-1-
dependent mechanism, although how tub-1 regulates fard-1 independent to daf-12 is not clear currently.

The current results suggest that cafestol increases worm speed, an indicator of energy expenditure, dependent on daf-12 and tub-1. Both FXR and TUBBY-like proteins have previously shown to regulate energy homeostasis [82, 138, 139, 151]. In C. elegans, the expression of DAF-12 is not specific to neurons, while TUB-1 is mostly expressed in ciliated neurons, involved in locomotor behavior [143–145]. In humans, TUB transcripts were found in the adipose tissue as well as in the hypothalamus, neuron region that regulates multiple metabolic pathways including energy expenditure [139]. Although there was no detection of cafestol in the brain of mice after oral treatment, there are reports that FXR pathway modulates neuron activity in the brain via both direct and indirect pathways [32, 152]. Other measurements of energy expenditure need to be used to further validate the effects of cafestol on energy expenditure, such as mitochondrial oxygen consumption and complexes activities. Moreover, other transcription factors, such as NHR-8, known to regulate DAF-12 activity, may be involved in the effects of cafestol [153]. Therefore, DAF-12/FXR activation by cafestol may increase energy expenditure via the neuroendocrine pathway TUB-1 in C. elegans.

It was reported that cafestol (1.5 mg/day) reduced fat accumulation in high-fat fed mice and cafestol (60 mg/day; equivalent to 10-20 cups of unfiltered coffee) altered cholesterol metabolism in humans [25, 32]. As filtered coffee contains less cafestol than unfiltered coffee, it is not clear whether cafestol’s effects on fatty acid metabolism seen in the current study are relevant from a moderate consumption of regular coffee (1.5-3.75 mg cafestol from daily intake of 2 to 5 cups of 250 mL) [132]. In addition, the current
study showed that cafestol at 30 and 60 µM (9.5 and 19 µg/mL, respectively) had shown to be effective on reduction of fat accumulation in *C. elegans*, which are relatively lower effective concentrations compared to other bioactive compounds on fat reduction in the same model [3, 89, 113, 115]. Compounds may be absorbed via cuticle or pharynx in *C. elegans*, thus, chemical structure and solubility contribute to its effectiveness in this model [105]. However, it is still not known how doses from *C. elegans* would be comparable to other mammals, which may be a limitation of using this model currently.

Other coffee compounds, such as caffeine and chlorogenic acids, have shown to reduce fat accumulation, however, with distinct mechanisms on fatty acid metabolism compared to that of cafestol [132]. In *C. elegans*, caffeine’s fat-lowering effects are related to changes on feeding and locomotor behavior via neurological pathways, while chlorogenic acids regulate fatty acid metabolism via IIS, lipogenesis, and lipid uptake and transport pathways [3, 154, 155]. Other coffee diterpene, kahweol, has shown to act on fatty acid metabolism via AMPK system [33]. Although there are limited studies on the interaction of coffee bioactive compounds on metabolism, a combination of cafestol, caffeic acid and trigonelline has shown to reduce plasma insulin and fatty liver in mice [30]. Overall, these reports suggest that cafestol is involved in the health effects of coffee, and it may further support the benefits of coffee on obesity [132].

### 5.5. Conclusion

In summary, we found that cafestol regulates fatty acid metabolism in *C. elegans* by regulating fatty acid β-oxidation and energy expenditure dependent on *daf-12/FXR*. 
CHAPTER 6
KAHWEOL REDUCES FOOD INTAKE OF Caenorhabditis elegans

6.1 Introduction

Coffee is one of the most popular drinks in the world and there are many reports about its association with reduced risk of obesity, diabetes and metabolic syndrome [1, 132]. However, there are controversies about the coffee’s effects on health, which may be linked to many minor bioactives present in coffee, including kahweol, a diterpene. Kahweol is found up to 0.5 mg in a regular cup of coffee from Arabica coffee beans and it has anti-cancer, anti-inflammatory and anti-adipogenesis properties in vitro [132, 156]. Studies with kahweol-enriched coffee products (i.e. unfiltered coffee and coffee oil) showed that kahweol has effects on lipid metabolism in humans [25, 157]. However, little is known about kahweol’s effects on lipid metabolism in vivo [33, 158].

Caenorhabditis elegans, a millimetric transparent worm, has been useful to understand the genetic influence on lipid metabolism, since more than 70% of C. elegans lipid metabolism-related genes have homologs in humans [159]. This invertebrate model allows the study of its feeding behavior, which it is used for the prospective studies of anti-obesity food bioactives that may act on food intake [160–162]. The aim of this study was to explore the kahweol’s effects on lipid metabolism in C. elegans.

6.2 Materials and methods

6.2.1 Materials

Kahweol was bought from Chengdu Biopurify Phytochemicals Ltd (purity ≥ 98%, CAS 6894-43-5, PubChem CID 114778, Chengdu, China). Carbenicillin and ampicillin
were purchased from Fisher Bioreagents (Pittsburgh, PA, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA), respectively. Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) unless it is otherwise specified in the methods. *C. elegans* stains (N2 wild-type and *eat-2(ad1116)* II) and *Escherichia coli* OP50 were obtained from *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA). The following TaqMan® gene expression primers (Thermo Fisher Scientific, Inc., Middletown, VA) were used to perform the relative quantitative RT-PCR: *ama-1* (Ce02462726_m1; internal control), *atgl-1* (Ce02406730_g1), *daf-2* (Ce02444336_m1), *daf-16* (Ce02422838_m1), *ech-1.1* (Ce02485968_g1), *hosl-1* (Ce02494529_m1), *nhr-49* (Ce02412667_m1), *sbp-1* (Ce02453000_m1) and *tub-1* (Ce02435686_m1).

### 6.2.2 *C. elegans* culture

Worms were cultured on nematode growth media (NGM) plates at 20°C on an incubator (model DT2-MP-47, Tritech Research Inc., Los Angeles, CA) until population synchronization by bleach solution was performed as previously described [3]. In liquid S-complete media, L1 stage worms (~1000 worms) were cultured in a 12-well plate with antibiotics (ampicillin and carbenicillin) and dead *E. coli* OP50 as food source. 120 µM 2’-deoxy-5-fluorouridine (TCI America, Portland, OR, USA) was put in the media before worms reached adulthood to stop laid eggs from hatching, since it inhibits the DNA synthesis and cell division [115]. Adult worms were treated with kahweol (30-120 µM in DMSO) or 0.2% dimethyl sulfoxide (DMSO; control) and incubated for 2 days.

### 6.2.3 Triglyceride quantification
After treatment worms were collected in tubes for sonication in 0.05% Tween 20 before triglycerides and protein assays [3]. Triglyceride content was determined by using Infinity™ Triglycerides Reagent (Fisher Diagnostics, Middletown, VA, USA) with glycerol as a standard. Then, triglycerides levels were normalized by protein, measured by using Bradford reagent (Sigma-Aldrich Co., St. Louis, MO, USA) with bovine serum albumin as a standard. Microplate reader and analysis software (SpectraMax i3 and SoftMax Pro 6.5, Molecular Devices, Sunnyvale, CA, USA) were used to measure absorbances in the colorimetric assays. The triglyceride and protein ratio is widely used to represent the total fat in C. elegans, especially for prospective studies with lipophilic molecules, such as kahweol [105].

### 6.2.4 Worm tracking

Worms were placed in low-peptone agar plate, acclimated under camera light for 20 min, then recorded in video (1 min) by using WormLab tracking system (model MSCOP-002, MBF Bioscience, MicroBrightField Inc., Williston, VT, USA) [117]. Videos were analyzed by WormLab software (3.1.0 64-bit, MBF Bioscience, Williston, VT, USA) to obtain length, width and moving speed [117].

### 6.2.5 Optical density measurements

The optical density at 600 nm (OD 600 nm), which measures turbidity in bacteria media, was measure in the sample supernatants at day 0 and day 2 as previously described [160]. There was no significant absorbance of kahweol in liquid media at 600 nm, consistently with previous report of kahweol’s highest absorbance at 290 nm [163].
Microplate reader and analysis software (SpectraMax i3 and SoftMax Pro 6.5, Molecular Devices, Sunnyvale, CA, USA) were used for these measurements.

6.2.6 Fluorescent *E. coli* quantification

The green fluorescence protein (GFP) expression after worms fed with the strain *E. coli* OP50-GFP was measured by a fluorescence imaging technique [89]. The fluorescence microscope Nikon Eclipse Ti-U (Nikon Instruments Inc., Melville, NY) was used to take the pictures of immobile nematodes on microscopic glass slides. Next, we used the NIS-Elements Basic Research software version 4.20.01 (Nikon Instruments Inc., Melville, NY) to collect data, then the fluorescence intensity of selected area (intestine) minus the background was represented as relative to the control.

6.2.7 Pharynx pumping rate

Pharynx pumping rate, used to estimate food intake, was measured in 30 s by using stereomicroscope [115, 160]. Worms were placed in low-peptone plates with live *E. coli*, then pharynx pumping rate of random worms were measured [115]. Alternatively, we used serotonin to induce pharynx pumping rate in liquid media without *E. coli*; after 2 days treatment, worms were placed in 96-well plates in liquid media with 10 mM serotonin before visual observations as previously described [160]. The observations started after 20 min of acclimation under stereomicroscope light. The differences on these protocols include the media (solid vs. liquid), *E. coli*, serotonin and kahweol, which was included in the liquid media for the serotonin-induced pharynx pumping rate.
6.2.8 Gene expression

Total RNA was extracted from nematode sample by using TRIZol® (Thermo Fisher Scientific, Middletown, VA, USA), then RNA samples were reverse-transcribed by using high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Inc., Middletown, VA) according with the respective manufacturer’s protocols. Gene signal intensity was detected by StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA), then comparative threshold cycle (Ct) method was used to express the results as fold change of gene expression ($2^{-\Delta\Delta Ct}$).

6.2.9 Statistical analysis

Data was analyzed by mixed one-way analysis of variance followed by Tukey’s test with SAS Software (version 9.4, SAS Institute, Cary, NC). Groups were considered statistically different when $P < 0.05$ compared to the control at each variable.

6.3 Results

Kahweol at 30, 60 and 120 µM reduced fat accumulation by 7, 14 and 17% (P=0.0038, 30 µM; P<0.0001, 60 and 120 µM), respectively, compared to the control in C. elegans (Fig. 6.1). However, there were no effects of kahweol treatment on body length, width or moving speed of C. elegans (Fig. 6.2), which suggest that the reduced fat accumulation by kahweol is not associated with changes on body size or energy expenditure in C. elegans.
Figure 6.1. Kahweol reduced fat accumulation of *Caenorhabditis elegans*. Worms were treated with kahweol for 2 days. Triglyceride levels were normalized by protein content. Data are means ± S.E. (n = 6-7). Means with different letters are significantly different at $P < 0.05$.

Next, we evaluated the effects of kahweol on food intake by measuring the optical density (OD 600 nm) of culture media since it is known that OD values are associated with bacteria concentrations [160]. Even though there were no significant differences on absorbance at day 0, treatment of kahweol at 60 and 120 µM had increased OD values by 34% ($P=0.0220$) and 105% ($P<0.0001$), respectively, compared to the control at day 2 (Fig. 6.3A). This indicates that there were more bacteria left on the media on kahweol’s group, representing reduced food intake by kahweol-treated nematodes compared to the control. We further determined the effects of kahweol at 120 µM on food intake by using *E. coli::GFP*. As shown in Fig. 6.3B, treatment of kahweol significantly reduced fluorescence intensity by 21% ($P=0.0026$) compared to the control (Fig. 6.3B).
Figure 6.2. Kahweol did not change body size and moving speed in *Caenorhabditis elegans*. Worms were treated with kahweol for 2 days. Randomly selected worms were recorded in 1 min video, then length (A), width (B) and moving speed (C) were analyzed (n = 21-24). Data are mean ± S.E.

Pharynx is the organ that pumps *E. coli* (diet) from the media to inside, thus, the measurements of pharynx contractions (pumping rate) is a behavioral assay that represents food intake of *C. elegans* [160, 164]. However, other behaviors may impact food intake without changes on pharynx pumping rate [165]. Our results showed that pharynx pumping rate of kahweol-treated worms were not different than the control in
solid media with *E. coli* (Fig. 6.3C). However, kahweol reduced pharynx pumping rate by 27% (*P* = 0.0013) compared to the control (Fig. 6.3C) with serotonin (10 mM), a neurotransmitter that induces pharynx pumping rate in absence of *E. coli* [160]. These results along with results in Fig. 6.3A and 6.3B indicate that kahweol reduced food intake, which may be associated with its presence in the media and/or serotonin pathway in *C. elegans*.

**Figure 6.3.** Kahweol reduced food intake in *Caenorhabditis elegans*. Worms were treated with kahweol for 2 days. A – Optical density at 600 nm of sample supernatant after treatment (n=4). B – Worms were fed with *E. coli* OP50-GFP, then fluorescence intensity of worms was measured in the worm intestine (n=17-18). C – Pharynx pumping rate was measured in low-peptone agar plates or liquid media with 10 mM serotonin (n=20-30 collected from two-three independent experiments). Data are means ± S.E. Means with different letters are significantly different at *P* < 0.05.
Since the overall fat accumulation is regulated by lipid metabolism, next we tested whether kahweol regulates lipid metabolism-related gene expressions. SBP-1, the homolog of the human sterol regulatory element-binding proteins (SREBPs), positively regulates fat accumulation via lipogenesis in *C. elegans*. Kahweol did not regulate *sbp-1* expression in *C. elegans* (Fig. 6.4).

NHR-49, a homolog of the human peroxisome proliferator-activated receptor alpha (PPARα), regulates fat accumulation negatively by inducing fatty acid β-oxidation in *C. elegans*. Kahweol did not regulate *nhr-49* expression in *C. elegans* (Fig. 6.4), which suggest that *nhr-49* is not involved in the kahweol’s effects on fat accumulation.

TUB-1, the homolog of the human tubby proteins (TUB), regulates fat accumulation negatively by inducing fatty acid β-oxidation via the enoyl-CoA hydratase, *ech-1.1*, in *C. elegans*. Kahweol downregulated *tub-1* expression by 31% (*P* = 0.0449) and *ech-1.1* expression by 47% (*P* = 0.0043) compared to the respective controls (Fig. 6.4), which suggest that the effects of kahweol on *tub-1* and *ech-1.1* may contribute for the reduced fat accumulation in *C. elegans*.

The lipases encoded by *atgl-1* (the homolog of the human adipose triglyceride lipase, ATGL) and *hosl-1* (the homolog of the human hormone-sensitive lipase, HSL) are involved in lipolysis in *C. elegans*. Kahweol downregulated *atgl-1* expression by 26% (*P* = 0.0377) compared to the control, while *hosl-1* expression was not changed by kahweol in *C. elegans* (Fig. 6.4). These results suggest that *atgl-1* downregulation may be a indicative of the overall reduced fat accumulation by kahweol in *C. elegans* [89].

The insulin/insulin-like growth factor signaling (IIS) pathway regulates lipid metabolism via *daf-2* (the homolog of the human insulin/insulin-like growth factor
receptor) and the transcription factor daf-16 (the homolog of the human Forkhead box O transcription factor) in C. elegans. Kahweol at 120 µM downregulated daf-2 expressions by 35% ($P=0.0019$) compared to the control in C. elegans (Fig. 6.4). Kahweol, only at 60 µM, downregulated daf-16 expression by 17% ($P=0.0292$) compared to the control in C. elegans (Fig. 6.4). Overall, these suggest that the kahweol has inhibitory effects on IIS pathway, which may contribute for the reduced fat accumulation in C. elegans.

**Figure 6.4.** Effects of kahweol on genes related to lipid metabolism in *Caenorhabditis elegans*. Worms were treated with kahweol for 2 days. Data are means ± S.E. (n = 4). Means with different letters are significantly different at $P < 0.05$ at each variable.

*eat-2*, which encodes an acetylcholine receptor subunit in pharyngeal muscle cells, starts the action potential in the pharynx muscle cells, thus *eat-2* mutant has reduced food intake due to disrupted pharynx contractions [164]. We determined if kahweol’s effects on fat accumulation were dependent on the reduced food intake by using *eat-2* mutant, known to have a reduced pharynx pumping rate [164]. Kahweol did not reduce fat accumulation in *eat-2* mutant (Fig. 6.5A), which suggests that kahweol’s effects on fat accumulation are dependent on food intake.
To evaluate whether kahweol regulates the lipid metabolism-related target genes (tub-1, ech-1.1, atgl-1, daf-2 and daf-16) dependently on reduced food intake, kahweol’s effects on the gene expressions were measured in eat-2 mutant. The expression of tub-1 was upregulated by 208% (P=0.0110) in eat-2 mutant when compared to the wild-type worms (Fig. 6.5), which suggest that the reduced food intake enhances TUB-1 activity. However, kahweol’s effects on tub-1 in eat-2 mutant were different than that of wild type; kahweol upregulated tub-1 by 44% (P=0.0406) in eat-2 mutant (Fig. 6.5). The expression of ech-1.1 was downregulated by 56% (P= 0.0006) in eat-2 mutant compared to wild-type worms (Fig. 6.5), which is consistent with the effects of fasting on ech-1.1 in C. elegans [120]. Not surprisingly, kahweol’s effect on ech-1.1 seen in wild-type was abolished in eat-2 mutant (Fig. 6.5), since ech-1.1 is downstream of TUB-1. Taken together, these results suggest that the kahweol’s effects on tub-1 and ech-1.1 are related to a reduced food intake, but the tub-1 upregulation by kahweol in eat-2 mutant needs further studies.

**Figure 6.5.** The reduced fat accumulation by kahweol is dependent on reduced food intake in Caenorhabditis elegans. eat-2 mutant, which has a reduced food intake due to disrupted pharynx function, were treated with kahweol for 2 days. A – Kahweol’s effects on fat accumulation were abolished in eat-2 mutant. Triglyceride levels were normalized by protein content. (n = 4). B – The effects of kahweol on genes related to lipid metabolism were dependent on food intake in eat-2 mutant (n = 3). Results in B were expressed as relative to the wild-type worm control (1.0, indicated with a trace line). Data
are means ± S.E. Means with different letters are significantly different at $P < 0.05$ at each variable.

*eat-2* mutant is known to have reduced fat accumulation as expected by reduced food intake and showed higher *atgl-1* expression with a 65% increase ($P=0.0022$) compared to the wild-type worms (Fig. 6.5), suggesting that lipolysis via *atgl-1* is enhanced by a reduced food intake similarly to fasting [166]. On the other hand, the *atgl-1* downregulation by kahweol seen in wild-type was abolished in *eat-2* mutant (Fig. 6.5), which suggest that reduced *atgl-1* by kahweol treatment seen in wild-type was dependent to reduced food intake.

IIS pathway seemed to be enhanced in response to the reduced food intake in *eat-2* mutants, since *daf-2* expression was upregulated by 144% ($P<0.0001$) in *eat-2* mutant compared to wild-type worms (Fig. 6.5). However, the expression of *daf-16* was not changed in *eat-2* mutant when compared to wild-type worms (Fig. 6.5), which suggest that DAF-16 post-translational changes are more important for the response to a reduced food intake [106, 167]. Kahweol did not regulate *daf-2* neither *daf-16* expressions in *eat-2* mutant (Fig. 6.5). These results suggest that kahweol’s inhibitory effects on IIS pathway are dependent on food intake, consistent with the role of IIS pathway as nutrient sensor [168].

6.4 Discussion

Kahweol is a coffee diterpene, found mainly in Arabica coffee beans, that has potential biological effects, such as antioxidant, anti-inflammatory, anti-cancer and anti-obesity properties [132, 156]. The previous studies have shown that kahweol reduces adipogenesis *in vitro*, but there are still limited evidence about kahweol’s effects on lipid
metabolism in vivo [33, 158]. In the current study, we showed that kahweol reduced fat accumulation dependent on reduced food intake in *C. elegans* by leading the downregulation of lipid metabolism-related genes involved in β-oxidation (*tub-1* and *ech-1.1*), insulin signaling (*daf-2* and *daf-16*) and lipolysis (*atgl-1*).

Appetite suppressors regulate the neurological control of hunger via distinct mechanisms; for example, lorcaserin and nicotine act on serotonin and acetylcholine pathway, respectively [169, 170]. The feeding behavior of *C. elegans* is also regulated by multiple neurological pathways that control the organ responsible for the food intake, pharynx [164]. The reduced pharynx pumping rate by kahweol was limited to serotonin-induced pharynx contractions suggesting that kahweol acts on serotonin pathway. SER-7, the human 5-hydroxytryptamine receptor 7 homolog, is responsible for the pharynx contractions in response to exogenous serotonin [160, 171, 172], thus, it is possible that kahweol reduces pharynx pumping rate dependent on *ser-7*. The direct effects of kahweol on *ser-7* and other pathways (e.g. acetylcholine, glutamate and dopamine) need further investigation since they may also contribute for the reduced food intake and/or fat accumulation in *C. elegans* [173–175].

It is known that motor neurons are activated by serotonin prior to acetylcholine release into the pharyngeal neuromuscular junction, then EAT-2, an acetylcholine receptor, together with ionic channels trigger the pharynx contractions in *C. elegans* [164, 170, 176–178]. Reports suggest that nicotine, an acetylcholine receptor agonist, at high concentrations reduces food intake by reducing pharynx pumping rate whether the contractions are induced by *E. coli* or serotonin in *C. elegans* [170, 177]. Even though kahweol’s effects on lipid metabolism were dependent on *eat-2*, we cannot rule out that
kahweol may reduce pharynx pumping rate due to other neurological pathway that controls food intake.

It is believed that Tubby proteins, expressed in the human brain and adipose tissue, are involved in a neuroendocrine pathway that regulates fatty acid β-oxidation [51, 139]. However, the effects of TUB-1 on fat accumulation are inconsistent; a gallotannin reduced fat accumulation via TUB-dependent pathway, but the effects of the gallotannin on tub-1 expression were dependent on diet (i.e. glucose) [179]. The interpretation of the effects of kahweol on tub-1 remain still elusive, even though it is unlikely that the kahweol’s fat-lowering effects are due to increased β-oxidation. In addition to ech-1.1, other TUB-1 targets, as the 3-ketoacyl-CoA thiolase kat-1, may be contribute for the effects of kahweol on lipid metabolism in *C. elegans* [51, 125]. Moreover, the kahweol’s effects on TUB-1 and serotonin may be linked, since it was previously reported that serotonin reduced lysosome-related fat content via TUB-1 pathway in *C. elegans* [180, 181]. Therefore, further studies on how the reduced food intake by kahweol may involve TUB-1 pathway are needed.

It is known that IIS pathway is activated when nutrients are abundant, which leads to insulin-like peptides to bind the insulin receptor that triggers a cascade of reactions that regulate multiple pathways, including lipid metabolism [105, 106]. The expression of the insulin receptor DAF-2 is reduced when worms are starved, which corroborates with the *daf*-2 downregulation by kahweol dependent on food intake [168]. Our results suggest that reduced food intake by kahweol may have little or null effects on the DAF-2 downstream target *daf*-16 at transcript level, since kahweol, only at 60µM, downregulated *daf*-16 and *daf*-16 was not regulated in *eat*-2 mutant. It is known that IIS
pathway regulates its downstream DAF-16 by post-translational changes; IIS inhibition induces DAF-16 nuclear translocation and subsequently DAF-16 can regulate its downstream targets [3, 106, 182]. Although it is still not known if kahweol induce DAF-16 nuclear translocation, kahweol’s effects on DAF-16 may be similar to other coffee bioactives (i.e. caffeine and chlorogenic acid), which was also found to induce DAF-16 nuclear translocation in *C. elegans* [3, 183].

A reduced fat accumulation may be caused by an increased lipolysis via ATGL-1, but ATGL-1 can also represent increased lipogenesis [89, 166, 184–186]. It was previously reported that *atgl-1* deletion reduced fat accumulation by inhibiting peroxisome proliferator-activated receptor gamma (PPARγ) and SREBP in addition to a reduced food intake in mice fed a high-fat diet [185]. Although the current study showed the kahweol does not change *sbp-1*, other lipogenesis-related factors may influence *atgl-1* in *C. elegans* [166, 185]. Moreover, it is known that ATGl-1 is regulated by serotonin and IIS pathways in *C. elegans* [166, 174, 175]. The influence of other pathways on kahweol’s effects on *atgl-1* is to be determined by further studies.

### 6.5 Conclusion

In conclusion, kahweol acts as appetite suppressor to reduce fat accumulation in *C. elegans*. Lipid metabolism was regulated by kahweol dependently on food intake, which may be due to an effect of kahweol on serotonin pathway. These results suggest that kahweol reduces food intake, which may be a useful tool to mitigate obesity.
CHAPTER 7
CONCLUDING REMARKS

Coffee drinks are popular around the world, but there are limited studies on the effects of coffee and its bioactives on lipid metabolism, including that how coffee consumption is associated with lower risk of metabolic syndrome, obesity and diabetes, which are conditions with altered lipid metabolism. This dissertation demonstrated the effects of green coffee bean extract and coffee bioactives (chlorogenic acid, cafestol and kahweol) on lipid metabolism in C. elegans, as an in vivo model. GCBE and the chlorogenic acid (5-O-caffeoylquinic acid) had similar effects on lipid metabolism dependent on sbp-1, daf-16 and far-6, which are involved in lipogenesis, insulin-signaling pathway and lipid transport, respectively. However, the coffee diterpene cafestol regulated fatty acid β-oxidation and energy expenditure dependent on the farnesoid X receptor homolog daf-12, while another coffee diterpene, kahweol, reduced fat accumulation dependent on food intake in C. elegans.

C. elegans can be used further to evaluate other metabolic pathways impacted by coffee bioactives. This dissertation focused on coffee’s effects on lipid metabolism, but it is possible to elucidate the mechanisms of action and the impact of coffee on lifespan, or even host-bacteria interactions by using C. elegans as well. Some of the targets for coffee’s fat-lowering effects elucidated in this dissertation, such as DAF-16 and DAF-12, may be related to its effects on lifespan, which may be independent of its effects on lipid metabolism, for example. Not only the evidence that coffee bioactives can modulate DAF-12, but also knowing that DAF-12 is regulated by bacteria show that further studies
in *C. elegans* may help us to understand the mechanisms of potential effects of coffee on host-bacteria interactions.

Coffee research has been focused on its main components, such as caffeine, but here it is showed that other coffee bioactives may also be important to be investigated. The variability and complexity of coffee composition still need to be explored, especially for other coffee-related products, such as extracts or oil, that may be included widely in our diet in the future. The expansion on the knowledge of coffee composition, which is highly dependent on species, environment, and processing, may help us to find new bioactives. Thus, future studies about coffee may need to consider other coffee bioactives that may have impact on human health in addition to caffeine.

Metabolic and high-throughput sequencing analysis, such as shotgun proteomics or RNA sequencing, may help to find other targets for coffee bioactives on metabolism. With the incorporation of these technologies, maybe we can also understand how coffee impacts metabolic pathways, including lipid metabolism, via non-coding RNAs for example. Although these tools have now some limitations to be applied in large scale, especially in humans, the availability of data provided by these -omics technologies would be useful to understand the effects of coffee on metabolism and find potential other targets. Thus, we can incorporate these tools in animal studies to suggest mechanisms of action of coffee bioactives for further consideration and validation in human studies in the future.

This dissertation corroborates with the fact that coffee has a potential to be used to improve human health, including metabolic syndrome, obesity, and diabetes. Although the current results are limited in a model system of *C. elegans*, the underlying
mechanisms of coffee bioactives on lipid metabolism may be applied in further animal and human studies to direct strategies against the metabolic conditions in future.
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