

Hypocholesterolemic effect of *Nostoc commune* var. *sphaeroides* Kützinger, an edible blue-green alga

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Abstract

Background Intake of an edible blue-green alga *Nostoc commune* var. *sphaeroides* Kützinger (*N. Commune*) has been shown to lower plasma total cholesterol concentration, but the mechanisms behind the hypocholesterolemic effect have not been elucidated.

Aim of the study To elucidate the mechanisms underlying the cholesterol-lowering effect of *N. commune* in mice.

Methods Male C57BL/6J mice were fed the AIN-93 M diet supplemented with 0 or 5% (wt/wt) dried *N. Commune* for 4 weeks. Lipid levels in the plasma and liver, intestinal cholesterol absorption and fecal sterol excretion were measured. Expression of hepatic and intestinal genes involved in cholesterol metabolism was evaluated by quantitative realtime PCR.

Results *N. commune* supplementation significantly reduced total plasma cholesterol and triglyceride concentrations by ~ 20% compared to controls. Intestinal cholesterol absorption was significantly decreased, while fecal neutral sterol output was significantly increased in *N. commune*-fed mice. mRNA levels of the cholesterol transporters such as Niemann Pick C1 Like 1, scavenger receptor class B type 1, ATP-binding cassette transporters G5 and A1 in small intestine were not significantly different between two

groups. Hepatic lipid contents including total cholesterol, triglyceride and free cholesterol in *N. commune*-fed mice were not significantly altered. However, the expression of cholesterol modulating genes including sterol regulatory element binding protein-2 and 3-hydroxy-3-methylglutaryl coenzyme A reductase were significantly increased in mice fed *N. commune*.

Conclusions *N. commune* supplementation exerted a hypocholesterolemic effect in mice, largely in part, by reducing intestinal cholesterol absorption and promoting fecal neutral sterol excretion.

Keywords Blue-green algae ·
Nostoc commune var. *sphaeroides* Kützinger ·
Cholesterol metabolism · Cholesterol absorption

Abbreviations

<i>N. commune</i>	<i>Nostoc commune</i> var. <i>sphaeroides</i> Kützinger
HMGR	3-Hydroxy-3-methylglutaryl CoA reductase
LDLR	Low-density lipoprotein receptor
FAS	Fatty acid synthase
CYP7A1	Cholesterol-7 α -hydroxylase
SREBP-1c	Sterol regulatory element binding protein-1c
SREBP-2	Sterol regulatory element binding protein-2
SR-B1	Scavenger receptor class B, type 1
NPC1L1	Niemann Pick C1-Like 1
ABCA1	ATP-binding cassette transporter A1
ABCG5	ATP-binding cassette transporter G5
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

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Introduction

Of all cardiovascular diseases, coronary heart disease (CHD) is the leading cause of death for both men and

women worldwide. In particular, a positive correlation between death from CHD and total plasma cholesterol (TPC) level exceeding 200 mg/dl prompted the National Cholesterol Education Program (NCEP) to recommend TPC level to remain at 200 mg/dl or lower for the general population [1]. Statin drugs are widely prescribed to lower TPC concentration. However, they can cause adverse effects such as liver dysfunction or myalgia [17, 27]. Lowering TPC through non-drug strategies, such as consuming foods containing bioactive compounds with hypocholesterolemic effects, would therefore be a desirable alternative. Many herbal and functional food supplements have been used for primary prevention of CHD. Soluble fibers from barley, beans, oat bran and psyllium reduced TPC concentration when consumed by healthy hyperlipidemic patients on a low-fat diet [21].

Recently, hypocholesterolemic and hypotriglyceridemic properties have been reported with intake of several strains of blue-green algae [20, 24, 36, 38]. Edible blue-green algae, such as *Spirulina platensis* and *Aphanizomenon flos-aquae*, are currently marketed as dietary supplements with various health claims for immune function, inflammation and heart disease. *Spirulina platensis* is harvested from controlled ponds around the world. In contrast, most of *Aphanizomenon flos-aquae* products are harvested from the Upper Klamath Lake in southern Oregon in the United States where it grows naturally. However, concerns over naturally grown blue-green algae have been raised because they are easily contaminated with hepatotoxin-producing *Microcystis aeruginosa*, as well as with heavy metals [14].

Nostoc commune var. *sphaeroides* Kützinger (*N. commune*), an edible blue-green alga, is a cyanobacterium that has been grown and cultivated for medicinal uses for centuries. *N. commune* is the only edible species of *Nostoc* listed in the Compendium of Materia Medica, a Chinese medicinal work from 1596. *N. commune* is currently consumed where it is grown naturally in spherical macrocolonies in both terrestrial and aquatic habitats. It has been historically suggested that *N. commune* can treat a variety of medical conditions, including inflammation, night blindness, burns, anxiety, and chronic fatigue. Recent research has indicated that *N. commune* contains cryptophycin, a compound that inhibits cancer cell growth [41], as well as anti-viral compounds [12, 23]. A cholesterol-lowering effect of *N. commune* was reported in rats fed a high cholesterol diet, the effects being attributed to its high fiber content [20]. Although studies have shown *N. commune* to confer health benefits, the investigation into the mechanisms behind the biological effects have been limited. We have previously shown that the lipid extract from *N. commune* exhibits potential cholesterol-lowering properties due to its inhibitory effects on the maturation of sterol regulatory element binding protein-2 (SREBP-2) and subsequent reduction of

cholesterol synthesis in HepG2 cells, a human hepatoma cell line [33]. The purposes of this study were to determine a hypocholesterolemic effect of cultivated *N. commune* in vivo and to elucidate a mechanism for such an effect. We show here that consumption of *N. commune* lowered TPC concentration by reducing intestinal cholesterol absorption.

Materials and methods

Animal feeding and care

Twenty-four C57BL/6J adult male mice (Jackson Laboratory) ranging in age from 11 to 24 weeks were randomly assigned to control or *N. commune* supplement group and housed individually in a polycarbonate cage under a 12-h light/dark cycle. Initial body weight of mice were not significantly different between two groups (control, 27.9 g; *N. commune*, 27.8 g; $P = 0.96$). Fresh *N. commune* obtained from Algean Corporation (Winston-Salem, NC, USA) was lyophilized and stored at -80°C until it was finely ground and incorporated with the remaining diet ingredients. AIN-93 M diet [34, 35] was supplemented with 5% dried *N. commune* (wt/wt) at the expense of casein, cornstarch and fiber on the basis of chemical composition of the alga (55.2% protein, 30% carbohydrate except fiber, 8.5% fiber, 6% fat) as determined by Southern Testing and Research Laboratories (Wilson, NC, USA; Table 1). Mice were fed an AIN-93 M control diet or *N. commune* supplemented diet for the 4-week experimental period with free access to food and water. Body weight and food intake were recorded weekly. At the end of the 4 weeks, mice were fasted for 4 h, anesthetized with ketamine HCl (50 mg/kg)/xylazine (10 mg/kg) and subsequently

Table 1 AIN-93 M diet supplemented with 5% dry *N. commune* (wt/wt)

Ingredient	Control, g/kg diet	5% <i>N. commune</i> , g/kg diet
Cornstarch	465.692	450.192
Casein	140.000	110.000
Dextrinized cornstarch	155.000	155.000
Sucrose	100.000	100.000
Soybean oil	40.000	40.000
Fiber	50.000	45.500
Mineral mix	35.000	35.000
Vitamin mix	10.000	10.000
L-Cystine	1.800	1.800
Choline bitartrate	2.500	2.500
Tert-butylhydroquinone	0.008	0.008
Dry <i>N. commune</i>	0.000	50.000
Total	1000.000	1000.000

euthanized by cardiac puncture and cervical dislocation. Blood sample collected by cardiac puncture was put into a 2 ml tube containing 3.6 mg EDTA (BD Vacutainer). Blood was centrifuged for 20 min at $5,000 \times g$ at 4 °C to remove red blood cells and plasma was stored at −80 °C. Liver and intestine were immediately frozen in liquid nitrogen and stored at −80 °C. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska-Lincoln.

N. commune safety assessment

Plasma samples were analyzed for concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) at Kansas State Veterinary Diagnostic Laboratory (Manhattan, KS, USA).

Plasma and liver lipids

Lipid from liver samples was extracted using the method of Folch et al. [13] and solubilized in Triton X-100 as previously described [6]. Liver and plasma lipids were determined using reagents for total cholesterol (Roche Diagnostics), triglycerides (Roche Diagnostics), free cholesterol (Free Cholesterol E, Wako Chemicals) and phospholipid (Phospholipid C, Wako chemicals) by enzymatic analysis [5]. Liver esterified cholesterol was calculated as the difference between total and free cholesterol.

Gene expression analysis

Approximately 1.0 g of liver or proximal intestinal sample was homogenized (Tissue Tearor, Biospec Products) in 1 ml of TRIzol reagent (Invitrogen) for total RNA isolation following manufacturer's protocol. Reverse transcription for cDNA synthesis and quantitative realtime PCR analysis were performed as previously described [32, 33]. Primers were designed according to GenBank database using the Primer Express software (Applied Biosystems). The following primers were used for realtime PCR analysis: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), forward (5'-CCCAGTTGTGCGTCTTCCA-3'), reverse (5'-TTCGAGCCAGGCTTTCACTT-3'); low-density lipoprotein receptor (LDLR), forward (5'-ACTGGGTTGACTC CAACTTCAC-3'), reverse (5'-GGTTGCCCCCGTTGACA-3'); fatty acid synthase (FAS), forward (5'-CGCTC GGCATGGCTATCT-3'), reverse (5'-CTCGTTGAAGA ACGCATCCA-3'); cholesterol 7 α hydroxylase (CYP7A1), forward (5'-AGCAACTAAACAACCTGCCAGTACTA-3'), reverse (5'-GTCCGGATATTCAAGGATGCA-3'); sterol regulatory element binding protein 2 (SREBP-2), forward (5'-TCCGCCTGTTCCGATGTAC-3'), reverse (5'-TGCA CATTACGCCAGGTTCA-3'); scavenger receptor class B

type 1 (SR-B1), forward (5'-GATGTGGGCACCCTTCAT G-3'), reverse (5'-CCGGGCTGAAGAATTCCA-3'); Niemann Pick C1 Like 1 (NPC1L1), forward (5'-CGTCTGTC CCCGCCTATACA-3'), reverse (5'-CTAATGACACCA GCTGCTTGGT-3'); ATP-binding cassette transporter A1 (ABCA1), forward (5'-CGTTTCCGGAAGTGTCTTA-3'), reverse (5'-GCTAGAGATGACAAGGAGGATGGA-3'); ATP binding cassette transporter G5 (ABCG5), forward (5'-CGTGCGGACCAAATGA-3'), reverse (5'-GCTCG CCACTGGAAATTCC-3'); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward (5'-GTGGTCTCTCT GACTTCAACA-3'), reverse (5'-GTTGCTGTAGCCAAA TTCGTTGT-3').

Cholesterol absorption efficiency

Fractional intestinal cholesterol absorption was measured using dual-isotope method [3]. In brief, at week 3, mice were orally administered 17 μ l of [3 H]- β -sitostanol and [14 C]-cholesterol (American Radiolabeled Chemicals) mixture in soybean oil for two consecutive days for an intake of 100,000 dpm of [3 H]- β -sitostanol and [14 C]-cholesterol each day. As our previous studies have shown that the majority of radiolabeled cholesterol is excreted at days 5–7, bedding and feces were collected at those days for analysis. Fecal sterol was extracted as previously described [39] and radioactivity was counted using a liquid scintillation counter (Packard). Cholesterol absorption efficiency was calculated as a % from the ratio of the two radiolabels in the dose and feces using the following equation: Percentage cholesterol absorption = $[(^{14}\text{C}^3\text{H in dose}) / (^{14}\text{C}^3\text{H in feces})] \times 100$.

Fecal sterols

Fecal neutral and plant sterols were determined by gas chromatography, and acidic sterols were determined by enzymatic analysis using Wako Bile Acid Kit (Wako Chemicals). The procedures were previously described [39].

Statistical analysis

Unpaired *T*-test was used to identify statistically significant differences between two groups with $P < 0.05$ considered significant by GraphPad InStat 3 (GraphPad Software, Inc.). Data are expressed as mean \pm SEM.

Results

Safety of *N. commune* consumption

Plasma levels of clinical indicators for tissue damage such as ALT, AST and BUN were measured to determine the

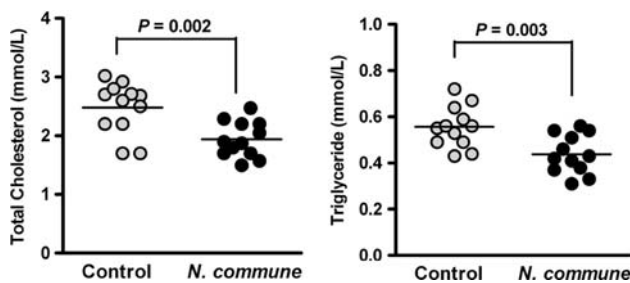


Fig. 1 Plasma total cholesterol and triglyceride concentrations were reduced in mice fed AIN-93 M diet supplemented with 5% dry *N. commune* (wt/wt). Plasma total cholesterol triglyceride concentrations were determined by enzymatic analysis. Data are expressed as mmol/l. Points represent data from individual mice and the horizontal lines denote the mean for each group, $n = 12$

safety of *N. commune* consumption. ALT is often used as an indicator of liver damage, and AST, while it is also indicative of liver damage, is used to assess damage to other body organs. BUN is a byproduct of protein catabolism and is an indicator of kidney function. Plasma concentrations of AST, ALT and BUN in *N. commune*-fed mice were not significantly different from those of control group (data not shown). No significant differences in food intake between two groups were seen throughout the 4-week experiment (control, 4.2 g/day; *N. commune*, 4.0 g/day; $P > 0.05$). Body weights of mice fed control or *N. commune* supplemented diet were not significantly different after 4-week dietary treatment (control, 30.3 g; *N. commune*, 29.2 g; $P = 0.38$). The data suggest that mice consuming 5% *N. commune* supplementation were healthy without manifesting signs of toxicity from algal supplementation when they were on *N. commune* supplemented diet for a month.

Effect of *N. commune* supplementation on plasma and liver lipids

Five percent *N. commune* supplementation for 4 weeks significantly decreased plasma concentrations of total cholesterol and triglycerides as compared to the control group (Fig. 1). Despite the reduction of plasma lipid concentrations, no significant differences were seen in hepatic total cholesterol, triglyceride and phospholipid between the two groups (Table 2). Free and esterified cholesterol

contents in the liver were not different in control and *N. commune*-fed mice (data not shown).

Alteration in hepatic gene expression by *N. commune* supplementation

To evaluate whether *N. commune* supplementation altered the expression of genes involved in cholesterol metabolism and transport in the liver, realtime PCR analysis was conducted utilizing GAPDH as a reference gene. A significant increase in mRNA abundance of both SREBP-2 and HMGCR was observed with *N. commune* supplementation compared with control animals (Fig. 2). While LDLR mRNA was not significantly increased, a trend toward increase was seen in the *N. commune*-fed group ($P = 0.098$). In addition, a trend toward decrease in FAS ($P = 0.061$) was seen in mice fed *N. commune*. CYP7A1, the rate-limiting enzyme for bile acid biosynthesis, mRNA levels were significantly increased ($P = 0.004$) in *N. commune*-fed mice but its protein levels were not altered (data not shown).

Inhibition of cholesterol absorption by *N. commune*

Cholesterol absorption plays a major role in the regulation of whole body cholesterol homeostasis [16, 45]. To determine if the hypocholesterolemic effect of *N. commune* is at least partly attributed to altered intestinal cholesterol absorption, cholesterol absorption efficiency was assessed by a fecal dual-isotope ratio method. Cholesterol absorption efficiency in control mice was 80% while *N. commune* fed mice exhibited a significant reduction at 68% (Table 3), possibly accounting for the reduction in TPC concentration seen in these mice. The expression of the intestinal transporters known to be involved in cholesterol absorption such as NPC1L1, SR-BI, ABCG5 and ABCA1 were not significantly different between two groups (data not shown) [2, 19, 42].

Increased sterol excretion by *N. commune* supplementation

Fecal sterol output was determined to investigate if *N. commune* supplementation could alter excretion of cholesterol and bile acid. As expected based on reduced

Table 2 Liver weight and lipid contents in mice fed an AIN-93 M diet supplemented with 5% dry *N. commune* (wt/wt) for 4 weeks

	Liver weight, mg/g body wt	Total cholesterol, $\mu\text{mol/g}$ wet wt	Triglyceride, $\mu\text{mol/g}$ wet wt	Phospholipid, $\mu\text{mol/g}$ wet wt
Control	41.89 \pm 1.71	5.60 \pm 0.30	34.56 \pm 4.02	23.67 \pm 0.85
<i>N. commune</i>	36.64 \pm 0.88*	5.88 \pm 0.20	30.08 \pm 3.75	23.97 \pm 0.59

Values represent mean \pm SEM ($n = 12$)

* Significantly different from control ($P < 0.05$)

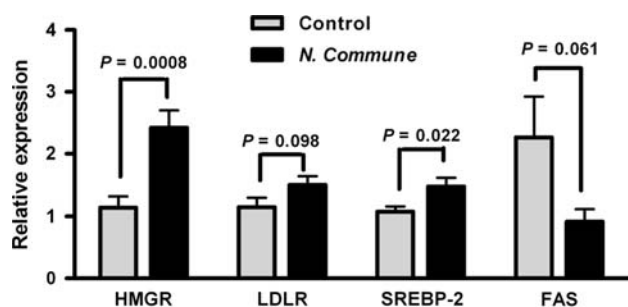


Fig. 2 mRNA abundance of hepatic genes were altered in mice fed an AIN-93 M diet supplemented with 5% dry *N. commune* (wt/wt). Total RNA was extracted from liver samples and realtime PCR analysis was performed to measure mRNA abundance. Data are expressed as fold change relative to control. Values are mean \pm SEM, $n = 12$

cholesterol absorption in *N. commune*-fed mice, cholesterol output was increased more than two-fold with *N. commune* supplementation compared with controls (Table 3). Plant sterols including campesterol, sitosterol and stigmasterol were excreted significantly more in *N. commune*-fed mice than controls. In contrast, output of acidic sterols, consisting of both bile acids and bile salts, remained unchanged between groups. Fecal output weight was also significantly increased in *N. commune*-fed mice (data not shown).

Discussion

Consumption of foods containing bioactive compounds as an alternative or a supplement to prescription drugs is gaining in popularity. In this study, we sought to unveil mechanisms for the cholesterol-lowering effects of *N. commune*. It was shown that intake of *N. commune* in rats reduced TPC concentration, which was attributed to the fiber component of the *N. commune* [20]. However, as *N. commune* used in that study was naturally grown, the macronutrient distribution would be unique to that harvest and would not be equated to the cultivated *N. commune* utilized in our study. Our results demonstrate that *N. commune*, at 5% of the diet by weight, reduces TPC concentration by reducing intestinal cholesterol absorption,

providing a mechanism for the cholesterol-lowering effect in *N. commune*-fed mice.

Intestinal cholesterol absorption is primarily protein-mediated. Several transporters have been suggested to directly or indirectly affect intestinal cholesterol absorption including NPC1L1, SR-B1, ABCA1 and ABCG5/G8. In particular, recent studies suggest a critical role of NPC1L1 in cholesterol and plant sterol absorption [2, 7, 37]. To determine if the reduction in cholesterol absorption by *N. commune* was due to a direct effect on these transporters, we measured mRNA abundance of intestinal cholesterol transporters such as NPC1L1, SR-B1, ABCG5 and ABCA1. There were no significant differences in the expression of the genes between control and *N. commune*-fed mice. Although we cannot rule out a possibility for the post-transcriptional regulation of the gene expression, the result suggests that the reduced cholesterol absorption by *N. commune* appears to be attributed to its effect within the intestinal lumen without a direct effect on the expression of the cholesterol transporter genes.

If cholesterol transporters do not directly play a role in the alteration of cholesterol absorption in *N. commune*-fed mice, an effect within the intestinal lumen may be occurring. Various dietary components have the capability to decrease cholesterol absorption including plant sterols [28, 31] and soluble fiber [22, 43]. Soluble polysaccharide from red microalga *Porphyridium* sp. and its biomass reduced serum cholesterol level in rats [10]. Even though the exact polysaccharide composition of *N. commune* is not known, compositional analysis of *N. commune* used in the current study revealed that it contained $\sim 9\%$ fiber. A significant increase in fecal output mass in *N. commune*-fed mice (data not shown) further supports a potential role of *N. commune* fiber in modulating the environment of the intestinal lumen. Additionally, GC analysis revealed that dry *N. commune* contains $3.25 \mu\text{g/g}$ of sitosterol with no detectible amount of cholesterol. Therefore, we speculate the fiber and plant sterol in *N. commune* are responsible, at least in part, for the diminished intestinal cholesterol absorption. In addition to fiber and plant sterols, protein from plant sources may affect cholesterol absorption. C-phycoerythrin, a phycobiliprotein in *Spirulina plantensis* with high levels of cystine, possesses hypocholesterolemic properties by

Table 3 Cholesterol absorption and sterol excretion in mice fed an AIN-93 M diet supplemented with 5% dry *N. commune* (wt/wt) for 4 weeks

	Cholesterol absorption, %	Sterol output, $\mu\text{mol/day/100 g body wt}$		
		Fecal neutral steroids	Fecal bile acids	Fecal plant sterols
Control	79.53 ± 1.75	1.85 ± 0.11	4.06 ± 0.28	1.12 ± 0.13
<i>N. commune</i>	$67.87 \pm 2.91^*$	$3.14 \pm 0.11^*$	4.15 ± 0.08	2.45 ± 0.06

Values represent mean \pm SEM ($n = 8$ for control, 9 for *N. commune*)

* Significantly different from control ($P < 0.05$)

reducing micellar solubility of cholesterol and cholesterol absorption, consequently increasing fecal neutral and acidic sterol output [29]. As *N. commune* is known to contain C-phycoyanin [15], it is possible that C-phycoyanin might contribute to the inhibition of cholesterol absorption by *N. commune*. In addition, the lipid fraction of *N. commune* equals ~ 6% of the dry weight. Analysis by thin-layer chromatography and gas chromatography showed that approximately 15% of the lipid fraction consists of fatty acids, 75% of which are unsaturated fatty acids [32]. While these fatty acids may have potential anti-inflammatory properties, their low amount in the diet is unlikely to affect plasma cholesterol levels.

Hepatic cholesterol synthesis is sensitive to the quantity of cholesterol that reaches the liver from the intestine via the chylomicron-remnant pathway [8]. While no significant changes in liver lipids were observed with *N. commune* supplementation, liver weights were significantly reduced in *N. commune*-fed mice, and a trend toward a decrease in hepatic lipid content was seen. Gene expression analysis by realtime PCR indicated a significant increase in several cholesterol-modulating genes, including SREBP-2 and HMGR. When cellular cholesterol is depleted, SREBP-2 is activated to increase the transcription of genes involved in cholesterol biosynthetic pathway and uptake, including HMGR, LDLR, HMG-CoA synthase, farnesyl diphosphate synthase and squalene synthase [11, 18, 30]. The increase in SREBP-2 and subsequently HMGR expression observed with *N. commune* supplementation could have been the result of minor but sufficient enough changes in hepatic cholesterol status to upregulate cholesterol synthesis for maintaining cholesterol homeostasis. Additionally, we cannot rule out the possibility that free cholesterol content in subcellular compartments such as endoplasmic reticulum membrane, where SREBP-2 and HMGR reside, could be altered by *N. commune* without a change in total free cholesterol contents.

LDLR is responsible for the uptake of LDL from circulation, and increased LDLR is associated with decreased plasma LDL cholesterol concentration [4]. Although it did not reach a statistical significance, *N. commune*-fed mice exhibited a slight increase in LDLR mRNA, which could contribute to the reduced TPC concentration. The decrease in TPC levels in *N. commune*-fed mice might also be due to its effect on a different aspect of lipoprotein metabolism such as very low density lipoprotein (VLDL) metabolism. Fatty acid and triglyceride synthesis in the liver regulate the assembly and secretion of VLDL [9, 44]. Enzymes involved fatty acid synthesis and uptake, such as acetyl-CoA carboxylase, FAS, and stearoyl CoA desaturase, are under the transcriptional regulation of SREBP-1c [25, 26, 40]. FAS expression in *N. commune*-fed animals showed a trend toward decrease ($P = 0.062$). Potential inhibition of

lipogenesis by *N. commune* may decrease VLDL formation, which could reduce plasma concentrations of total cholesterol and triglyceride as seen in *N. commune*-fed animals.

Inflammation and dyslipidemia are two major contributing factors involved in the progression of atherosclerosis. Lifestyle factors, including diet, influence atherosclerosis development which leads to CHD as a significant cause of death worldwide. While drugs are commonly used to control dyslipidemia such as high plasma cholesterol and triglyceride levels, consumption of natural products for dietary regulation of plasma lipids are becoming increasingly popular. Our previous research has shown *N. commune* lipid extract to have an anti-inflammatory effect in RAW 264.7 macrophages [32], as well as an inhibitory role in SREBP-1 and 2 maturation [33], indicating a potential athero-protective role of *N. commune*. Additionally, our current study indicates that whole *N. commune* supplementation decreases the absorption of cholesterol within the intestinal lumen, reducing TPC concentration. The combination of these beneficial effects of *N. commune* intake on lipid metabolism may provide a beneficial alternative for lowering plasma cholesterol concentration in the human population.

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Conflict of interest statement None declared.

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