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Cholesterol-lowering effect of whole lupin (*Lupinus albus*) seed and its protein isolate

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ABSTRACT

This study describes the hypocholesterolaemic effect of whole lupin and its protein in hamsters. The diets were: casein (control group HC), lupin protein isolate (group HPI) and whole lupin seed (group HWS). Diets from HPI and HWS promoted a significant reduction of total cholesterol and non-HDL cholesterol in the hamsters' plasma as compared with HC. The true digestibility of HPI and HC groups were similar and differed significantly from the HWS one, which in turn showed a significant difference in total sterol excretion as compared to the former groups. Histological analysis of the liver revealed that animals fed on HPI and HWS diets presented a low level of steatosis (level 1) as compared to the ones fed on HC diet (level 4). Our findings demonstrate that protein isolate from *Lupinus albus* from Brazil has a metabolic effect on endogenous cholesterol metabolism and a protector effect on development of hepatic steatosis.

1. Introduction

Currently, the implications of hypercholesterolaemia and cardiovascular disease such as atherosclerosis, are the main problems facing the public health system and deserve more attention as dietary intervention is a very important procedure for preventing and even controlling this disease (Johnson, Chua, Hall, & Baxter, 2006; Kerckhoffs, Brouns, Hornstra, & Mesink, 2002). An unbalanced diet can have an impact of over 30% on the development of cardiovascular diseases, 35% in the development of cancer and 50% in the development of obesity (Holm, 2003).

Epidemiological studies and *in vitro* and *in vivo* tests in animals and humans show that diets based on the consumption of vegetables can have a hypocholesterolaemic effect and reduce the risk of chronic diseases (Anderson & Major, 2002; Craig, 1997; Frota, Mendonça, Saldiva, Cruz, & Arêas, 2008; Jones, 2002; Macarulla et al., 2001), especially for cardiovascular diseases. This action is exerted by biologically active substances such as proteins, oils, dietary fibres, phytosterols and saponins (Duranti, 2006; Potter, 1995; Roy, Boye, & Simpson, 2010; Sirtori, Galli, Anderson, & Arnoldi, 2009).

Among the legumes, soya bean and its protein fractions 7S and 11S, are those that are most studied because of their effect in reducing total serum cholesterol and LDL-c through the modulation of genes related to the lipid metabolism (Anderson, Johnstone, & Cook-Newell, 1995; Fukui, Tachibana, Fukuda, Takamatsu, & Sugano, 2004; Reynolds et al., 2006). Other seeds such as peas, lentils, chick peas, beans and lupin are also being investigated due to their chemical composition and great potential in the prevention of lipid disorders (Duranti, 2006; Sirtori et al., 2004; Smith et al., 2006). However, no study has been undertaken to demonstrate the cholesterol-lowering effect of the whole lupin (*Lupinus albus*) and/or its protein isolate using hamsters, the best type of animal for use in experiments involving a lipid metabolism (Frota et al., 2008).

Among legumes, lupin is the one that has the highest protein content in its composition apart from being a good source of fibres (Martínez-Villaluenga, Frías, & Valverde, 2006), giving it great potential for consumption. The lupin protein has two major fractions, albumins and globulins in a ratio of 1:9, respectively. The globulin is characterised by two dominant classes, fractions 7S globulin and 11S globulin, followed by fractions of α and β conglutinins, respectively (Nadal, Canela, Katakis, & ÓSullivan, 2011), similar to the same fractions present in soybeans that exert physiological features.

The amount of lipids present in the grain varies between the species, from 6% to 15%, with a high concentration of polyunsaturated

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fatty acids (Beneytout, Desmaison, Najid, & Rigud, 1987; Musquiz, Burbano, Rey, & Cassinello, 1989). The fractions of soluble and insoluble fibre range from 30% to 40%, practically double that of soybean (21.7%), peas (18%) and faba beans (19%) (Van Barneveld, 1999).

Considering the potential cholesterol-lowering effect of lupin due to its constituents and the lack of tests using an experimental model of the lipid metabolism similar to that of humans, the objective of this work was to investigate whether the whole lupin and its protein isolate have an effect on the reduction of cholesterol in hypercholesterolaemic hamsters fed on a diet containing high levels of saturated fats and cholesterol and which possible mechanisms were involved in this process. Our group, using the same protocol, has already reported different mechanisms for these effects in the reduction of cholesterol and has been undertaking studies of bioavailability to show which fractions could be responsible for them.

2. Materials and methods

2.1. Materials

Mature seeds of a sweet variety of white lupin were obtained from IAPAR (Agronomic Institute of Paraná), Londrina, PR, Brazil. Alkaloids were removed by soaking the seeds in water at 50 °C, three times a day, for five days. Afterwards, the seeds were oven dried at 50 °C and pulverised in a hammer grinder with a 0.4 mm sieve (whole flour). The dried alkaloid free seeds were manually decorticated, pulverised in a hammer grinder with a 0.4 mm sieve, defatted with hexane (1:5 w/v) for 4 h, under constant shaking, and oven dried at 50 °C to remove the solvent residues. The resulting lupin seed flour (LSF) was finally homogenised, stored in polyethylene bags and frozen (−18 °C) until analysis.

2.2. Preparation of protein isolates

Lupin protein isolates (PI) were obtained from lupin seed flour (LSF) as described by Liadakis, Tzia, Oreopoulou, and Thomopoulos (1995), with modifications to the extraction step. LSF was extracted (in the proportion 2:40, w/v) with 0.5 M NaCl at pH 10.0. The suspension was stirred for 30 min at room temperature and then centrifuged for 30 min at 10,000*g*. The supernatant volume obtained was further subjected to ultrafiltration (5 kDa pore size) and the permeate was discarded to reduce the initial volume to a few millilitres. Next, the supernatant was adjusted with 0.1 M HCl, to pH 5.0, for the isoelectric precipitation (pI) of protein, and centrifuged at 10,000*g* for 30 min. The protein pellet was re-suspended in water, adjusted to pH 7.0 with 0.5 M NaOH, freeze-dried and homogenised. This procedure guaranteed that all the protein fractions present in the whole grain were also in the isolated protein, verified by SDS-PAGE, and with less damage possible to its structure, confirmed by DSC (Fontanari et al., 2011).

2.3. Lupin seed and protein isolate composition analysis

The official methods of AOAC (1995) were used for proximate analysis. Water and ash content were determined gravimetrically, total protein by means of the micro-Kjeldahl method (N × 6.25), fat by diethyl ether extraction in a Soxhlet apparatus, crude fibre by an enzymatic–gravimetric method (Prosky, Asp, Schweizer, De Vries, & Furda, 1988), and carbohydrates by difference calculation.

2.4. Animals, diets and feeding procedures

Four-week-old male Golden Syrian hamsters ($n = 32$) were purchased from the animal house of the School of Medicine, University of São Paulo, São Paulo, Brazil. They were housed individually in stainless steel mesh cages under controlled conditions: temperature 23 ± 1 °C; 12-h periods of darkness and light (lights on from 8:00 a.m. to 8:00 p.m.); as well as free access to water and food. Preliminary tests were performed to assess all the methodologies employed in the animal assay. Thus, we decided to increase the group size and to sacrifice some animals for the baseline of cholesterol and fractions. After 7 days of adaptation time to a commercial diet (Nuvilab CR1, Brazil), 4 animals were killed to determine the basal levels of blood lipids. The hamsters (84.3 ± 7.4 g) were fed for 3 week *ad libitum* on a diet rich in saturated fatty acids (13.5%) and cholesterol (0.1%), containing 20% casein, to induce hypercholesterolaemia. At the end of this period, 4 animals were killed to check whether hypercholesterolaemia had been achieved. The remaining ($n = 24$) were randomized and assigned to 1 of 3 groups receiving the following diets *ad libitum* for 4 week: the casein group ($n = 8$), which was kept on the hypercholesterolaemic casein diet (HC); the hypercholesterolaemic whole seed group (HWS) ($n = 8$), which received a diet rich in saturated fatty acids and cholesterol but containing whole lupin seed; and the hypercholesterolaemic protein isolate (HPI) group ($n = 8$), which received a diet rich in saturated fatty acids and cholesterol but containing the lupin protein isolate.

The diets were formulated based on seed and protein isolate composition analyses (Table 1), and were designed to be isocaloric and identical in composition (including dietary fibre content) except for the protein source. The compositions of the experimental diets (HC, HWS, and HPI) are shown in Table 2.

Food intake was monitored daily and body weight, weekly. The food efficiency ratio (FER) was calculated as the ratio between body weight gained during the 4 week of experimental diets and the amount of food consumed over the same period. All the experimental protocols and procedures were approved by the Research Ethics Committees of the School of Pharmaceutical Sciences from São Paulo State University (Research Protocol N° 16/2009) and the Institute of Tropical Medicine (Research Protocol N° 54/2009), within the University of São Paulo, where this trial was performed.

2.5. Sample collection

During the last week of the feeding period (5 consecutive days), faecal samples were collected from the hamsters, which were housed in wired-bottomed cages. The samples were then weighed, dried at 50 °C overnight, weighed again and ground into a fine powder. At the end of the study, the hamsters were subjected to overnight fasting (14 h) and then their blood was withdrawn by cardiac puncture under anaesthesia, using ketamine (85 mg kg^{-1} of animal weight) and xylazine (8.3 mg kg^{-1} of animal weight) and the animals sacrificed. The blood samples were collected into heparin-moistened syringes, and plasma was obtained after centrifugation at 1500*g* for 15 min. The liver was excised, weighed, and washed with cold saline solution (9 g NaCl l^{-1}), and was kept in buffered formol. The animals were sacrificed under anaesthesia by hypovolemia.

Table 1
Composition of *Lupinus albus* whole grain and it protein isolate (g/100g on dry basis).

	Whole grain	Protein isolate
Moisture	5.53 ± 0.10	8.41 ± 0.45
Ash	1.17 ± 0.03	1.91 ± 0.07
Protein	36.47 ± 0.2	92.41 ± 0.4
Lipids	12.37 + 0.19	0.18 ± 0.4
Dietary fibre	49.99 ± 0.1	n.a.

n.a. not analysed.

Table 2
Formulation and composition analyses on experimental diets (g/kg of diet).

Ingredients	HC	HPI	HWS
Casein[b]	237.2	0	0
Lupin seed	0	0	548.3
Lupin protein isolate	0	216.14	0
L-methionine[c]	4.2	4.2	4.2
Tryptophan[d]	1.8	1.8	1.8
Lysine[d]	4.3	4.3	4.3
Sucrose	50	50	50
Corn starch	155.88	171.45	156.1
Maltodextrin	51.9	57.15	52
Cellulose	244	244	0
Soy oil	67.5	67.5	0
Coconut oil	134.3	134.3	134.3
Cholesterol	1	1	1
Choline chloride	3	3	3
Vitamin mix[c]	10	10	10
Mineral mix[c]	35	35	35
Nutrient content (dry basis)			
Crude protein ($N \times 6.25$)	211.4[a]	210.8[a]	213.1[a]
Crude fat	201.3[a]	199.6[a]	199.7[a]
Carbohydrate[e]	562. 1[a]	563.2[a]	560.7[a]
Cholesterol	1.0[a]	0.99[a]	1.0[a]
Ash	25.2[a]	26.4[a]	26.5[a]
Total energy (kCal/kg diet)[f]	4905.7[a]	4892.4[a]	4892.5[a]

HC = diet with casein: HPI = diet with lupin protein isolate; HWS = diet with whole lupin seed.
[a] Values in the same row followed by different letters differ significantly ($P < 0.05$).
[b] 84.3% of protein.
[c] In accordance with AIN-93 recommendations for growing rodents (Reeves, Nielsen, & Tahey, 1993).
[d] Amino acids limiting in this lupin (data not shown).
[e] Includes dietary fibre.
[f] Protein × 4, carbohydrate × 4, lipid × 9.

2.6. Plasma lipid lipoprotein analyses

Plasma total cholesterol (TC) and triacylglycerol (TAG) concentrations were measured with commercial enzymatic assay kits (Labtest, Brazil). HDL-cholesterol was measured subsequent to the precipitation of the apo B-containing lipoproteins with sodium phosphotungstate magnesium chloride (Labtest, Brazil, catalogue number Cat 13). The supernatant fraction was assayed for total cholesterol using the enzymatic kit for total cholesterol (Weingard & Daggy, 1990). Cholesterol concentration in the VLDL + LDL fractions was expressed as non-HDL-cholesterol and calculated as the difference between total plasma cholesterol and HDL-cholesterol. Calculation of LDL-cholesterol using Friedewald's equation was inappropriate in this case due to the different distribution of lipids across lipoprotein groups in hamsters as compared to humans (Goulinet & Chapman, 1993).

2.7. Analysis of faecal sterol and bile acids

The cholesterol concentrations in the oven-dried faeces were analysed after performing cholesterol extraction using petroleum ether. The solvent extract was dried, resolubilised in 500–3000 μl of hexane/isopropanol (97:3), filtered through a 0.45-μm membrane, and injected into an HPLC system, as described by Chen and Chen (1994). This was a Shimadzu HPLC with PDA detector, equipped with a Luna Phenomenex linked to a cyan column (5 μm) of 4.6 × 150 mm. The mobile phase used was hexane/isopropanol (97:3, v/v) solution flowing at 1 ml/min. Each run took about 7 min and spectra were recorded from 190 to 300 nm and chromatograms at 206 nm. Quantification was carried out by means of daily external standardisation, using an external reference curve for standard cholesterol (Sigma nr C-8667; Sigma–Aldrich do Brasil Ltd., São Paulo, SP, Brazil). The cholesterol peak was identified and also checked for its purity by means of spectra obtained using the photodiode array detector. The software used was Class-VP 10 (Shimadzu do Brasil, São Paulo, SP, Brazil). Total faecal bile acid was measured from faeces extracts using an enzyme recycling rate assay kit (DZ042A, Diazyme, San Diego, Calif., U.S.A.). The oven-dried faeces were extracted using a mixture of tert-butanol and water (50:50) for 15 min at 37 °C. Each sample was centrifuged at 10,000*g* for 2 min (Van Der Meer, Vries, & Glatz, 1985).

2.8. True digestibility of proteins

True protein digestibility (TD%) was calculated according to the FAO/WHO (1991) method. The faeces of animals assigned to each dietary group, housed in wired-bottom cages as described previously, were collected after day 23, over 5 d. Another group (10 hamsters) receiving a similar diet to those of the other groups, except for the absence of protein, was used as the reference. Nitrogen of all samples was determined through the micro-Kjeldahl method. The true digestibility for each hamster was calculated as follows:

$$\mathrm{TD}\% = \frac{I - (F - F_K)}{I} \times 100$$

where TD is true digestibility, I is nitrogen intake (g), F is nitrogen excreted in faeces by hamsters of experimental diet, and F_k is the nitrogen excreted in faeces by hamsters in a diet free of protein.

2.9. Histological evaluation

Specimens for histological examination of liver were fixed in 10% formalin and stained with haematoxylin–eosin (H&E). The histological evaluation included semi-quantitative analysis of vesicular fat presence. All sections were coded and analysed blindly by the pathologist, who had no knowledge of the diet consumed by the animals. The fat accumulation (steatosis) was categorised by visual assessment as: 0 = absence of steatosis, 1 = focal steatosis (<50% of lobule central veins), 2 = steatosis in >50% of lobule central veins, 3 = comprehensive steatosis, 4 = comprehensive and intense steatosis.

2.10. Statistical analyses

The values were presented as means with their standard errors (±SE). Statistical analyses using one-way analysis of variance (ANOVA) were performed to test the significance of differences ($P < 0.05$) between groups. When the interaction was significant, the Tukey multiple comparison test was used as a *post hoc* test. The statistical analyses were performed using the SPSS software, version 13.0 for Windows (SPSS, Inc., Chicago, Ill., U.S.A.).

3. Results and discussion

To answer the question about the hypocholesterolaemic effect of whole lupin seed and its protein isolate, it was necessary to undertake a process of isolating the protein without damaging the composition and structure of the protein in relation to the protein in the whole seed. Various conditions for obtaining the protein isolate were tested (Fontanari et al., 2011) and the electrophoresis profile and DSC (differential scanning calorimetrical) were adopted as parameters of quality control for the protein content.

Proximal composition of lupin and its protein isolate are presented in Table 1. The results show that the process of isolating the lupin protein was efficient, presenting 92% purity and only traces of fat. It can be seen from these results that the major component of lupin was the dietary fibre fraction followed by protein and lipids.

The compositions of the experimental diets (HC, HWS, and HPI) are shown in Table 2. The diets were formulated based on seed and protein isolate composition analyses (Table 1), and were designed to be isocaloric and identical in composition except for the protein source. All diets presented the same energy density, ash, crude fat, carbohydrate, and cholesterol contents, not differing statistically ($P > 0.05$). The growth parameters for the hamsters and the true digestibility of the experimental diets are summarised in Table 3.

A daily ingestion and total consumption of the diets was equal for all the groups. The weight increase of the animals after 4 weeks did not ($P > 0.05$) significantly differ between the HC and HPI groups, however, weight gain in the HWS group was significantly ($P < 0.05$) higher. There was no correlation of weight of the liver for every 100 g of body weight to the weight gain. The HWS group presented the lowest liver weight and differed ($P < 0.05$) significantly from the HC group, which was the heaviest. HPI group showed an intermediary weight not differing ($P > 0.05$) between the other two groups.

Frota et al. (2008) investigated the cowpea bean, in a similar protocol experiment and reported that the lowest weight of the liver was for the group that consumed whole beans in relation to the other groups, also observing that in this same group there was a greater excretion of total sterols in the faeces, this being a possible mechanism which could explain the hypocholesterolaemic effect of whole legumes.

Fig. 1 shows the results obtained at the end of the experiment for the lipid profile of the animals. The reduction of total cholesterol can be seen from the chart for the HPI and HWS groups in relation to the HC group ($P < 0.05$), reduced to 15.3% and 16.88%, respectively. The same behaviour was observed for the values of non-HDL cholesterol with a significant ($P < 0.05$) reduction for HPI and HWS groups compared to the HC group. On the other hand an increase in the fraction HDL-c for the HWS group was observed, differing ($P < 0.05$) significantly from the other groups. A reduction was observed in the triglycerides levels for the HPI group in comparison to the HWS group although both groups were no different to the HC group.

This behaviour can be attributed to the mechanism reported by other authors studying lupin proteins and their effects on metabolism. Sirtori et al. (2004) reported that lupin proteins are capable of stimulating the activity of LDL receptors, increasing the capture of LDL from the plasma to the cells. On the other hand, the inhibition of HMG-CoA reductase, a key enzyme in the synthesis of cholesterol, regulated by the action of SREBP-2, could also reduce the concentration of LDL cholesterol in plasma (Goméz-Pérez et al., 1992). Bettzieche et al. (2008) described distinctive effects for different species of lupin proteins in the lipid metabolism. The cultivar Vitabor of lupin (*Lupinus angustifolius* L.) administered to rats, reduced the triglycerides and total cholesterol through the reduction of the expression of genes SREBP-1c and HMG-CoA reductase.

Martins et al. (2005) who administered whole lupin (*L. angustifolius*) to pigs as experimental models, also reported a reduction of cholesterol and an increase in the excretion of cholesterol and bile acids in the faeces caused by the components of legumes such as fibres and phytosterols. Frota et al. (2008) studying another type of legume (cowpea bean) and its protein isolate, also described a reduction of total cholesterol and non-HDL in hamsters and an increase in cholesterol excreted in the faeces, this being a probable mechanism of cholesterol homoeostases.

Table shows the values of the faecal excretion of total sterols by the experimental groups. According to Table 4, it can be seen that there was a greater faecal excretion for the group that consumed a diet containing whole lupin. It can also be seen that for the same group the total excretion of sterols was greater and differed ($P < 0.05$) significantly from the other groups corroborating the statements above about the action of the constituents of whole legumes.

Table also shows that the HWS group showed a greater excretion of bile acids in relation to the HC group, with a significant ($P < 0.05$) difference between them, but there was no significant difference between the HC and HPI groups.

An increase in the faecal excretion of cholesterol and bile acids was reported for diets containing whole legumes with a high protein content, fibres, bio-active components, phytosterols and saponins (Frota et al., 2008; Macarulla et al., 2001; Martins et al., 2005).

These bio-active compounds act in the intestinal tract and are generally considered as a primary complexing of the mixed micelles of dietary cholesterol and bile acids (Carr, Cornelison, Illston,

Table 3
Physiological data and relative liver weights in hamsters fed with different experimental diets for 4 week (mean ± SEM).

	HC	HPI	HWS
Daily diet intake (g/day)	6.62 ± 0.06[a]	6.37 ± 0.30[a]	6.57 ± 0.68[a]
Total diet intake (g)	185.24 ± l.79[a]	178.47 ± 0.86[a]	184.2 ± 9.55[a]
Weight gain (g)	12.68 ± 4.35[b]	11.82 ± 1.32[b]	19.03 ± 3.18[a]
FER (%)[c]	6.87 ± 2.38[b]	6.63 ± 0.75[b]	10.35 ± l.6[a]
True digestibility	99.3 ± 0.3[a]	95.3 ± 1.1[a]	88.3 ± 3.3[b]
Liver g/100 g BW[d]	4.91 ± 0.21[a]	4.46 ± 0.26[a,b]	4.09 ± 0.14[b]

[a,b] Values in the same row followed by different letters differ significantly ($P < 0.05$).
[c] FER = food efficiency ratio = weight gain/total diet intake × 100.
[d] BW = body weight; HC = diet with casein: HPI = diet with lupin protein isolate: HWS = diet with lupin whole seed.

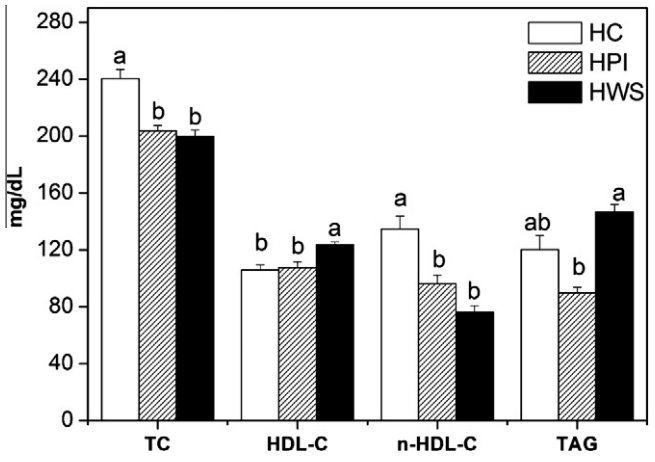

Fig. 1. Plasma lipid and lipoprotein cholesterol concentration in hamsters fed with different diets for 4 week. TAG = triacylglycerol; TC = total cholesterol; LDL-C = LDL cholesterol; HDL-C = HDL cholesterol; n-HDL = non-HDL cholesterol. Means (±SEM) in the same group not sharing common superscript letters are significantly different ($P < 0.05$). HC = diet with casein; HPI = diet with lupin protein isolate; HWS = diet with lupin whole seed.

Table 4
Faecal excretion of cholesterol and total bile acids among hamsters fed with experimental diets for 4 week, collected over last 5 d of experimental period.

	HC	HPI	HWS
Weight of dried faeces (g/days)	1.13 ± 0.10[b]	1.26 ± 0.20[b]	1.65 ± 0.30[a]
Cholesterol (μmol/d/BW)[c]	1.7 ± 0.11[a]	2.l9 ± 0.08[a]	4.79 ± 0.28[b]
Total bile acid (μmol/d/BW)[c]	1.14 ± 0.10[a]	1.88 ± 0.20[a,b]	3 ± 0.9[b]
Total steroid (μmol/d/BW)[c,d]	2.84 ± 0.21[a]	4.07 ± 0.13[a]	7.79 ± 0.35[b]

HC = diet with casein; HPI = diet with lupin protein isolate: HWS = diet with lupin whole seed.
[a,b] Values in the same row followed by different letters differ significantly ($P < 0.05$).
[c] Micromolecules excreted in faeces per day per 100 g of animal weight.
[d] Total steroid = cholesterol + total bile acid.

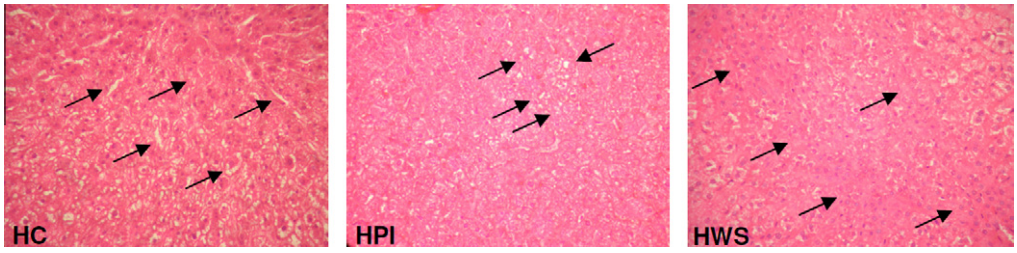

Fig. 2. Photomicrographs of oil-red-O stained 5-micron sections through liver at 400× magnification illustrating periportal lipid accumulation in hamsters fed with casein (HC), lupin protein isolate (HPI), or lupin whole seed (HWS). 0 = absence of steatosis, 1 = focal steatosis (<50% of lobule central veins), 2 = steatosis in >50% of lobule central veins, 3 = comprehensive steatosis, 4 = comprehensive and intense steatosis. Arrows indicate lipid pockets in HC and HPI, and preservated area, in HWS. Average steatosis observed in 5 microscopic evaluations: HC:4; HPI:1+; HWS:1−.

Stuefer-Powell, and Gallaher, 2002), and are able to modulate the expression of exogenous cholesterol transporters ABCG5 e ABCG8 e NPC1L1, reducing the absorption of cholesterol and the re-absorption of bile acids (Jong, Plat, & Mensink, 2003; Turley & Dietschy, 2003) with an increase in cholesterol synthesis, in order to increase the conversion of bile acids and cholesterol that were excreted in the faeces. The increase in the synthesis of endogenous cholesterol after the consumption of whole legumes was reported in experiments undertaken with peas (Martins et al., 2004) and lupin (Martins et al., 2005) where the increase of HMG-CoA reductase activity was responsible for the endogenous cholesterol synthesis.

The fibre in the diet from the HWS group comes from the whole lupin itself, differently from the fibre added in the other diets, which was cellulose. This may have caused a positive effect on sterol excretion. Turnbull, Baxter, and Jonhson (2005) reported that the lupin kernel fibre contains both soluble and insoluble fractions and has high water binding capacity with a range of 8.47–11.07 g water/g dry solids. This characteristic could promote an increase in faecal weight (Table 4) and in the weight of the animals (Table 3). These physical–chemical properties could underlie a possible mechanism for the beneficial effect of fibre from lupin on serum lipids, through inhibition of cholesterol absorption and modification to the enterohepatic circulation of bile acids. In the present study, the lupine's fibre is mostly insoluble, which has a limited effect on cholesterol absorption. Thus, the higher sterol excretion in the HWS group deserves further investigation.

A hypocholesterolaemic effect was found for the group that received the lupin protein isolate, which showed the same total excretion of sterols as the casein group but without correlation to the increase of sterols in the faeces. Proteins isolated from food are being studied and reported on in literature as presenting a hypocholesterolaemic action (Frota et al., 2008; Mendonça, Saldiva, Cruz, & Arêas, 2009). Peptides formed by the incomplete digestion of these isolates can play a metabolic role in the reduction of the levels of cholesterol, probably by regulating the genes involved in the synthesis of cholesterol (such as HMG-CoA reductase) and the absorption of cholesterol (as the LDL receptor) through a reduction in the expression of m-RNA that codifies SREBP-2 as shown for the soybean protein free of isoflavones (Asato, Kina, Sugiyama, Shimabukuro, & Yamamoto, 1994; Cho, Juillerat, & Lee, 2008; Nagaoka et al., 1999; Shukla et al., 2007; Wang & Ng, 1999).

Another parameter of interest found in the lipid metabolism is the accumulation of fat in the liver (steatosis). The consumption of diets with an elevated content of saturated fats and cholesterol have a tendency to develop a non-alcoholic fatty liver disease, apart from other clinical states such as a resistance to insulin, diabetes type II and obesity (Ferré & Foufelle, 2010). Marchesi et al. (2008) studied the hypolipidemic and anti-atherogenic effect of lupin protein isolates (*Lupinus albus*) in rabbits and reported a significant reduction of cholesterol and a reduction of the risk of developing atherosclerosis. A point raised in this study was the capacity of intervention of this legume and its protein isolate in the case of hypercholesterolaemia and steatosis of the liver.

Fig. 2 shows the semi-quantitative histological analysis of the presence of micro- and macro-bladder fats; the slides were coded and the degree of steatosis was assessed on a scale of 0–4. According to Fig. 2, it can be seen that the HC group showed diffuse steatosis and a scale intensity of 4, with deposition of fat globules of different sizes within the parenchyma cells being present both in the periportal hepatocytes and in the pericentre. The other groups showed a degree of steatosis of 1, but the HPI group showed a more focal fat accumulation showing a scale of 1+, while the HWS group showed a lower scale of steatosis (1−) which was a focal of less intensity than that of the HPI group.

These data suggest a higher accumulation of fat in the liver for the group that consumed the HC compared to the experimental HWS and HPI groups.

Spielmann et al. (2007) reported a lower liver weight for rats that consumed lupin protein compared to casein, attributing it to a reduction of triglycerides present in the liver. The reduction of triglycerides in the liver of rats in a study using lupin protein has already been reported by Sirtori et al. (2004). In this case, a process of alteration was attributed to the expression of the genes of the enzyme SREBP-1c, which is responsible for regulating the synthesis of fatty acids and triglycerides in the liver. In hamsters, the cowpea bean and its protein isolate were able to reduce the total cholesterol of plasma. However, no significant difference was observed in the levels of plasma triglycerides (Frota et al., 2008). These authors also observed a hepatoprotective effect in the groups that consumed whole grain and its protein isolate as demonstrated in this study.

4. Conclusions

It can be concluded that whole lupin and its protein isolate have the potential to be used as functional foods and are efficient in the reduction of total cholesterol and plasma non-HDL cholesterol. The protein component of this grain is responsible for the greater part of the hypocholesterolaemic effect. Apparently, there is a synergy between other components of the whole grain such as fibres, saponins and phytosterols.

The protein isolate and whole lupin seed also showed a hepatoprotective effect, reducing the accumulation of fat in the hepatocytes, even in the presence of hypercholesterolaemic diets, containing high levels of fats and cholesterol. The mechanism through which the lupin protein isolate provided a hepatoprotective and hypocholesterolaemic effect seems to be related to bioactive peptides which are bio-available in the protein isolate that act on enzymes related to the metabolism and not on the excretion of total sterols.

Studies are being conducted in our laboratory to show which peptides in this protein isolate are the bio-available bioactive and

to better understand the mechanism of the action of the protein isolate in the hypocholesterolaemic effect.

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References

Anderson, J. W., & Major, A. M. (2002). Pulses and lipaemia, short and long term effect: potential in prevention on cardiovascular disease. *British Journal of Nutrition, 88*(3), 263–271.

Anderson, J. W., Johnstone, B. M., & Cook-Newell, M. E. (1995). Meta-analysis of the effects of soy protein intake on serum lipid. *The New England Journal of Medicine, 333*, 276–282.

AOAC. (1995). *Official methods of analysis* (16th ed.). Washington, DC: Association of Official Analytical Chemists.

Asato, L., Kina, T., Sugiyama, M., Shimabukuro, T., & Yamamoto, S. (1994). Effect of dietary peptides on plasma lipids and its mechanism studied in rats and mice. *Nutrition Research, 14*, 1661–1669.

Beneytout, J. L., Desmaison, A. M., Najid, A., & Rigud, M. (1987). Composition en acides gras de dix espèces et variétés de lupin. *Science de Aliments, 7*(Hors Série VIII), 337–345.

Bettzieche, A., Brandsch, C., Schmidt, M., Weibe, K., Eder, K., & Stangl, G. I. (2008). Differing effect of protein isolates from different cultivars of blue lupin on plasma lipoproteins of hypercholesterolaemic rats. *Bioscience, Biotechnology and Biochemistry, 72*(12), 3114–3121.

Carr, T. P., Cornelison, R. M., Illston, B. J., Stuefer-Powell, C. L., & Gallaher, D. D. (2002). Plant sterols alter bile acid metabolism and reduce cholesterol absorption in hamsters fed a beef-based diet. *Nutrition Research, 22*, 745–754.

Chen, B. H., & Chen, Y. C. (1994). Evaluation of the analysis of cholesterol oxides by liquid chromatography. *Journal of Chromatography A, 661*, 127–136.

Cho, S. J., Juillerat, M. A., & Lee, C. H. (2008). Identification of LDL-receptor transcription stimulating peptides from soybean hydrolysate in human hepatocytes. *Journal of Agricultural and Food Chemistry, 56*, 4372–4376.

Craig, W. J. (1997). Phytochemicals: Guardians of our health. *Journal of the American Dietetic Association, 97*, s199–s204.

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia, 77*, 67–82.

[FAO/WHO] Food and Agriculture Organization/World Health Organization. 1991. Protein quality evaluation. Rome, Italy: (FAO Food and Nutrition Paper, 51). 66p.

Ferré, P., & Foufelle, F. (2010). Hepatic steatosis: A role for de *novo* lipogenesis and the transcription factor SREBP-1c. *Diabetes, Obesity and Metabolism, 12*(Suppl. 2), 83–92.

Fontanari, G. G., Martins, J. M., Kobelnik, M., Pastre, I. A., Arêas, J. A. G., Batistuti, J. P., & Fertonani, F. L. in press. Thermal studies on protein isolates of white lupin seeds (*Lupinus albus*). *Journal of Thermal analysis and Calorimetry*, (doi:10.1007/s10973-011-1898-6).

Frota, K. M. G., Mendonça, S., Saldiva, P. H. N., Cruz, R. J., & Arêas, J. A. G. (2008). Cholesterol-Lowering properties of whole cowpea seed and its protein isolate in hamsters. *Journal of Food Science, 73*(9), 235–240.

Fukui, K., Tachibana, N., Fukuda, Y., Takamatsu, K., & Sugano, M. (2004). Ethanol washing does not attenuate the hypocholesterolaemic potential of soy protein. *Nutrition, 20*, 984–990.

Goméz-Pérez, F. J., Bustamante, F., Vergara, A., Villaseñor, J., Wong, B., & Rull, J. A. (1992). A controlled trial of provastatin vs. Probucol in the treatment of primary hypercholesterolaemia. *Revista de Investigación Clínica, 44*, 53–61.

Goulinet, S., & Chapman, M. J. (1993). Plasma lipoprotein inn the golden Syrian hamsters (*Mesocricetus auratus*): Heterogeneity of ApoB- and ApoA-I-containing particles. *Journal of Lipid Research, 34*, 943–959.

Holm, F. (2003). New functional food ingredients. *Cardiovascular Health*. SMEs n. 5, Flair-flow 4.

Johnson, S. K., Chua, V., Hall, R. S., & Baxter, A. L. (2006). Lupin kernel fibre foods improve bowel function and beneficially modify some putative faecal risk factors for colon cancer in men. *British Journal of Nutrition, 95*, 372–378.

Jones, P. J. (2002). Clinical Nutrition: 7. Functional foods – more than just nutrition. *Canadian Medical Association Journal, 166*, 1555–1563.

Jong, A., Plat, J., & Mensink, R. P. (2003). Metabolic effects of plant sterols and stanols (Review). *Journal of Nutrition and Biochemistry, 14*, 362–369.

Kerckhoffs, D. A. J. M., Brouns, F., Hornstra, G., & Mesink, R. P. (2002). Effects on the humans serum lipoprotein profile of β-glucan, soy protein and isoflavones, plant sterols and stanols, garlic and tocotrienols. *Journal of Nutrition, 132*, 2494–2505.

Liadakis, G. N., Tzia, C., Oreopoulou, V., & Thomopoulos, C. D. (1995). Protein isolation from tomato seed meal, extraction optimization. *Journal of Food Science, 60*, 477–482.

Macarulla, M. T., Medina, C., De Diego, M. A., Chávarri, M., Zulet, M. A., Martínez, J. A., et al. (2001). Effects of the whole seed and a protein isolate of faba bean (*Vicia faba*) on the cholesterol metabolism of hypercholesterolaemic rats. *British Journal of Nutrition, 85*, 607–614.

Marchesi, M., Parolini, C., Diani, E., Rigamonti, E., Cornelli, L., Arnoldi, A., et al. (2008). Hypolipidaemic and anti-atherosclerotic effects of lupin proteins in a rabbit model. *British Journal of Nutrition, 100*, 707–710.

Martínez-Villaluenga, C., Frías, J., & Valverde, C. V. (2006). Functional lupin seeds (*Lupinus albus* L. and *Lupinus luteus* L.) after extraction of α-galactosides. *Food Chemistry, 98*, 291–299.

Martins, J. M., Riotto, M., Abreu, M. C., Lança, M. J., Viegas-Crespo, A. M., Almeida, J. A., et al. (2004). Dietary raw pea (*Pisum sativum* L.) reduce plasma total and LDL cholesterol and hepatic esterified cholesterol in intact and ileorectal anastomosed pigs fed cholesterol-rich diets. *Journal Nutrition, 134*, 3305–3312.

Martins, J. M., Riotto, M., Abreu, M. C., Viegas-Crespo, A. M., Lança, M. J., Almeida, J. A., et al. (2005). Cholesterol-lowering effects of dietary blue lupin (*Lupinus angustifolius* L.) in intact and ileorectal anastomosed pigs. *Journal of Lipid Research, 46*, 1539–1547.

Mendonça, S., Saldiva, P. H., Cruz, R. J., & Arêas, J. A. G. (2009). Amaranth protein presents cholesterol-lowering effect. *Food Chemistry, 116*, 738–742.

Musquiz, M., Burbano, C., Rey, C., & Cassinello, M. (1989). A chemical study of *Lupinus hispanicus* seed. Nutritional components. *Journal of the Science of Food and Agriculture, 47*, 197–204.

Nadal, P., Canela, N., Katakis, I., & ÓSullivan, C. K. (2011). Extraction, isolation, and characterization of globulin proteins from *Lupinus albus*. *Journal of Agriculture and Food Chemistry, 59*, 2752.

Nagaoka, S., Miwa, K., Eto, M., Kuzuya, Y., Hori, G., & Yamamoto, K. (1999). Soy protein peptic hydrolysate with bound phospholipids decreases micellar solubility and cholesterol absorption in rats and Caco-2 cells. *Journal of Nutrition, 129*, 1725–1730.

Potter, S. M. (1995). Overview of proposed mechanisms for the hipocholesterolemic effect of soy. *Journal of Nutrition, 125*, 606S–611S.

Prosky, L., Asp, N. G., Schweizer, T. F., De Vries, J. W., & Furda, I. (1988). Determination of insoluble, soluble and total dietary fibre in foods and products: Intelaboratory study. *Journal - Association of Official Analytical Chemists, 71*, 1017–1023.

Reeves, P. G., Nielsen, F. H., & Tahey, G. C. (1993). AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc writing committee on the reformulation of the AIN-76A rodent diet. *Journal of Nutrition, 123*(11), 1939–1951.

Reynolds, K., Chin, A., Lees, K. A., Nguyen, A., Bujnowski, D., & He, J. (2006). A meta-analysis of the effect of soy protein supplementation on serum lipids. *American Journal of Cardiology, 98*, 633–640.

Roy, F., Boye, J. I., & Simpson, B. K. (2010). Bioactive proteins and peptides in pulse crops: Pea, checkpea and lentil. *Food Research International, 43*, 432–442.

Shukla, A., Brandsch, C., Bettzieche, A., Hirche, F., Stangl, G. I., & Eder, K. (2007). Isoflavone-poor soy protein alters the lipid metabolism of rats by SREBO-mediated down-regulation of hepatic genes. *Journal of Nutrition and Biochemistry, 18*, 313–321.

Sirtori, C. R., Galli, C., Anderson, J. W., & Arnoldi, A. (2009). Nutritional and nutraceutical approaches to dyslipidemia and atherosclerosis prevention: Focus on dietary proteins. Review. *Atherosclerosis, 23*(1), 8–17.

Sirtori, C. R., Lovati, M. R., Manzoni, C., Castiglioni, S., Duranti, M., Magni, C., et al. (2004). Proteins of white lupin seed, a naturally isoflavone-poor legume, reduce cholesterolemia in rats and increase LDL-receptor activity in hepG2 cells. *Journal of Nutrition, 134*, 18–23.

Smith, S. C., Choy, R., Johnson, S. K., Hall, R. S., Wildeboer-Veloo, A. C. M., & Welling, G. W. (2006). Lupin kernel fibre consumption modifies faecal microbiota in healthy men as determined by rRNA gene fluorescent *in situ* hybridisation. *European Journal of Nutrition, 45*, 335–341.

Spielmann, J., Shukla, A., Brandsch, C., Hirche, F., Stangl, G. I., & Eder, K. (2007). Dietary lupin protein lowers triglyceride concentrations in liver and plasma in rats by reducing hepatic gene expression of sterol regulatory element-binding protein-1c. *Annals of Nutrition & Metabolism, 51*, 387–392.

Turley, S. D., & Dietschy, J. M. (2003). Sterol absorption by the small intestine. *Current Opinion in Lipidology, 14*, 233–240.

Turnbull, C. M., Baxter, A. L., & Jonhson, S. K. (2005). Water binding capacity and viscosity of Australian sweet lupin kernel fibre under *in vitro* conditions simulating the human upper gastrointestinal tract. *International Journal of Food Science and Nutrition, 56*, 87–94.

Van Barneveld, R. J. (1999). Understanding the nutritional chemistry of lupin (*Lupinus* sp.) seed to improve livestock production efficiency. *Nutrition Research Reviews, 12*, 1–30.

Van Der Meer, R., Vries, H. D., & Glatz, J. F. C. (1985). *t-Butanol extraction of faeces: Rapid procedure for enzimac determination of faecal bile acids. Cholesterol metabolism in health and disease studies in the Netherlands*. Wageningen: Posen and Looijen, pp. 113–119.

Wang, H. X., & Ng, T. B. (1999). Natural products with hypoglycemic, hypotensive, hypocholesterolaemic, antiatherosclerotic and antithrombotic activities. *Life Sciences, 65*, 2663–2677.

Weingard, K. W., & Daggy, B. P. (1990). Quantification of high-density-lipoprotein cholesterol in plasma from hamsters by differential precipitation. *Clinical Chemistry, 36*, 575.