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A *Lupinus angustifolius* protein hydrolysate exerts hypocholesterolemic effect in western diet-fed ApoE$^{-/-}$ mice through the modulation of LDLR and PCSK9 pathways

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Lupin protein hydrolysate (LPH) is gaining attention in the food and nutraceutical industries due to several beneficial health effects. Recently, we have shown that LPH treatment reduces liver cholesterol and triglyceride levels in hypercholesterolemic mice. The aim of this study was to elucidate the effects of LPH treatment on the molecular mechanism underlying liver cholesterol metabolism in ApoE$^{-/-}$ mice fed the Western diet. After identifying the composition of the peptide within the LPH mixture and determining its ability to reduce HMGCoAR activity *in vitro,* the evaluation of its effect on the LDLR and PCSK9 pathways was measured in liver tissue from the same mice. Thus, LPH reduced the protein levels of HMGCoAR, increased the phosphorylated inactive form of HMGCoAR and the pHMGCoAR/HMGCoAR ratio, which shows the deactivation of *de novo* cholesterol synthesis. Furthermore, LPH decreased the protein levels of SREBP2, a key upstream transcription factor involved in the expression of HMGCoAR and LDLR. Consequently, LDLR protein levels decreased in the liver of LPH-treated animals. Interestingly, LPH also increased the protein levels of pAMPK responsible for HMGCoAR phosphorylation. Furthermore, LPH controlled the PSCK9 signal pathway by decreasing its transcription factor, the HNF1-α protein. Consequently, lower PSCK9 protein levels of PSCK9 were found in the liver of LPH-treated mice. This is the first study elucidating the molecular mechanism at the basis of the hypocholesterolemic effects exerted by LPH in the *in vivo* model. All these findings point out LPH as a future lipid-lowering ingredient to develop new functional foods.

Introduction

Lupin is a leguminous plant belonging to the *Fabaceae* family. Among the *Lupinus* spp., *L. albus* (white lupin), *L. angustifolius* (narrow-leaf lupin), and *L. luteus* (annual-yellow lupin) are the most cultivated for either animal or human nutrition [1]. Proteins derived from lupin seeds are gaining attention as a source of bioactive peptides. In fact, several pieces of evidence highlight the biological activities of lupin protein hydrolysates and peptides, including hypocholesterolemic, hypoglycemic, antimicrobial, anti-inflammatory, and immunomodulatory effects [2-9]. The health-promoting effects of lupin peptides have been tested in different animal models and clinical trials. Briefly, diets containing protein from *L. albus* [10, 11] or *L. angustifolius* [12, 13 14] significantly reduce both total cholesterol and low-density lipoprotein (LDL) cholesterol (LDL-C) levels versus control diets containing casein in a rat model of hypercholesterolemia. In particular, the cultivar Vitabor of lupin (*L. angustifolius*) administered to rats, reduces the triglycerides and total cholesterol through the reduction of the expression of genes SREBP-1c and HMG-CoA reductase [12]. In agreement with these studies, it was also demonstrated that lupin protein isolate from *L.albus* provides hepatoprotective and hypocholesterolaemic effects in a hamster model with a similar mechanism of action [15].

An uncontrolled clinical trial on *L. albus* [16] and two controlled ones on *L. angustifolius* have confirmed the hypocholesterolemic activity in humans [17, 18]. Lupin protein-enriched diets including foods such as bread, biscuits, and pasta suppress the lipid profile and blood pressure in both normal and hypertensive human subjects [19-21]. Similarly, lupin-based diets improved glycemic control in diabetic and normal human subjects [22]. In addition, a recent study supports the pleiotropic actions of a lupin bioactive peptides-based functional food on critical steps of atherosclerosis, including inflammation, oxidative stress, and cholesterol metabolism [23].

Recently, we have demonstrated that lupin protein hydrolysate (LPH) obtained after the hydrolysis with Alcalase®, reduce hepatic steatosis and abdominal adiposity, as well as total

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Electronic Supplementary Information (ESI) available: **Table S1**. Composition of the experimental Western diet; **Table S2.** Composition of the standard diet; **Table S3**. Medium-sized peptide identification; **Table S4**. Short-sized peptide identification. See DOI: 10.1039/x0xx00000x

cholesterol levels in apolipoprotein E-deficient ($ApoE^{-/-}$) mice. Furthermore, LPH increases the hepatic total antioxidant capacity and reduces the hepatic inflammatory status in these mice [24]. In addition, a LPH-based beverage decreases the LDL-C/HDL-C ratio in healthy subjects (Lupine-1 study), a significant predictory marker of cardiovascular disease [25].

In light of these evidences, the present study was aimed at assessing the underlying molecular mechanism of the LPH cholesterol-lowering properties in the same animal model using a multidisciplinary strategy. Firstly, a detailed characterization of the LPH composition was performed by using nano-HPLC-MS/MS and UHPLC-HRMS to identify medium- and short-sized peptides, respectively. Afterwards, *in vitro* experiments were carried out to evaluate the effect of LPH on the 3-Hydroxy-3-methylglutaryl-CoA Reductase (HMGCoAR) activity, the key enzyme involved in the cholesterol synthesis and target of statins, the primary drugs used for the hypercholesterolemia treatment [26]. Finally, the effects of LPH on the cholesterol metabolism pathway in liver tissue of $ApoE^{-/-}$ mice were investigated by performing protein quantification of sterol regulatory element-binding protein 2 (SREBP2), LDL receptor (LDLR), HMCCoAR, phospho-adenosine monophosphate (AMP) activated protein kinase (pAMPK), proprotein convertase subtilisin/kexin type 9 (PCSK9), and hepatocyte nuclear factor 1-α (HNF1-α).

Materials and methods

Reagents

All reagents and solvents employed were from commercial sources (see "Supplementary Materials").

LPH preparation

The preparation and characterization of the LPH was carried out as previously described [27]. Briefly, lupin protein isolate was dissolved in distilled water and subsequently hydrolyzed with Alcalase® (Novozymes, Bagsvaerd, DK) for 15 min at pH 8, 50 °C. The protein hydrolysis was stopped by heating at 85 °C for 15 min. The solution was centrifuged (10,437 g for 15 min.), and the supernatant was lyophilized and stored at room temperature, constituting the LPH. The absolute yield of LPH was > 50% respect to the lupin protein isolate.

Peptide purification and concentration

As previously described [28], solid-phase extraction (SPE) on C18 was used to concentrate medium-sized peptides. In this case, 1 mg of LPH was loaded on a 50 mg Bond Elut C18 EWP cartridge (Agilent, Santa Clara, USA). The cartridge was preliminary washed with 3 mL of acetonitrile (ACN) and conditioned with 3 mL of 0.1% trifluoroacetic acid (TFA); the sample was acidified with aqueous TFA to pH 2.5, then loaded, and the cartridge washed with 3 mL of 0.1% TFA. Peptides were eluted from the SPE column with 0.5 mL ACN/H_2O (50:50, v/v) containing 0.1% TFA, and were dried in a SpeedVac SC250 Express (Thermo Savant, Holbrook, NY, USA). The residue was reconstituted in 150 µL of 0.1% formic acid in H_2O for analysis.

Analysis of medium-sized peptides by nano high-performance liquid chromatography-MS/MS and peptide identification

Medium-sized peptides were analysed by nano HPLC on an Ultimate 3000 (Thermo Fisher Scientific, Bremen, Germany) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific), as previously described [29]. The samples (20 µL) were on-line preconcentrated on a µ-precolumn (Thermo Fisher Scientific, 300 µm i.d. 5 mm Acclaim PepMap 100 C18, 5 µm particle size, 100 Å pore size) at 10 µL/min flow rate of H_2O/ACN (99:1, v/v) containing 0.1% TFA (v/v). Then, they were separated on an EASY-Spray column (Thermo Fisher Scientific, 15 cm × 75 µm i.d. PepMap C18, 3 µm particles, 100 Å pore size) operated at 300 nL/min and 35 °C. Mobile phases were H_2O (solvent A) and ACN (solvent B), both with 0.1% formic acid. The following linear gradient was used: 1% B for 5 min; 1–5% B in 2 min; 5–35% B in 90 min; 35–90% B in 3 min; finally, the column was washed at 90% B for 10 min and then equilibrated at 1% B for 29 min.

Peptide spectra were acquired in the 300–2000 m/z range at 30,000 resolution (full width at half maximum, FWHM, at m/z 400) for the full scan. MS/MS spectra were acquired at 15,000 resolution (FWHM, at m/z 400) in top 10 data-dependent acquisition mode with the rejection of singly charged ions and unassigned charge states. Precursors were fragmented by higher-energy collisional dissociation with 35% normalized collision energy and 2 m/z isolation window. Dynamic exclusion was enabled with a repeat count of 1 and a repeat duration of 30 s with an exclusion duration of 20 s. For each sample, three technical replicates were performed. Raw data files were acquired by Xcalibur software (version 2.2, Thermo Fisher Scientific).

Raw data spectra were identified using Proteome Discoverer (version 1.3, Thermo Fisher Scientific) with the Mascot search engine (v2.3.02) using a protein sequence database downloaded from Uniprot for the taxonomy *L. angustifolius* (31,386 sequences). Precursor ion tolerance was 10 ppm, fragment ion tolerance of 0.05 Da; no enzyme was used for digestion and methionine oxidation was selected as dynamic modification. Decoy function was used for false discovery rate calculation, which was set at 1%.

Purification of short-sized peptide sequences

SPE and cleanup of short peptides were carried out by cartridges packed with 500 mg Carbograph 4. The applied procedure has been optimized in a previous paper [30]. Briefly, the cartridge was washed with 5 mL of dichloromethane (DCM)/MetOH, 80:20 (v/v) with 20 mmol/L TFA and 5 mL of MetOH with 20 mmol/L TFA. The activation was carried out by flushing with 10 mL of 0.1 mol/L HCl and finally conditioned with 10 mL of 20 mmol/L TFA. Then, samples (1 mg/mL of proteins) were diluted in 10 mL H_2O with 20 mmol/L TFA and loaded into the cartridge, which was sequentially washed with 2 mL of 20 mmol/L TFA and 0.5 mL MetOH. Finally, analytes were eluted by backflushing elution with 10 mL of DCM/MetOH and 80:20 (v/v) with 20 mmol/L TFA. The eluate was evaporated at room temperature in a Speed-Vac SC250 Express (Thermo Savant,

Holbrook, NY, USA), and the residue was reconstituted in 200 µL water for subsequent reversed-phase separation.

UHPLC-HRMS analysis and short-sized peptide identification

Short peptides were analysed by Vanquish binary pump H (Thermo Fisher Scientific) coupled to a hybrid quadrupole-Orbitrap mass spectrometer Q Exactive (Thermo Fisher Scientific) using a heated ESI source operating in positive ion mode. The mass-spectrometric strategy was set up as previously reported [31]. Each sample (20 µL) was injected onto a Kinetex XB-C18 (100 × 2.1 mm, 2.6 µm particle size, Phenomenex, Torrance, USA). Chosen flow, column temperature, and gradient parameters are reported in our previous work without any modification [32]. Untargeted suspect screening analysis was performed in top 5 data-dependent acquisition mode in the range m/z 150-750 with a resolution (FWHM, m/z 200) of 70,000. Higher-energy collisional dissociation fragmentation was performed at 40% normalized collision energy at resolution of 35,000 (FWHM, m/z 200). An inclusion list, in which all precursor ion deriving from the combination of the 20 amino acids in di-, tri-, and tetrapeptides were listed, was included in the method [31]. Raw data files from three experimental replicates and a blank sample were processed by Compound Discoverer using a workflow specifically dedicated to short peptide analysis. The database of short peptide sequences with IDs, masses, and molecular formulas, was implemented for the automatic matching of extracted m/z ratios. Extracted masses from the chromatograms were aligned and filtered to remove background compounds present in the blank sample, features whose masses were not present in the databases, and those not fragmented. Filtered features were manually validated, matching experimental spectra to those generated *in silico* by mMass 5.5 [33].

Database searching, protein identification, and validation

The UniProt database (UniProt Consortium) [34] was used to identify the origin proteins of medium-sized peptides, while the BIOPEP-UWM database [35] was used to identify the peptides with a described hypolipidemic activity.

HMGCoAR activity assay

The HMGCoAR enzyme activity was measured using the commercial kit HMGCoAR assay kit (Sigma-Aldrich, St. Louis, MO, USA). The experiments were carried out at 37 °C following the manufacturer's instructions. Each reaction (200 µL) was prepared adding the reagents in the following order: assay buffer (162 µL); LPH (20 µL at final concentration of 0.1, 0.5, 1.0, and 2.5 mg/mL) or vehicle; NADPH (4 µL); substrate solution (12 µL); and finally, HMGCoAR (2 µL). Then, the samples were mixed, and the absorbance was measured at 340 nm using a Synergy H1 microplate reader (Biotek Instruments, Winooski, VT, USA) at 0 and 10 min. The inhibition generated by LPH on the activity of HMGCoAR was calculated through the reduction of absorbance, which is directly proportional to the activity of the enzyme. 1.0 µM pravastatin (Pravastat, Sigma-Aldrich) was used as a positive control.

***in vivo* study design**

LPH was suspended in a physiological saline solution containing 0.25% of carboxymethylcellulose (Sigma Aldrich, St. Louis, MO, USA). The experimental study design was detailed described in a previous paper [24]. Briefly, 19 ApoE$^{-/-}$ mice were initially classified in two groups: mice that were fed with a standard diet (SD, n = 5) or Western diet (WD) (n = 14). The composition of the SD (Envigo Teklad Global, 13% energy from fat)[36] and the WD (TestDiet 58v8, 45% energy from fat) are shown in Supplemental Table S1 and S2, respectively. When mice from the WD group turned 6 weeks old, they were randomly divided in two groups and daily intragastrically treated with 100 mg/kg LPH (WD+LPH, n = 7) or vehicle (WD, n = 7) for 12 weeks. Mice were housed in the animal facility of the Instituto de Biomedicina de Sevilla (IBiS) under standard conditions (12/12 light/day cycles, temperature 22 ± 2°C and humidity < 55%) with free access to water and diet. The aim of this study was to determine whether LPH was able to restore the altered lipid profile by WD, for this motif, the SD group was necessary only to confirm that the WD ingestion induces an alteration of this profile, according to other study designs [24, 37-42]. At the end of the experiment, fasted mice were euthanized by an overdose intraperitoneal injection, set for each mouse, of sodium thiopental (50 mg/kg, B. Braun Medical SA, Barcelona, Spain) that was effective within 1 min., and liver was subsequently collected, rapidly frozen, and stored at -80°C until use. Plasma and hepatic lipid profile parameters were quantified as is described in Supplementary Materials. The experimental protocol was performed under the Spanish legislation and the EU Directive 2010/63/EU for animal experiments and was approved by the Virgen Macarena and Virgen del Rocío University Hospitals ethical committee (reference 21/06/2016/105).

Western Blot analysis

Tissues were homogenized in a lysis buffer (RIPA buffer + protease inhibitor cocktail + 1:100 PMSF + 1:100 Na-orthovanadate + 1:1000 β-mercaptoethanol). Protein concentration was determined by the Bradford's method and 50 µg of total proteins were run into a pre-cast 7.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) at 130 V for 45 min and then transferred to a nitrocellulose membrane (Mini nitrocellulose Transfer Packs, BioRad, Hercules, CA, USA) using a Trans-Blot Turbo (BioRad) at 1.3 A, 25 V for 7 min, and stained with Ponceau red solution (Sigma-Aldrich). To investigate proteins of different sizes on the same membrane, the membrane was cut into horizontal stripes. The milk or BSA blocked membrane were overnight incubated at 4 °C with primary antibodies against SREBP2, HMGCoAR, LDLR, phospho AMPK (Thr172), phospho HMGCoAR (Ser872), PCSK9, HNF1-α, and β-actin. After washing, the blots were incubated with secondary antibodies conjugated with horseradish peroxidase. A chemiluminescent reagent (Euroclone, Milan, Italy) was used to visualize target proteins and the densitometry analysis was performed using the software Image Lab 6.1 (BioRad). The internal control β-actin was used to normalize loading

variations. Supplemental Figure S1 shows that there are not exist differences in β-actin levels concentration among the experimental groups.

Statistical analysis

All the data sets were checked for normal distribution by D'Agostino and Pearson test. Since they are all normally distributed with *p*-values < 0.05, statistical analysis was carried out by One-way ANOVA followed by Tukey's post-hoc analysis (GraphPad Software, San Diego, CA, USA). Values were expressed as means ± SD; *p*-values ≤ 0.05 were considered to be significant.

Results

Peptidomics characterization of LPH

Analysis of medium- and short-sized peptides in LPH

To identify the medium-sized peptide composition, LPH underwent to nano HPLC–MS/MS analysis. Then, the peptide identification was performed using Proteome Discoverer with the Mascot search engine using a protein sequence database downloaded from Uniprot for the taxonomy *L. angustifolius*. A total of 278 medium-sized peptides with an area greater than 10^7 were identified containing 7 to 26 amino acid residues with a molecular weight ranging from 755.5 to 3,111.5 Da (Supplemental Table S3).

At the same time, short peptide identification was carried out using a previously developed method following an HRMS-based suspect screening approach [43]. Notably, using a list containing the exact masses of precursors relative to all the possible combinations of the 20 natural amino acids (from two to four, resulting in 168,400 unique combinations), a total of 282 unique amino acid sequences were identified in LPH sample. However, under the operating conditions, it was not possible to discriminate the isobaric leucine and isoleucine by tandem mass spectra [44]; therefore, the occurrence of the two amino acids within the identified sequence was retained as equally probable (Supplemental Table S4).

Hypocholesterolemic activity of LPH sequences

The analysis of LPH sequences using BIOPEP permitted to identify 55 sequences that contained a peptide motif associated with a hypolipidemic effect (Table 1). In detail, 28 sequences (50.9%) proceeded from conglutin proteins (α- and β-conglutin), and 27 sequences (49.1%) proceeded from non-conglutin proteins. Of these, 20 sequences contained the peptide GGV or IVG, identified with IDs 9383 and 9384, respectively.

Biological effects of LPH

LPH inhibits the catalytic activity of HMGCoAR

To evaluate the capacity of LPH to inhibit the HMGCoAR activity, a preliminary *in vitro* assay was performed. As shown in Figure 1, LPH was shown to reduce the activity of the HMGCoAR enzyme in a dose-dependent manner. Although there were no significant differences at concentrations of 0.1 mg/mL ($p > 0.05$), LPH decreases the HMGCoAR enzyme activity by 16.1 ± 0.4 ($p < 0.01$), 33.9 ± 5.4 ($p < 0.0001$) and 51.5 ± 0.6% ($p < 0.0001$) at 0.5, 1.0, and 2.5 mg/mL, respectively. Pravastatin (1.0 μM, positive control), as expected, reduced the HMGCoAR activity by 92.0 ± 2.6% ($p < 0.0001$).

LPH ingestion does not alter the body weight gain, and improves the lipid profile

As shown in Figures 2A-B, no differences were observed either in the baseline body weights (SD: 20.83 ± 0.67g; WD: 21.56 ± 2.14 g; WD+LPH: 21.32 ± 2.26g; $p = 0.838$) and final body weight (SD: 26.10 ± 2.26; WD: 31.51 ± 4.72g; WD+LPH: 30.31 ± 5.98g; $p = 0.222$) among the three experimental groups. However, a significant increase ($p = 0.046$) in the body weight gain was observed in the WD group (+9.96 ± 2.71 g) in comparison with the SD group (+5.28 ± 1.91 g) (Figure 2C). Furthermore, no significant differences ($p = 0.949$) were observed in the body weight gain when mice were fed with the WD and treated with the LPH (+9.51 ± 3.65 g) compared to the WD group (Figure 2C). Additionally, significant differences ($p < 0.0001$) in the daily food intake were observed between the SD group (2.87 ± 0.34 g/mouse) and WD group (2.27 ± 0.18 g/mouse), as well as, between the SD group and WD+LPH group (2.21 ± 0.17 g/mouse) (Figure 2D). However, no changes in daily food intake were observed between the treated mice ($p = 0.704$) in comparison to WD group (Figure 2D). On the other hand, the lipid profile was analysed in plasma and liver of all experimental groups. In the group WD+LPH, a reduction in the plasma levels of total cholesterol (TC) (-60 mg/dL = -19.7%, $p = 0.020$), LDL-C (-66 mg/dL = -21.6%, $p = 0.026$), and TG (-27mg/dL = -27.5%, $p = 0.036$) was observed compared to the WD, while no modifications were observed in comparison to SD group (Table 2). Moreover, in the group WD+LPH a mitigation of the significant increase in TC ($p = 0.008$), LDL-C ($p = 0.006$) and TG ($p = 0.005$) generated by the consumption of the WD, compared to the consumption of a SD was detected. In addition, HDL-C did not show significant differences between the three experimental groups ($p = 0.373$) (Table 2). In a similar way, LPH intake, in the group WD+LPH, reduced the hepatic levels of TC (-0.5 mg/g tissue = -19.0%, $p < 0.05$) and TG (-13.1 mg/g tissue = 26.0%, $p < 0.01$) in comparison to the WD group, reaching similar values to SD-fed mice (Table 2).

LPH modulates the activation of the LDLR pathway induced by a WD

SREBP2 and LDLR protein were quantified in the liver of mice of the three experimental groups for investigating the effects of LPH on the LDLR pathway. Figure 3 indicates that the LDLR pathway is activated when mice are fed with a WD. More in detail, the WD ingestion increased the protein level of LDLR (Figure 3A) and SREBP2 transcription factor (Figure 3B) by 47.1% ± 35.9% ($p < 0.05$) and 118.6% ± 42.7% ($p < 0.01$), respectively. In particular, in the Figure 3B, the qualitative differences of β-actin bands among the groups are not statistically significant from a quantitative point of view as indicated by Figure S1, where β-actin level concentrations among the groups are illustrated (see Supplementary Material). The treatment with

LPH for 12 weeks reduced both LDLR by 48.8% ± 21.2% ($p < 0.05$) and the protein levels of SREBP2 by 83.1% ± 18.7% ($p < 0.01$) comparing with the WD group. Interestingly, values in the WD+LPH group were similar to the SD-fed mice group.

LPH reduces the WD-induced HMGCoAR activation

The LPH effects on the HMGCoAR protein levels were evaluated by Western Blot. As shown in Figure 4, mice fed with the WD increased their HMGCoAR protein levels by 53.0% ± 33.7% compared to SD fed mice ($p < 0.05$). LPH treatment reduced 61.8% ± 12.4% of the HMGCoAR protein levels ($p < 0.05$) with respect to WD-fed mice, obtaining similar values to the SD group ($p = 0.99$) (Figure 4A). Moreover, the treatment with LPH significantly increased the phosphorylation levels of HMGCoAR (serine 872, AMPK phosphorylation site) by 22.8% ± 4.2% compared with the WD group ($p < 0.05$) (Figure 4B). This result was in line with the increase observed in pAMPK (threonine 172) levels, which is increased by 38.7% ± 2.5% in LPH-treated mice in comparison to the WD group ($p < 0.05$) (Figure 4C).

Furthermore, the pHMGCoAR/HMGCoAR ratio was calculated. As shown in Figure 3D, the ratio was reduced by 36.8% ± 1.0% in WD-fed mice compared to SD-fed mice ($p < 0.05$). The treatment with LPH overcomes this effect by increasing 25.7% ± 8.7% the pHMGCoAR/HMGCoAR ratio compared to the WD group ($p < 0.01$). No differences between WD+LPH and SD groups were observed ($p = 0.581$).

LPH treatment reduces the PCSK9 protein levels increased by WD ingestion

To elucidate the effects of the LPH treatment on the PCSK9 pathway, PCSK9 and HNF1-α protein levels were analysed by Western Blot. As shown in Figure 4, the ingestion of the WD increased the protein levels of PCSK9 (Figure 4A) and HNF1-α (Figure 4B) by 17.1% ± 8.7% ($p < 0.05$) and 39.3% ± 0.3% ($p < 0.05$), respectively, compared to SD-fed mice. The LPH treatment for 12 weeks palliates these effects. Mice treated with LPH showed a significant reduction of PCSK9 by 21.5% ± 6.4% ($p < 0.05$) and a significant reduction of HNF1-α by 43.8% ± 7.6% ($p < 0.05$) in comparison to the WD group, with no differences between WD + LPH and SD-fed mice group.

Discussion

LPH are a peptide mixture obtained after the hydrolysis of *L. angustifolius* protein using Alcalase®, which exerts both *in vitro* and *in vivo* anti-inflammatory and immunomodulatory effects [25, 27, 45, 46].

In addition LPH intake reduces abdominal adiposity, steatosis, and increases the hepatic total antioxidant capacity in WD-ApoE$^{-/-}$ mice [24]. These pieces of evidence prompted us to study the molecular events behinds the LPH cholesterol-lowering properties. To achieve this goal, a multidisciplinary study has been conducted through the combination of analytical, molecular, and biochemical techniques.

More in detail, since the biological activity of a specific protein hydrolysate is strictly correlated to its chemical composition, the identification of LPH peptide sequences was carried out using the most updated analytical techniques. Indeed, the characterization of the medium-sized peptide revealed the presence of 278 peptides. In total, 58% of these peptides derive from conglutins, the most abundant storage proteins in lupin seeds [47]. Notably, 89, 71, and 1 peptide belong to α, β, and δ conglutin, respectively, while no peptides from γ-conglutin were identified. It is known that the identification of short peptides is a challenging topic for several reasons [48]. Standard proteomics database search engines cannot efficiently annotate short-sized peptides, since their short sequence cannot be confidently associated with single proteins [49]. Moreover, the short sequences generate mostly singly charged ions, which are non-compatible with proteomics workflows and generate noisier MS/MS spectra. Finally, the fragmentation pathways of short peptide sequences are more strongly affected by the nature of the single amino acid that constitutes the sequences [31].

Nevertheless, applying a high-resolution mass spectrometry-based suspect screening approach, these particular issues were overcome and used to identify, for the first time, the sequence of short peptides within a lupin protein hydrolysate.

The parallel analysis of medium and short peptide sequences using BIOPEP database allowed us to identify some sequences containing some known hypocholesterolemic motives. More in detail, GGV and IVG motives, described for the first time in *Amaranthus cruentus* protein, show an HMGCoAR activity inhibition [50]. In total, 24 sequences contained the EF motif (ID 9580), identified in *Allium cepa*, which attenuates the lipogenesis in hepatocytes [51]. Known peptides were also identified: P7a (LTFPG) has been previously demonstrated to be able to reduce the *in vitro* HMGCoAR activity and inhibiting PCSK9/LDLR protein−protein interaction *in silico* analysis [52]; whereas T9 (GQEQSHQDEGVIVR, ID 9676) possesses the capacity to directly inhibit these interactions [53].

In light of these observations, our results suggest that LPH drops the *in vitro* HMGCoAR activity with a dose-response trend. Literature evidences suggest that LPH displays a similar activity of a *L. albus* protein hydrolysate generated using trypsin (-37% at 0.25 mg/mL, -57% at 0.5 mg/mL, and -61% at 1.0 and 2.5 mg/mL), and both are more active than a *L. albus* protein hydrolysate generated digesting with pepsin, i.e. only −17% at 2.5 mg/mL [9].

In accordance with this evidence, we confirmed a decrease in lipid concentration both in plasma and liver tissue in WD +LPH-treated mice compared to the WD-fed mice, reestablishing the normal values observed in SD-fed mice.

Basis on these results, LPH effects were evaluated on the hepatic cholesterol metabolic pathway. Our findings show that mice fed with WD presented higher protein levels of LDLR, HMGCoAR, and SREBP2 than SD-fed mice, in agreement with previous studies [54-56]. Specifically, the higher intake of dietary fatty acids can cause the activation of cholesterol pathway in these mice for two reasons: 1) in situations of excess fatty acids, the generation of cholesterol activated by fatty acids gains priority over cholesterol downregulation; 2) regulation of cholesterol synthesis can be disrupted in the liver as a consequence of WD feeding [54, 56]. In this context, it is important to underline that even though dietary fat is known to affect

serum concentrations of total and lipoprotein cholesterol, all components of dietary triglycerides -saturated, monounsaturated, and polyunsaturated fatty acids- do not have identical effects on serum cholesterol levels [57]. Notably, saturated fatty acids are mainly responsible for raising cholesterol levels, whereas monounsaturated and mainly polyunsaturated fatty acids (i.e., omega-3), are able to lower cholesterol levels [58-60].

Our results shown that LPH-treated mice had similar levels of LDLR, HMGCoAR, and SREBP2 than SD-fed mice, supporting the LPH ability on counteracting the hepatic effects related to cholesterol metabolism caused by the WD consumption. Similar results have been previously observed with other natural compounds, i.e., polyphenols [54] and spirulina lipids [55].

HMGCoA reductase activity can be modulated by a reversible phosphorylation-dephosphorylation, with the phosphorylated form of the enzyme being inactive (70%) and the dephosphorylated form active (30%). Thus, when HMGCoAR is phosphorylated the synthesis of *de novo* cholesterol is reduced. This short-term phosphorylation of HMGCoAR is induced by pAMPK that deactive HMGCoAR by phosphorylation on Ser872. In this regard, statins can activate (phosphorylate) AMPK, inhibiting HMGCoAR. In this study, we observed an increase of the phosphorylated form of HMGCoAR and AMPK in LPH-treated mice, compared to the WD group, which suggest that LPH acts on HMGCoAR activity through the AMPK phosphorylation. The increase in the pHMGCoAR/HMGCoAR ratio, observed in LPH-treated mice, support the positive effect of LPH on the exacerbated synthesis of cholesterol in WD-fed mice. These results are in line with other results of our group carried out with peptides from *L. albus* in human hepatic (HepG2) cell line [61, 62].

In addition, LPH was able to counteract the increase of hepatic PCSK9 and HNF1-α protein levels observed in WD-treated mice. The modulation of the PCSK9 pathway is highly significant due to PCSK9 is a new important target for treating hypercholesterolemia [63]. Therefore, these results confirm for the first time in an *in vivo* model the *in vitro* action mechanism previously disclosed for other *L. albus* peptides, through which lupin peptides synergistically modulate the cholesterol metabolism pathway [5, 53, 61, 62, 64]. These results are also in line with previous studies that show a decrease of circulating PCSK9 levels in humans after lupin protein consumption [64, 65]. In this respect, several pieces of evidence indicate that diets containing either *L. albus* [10, 11] or *L. angustifolius* proteins [12, 13] significantly reduced both total cholesterol and LDL-C levels *in vivo*. In particular, specific studies have clearly underlined that the consumption of lupin proteins modulate the cholesterol pathway in both rat [14] and hamster [15] models, determining a hypocholesterolemic effect. In fact, with a more updated perception of the phenomenon, our study suggests that the observed health-promoting activity does not lie in the native protein, but the mixture of peptides generated from the physiological hydrolysis of proteins during digestion. Therefore, our study strongly suggests that LPH might be directly used as potential bioactive ingredient for the development of new dietary supplements and/or functional foods.

Conclusions

In conclusion, this is the first study to show the *in vivo* effects of lupin protein hydrolysate on the hepatic cholesterol metabolic pathway (action mechanisms summarized in Figure 5). Moreover, several sequences containing peptide motifs associated with hypolipidemic effects, HMGCoAR inhibition, and PCSK9/LDLR binding inhibition were identified within the LPH mixture. Therefore, this study confirms the pleotropic effects of the peptide mixture as a new possible ingredient for the development of supplements or functional foods with multifunctional activity including cholesterol lowering.

Abbreviation

AMP	adenosine monophosphate
AMPK	AMP activated protein kinase
C	WD-fed mice control group
CoA	Coenzyme-A
HMGCoAR	3-hydroxy-3-methylglutaryl CoA reductase
HNF1-α	hepatocyte nuclear factor 1-α
LDL-C	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
LPH	lupine protein hydrolysate
PCSK9	proprotein convertase subtilisin/kexin type 9
pHMGCoAR	phosphoHMGCoAR
pAMPK	phospho-AMP-activated protein kinase
SD	standard diet-fed mice
SREBP2	sterol regulatory element-binding protein 2
WD	western diet.

Author Contributions

The following are the authors' contributions: Conceptualization: C.L. and A.C.-V.; Methodology: G.S.-S., I.C.-C., C.B., M.B., A.L.C., A.C., and A.L.; Data curation: G.S.-S., I.C.-C., C.B., M.B., AL.C., A.C., and A.L.; Formal analysis: G.S.-S., I.C.-C., C.B., M.B., AL.C., A.C., and A.L. Resources: C.L., A.C.-V., A.A., J.P., F.M., and M.C.M.-L.; Writing – original draft: G.S.-S., I.C.-C., C.L., and A.C.-V.; Supervision: C.L. and A.C.-V.; Funding acquisition: C.L., A.C.-V., and A.A.

Conflicts of interest

There are no conflicts to declare.

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Figure captions

Figure 1. In vitro HMGoAR activity inhibition. Inhibition ability of LPH, tested at different concentrations (0.1 – 2.5 mg/mL), in in vitro experiments on HMGoAR activity. Data were expressed as a percentage of the control group and represented as mean ± SD. ****, $p < 0.0001$. Different letters represented a statistical difference ($p \leq 0.05$). C, control group; LPH, lupine protein hydrolysate; PRAV, pravastatin (1.0 μM).

Figure 2. Body weight parameters and daily food intake. Baseline body weight (A), final body weight (B), body weight gain (C), and daily food intake (D) of all experimental groups. Values are shown as the mean ± SD. *, $p \leq 0.05$. Different letters represented a statistical difference ($p \leq 0.05$). n.s., not significant; LPH, lupine protein hydrolysate.

Figure 3. **Modulation of LDLR pathway by LPH**. LPH modulates the increase of LDLR (A) and SREBP2 transcription factor (B) induced by WD fed. Data were represented as mean ± SD. *, $p < 0.05$; ** $p < 0.01$. Different letters represented a statistical difference ($p \leq 0.05$). LPH, lupine protein hydrolysate; LDLR, low-density lipoprotein receptor; SREBP2, sterol regulatory element-binding protein 2.

Figure 4. Effects of LPH on HMGCoAR, pHMGCoAR, and pAMPK protein levels. Representative Western Blots and densitometric analyses of the HMGCoAR (A), pHMGCoAR (B), and pAMPK (C) protein levels. Ratio of the inactive phosphorylated and active non-phosphorylated forms of the HMGCoAR (D). Data were represented as mean ± SD. *, $p < 0.05$. Different letters represented a statistical difference ($p \leq 0.05$). LPH, lupine protein hydrolysate; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; pHMGCoAR, phosphoHMGCoAR; pAMPK, phospho adenosine monophosphate activated protein kinase.

Figure 5. Effects of LPH treatment on PCSK9 pathway. LPH counters the increase of PCSK9 (A), and its transcription factor HNF1-α (B) caused by the ingestion of WD. Data were represented as mean ± SD. *, $p < 0.05$. Different letters represented a statistical difference ($p \leq 0.05$). LPH, lupine protein hydrolysate; HNF1-α, hepatocyte nuclear factor 1-α; PCSK9, proprotein convertase subtilisin/kexin type 9.

Figure 6. Schematic representation of hypocholesterolemic mechanism of action mediated by lupin protein hydrolysate in ApoE$^{-/-}$ mice. Green arrows show hepatic lupin protein hydrolysate (LPH) effects. AMPK, adenosine monophosphate (AMP) activated protein kinase; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HNF1-α, hepatocyte nuclear factor 1-α; LDL-C, low-density lipoprotein cholesterol; LDLR, LDL-C receptor; P, inorganic phosphate; PCSK9, proprotein convertase subtilisin/kexin type 9; SREBP2, sterol regulatory element-binding protein 2.

ARTICLE

Table 1. Sequences of identified peptides from LPH with hypolipidemic activity.

Effect	Ref.	Bioactive peptide motif	ID [35]	Origin protein[a]	Accession number[a]	N. sequences	Sequences
HMGCoA reductase activity inhibitor	[50]	GGV IVG	9383 9384	α-Conglutin	F5B8V7 F5B8V8	11	ALEPDNRIESEGGVT ALEPDNRIESEGGVTE ALEPDNRVESEGGVTE LEPDNRIESEGGVT LEPDNRIESEGGVTE LEPDNRVESEGGVT LEPDNRVESEGGVTE NALEPDNRIESEGGVT NALEPDNRIESEGGVTE NALEPDNRVESEGGVT NALEPDNRVESEGGVTE
				Non-conglutin proteins		9	ADKEGGGGGVAPNVGKF GRGREDKEVVEGRDKAGGVT GVEGSMRPPGQNPGGVL HIVGPDKKIKL HIVGPDKKIKLS KEGGVLPGIK LVIVGDGGTGKTT RYDTPTNPQGRYRSGGSGGVGGVN SGREKEEEGERGRRGGVRPH
Hypolipidemic peptide	[51]	EF	9580	α-Conglutin	F5B8V7 F5B8V8 F5B8V6	3	HQAGDEGFEF HQAGDEGFEFIAF SGFNDEFLEE
				β-Conglutin	F5B8V9 F5B8W0 F5B8W1 F5B8W2 F5B8W3 F5B8W4	4	EFQSKPNTL EFQSKPNTLILPK EFQSRPNTL EFQSRPNTLILPK
				Non-conglutin proteins		17	AEFPIFDKV DSKDEFLAIL FKAEFPIFDKV GGRVILHPITSPASEFE GGRVILHPITSPASEFEHVE KAEFPIFDKV NTRGGRVILHPITSPASEF NTRGGRVILHPITSPASEFE NTRGGRVILHPITSPASEFEHVE NVVLDEFGNPR QEFPPRSNLDPETY QEFPSRSKLDSK RVRENPKSDVDLVEF

							SIIEEFPRLV SIIEEFPRLVTW VGVPKKDAEFK VLVGVPKKDAEFK
PCSK9/LDLR binding inhibition	[53]	GQEQSHQDEGVIVR (T9)	9676	β-Conglutin	F5B8V9 F5B8W1 F5B8W2 F5B8W4	4	DEQRHGQEQSHQDEGVIVR EKQRRGQEQSHQDEGVIVR EQRHGQEQSHQDEGVIVR GQEQSHQDEGVIVR
				Non-conglutin proteins		1	EEQSRGQEQSHQDEGVIVR
Computational PCSK9/LDLR binding inhibition	[52, 66]	LTFPG (P7)		β-Conglutin	F5B8V9 F5B8W0 F5B8W2 F5B8W5	6	GLTFPGSTEDVERL LTFPGSAQDVERL LTFPGSIEDVERL LTFPGSIEDVERLIK LTFPGSTEDVERL LTFPGSVEDVERL
Total						**55**	

[a] According to "UniProtKB" (http://www.uniprot.org/).

Table 2. Plasma and hepatic lipidic profile in ApoE$^{-/-}$ mice.

Parameters	Plasma (mg/dL)				Liver (mg/g tissue)			
	SD	WD	WD+LPH	*p*-value	SD	WD	WD+LPH	*p*-value
TC	254.3 ± 27.21^{a}	337.2 ± 28.45^{b}	270.8 ± 39.87^{a}	**0.006**	1.50 ± 0.31^{a}	2.42 ± 0.56^{b}	1.96 ± 0.31^{a}	**0.003**
TG	56.17 ± 9.52^{a}	98.60 ±16.99^{b}	71.50 ± 9.88^{a}	**0.005**	27.83 ± 2.90^{a}	50.37 ± 14.42^{b}	37.26 ± 4.88^{a}	**0.003**
LDL-C	199.8 ± 32.77^{a}	281.6 ± 25.56^{b}	220.8 ± 34.61^{a}	**0.005**				
HDL-C	41.88 ± 12.00^{a}	35.80 ± 4.76^{a}	35.67 ± 4.41^{a}	0.373				

Data are expressed as mean ± standard deviation. Total cholesterol (TC) and triglycerides (TG) concentration were quantified in the plasma and hepatic tissue. Low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) concentration were quantified in the plasma. WD: western diet-fed mice group; LPH, lupine protein hydrolysate-treated mice group. The *p*-value indicates the statistical analysis carried out by One-way ANOVA between the three experimental groups within each tissue. Different letters represented a statistical difference ($p \leq 0.05$).

Fig1