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# A casein hydrolysate based formulation attenuates obesity and associated non-alcoholic fatty liver disease and atherosclerosis in LDLr-/-.Leiden mice

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## Abstract

### Background

Obesity frequently associates with the development of non-alcoholic fatty liver disease (NAFLD) and atherosclerosis. Chronic inflammation in white adipose tissue (WAT) seems to be an important driver of these manifestations.

### Objective

This study investigated a combination of an extensively hydrolyzed casein (eHC), docosahexaenoic acid (DHA), arachidonic acid (ARA), and Lactobacillus Rhamnosus *GG* (LGG) (together referred to as nutritional ingredients, NI) on the development of obesity, metabolic risk factors, WAT inflammation, NAFLD and atherosclerosis in high-fat diet-fed LDLr-/-.Leiden mice, a model that mimics disease development in humans.

### Methods

LDLr-/-.Leiden male mice (n = 15/group) received a high-fat diet (HFD, 45 Kcal%) for 21 weeks with or without the NI (23.7% eHC, 0.083% DHA, 0.166% ARA; all w/w and $1x10^9$ CFU LGG gavage 3 times/week). HFD and HFD+NI diets were isocaloric. A low fat diet (LFD, 10 Kcal%) was used for reference. Body weight, food intake and metabolic risk factors were assessed over time. At week 21, tissues were analyzed for WAT inflammation (crown-like structures), NAFLD and atherosclerosis. Effects of the individual NI components were explored in a follow-up experiment (n = 7/group).

### Results

When compared to HFD control, treatment with the NI strongly reduced body weight to levels of the LFD group, and significantly lowered (P<0.01) plasma insulin, cholesterol,

consequent data collection and analysis. No other authors declare a conflict of interest. The specific roles of these authors are articulated in the 'author contributions' section.

**Competing interests:** M.H. Schoemaker and E.A.F. van Tol are employees of Mead Johnson Nutrition. No commercial products were tested in the current study, which was financially supported by MJN. In general, research on these ingredients are subject to patent filings. We hereby confirm that this commercial affiliation does not alter our adherence to all PLOS ONE policies on sharing data and materials.

**Abbreviations:** ALAT, Alanine aminotransferase; ARA, Arachidonic acid; CVD, Cardiovascular disease; DHA, Docosahexaenoic acid; eHC, Extensively hydrolyzed casein; HFD, High fat diet; LCPUFA, Long chain polyunsaturated fatty acid; LFD, Low fat diet; LGG, Lactobacillus Rhamnosus *GG*; NAFLD, Non-alcohol fatty liver disease; NI, Nutritional ingredients; SAA, Serum Amyloid A; VCAM, Vascular cell adhesion molecule.

triglycerides, leptin and serum amyloid A (P<0.01). NI also reduced WAT mass and inflammation. Strikingly, NI treatment significantly reduced macrovesicular steatosis, lobular inflammation and liver collagen (P<0.05), and attenuated atherosclerosis development (P<0.01). Of the individual components, the effects of eHC were most pronounced but could not explain the entire effects of the NI formulation.

## Conclusions

A combination of eHC, ARA, DHA and LGG attenuates obesity and associated cardiometabolic diseases (NAFLD, atherosclerosis) in LDLr-/-.Leiden mice. The observed reduction of inflammation in adipose tissue and in the liver provides a rationale for these comprehensive health effects.

## Introduction

The prevalence of overweight and obesity has risen dramatically in the past decades worldwide.[1] Obesity is a hallmark of the metabolic syndrome which represents a major global health problem that frequently associates with the development of non-alcohol fatty liver disease (NAFLD), and cardiovascular disease (CVD) as complications.

There are at least three critical periods for the development of obesity and its complications [2, 3]: during gestation and early infancy, in the period of adiposity rebound that occurs between 5 and 7 years of age, and in adolescence. The processes that contribute to the development of obesity have been extensively investigated and include increased calorie intake, nutrient overload, decreased physical activity, and changes in the gut microbiome.[4–7] It is thought that these processes contribute to the development of chronic inflammation in distinct adipose tissue depots, and that adipose tissue inflammation is a driving force for NAFLD and CVD development.[8–10]

Preventive nutritional strategies during critical periods in life seem promising to reduce the risk of obesity development.[11] A combination of dietary compounds that can affect multiple disease processes may be effective. We herein examined the impact of a combination of three specific nutritional ingredients (NI) on the development of obesity and its comorbidities in liver and vasculature. The specific NI are extensively hydrolyzed casein (eHC), long-chain polyunsaturated fatty acids (LC-PUFAs) Docosahexaenoic acid (DHA) and Arachidonic acid (ARA) and the probiotic strain *Lactobacillus Rhamnosus* GG (LGG). These NI are also present in infant formulas and combination treatment in early life has been shown to improve clinical health outcomes.[12–15] However, it is not known whether combination treatment with NI would counteract the detrimental effects of an obesogenic diet and prevent NAFLD and CVD on the long run.

Protein can have an impact on metabolism [16] and there are indications that casein hydrolysates exert anti-obesity effects in mice [17] and may alleviate inflammation in children.[12] Extensive casein hydrolysates are used for the dietary management of cow milk allergy, and hence are devoid of allergenic protein isotopes. But despite lacking proteins, these eCH contain smaller functional peptide sequences that may have a variety of biological effects, which are poorly understood. Extensive enzyme hydrolysis generates bioactive sequences and predominantly produces smaller peptides (95% lower than 1 kDa). As such, extensive casein hydrolysates can vary in peptide moieties and in milk proteins with unique bioactive sequences.[18]

Because of the unique properties of each hydrolysate, general health claims should not be made and dedicated studies are therefore required to better describe the functional peptide composition of these eCH in relation to their broader biological activity. In the case of LC-PUFAs, it has been proposed that their intake lowers adiposity and attenuates related complications, both in pre-clinical and clinical studies. However, most of these studies investigated the effects of the n-3 LC-PUFA DHA [19–21], whereas supplementation with n-6/n-3 LC-PUFAs at a particular ratio (2:1) has been associated with health effects in humans and animal studies. [14, 15, 22, 23] We therefore tested a well-defined n-6/n-3 LCPUFA mixture of ARA/DHA as present in breast milk. In addition to eHC and LC-PUFAs, we also employed a well characterized probiotic LGG that has been shown to affect inflammatory responses in children [24] and that may ameliorate experimental NAFLD.[25]

The aim of the present study is to investigate whether this combination of NI (eHC, ARA/DHA, LGG) may counteract the development of obesity and associated NAFLD and atherosclerosis. To do so, we used LDLr-/-.Leiden mice, which develop obesity and risk factors of the metabolic syndrome, and pathological endpoints akin to humans.

## Methods

### Animals and diets

Experiments were performed conform to the rules and regulations set forward by the Netherlands Law on Animal Experiments and were approved by an independent Committee on Animal Care and Experimentation (Dierexperimentencommissie Zeist, Netherlands; approval number 3277). Male low-density lipoprotein receptor-deficient LDLr-/-.Leiden mice were obtained from the breeding facility at TNO. Animals were housed in macrolon cages (3–5 mice per cage) during the experiment in clean-conventional animal rooms (relative humidity 50–60%, temperature ~21˚C, light cycle 7 am to 7 pm). Food and acidified tap water were supplied *ad lib*. Mice were fed standard lab chow (Ssniff R/M diet V1530, Uden, The Netherlands) until the start of the study at 12–15 weeks of age (that is when mice were young adolescent). Mice were divided (at t = 0) into three experimental groups ($n$ = 15 per group) that were matched based on blood glucose (primary matching parameter) and body weight (secondary matching parameter). Animals were fed for 21 weeks a high fat diet (HFD, 45 Kcal%) with or without specific NI or a low fat diet (LFD, 10%Kcal) as a reference. The LFD (diet D12450B, Research Diets, New Brunswick, USA) contained 19.2% w/w protein, 67.3% w/w carbohydrates and 4.3% w/w fat. The high fat diet (HFD) control group was fed a lard based diet (diet D12451 Research Diets, New Brunswick, USA) containing 23.7% w/w protein, 41.4% w/w carbohydrates and 23.6% w/w fat. Control mice were treated three times a week with PBS gavage (200 μL) to control for the effect of gavage treatment in the experimental group treated with NI. This group was fed the same high fat diet as the HFD control group but the intact casein protein was fully replaced by extensively hydrolyzed casein (HFD+NI diet, MJN, Evansville, IN), and the diet was supplemented with 0.083% w/w DHA and 0.166% w/w ARA (both from DSM Nutritional Products North America, Columbia, MD, USA). Three times per week (Monday, Wednesday, Friday at 11 a.m.), mice received *Lactobacillus Rhamnosus GG* (LGG, Chr. Hansen Holding A/S. Denmark) at $1x10^9$ CFU in 200 μL of PBS through oral gavage. The composition of the diets of the three groups is specified in S1 Table.

Additionally, an explorative follow-up study was performed (n = 7/group) under the same experimental conditions as described above, i.e. groups of mice were treated with HFD and a single component of the NI as well as the NI mixture for 21 weeks to explore the effects of the single components on body weight, metabolic risk factors and liver pathology.

## Body composition and food intake

Body weight (individually) and food intake (at cage level, n = 3–5 mice per cage) were monitored over time. Food intake was analyzed in week 3, 6, 9, 12, 15, 18 and 21 on the diets. In each week, food intake was assessed over a period of five days and then the average daily food intake in gram per mouse was calculated.

After 21 weeks on the diets, total body fat and lean body mass were assessed non-invasively by EchoMRI (EchoMRI LLC, Houston TX, USA). Conscious mice were placed in a constraint tube which was inserted into the EchoMRI for a period of approximately 30 s. During that time, total body fat and lean body mass were measured.

## Plasma and urine measurements

Blood samples were taken by tail incision in week 0, 3, 6, 9, 12, 15, 18 and 21 after 5 h fasting allowing a collection of approximately 50 μl plasma. Blood glucose was measured immediately using a hand-held glucometer in tail blood (FreeStyle Lite, Abbott, Alameda CA, USA). The remainder of the plasma was used for plasma lipid analysis, or stored at -80˚C for further analysis. Total plasma cholesterol and triglyceride levels were measured using kits No. 11489437 and 11488872 (Roche Diagnostics, Almere, The Netherlands), respectively. Plasma alanine aminotransferase (ALAT) levels were measured using a spectrophotometric activity assay (Reflotron-Plus, Roche). Fasting plasma insulin (Ultrasensitive mouse insulin ELISA, Mercodia, Uppsala, Sweden), soluble vascular cell adhesion molecule 1 (sVCAM-1; R&D Systems), leptin (R&D Systems), adiponectin (R&D Systems) and serum amyloid A (SAA; Biosource) were determined by ELISA in EDTA plasma. Serum C-peptide, resistin and GIP were determined by the 'Millipore metabolic hormones Multiplex kit' (MMHMAG-44K). The beads were read on a LiquiChip 200, (Qiagen, Hombrechtikon, Switzerland), and data were analyzed by the five parameter curve fitting in Luminex100 IS Software. HOMA-index was calculated as previously described.[26] To assess glomerular function, urine was collected during the study and urinary albumin (Exocell Inc. Philadelphia, PA, USA) and creatinine concentrations were determined (Bethyl Laboratories Inc. Montgomery, TX, USA) according to the instructions of the manufacturers.

## Sacrifice

Animals were not fasted at sacrifice. The mice were sacrificed after 21 weeks between 10 a.m. and 2 p.m. and liver and adipose tissue from inguinal, omental and epididymal depots were isolated for further analyses. Blood was collected by heart puncture to prepare serum. The heart including aortic root was used for atherosclerosis analysis.

## Adipose tissue analysis

From all three adipose tissue depots (inguinal, omental and epididymal), cross-sections were prepared from paraffin-embedded samples and stained with hematoxylin-phloxine-saffron. From each mouse and each fat depot, three cross-sections were evaluated for the presence of crown-like structures. The analyzed surface area of each cross-section was 580.000 $\mu m^2$, resulting in an analyzed area of in total 1.74 $mm^2$.

## Liver tissue analyses

Formalin-fixed and paraffin-embedded cross-sections (5μm) of the median lobe were stained with haematoxylin and eosin and scored blindly by a board-certified pathologist using an adapted grading method for human NASH [27]. Briefly, two HE-stained cross-sections/mouse

were examined and the level of macrovesicular steatosis was determined relative to the liver area analyzed (expressed as a percentage). In addition, liver lipids were analyzed biochemically by high-performance thin-layer chromatography (HPTLC) as described previously.[28] Briefly, lipids were extracted from freshly prepared liver homogenates following the Bligh and Dyer method [29] after which they were separated by HPTLC on silica gel plates. Then, lipid spots were stained with color reagent (5 g of $MnCl_24H_2O$, 32 ml of 95–97% $H_2SO_4$ added to 960 ml of $CH_3OH/H_2O$ = 1:1 (v/v)). Hepatic triglycerides, cholesteryl esters and free cholesterol were quantified using Image Lab, version 5.2.1 (Bio-Rad Laboratories, Veenendaal, the Netherlands) and expressed per mg liver protein, which was measured in the same homogenates used for the HPTLC analysis using the Lowry Protein assay.[30]

Hepatic inflammation was assessed by counting the number of inflammatory foci per field at a 100× magnification (view size 3.1 $mm^2$) in five non-overlapping fields per HE-stained specimen, expressed as the average number of foci per field. Fibrosis was assessed histochemically by Picro-Sirius Red staining (Chroma, WALDECK-Gmbh, Münster, Germany) and visualized using ImageJ software (version 1.48, NIH, Bethesda, MD, USA). The hepatic collagen content was quantified biochemically in freshly prepared homogenates of liver tissue using a total collagen assay (Quickzyme, Leiden, The Netherlands). This assay does not discriminate between different collagen types, and reflects the total hepatic collagen content. To further investigate effects on inflammation and fibrosis, hepatic gene expression was assessed by RT-qPCR. For this, RNA was extracted from snap-frozen liver tissue using RNA-Bee Total-RNA Isolation Kit (Bio-Connect, Huissen, the Netherlands). Nanodrop 1000 (Isogen Life Science, De Meern, the Netherlands) was used for spectrophotometric assessment of RNA concentration and RNA integrity was evaluated using 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). cDNA was synthesized from 1 μg of RNA using a High-Capacity RNA-to-cDNA Kit (Life Technologies, Bleiswijk, the Netherlands). Transcripts were quantified using TaqMan Gene Expression Assays (Life Technologies) and the following primer/probe-sets: *Tnf* (Mm00443258_m1), *Cd68* (Mm03047340_m1), *Ccl2* (Mm00441242_m1), *Tgfb1* (Mm00441724_m1), *Col1a1* (Mm00801666_g1), and *Col5a1* (Mm00489342_m1). *Ppif* (Mm01273726_m1) and *Hprt* (Mm00446968_m1) were used as endogenous controls. Changes in gene expression were calculated using the comparative Ct (ΔΔCt) method and expressed as fold-change relative to LFD as described previously.[31]

## Atherosclerosis analysis

Atherosclerosis was analyzed blindly in hematoxylin-phloxinesaffron-stained serial cross-sections (n = 4 of each mouse) of the aortic arch (40 mm intervals) and scored essentially as described [32] using an Olympus BX51 microscope and CelîD software (Olympus, Zoeterwoude, The Netherlands).

## Fecal energy content

Fecal samples at cage level (n = 3–5 mice per cage) were collected over a fixed period of one week in week 1, week 9 and week 21 of dietary exposure. Samples were dried and homogenized and energy content was assessed as a measure of reduced intestinal energy uptake. This was performed by using bomb calorimetry (IKA oxygen bomb calorimetry, company, country). Data were expressed as kJ/g feces and compared to food intake and body weight.

## Statistical analysis

SPSS Version 20 was used for statistical evaluation of the data. Two-Way repeated measures ANOVA with factors time and diet were applied to analyze for a diet, time and interaction

effect. One-Way ANOVA for individual time points were applied to analyze for differences at specific time points between groups. LSD post hoc test was used to compare groups. For non-parametric comparison, Mann-Whitney U tests were performed. One mouse in the LFD and one mouse in the experimental diet group were excluded from the data set and all analyses because they were statistical and biological outliers. $P<0.05$ was considered significant. All data are presented as mean ± SEM.

## Results

### Reduced body weight gain at higher food intake in mice fed HFD+NI

The dietary interventions with HFD or HFD+NI started when LDLr-/-.Leiden mice were young adolescent (12 weeks of age). Twenty-one weeks of HFD feeding with PBS gavage control resulted in a body weight gain of 25 grams in the HFD control group while LFD fed mice gained approximately 10 grams (Fig 1A). Body weight and body weight gain of mice fed HFD+NI were significantly lower than HFD fed mice and comparable to LFD fed mice.

Food intake in the HFD+NI group was significantly higher compared to HFD (Fig 1B). There was no effect on the fecal energy content which was determined as a measure of intestinal energy uptake (data not shown). Together these data indicate that animals fed HFD+NI can maintain a low body weight under obesogenic conditions, despite higher food intake.

### Intervention with NI reduces metabolic and cardiovascular risk factors

Plasma cholesterol increased gradually over time in the HFD group whereas plasma cholesterol in the LFD group increased modestly until week 9 and remained stable for the remainder of the study (Fig 2A). The HFD+NI group had low levels of plasma cholesterol throughout the study until 21 weeks (Fig 2A). Similarly, plasma triglycerides remained low in the HFD+NI group (Fig 2B) which underscored the pronounced effects on plasma lipids. Fasting blood glucose levels increased in the HFD group while they remained low in the LFD group. At several time points, blood glucose in the HFD+NI group was significantly lower than in the HFD group (Fig 2C). Plasma fasting insulin levels gradually increased over time in the HFD group

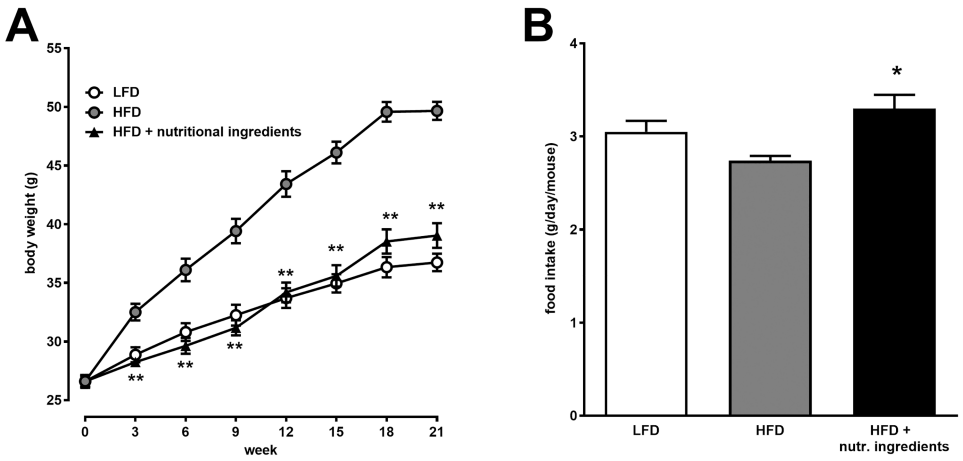


**Fig 1. Effects of nutritional ingredients on body composition and food intake in LDLr-/-.Leiden mice.** Changes in Body weight over time (A) and average daily food intake (B). Data are presented as mean ± SEM, n = 15. Significant diet effects are shown by *P<0.05 or **P<0.01 compared to HFD. LFD = Low Fat Diet; HFD = High Fat Diet + control gavage; HFD + nutritional ingredients (including eHC, ARA, DHA, and gavage with LGG).

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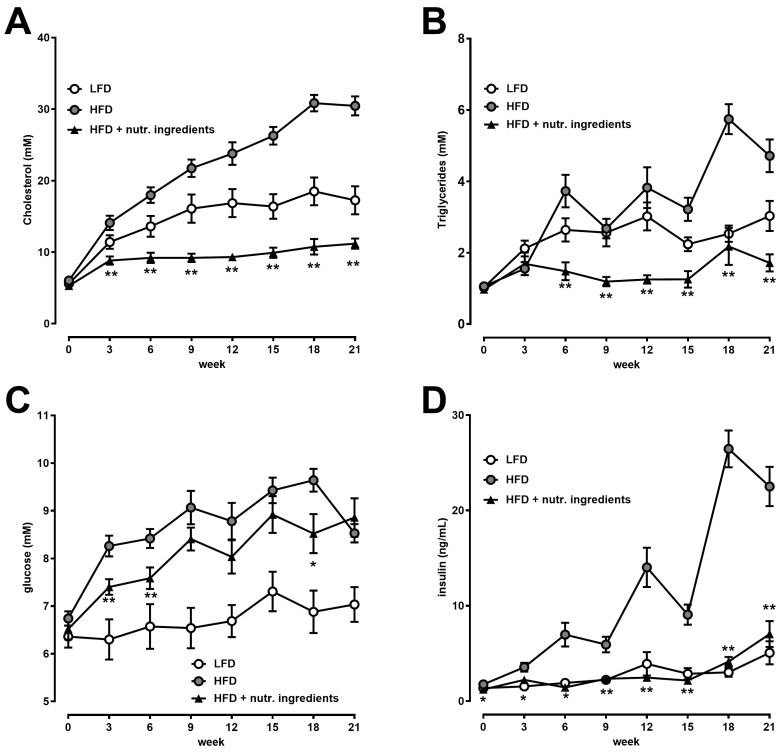


**Fig 2. Effects of nutritional ingredients on cardiovascular risk factors in LDLr-/-.Leiden mice.** Plasma levels at 21 weeks of dietary intervention for cholesterol (A), triglycerides (B), glucose (C), insulin (D). Values are presented as mean ± SEM, n = 15. Significant diet effects are shown by *P<0.05 or **P<0.01 compared to HFD. LFD = Low Fat Diet; HFD = High Fat Diet + control gavage; HFD + nutritional ingredients (including eHC, ARA, DHA, and gavage with LGG).

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whereas they remained low in the HFD+NI group comparable to the LFD group showing a significant difference versus HFD throughout the entire experiment (Fig 2D). In line with this, C-peptide, which is co-secreted with insulin, was significantly lower in the HFD+NI group compared to the HFD control group (Table 1). Also the HOMA-index data indicated that NI significantly reduced insulin resistance relative to HFD (Table 1).

Serum Amyloid A (SAA), a marker of systemic inflammation, and VCAM-1, a marker for vascular activation were comparable in LFD and HFD. SAA was significantly lower in the HFD+NI group compared to HFD (Fig 3A) and VCAM-1 tended to be lower (Fig 3B). Additionally metabolic hormones GIP and leptin were significantly lower in the HFD+NI group (Table 1). Overall, NI did not correct HFD-induced metabolic parameters to the level of LFD but typically attenuated the effect of HFD. Adiponectin plasma levels were higher in the HFD+NI group when compared to HFD with borderline significance (P = 0.07). No significant effects were observed for resistin plasma levels (Table 1).

## NI attenuates HFD-induced adiposity and adipose inflammation

Total body composition after 21 weeks of dietary intervention was analyzed non-invasively by EchoMRI (Fig 4A). Mice in the HFD+NI group had 39% less body fat and 10% less lean body mass compared with mice fed a HFD, showing a body composition similar to LFD fed mice. After sacrifice, adipose tissues were isolated and weighed to investigate the impact of dietary regimens on fat distribution between these depots. The epididymal fat depot was comparable

**Table 1. Effects of nutritional ingredients on metabolic hormones in LDLr-/-.Leiden mice.**

| | LFD | HFD | HFD + NI |
|---|---|---|---|
| **C-Peptide (ng/mL)** | 6.7 ± 1.6 | 38.2 ± 5.2 | 10.1 ± 2.0** |
| **GIP (pg/mL)** | 151 ± 35 | 194 ± 18 | 142 ± 11* |
| **Resistin (ng/mL)** | 9.7 ± 1.1 | 22.9 ± 1.8 | 20.1 ± 1.6 |
| **Leptin (ng/mL)** | 12.6 ± 2.0 | 52.5 ± 2.7 | 20.6 ± 2.5** |
| **Adiponectin (μg/mL)** | 6.9 ± 2.6 | 4.4 ± 1.0 | 5.3 ± 1.3$^{\$}$ |
| **HOMA-index** | 1.64 ± 1.54 | 8.65 ± 3.54 | 3.0 ± 2.59*** |

LFD = Low Fat Diet; HFD = High Fat Diet + control gavage; HFD + nutritional ingredients (NI, including eHC, ARA, DHA, and gavage with LGG). Significant diet effects are shown by

*P<0.05 or

**P<0.01 or

***P<0.001 or

$^{\$}$P = 0.07 compared to HFD.

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between the HFD+NI group and the HFD group, while tissue weights from the mesenteric and inguinal fat depots were strongly reduced by the nutritional ingredients (reduction of 52% and 54% respectively) and more closely matching adipose tissues from LFD fed mice (Fig 4B). Additionally, the number of crown-like structures were counted as a measure of inflammatory cell infiltration in the adipose tissue depots (Fig 4D and representative photomicrographs in Fig 4C). The HFD group developed a high number of crown like structures in the epididymal adipose tissue, while mice fed HFD+NI showed hardly any crown-like structures in this depot. Although in the mesenteric and inguinal depots only few crown-like structures were induced by HFD, still these were significantly lowered and hardly present in the HFD+NI group.

## NI protects against development of hepatosteatosis and liver inflammation

Liver integrity was assessed by analysis of circulating ALAT levels. HFD mice showed a substantial increase in ALAT levels over time, indicating liver damage. ALAT levels of the HFD+NI group remained low throughout the experiment and were significantly lower

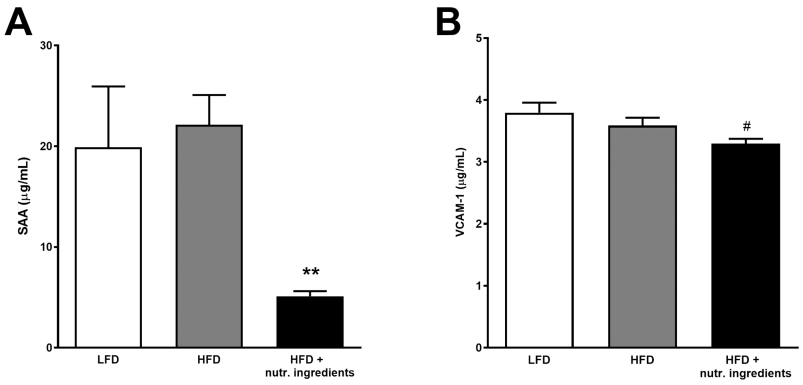


**Fig 3. Effects of nutritional ingredients on markers of systemic inflammation and vascular activation in LDLr-/-.Leiden mice.** Plasma levels of SAA (A) and VCAM-1 (B) at 21 weeks of dietary exposure. Data are presented as mean ± SEM, n = 15 Significant diet effects are shown by **P<0.01 or $^{\#}$P = 0.06 compared to HFD. LFD = Low Fat Diet; HFD = High Fat Diet + control gavage; HFD + nutritional ingredients (including eHC, ARA, DHA, and gavage with LGG).

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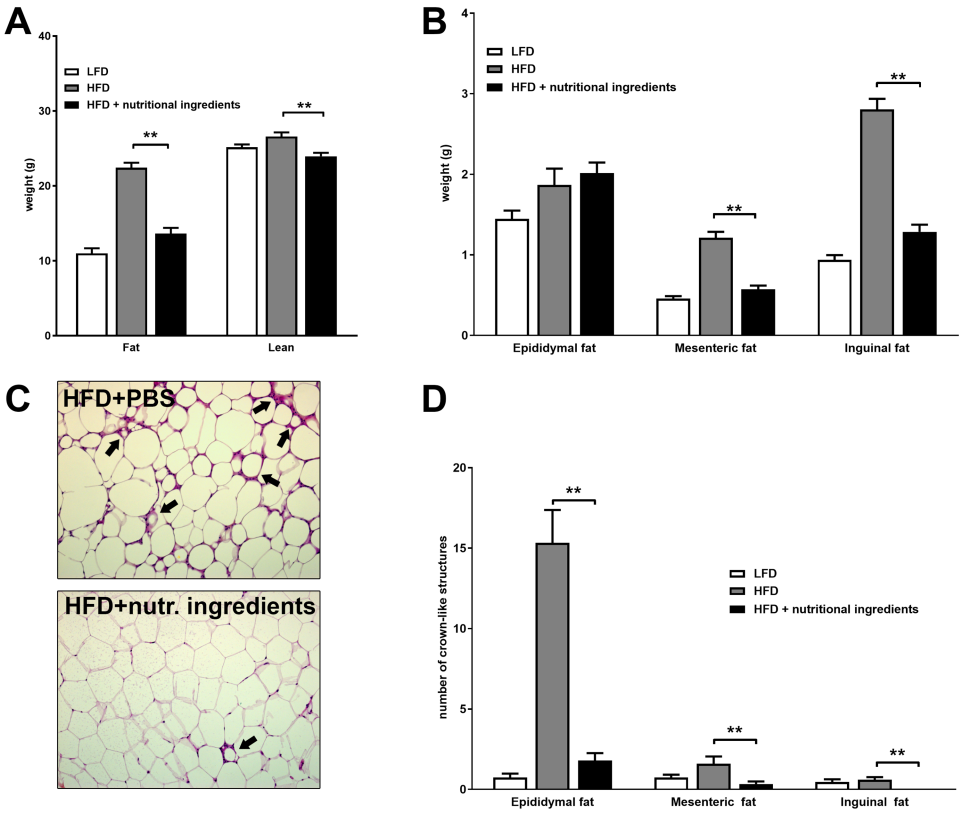


**Fig 4. Effects of nutritional ingredients on adipose tissue quantity and quality in LDLr-/-.Leiden mice.** Body composition analysis at 21 weeks of feeding regimes with fat and lean mass (A), with weights of epididymal fat, mesenteric fat and inguinal fat depots (B), representative histology of epidydimal WAT with inflammatory cells forming crown like structures (CLS, black arrows) (C) and quantitative analysis of CLS in different adipose tissue depots (D) (C). Data are presented as mean ± SEM, n = 15. Significant diet effects are shown by **P<0.01 compared to HFD. LFD = Low Fat Diet; HFD = High Fat Diet + control gavage; HFD + nutritional ingredients (including eHC, ARA, DHA, and gavage with LGG).

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compared to HFD control at all time points (Fig 5B). The liver weight of the HFD control group was markedly higher, while liver weight in the HFD+NI group was significantly lower and comparable to LFD reference animals (Fig 5A). Macrovesicular steatosis and lobular inflammation, both hall marks of non-alcoholic fatty liver disease were markedly and significantly lower in HFD+NI fed mice compared to HFD (Fig 5C and 5D respectively). Also the formation of total collagen (an indicator of fibrosis) was significantly attenuated in the HFD+NI group (Fig 5E and 5F).

These anti-steatotic, anti-inflammatory and anti-fibrotic effects were confirmed with independent biochemical measurement of hepatic lipids (triglycerides, cholesteryl esters and free cholesterol) and gene expression analysis of inflammation and fibrosis-related genes (i.e.. TNFα, CD68, MCP1 and TGFβ, Col1A1, Col5A1) (Table 2). These analyses showed that the anti-steatotic effects of NI were attributable to significant reductions in hepatic triglyceride levels as well as reduced levels of both unesterified (free) cholesterol and esterified cholesterol (Table 2). Hepatic gene expression analyses showed that NI attenuated expression of the pro-inflammatory cytokine TNFα and the macrophage marker CD68, while expression of the chemokine MCP-1 was not affected by NI. Addition of NI to the diet significantly reduced

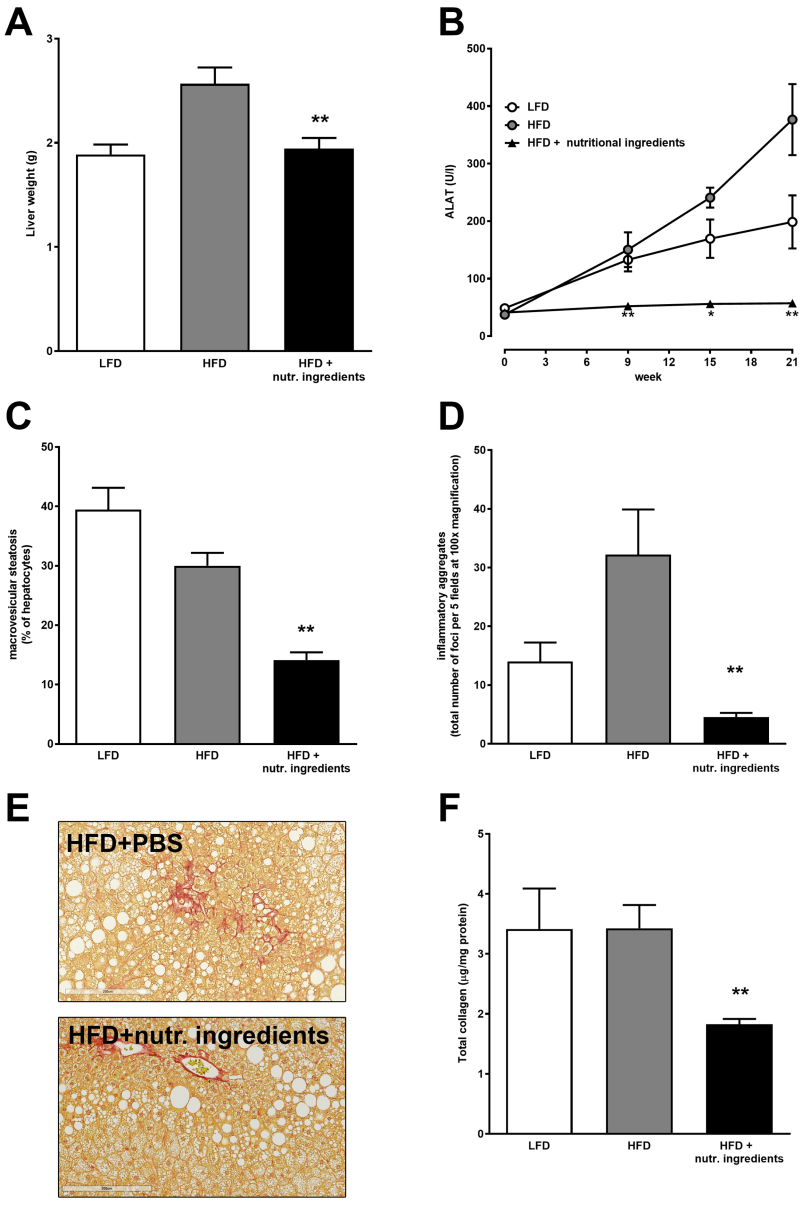


**Fig 5. Liver integrity and non-alcoholic fatty liver disease in LDLr-/-.Leiden mice.** Liver mass at 21 weeks of feeding regimes (A), alanine aminotransferase (ALAT) over time (B), liver macrovascular steatosis (C) and lobular inflammation (D). Representative Sirius Red staining of liver (E) and quantitative biochemical analysis of hepatic total collagen (F) at 21 weeks of dietary interventions. Data are presented as mean ± SEM, n = 15. Significant diet effects are shown by *P<0.05 or **P<0.01. Low Fat Diet; HFD = High Fat Diet + control gavage; HFD + nutritional ingredients (including eHC, ARA, DHA, and gavage with LGG).

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expression of the pro-fibrotic cytokine TGFβ and alpha-1 type I collagen. Expression of alpha-1 type V collagen was not affected.

## NI exerts anti-atherosclerotic vasculoprotective effects

Analysis of the urinary albumin/creatinine ratio showed higher levels in HFD relative to LFD. Treatment with the nutritional ingredients reduced microalbuminuria significantly (Fig 6A) suggesting a beneficial effect on the microvasculature. We next analyzed atherosclerosis

**Table 2. Effects of nutritional ingredients on hepatic lipids and expression of hepatic genes.**

| | LFD | HFD | HFD + NI |
|---|---|---|---|
| **Liver lipids (μg/mg protein)** | | | |
| liver triglycerides | 181.8 ± 23.2** | 227.7 ± 18.2 | 176.0 ± 25.3** |
| liver cholesteryl ester | 21.9 ± 3.1* | 27.4 ± 6.1 | 14.7 ± 3.4** |
| liver free cholesterol | 16.8 ± 1.9 | 17.5 ± 1.8 | 15.1 ± 0.8* |
| **Hepatic gene expression (fold change relative to LFD)** | | | |
| tumor necrosis factor alpha (*Tnf*) | 1.00 ± 0.23 | 1.32 ± 0.53 | 0.61 ± 0.17** |
| CD68 (*Cd68*) | 1.00 ± 0.20 | 1.10 ± 0.25 | 0.78 ± 0.53** |
| monocyte chemoattractant protein 1 (*Ccl2*) | 1.00 ± 0.42 | 0.84 ± 0.74 | 1.55 ± 1.22 |
| TGF-β (*Tgfb1*) | 1.00 ± 0.20 | 1.30 ± 0.42 | 0.89 ± 0.20* |
| alpha-1 type I collagen (*Col1a1*) | 1.00 ± 0.12** | 3.45 ± 2.92 | 0.56 ± 0.13*** |
| alpha-1 type V collagen (*Col5a1*) | 1.00 ± 0.18 | 1.15 ± 0.80 | 0.89 ± 0.20 |

LFD = Low Fat Diet; HFD = High Fat Diet + control gavage; HFD + nutritional ingredients (NI, including eCH, ARA, DHA, and gavage with LGG). Significant diet effects are shown by
*P<0.05,
**P<0.01,
***P<0.001 compared to HFD.

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development in the aortic valve area and observed pronounced atherosclerosis development in both HFD and LFD treated groups with total lesion areas of 211138 $\mu m^2$ and 180795 $\mu m^2$, respectively. Intervention with the nutritional ingredients strongly attenuated atherosclerosis development (39083 $\mu m^2$) further supporting a vasculoprotective effect of these specific nutritional compounds (Fig 6B and representative photomicrographs in S1 Fig).

## Health effects of NI are attributable to the combined effects of its constituents

To assess whether the observed health effects of NI can be explained by a specific constituent of NI or the combination of constituents, a small-scaled (n = 7/group) experiment was performed with its single components. As shown in Table 3, the extensively hydrolyzed casein

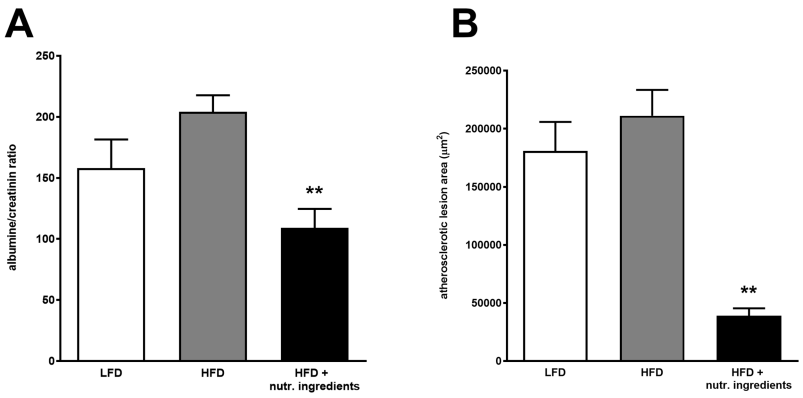


**Fig 6. Vasculoprotective effects of nutritional ingredients in LDLr-/-.Leiden mice.** Albuminurea at 15 weeks (A) and Atherosclerosis (B) at 21 weeks of dietary interventions. Data are presented as mean ± SEM, n = 15. Significant diet effects are shown by *P<0.05 or **P<0.01 compared to HFD. Low Fat Diet; HFD = High Fat Diet + control gavage; HFD + nutritional ingredients (including eHC, ARA, DHA, and gavage with LGG).

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**Table 3. Effects of individual nutritional ingredients.**

| 21 weeks | HFD | HFD + NI | HFD + eHC | HFD + LCPUFAs | HFD + LGG |
|---|---|---|---|---|---|
| **Body weight (g)** | 48.2 ± 6.3 | 42.1 ± 4.8** | 44.3 ± 5.0* | 50.2 ± 5.4 | 45.8 ± 5.3 |
| **Body composition (g)** | | | | | |
| Fat mass | 18.4 ± 5.7 | 14.4 ± 3.4* | 17.1 ± 3.1 | 21.3 ± 4.0 | 17.1 ± 4.6 |
| Lean mass | 28.1 ± 2.1 | 26.0 ± 1.7* | 25.4 ± 1.8** | 26.8 ± 1.3 | 27.1 ± 3.0 |
| **Insulin (ng/ml)** | 16.4 ± 9.1 | 8.2 ± 4.3$^{\$}$ | 12.1 ± 9.1 | 18.9 ± 11.9 | 17.6 ± 14.1 |
| **Triglycerides (mM)** | 3.5 ± 3.0 | 1.1 ± 0.6** | 1.5 ± 1.0* | 4.6 ± 3.0 | 3.0 ± 1.7 |
| **Cholesterol (mM)** | 24.3 ± 13.7 | 11.1 ± 3.3*** | 11.9 ± 3.1** | 28.8 ± 9.9 | 21.4 ± 7.3 |
| **ALT (U/L)** | 187 | 54 | 67 | 81 | 124 |
| **Liver histology** | | | | | |
| Macrovesicular steatosis (%) | 30.3 ± 12.6 | 11.3 ± 6.4*** | 19.3 ± 5.3** | 29.8 ± 10.6 | 23.6 ± 5.4 |
| Inflammatory aggregates (per 5 fields) | 29.4 ± 67.5 | 10.6 ± 6.6 | 7.5 ± 3.9 | 21.7 ± 21.5 | 15.6 ± 23.0 |

LFD = Low Fat Diet; HFD = High Fat Diet + control gavage; HFD + nutritional ingredients (NI, including eCH, ARA, DHA, and gavage with LGG). Significant diet effects are shown by

*P<0.05 or

**P<0.01 or

***P<0.001 or

$^{\$}$P = 0.07 compared to HFD (n = 7/group).

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component is largely responsible for the observed lowering effect of atherogenic plasma lipids (triglycerides and cholesterol). Other effects of the NI mixture cannot be explained by a single constituent (e.g. body weight, fat mass, insulin, ALT and liver histology). These data suggests that complimentary underlying mechanisms of action of the constituents may contribute to the health effects observed with NI.

## Discussion

In this study, we investigated the combined effect of specific nutritional ingredients (NI) with beneficial health effects, i.e. an extensively hydrolyzed casein, the LC-PUFAs DHA and ARA and the probiotic LGG, on the development of obesity and its comorbidities NAFLD and atherosclerosis. The study was performed in LDLr-/-.Leiden mice which develop obesity and dyslipidemia and associated NAFLD and atherosclerosis when treated with HFD. In this translational model, the applied concentration of fat is also reached in human diets [33], and NAFLD and atherosclerosis were scored using human-based grading systems.[27, 34, 35] The tested combination of NI mitigated HFD-induced obesity and the development of associated complications, NAFLD and atherosclerosis.

The combined NI strongly attenuated body weight gain, which could at least in part be attributed to a reduction of adipose tissue mass. Since the food intake in the HFD+NI group was even higher than in the HFD group, the effect on adiposity cannot be explained by a reduced caloric intake. Analysis of fecal energy content indicated that a comparable amount of energy was excreted, suggesting that the NI may have increased energy expenditure or locomotor activity which should be explored in dedicated future studies.

In humans increased adiposity is associated with elevated leptin and decreased adiponectin plasma concentrations [36], and HFD-treated obese Ldlr.Leiden mice reflected this change in adipokines. Anti-obesogenic dietary or lifestyle regimens can improve adipokine levels [36], and consistent with the anti-obesogenic effects, plasma leptin levels were decreased and adiponectin levels were increased. Previously, we showed that the eHC used herein is a potent

nutritional factor that stimulates adiponectin secretion from primary human adipocytes.[13] The observed glucose, insulin and C-peptide lowering effects in mice treated with NI that contains eHC could be in line with observations for other casein hydrolysates [17]. The suggested insulin sensitizing effect may possibly be related to the observed increase in adiponectin. It is well-established that adiponectin also reflect the quality and inflammatory state of fat tissue. [37, 38] It has become obvious that attenuation of chronic inflammation is an important aspect in driving health outcomes such as improving insulin sensitivity, liver function and cardiovascular risk.[39] Consistent with this notion, circulating SAA, a marker of low-grade inflammation [40] SAA was strongly reduced with the NI. SAA is produced and secreted by the liver as well as adipose tissue [41] and SAA directly participates in several processes that contribute to atherosclerosis (e.g. increased oxLDL retention time, stimulation of vascular remodeling).[42, 43] Consistent with the observed reduction of SAA and the reduction in VCAM-1, a vascular marker of inflammation, we found reduced atherosclerotic plaque area in mice that were treated with NI. It is well-established that Ldlr-/- mice develop atherosclerosis after prolonged periods of time, even on a low fat or chow diet.[26] The development of atherosclerosis is driven by LDL cholesterol (which is the primary lipoprotein species in Ldlr-/- mice), and inflammation. Since plasma lipids and the inflammatory state were elevated (typical baseline levels for SAA and VCAM-1 are $<$10μg/ml and $<$2μg/ml respectively) in both LFD- and HFD-fed mice, both groups developed atherosclerosis. An important finding of this study is that NI strongly attenuated inflammation in all adipose tissues analyzed. Reduced adipose tissue inflammation may provide a rationale for the attenuated NAFLD and atherosclerosis development since inflammatory factors released from WAT can drive these pathologies.[9, 44] An extensive time-resolved histological analysis of several adipose tissue depots during HFD-induced obesity revealed that inguinal and mesenteric adipose tissue depots of mice are less susceptible to develop HFD induced inflammation when compared to epidydimal WAT because they have a greater ability to expand [9]. Even these less susceptible depots showed a marked reduction of crown like structures upon NI treatment indicating a more generic anti-inflammatory effect of NI that applies to multiple depots. Reduced inflammation of WAT in absence of an effect on its mass was also observed with other anti-inflammatory interventions for NAFLD/NASH such as an inhibitor of caspase-1 [28] or inhibitor of Ccr2.[45]

Our finding that a dietary intervention can attenuate NAFLD development is relevant in light of the rise of obesity and NAFLD as the most common chronic liver disease worldwide which requires preventive (nutrition-based) strategies as an alternative to pharmacotherapy. [46] The beneficial role of extensively casein hydrolysate and LGG has been reported in other studies.[47] For example, the treatment of cow's milk allergic infants with extensively hydrolyzed casein formula containing LGG resulted in more butyrate-producing bacteria strains. [47] This short-chain fatty acid has been shown to improve intestinal barrier integrity and mucus synthesis [48, 49] which may contribute to observations of attenuated NAFLD and neurodegenerative disorders.[50] With the reported potency of butyrate and producing bacteria, the contribution of butyrate to the hepatoprotective effects of NI merits further investigation.

The explorative follow-up experiment with individual components indicated that the observed hepatoprotective effects of NI cannot be explained by one single constituent, but rather by the combined effects of all constituents, i.e. eHC, LC-PUFAs and LGG. The eHC appeared to exert more pronounced effects when compared to LC-PUFA and LGG at the dose tested, and affected metabolic readouts such as body composition, plasma lipids and insulin. Since the tested NI are constituents of infant formulas, future studies may investigate whether such dietary regimens will prevent obesity and disease development in children, the more so because complications such as NAFLD may already develop in childhood.[51]

## Conclusions

In conclusion, our data provide evidence that a combination of specific NI (extensively hydrolyzed casein, LC-PUFAs ARA and DHA, LGG) can reduce obesity and metabolic diet-induced inflammation at organ level (adipose tissue, liver) with pronounced attenuating effects on NAFLD and atherosclerosis. These results advocate research on dietary approaches for the management of obesity and its comorbidities and warrants further studies in humans.

## Supporting information

**S1 Table. Compositions of experimental diets.** Rodent low fat diet composition with 10 kcal % fat, a high fat diet composition with 46 kcal% fat and an isocaloric high fat diet composition with an extensively hydrolyzed casein and long-chain polyunsaturated fatty acids Docosahexaenoic acid (0.083%) and Arachidonic acid (0.166%).
(TIF)

**S1 Fig. Representative photomicrographs of cross-sections in the aortic valve area with lumen (L) and representative atherosclerotic lesions.** The LFD and HFD groups showed intimal thickening with pronounced atherosclerotic lesions as indicated (black arrows). Intervention with the combination of nutritional ingredients (extensively hydrolyzed casein, long-chain polyunsaturated fatty acids Docosahexaenoic acid, Arachidonic acid and probiotic *Lactobacillus Rhamnosus* GG) attenuated atherosclerosis development with mild intimal thickening and smaller lesions (white arrow).
(TIF)

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## Author Contributions

**Conceptualization:** MHS RK MCM JV KS EAFvT TK PYW.

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**Writing – original draft:** MHS RK MCM KS JV EAFvT TK PYW.

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