RESEARCH ARTICLE

# Consumption of plant sterols reduces plasma and hepatic triglycerides and modulates the expression of lipid regulatory genes and *de novo* lipogenesis in C57BL/6J mice

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To investigate emerging clinical data suggesting a triglyceride (TAG)-lowering response to plant sterol (PS) therapy, we characterized changes in TAG metabolism in 16 C57BL/6J mice fed a basal control diet (CON) or the CON diet supplemented with 2% PS for 6 wk. PS consumption reduced ($p<0.05$) plasma (−28%) and hepatic (−30%) TAG concentrations compared with CON mice. PS consumption increased ($p<0.05$) hepatic lipogenic gene expression (sterol-regulatory-element-binding protein 1c, 2.4-fold of CON; fatty acid synthase, 6.5-fold of CON) and *de novo* lipogenesis ($4.51\pm0.72$ *versus* $2.82\pm0.61$%/day) compared with CON. PS consumption increased ($p<0.05$) fecal palmitate and stearate excretion and reduced body weight gain compared with CON mice. Although no change in the transcription of intestinal fatty acid absorptive genes was observed, peroxisome proliferator-activated receptor α mRNA was reduced ($p<0.05$, 2.0-fold of CON) in the PS-fed mice. In conclusion, PS-fed C57BL/6J mice showed pronounced reductions in plasma and hepatic TAG concentrations despite increases in hepatic lipogenic gene expression and *de novo* lipogenesis. Interference with intestinal fatty acid/TAG metabolism as suggested by increased fecal fatty acid loss and reduced weight gain may be associated with the TAG-lowering response to PS consumption.

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## 1 Introduction

Plant sterols (PS) have a long-standing history as effective dietary cholesterol-lowering agents [1]. Recent meta-analyses suggest that consumption of PS lowers circulating cholesterol concentrations by 10% and may reduce heart disease risk [2, 3]. Although PS are not generally recognized as effective triglyceride (TAG)-lowering agents, assessment of previously published clinical PS interventions reveals a variable, yet often-overlooked response of plasma TAG concentrations [4–8]. Furthermore, a recent meta-analysis supports this potentially significant and emerging TAG-lowering response to PS therapy [9], although previously meta-analyses have failed to establish this relationship [2, 10].

Similarly, PS consumption has been associated with modulations in TAG metabolism in both hamster [11, 12] and C57BL/6J mouse models [13–15]. Therefore, we hypothesized that the C57BL/6J mouse model may be beneficial in exploring potential mechanisms associated with TAG reductions in response to PS consumption. The objectives of the present study were to examine *de novo* lipogenesis and differential mRNA and protein expression patterns of intestinal and hepatic regulators of fatty acid/TAG metabolism in

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**Abbreviations: ASBT,** apical sodium-dependent bile salt transporter; **Dgat,** diacylglycerol acyltransferase; **FAS,** fatty acid synthase; **FABP2,** fatty acid-binding protein 2; **HDL-C,** high-density lipoprotein cholesterol; **PPARα,** peroxisome proliferator-activated receptor alpha; **PS,** plant sterol; **TAG,** triglyceride; **Scd1,** stearoyl-CoA desaturase; **SREBP1c,** sterol-regulatory-element-binding transcription factor 1

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response to PS consumption in C57BL/6J mice. Intestinal and hepatic molecular targets included: diacylglycerol acyltransferase (Dgat), peroxisome proliferator-activated receptor alpha (PPARα), sterol-regulatory-element-binding transcription factor 1 (SREBP1c), apical sodium-dependent bile salt transporter (ASBT), fatty acid synthase (FAS), and stearoyl-CoA desaturase (Scd1).

## 2 Materials and methods

### 2.1 Animals, diet, and experimental design

Sixteen male C57BL/6J mice were acquired from Charles Rivers and brought to the Animal Model Research Facility at the Richardson Centre for Functional Foods and Nutraceuticals at the University of Manitoba. Mice were pair-housed in plastic cages with shavings in a temperature-controlled room (20°C) with a 12-h light/dark cycle. Mice had free access to water and were acclimatized to the facility and research staff for 1 wk on normal rodent chow prior to commencement of the experiment. At the initiation of the experiment, mice were fed a control diet (AIN 76A, Western diet) or the control diet supplemented with a 2% PS mix (Reducol, Forbes Meditech) for 6 wk (Table 1). This diet was chosen as it is representative of a typical ''Western diet''. The study was designed as a randomized complete block design with four animals per block (two control and two PS mice) and a total of four blocks. All procedures were reviewed and approved by the Animal Care Committee at the University of Manitoba (protocol number F06-013/1/2). The animals used in this experiment were cared for in accordance with the guidelines established by Canadian Council on Animal Care [16].

### 2.2 Sample collection and processing

Following the 6-wk feeding, mice were anesthetized with isoflurane for blood and tissue collection. Fasting blood was collected by cardiac puncture into EDTA tubes. Plasma was separated from whole blood by centrifugation at 1000 × *g* for 10 min and stored in aliquots at −20°C. Livers were quickly removed, rinsed in chilled saline (154 mM containing 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4), flash frozen in liquid nitrogen, and stored at −80°C.

### 2.3 Plasma lipid analyses

Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C), non-HDL cholesterol, and TAG were determined by automated enzymatic methods on a Vitros 350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada). Liver TAG were determined in a similar manner following an initial lipid extraction [17].

**Table 1.** Formulation of control and PS supplemented diets for C57BL/6J mice

| Item[a)] | Control diet | PS diet |
|---|---|---|
| Sucrose | 34.13 | 34.13 |
| Milk fat | 19.96 | 19.96 |
| Casein | 19.47 | 19.47 |
| Maltodextrin | 9.98 | 9.98 |
| PS mix[b)] | – | 2.0 |
| Corn starch | 4.99 | 2.99 |
| Powdered cellulose | 4.99 | 4.99 |
| AIN76 – mineral mix | 3.49 | 3.49 |
| AIN76 – vitamin mix | 0.99 | 0.99 |
| Corn oil | 0.99 | 0.99 |
| Calcium carbonate | 0.39 | 0.39 |
| Choline bitartrate | 0.19 | 0.19 |
| DL-methionine | 0.29 | 0.29 |
| Cholesterol | 0.14 | 0.14 |
| Ethoxyquin | 0.004 | 0.004 |
| Total | 100 | 100 |

a) All ingredients (with exception of the PS mix) were purchased from Dyets, Bethlehem, PA, USA.
b) Forbes Meditech (composition (w/w, as is basis): total sterols, 98%; campestanol, 7%; stigmasterol, <1%; sitosterol, 71%; sitostanol, 15%).

### 2.4 Liver sterol and fecal fatty acid concentrations

Liver sterol and fecal fatty acid concentrations were determined by gas–liquid chromatography according to previously established procedures [18].

### 2.5 RNA preparation and real-time RT-PCR

Total RNA was isolated from whole liver tissue using TRIzol reagent (Invitrogen Canada, Burlington, ON, Canada). RNA concentration and integrity were determined with spectrophotometry (260 nm) and agarose gel electrophoresis, respectively. RNA preparation and real-time RT-PCR was conducted using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Mississauga, ON, Canada) on an Applied Biosystems 7500 system according to previously established protocols [19]. Sequences of sense and antisense primers for target and housekeeping genes were based on previously published reports for fatty acid translocase (CD36), fatty acid-binding protein 2 (FABP2) [20], Dgat [21], SREBP1c, FAS, PPARα [22], ASBT [23], Scd1, and β-actin [24].

### 2.6 Immunoblot analysis of hepatic-regulatory proteins

Immunoblots were prepared as previously described [19]. Nuclear extracts for immunoblot analyses of SREBP1c (antibody SC-13551, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and PPARα (antibody SC-9000, Santa Cruz

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Biotechnology) were separated using CelLytic™ NuCLEAR™ extraction kit (Sigma, Saint Louis, MO, USA), respectively.

### 2.7 Stable isotope analyses

Mice were given an intraperitoneal injection of deuterium (100 μL) 2 h prior to euthanization. Lipogenesis rates (%/day) were quantified using the uptake rate of deuterium from body water into the newly synthesized VLDL-palmitate extracts over 2 h at the end of the feeding experiment [25, 26]. Plasma VLDL was isolated by ultracentrifugation and lipids were extracted from the VLDL fraction by previously established methods [17]. Deuterium enrichment of VLDL palmitate was quantified using online gas chromatography/combustion/isotope ratio mass spectrometry approach (Agilent 6890N chromatograph interfaced with a Finnigan Delta V Pulse isotope ratio mass spectrometer, Bremen, Germany). Isotope abundance, expressed in delta (δ) per mil (‰), was calculated in plasma VLDL palmitate and plasma water (precursor pool) using $H_2$ as a reference gas and further corrected against the international reference, standard mean ocean water. Fractional synthesis rates (FSR) were calculated with the following equation:

$$\text{FSR (per day)} = \Delta\text{TGFA (‰)}/\Delta\text{Plasma (‰)} \times 0.477$$

where ΔTGFA is the change in deuterium enrichment in VLDL palmitate; ΔPlasma is the change in the deuterium enrichment of the precursor plasma water; and 0.477 is derived from 0.87g-atom $^3H$ per g-atom carbon incorporated into adipose tissue fatty acids and a correction factor to account for the glycerol moiety as previously described [27].

### 2.8 Statistical analyses

Data were analyzed with a general linear model according to a randomized block design using experimental block as a fixed factor [28]. Data were analyzed with SPSS 16 for Mac (SPSS, Chicago, IL, USA). Data are presented as mean ± SEM. All the results are the mean values from eight animals ($n = 8$). Differences from the control were considered significant at $p \leqslant 0.05$.

## 3 Results

### 3.1 Feed intake and body weight

The animals remained visibly healthy throughout the duration of the study. Percent change in body weight was different ($p < 0.05$) between the two groups with PS-fed mice gaining less weight than control mice after the 6-wk feeding period (Table 2).

### 3.2 Plasma biochemistry

Consumption of PS did not reduce ($p > 0.05$) plasma total cholesterol, HDL-cholesterol, or non-HDL cholesterol concentrations in comparison with the control group (Table 2). However, compared with the control group, PS consumption reduced ($p < 0.05$) plasma TAG by 28% (0.91 ± 0.06 *versus* 0.68 ± 0.02 mmol/L) (Table 2).

### 3.3 Liver sterol, TAG and fecal fatty acid concentrations

Hepatic β-sitosterol and campesterol concentrations were higher ($p < 0.05$) in the PS-supplemented group compared with the control group (Table 3). Hepatic TAG concentrations were reduced ($p < 0.05$) in the PS-fed mice in comparison with the control group (27.57 ± 2.16 *versus* 37.18 ± 3.06 mmol/g tissue) (Table 3). Compared with controls, fecal palmitate and stearate excretion (mg/day) were increased ($p < 0.05$) in the PS-supplemented mice. Fecal oleate excretion did not differ between the PS and control groups (Fig. 1).

**Table 2.** Feed intake, body weight, and endpoint plasma lipid parameters in C57BL/6J mice fed control and PS supplemented diets[a)]

| Item | Control diet | PS diet | *p*-value |
|---|---|---|---|
| Feed intake (g/day) | 2.89 ± 0.11 | 2.95 ± 0.09 | 0.69 |
| *Body weight (g)* | | | |
| Start weight | 21.14 ± 0.34 | 22.10 ± 0.30 | 0.25 |
| Final weight | 31.17 ± 0.55 | 29.77 ± 0.50 | 0.12 |
| % Change from start | 40.75 ± 2.36 | 33.10 ± 3.42 | 0.01 |
| *Plasma parameters (mmol/L)* | | | |
| Total cholesterol | 3.63 ± 0.05 | 3.42 ± 0.09 | 0.15 |
| HDL-cholesterol | 3.05 ± 0.07 | 2.83 ± 0.08 | 0.09 |
| Non-HDL cholesterol | 0.54 ± 0.04 | 0.67 ± 0.05 | 0.08 |
| Triglycerides | 0.91 ± 0.06 | 0.68 ± 0.02 | 0.00 |

a) Values represent mean ± SEM ($n = 8$).

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**Table 3.** Hepatic sterol and triglyceride concentration in C57BL/6J mice fed control and PS supplemented diets[a)]

| Item | Control diet | PS diet | *p*-value |
|---|---|---|---|
| Hepatic cholesterol (μmol/g tissue) | | | |
| Total cholesterol | 8.62±0.46 | 1.81±0.37 | 0.00 |
| Free cholesterol | 0.59±0.14 | 0.49±0.11 | 0.62 |
| Esterified cholesterol | 8.03±0.52 | 1.32±0.41 | 0.00 |
| PS (μmole/g cholesterol) | | | |
| β-sitosterol | 2.23±0.49 | 18.07±3.56 | 0.02 |
| Campesterol | 2.62±0.65 | 24.22±3.46 | 0.00 |
| Hepatic TAG (mmol/g tissue) | 37.18±3.06 | 27.57±2.16 | 0.02 |

a) Values represent mean±SEM ($n = 8$).

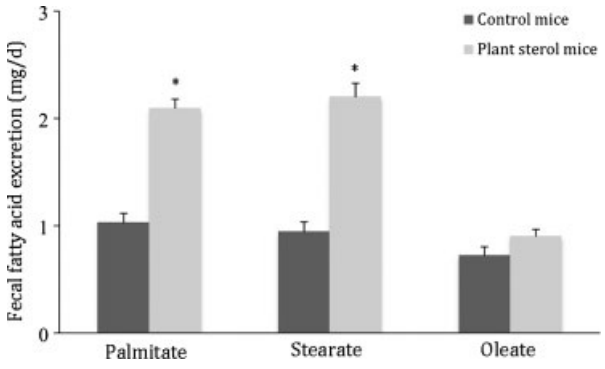


**Figure 1.** Fecal fatty acid excretion (mg/day) in C57BL/6J mice fed the control and PS supplemented diets. Values represent mean±SEM ($n = 8$). *Significantly different from the control group, $p < 0.05$.

### 3.4 Intestinal and hepatic mRNA expression

PS consumption did not affect ($p > 0.05$) the mRNA expression of intestinal CD36, FABP2, Dgat, ASBT, or SREBP1c in comparison with control mice. However, mRNA expression of intestinal PPARα was decreased ($p = 0.001$, 2.0-fold of control) in PS-fed mice compared with control mice (Fig. 2A).

PS consumption increased hepatic mRNA expression of FAS ($p = 0.01$, 6.5-fold of control) and tended to increase SREBP1c ($p = 0.07$, 2.3-fold of control) in comparison with the control group. Alternatively, PS consumption reduced mRNA expression of hepatic Scd1 ($p = 0.02$, 1.7-fold of control) but did not affect ($p = 0.29$) mRNA expressionof hepatic PPARα in comparison with the control group (Fig. 2B).

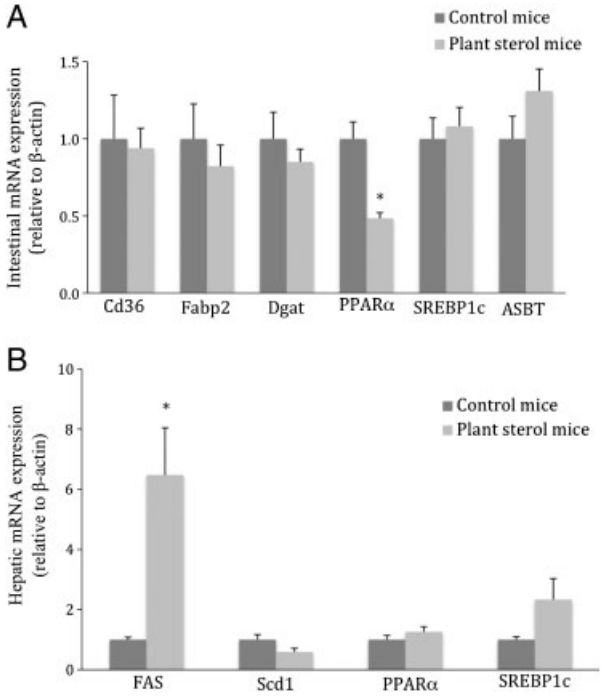


**Figure 2.** Intestinal and hepatic mRNA expression in C57BL/6J mice fed the control and plant sterol supplemented diets. All data was normalized to β-actin. Values represent means ± SEM (n = 8); *, significantly different from control group, $P < 0.05$. (2A) Intestinal mRNA expression including: Cd36, fatty acid translocase; Fabp2, fatty acid binding protein 2; Dgat, diacylglycerol acyltransferase, PPARα, peroxisome proliferator activated receptor alpha, SREBP1c, sterol regulatory element binding transcription factor 1; ASBT, apical sodium dependent bile salt transporter. (2B) Hepatic mRNA expression including: FAS, fatty acid synthase; Scd1, stearoyl-CoA desaturase; PPARα and SREBP1c.

### 3.5 Hepatic-regulatory protein expression

PS consumption did not affect ($p > 0.05$) hepatic SREBP1c and PPARα protein expression in comparison with the control mice (Fig. 3).

### 3.6 *De novo* lipogenesis

PS consumption increased *de novo* lipogenesis by 23% compared with the control group (4.51±0.72 *versus* 2.82±0.61 %/day, Fig. 4).

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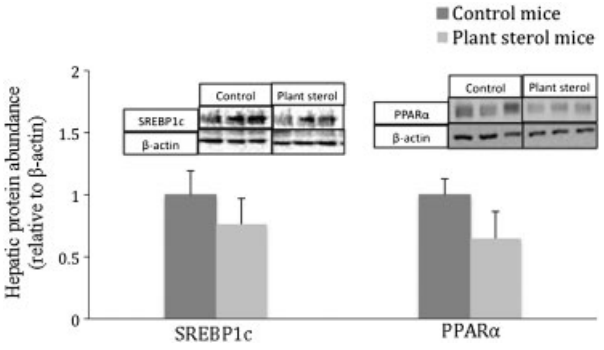


**Figure 3.** Hepatic protein abundance of SREBP1c and PPARα in C57BL/6J mice fed the control and PS supplemented diets. Values represent mean ± SEM ($n = 8$). Blots were run in triplicate.

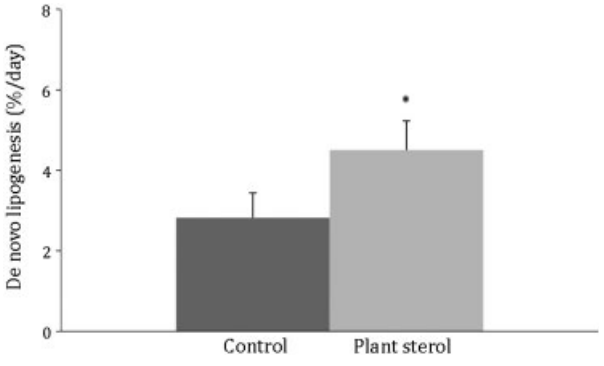


**Figure 4.** Lipogenesis (%/day) rates in C57BL/6J mice fed the control and PS supplemented diets. Values represent mean ± SEM ($n = 8$). *Significantly different from the control group, $p < 0.05$.

## 4 Discussion

Two principle findings are evident from this study. First, in support of recent clinical investigations suggesting an emerging effect of PS consumption on plasma TAG concentrations [8, 29], considerable reductions in plasma (−28%) and hepatic (−30%) TAG concentrations were observed in the PS-fed mice compared with the control mice. Similar to previous investigations in C57BL/6J mice, plasma cholesterol concentrations were unaffected by PS consumption [13, 15, 30]. Secondly, our data suggest that the decrease in systemic TAG concentrations in response to PS consumption are associated with increased fecal saturated fatty acid loss and may be partially compensated for with an increase in *de novo* lipogenesis.

The observed increase in *de novo* lipogenesis in the PS-supplemented mice was likely the result of an upregulation in hepatic SREBP1c and FAS mRNA expression compared with the control mice. Contrary to our findings, campestanone, a 3-oxo derivative of campesterol, has been reported to reduce serum and liver TAG concentrations by suppressing hepatic SREBP1c expression and reducing the mRNA expression and activities of hepatic lipogenic enzymes in rats [31]. Therefore, the increase in lipogenic gene expression and lipogenesis in the PS-fed mice in the current study may have been compensatory responses associated with interference with intestinal fatty acid absorption, as suggested by the observed increase in fecal palmitate and stearate excretion. Furthermore, the reduced weight gain observed in the PS-fed mice compared with control mice lend support to the suggestion of reduced fat absorption in response to PS consumption. Although our results agree with an earlier report suggesting an increase in fecal lipid excretion in response to PS consumption [32], previous studies have observed no change in intestinal lipid absorption in apoE$^{-/-}$ mice fed 2% PS [15] and in male rats fed 0.5% campestanone [31]. However, if intestinal lipid absorption was indeed reduced in response to PS feeding in the current study, this reduction did not involve alterations in the mRNA expression of genes that regulate intestinal fatty acid uptake (CD36), intracellular fatty acid translocation (FABP2), or TAG synthesis (Dgat). Future studies examining the association between PS consumption and TAG metabolism should include a direct measure of intestinal fatty acid absorption.

Although the specific mechanism(s) whereby PS may have affected the intestinal digestion and absorption of dietary TAG is unknown, a possible explanation may involve modulation of enterohepatic bile acid metabolism in the PS-fed mice. PS consumption has been reported to reduce bile acid synthesis [33, 34], a scenario that would likely reduce the total bile acid pool size and ultimately impair intestinal lipid absorption. Furthermore, we observed an unexpected reduction in intestinal PPARα mRNA expression in response to PS consumption compared with control mice. PPARα is highly expressed within intestinal enterocytes and has recently been identified as a key mediator of intestinal bile acid transport and intracellular translocation through ASBT and ileal bile acid-binding protein, respectively [35, 36]. However, we failed to observe modulation in the mRNA expression of ASBT, the primary intestinal bile salt transporter whose expression is closely linked to plasma TAG concentrations [35]. It is also possible that reduction in hepatic cholesterol concentration following PS consumption may interfere with hepatic VLDL secretion and ultimately reduce circulating TAG concentrations. Accordingly, Ho *et al.* have suggested that exposure of HepG2 cells to PS limits cellular lipid availability and reduces apoB100 secretion [37].

Multiple clinical trials have reported variable reductions in circulating TAG concentrations following PS consumption in the range of 6–27% [4–9]. Furthermore, although a recent meta-analysis reports reductions in plasma TAG in response to PS consumption [9], previous meta-analyses have failed to establish this relationship [2, 10]. What factors contribute to this seemingly inconsistent and variable TAG-lowering response to PS? Two recent studies suggest that this TAG-lowering response to PS therapy may be more pronounced in populations with high baseline plasma TAG concentrations [8, 9]. In agreement with these findings, we

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recently reported a 9% reduction in plasma TAG in hypertriglycerolemic subjects, consuming PS-enriched soymilk [38]. Similarly, it is possible that PS-induced TAG reductions may be more readily detected in animal models with higher plasma TAG concentrations (mice) in comparison with animal models with relatively lower circulating TAG concentrations (rats and guinea pigs) [39]. Alternatively, a genetic component may underscore the variable TAG-lowering responses to PS therapy. A clinical study by Sanchez-Muniz *et al.* recently reported TAG reductions in apoE2 but not in apoE3 or E4 subjects following PS intervention in hypercholesterolemic adults [29].

Although a 2% PS supplementation level is typical of most animal model experiments investigating the molecular effects of PS, it is important to recognize that this level of PS intake is higher than what is used in human clinical investigations. As the effects of high PS consumption on intestinal fat metabolism are unknown, further investigation into how the recommended daily intake of PS modulates the kinetics of intestinal fat absorption in humans is warranted to clarify the clinical utility of PS in reducing circulating TAG concentrations.

In summary, PS-fed C57BL/6J mice display pronounced reductions in plasma and hepatic TAG concentrations despite compensatory increases in hepatic lipogenic gene expression and *de novo* lipogenesis. This TAG-lowering response in C57BL/6J mice to PS consumption is likely associated with an interference with intestinal fatty acid/TAG metabolism as suggested by increased fecal fatty acid loss.

*T. C. R. designed and conducted the research, analyzed the data, and wrote the initial draft manuscript. S. V. H. contributed to stable isotope analyses and revised the draft manuscript. P. J. H. J. assisted in project conception and design and revised the draft manuscript. All the authors read and approved the final manuscript. Funded by NSERC.*

*The authors have declared no conflicts of interest.*

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