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Cranberry Product Decreases Fat Accumulation in *Caenorhabditis elegans*

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ABSTRACT Cranberry phenolic compounds have been linked to many health benefits. A recent report suggested that cranberry bioactives inhibit adipogenesis in 3T3-L1 adipocytes. Thus, we investigated the effects and mechanisms of the cranberry product (CP) on lipid metabolism using the *Caenorhabditis elegans* (*C. elegans*) model. CP (0.016% and 0.08%) dose-dependently reduced overall fat accumulation in *C. elegans* (N2, wild type) by 43% and 74%, respectively, without affecting its pumping rates or locomotive activities. CP decreased fat accumulation in *aak*-2 (an ortholog of AMP-activated kinase α) and *tub-1* (an ortholog of TUBBY) mutants significantly, but only minimal effects were observed in *sbp-1* (an ortholog of sterol response element-binding protein-1) and *nhr-49* (an ortholog of peroxisome proliferator-activated receptor-α) mutant strains. We further confirmed that CP downregulated *sbp-1*, *cebp*, and *hosl-1* (an ortholog of hormone-sensitive lipase homolog) expression, while increasing the expression of *nhr-49* in wild-type *C. elegans*. These results suggest that CP could effectively reduce fat accumulation in *C. elegans* dependent on *sbp-1*, *cebp*, and *nhr-49*, but not *aak-2* and *tub-1*.

KEY WORDS: • **C. elegans** • ***cranberry*** • ***fat accumulation*** • **nhr-49** • **sbp-1**

INTRODUCTION

OBESITY HAS BECOME one of the leading contributors to a number of chronic illnesses all over the world, such as diabetes, cardiovascular diseases, and hypertension.[1] It is well known that excessive intake of food and decreased physical activity are important factors contributing to obesity; however, there are many other factors that contribute to the development of obesity.[2] Treatment of obesity using drugs is possible, but rather limited, thus using a food-based approach to control obesity is more desirable.

Cranberries are a well-known food with high phytochemical content of natural antioxidants and have many known health benefits, including prevention of urinary tract infection and chronic diseases, such as diabetes mellitus, cardiovascular diseases, and cancers.[3–5] Cranberry products (CPs) are very popular in the United States. More than 8 million barrels of cranberries, with a value of 385.5 million, were produced in the United States in 2012.[6] Recently, there were reports of cranberries inhibiting adipogenesis in 3T3-L1 cells.[7] However, no reports have investigated the mechanisms of cranberries modulating fat accumulation in animal models.

As a model system to test the effects of food components on obesity, we have used *Caenorhabditis elegans* (*C. elegans*). *C. elegans* has been used intensively in biological and medical studies due to their short life span of 30 days and rapid reproduction cycles.[8] Moreover, various mutants of *C. elegans* are available at minimum cost, which makes it a great *in vivo* model for cellular, genetic, or behavior studies. It is also known that *C. elegans*'s lipid metabolism is conserved in mammalian cells.[9] Therefore, studying antiobesity effects using *C. elegans* is not only efficient and easier to handle but also provides an ideal model to study the genes involved in the process. In fact, Martorell *et al.*[10] suggested that *C. elegans* could be a useful *in vivo* model to investigate as well as screen antiobesity drugs or food bioactives. Thus, we chose *C. elegans* as an *in vivo* model to investigate the effect and mechanisms of the CPs on fat accumulation in the current study.

MATERIALS AND METHODS

Materials

A water-soluble cranberry extract powder standardized to 4% proanthocyanidins (HI-PAC 4.0) was provided by Decas botanical Synergies (Carver, MA, USA). N2, bristal (wild-type); CE541, *sbp-1* (*ep79*); RB754, *aak-2* (*ok524*); DG2179, *tub-1* (*nr2044*); GR1307, *daf-16 (mgDf50)*; RB1716, *nhr-49* (*ok2165*); CE548, *sbp-1* (*ep79*) III; epEx141; and *Escherichia coli* OP50 were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA). The amounts of triglyceride (TG) and protein were quantified using kits from Thermo Scientific (Middletown, VA, USA) and Bio-Rad Co. (Hercules, CA, USA), respectively. TaqMan gene expression assays used for *sbp-1*, *cebp*, *hosl-1*, *atgl-1*, *nhr-49*, and *daf-16* were purchased from

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Applied Biosystems (Carlsbad, CA, USA). Fluorodeoxyuridine (FUDR) and carbenicillin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fluoromount-G was from Southern Biotechnology Associates (Birmingham, AL, USA). TRIzol was from Thermo Scientific (Rockford, IL, USA), and other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Preparation of cranberry in S-complete solution

The CP was previously reported to contain ~6.6% phenolic acids, 2.2% flavonoids, and 0.6% anthocyanidins.[11] The CP was dissolved in S-complete and filtered through a 0.45-μm membrane to make 24 mg of the CP/mL stock solution (2.4%). Previously, it was reported that 0.2% (2 mg/mL) CP has beneficial effects on *C. elegans*.[12,13] Our preliminary tests showed that in the range of 0.016% (0.16 mg/mL) to 0.2% (2 mg/mL) CP could reduce triacylglyceride in wild-type *C. elegans* dose-dependently (data not shown). Thus, we chose 0.016% and 0.08% CPs to study the mechanisms underlying the fat reduction effect induced by the CP in the current study.

C. elegans *culture*

M9 buffer, S-basal, S-complete, and nematode growth media agar used in *C. elegans* culture were prepared as previously described.[14] A synchronous worm culture was obtained using the previously described method.[14] All *C. elegans* strains were raised at 20°C in S-complete media supplemented with the CP in 12-well plates. Treatments started from the L4 stage (3 days old) and treatment periods were 2–4 days with FUDR treatment (DNA synthesis inhibitor[15]) to prevent eggs from hatching during the treatment period. For experiments with green fluorescent protein (GFP) detection and quantitative reverse transcriptase–polymerase chain reaction, we treated nematodes with CP, which were 1 day old, for 3 days (before eggs were produced). This was done because FUDR treatment during the egg-producing period might influence gene expression levels.[16] We further confirmed that effects of CP on TG accumulation (treated when either 1 day old or 3 days old for 2–4 days) were comparable (data not shown).

Triacylglyceride quantification

At the end of the treatment, *C. elegans* were collected and washed twice with M9 buffer to remove bacteria and S-complete media. *C. elegans* samples were dissolved in 0.05% Tween 20 solution. After sonication, *C. elegans* samples were used for the TG and protein measurements. The TG assay was conducted with a commercial assay kit (Infinity™ Triglyceride Reagent; Thermo Scientific) and the protein content was measured with the Bio-Rad DC protein assay kit according to the manufacturer's instructions. TG content was normalized with protein concentration.

Pharyngeal pumping rate and locomotion assay

Food intake was measured by counting the rate of pharyngeal muscle contraction from *C. elegans* under an optical microscope (Olympus Corporation, Tokyo, Japan).[17] Locomotion activity was measured by using the Wormlab tracking system (Allied Vision Technologies, Stadtroda, Germany, and Wormlab Software; MBF Bioscience, Williston, VT, USA) as previously reported with minor modifications.[18] Each video used for tracking analysis lasted for 1 min. Data for average moving speed [(forward distance + reverse distance)/time] and the width and length of wild-type *C. elegans* were collected from the tracking system. Body size and locomotive activity of *C. elegans* were measured after treatment with CP for both 2 and 4 days.

Detection of GFP-labeled sbp-1 expression

The CE548 strain was washed twice with S-basal solution at 1,000 *g* for 20 s. Then, *C. elegans* samples were fixed with 4% paraformaldehyde for 2 h at 4°C. Fixed *C. elegans* samples were washed thrice with phosphate-buffered saline. The fluoromount-G was put onto a glass slide, followed by addition of the fixed *C. elegans*. Pictures were taken under confocal microscopy (Nikon microscope D-Eclipse C1 80i; Nikon Corporation, Melville, NY, USA).

mRNA expression analysis

Total RNA was extracted from *C. elegans* using TRIzol® reagent under RNase-free conditions. Total RNA was reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed on a StepOne Plus real-time PCR system (Applied Biosystems). Integrated sequences for TaqMan gene expression assays used for *sbp-1*, *cebp*, *hosl-1*, *atgl-1*, *nhr-49*, and *daf-16* were NM_067071.5, NM_182035.3, NM_001047763.3, NM_171167.4, NM_181998.4, and NM_001264561.1. Threshold values were analyzed using the comparative CT method. The RNA polymerase II large subunit *ama-1* gene (NM_068122.6) was used as an internal standard.

Statistical analyses

Data are expressed as means ± standard errors and analyzed with the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences between groups were assessed with one-way analysis of variance, followed by Tukey's multiple range test. Significance of differences was defined at the $P < .05$ level.

RESULTS

Figure 1 shows the effects of CP on the TG content of wild-type *C. elegans.* The results showed that the TG content was reduced by treatment with CP in a dose-dependent manner. This is consistent with a previous report that cranberry treatments reduce fat accumulation in 3T3-L1 adipocytes.[7]

As it is known that either increased energy intake or decreased energy expenditure can result in increased fat accumulation, we further determined pumping rates (food intake)[19] and locomotive activity (energy expenditure) from

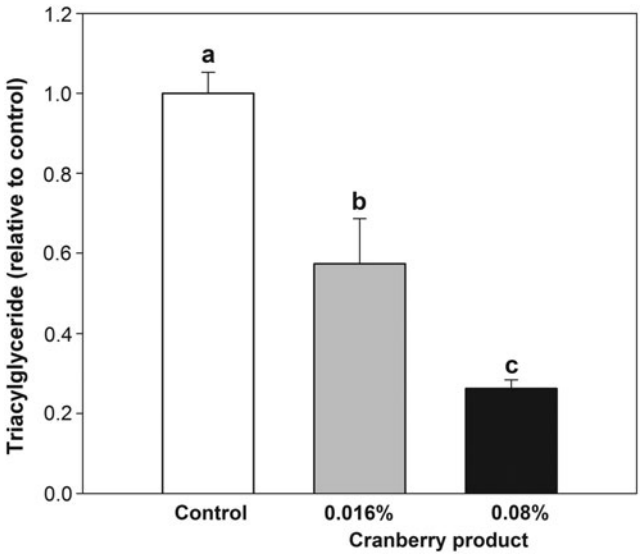

FIG. 1. Effects of the cranberry product on triacylglyceride accumulation in wild-type (N2) *Caenorhabditis elegans.* Cranberry treatment of *C. elegans* started from the L4 stage and treated *C. elegans* samples were harvested for analysis after a 4-day treatment. Data are expressed as means ± standard errors ($n = 3$). Means with different letters are significantly different at $P < .05$.

these nematodes after treatments with the CP (Table 1). Genetic and environmental factors, including food, are also known to affect the body size of *C. elegans*, which can also influence overall fat accumulation.[20] Thus, body sizes after treatment with CP were also measured (Table 1). No significant difference was observed for pumping rates among control and cranberry-treated groups. Cranberry also did not affect the locomotive activity of *C. elegans* (Table 1). No significant differences were observed for body size between control and CP-treated groups (Table 1). These results suggest that CPs have no influence on food intake, energy expenditure, or body size of *C. elegans*, suggesting that another mechanism may be responsible for its effect on fat accumulation, such as altering lipid metabolism.

Table 1. Effects of the Cranberry Product on Pumping Rates, Locomotion Activity, and Body Size of *Caenorhabditis elegans*

	Pumping rate (times/min)	*Speed (μm/s)*	*Body size*	
			Length (μm)	*Width (μm)*
2-day treatment				
Control	222.6 ± 5.6	39.6 ± 2.8	542.2 ± 11.5	56.0 ± 3.8
Cranberry product				
0.016%	247.8 ± 8.1	38.0 ± 2.0	522.5 ± 10.3	51.9 ± 1.3
0.08%	219.3 ± 12.0	39.3 ± 2.5	513.9 ± 23.1	57.1 ± 5.4
4-day treatment				
Control	221.6 ± 7.9	31.2 ± 2.0	538.9 ± 14.2	52.7 ± 1.5
Cranberry product				
0.016%	245.8 ± 14.9	31.2 ± 2.2	528.2 ± 24.5	51.1 ± 2.8
0.08%	218.5 ± 14.7	34.2 ± 2.0	565.8 ± 18.7	54.0 ± 1.9

Numbers are means ± standard errors ($n = 10$–15 for pumping rates and $n = 15$–35 for speed and body size measurements). There are no significant differences between control and the cranberry product treatment group.

Next, we completed genetic epistasis assays to determine if cranberry compounds influence key genes in lipid metabolism using various available mutants, particularly genes known to be associated with lipid metabolism, such as *aak-2*, *tub-1*, *sbp-1*, *nhr-49*, or *daf-16*. The AMP-activated kinase (AMPK) is an important cellular fuel gauge, which responds to the cellular AMP:ATP ratio as well as upstream kinase cascades.[21,22] The activation of AMPK promotes energy-generating pathways and inhibits energy-consumptive pathways. AMPK is conserved in *C. elegans*, and *aak-2* is a homolog of α subunit of AMPK in mammals.[23] To understand whether *aak-2* is involved in the fat accumulation attenuation effect of cranberry in *C. elegans*, the *aak-2* deficiency mutant was studied. These mutants had greater amounts of fat accumulation compared with wild-type animals, suggesting that this is one of the important genes involved in fat metabolism (Fig. 2). CPs (0.016% and 0.08%) significantly reduced fat accumulation in *aak-2* mutants (62% and 73% reduction compared with control, respectively), which were not different to the triglyceride reduction trend in wild-type *C. elegans* (43% and 74%, respectively, compared with control). This suggests that the fat reduction by CP in *C. elegans* might be independent of *aak-2*.

Tubby is broadly expressed in the central nervous system; mutations in rodent *tubby* cause adult-onset obesity with insulin resistance.[24] Similarly, loss of function in *tub-1* in *C. elegans* (ortholog of *Tubby*) caused fat accumulation as seen in Figure 2 and previously.[25] CPs (0.016% and 0.08%) significantly reduced fat accumulation in the *tub-1* deficiency mutant (Fig. 2), suggesting that the fat reduction effect of cranberry in *C. elegans* might be independent of *tub-1*.

DAF-16 is known to be involved in lipid metabolism along with other functions.[26] DAF-16 is known to be the main target of DAF-2 in *C. elegans*, which is a homolog to insulin-like receptors in mammals.[26] It is reported that *daf-2* inhibits DAF-16 (Forkhead family of transcription factors), resulting in shortened life span in *C. elegans*,[25,27] although responses to insulin between mammals and *C. elegans* are somewhat inconsistent.[28,29] To determine whether *daf-16* is involved in CP's effect on reduced fat accumulation in *C. elegans*, we studied the effect of CP on fat accumulation in *daf-16* mutant. The TG content of *daf-16* mutant is no different from wild type, which might suggest that *daf-16* is not critical in regulating overall TG content in *C. elegans*. We observed that CP decreased TG content in *daf-16* mutant at 0.08% only (Fig. 2), and the percentage of fat reduction induced by CP in this mutant was less than that seen in wild-type *C. elegans*. These results indicate that reduction of TG by CP may not mediate *daf-16* in *C. elegans*.

Next, we tested CP on the sterol response element-binding protein (SREBP) deficiency mutant. SREBP is a key transcriptional regulator of fat and sterol synthesis pathways in mammals[30,31] as *C. elegans* SREBP homolog *sbp-1*

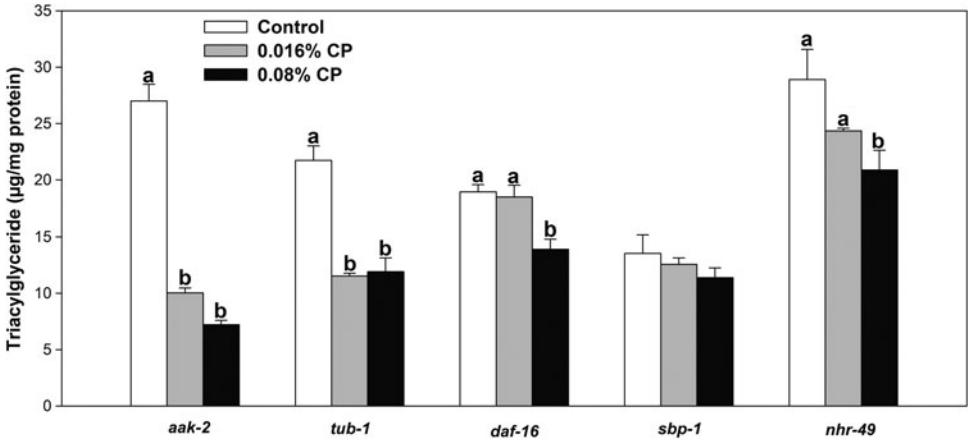

FIG. 2. Effects of the CP on triacylglyceride accumulation in *various* mutants. CP treatment of *C. elegans* started from the L4 stage and *C. elegans* samples were harvested for analysis after a 4-day treatment. Control (*white bars*) and CPs (*gray bars* for 0.016% and *black bars* for 0.08%). Numbers are means ± standard errors ($n = 3$). Means with different letters are significantly different at $P < .05$ at each gene. CP, cranberry product.

deletion mutants exhibit significant reductions in fat content (Fig. 2).[32] No significant differences between the triglyceride content of the control and cranberry treatment groups in *sbp-1* mutants were detected (Fig. 2). This suggests that *sbp-1* is an important gene involved in reduced TG accumulation due to cranberry treatment.

The peroxisome proliferator-activated receptor (PPAR)[33] family is also known to be a key regulator of fat, cholesterol, and glucose homeostasis. In *C. elegans*, *nhr-49* has similar functions to the PPAR family, particularly subtype α, and increased fatty acid β-oxidation.[25,34,35] As expected, there were greater total fat contents in *nhr-49* mutant compared with wild type (Fig. 2). When cranberry compounds were used for treatment in *nhr-49* mutant, no significant difference was observed between the cranberry treatment groups and the control group in *nhr-49* mutant (Fig. 2). These data suggest that cranberry could act on *nhr-49* to mediate fat accumulation in *C. elegans*.

The above data suggest that *sbp-1* and *nhr-49* genes, but not *aak-2*, *tub-1*, and *daf-16*, may mediate the effects of CP on fat reduction in *C. elegans*. To further support this conclusion, we measured expression of these genes in wild-type *C. elegans* (Fig. 3A). CP treatment significantly reduced the expression of *sbp-1* and *daf-16*, while increased *nhr-49* gene expression. Reduced *sbp-1* was further shown from fluorescence detection of GFP-labeled *sbp-1* in CE548 *C. elegans* (Fig. 3B–D). However, the fluorescence of GFP-labeled *sbp-1* expression in the 0.016% CP group (Fig. 3C) apparently was similar to that of control CE548 (Fig. 3B). This is different from the results of *sbp-1* expression, which

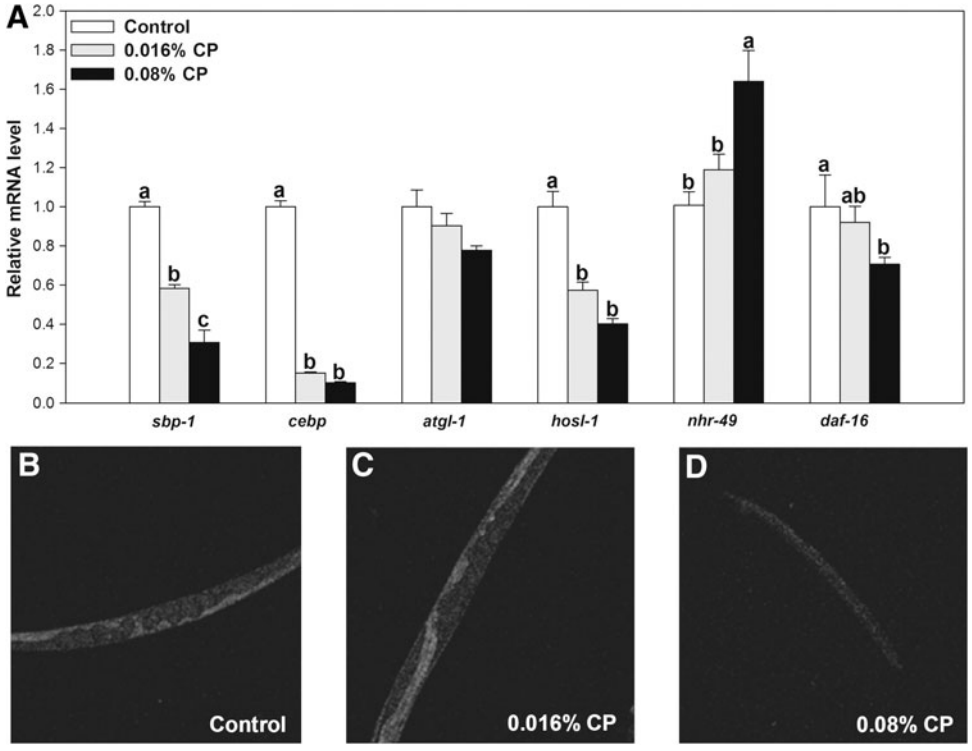

FIG. 3. Effects of the CP on *sbp-1*, *cebp*, *atgl-1*, *hosl-1*, *nhr-49*, and *daf-16* gene expression levels in N2 *C. elegans* **(A)**. Green fluorescent protein-labeled *sbp-1* expression level in CE548 strain: **(B)** control; **(C)** 0.016% CP; and **(D)** 0.08% CP. These images were magnified by 200 times. Cranberry treatment started from the L1 stage and *C. elegans* samples were harvested for analysis after a 3-day treatment. Numbers are means ± standard errors ($n = 3$–4). Means with different letters are significantly different at $P < .05$.

were significantly reduced in wild-type *C. elegans* after 0.016% CP treatment (Fig. 3A). Since we have not quantified the total fluorescence from these strains, this may not represent the overall effects. Alternatively, this discrepancy might be due to the fact that the CE548 strain is less sensitive to CP treatment than the wild-type strain or the GFP expression method may not be as sensitive as the reverse transcriptase–polymerase chain reaction method.

We then determined other genes involved in lipogenesis, *cebp* (ortholog of C/EBP, required for fat storage[36]), *hosl-1*, and *atgl-1* (orthologs of hormone-sensitive lipase and adipose triglyceride lipase, respectively, key genes for lipolysis), since mutants for *cebp*, *hosl-1*, or *atgl-1* are not available currently. CP treatment had significantly inhibited the expression of *cebp* and *hosl-1*, but not *atgl-1* (Fig. 3A). These results also suggest that CP may inhibit adipogenesis in *C. elegans*.

DISCUSSION

In this study, we observed that CP dose-dependently reduced triacylglyceride content in wild-type *C. elegans* without affecting food intake, body size, or locomotive activity. The current results are consistent with others suggesting that CP reduces fat accumulation without adversely influencing other physiological functions.[37]

The fat reduction by cranberry treatment was completely attenuated by *sbp-1* deficiency, while in *nhr-49* mutant, the fat reduction effect of cranberry was partly attenuated. Thus, the *sbp-1* gene might play a more significant role in the CP's effect on fat reduction compared with *nhr-49*. Moreover, CP may inhibit fat accumulation by inhibiting *cebp.* These results are consistent with Kowalska *et al.*[7] where significant effects of CPs on C/EBPα (*cebp* ortholog) and SREBP (*sbp-1* ortholog) were reported in a 3T3-L1 adipocyte model. The current results further suggest that *sbp-1* may play a greater role compared with *nhr-49* on CP's effect on overall fat accumulation.

In addition to being involved in life span regulation, *daf-16* is reported to regulate fat accumulation in *C. elegans.*[38] However, no differences of triglyceride contents between wild-type and *daf-16* mutants were observed in the current study. Although we did not determine overall fat contents of wild-type and *daf-16* mutants at the same study, we observed the relatively small difference on overall fat contents between experiments (5% of difference between studies). This discrepancy might be, in part, due to different quantification methods used (Triglyceride kit measurement in the current study versus Nile Red O staining in previous). Alternatively, the current results suggest that *daf-16* may not contribute to overall TG accumulation. It is also necessary to point out that CP decreased gene expression of *daf-16* in the current study, which is different from a previous publication (0.2% CP in water).[37] Based on these observations, we can infer that *daf-16* is unlikely to contribute to CP's effect on fat accumulation; however, *daf-16* may play an important role in other functions of CP, such as its effect on life span. Therefore, additional studies are needed to clarify the role of *daf-16* in *C. elegans*.

Unlike Kowalska *et al.*[7] who reported increased lipolysis by CP in 3T3-L1 adipocytes, the current results with decreased expression of *hosl-1*, but not *atgl-1*, are inconsistent effects of CP on lipolysis. It is possible that there might be other mechanisms, such as post-translational regulation of these genes[39,40] or *sirt* (SIRT1 ortholog), responsible for the CP's fat reduction effect, which we have not explored in the current study.[41]

The CP (HI-PAC 4.0) used was reported to contain ~6.6% phenolic acids, 2.2% flavonoids, 0.6% anthocyanidins, 38% sugars, and 4.1% dietary fiber.[11,42] Several components in CPs were previously reported to have antiobesity functions. First, quercetin, one flavonoid found in CP, was found to inhibit adipogenesis by activation of AMPK genes and downregulating the expression of C/EBPα and SREBP-1.[34,43,44] The current results suggest that cranberry bioactive may influence C/EBPα and SREBP-1, but not AMPK.[45,46] This inconsistency may derive from differences of CPs used (pure quercetin vs. mixture of compounds in CP), doses of CPs used, or models used (such as potential different sensitivity to AMPKα between *C. elegans* and mammals).

Next, anthocyanins, another flavonoid found in CP, were also reported to have potential antiadipogenic effects.[47] Anthocyanins from purple corn reduced high-fat diet-induced weight gain, but also decreased white and brown adipose tissue weights significantly in mice.[47] These effects were further supported by the finding that purple corn reduced the mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis, as well as the SREBP-1 mRNA level in white adipose tissue.[47]

Pterostilbene, a stilbenoid found in cranberry, was reported to activate PPAR-α, an *nhr-49* homolog. NHR-49 serves a similar function to mammalian PPARα, which modulates pathways controlling the increased fatty acid β-oxidation and decreased triglyceride content in the liver and lowers plasma lipid levels when fed to hamsters.[48] Consistently, it has also been reported that deletion mutation of *nhr-49* causes a higher fat content in *C. elegans.*[35] It is possible that pterostilbene in CP may have contributed to the current results of CP and *nhr-49*, which needs to be further confirmed.

Dietary fiber, which exists in the CP, is also reported to reduce fat accumulation in *C. elegans*.[8] Since the CP used contained ~4.1% fiber, the final concentrations of fiber in current experiments were 0.0007% and 0.003%, respectively. Thus, we speculate that fiber in the CP used has a minimal effect in *C. elegans* on fat accumulation; however, we cannot rule out the possibility that fiber present in the CP used played a role in the overall effects of CP in the current study. In addition to fiber, there are about 38% sugars, mainly fructose and glucose (per Naturex; South Hackensack, NJ, USA), in the CP, resulting in final sugar concentrations of 0.006% and 0.03% in the treatment used, respectively. Previously, we determined the role of glucose on fat accumulation in *C. elegans* and found that 0.5% glucose supplemented with *C. elegans* had no effect on its triacylglyceride content, while 1% glucose increased

triacylglyceride content in *C. elegans* (data not shown). Thus, it is unlikely that sugars in the CP used have a significant effect on *C. elegans* fat accumulation.

Compared with rodent models, *C. elegans* have unique advantages. The short life span, rapid reproduction cycles, and large brood sizes, as well as various available mutants, allow for a variety of cellular, molecular, genetic, and behavioral analyses. Core fat metabolic pathways are conserved in *C. elegans* as many fat regulatory pathways found in *C. elegans* play similar roles in mammals.[50] Thus, *C. elegans* is a great *in vivo* model for obesity research.

In conclusion, the current results conclude that the reduction of fat accumulation by CP is dependent on *sbp-1* and *nhr-49*, while independent of *aak-2* and *tub-1*. CP might also regulate adipogenesis in *C. elegans*, as shown by the decreased *cebp* gene expression after CP treatment. The current findings may provide evidence to promote the application of cranberries as natural products in the prevention and treatment of obesity.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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