

Research Article

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Inhibition of atherosclerotic plaque formation in ApoE-deficient mice by dietary supplementation with *Lactobacillus casei*

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ABSTRACT

Background: Elevated serum cholesterol in humans is generally a risk factor correlated with the development of atherosclerosis (AS). *Lactobacillus casei* has been demonstrated to have the potential to reduce human serum cholesterol levels. The purpose of this study was to evaluate the anti-atherosclerotic effect of *Lactobacillus casei* (Strain Shirota) in apoE-deficient mice.

Methods: A total of 60 male ApoE-deficient mice of 4 weeks age, were randomly divided into 4 groups of 15 each group and matched for body weight. Four groups of apoE-deficient mice consumed one of the following diet: AIN-93G purified diet (n=15); AIN-93G purified diet with *Lactobacillus casei* (Strain Shirota; 0.5 mL of 10^8 cfu/mL, n=15); AIN-93G purified diet with *Lactobacillus casei* (Strain Shirota; 0.5 mL of 10^{10} cfu/mL, n=15); AIN-93G purified diet with *Lactobacillus casei* (Strain Shirota; 0.5 mL of 10^{12} cfu/mL, n=15).

Results: After 16 weeks intervention, the areas of atherosclerotic plaques in the aortic sinus were determined. Plaques were much more severe in control group than in *lactobacillus casei*-treated groups ($P < 0.05$). The plaque area of aortic sinus in mice fed *lactobacillus casei* with 0.5 mL of 10^8 , 10^{10} , or 10^{12} cfu/mL was 44.61%, 56.01%, 82.58% less compared with control group, respectively. Compared with control group, total cholesterol accumulation in aortas and livers showed a significant reduction in mice fed with *lactobacillus casei* ($P < 0.05$). Addition of *lactobacillus casei* also ameliorated serum lipid profile by decreasing total serum cholesterol and increasing HDL cholesterol concentration.

Conclusions: *lactobacillus casei* significantly improved lipid profile and reduced cholesterol

accumulation in liver and aorta, leading the inhibition of the formation of atherosclerotic lesion.

Keywords: *Lactobacillus casei*, atherosclerosis, apoE-deficient mice, cholesterol

INTRODUCTION:

Atherosclerosis (AS) is a complex and multifactorial disease, the etiology of which involves both genetic and environmental factors. A large number of studies revealed that atherosclerotic cardiovascular disease is the major cause of death in the United States, Europe and some of Asia society [1-2]. Atherosclerosis is the main etiopathogenic process that causes Coronary artery disease (CHD). CHD is the principal individual cause of mortality and morbidity worldwide. Disability-adjusted life years (DALYs) which is calculated as the sum of years of life lost and years lived with disability is considered as a new metric to measure disease burden. A recent report on the Global Burden of Disease, indicates that CHD accounted for the largest proportion of DALYs due to a single cause worldwide in 2010, explaining 5% of the total number of DALYS[3]. Hypercholesterolemia is an important risk factor associated with atherosclerosis and CHD [4]. In the United States, with the ≥ 5.18 mmol/L definition, the age-adjusted mean hypercholesterolemia prevalence in 2000 to 2002 was 54.9% for men and 46.5% for women [5].

Few therapeutic measures were effective in treatment of AS due to its pathological complexity until now. Thus, it is essential to control the major risk factors for AS. Dietary mode and life style are now considered tightly related to the atherogenesis, in which the improvement of eating habits and dietary model may lead to the decline in morbidity of AS [4,6]. The lactobacilli as the beneficial probiotics, are important inhabitants of the intestinal tract of human and animals, affecting host metabolic reactions, supporting a balance of human ecosystem. Considerable evidence has implicated lactobacilli in a number of potentially beneficial roles. Several studies reported that lactobacilli have the hypocholesterolemia effects in animals [7-8]. Some studies in vitro have shown that beneficial bacteria can remove cholesterol from culture medium, much attention has been given to the cholesterol-lowering potential of probiotics in humans [9-10]. Furthermore, lactobacilli strains were considered to have the property of inhibiting oxidation [10-11].

As it is well known that hypercholesterolemia is involved in the progression of atherosclerosis or CHD. Lowering hypercholesterolemia and antioxidative effects by lactobacilli is expected to contribute to the inhibition of atherosclerosis. However, there is so far no study demonstrating that supplementation of lactobacilli could reduce the atherosclerotic plaque formation in human or animals. The present study is to investigate the influence of supplementation of *Lactobacillus casei* on atherosclerotic plaque formation in apoE-deficient mice, and further to explore whether *Lactobacillus casei* supplementation induced-reduction of hypercholesterolemia, total cholesterol accumulation in aortas and livers contribute to its inhibition of atherosclerotic lessons in the animals.

MATERIALS AND METHODS

Animals and Diets: ApoE-deficient mice on a C57BL/6J background were purchased from Jackson Laboratories in USA, bred, and maintained under conventional housing conditions in our animal facility. *Lactobacillus casei* Shirota strain YIT9029 was from Yakult Honsha Co., Ltd (Japan). A total of 60 male ApoE-deficient mice of 4 weeks age, were randomly divided into 4 groups of 15 each group and matched for body weight. All the groups received purified diet based on the AIN-93G formulation [11]. The three experimental groups were given by gavage once a day with same volume(0.5ml) of different doses of *Lactobacillus casei*(10^8 , 10^{10} and 10^{12} cfu/mL). The control group didn't receive gavage. All procedures using animals were in accordance with the protocol approved by the standing committee of Sun Yat-sen University. All the mice were housed in sterile filter-top cages at 24 °C with a 12 h light-dark cycle. Animals had ad libitum access to food and autoclaved water. The experiment lasted 16 weeks and animal body-weight were recorded weekly throughout the experiment. The average amount of dietary intake by each mouse was \approx 2.8 g/d in each group. At the end of experiment, all mice were deprived of food overnight and kindly killed by withdrawing blood from retro orbital plexus under anesthesia. Serum was prepared by low speed centrifugation at 2800g for 20 min at 4 °C and used to determine the levels of serum lipids profile. The major organs and aortas were harvested, washed with ice-cold PBS and weighed. Aortas and hearts were prepared for morphological and biochemical analysis. The serum samples were stored at -80 °C, heart and aorta samples were stored in liquid nitrogen until used for assay.

Atherosclerotic Lesion Area: Quantification of atherosclerotic fatty streaks was done by calculating the lesion size in the aortic sinus as previously described [13] with a few modifications. Briefly, the heart and upper section of the aorta were removed from the animals and the peripheral fat cleaned carefully. The upper section was embedded in OCT compound and frozen at -20 °C. Every other section (10 μ m thick) throughout the aortic sinus (400 μ m) was taken for analysis. The distal portion of the aortic sinus was recognized by the three valve cusps which are the junctions of the aorta and the heart. Cryostat sections were evaluated for fatty streak lesions after staining with Oil red O and counterstaining with hematoxylin. Each section of the aortic valve was evaluated for Oil red O staining area by capturing images directly from an RGB camera attached to an Olympus BX-50 light microscope and displaying them on a Trinitron RGB monitor. Image analysis was determined using Optimas 4.1 software (Image Processing Solutions). Results were expressed as the percent of the total cross-sectional vessel wall area (normal+diseased area/section, excluding the lumen) stained with Oil red O.

Serum Lipid Profile: Cholesterol is insoluble in water but soluble in lipid, and is transported in the blood among tissues by four lipoproteins: chylomicron (CM), very low-density

lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Blood samples were obtained from animals fasted overnight by retroorbital bleeding under anesthesia. Serum total cholesterol (TC) and HDL-C were measured using a cholesterol esterase and cholesterol oxidase assay [14]. Serum LDL-C were determined by Direct LDL™ assay (Sigma Diagnostics, St. Louis, MO, USA), measured on a Hitachi 7060 Automatic Biochemical Analyser [15].

Cholesterol Content in Liver: Immediately after animal sacrifice, fresh livers were removed from the mice and heat processed at 110 °C for 11 h. Weighed dried livers (300 mg) were extracted by Folch reagent (chloroform: methanol (2:1, vol:vol)) and stored at -20 °C until analysis [16].

Cholesterol Accumulation in Aorta: Immediately following animal sacrifice, the heart of each mice was exercised which the aortic arch was dissected from the aortic root to the right renal artery and adventitial fat was removed. The aortas were stored on ice in PBS for a period not longer than 8 hours and not less than 2 h. Aortic tissue was dried, minced, and weighed before removing the lipids by the method of Folch et al [16]. In brief, the aortas were homogenized in 2 mL of chloroform: methanol (2:1, vol:vol) and centrifuged at 1700 g for 5 min. Two milliliters of supernatant was transferred to a clean test tube, and 0.4 mL of 0.88% KCl was added to separate the aqueous and lipid layers. The lipid layer was transferred to another tube, completely dried under N² at 37 °C, redissolved in 0.2 mL of 100% reagent-grade ethanol and stored at -20 °C until the time of assay. The total cholesterol content of aortic extracts was determined using an enzymatic fluorometric assay based on a modification of previously described methods [17]. Briefly, for total cholesterol determination, 0.1 mL of the sample was added to 0.9 mL assay solution (0.1 U/mL cholesterol oxidase, 1 U/mL peroxidase, 0.01 U/mL cholesterol ester hydrolase, 0.05% triton X-100, 1 mL sodium cholate and 0.6 mL potassium phosphate buffer, PH 7.4) and incubated at 37 °C for 1 hour. Fluorescence was measured using a Shimadzu R-540 spectrofluorophotometer (excitation, 325 nm; emission, 415 nm). Samples for each aorta were run in duplicate. All values are expressed as nmol/mg aorta (wet weight) for each mouse. Means and SD were determined for each group.

Statistics: Results are expressed as means ± SD. Data were analyzed by one-factor ANOVA. Differences between groups were considered significant if $p < 0.05$. SPSS10.0 software was used for all statistical analysis.

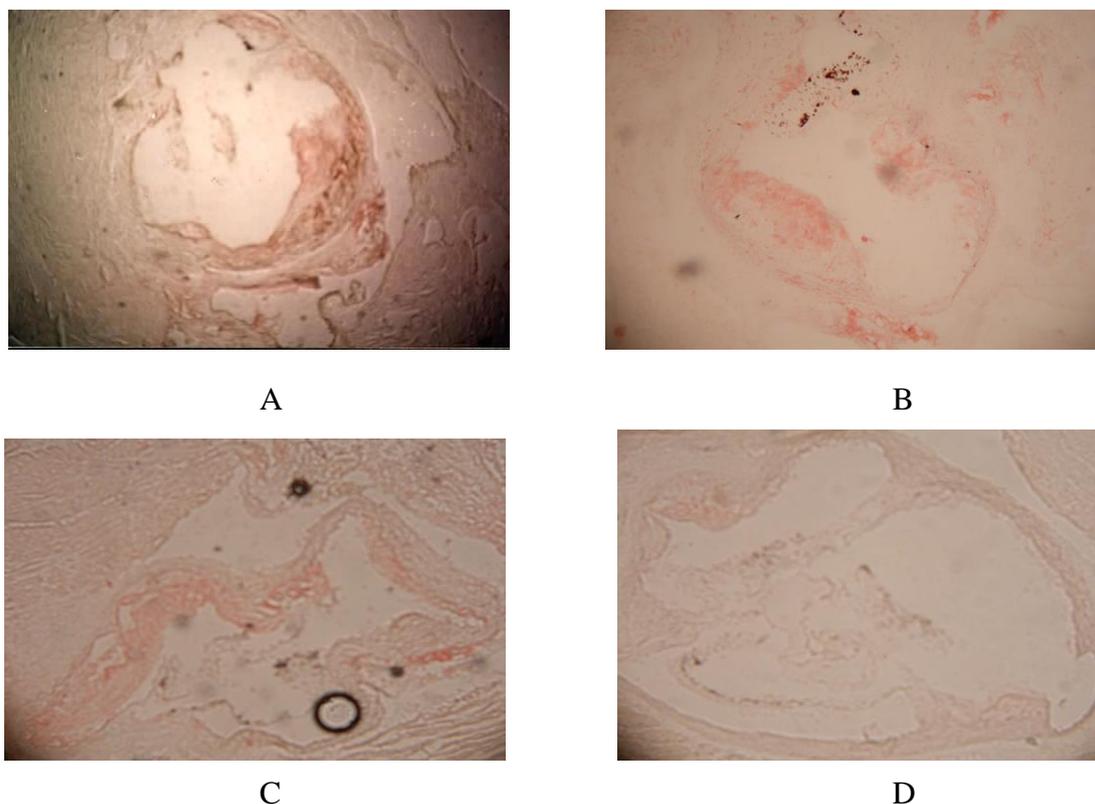
RESULTS:

Lactobacillus casei Strain Shirota supplementation inhibits atherosclerotic plaque formation in apoE-deficient mice: After 16 weeks intervention, the areas of atherosclerotic plaques in the aortic sinus were determined. Plaques were much more severe in control group than in lactobacillus casei-treated groups. The average plaque areas in mice

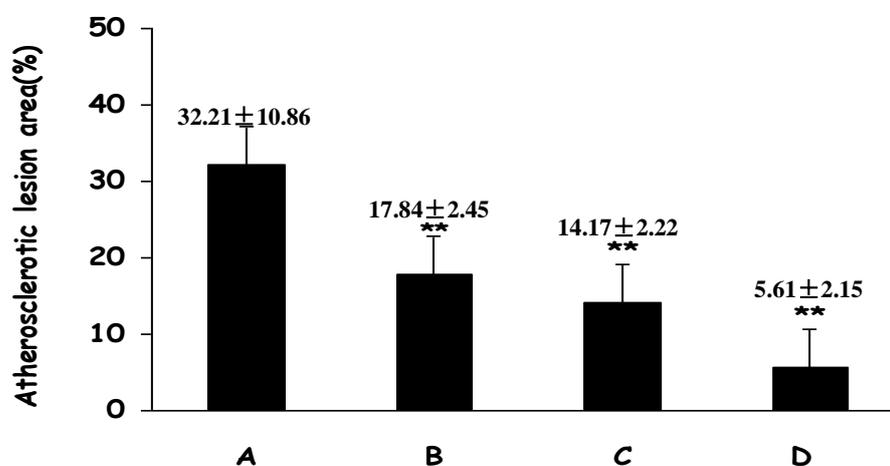
fed *Lactobacillus casei* (Strain Shirota) with 10^8 , 10^{10} , 10^{12} cfu/mL were lower than that of untreated control group ($P < 0.05$). The plaque area of aortic sinus in mice fed *Lactobacillus casei* was 44.61%, 56.01%, 82.58% less compared with control group, respectively. This result suggests that *Lactobacillus casei* exhibit anti-atherogenesis (Figure 1).

Figure 1

(A) Oil red O Staining of Aortic Sinus Sections



(B) Areas of Atherosclerotic Plaque in Different Groups.



A. Control; B. *Lactobacillus casei* 10^8 ;
 C. *Lactobacillus casei* 10^{10} D. *Lactobacillus casei* 10^{12}

(A) Mice from different groups were sacrificed after 16 weeks on different diets and their hearts were embedded in OCT. Section from the aortic sinus and stained with Oil red O. Original magnification×40.

(B) The extent of atherosclerosis was quantified after 16 weeks. Atherosclerotic lesion area in the sinus was measured by staining lesions with Oil-red O. Values are means ± SD, n=15. **P < 0.01.

Lactobacillus casei Strain Shirota supplementation reduces serum lipid levels: Compared with control group, plasma levels of total cholesterol, triglycerides, LDL significantly decreased, while HDL dramatically increased in mice fed with *Lactobacillus casei* (Strain Shirota) with 0.5 mL of 10^8 , 10^{10} , and 10^{12} cfu/mL (P < 0.05). There was no significant difference in the plasma levels of TC, TG, LDL and HDL among *Lactobacillus casei* treated groups (P > 0.05). (Figure 2)

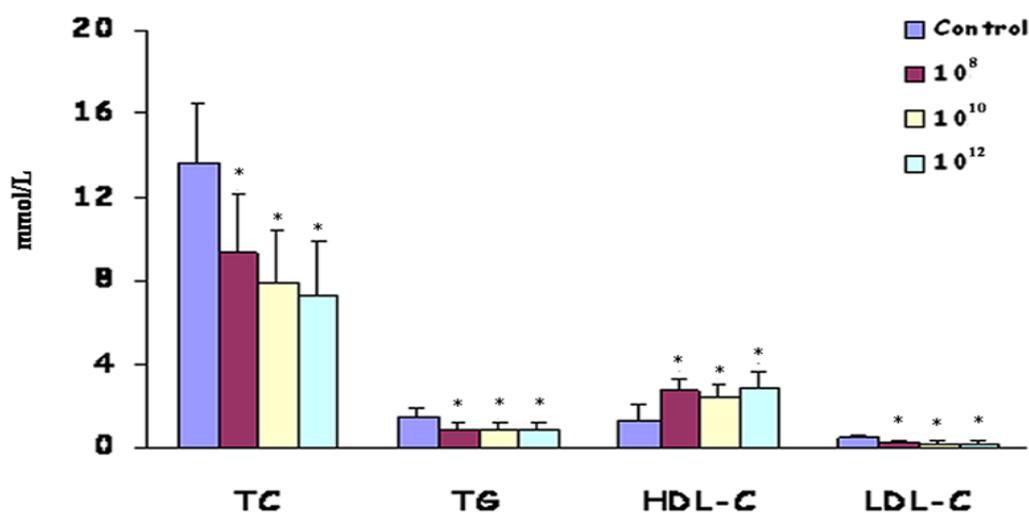


Figure 2. Serum Lipid Concentrations in Apolipoprotein E-deficient Mice Fed AIN-93G Diet or AIN-93G Diet Supplemented with *Lactobacillus casei*.

After 16 weeks, compared with control group, plasma levels of total cholesterol, triglycerides, LDL significantly decreased, while HDL dramatically increased in mice fed with *Lactobacillus casei* (Strain Shirota) with 0.5 mL of 10^8 , 10^{10} , and 10^{12} cfu/mL (P < 0.05).

Lactobacillus casei Strain Shirota supplementation decreases total cholesterol accumulation in aortas and livers: Aortic and hepatic cholesterol contents were lower in the *Lactobacillus casei*-treated groups as compared with the untreated control group. Compared with the control group, total cholesterol accumulation in aortas and livers showed a significant reduction in mice fed with *Lactobacillus casei* (P < 0.05). There was no significant difference among *Lactobacillus casei* treated groups (P > 0.05). (Figure 3-4)

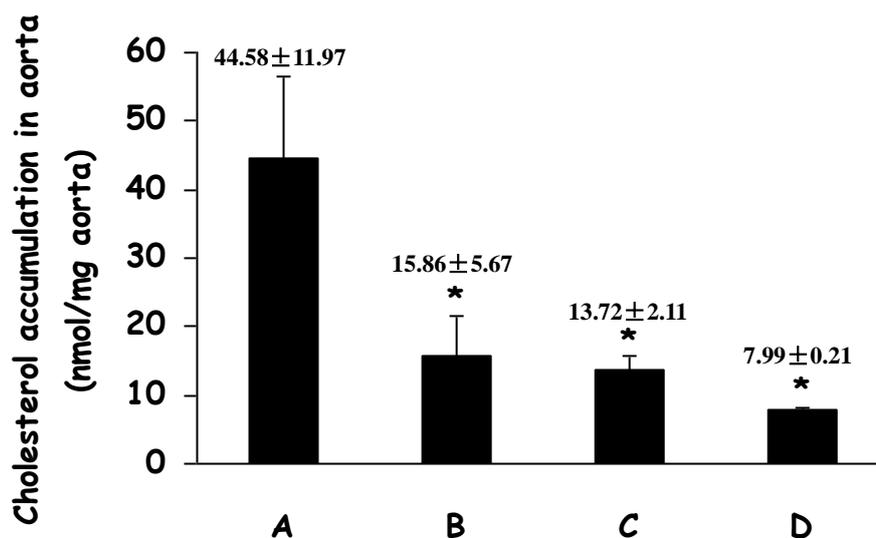


Figure 3. Cholesterol Accumulation in Aortas

After dissected extending from the aortic root to the right renal artery, the aortas were subjected to lipid extraction by a modified Folch method with chloroform/methanol (2:1, vol/vol). Total cholesterol content of the lipid extract was quantified with a fluorometric enzymatic assay. Results are expressed as means \pm SD, n=6. *P < 0.05 compared with Control.

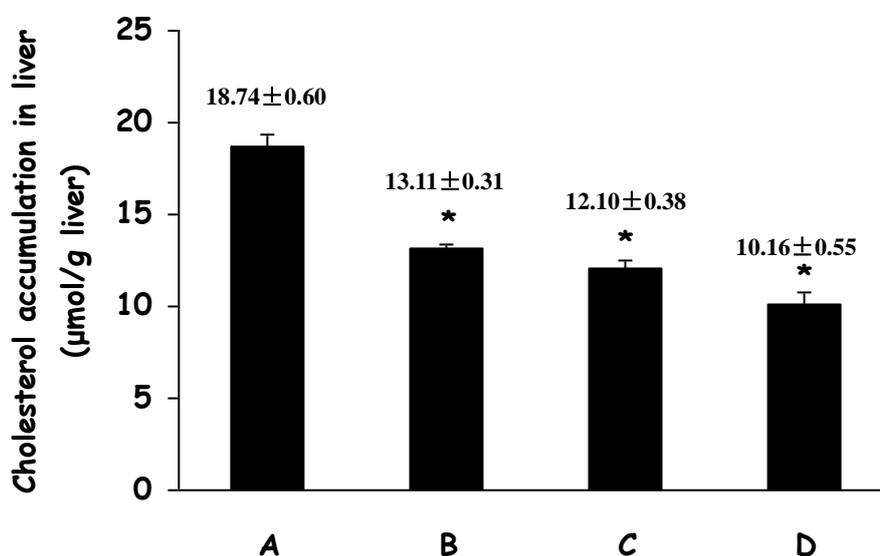


Figure 4. Cholesterol Accumulation in Livers

Immediately after animal sacrificed, livers were removed and heat processed. Weighed dried livers (300 mg) were extracted by chloroform: methanol (2:1, vol: vol) and stored at -20 °C until analysis. Results are expressed as means \pm SD, n=8. *P < 0.05 compared with Control.

DISCUSSION:

The results of the present study show that *Lactobacillus casei* dose-dependently reduced

atherosclerotic plaque areas in apoE-deficient mice. The *Lactobacillus casei* thus has the capacity to prevent atherosclerotic plaque formation and progression in apoE-deficient mice. Genetic knockout of apoE gene in mice has shown to result in accumulation of plasma remnant lipoproteins and development of atherosclerosis. ApoE-deficient mice develop severe hypercholesterolemia and atherosclerosis on a regular chow diet [18-19]. Thus this mouse model has been widely used to investigate the atherogenesis and dietary intervention. The present study showed that after 16 weeks of dietary intervention, apoE-deficient mice group exhibited higher serum cholesterol concentrations which in turn corresponded to greater cholesterol accumulation in both liver and aortic tissue. Supplementation of diets with *lactobacillus casei* significantly lowered serum TC, TG, LDL-C, and simultaneously enhanced HDL-C concentrations compared with the control group. In addition, Ataie-Jafari et al also evaluated the consumption of yoghurt containing *L.acidophilus* can decrease serum total cholesterol levels in mildly to moderately hypercholesterolemic subjects after 12 weeks [8].

Mann and Spoerry were the first to report a hypocholesterolemic activity of fermented milk in Kenya's Maasai tribe in 1974 [20]. Thereafter, dairy products with some species of *Lactobacillus acidophilus* ATCC 43121 in rats [21], *Bifidobacterium bifidum* NRRL 1976 [22] and *Lactobacillus* in hamsters were reported [23]. In addition to endogenously synthesised cholesterol, the absorption of dietary cholesterol and the reabsorption of biliary cholesterol in the small intestine also contribute to the regulation of plasma cholesterol concentrations. Moreover, reducing the intestinal absorption of dietary and biliary cholesterol can decrease plasma cholesterol concentrations [24].

It has been proposed that the mechanisms underlying the hypocholesterolemic activity of probiotics can be attributed to the inhibition of the absorption of exogenous cholesterol in the small intestine by following mechanisms [25-26]. First, some strains of *Lactobacilli casei* may survive in passage through the acidic stomach, particularly when they are ingested together with other foods. The live *Lactobacilli casei* may change the gut bacterial content and colonization due to their adhesion to host intestinal epithelium, promote the proliferation of beneficial gastrointestinal indigenous microflora and prevent the growth or invasion of pathogenic bacteria into the animal intestine [27]. Second, these probiotic bacteria in the large colon may ferment unabsorbed carbohydrates to produce shortchain fatty acids, including propionate, which lowers cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA R) [10]. Cholesterol synthesis is a multienzyme pathway in which HMG-CoA reductase mediates the rate-limiting step. Third, live lactobacilli casei cells may bind and absorb cholesterol, reducing cholesterol absorption in the intestine and leading to reduced serum cholesterol levels [28]. Liong and Shah reported that not only growing cells, non-growing but also dead lactobacilli cells could remove cholesterol from media [29]. Fourth, these probiotic bacteria may suppress the reabsorption of bile acids mediated by bacterial bile salt hydrolysis (BSH) through enterohepatic circulation [30]. BSH is an enzyme that catalyses the hydrolysis of glycine- and taurine-conjugated bile salts into amino acid residues and free bile acids. Live lactobacilli and

bifidobacteria cells can produce BSH, hydrolyze the conjugated bile acids, excrete them more rapidly, and reduce the extent to which they are reabsorbed [31]. The increased fecal excretion of bile acids leads to a decreased enterohepatic circulation of bile acids, followed by an increase in conversion of cholesterol to bile acids in the liver and an increase in cholesterol uptake from the circulation. Furthermore, a very recent study showed that the hypocholesterolemic effects of *Lactobacillus acidophilus* in rats are due to the reduction of cholesterol absorption mediated by the down-regulation of Niemann-Pick C1-Like 1 protein which is required for cholesterol absorption [7]. Whether hypercholesterolemic effect of *lactobacillus casei* in present study is associated with decreased cholesterol absorption needs further investigation.

Elevated cholesterol concentrations in the serum are associated with an increased risk of atherosclerosis and CHD, whereas a high concentration of serum HDL-C and a low ratio of TC to HDL-C are protective against CHD. A reduction as small as 1% in serum cholesterol concentrations has been shown to decrease the risk of CHD in human subjects by 2-3% [29]. Elevated serum HDL has been shown negatively correlated with the risk of atherosclerosis as HDL has an important role in cholesterol reverse transport by promoting cholesterol or cholesterol ester from peripheral tissues to the liver where cholesterol was metabolized and transformed into bile acids [32]. This pathway represents one critically important mechanism for reducing cholesterol concentrations in both blood and peripheral tissues, thus protecting against atherosclerotic plaque formation. In addition, it is also noteworthy that *Lactobacillus casei* supplementation decreased cholesterol deposition in tissues including liver and aorta which may explain for the reduced atherosclerotic plaque formation by *lactobacillus casei*. However, the possible molecular mechanism of *lactobacillus casei* affecting HDL and cholesterol accumulation in aorta and liver is still unknown and need further studies.

In addition to the dyslipidemia, oxidative and elevated inflammatory response are also the important risk factor of atherosclerosis [33]. Several studies demonstrated that lactobacilli strains have the properties of inhibiting oxidative stress and elevated inflammation response. Amal S et al evaluated that *lactobacillus casei* and *lactobacillus reuteri* could protect against oxidative stress in rats which fed aflatoxins- contaminated diet [34]. And other research in antioxidant ability of lactic acid bacteria suggests that some lactic acid bacteria strains not only could decrease the risk of reactive oxygen species accumulation through food ingestion but also degrade the superoxide anion and hydrogen peroxide [35]. Suman Kapila reported that rats supplementation of 5% lyophilized culture or fermented milk prepared using *L. casei* ssp *casei* for a period of 90 days decreased cholesterol levels by 15-25%, simultaneously decreased the levels of TBARS in the LDL fraction of plasma [9]. In a pilot study of elderly person, the intestinal lactobacilli are tightly bound to WBC count, blood glucose and content of oxidized lipoprotein which all serve as risk markers in pathogenesis of inflammation, metabolic syndrome and CHD. The particular species of probiotic lactobacilli could perform the benefits in terms of the health care of elderly people concerning the complex control of their metabolic and systemic inflammatory and oxidative reactions [36]. Therefore,

Lactobacillus casei supplemented to apoE ^{-/-} mice in the present study may also inhibit the atherosclerotic plaque through improvement of oxidative stress and inflammatory response in addition to the improvement in lipid profile.

CONCLUSIONS:

The presence of *Lactobacillus casei* in diet was effective at decreasing atherosclerotic plaque development in aortic sinus in apoE-deficient mice. This cardio-protective effect was related to several mechanisms that corresponded to improving serum total cholesterol concentration and decreasing cholesterol accumulation in the liver and aortic arterial tissue.

Conflict of interest: The authors have declared no conflicts of interest for this article.

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Authors' Contributions: The work presented here was carried out in collaboration between all authors. Zhihong Tang and Jing Ma defined the research theme, designed methods and experiments. Zhihong Tang and Zhen Zeng carried out the laboratory experiments, analyzed the data, interpreted the results and wrote the paper. Siping Wu and Mengjun Hou co-designed experiments, discussed analyses and interpretation. All authors have contributed to, seen and approved the manuscript.

Abbreviations: CHD, Coronary heart disease; AS, Atherosclerosis; CM, chylomicron; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TC, total cholesterol.

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