Kefir Supplementation Improves Lipid Profile and Oxidative Stress but does not Reduce Atherosclerotic Lesion in apoE Deficient Mice

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Abstract
Aim: Cardiovascular diseases are related to several risk factors, and diet and life style modifications are among the targets of the treatment and prevention. Consequently, natural compounds that act as protective agents are continuously studied. In this context, kefir solutions have been proposed as a potential adjuvant of treatment, since some studies showed its role in reducing oxidative stress, and blood lipids. The present study investigated the effects of brown sugar-fermented kefir solution on the associate risk factor and development of atherosclerosis.

Methods: Twenty-four Apo E KO mice received Kefir solution (50 g of kefir grains per liter of brown sugar solution (30% in water), or water for 4 weeks. Food and solution intakes were assessed weekly. At the end of experiment, blood, liver, aorta and heart were collected.

Results: The results showed that in Kefir group, HDL increased and triacylglycerols decreased significantly, as compared to the control group. Lipid peroxidation and catalase activity were also reduced in liver of Kefir supplemented mice. Despite those improvements, oxidized LDL and atherosclerotic lesions size in the aortic valve and aorta were similar between groups. Macrophage derived foam cells were also similar in aortic valve lesions of both groups.

Conclusion: Kefir supplementation, despite increasing HDL-c, is not associated to reductions of oxidized lipoproteins or atherosclerosis development.

Keywords
Kefir; Atherosclerosis; Probiotic; Lipids; Cholesterol; Oxidative stress

Introduction
Atherosclerosis is a multifactorial chronic inflammatory disease characterized by a prothrombotic status, lipid accumulation and fibrosis on sub-endothelium of arteries [1]. Therefore, the search for prevention and control of atherosclerosis by both drug therapy and life style modifications—including diet-is mandatory. In this context, foods that mitigate dyslipidemia, oxidative stress, as well as delay the inflammatory process are of great interest.

Kefir grains are made up of a complex mixture of bacteria and yeast encapsulated in a polysaccharide matrix [2,3]. Several species of bacteria (genera Lactobacillus, Lactococcus, Acetobacter and Leuconostoc) and yeasts (genera Kluyveromyces, Candida, Torula and Saccharomyces) were found in the kefir grains [4,5]. Kefir, known for thousands of years, is a fermented drink from kefir grains.

Several studies suggest different beneficial properties for health of kefir grains, including antitumor [6], hypoallergenic [7], antimicrobial, antioxidant [8], immunomodulatory [9-11], anti-inflammatory [2,3] and gastrointestinal regulatory activities [12]. Although some studies aimed to assess the effect of kefir on blood lipids [13,14], few of them analyzed the atherosclerosis development after kefir ingestion [15].

The apoE KO mice spontaneously develop hypercholesterolemia due to impairment of very low density (VLDL) and intermediate density lipoprotein (IDL) clearance. This effect acts as the atherosclerosis stimulus, increasing lipid deposition on arteries and immune cell migration to lesion site [16,17]. Differently of wild type mice, apoE KO mice present lesions similar to those seen in human, becoming an important model for the study of factors that influence atherogenesis [18], and consumption of diets high in fat and cholesterol [1,17].

Thus, our objective was to investigate the effects of kefir ingestion on the risk factors and in the atherosclerosis development, particularly on lipid profile and oxidative stress, using a well-known model of atherosclerosis, the ApoE deficient (ApoE KO) mice.

Methods
Preparation of kefir
The kefir grains were kindly donated by the University of Rosario Jose Velan (Unifenas-MG, Brazil). The qualitative and quantitative microbiological composition of these grains has been previously determined, and the predominant species of microorganisms (over 10⁶ cfu/mL) were Lactobacillus casei, Lactobacillus plantarum and Lactobacillus nagelei for bacteria, and Zygosaccharomyces fermentati, Saccharomyces cerevisiae and Torulaspora delbrueckii for yeasts [19].

Production of kefir solution
Kefir was obtained by the fermentation of kefir grains in brown sugar containing distilled water. This method was chosen because sugar fermentation is more easily obtained and preserved at room temperature, and also because this is the most popular fermentation technique in Brazil. The original volume of kefir grains was expanded by keeping an initial suspension in brown sugar containing distilled water. For that, suspension of kefir grains (50 g of kefir grains added to a broth of 1000 mL of distilled water containing 3% of brown sugar) were incubated into glass flasks at 25°C during 15 days. After that, samples
of this cultivated kefir were subdivided and incubated for 24 hours at room temperature. Flasks were not sealed to allow fermentation gas release. After 24 h fermentation, the suspension was carefully filtered in sterile quantitative (fast filtration) filter paper. The fermented (filtered) solution was used, while kefir grains were transferred to other flasks containing 1000 mL of distilled water, containing 3% brown sugar for more 24 hours. This procedure was repeated each 24 hours, until the end of the experiment. The pH, lactic acid bacteria and yeasts were measured after every cycle of 24 h fermentation.

**Experimental design**

The experimental protocol was approved by the animal care committee of the Federal University of Minas Gerais (CETEA #224/2008). Twenty-three 10-week-old female ApoE KO mice from the animal facility of the Department of Biochemistry and Immunology (UFGM, Brazil) were used. At the beginning of experiment, body weight and blood cholesterol (collected by tail puncture) were obtained. Mice were then divided into Control (CT) and Kefir (KF) groups, according to body weight (about 18 g), and total cholesterol about (350 mg/dL). Mice from CT group (n=11) were fed on chow diet (Labina), Brazil, and received water in the same pH of kefir solution. Animals from KF group (n=12) were fed on the same chow diet and received kefir solution replacing drinking water. Chow diet composition follows the recommendation of Institute for Laboratory Animal Research [20], containing 50.3%, 41.9% and 7.8% of carbohydrate, protein and lipids, respectively; and energy density of 2.18 kcal/g.

Water and kefir solutions were changed each two days to preserve its characteristics. Mice were housed six per cage in a temperature-controlled environment with a 12-h light-dark cycle, and fed food and water ad libitum. The mice were kept on their respective diets for 4 weeks. Body weight as well as solutions and food intakes were evaluated weekly. Food and solution intakes were measured considering the amount consumed in each cage divided by the number of animals per cage. The result represents the average intake calculated in each experimental week.

At the 4th experimental week, after overnight fasting and anesthesia, all mice were euthanized. Blood was collected by cardiac punction, and animals were gently perfused with 10 mL of PBS before collection of heart, liver, aorta and cecal content.

**Microorganism counts:** The kefir solution was submitted to microbiological analysis for total counts of lactic acid bacteria and yeasts, by surface plating on culture media after decimal dilutions (10⁻³, 10⁻⁴ and 10⁻⁵) of kefir suspension were spread inoculated onto Petri dishes containing de Man, Rogosa and Sharpe agar medium (MRS, Acumedia, Michigan, USA), supplemented with 100 mg/L of cycloheximide (Sigma, St. Louis, USA), and incubated under aerobic conditions for 72 hours at room temperature. The yeast count was performed using the same decimal dilutions to inoculate plates containing yeast malt extract agar medium (YME, Difco, USA), supplemented with 100 mg/L of chloramphenicol (Inlab, USA). Plates were incubated aerobically for 72-96 hours at room temperature. Result was given as number of colony forming unit (cfu)/mL of kefir solution.

**Blood analysis:** Sera were collected for lipid profile determination and plasma (EDTA, 6 g/L and potassium fluoride, 12 g/L, Gistab, Labtest, Brazil) were collected for glucose determination. Blood samples were centrifugated at 3,000×g for 10 min (Famem Centrimec 243, Brazil). Blood glucose, total cholesterol (TC), HDL cholesterol (HDL-c) and triacylglycerols were determined using commercial kits (Labtest, Brazil).

**Hepatic and cecal lipids determination:** Hepatic and cecal lipids were weighted and extracted by organic solvents according to Folch et al. [21]. Lipid extracts were dried overnight at 37°C, and total lipids gravimetrically quantified. Total cholesterol and triacylglycerols concentrations were measured by commercial kits (Labtest, Brazil), in lipid extracts diluted in 500 mL of isopropanol.

**Assessment of atherosclerotic lesions:** Hearts and aortas were first gently perfused with phosphate-buffered saline (PBS), and then with phosphate-buffer formalin (4%, pH 7.4) before collection. The hearts were removed and fixed with 4% paraformaldehyde for 6 hours, and then stored overnight in a 30% sucrose solution at 4°C. Next, the hearts were embedded in tissue freezing medium (Leica microsystems, Wetzlar–Germany), and 10 μm thickness sections for hematoxylin–eosin staining and immunohistochemistry assay.

**Immunohistochemistry:** Slides were treated using rat anti mouse macrophage MCA (Serotec, USA) as primary antibody and goat anti rat IgG: HRP (Serotec Starter, Serotec, USA). The stain was revealed by adding the substrate 3,3′-diaminobenzidine (DAB, sigma). Eight sections per heart, with 40 μm intervals between sections, were used to quantify lesion areas in the aortic valve using the Image-Pro Plus software (Media Cybernetics, USA).

Atherosclerosis in aorta was also assessed. The aortas were cleaned of adventitial fat, opened in the longitudinal sense and stained with Sudan IV. Lesion areas were assessed using Image-Pro Plus software (Media Cybernetics), and expressed as a percentage of the total luminal aorta surface [22].

**Determination of lipid peroxidation:** Lipid peroxidation was determined by the detection of thiobarbituric acid reactive substances (TBARS) technique [23]. TBARS levels are represented as malondialdehyde acid (MDA)/mg protein. Protein was assessed according to the method of Lowry et al. [24]. Hydrogen peroxide and hydroperoxide concentrations were determined according to Banerjee et al. [25].

**Anti-oxidized LDL autoantibody:** Levels of anti-oxidized LDL (oxLDL) autoantibody in the plasma were evaluated using the ELISA method, according to a previously described study by Lee et al. [26].

**Antioxidant enzyme assay:** Determination of superoxide dismutase (SOD) activity in the liver homogenates was based on its ability to scavenge superoxide (O₂⁻) radicals, decreasing the rate of auto-oxidation of pirogallol, according to the method of Dieterich et al. [27]. Catalase activity was assayed using the Nelson and Kiesow [28] protocol. The decomposition of H₂O₂ due to catalase activity was assessed by the decrease in the absorbance of H₂O₂ at 240 nm.

**Statistical analysis:** Data were analyzed by the Kolmogorov-Smirnov test to verify normal distributions, and Grubbs’ test was used for detection of outliers. Student’s t and Mann Whitney tests were used for parametric and non-parametric data, respectively. Statistical significance was accepted at level of p < 0.05. The analysis was made using Prism software version 5.0 (Graphpad Software, San Diego California - USA). Data were showed as means ± standard error (SE).
Results

pH and total lactic acid bacteria and yeast counts

The pH of kefir solution was 4.17 (± 0.6), at the end of 24 h period of fermentation. The results of microbiological counts showed that, after 24 hours of fermentation, lactic acid bacteria and yeasts reached population levels of 4.3×10⁸ and 6.6×10⁵ cfu/ml of kefir suspension, respectively.

Weight gain, liver and cecal lipids

Kefir supplementation did not affect food (CT=2.97 ± 0.10; Kefir=2.89 ± 0.14 mg/mouse/d) and liquid (CT=3.41 ± 0.14; 3.46 ± Kefir=0.29 mL/mouse/) intakes. Initial body weight (CT=21.38 ± 0.51 g; Kefir=21.24 ± 0.26 g), and weight after 4 experimental weeks are also similar between groups. The same occurred with cecal content (CT=94.10 ± 5.12; kefir=78.54 ± 11.88 mg/g) and liver (CT=114.0 ± 7.7 mg/g; kefir=126.3 ± 11.9 mg/g) lipids, suggesting that absorption of dietary lipids is not altered by kefir.

We also analyzed hepatic cholesterol (CT=3.09 ± 0.26; kefir=2.99 ± 0.34 mg/g) and triacylglycerols (CT=12.54 ± 0.31; kefir=13.61 ± 0.47 mg/g) concentrations that were similar between groups.

In caecal content, both cholesterol (CT=3.88 ± 0.87; kefir=4.71 ± 0.66 mg/g) and triacylglycerols (CT=4.65 ± 0.87; kefir=3.76 ± 0.60 mg/g) were unaffected by kefir supplementation.

Blood glucose, total cholesterol and LDL-c levels were not changed by kefir treatment. In Kefir group, HDL increased and TG decreased significantly, as compared to control group (Figure 1). These results suggest some improvement in lipid profile due to kefir supplementation.

Oxidative stress

Liver antioxidant capacity is improved by kefir. Although,
hydrogen peroxide, SOD activity and TBARS did not change in KF compared to CT group, hydroperoxides and catalase activity were reduced in liver of KF mice (Figure 2). Blood anti oxLDL antibodies were also similar in both groups, suggesting that kefir do not affect oxLDL formation in blood.

Atherosclerosis lesion evaluation

Our next step was to determine whether this improvement resulted in an impaired atherosclerosis development, as assessed in two different sites: abdominal aorta (Figure 3), and aortic valve (Figure 4). Data revealed no reduction in the area of aorta affected by atherosclerosis after kefir treatment (Figures 3A-3C). Lesion size in aortic valve was also similar in Control and Kefir groups (Figure 4A, 4C and 4E). Both groups showed lesions on intermediate stages, with a predominance of macrophage-derived foam cells. The presence of foam cell in lesion area was also assessed by showing similar results between groups (Figure 4B, 4D and 4F).

Discussion

Our results showed that kefir supplementation exerted some beneficial effects on lipoprotein profile, increasing HDL-c and reducing triacylglycerols. The increase in HDL-c seen in our study is in agreement with previous studies using milk-fermented or sugar-fermented kefir preparations [14,29]. In contrast, kefir was not effective in improving lipid profile in moderately hypercholesterolemic individuals after 4 weeks of supplementation [13]. These results may be due to differences between animal and human lipid metabolism, since in mice, rather than in humans, HDL-c represents about 70% of cholesterol-rich lipoproteins, while LDL-c represents only a minor fraction of cholesterol carrier in those animals. Alternatively, the duration of this clinical study (4 weeks) can be too short to detect changes in cholesterolemia in those individuals.

It is well known that atherogenesis is enhanced not only due to quantitative, but also to qualitative modifications of lipoproteins such oxidation. For this reason, we evaluated the possible effects of

![Figure 2: Measure of hydroperoxide (A), hydrogen peroxide (B), SOD (C) and catalase (D) activities and TBARS (E) in liver; and anti oxLDL antibodies (F) in serum of Apo E KO mice fed chow diet and drinking water (Control), or brown-sugar fermented kefir solution (Kefir) for 4 weeks. Bars represent average, and vertical lines standard deviation, n=11-12/group.](image-url)
kefir solution on oxidative stress in two sites: liver, the key organ on lipid production and lipoprotein metabolism, and in blood (through anti-oxLDL antibodies). Our results showed that liver from KF group mice showed a lower concentration of hydroperoxides compared to CT ones, but there were no differences in MDA concentration. It well known that hydroperoxide is a more specific marker of lipid peroxide than TBARS, since several primary and secondary non-lipid products (such as proteins and carbohydrates) can also react with thiobarbituric acid. This effect on lipid oxidation was also seen in previous in vitro studies that showed protective effects of kefir against oxidative damage of DNA, proteins and lipids [30,31]. Since kefir supplementation reduced hydroperoxide concentration, activity of two antioxidant enzymes, superoxide dismutase (SOD) and catalase were also evaluated. Although results showed no differences on SOD activity, catalase activity was reduced in KF mice. Catalase activity is regulated by several factors, including oxidized lipids that induce catalase expression [32]. We believe that the lower concentration of hydroperoxides in KF group down regulated catalase expression, and consequently, its activity as seen in our study.

Since non-HDL-c fraction, representing the atherogenic lipoproteins LDL, IDL and VLDL was not affected by kefir intake, we investigate levels of oxLDL. However, lipoprotein oxidation, rather than liver oxidation, is a central event on atherogenesis. In our study, anti-oxLDL antibodies were similar in both groups, suggesting the inefficacy of kefir supplementation in reducing oxidation of lipoproteins. The lack of effects of kefir in reducing LDL oxidation may be due to the more potent antioxidant defenses in blood than in liver. Human plasma is donated with an array of antioxidant defense agents, including ascorbate, urate and alpha-tocopherol among others. This antioxidant defense could mask a possible effect of Kefir on blood. Moreover, after oral supplementation, the amount of kefir antioxidant components that reaches blood is smaller than that arriving to liver via portal system. These results suggest that although kefir antioxidant properties are present in liver, it is not sufficient to alter levels of circulating oxLDL.

Despite reducing triacylglycerols and increasing HDL-c, kefir supplementation was not associated to decreases in atherosclerosis area in two sites, aorta and aortic valve. Since foam cells are the main cell population triggering lesion development, we also evaluated the proportion of macrophage-derived foam cells inside atherosclerosis lesion. Likely lesion area, foam cell infiltration in aortic valve was similar between groups. These results did not support beneficial effects of kefir on atherosclerosis development. This result was not expected, since HDL-c is an important atheroprotective factor. However, other important risk factors, such as concentration of native and oxidized LDL were not affected by kefir supplementation. LDL is.

Figure 3: Histopathological aspect and morphometry of aorta lesion area of Apo E KO mice fed chow diet and drinking water (Control), or brown-sugar fermented kefir solution (Kefir) for 4 weeks. Sudan IV staining of aorta for fatty streaks of Control (A) and kefir (B) groups. (C) Measure of fatty streak in aorta as percentage of total area. Bars represent average, and vertical lines standard deviation, n=10/group. In A and B, magnification= 4X.
assumed as *sine qua non* condition for atherosclerosis development and oxLDL is a key factor on atherogenesis. The similarity between groups of both native and oxLDL is in consonance with the lack of difference in atherosclerosis lesion. Similar result was observed in a previous study of our group studying Apo E KO mice orally treated with *Lactobacillus delbrueckii*, a lactic acid bacteria also isolated from kefir grains [2]. This probiotic lactobacillus increased HDL-c without reduce atherosclerotic plaque formation [33].

Uchida et al. [15] studied the effect of kefiran, an exopolysaccharide produced by *Lactobacillus kefiranofaciens* isolated from kefir grain, on atherosclerosis development in New Zealand white rabbits fed cholesterol-rich diets. The authors observed that atherosclerotic lesions were reduced after kefiran supplementation and concluded that kefiran prevents the onset and development of atherosclerosis in hypercholesterolemic rabbits by anti-inflammatory and anti-oxidant actions. Contrarily to Uchida et al. [15] that supplemented a single component of kefir, our animals received a filtered solution of kefir grains, which was composed by several sub products of lactobacillus and yeast fermentation. Nonetheless, *Lactobacillus kefiranofaciens* was not found among the predominant lactic bacteria of our kefir grain [19]. We believe that kefiran concentration in our kefir solution was irrelevent or insufficient to induce beneficial effects on atherosclerosis lesion. Moreover, there are several differences between atherosclerosis development between New Zealand white rabbits and Apo E KO mice. Although rabbits present early atherosclerotic lesions similar to human fatty streaks, they do not develop as tissue plaques, which are the trademarks of atherosclerosis in humans. On the other hand, Apo E KO mice plaques resemble human lesions, justifying the use of this
model in studies of factors affecting plaque size and composition [18].

The use of this well-defined murine model of atherosclerosis give us results closer to the possible effects of kefir on humans.

Altogether, our results suggest that although kefir supplementation increases HDL-c levels, it does not reduce major risk factors, such as native and oxidized LDL. As result, no beneficial effect was linked to its supplementation when atherosclerosis development is taken in consideration.

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