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Research Paper

Additive effects of lupin protein and phytic acid on aortic calcification in ApoE deficient mice☆

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ABSTRACT

Lupin proteins have repeatedly been shown to exhibit lipid lowering properties and reduce aortic calcification in atherosclerosis models. Despite many efforts on its identification, the component which is responsible for the observed effects is still under debate. Phytic acid which is generally associated with lupin protein isolates has currently been described as bioactive plant compound. The objective of the study was to determine the role of associated phytic acid for the described lupin protein effects.

A two-factorial study with ApoE knockout mice was conducted in which mice received lupin protein isolate or casein with or without phytase. Phytic acid was added to the casein diets to a final concentration identical to the lupin protein diets. Here we show that the serum concentrations of cholesterol, lathosterol and desmosterol were lower and the faecal bile acid excretion was higher in the groups fed lupin proteins than in the groups fed casein ($p < 0.05$). Mice that received the lupin protein diet containing phytic acid were characterized by a lower aortic calcification than mice of the other three groups ($p < 0.05$). In conclusion, our results show that the cholesterol lowering properties of lupin protein isolate were not caused by phytic acid. However, the hypocalcific action of lupin proteins appears to depend on the combination of lupin proteins and phytic acid.

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Introduction

Grain legumes such as soy, lupin, pea and faba beans are important protein sources in the human nutrition, particularly in the case of low meat or vegetarian diets, as they contain high amounts of proteins and show a favorable amino acid composition. Among them, soy and lupin have been assumed to exert cholesterol-lowering effects. Several studies that were conducted with mice, rats, rabbits, nonhuman primates and humans have shown that protein isolates from both legumes compared to casein may lower the plasma cholesterol concentration, especially under hypercholesterolemic conditions [1–7]. A few studies found also reduced aortic calcification and lesion size in rabbits and mouse models of atherosclerosis in response to soy and lupin proteins [2,6,8,9].

The plant protein components and the mechanisms that are responsible for the cholesterol lowering effects are still under debate. Several mechanisms of action have been proposed. Different components of the plant protein isolates have been tested for their lipid lowering properties. Single peptides and amino acids, but also plant compounds associated with the plant proteins are suggested to have an impact on plasma cholesterol (reviewed in [10–12]). Lupin protein isolates are associated with 14–25 g/kg of phytic acid [13,14]. Although phytic acid is considered as an anti-nutritive component due to its mineral binding [15], there has been increasing evidence for beneficial effects of phytic acid in the body. Grases et al. demonstrated that phytic acid reduces the renal and cardiovascular calcification in rats [16,17] and Lee et al. found dietary phytic acid associated with low cholesterol levels in diabetic KK mice [18]. Both effects of phytic acid are in many ways similar to those which are observed in lupin protein studies. The current

Abbreviations: 2WA, two-way ANOVA; ApoE, apolipoprotein E; C, casein; CD68, cluster of differentiation 68; KO, knockout; L, lupin protein isolate; PA, phytic acid.

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Author contributions: GIS supervised the study. GIS and AS wrote the manuscript with assistance from all other authors. AS carried out the animal experiment and performed the immunhistochemical analyses, quantified cholesterol, triglycerides, bile acids and minerals. FH, SG and JR analyzed cholesterol derivatives in serum samples.

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study aimed to test whether some of the beneficial effects of lupin protein isolate on plasma cholesterol and cardiovascular system are caused by the associated phytic acid. To this end, lupin protein isolate with and without the phytic acid-cleaving enzyme phytase was used and compared with phytic acid supplemented casein treated or nontreated with phytase. The study was conducted with ApoE knockout (KO) mice as a hypercholesterolemic atherosclerosis model.

Experimental procedures

Animals, diets and design

The experiment was conducted with 48 six-week old male ApoE KO mice with an initial weight of 19.0 ± 1.5 g (mean ± SEM). The ApoE KO (B6.129P2-Apoe^{tm1Unc}/J) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA), and were kept pair-wise in Macrolon cages in a room maintained with controlled temperature (21 ± 1 °C), humidity (50–60%), and lighting (6.00 am to 6.00 pm). Water was available ad libitum from nipple drinkers. All experimental procedures described followed established guidelines for the care and handling of laboratory animals.

All mice received a semi-synthetic western diet, containing (in g/kg diet) starch (264.5), sucrose (200), lard (200), cellulose (50), soybean oil (5), cholesterol (0.5) and vitamin and mineral mixture (60). Minerals and vitamins were added according to the recommendations of the NRC [19].

The study was conducted as a two-factorial design with the factors dietary protein and phytic acid. From the 48 ApoE KO mice 24 received a diet with defatted total lupin protein isolate (200 g/kg) from *L. angustifolius* (Fraunhofer Institute, Freising, Germany) as protein source. The lupin protein diets were supplemented with 4.4 g/kg of DL-methionine at the expense of sucrose to meet the requirements for methionine for growing mice and to avoid adverse effects on food intake and body weight. Our analysis showed that lupin protein isolates are associated with 20 g/kg phytic acid. To differentiate between effects of lupin protein and the concomitant phytic acid, phytase (Natuphos 10 000, BASF, Ludwigshafen, Germany) was added for phytic acid hydrolysis. Thus, 12 mice received a diet containing lupin protein isolate including associated phytic acid (lupin proteins + phytic acid group (L + PA)) and 12 mice received the same protein with added phytase to hydrolyse phytic acid (lupin proteins − phytic acid group (L − PA)). 24 mice received a diet containing casein (Meggle, Wasserburg, Germany) which served as control protein (200 g/kg diet). Since casein protein does not contain measurable amounts of phytic acid, phytic acid (Sigma Aldrich, Taufkirchen, Germany) was added to casein to a final concentration of 20 g/kg which was identical to the phytic acid content of lupin protein isolate. 12 mice received a casein diet containing phytic acid (casein group (C + PA)) and 12 mice received a casein diet where the phytic acid was hydrolyzed by phytase incubation (casein − phytic acid group (C − PA)) as described for the L-PA group. The diets were fed for 16 weeks. Food intake and weight of the mice were recorded weekly.

Characterization of the experimental proteins

The crude components of the dietary proteins were determined by official methods [20] and are shown in Supplementary Table S1. The amino acid contents of the proteins were analyzed as described elsewhere [14], and are shown in Table 1. Total phosphate and Mg^{2+} concentrations were determined after digestion in HNO_3 for 8 h at 170 °C using inductively coupled plasma optical emission spectrometry (ICP-OES) (Ultima 2, Horiba Jobin Yvon S.A.S, Longjumeau, France).

Table 1
Analyzed contents of amino acids in the experimental diets (g/kg diet)

	Casein diets (20% protein)	Lupin protein diets (20% protein)
Alanine	4.9	5.1
Arginine	5.8	18.4
Aspartic acid	11.4	17.3
Cysteine	0.6	2.3
Glutamic acid	33.8	36.7
Glycine	3.0	6.5
Histidine	4.6	4.1
Isoleucine	8.2	7.3
Leucine	15.7	13.1
Lysine	13.3	6.9
Methionine	4.5	0.6
Phenylalanine	8.6	7.1
Proline	18.0	7.3
Serine	9.3	8.2
Threonine	6.7	5.0
Tryptophane	2.2	1.5
Tyrosine	7.3	4.8
Valine	10.9	6.5

Analysis of phytic acid content

The content of phytate phosphate in the dietary proteins was analyzed according to Harland and Oberleas [21]. A description of the protocol is published elsewhere [14].

Phytase incubation of the experimental proteins

For all experimental diets 200 g of casein or lupin protein isolate, respectively, were mixed with 600 ml H_2O. The proteins for the C-PA and L-PA diets were treated with 40 mg phytase/200 g protein. Then, all protein-H_2O mixtures were incubated at 35 °C for 18 h. After 18 h no remaining phytic acid could be detected in the C-PA and L-PA diets, whereas in the C and L groups no changes in the phytic acid concentration were seen. The treated proteins were then added to the diets.

Sample collection

Faeces were collected over a period of 3 days during the last experimental week. Pooled faeces from two mice were frozen at −20 °C pending analysis. At the end of the experiment, the mice were anaesthetized with diethyl ether and killed by decapitation. Serum was collected. The liver was harvested. Samples of liver were snap-frozen in liquid nitrogen and stored at −80 °C pending further analysis. For preparation of the aortic root sections, the vasculature was perfused with 0.9% NaCl, and the ventricular edge and approximately 1 mm of the aortic root were immediately dissected under a stereomicroscope and cryomounted (Neg50, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Analysis of cholesterol, triglycerides and bile acids

Lipid extracts from liver and faeces were prepared as described recently [14]. Concentrations of cholesterol in lipid extracts and cholesterol and triglycerides in serum samples were quantified using enzymatic reagent kits according to the manufacturer's protocols (Diagnostic Systems, Holzheim, Germany).

Faecal bile acid contents were quantified using an enzymatic reagent kit (DiaSys Greiner, Flacht, Germany).

Quantification of serum cholesterol derivatives

Serum samples were saponified with ethanolic KOH. A mixture of deuterated cholesterol and ergosterol was used as internal

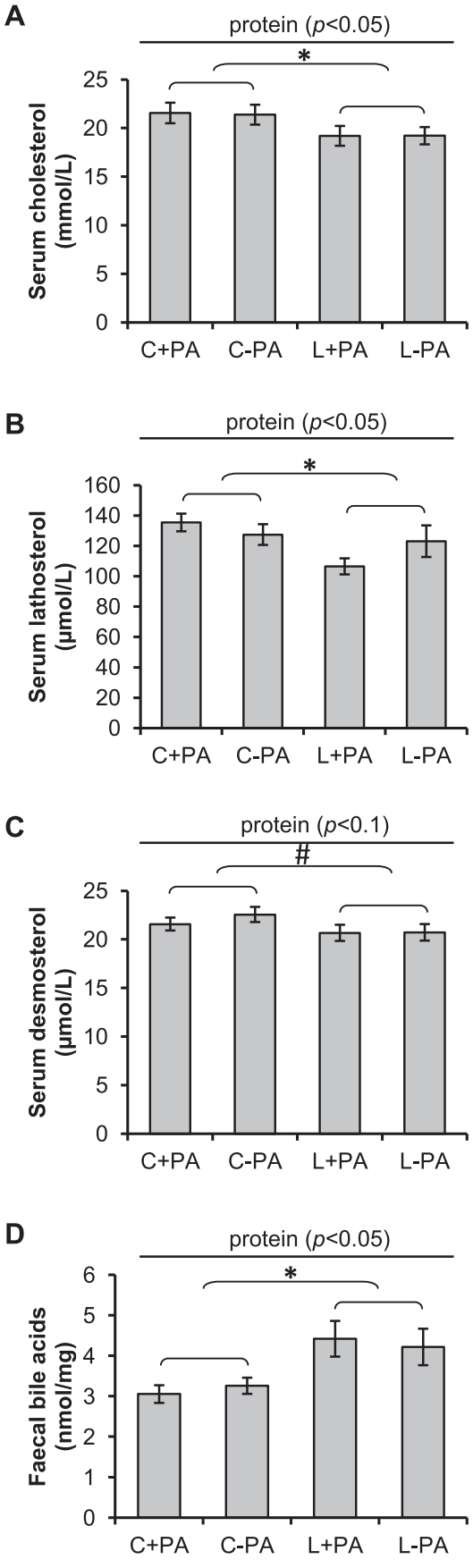

Figure 1. Effects of dietary protein and phytic acid on serum sterols and faecal bile acid contents. The serum samples were analyzed for cholesterol (A), lathosterol (B) and desmosterol (C) concentrations and also the faecal bile acid content (D) was analyzed. Quantification was performed as described in the materials and methods section. Values are means ± SEM of 12 mice per group for serum parameters and 6 faeces samples per group for bile acid content. 2WA = two-way ANOVA, $^{*}p < 0.05$.

standard. Samples were gassed with nitrogen and hydrolyzed at 37 °C for 3 h. Sterols were extracted twice with 0.5 mL hexane and washed with distilled H_2O. The solvent was evaporated and residues solved in hexane:isopropanol (99:1). For determination of cholesterol, desmosterol and lathosterol, sterols were derivatized to picolinyl esters according to the method of Honda et al. [22]. Dehydrocholesterol was derivatized with PTAD (4-phenyl-1,2,4-triazole-3,5-dione). The analysis of the cholesterol derivatives was performed by HPLC (Agilent 1100, Agilent Technologies, Waldbronn, Germany), coupled to an MS system (API 2000, Applied Biosystems, Darmstadt, Germany).

Serum concentrations of magnesium, inorganic phosphate and transferrin

The serum concentrations of Mg^{2+} and inorganic phosphate were measured spectrophotometrically according to the manufacturer's protocols (Analyticon Biotechnologies AG, Lichtenfels, Germany). Prior to analysis of Mg^{2+}, serum was diluted 1:4 with 0.9% NaCl to avoid interferences with triglycerides.

Transferrin concentration in serum was analyzed by means of a mouse-specific Elisa kit (E−90TX, BIOTREND Chemikalien GmbH, Koeln, Germany) according to the manufacturer's protocol.

Morphometric determination of atherosclerosis

Serial 7 µm thick slices (CM 1850 UV microtome, Leica, Nussloch, Germany) were collected, beginning at the aortic valve area. The following staining methods were used for the morphometric determination of the atherosclerosis. Oil red O staining was used to visualize vascular lipids and von-Kossa-silver was used to stain vascular calcification. The atherosclerotic lesions were characterized and quantified histomorphometrically (Axiovert 200 microscope and AxioCamMRc by use of Axiovision Rel. 4.8.2 software, Carl Zeiss, Jena, Germany). The cross-sectional surface area of the total vessel, the atherosclerotic lesion area as well as the lipid and calcification area were assessed to characterize atherosclerosis development in the aortic root. Movat-pentachrom staining kit (Morphisto GmbH, Frankfurt am Main, Germany) was used for further characterization of plaque composition.

Immunohistochemical analysis

The sections were fixed in acetone, and subsequently pretreated with 0.3% H_2O_2 in methanol to reduce non-specific background staining due to the activity of endogenous peroxidase. After blocking with 5% BSA in PBS anti-CD68 antibody (AbDSerotec, Oxford, UK) was used as primary antibody to detect macrophages. Afterward the sections were incubated with horseradish peroxidase-labeled secondary antibody (AbDSerotec). The immunocomplex was visualized using diaminobenzidinechromogen (Dako, Hamburg, Germany). Subsequently, sections were counterstained with Harris haematoxylin solution. The immunhistochemically stained slices were evaluated for the size and intensity of positive reaction using Axiovision software.

Statistics

Values are expressed as means ± SEM. Statistical analyses were performed using SPSS 20 (IBM, Armonk,

Table 2
Serum triglyceride, mineral and transferrin concentrations and cholesterol content of liver and faeces of ApoE KO mice treated with one of the four diets. Values are expressed as means ± SEM. Data were analyzed by two-way ANOVA

Dietary protein	Casein	Casein	Lupin proteins	Lupin proteins	Two-way ANOVA		
PA	(+)	(−)	(+)	(−)	Protein	PA	Protein × PA
Serum (n = 12/group)							
Triglycerides (mmol/L)	1.42 ± 0.22	1.56 ± 0.22	1.64 ± 0.15	1.25 ± 0.16	ns	ns	ns
Mg^{2+} (mmol/L)	1.43 ± 0.05	1.45 ± 0.05	1.72 ± 0.09	1.51 ± 0.08	$p < 0.05$	ns	ns
P_i (mmol/L)	3.76 ± 0.16	3.78 ± 0.21	4.46 ± 0.28	4.34 ± 0.20	$p < 0.05$	ns	ns
Transferrin (μmol/L)	322.81 ± 23.03	321.41 ± 22.14	322.03 ± 15.43	325.30 ± 13.22	ns	ns	ns
Liver (n = 12/group)							
Cholesterol (mg/g)	5.02 ± 0.29	5.20 ± 0.32	5.80 ± 0.34	5.11 ± 0.26	ns	ns	ns
Faeces (n = 6/group)							
Cholesterol (mg/g)	3.77 ± 0.34	3.69 ± 0.18	3.89 ± 0.24	4.01 ± 0.17	ns	ns	ns

Ns, no significant difference, PA, phytic acid.

NY, USA). Two-way ANOVA was used to compare the effects of dietary protein (casein vs. lupin protein isolate), phytic acid (+PA vs. −PA), and their interaction. When two-way ANOVA revealed a significant interaction between protein and phytic acid, a post-hoc comparison was performed. As all data showed variance homogeneity, means of the four groups were

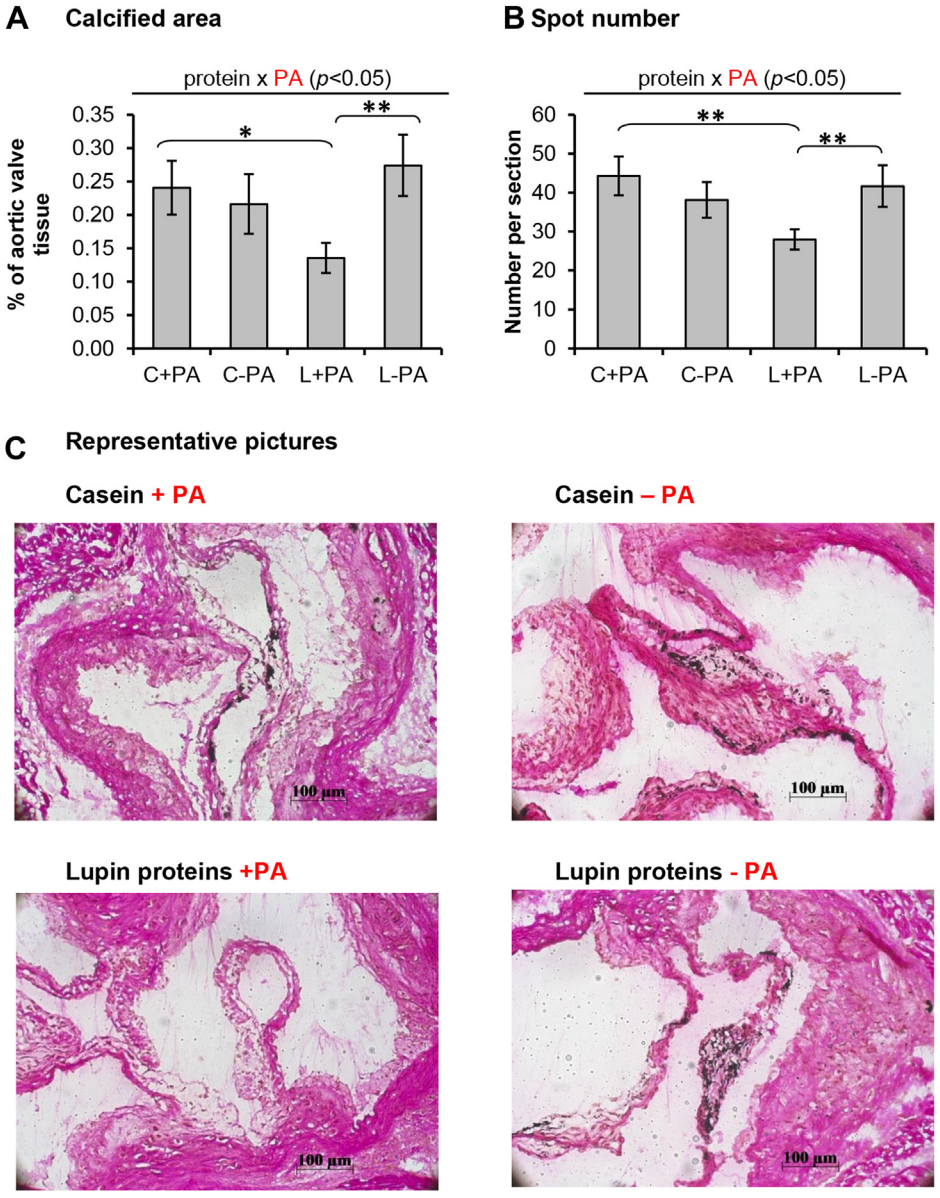

Figure 2. The impact of proteins and phytic acid on aortic calcification. The relative calcified area (A) and the number of spots per section (B) were analyzed in aortic root sections after von-Kossa silver staining. (C) Representative pictures of von-Kossa stained aortic root section of each group. The black staining shows aortic calcification. Values are means ± SEM of 12 mice per group. 2WA = two-way ANOVA, $^{**}p < 0.001$, $^{*}p < 0.05$.

compared by LSD test. Means were considered significantly different at $p < 0.05$.

Results

Amino acid composition of the experimental diets

Based on the amino acid analysis of the dietary proteins the amino acid contents of the experimental diets containing 20% protein was calculated (Table 1). The lupin protein diets were mainly characterized by higher contents of arginine and lower contents of lysine, methionine, tyrosine and valine than the casein diets. Minor differences were seen for aspartic acid, glycine and proline.

Food intake and body weight

Two-way ANOVA did not reveal main and interactive effects of dietary protein or phytic acid on final body weight (C + PA, 31.2 ± 1.3 g; C − PA, 31.5 ± 3.6 g; L + PA, 32.2 ± 2.5 g; L − PA, 32.4 ± 2.6 g; mean ± SEM) and food intake (C + PA, 5.10 ± 0.38 g/d per cage; C − PA, 5.48 ± 0.44 g/d per cage; L + PA, 5.24 ± 0.62 g/d per cage; L − PA, 5.26 ± 0.44 g/d per cage; mean ± SEM).

Lipid concentrations in serum, liver and faeces

Two-way ANOVA revealed a significant effect of the dietary protein on serum cholesterol and lathosterol concentrations. The concentrations of cholesterol and lathosterol in the serum of the mice that received lupin proteins (L + PA and L − PA) were significantly lower compared to the groups that were fed casein (C + PA and C − PA) (Fig. 1A, B). There was also a trend towards lower serum concentration of desmosterol in the lupin proteins vs. casein fed mice (Fig. 1C). Data did not reveal any phytic acid effect on serum lipids. Two-way ANOVA analysis found an effect of the dietary protein on faecal bile acid content (Fig. 1D). Bile acid content of faeces was lower in the groups fed lupin proteins compared to those fed casein. No effects of phytic acid on faecal bile acid contents were observed. Cholesterol contents of liver and faeces and also serum concentrations of triglycerides did not change in response to the dietary treatments (Table 2).

Serum mineral concentrations

Data revealed an effect of the dietary protein on serum mineral concentrations. Irrespective of phytic acid content, groups that received lupin proteins had higher concentrations of Mg^{2+} and inorganic phosphate in serum than groups that received casein. Phytic acid did not show any impact on serum concentrations of Mg^{2+} and inorganic phosphate (Table 2).

As shown in Table 2 the concentration of transferrin in the serum was neither influenced by the dietary protein nor by phytic acid.

Aortic atherosclerosis

At the end of the experiment all mice showed severe atherosclerosis. Two-way ANOVA analysis revealed a significant interaction between the dietary protein and phytic acid on the calcified area and the number of calcified spots in the aortic root sections (Fig. 2A, B). Mice that were fed the lupin protein diet with phytic acid developed less calcification than the L − PA group and tended to have a lower calcification compared to the C + PA group. The number of calcified spots per section was smaller in the L − PA group than in the C + PA group (Fig. 2B). Representative pictures are shown in Fig. 2C. The atherosclerotic lesion size, the lipid content and CD68 expression were neither influenced by the dietary protein nor by phytic acid (Fig. 3A, B, C). Lupin proteins fed mice were further characterized by a higher lumen size than those fed casein (Fig. 4A). The phytic acid displayed no effect on the lumen size. The relative size of the necrotic area visualized by the Movat-staining varied strongly between the mice, without showing any differences between the four treatment groups (Fig. 4B). Representative pictures are shown in Fig. 4C.

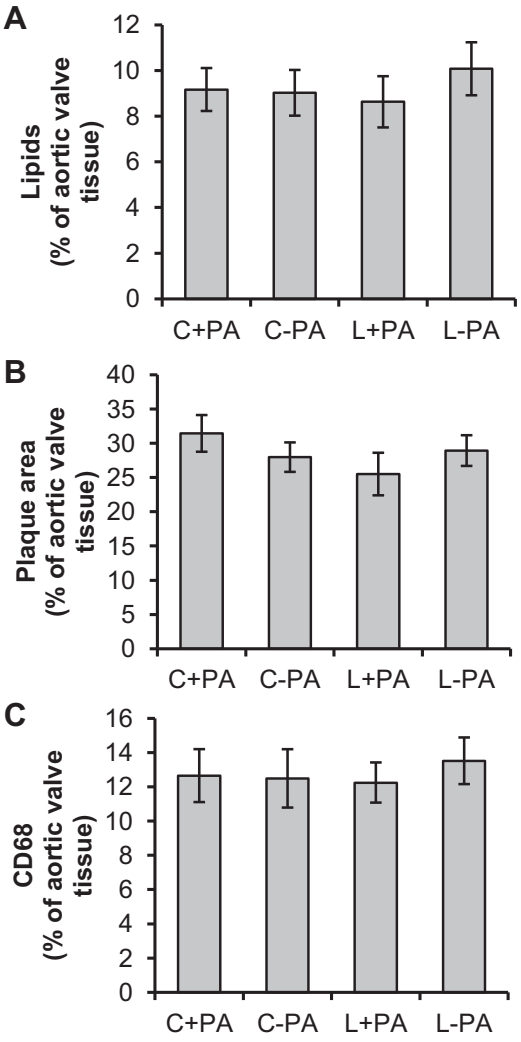

Figure 3. Effects of dietary protein and phytic acid on lipid content (A), plaque area (B) and CD68 expression, which is a marker protein of macrophages (C). Quantification was performed as described in the materials and methods section. Values are means ± SEM of 12 mice per group for all parameters. 2WA = two-way ANOVA, $^{*}p < 0.05$.

Discussion

Lupin proteins isolated from lupin seed by common food technology processes to produce food or to design experimental diets mostly contain considerable amounts of phytic acid [13]. Therefore the question arose whether the cholesterol-lowering and anticalcific effects of lupin protein isolates may result from the proteins and/or from the associated phytic acid. The current study showed that lupin protein isolate compared to casein was capable of lowering the serum cholesterol levels irrespective of phytic acid. These findings suggest that the cholesterol-lowering effect of lupin protein isolate was caused by the proteins, not by

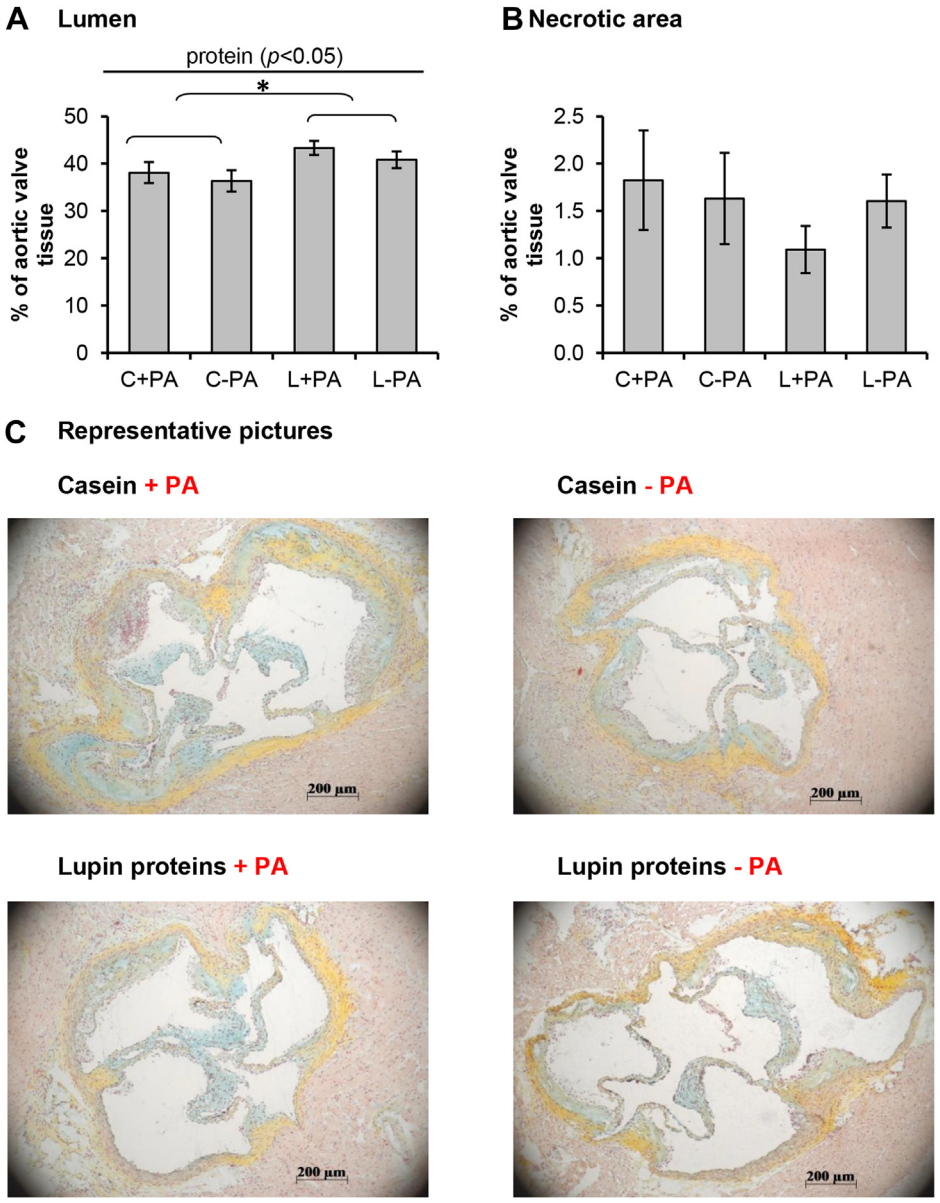

Figure 4. The impact of proteins and phytic acid on lumen size and necrotic area. The relative lumen size area (A) and the necrotic area (B) were analyzed in aortic root sections after Movat staining. (C) Representative pictures of Movat stained aortic root section of each group. The black staining shows aortic calcification. Values are means ± SEM of 12 mice per group. 2WA = two-way ANOVA, $^{*}p < 0.05$.

the phytic acid. If phytic acid alone would have influenced serum cholesterol we would have also seen a cholesterol-lowering effect in the C + PA group which received phytic acid. Recently, we found that pigs that were fed a lupin protein rich diet had a higher faecal output of cholesterol than pigs fed casein, which could have caused the lower cholesterol levels observed in these animals [14]. The current findings reveal an increased faecal output of bile acids in the lupin protein fed groups compared to the casein groups. We therefore assume that the cholesterol-lowering impact of lupin protein isolate was caused by an enhanced bile acid excretion. We assume that the enhanced bile acid output could have been caused by an altered expression of sterol transporters in the intestine upon lupin protein as found in a recent study with pigs in which lupin protein effects were compared to those of casein [14]. Quite a few studies reported on plant peptides with bile acid capacity. Those bile acid-binding activities have been described for corn protein [23], buckwheat protein [24], and also for lupin protein and their hydrolysates [25] which may explain the observed current findings.

The reduction in serum desmosterol levels, a precursor of cholesterol synthesis [26] in the lupin proteins groups compared to the casein groups is indicative of a reduced cholesterol synthesis in the lupin proteins treated mice. We conclude that the cholesterol-lowering properties of lupin proteins are independent of phytic acid and are caused by an increased sterol output and a diminished cholesterol synthesis.

All ApoE KO mice developed severe atherosclerotic changes in the aortic valve during the experimental period of 16 weeks. A particularly interesting finding of the study is that apparently only the combination of lupin proteins and associated phytic acid decreased the quantity of calcifications in the aortic valve. Mice that received lupin proteins associated with phytic acid were characterized by 44% less calcification compared to the C + PA group, 37% less compared to the C − PA group and 46% less calcification compared to the L − PA

group. The described anticalcific effects of lupin proteins confirm findings from a previous study with ApoE KO mice [2]. Additionally Grases et al. showed that phytate inhibits vascular calcification in different rat models [16,27]. The current data show that phytic acid evolved the anticalcific effect only in combination with the lupin proteins but not in the combination with casein, indicating a synergistic effect of both components on vascular calcification. Considering the fact that aortic calcifications are normally associated with reduced aortic elastance and cardiovascular mortality [28], the current data assume a preventive potential of lupin protein isolate only in the case of a lupin protein phytic acid-combination. However, the vascular effects of the lupin protein treatment was restricted to the effects on calcification, because the plaque area, the proportion of lipids and the macrophage content of the aortic roots remained unaffected in this group compared to the other groups.

On the basis of the current data, we can only speculate on the mechanisms of the observed synergistic actions of lupin proteins and phytic acid on vascular calcification. The emergence of vascular calcification could be favored by numerous factors such as oxidative stress, modified lipoproteins, oxysterols or high plasma phosphate levels [29–31]. Based on the fact that both lupin proteins groups had comparable serum concentrations of inorganic phosphate we assume that phosphate can be ruled out as a causal factor. Due to the failing differences between the L + PA and the L − PA group on transferrin level, which serves as a biomarker for iron status, we do not believe that pro-oxidative iron may explain the differences on vasculature. However, in the L + PA group we observed moderately higher serum levels of magnesium than in the L − PA group. As increased magnesium intake has been considered to slow arterial calcification [32], differences in magnesium may provide an explanation for the anti-calcific effect observed in the L + PA group.

Collectively, the current findings show that the cholesterol-lowering properties of lupin proteins are not caused by the lupin-associated phytic acid. However, the hypocalcific action of lupin protein on the vasculature was seen only in mice fed the combination of lupin proteins and phytic acid. Those interactions between these two biofunctional compounds of lupin proteins should be considered in future studies and in practical human nutrition.

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Supplementary Table1
Analyzed composition of the dietary proteins

	Casein	Lupin protein isolate
Dry matter	91.4%	94.2%
Crude protein	94.2%	95.7%
Crude ash	2.2%	4.6%
Crude fat	0.43%	2.7%
NDF	n.n.	0.99%
ADF	0.6%	0.43%
Lignin	n.n.	0.07%
Hemicellulose	n.n.	0.56%
Cellulose	n.n.	0.36%
P	0.78%	0.90%
Mg	0.005%	0.035%

NDF, Neutral detergent fibre; ADF, acid detergent fibre.