Bioscience of Microbiota, Food and Health Vol. 42 (1), 56–64, 2023

Full Paper

Camellia oil (*Camellia oleifera* Abel.) treatment improves high-fat diet-induced atherosclerosis in apolipoprotein E (ApoE)$^{-/-}$ mice

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Received February 2, 2022; Accepted August 12, 2022; Published online in J-STAGE September 2, 2022

Atherosclerosis is the main cause of cardiovascular diseases, and healthy dietary habits are a feasible strategy to prevent atherosclerosis development. Camellia oil, an edible plant oil, exhibits multiple beneficial cardiovascular effects. Our previous study showed that oral administration of camellia oil attenuated hyperglycemia, fat deposits in the liver, and the atherosclerosis index in high-fat diet (HFD)-induced obese mice. Here, an atherosclerosis model of apolipoprotein E (ApoE)$^{-/-}$ mice induced by HFD was used to study the effect of camellia oil on atherosclerosis, and 16S rRNA gene sequencing was used to analyze the changes in gut microbiota composition. The results showed that camellia oil significantly inhibited the formation of atherosclerotic plaques in ApoE$^{-/-}$ mice, which were characterized by significantly reduced levels of serum total cholesterol and enhanced levels of serum high-density lipoprotein cholesterol. The aortic levels of interleukin-6 and tumor necrosis factor were decreased. The results of the 16S rRNA analysis showed that after camellia oil interventions, the intestinal flora of ApoE$^{-/-}$ mice changed significantly, with the diversity of intestinal flora especially increasing, the relative abundances of Bacteroides, *Faecalibaculum, Bilophila*, and *Leuconostoc* increasing, and the Firmicutes/Bacteroidetes ratio and Firmicutes abundance decreasing. Collectively, our findings confirmed the promising value of camellia oil in preventing the development of atherosclerosis in ApoE$^{-/-}$ mice. Mechanistically, this preventive effect of camellia oil was probably due to its lipid-lowering activity, anti-inflammatory effects, and alteration of the gut microbiota composition in the mice.

Key words: camellia oil, atherosclerosis, apolipoprotein E (ApoE), high-fat diet, gut microbiota

INTRODUCTION

Atherosclerosis is a serious threat to human health and is considered to be the main cause of cardiovascular diseases. According to a survey in 2020, the incidence rate of atherosclerosis in the general population aged 30 to 79 worldwide was 28% [1]. The typical features of atherosclerosis are abnormal cholesterol levels, the formation of foam cells, and calcium deposition in the arteries, and these features can lead to the gradual disintegration and calcification of the middle artery [2]. The most common complications of atherosclerosis are myocardial infarction and stroke, both of which are the most common causes of death worldwide [3, 4].

Currently, several lipid-lowering drugs are widely used to reduce the threat of atherosclerosis to human health and display certain efficacy, but side effects such as statin-associated muscle symptoms limit their further clinical use [5]. The development of prevention approaches to reduce the risk of atherosclerosis has always been the focus of attention [6]. Epidemiological evidence has indicated that eating habits are also one of the main risk factors leading to pathological conditions such as atherosclerosis [7]. Therefore, eating safe and effective functional foods in one's daily diet to prevent or treat vascular diseases is an effective, healthy, and convenient treatment method.

Camellia oil (*Camellia oleifera* Abel.) is a kind of edible oil with high medicinal and nutritional value in China [8]. Studies have shown that it contains a variety of fatty acids, including oleic acid (764 mg/g), linoleic acid (108 mg/g), and palmitic acid (96 mg/g) [9]. Interestingly, previous reports have shown that diets rich in polyunsaturated fatty acids can reduce the risk of cardiovascular disease and diabetes [10]. The preclinical studies of camellia oil accumulated to date also show that it exhibits various potential pharmacological effects, including anti-inflammatory, antioxidative, antidyslipidemic, hypoglycemic, and hepatoprotective activities [11, 12].

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doi: 10.12938/bmfh.2022-005

Our previous research results showed that oral administration of camellia oil exhibited beneficial effects, which were characterized by attenuating hyperglycemia, fat deposits, and the atherosclerosis index in high-fat diet-induced obese mice [13]. Therefore, it is logical to assume that camellia oil should take effect in the atherosclerotic mouse model. However, this speculation needs to be confirmed experimentally. Here, we investigated the beneficial effects of camellia oil in an ApoE$^{-/-}$ mouse model of atherosclerosis and explored its potential underlying mechanisms.

MATERIALS AND METHODS

Materials

Commercial camellia oil was obtained from Guizhou Yi Hang Ecological Agriculture and Animal Husbandry Technology Development Co., Ltd. (Nansi, Guizhou, China) and stored at 4°C in a sealed container until use. Simvastatin and the rest of the reagents were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

Animal study

Male 8-week-old C57BL/6J mice and apolipoprotein E (ApoE)$^{-/-}$ mice (weight 22 ± 1 g) were purchased from Guangdong Medical Animal Experimental Center (Guangdong, China). The animals were fed at room temperature (23 ± 2°C) with a relative humidity of 50% and had a 12-hr light/dark cycle. All animal experiments were performed according to the guidelines issued by the Animal Care and Use Committee of Zunyi Medical University.

After one week of acclimatization, male C57BL/6J mice were used as the control group (Con; n=8). ApoE$^{-/-}$ mice were randomly divided into four groups: vehicle group (Veh; n=8), simvastatin group (Svt; 5.3 mg/kg; n=8), low-dose camellia oil group (Cam 3 mL/kg; n=8), and high-dose camellia oil group (Cam 6 mL/kg; n=8). Mice in the control and vehicle groups were treated with sterile water, and other administrative groups were treated with the indicated dose of simvastatin or camellia oil once a day by gavage. Except for the control group, which was given normal feed, the other experimental groups were given high-fat diet (HFD) feed. The body weights of the mice were measured every week, and the experimental period was 8 weeks.

Analysis of atherosclerotic lesions

The complete brachiocephalic artery (from the arch of the aorta to the bifurcation into the right common carotid artery) was removed, fixed with 10% formalin for 10 min, and then stained with Oil Red O to determine the total positive area. Meanwhile, the other part of the arch of the aorta was fixed in buffered chloral hydrate for 24 hr, embedded in paraffin, and then sectioned at 3 μm. These sections were stained with hematoxylin-eosin (HE) for further plaque area calculation. Finally, quantitative analysis of the Oil Red O-positive area and HE-stained plaque area was carried out using Image-Pro Plus 6.0 software.

A magnetic bead homogenizer was used to homogenize arterial tissue of the same position and weight in sodium chloride solution (g:mL=1:9), and then the homogenate was centrifuged at 4,000 g for 10 min. Finally, the clear supernatant was collected. The levels of tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) in the arterial tissue were detected by enzyme-linked immunosorbent assay (ELISA) kits (NanJing SenBeiJia Biotechnology Co., Ltd., Nanjing, China).

Biological analysis

Serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and glucose levels were determined using the indicated assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). Liver tissue was homogenized using a magnetic bead homogenizer, and then the homogenate was centrifuged at 3,000 g for 10 min. Finally, the clear supernatant was collected, and the TG and TC levels were measured according to the manufacturer's instructions.

16S rRNA analysis

Microbial DNA (with a total mass of 1.2–10.0 ng) was isolated from each mouse stool sample with a PowerSoil DNA Isolation Kit (Qiagen, Carlsbad, CA, USA) and quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V4–V5 regions of the 16S rRNA genes were amplified by using specific primers (515F, 5′-CCGTCAATTCMTTTRAGTTT-3′, 806R, 5′-GTGCCAGCMGCCGCGGTAA-3′). After amplification and detection, the polymerase chain reaction (PCR) products were mixed in equidensity ratios according to the GeneTools Analysis Software (version 4.03.05.0, Syngene, Synoptics Ltd., Cambridge, UK). The sequencing libraries were generated using a NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's recommendations, and the index was added. Finally, the library was sequenced on an Illumina HiSeq 2500 platform, and 250 bp paired-end reads were generated. Sequences with ≥97% similarity were clustered into the same operational taxonomic units (OTUs) using the UPARSE method.

Microbial bioinformatics analysis

A Venn diagram was employed to show the core and shared gut microbiota species of the mice in different groups. A bacterial taxonomy and cluster heatmap were generated to represent the abundance of bacteria among different groups. The alpha diversity and beta diversity were calculated with QIIME (V1.9.1) and then displayed with R software (V2.15.3). The Wilcoxon rank sum test was performed to compare the difference in alpha diversity among different groups. Additionally, to identify the genomic features in different classes, we applied the linear discriminant analysis effect size (LEfSe) method for biomarker discovery. A nonparametric factorial Kruskal–Wallis (KW) sum-rank test and pairwise Wilcoxon tests were carried out to detect significantly different species among the various groups, followed by a linear discriminant analysis (LDA) to assess the effect size of each differentially abundant taxon. Finally, those species with LDA scores (-log10) ≥3 were defined as biomarkers.

Statistical analysis

All experimental data are expressed as the mean ± SD. The data were analyzed using GraphPad Prism 8.0 statistical software, and one-way analysis of variance was used to compare the differences among multiple groups. $p \leq 0.05$ indicated that differences were statistically significant.

doi: 10.12938/bmfh.2022-005

RESULTS

Effects of camellia oil on aortic plaques in ApoE$^{-/-}$ mice

We first examined the antiatherosclerotic effects of camellia oil treatment on aortic plaques in ApoE$^{-/-}$ mice. The results of Oil Red O staining and HE staining of the aortas showed that HFD exposure for 8 weeks caused severe atherosclerotic lesions and inflammatory reactions in the whole blood vessels of ApoE$^{-/-}$ mice. After camellia oil interventions, the atherosclerotic symptoms of ApoE$^{-/-}$ mice were improved (Fig. 1A, 1B). This observation was further confirmed by quantification of the Oil Red O-positive area and HE-stained plaque area. Compared with the vehicle group, the proportions of the Oil Red O-positive area relative to the complete artery and plaque area in the camellia oil groups were significantly reduced (Fig. 1C, 1D, $p<0.05$). This finding indicated that camellia oil could improve atherosclerosis in ApoE$^{-/-}$ mice fed a HFD.

Effects of camellia oil on serum and blood biochemical parameters in ApoE$^{-/-}$ mice

As expected, the serum TC, TG, LDL-C, TNF-α, and blood glucose levels in the vehicle group were significantly increased compared with those in the control group ($p<0.05$). After camellia oil (6 mL/kg) intervention, the levels of TC in the serum of atherosclerotic mice were significantly decreased (Fig. 2A, $p<0.05$). For TG, LDL-C, TNF-α, and blood glucose levels, although there was a trend toward a decrease, there were no significant differences after camellia oil treatment (Fig. 2B, 2C and 2E, 2F, $p>0.05$). In contrast, camellia oil at both 3 mL/kg and 6 mL/kg significantly increased serum HDL-C levels in ApoE$^{-/-}$ mice (Fig. 2D, $p<0.05$).

Effects of camellia oil on the levels of arterial inflammatory cytokines and the arteriosclerosis index in ApoE$^{-/-}$ mice

The enzyme-linked immunoassays showed that arterial IL-6, TNF-α levels, and the arteriosclerosis index in the vehicle group were significantly increased compared with the control group (Fig. 3, $p<0.05$). After camellia oil interventions, especially at 6 mL/kg, the inflammatory cytokine expression and arteriosclerosis index in ApoE$^{-/-}$ mice were significantly decreased ($p<0.05$), which indicated that camellia oil may function by inhibiting inflammatory cytokines.

Effects of camellia oil on the lipid parameters of liver homogenate and body weight in ApoE$^{-/-}$ mice

As shown in Fig. 4A, 4B, the hepatic TG, TC, and liver index of mice in the vehicle group were significantly increased ($p<0.05$) compared with the control group. In contrast, compared with the vehicle group, camellia oil decreased the levels of TG (Cam 3 mL/kg, $p<0.05$) and TC (Cam 6 mL/kg, $p<0.05$) in liver homogenate. Meanwhile, camellia oil showed a slight trend toward a decrease in the liver index and body weight of mice, but there were no significant differences ($p>0.05$, Fig. 4C, 4D) compared with the control group. These data suggest that camellia oil improved nonalcoholic fatty liver disease induced by the HFD in ApoE$^{-/-}$ mice, with little effect on body weight.

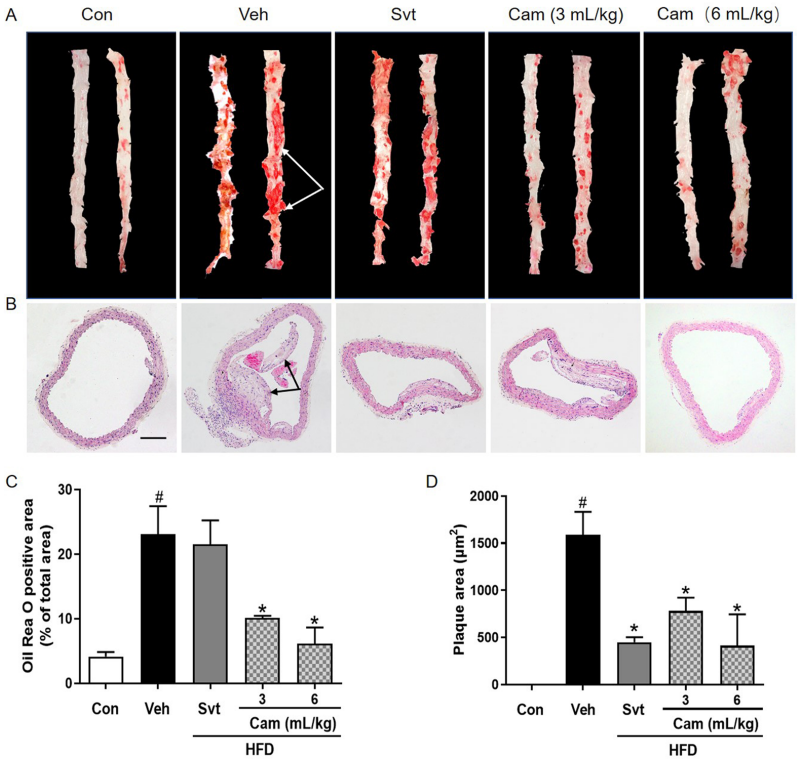

Fig. 1. Effects of camellia oil on vascular atherosclerosis in ApoE$^{-/-}$ mice. (A) Oil Red O staining of the complete brachiocephalic artery. White arrows indicate the positive lesion (magnification: 8×). (B) HE staining of the aortic arch. Black arrows indicate plaque lesions (scale bar: 25 μm). (C) Quantification of Oil Red O staining based on the percentage of the positive area relative to the complete artery area. (D) Quantification of HE staining based on plaque size. #$p<0.05$ compared with the control group. *$p<0.05$ compared with the vehicle group. Con: control group; Veh: vehicle group; Svt: simvastatin group; Cam: camellia oil group; HFD: high-fat diet.

doi: 10.12938/bmfh.2022-005

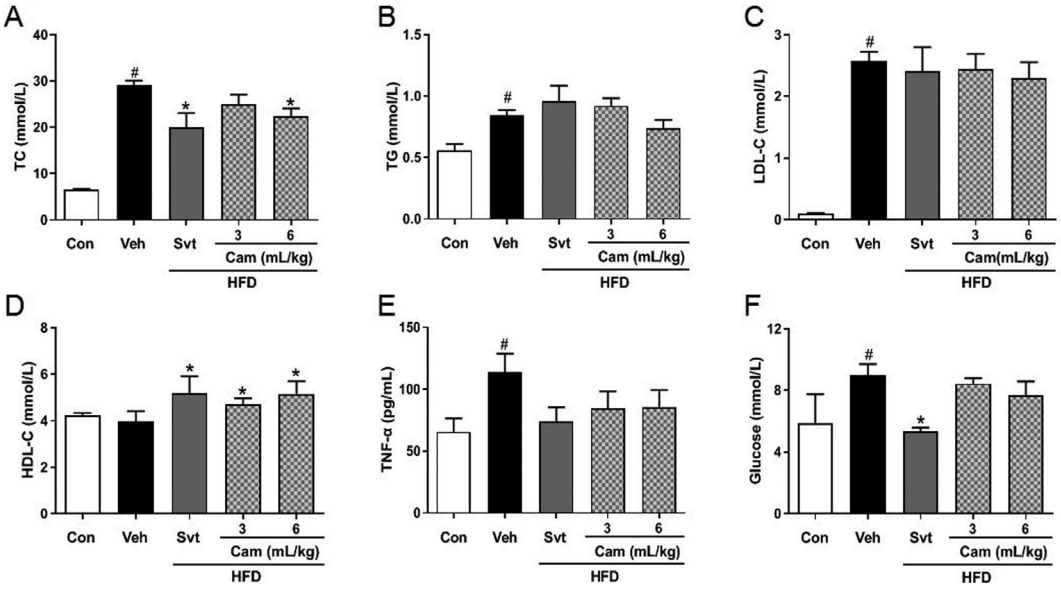

Fig. 2. Effects of camellia oil treatment on serum and blood biochemical parameters in ApoE$^{-/-}$ mice. (A) TC, (B) TG, (C) LDL-C, (D) HDL-C, (E) TNF-α, and (F) glucose levels in ApoE$^{-/-}$ mice. $^{\#}p<0.05$ compared with the control group. *p<0.05 compared with the vehicle group. Con: control group; Veh: vehicle group; Svt: simvastatin group; Cam: camellia oil group; HFD: high-fat diet; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TNF-α: tumor necrosis factor α.

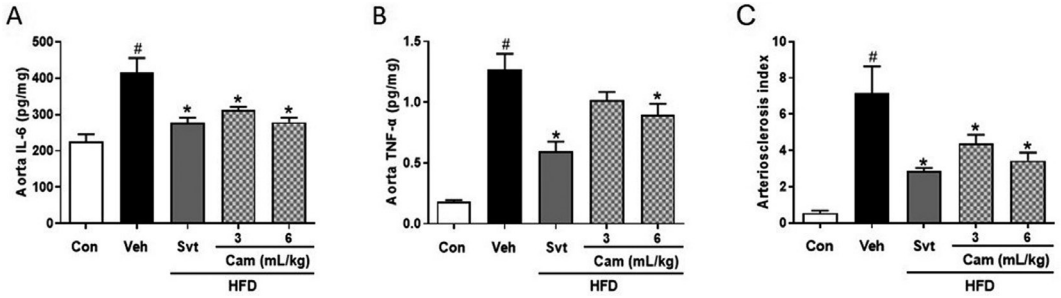

Fig. 3. Effects of camellia oil treatment on the levels of arterial inflammatory cytokines and the arteriosclerosis index in ApoE$^{-/-}$ mice. (A) IL-6, (B) TNF-α, and (C) arteriosclerosis index. $^{\#}p<0.05$ compared with the control group. *p<0.05 compared with the vehicle group. Con: control group; Veh: vehicle group; Svt: simvastatin group; Cam: camellia oil group; HFD: high-fat diet; TNF-α: tumor necrosis factor α.

Effects of camellia oil on gut microbiota alteration in ApoE$^{-/-}$ mice

To reveal the roles of the intestinal flora underlying the potential mechanisms involved in the antiatherosclerotic effects of camellia oil, 16S rRNA gene sequencing was performed using feces from the mice. The Venn diagram showed that the unique OTUs of the control, vehicle, and camellia oil 6 mL/kg groups were 694, 270, and 552 (Fig. 5A), respectively, indicating that the OTU diversity of the intestinal microflora of mice was significantly increased after the intervention with camellia oil. In addition, camellia oil-treated mice displayed a trend toward increased intestinal α-diversity, which was indicated by a slightly increased trend in the richness index as well as a significantly increased Shannon index compared with the vehicle group (Fig. 5B, 5C; richness index, p>0.05; Shannon index, p<0.05).

At the phylum level, the relative abundance of Firmicutes in the vehicle group was significantly increased compared with that in the control group, while the relative abundances of Bacteroidetes and Tenericutes were significantly decreased. However, camellia oil reversed this trend. The relative abundances of Firmicutes in the camellia oil groups were significantly reduced compared with that in the vehicle group, while the relative abundances of Bacteroidetes and Tenericutes were significantly increased (Fig. 5E). Furthermore, the F/B ratio of the vehicle group was significantly increased (p<0.05) compared with that in the control group (Fig. 5D), while camellia oil significantly reduced the F/B ratio of the intestinal microflora of ApoE$^{-/-}$ mice (p<0.05).

At the genus level, the relative abundances of *Faecalibaculum*, *Dubosiella*, *Coriobacteriaceae* UCG-002, *Lactobacillus,* and *Alloprevotella* in the vehicle group increased compared with those in the control group. The relative abundances of the *Alloprevotella* and *Lachnospiraceae* NK4A136 groups decreased. However, after treatment with camellia oil, the relative abundances of *Faecalibaculum*, *Dubosiella*, *Coriobacteriaceae* UCG-002, *Lactobacillus*, and *Alloprevotella* decreased compared with those in the vehicle group. The relative abundances of the *Alloprevotella* and *Lachnospiraceae* NK4A136 groups increased (Fig. 5F). At the same time, these results at the genus level were

doi: 10.12938/bmfh.2022-005

further confirmed by the cluster analysis diagram, and the cluster analysis results showed that the structure of the intestinal flora in the camellia oil group (6 mL/kg) was more similar to that in the control group (Fig. 5G), indicating that camellia oil improved the intestinal flora composition.

Effect of camellia oil on intestinal bacterial biomarkers in ApoE$^{-/-}$ mice

To identify the main active microflora, an LEfSe analysis was performed to distinguish biomarkers (LDA score [-log10] >3) in each group at the different unit levels. Figure 6A shows 33, 7, and 11 biomarkers (LDA score >35) in the control, vehicle,

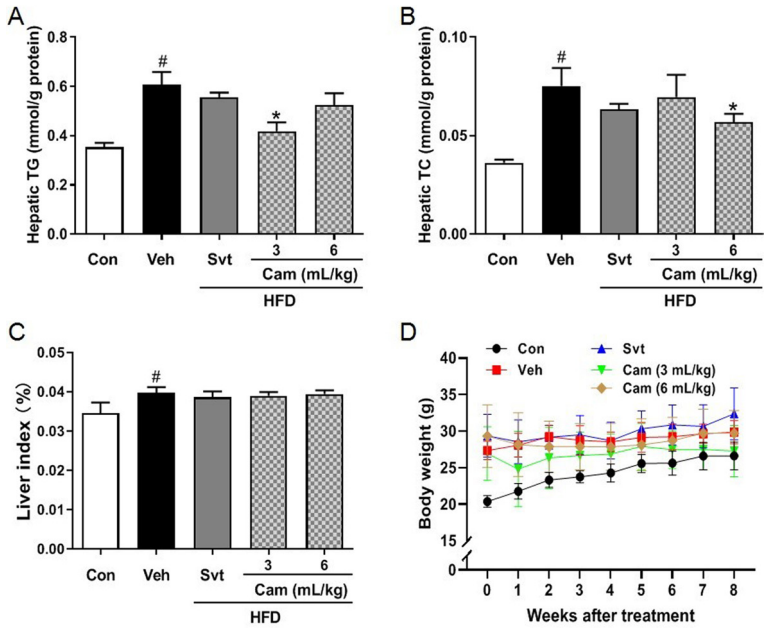

Fig. 4. Effects of camellia oil treatment on liver homogenate and body weight in ApoE$^{-/-}$ mice. (A) Hepatic TG, (B) hepatic TC, (C) liver index, and (D) body weight. $^{\#}p<0.05$ compared with the control group. *p<0.05 compared with the vehicle group. Con: control group; Veh: vehicle group; Svt: simvastatin group; Cam: camellia oil group; HFD: high-fat diet; TG: triglyceride; TC: total cholesterol.

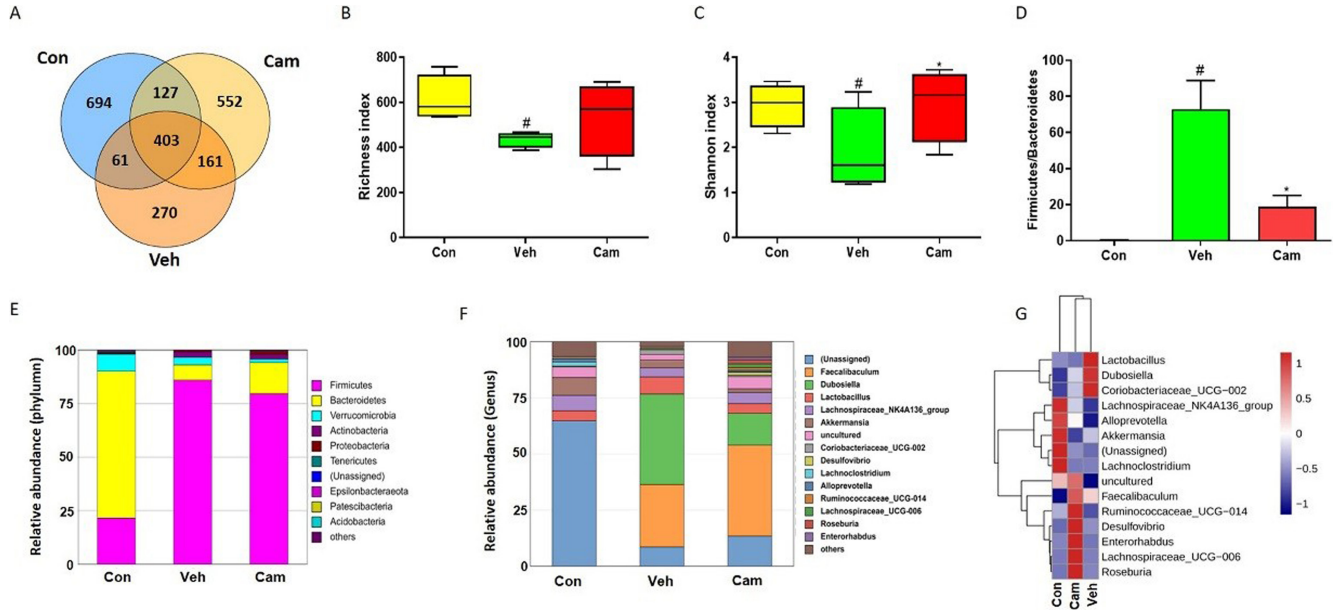

Fig. 5. Effects of camellia oil treatment on gut microbiota diversity and special bacteria in ApoE$^{-/-}$ mice. (A) Venn diagram of overlap showing the shared species from each group. (B, C) The α diversity indices included the richness index (B) and Shannon index (C) after camellia oil treatment. (D–G) Effects of camellia oil on the gut microbiota structure in ApoE$^{-/-}$ mice. (D) The Firmicutes/Bacteroidetes ratio was calculated. (E, F) The microbiota composition was analyzed at the phylum (E) and genus (F) levels. (G) A heatmap for the significantly different genera was generated by microbiota community analysis. $^{\#}p<0.05$ compared with the control group. *p<0.05 compared with the vehicle group. Con: control group; Veh: vehicle group; Cam: camellia oil group.

doi: 10.12938/bmfh.2022-005

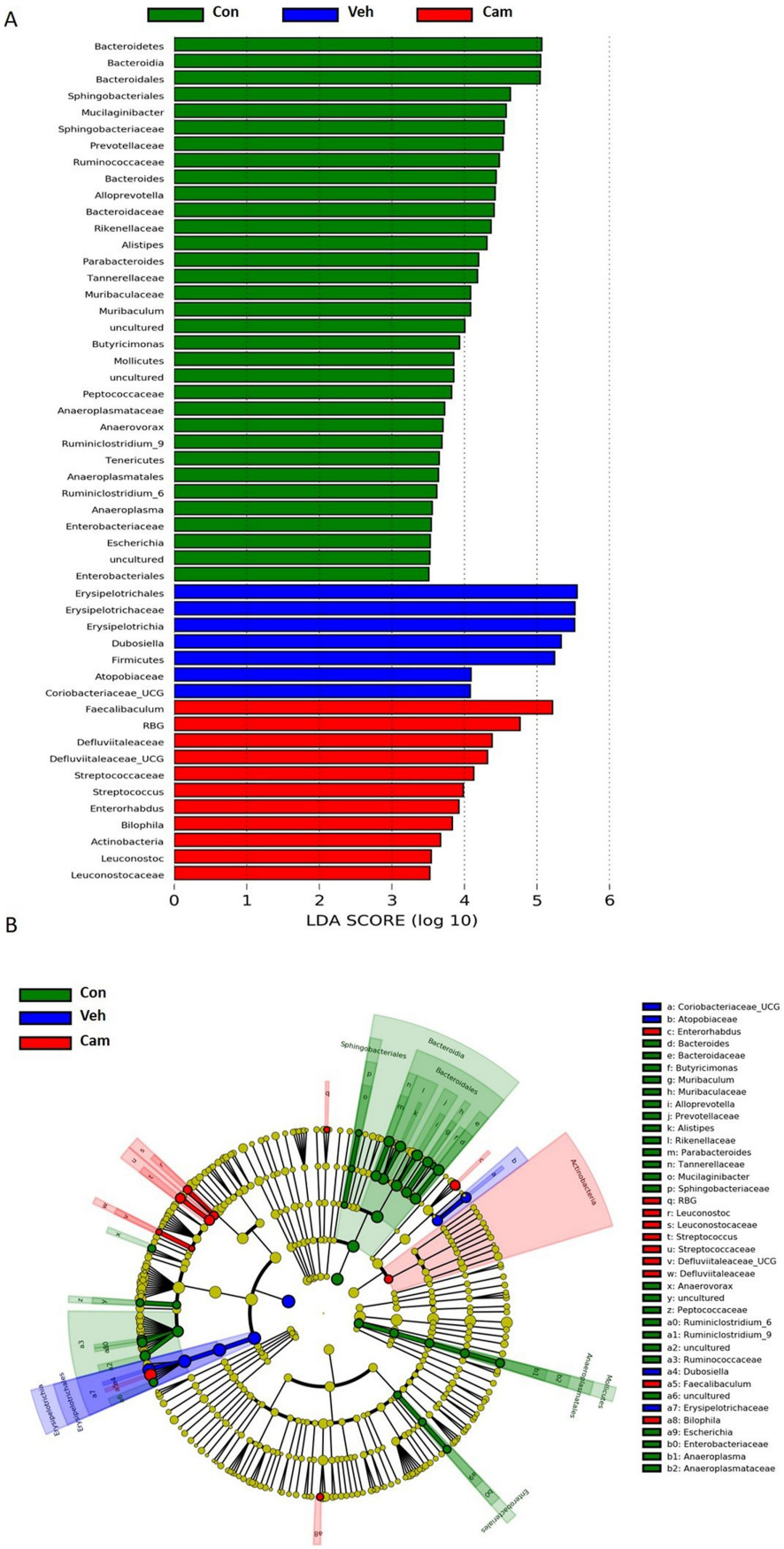

Fig. 6. Linear discriminant analysis effect size (LEfSe) analysis and cladogram. (A) LEfSe analysis revealed the different functional biomarkers among various groups. (B) The cladogram shows the phylogenetic distribution of functional biomarkers responding to camellia oil treatment. Linear discriminant analysis (LDA) scores >3 are shown. Con: control group; Veh: vehicle group; Cam: camellia oil group.

doi: 10.12938/bmfh.2022-005

and camellia oil group (6 mL/kg), respectively. Figure 6B shows a map of different species at different levels. The circle area is proportional to richness. This study focused on the analysis of biomarkers at the phylum and genus levels. Based on a phylum-level comparison, the biomarkers in the control group included Bacteroidetes (LDA score=5.06) and Tenericutes (LDA score=3.65). For the vehicle group, Firmicutes was a biomarker (LDA score=5.24), while there were no differences at the phylum level in the camellia oil group (6 mL/kg). At the genus level, the biomarkers of the control group included *Mucilaginibacter* (LDA score=4.58) and *Bacteroides* (LDA score=4.44). *Alloprevotella* (LDA score=4.42), *Alistipes* (LDA score=4.31), *Parabacteroides* (LDA score=4.19), *Muribaculum* (LDA score=4.08), *Butyricimonas* (LDA score=3.93), uncultured (LDA score=3.85), *Anaerovorax* (LDA score=3.70), *Ruminiclostridium 6* (LDA score=3.62), *Ruminiclostridium 9* (LDA score=3.62), *Anaeroplasma* (LDA score=3.56), and *Escherichia* (LDA score=3.53). The biomarkers of the vehicle group included *Dubosiella* (LDA score=5.33) and *Coriobacteriaceae UCG* (LDA score=4.08). The biomarkers of the camellia oil group (6 mL/kg) included *Faecalibaculum* (LDA score=5.21), *Defluviitaleaceae UCG* (LDA score=4.32), *Streptococcus* (LDA score=3.99), *Enterorhabdus* (LDA score=3.92), *Bilophila* (LDA score=3.83), and *Leuconostoc* (LDA score=3.54).

DISCUSSION

Our study highlights an interesting beneficial effect of camellia oil, an edible plant oil extracted from the seeds of *C. oleifera*, in an ApoE$^{-/-}$ mouse model of atherosclerosis. The key findings of the current study are that oral camellia oil administration effectively inhibited the formation and enlargement of arterial plaques in ApoE$^{-/-}$ mice, reduced serum TG, TC, LDL-C, and blood glucose levels, increased serum HDL-C levels, suppressed the levels of the arterial inflammatory factors TNF-α and IL-6, and reduced hepatic TG and TC levels. Furthermore, the extensive results from 16S rRNA analysis showed that the intestinal microflora composition changed significantly after the camellia oil interventions, especially increasing the diversity of intestinal microflora and the relative abundances of Bacteroides, *Faecalibaculum, Bilophila,* and *Leuconostoc* while decreasing the Firmicutes/Bacteroidetes ratio and Firmicutes abundance. These findings are vital and suggest that camellia oil exhibits potential as a dietary supplement and as a functional food against atherosclerosis.

Currently, the pathogenic mechanisms of atherosclerosis have not been fully elucidated, but they are likely multifactorial. In particular, abnormal elevation of blood lipids is one of the main causes of atherosclerosis, and lipid-lowering therapy was associated with less progression of atherosclerosis. The oxidation of lipids in LDL-C can activate a series of reactions, including the inflammatory response in arterial cells. Therefore, this process can cause subsequent fatty streak formation and arterial calcification, and it has been considered one of the key mediators of the pathogenesis of atherosclerosis [14]. It is well accepted that ApoE, a blood plasma protein, promotes the uptake of cholesterol and lipids through its high-affinity binding with various receptors, including LDL receptors [15]. Once the ApoE gene is knocked out in mice, a large amount of cholesterol is mainly distributed in very low-density lipoprotein and cannot be degraded due to the lack of recognition of cell-surface lipoprotein receptors. A high-fat/high-cholesterol atherogenic diet can increase plasma cholesterol levels (1,821 mg/dL) and accelerate the progression of atherosclerosis [16]. Correspondingly, compared with our previous research, the current study demonstrated that the serum TC level in ApoE$^{-/-}$ mice (C57 BL/6 genetic background) was four times higher than that in C57BL/6 mice fed the same HFD. Furthermore, the findings of our previous study showed that oral administration of camellia oil ameliorated obesity in C57BL/6 mice fed a HFD [13]. Notably, the results of our present study demonstrated that camellia oil administration resulted in similar decreasing trends in hyperlipidemia, hyperglycemia, and hepatic TG and TC levels in ApoE$^{-/-}$ mice. Collectively, these results indicated that camellia oil, at least in part, acted directly or indirectly through its lipid-lowering effects.

In addition to the suppression of lipid levels, other molecular mechanisms, such as inhibition of the overgeneration of TNF-α, which is associated with proinflammation, have been implicated in interventions against the progression of atherosclerosis, where suppression of TNF-α could reduce atherosclerosis in ApoE$^{-/-}$ mice [17, 18]. In line with these studies, we found that camellia oil treatment significantly reduced TNF-α expression levels in mouse artery homogenate. In human clinical trials, Bumrungpert *et al.* [19] also found that a camellia oil diet reduced inflammatory markers in subjects with hypercholesterolemia. These results suggest that camellia oil administration can be used as a potential intervention approach for inflammatory diseases. De Oliveira *et al.* [20] found that supplementation with unsaturated fatty acids in vegetable oils reduced proinflammatory properties and improved thrombosis conditions. This result indicated that the antiatherosclerotic effects of camellia oil may be due to its function in suppressing proinflammatory responses.

The intestinal flora also plays an important role in atherosclerosis. Recent studies have shown that there are significant differences in the intestinal flora of atherosclerosis patients and healthy people [21]. The present study further showed that the development of atherosclerosis was associated with intestinal microbiota, revealing differences between HFD-fed ApoE$^{-/-}$ mice and mice fed a normal diet. Meanwhile, it has been shown that the development of atherosclerosis can be effectively inhibited by altering the intestinal flora of ApoE$^{-/-}$ mice [22, 23]. In this study, the results of a PCoA analysis showed that the intestinal microflora of the mice in the vehicle group and those in the camellia oil groups were distributed in different regions, suggesting that camellia oil could change the intestinal microflora of ApoE gene knockout mice. Camellia oil (6 mL/kg) significantly increased the unique OTUs, richness index, and Shannon index of ApoE$^{-/-}$ mice, suggesting that it can improve intestinal microflora diversity. Therefore, the antiatherosclerotic function of camellia oil may be partly related to the increased diversity of intestinal flora.

Other reports have shown that camellia oil increased the Firmicutes/Bacteroidetes ratio of intestinal microflora and the α-diversity and relative abundance of *Bifidobacterium*. The model of colitis induced by acetic acid in rats was improved with camellia oil [24]. As an important "organ" for the body's nutrient intake, the intestinal flora is closely related to obesity and related metabolic diseases. Animal models of obesity have shown that a deficiency in intestinal *Bifidobacterium* species can significantly increase serum lipopolysaccharide levels in mice, leading to

weight gain, adipose tissue accumulation, and worsening of hepatitis [25]. In addition, some studies have shown that in obese ob/ob mice and obese people, the intestinal flora contains more Firmicutes and less Bacteroidetes [26, 27]. Other studies have suggested that gut microbiota may influence atherosclerosis by activating the immune system, altering cholesterol metabolism, and producing bacterial metabolites, such as trimethylamine N-oxide (TMAO), that promote plaque development [28]. Furthermore, the intestinal microbiota has been reported to influence the development of atherosclerosis by regulating plasma cholesterol levels in ApoE$^{-/-}$ mice [23]. The regulation of intestinal microbiota appears to be a new method for preventing and treating metabolic diseases [29]. Hence, the results of the current study, combined with those in the literature, highlight the promising role of the intestinal microbiota in camellia oil interventions for managing atherosclerosis.

LEfSe showed that there were significant differences in the main active flora among the different groups. It was found that the changes in Firmicutes and Bacteroidetes in the intestinal flora were closely related to the individual's metabolism. After the camellia oil (6 mL/kg) intervention, the Firmicutes/Bacteroidetes (F/B) ratios of the intestinal flora of ApoE$^{-/-}$ mice were consistent with that of obese mice, showing an obvious downward trend. Elevated levels of Firmicutes have been reported to lead to the accumulation of toxins and inflammation in the body [21]. In this study, the level of intestinal Firmicutes in ApoE$^{-/-}$ mice fed a HFD was significantly increased, whereas it was significantly reduced by camellia oil, and the levels of the inflammatory cytokines TNF-α and IL-6 in the arteries were also significantly decreased. These results suggested that camellia oil may regulate the inflammatory response of ApoE$^{-/-}$ mice by changing the level of Firmicutes in the intestinal flora. Notably, in this study, we found that camellia oil significantly increased the abundance of *Bacteroides*, a type of short-chain fatty acid-producing bacteria that helps to combat obesity induced by a HFD [30]. Studies have shown that *Bacteroides* are beneficial for the immune system [31]. Shi *et al.* [32] found that an increased abundance of *Faecalibaculum* improved fatty liver in atherosclerosis model mice, indicating its potential hepatoprotective function. Our study showed that camellia oil significantly increased the abundance of *Faecalibaculum* in the intestinal flora and significantly reduced the accumulation of hepatic TG and TC in ApoE$^{-/-}$ mice. Therefore, it is logical to propose that the antisteatogenic effect of camellia oil may be related to *Faecalibaculum*. In addition, *Bilophila* was reported to be negatively correlated with the plasma glucose concentration in male broilers. Interestingly, in this study, *Bilophila* increased significantly after camellia oil treatment, so the glucose-lowering effect of camellia oil was likely attributable to *Bilophila* [33]. In addition, *Leuconostoc pseudomesenteroides* has been shown to regulate metabolic imbalance and microbiota dysbiosis [34]. Correspondingly, in the current study, *Leuconostoc* was a biomarker in the camellia oil group (6 mL/kg), which implied that the efficacy of camellia oil may result from the increase in *Leuconostoc* in the gut microbiota. Furthermore, the current results regarding the alteration of the gut microbiota composition, especially the decrease in the F/B ratio and enhancement of the flora diversities, were consistent with our previous study [13]. Based on the above evidence, the antiatherosclerotic effects of camellia oil are likely attributable to changes in the diversity and composition of the intestinal flora in ApoE$^{-/-}$ mice.

Interestingly, prospective cohort studies have shown that long-term consumption of unsaturated vegetable oil instead of animal fat can prevent chronic diseases and premature death [35]. Similarly, Han *et al.* [36] found that monounsaturated fatty acids (MUFAs) can promote longevity by promoting cell membrane fluidity, reducing oxidative stress, enhancing energy storage, or activating signaling pathways in *Caenorhabditis elegans*. The results by Yang *et al.* [37] showed that a diet rich in monounsaturated fatty acids can lead to the enrichment of unsaturated fatty acids in the liver. Both isomers of MUFAs can inhibit the progression of atherosclerosis in LDLr$^{-/-}$ mice by stimulating the peroxisome proliferator-activated receptor alpha (PPAR) pathway. This is also consistent with our current findings showing that camellia oil interventions reduced the glycemic index and the risk of atherosclerotic complications in ApoE$^{-/-}$ mice. Whether the lifespan and other beneficial effects observed in experiments can also translate to humans after camellia oil administration is worthy of further study.

In conclusion, this is the first study to demonstrate that camellia oil significantly inhibited the formation of atherosclerotic plaques in ApoE$^{-/-}$ mice. Mechanistically, this preventive effect of camellia oil was probably due to the reduced levels of serum total cholesterol, triacylglycerol, and low-density lipoprotein cholesterol and the improved levels of serum high-density lipoprotein cholesterol. In addition, the decreased levels of TNF-α and alteration of the gut microbiota composition induced by camellia oil might contribute to its preventive effect against atherosclerosis in mice.

ACKNOWLEDGMENT

This work was supported by grants from the Science and Technology Foundation of Guizhou Province (ZK [2022]587), Science and Technology Corporation Platform Talents Fund of Guizhou Province ([2018]5252), National Innovation and Entrepreneurship Training Program (202010661007), and Zunyi Medical University Innovation and Entrepreneurship Training Program (ZHCX201934) for College Students.

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