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Safety and Efficacy of a Beverage Containing Lupine Protein Hydrolysates on the Immune, Oxidative and Lipid Status in Healthy Subjects: An Intervention Study (the Lupine-1 Trial)

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Scope: We have previously demonstrated the anti-inflammatory and antioxidant properties of in vitro administered *Lupinus angustifolius* protein hydrolysates (LPHs) on human peripheral blood mononuclear cells (PBMCs). This study aims to evaluate the safety and efficacy of a beverage containing LPHs (LPHb) on the immune, oxidative and metabolic status of healthy subjects.
Methods and Results: In this open-label intervention, 33 participants daily ingest a LPHb containing 1 g LPHs for 28 days. Biochemical parameters are assayed in fasting peripheral blood and urine samples before, during (14 days) and after LPHb ingestion. Participants' health status and the immune and antioxidant responses of PBMCs are also evaluated throughout the trial. The LPHb ingestion is safe and effective in both increasing the anti-/pro-inflammatory response of PBMCs and improving the cellular anti-oxidant capacity. LPHb also reduces the low-density lipoprotein-cholesterol (LDL-C)/high-density lipoprotein-cholesterol (HDL-C) atherogenic index. LPHb effect is particularly beneficial on decreasing not only the LDL-C/HDL-C index but also serum total cholesterol levels in the male cohort that shows the highest baseline levels of well-known cardiovascular risk factors.
Conclusion: This is the first study to show the pleiotropic actions of a lupine bioactive peptides-based functional food on key steps of atherosclerosis including inflammation, oxidative stress, and cholesterol metabolism.

1. Introduction

There is an increasing awareness of the importance of healthy nutrition, and the search for new food products, that not only provide the balanced nutrients but also beneficial health effects, has become in a cutting-edge field in the frame of human and animal nutrition. In this way, many commercial products are enriched with different components to improve their beneficial properties. In particular, the number of studies based on the bio-functionality of protein hydrolysates has dramatically increased over the last few years.[1,2] An additional potential benefit of protein hydrolysates resides in their low immunogenicity,[3] given that the protein antigenic epitopes can be also hydrolyzed.[4] Due to an increasing concern in animal welfare and the rising environmental awareness,[5] the consumption of vegetable products is becoming a common trend. In this way, a growing body of evidence has underlined several biological actions such as

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immune,[6,7] antioxidant[6,8] and metabolic [9–11] effects of different vegetable protein hydrolysates by in vitro or pre-clinical approaches. We have previously shown that *Lupinus angustifolius* protein hydrolysates (LPHs), generated by hydrolyzation with the commonly used food-grade endopeptidase Alcalase 2.4 L, inhibit the enzymatic activity inflammatory enzymes phospholipase A2, cyclooxygenase 2, thrombin, and transglutaminase in a cell-free system.[12] Moreover, in vitro administration of LPHs attenuated the pro-inflammatory response of THP-1-derived macrophages.[13] Likewise, we have shown for the first time that LPHs improve the anti-inflammatory/pro-inflammatory cytokine balance and total antioxidant capacity in ex vivo cultured human peripheral blood mononuclear cells (PBMCs).[14]

Once studied the biological activity of LPHs under in vitro system, the aim of the present study was to evaluate the safety and efficacy of a beverage containing LPHs (LPHb) on the immune, oxidative and metabolic status in healthy subjects by conducting an open-label longitudinal study (Lupine-1 trial).

2. Experimental Section

2.1. Subjects

Thirty five healthy participants, 18–50 years, were recruited via mass advertising from Seville Urban Community (Figure S1, Supporting Information) according to the inclusion/exclusion criteria (Table S1, Supporting Information). The sample size of 35 subjects was calculated using the nQuery Sample Size Calculator tool based on the normal distribution of the studied parameters in the population. A minimum expected difference of 5% has been set between before and after the intervention, with an expected standard deviation between the means of 10%. Considering an error $\alpha = 5\%$ and a β error of 85% (power) an estimated loss percentage of 5%, the minimum number of subjects required for the study was 35. The study was conducted between October 2015 and December 2015, and all the procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983. The clinical trial "Lupine-1" was approved by the ethical review board of Virgen Macarena and Virgen del Rocío University Hospital (reference number 2015/110). Written informed consent was obtained from all participants. This clinical study was declared to the website ClinicalTrials.gov under the number NCT02590887 (https://clinicaltrials.gov/ct2/show/NCT02590887).

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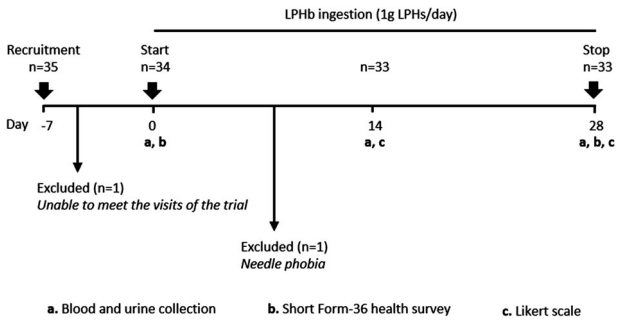

Figure 1. Experimental study scheme. Schematic representation of the different phases of the study. The open-label longitudinal study lasted 28 days in which the volunteers daily consumed 200 mL of experimental beverage containing 1 g LPHs. On days 0, 14, and 28, the volunteers were called on different experimental approaches. LPHb, LPHs-based functional beverage; LPHs, lupine protein hydrolysates; n, number of volunteers.

2.2. Study Design

Thirty five healthy participants were enrolled in an open-label longitudinal study. Thirty three subjects [16 males; mean age 30.53 (±2.71); 17 females; mean age 30.47 (±2.27)] completed the trial (**Figure 1**). Before the first LPHb intake, fasting peripheral blood and urine samples as well as the Short Form-36 (SF-36) health survey were collected from every participant to establish the baseline parameters of the study. Biological samples and Likert scale were also collected on days 14 and 28 (end of study), whereas SF-36 health survey was collected at the end of the study. Blood collection was carried out between 08:00 and 09:00 am in the Department of Clinical Biochemistry of Virgen del Rocío University Hospital, Seville. Blood samples were drawn in VACUETTE Z Serum Sep Clot Activator tubes (Greiner Bio-one, Kremsmünster, Austria) for analysis of biochemical parameters; in VACUETTE K3EDTA tubes (Greiner Bio-one) for a white blood cell count; and in Vacutainer CPT tubes (BD Biosciences, San Jose, CA, USA) for the subsequent PBMCs isolation. First morning urine was collected in VACUETTE urine tubes (Greiner Bio-one). In order to prevent a bias in the results caused by changing the volunteers' habits, subjects were instructed not to modify their dietary and physical habits.

2.3. Preparation of the Study Product

LPHs were obtained and characterized (chemical composition and the degree of hydrolysis) as previously described in details.[14] The LPHb consisted in an emulsion of 95% (w/w) aqueous phase (AP) and 5% oil phase (OP). The AP contained equal volumes of solution A and B. Solution A was an aqueous solution made up of LPHs (1% w/w), sucrose (12% w/w), vanilla flavor (0.36% w/w) and drinking water (86.64% w/w) whereas Solution B was an aqueous solution composed of xanthan gum (0.25% w/w) and drinking water (99.75% w/w). Solution B was prepared aiming to disperse xanthan gum in hot water (80°C) with a gentle agitation for 1 h. Once completely dissolved, xanthan gum was cooled down at room temperature and preserved during 24 h for an optimum hydration. OP was entirely composed of refined sunflower oil. The final emulsion was obtained

from two steps. First, pre-emulsion was made by slowly adding the OP into the AP and homogenizing by a T-25 basic ULTRA-TURRAX (IKA-Werke GmbH & Co. KG, Staufen, Germany) at 8000 rpm. Subsequently, the pre-emulsion was passed through an Emulsiflex-2000-B3 high-pressure homogenizer (Avestin Inc., Ottawa, Canada) at 70,000 psi (4.83×10^8 Pa) to make the final beverage. The LPHb was packaged by PULEVA (Lactalis, France) in 200 mL sterile tetrabricks, according to Spanish and European food regulation (RD 135/2010). Table S2, Supporting Information shows the composition of the administered formulation to volunteers.

2.4. Dosage Information

Volunteers daily consumed a fasting single dose of 200 mL of LPHb (one tetrabrick) for a period of 28 days between 08:00 and and 10:00 am. LPHs concentration was selected based on previous internal tests. The concentration of LPHs was not achievable through a regular diet or food supplements.

2.5. Biochemical Measurements

Biochemical parameters were quantified in serum from fasting blood samples as previously described,[15,16] by an automated immunoassay electrochemiluminescence systems (COBAS c702 and e602 analyzers; Roche Diagnostic, Basel, Switzerland); white blood cells were quantified in blood samples by fluorescence flow cytometer SYSMEX XE 5000 Hematology Analyzer (Sysmex Europe GmbH, Norderstedt, Germany). Urine samples were analyzed by Beckman Coulter AU2700 (Beckman Coulter, Brea, USA).

2.6. Participants' Health Status

SF-36 health survey was filled out by all participants to evaluate their physical and mental health status before (day 0, baseline) and at the end of the study (day 28). The SF-36 measured the health status in eight dimensions that evaluated physical health (physical functioning, role limitation due to physical health, pain, and general health) and mental health (social functioning, role limitation due to emotional problems, energy/fatigue, and emotional well-being). Total score on each SF-36 dimension ranged between 0 and 100, a higher score indicated a healthier state.[17]

2.7. Analysis of LPHb Palatability

A Likert type scale was conducted to estimate the degree of palatability of LPHb. The scale was applied to all participants on days 14 and 28. A 7-items scale was performed with the following scoring system: 1, extremely unpalatable; 2, quite unpalatable; 3 slightly unpalatable; 4, neutral taste; 5, slightly palatable; 6, quite palatable; 7, very palatable.

2.8. Cell Cultures

PBMCs were isolated by centrifugation of Vacutainer CPT tubes, and then cultured at a density of 1×10^6 cells mL^{-1} in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50 U mL^{-1} of penicillin/streptomycin (all from BioWest, Nuaillé, France). To evaluate the antioxidant activity, cells were cultured overnight, while they were cultured for cytokine quantification for 48 h in the presence of 8 µg mL^{-1} phytohemagglutinin-P (PHA; Sigma-Aldrich, St. Louis, MO, USA), a well-known selective T cell mitogen, and incubated at 37 °C in a 5% CO_2 humidified atmosphere. Supernatants were collected and stored at −20 °C until use.

2.9. Extracellular Cytokine Quantification

Cytokine production was quantified in supernatants of cultured PBMCs by using a fluorescent bead multiples assay (Human 13plex Kit FlowCytomix; eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Median fluorescence intensity data were obtained using a Canto II flow cytometer (BD Biosciences) and analyzed with FlowCytomix Pro 2.4 software (eBioscience).

2.10. Antioxidant Capacity

TAC was quantified in the supernatants from overnight cultured PBMCs by the OxiSelect Total Antioxidant Capacity Assay Kit (Cell Biolabs, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, this assay was based on the reduction of copper (II) due to the presence of antioxidant molecules. The absorbance was measured at 490 nm with a Tecan Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland). Data were expressed as "µM copper reducing equivalents" (CRE), which were proportional to the TAC sample.

Oxygen radical absorbance capacity (ORAC) assay was performed in supernatants from overnight-cultured PBMCs (sample dilution was 1:400 in phosphate buffer; 75 mM, pH 7.4) according to Ou et al.[18] The fluorescence (excitation: 485/20 nm, emission: 528/20 nm) was calculated every 5 min for 90 min with the Synergy HT-multimode microplate reader (Biotek Instruments, Winooski, VT, USA). Final ORAC values were expressed as "mmoles of Trolox equivalents/mL".

2.11. Statistical Analysis

Results were expressed as the mean and standard error of the mean (SEM). Non-normally distributed variables were analyzed by non-parametric Friedman or Wilcoxon paired tests, whereas normally-distributed ones (TAC and ORAC) were analyzed by the parametric Student's *t*-test, using IBM SPSS Statistics software v.24 (IBM, Armonk, NY, USA). Values of $p \leq 0.05$ were considered statistically significant.

3. Results

3.1. Participant Characteristics

This study was conducted in a final sample of 33 subjects out of the 35 subjects recruited, encompassing 16 males (48.48%) and

Table 1. Demographic characteristics of the study participants.

	Males	Females	Total
Number	16	17	33
Age	30.53 ± 2.71	30.47 ± 2.27	30.27 ± 1.72
BMI	23.77 ± 0.76	21.71 ± 0.31	22.71 ± 0.43

Number of participants, age (years), and body mass index (BMI) of the study participants. Data present the mean ± standard error of the mean for each group.

17 females (51.52%). No significant differences ($p > 0.05$) in the age and body mass index (BMI) between males and females were observed at baseline (**Table 1**). After 28 days of intake no impact in the weight of volunteers was observed (day 0: 65.81 ± 1.94 kg; day 28: 66.16 ± 1.95 kg; $p = 0.082$).

3.2. Safety Study

To test the LPHb safety (side effects or discomfort) we have evaluated the change (between baseline, 14 days and after LPHb ingestion) of the general, hepatic, renal and immune biochemical markers (**Tables 2–6**) as well as the white blood cell counts (hemogram) (**Table 7**). In addition, SF-36 health survey was performed to test the volunteers´ perception about their physical and mental health before and after 28d LPHb ingestion (Table 7). The baseline levels of the studied biochemical markers were within the normal range and no changes ($p > 0.05$) were observed either in the general (Table 2), hepatic (Table 3), renal (Table 4) and immune (Table 5) biochemical profiles, or in the white blood cell counts (Table 6) after 28 days of LPHb ingestion. In addition, no variations ($p > 0.05$) in the SF-36 health survey (physical and mental health) of the volunteers between the baseline and the endpoint of the study were observed (Table 7). Thus, no side effects on their metabolism (biochemical markers) or in the global health status perception (SF-36) were observed. Moreover, no adverse effect or discomfort was reported by any of the participants during or after the end of the study.

3.3. LPHb Palatability

To assess the acceptability of the LPHb, the widely used Likert scale was completed to estimate the degree of palatability of the beverage. As is shown in Table S3, Supporting Information, Likert scale scoring indicated an increase in the LPHb acceptance over the course of the study with a mean Likert score of 4.06 ± 0.21 at the end of the study ($p = 0.012$).

3.4. LPHb Effects on Blood Lipid Biochemical Markers

LPHb ingestion for 28 days did not modify ($p > 0.05$) the serum concentrations of total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) and triglycerides (TG). However, the LPHb treatment significantly reduced ($p \leq 0.05$) the LDL-C/HDL-C ratio in the total population. This was due to the significant decrease ($p \leq 0.01$) of LDL-C/HDL-C levels in the male participants (**Table 8**). We also found that the LPHb effect on the reduction of LDL-C/HDL-C ratio was significant in males with high baseline levels (defined as baseline values above the median; M2) of cardiovascular risk factors such as BMI ($p \leq 0.05$), TC ($p \leq 0.05$), LDL-C ($p \leq 0.05$), Castelli risk index I (TC/HDL-C ratio) ($p \leq 0.05$) and II (LDL-C/HDL-C ratio) ($p \leq 0.01$), and atherogenic index of plasma [(Log(TG/HDL)] ($p \leq 0.05$), whereas no differences ($p > 0.05$) were observed in those whose baseline levels were under the median (M1) (**Figure 2**). Moreover, LPHb consumption significantly decreased the TC levels in males with higher baseline values of BMI ($p \leq 0.05$), TC ($p \leq 0.05$), LDL-C ($p \leq 0.05$), and Castelli risk index II ($p \leq 0.05$), (Figure 2A–C,E, respectively). Likewise, males with baseline levels of LDL-C and Castelli risk index II in the M2 showed significantly lower ($p \leq 0.05$) serum concentrations of LDL-C after 28 days of LPHb consumption (Figure 2C,E).

3.5. LPHb Efficacy on PBMCs Immune Response

To analyze the immune effects of the 28 days-ingestion of LPHb on PBMCs from participants, the levels of key pro-inflammatory Th1 [interleukin (IL)-2, interferon-γ (IFN-γ) and tumor necrosis factor (TNF)] and anti-inflammatory Th2 (IL-4 and IL-10) cytokines were quantified in the supernatants of PHA-stimulated PBMCs. As shown in **Figure 3**, LPHb significantly diminished the Th1 pro-inflammatory response by reducing the production of IL-2 ($p \leq 0.01$) (Figure 3A), IFN-γ ($p \leq 0.05$) (Figure 3B), and TNF ($p \leq 0.01$) (Figure 3C). Although the production of the Th2 cytokines, IL-4 and IL-10 was not modified by LPHb treatment ($p > 0.05$) (Figure 3D,E, respectively), a significant increase in the ratios of IL-10/IFN-γ ($p \leq 0.05$), IL-10/TNF ($p \leq 0.05$), and IL-4/TNF ($p \leq 0.01$) was observed after the 28 days-LPHb consumption (Figure 3G,H,K, respectively).

Table 2. General biochemical parameters.

	Range	Males			Females			Total		
		Baseline	Day 14	Day 28	Baseline	Day 14	Day 28	Baseline	Day 14	Day 28
Glucose (mg dL^{-1})	70-110	89.13 ± 1.82	87.87 ± 1.85	90.44 ± 1.91	83.35 ± 2.56	84.94 ± 2.02	85.59 ± 2.00	86.06 ± 1.66	86.36 ± 1.38	87.94 ± 1.43
Urea (mg dL^{-1})	10-40	33.33 ± 1.83	31.06 ± 1.23	32.75 ± 1.58	29.94 ± 1.76	27.23 ± 1.74	28.29 ± 1.73	31.58 ± 1.29	29.09 ± 1.11*	30.45 ± 1.22
Creatinine (mg dL^{-1})	0.5-1.1	0.90 ± 0.02	0.91 ± 0.02	0.90 ± 0.25	0.74 ± 0.02	0.75 ± 0.02	0.77 ± 0.02	0.82 ± 0.02	0.83 ± 0.02	0.84 ± 0.02
Insulin (μU mL^{-1})	2.5-25	8.21 ± 1.50	7.13 ± 0.68	8.90 ± 0.97	8.04 ± 0.97	7.29 ± 0.64	8.82 ± 0.90	8.13 ± 0.88	7.21 ± 0.46	8.86 ± 0.65

Data present the mean ± standard error of the mean of the basic biochemical parameters for each group and time point. Friedman test was applied to determine intergroup differences for the values obtained at each time point in all volunteers and discriminated by sex. Statistical significance with respect to day 0 (baseline). *$p \leq 0.05$.

Table 3. Hepatic biochemical parameters.

	Range	Males			Females			Total		
		Baseline	Day 14	Day 28	Baseline	Day 14	Day 28	Baseline	Day 14	Day 28
Total Protein (g dL^{-1})	6.5-8.0	7.71 ± 0.09	7.32 ± 0.09**	7.57 ± 0.10	7.25 ± 0.08	7.11 ± 0.92	7.16 ± 0.06	7.46 ± 0.07	7.21 ± 0.06**	7.36 ± 0.07
ALP (U L^{-1})	40-130	63.00 ± 4.41	62.06 ± 3.49	65.06 ± 3.75	48.00 ± 3.02	50.00 ± 3.53	49.47 ± 3.13	55.27 ± 2.92	55.85 ± 2.67	57.03 ± 2.76
GGT (UI L^{-1})	10-50	16.60 ± 1.21	14.94 ± 0.99**	15.50 ± 1.10	12.23 ± 1.15	11.06 ± 1.18*	11.71 ± 1.23	14.28 ± 0.91	12.94 ± 0.84***	13.54 ± 0.88
GOT (U L^{-1})	10-37	19.31 ± 1.09	18.27 ± 1.14	20.60 ± 1.85	17.29 ± 0.95	16.59 ± 0.85	15.65 ± 1.04	18.27 ± 0.73	17.37 ± 0.71	17.97 ± 1.10
GPT (UI L^{-1})	10-40	17.93 ± 1.53	17.94 ± 2.12	19.64 ± 1.97	13.59 ± 1.41	13.53 ± 1.07	14.12 ± 1.76	15.62 ± 1.09	15.67 ± 1.21	16.61 ± 1.39

Data present the mean ± standard error of the mean of the total protein and the transaminases values for each group and time point. Friedman test was applied to determine intergroup differences for the values obtained at each time point in all volunteers and discriminated by sex. Statistical significance with respect to day 0 (baseline). ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; GOT, glutamic-oxaloacetic transaminase; GPT glutamic-pyruvic transaminase. *$p \leq 0.05$; **$p \leq 0.01$ and ***$p \leq 0.001$.

Table 4. Renal biochemical parameters.

	Range	Males			Females			Total		
		Baseline	Day 14	Day 28	Baseline	Day 14	Day 28	Baseline	Day 14	Day 28
Protein/Creatinine (mg dL^{-1})	0–0.2	0.054 ± 0.003	0.052 ± 0.002	0.053 ± 0.004	0.065 ± 0.003	0.062 ± 0.004	0.062 ± 0.003	0.062 ± 0.003	0.062 ± 0.003	0.058 ± 0.002
Urea/Creatinine (mg dL^{-1})	12-20	13.93 ± 0.89	12.77 ± 0.81	13.61 ± 0.82	13.98 ± 1.04	12.97 ± 1.08	13.72 ± 0.88	13.95 ± 0.67	12.87 ± 0.67	13.67 ± 0.59

Data present the mean ± standard error of the mean of the renal biochemical parameters ratios for each group and time point. Friedman test was applied to determine intergroup differences for the values obtained at each time point in all volunteers and discriminated by sex. Statistical significance with respect to day 0 (baseline).

3.6. LPHb Efficacy on Antioxidant Capacity

To evaluate whether the LPHb ingestion was able to modify the PBMCs antioxidant status, TAC and ORAC assays were performed in the supernatant of overnight-cultured cells. After 28 days of daily LPHb consumption, a significant increase in both TAC ($p \leq 0.05$) (**Figure 4**A) and ORAC ($p \leq 0.05$) (Figure 4B) activities was observed in the PBMCs of participant subjects.

4. Discussion

This study shows that 4 weeks of daily ingestion of LPHb is safe and effective at increasing the anti-inflammatory and antioxidant responses in circulating blood leukocytes from healthy subjects. In addition, LPHb consumption reduces the LDL-C/HDL-C atherogenic index, a predictive factor for atherosclerosis effects.[19,20] Interestingly, the LPHb intake was particularly beneficial in decreasing not only the LDL-C/HDL-C index but also serum TC levels in males who showed high baseline levels of well-known cardiovascular risk factors such as BMI, TC, LDL-C, Castelli risk index I and II and the atherogenic index of plasma. Moreover, the good sensorial acceptability of the LPHb, tested by the Likert scale, allowed to successfully comply with the study.

Despite the presence of the fatty and glucidic content (5% oil and 6% sucrose, respectively) in the LPHb, after 28 days of intake no impact in the weight of volunteers was observed. Also, neither lipid nor glucidic biochemical parameters increased after LPHb consumption. Although each LPHb tetrabrick contained 1 g of LPHs, no changes in the total protein levels were observed after the beverage ingestion. Additionally, hepatic, renal, and immune biochemical markers were not altered throughout the 28 days. Although some proteins from *Lupinus* spp. have been described to trigger an allergic response,[21] no effect on the blood levels of C-reactive protein and immunoglobulin (Ig) G, IgA, IgM or IgE, a marker of food allergy,[22] was observed after LHPb consumption.

In accordance with these findings, the consumption of LPHb did not affect the volunteers' perception of their own health status, manifesting a general healthy self-perception at the end of

Table 5. Immune biochemical parameters.

	Range	Males			Females			Total		
		Baseline	Day 14	Day 28	Baseline	Day 14	Day 28	Baseline	Day 14	Day 28
CRP (mg L^{-1})	0-5	0.73 ± 0.12	0.72 ± 0.14	0.77 ± 0.15	1.49 ± 0.40	0.78 ± 0.15	2.82 ± 0.67	1.09 ± 0.20	0.75 ± 0.10	1.79 ± 0.39
IgG (mg dL^{-1})	700-1600	1258.64 ± 51.98	1229.69 ± 52.74	1259.89 ± 52.52	1214.12 ± 43.45	1196.09 ± 51.93	1185.97 ± 49.83	1234.99 ± 33.25	1212.38 ± 42.35	1221.81 ± 36.18
IgA (mg dL^{-1})	70-400	282.33 ± 21.00	276.05 ± 20.26	282.81 ± 21.19	239.78 ± 21.86	241.38 ± 21.35	241.57 ± 20.96	259.72 ± 15.46	258.19 ± 14.84	261.56 ± 15.12
IgM (mg dL^{-1})	40-230	111.37 ± 9.45	108.36 ± 8.82	111.62 ± 9.05	145.54 ± 11.37	139.75 ± 9.84	139.79 ± 9.69	129.52 ± 7.98	124.53 ± 7.09	126.13 ± 7.00
IgE (mg dL^{-1})	0-100	58.23 ± 16.95	54.65 ± 13.75	60.83 ± 14.76	52.85 ± 18.46	48.69 ± 16.33**	48.80 ± 16.49	55.54 ± 12.32	51.67 ± 10.50**	54.82 ± 10.93

Data present the mean ± standard error of the mean of the C-reactive protein (CRP) and the immunoglobulins (Ig) values for each group and time point. Friedman test was applied to determine intergroup differences for the values obtained at each time point in all volunteers and discriminated by sex. Statistical significance with respect to day 0 (baseline). **$p \leq 0.01$.

Table 6. Hemogram.

	Range	Males			Females			Total		
		Baseline	Day 14	Day 28	Baseline	Day 14	Day 28	Baseline	Day 14	Day 28
Leukocyte ($\times 10^9$/L)	3.8-11.5	5.52 ± 0.26	5.53 ± 0.33	5.94 ± 0.33	5.83 ± 0.40	5.44 ± 0.31	6.14 ± 0.34	5.68 ± 0.24	5.48 ± 0.22	6.04 ± 0.23
Neutrophil ($\times 10^9$/L)	2.5-7.5	3.21 ± 0.17	3.15 ± 0.25	3.46 ± 0.24	3.54 ± 0.31	2.96 ± 0.21	3.71 ± 0.25	3.38 ± 0.18	3.05 ± 0.16	3.59 ± 1.73
Lymphocyte ($\times 10^9$/L)	1.5-4	1.70 ± 0.11	1.74 ± 0.12	1.80 ± 0.10	1.74 ± 0.12	1.92 ± 0.10	1.82 ± 0.13	1.72 ± 0.08	1.83 ± 0.08	1.81 ± 0.08
Monocyte ($\times 10^9$/L)	0.2-0.8	0.33 ± 0.02	0.33 ± 0.30	0.35 ± 0.02	0.29 ± 0.02	0.27 ± 0.20	0.30 ± 0.03	0.31 ± 0.01	0.30 ± 0.02	0.33 ± 0.02
Eosinophil ($\times 10^9$/L)	0.05-0.5	0.15 ± 0.02	0.18 ± 0.03	0.16 ± 0.02	0.12 ± 0.02	0.16 ± 0.03	0.13 ± 0.02	0.14 ± 0.01	0.17 ± 0.02	0.14 ± 0.01
Basophil ($\times 10^9$/L)	0.01-0.15	0.017 ± 0.00	0.017 ± 0.00	0.025 ± 0.00	0.015 ± 0.00	0.015 ± 0.00	0.025 ± 0.00	0.016 ± 0.00	0.016 ± 0.00	0.025 ± 0.00*

Data present the mean ± standard error of the mean of the white blood cells numbers for each group and time point. Friedman test was applied to determine intergroup differences for the values obtained at each time point in all volunteers and discriminated by sex. Statistical significance with respect to day 0 (baseline). *$p \leq 0.05$.

Table 7. Physical and mental self-perception (SF-36 health survey).

	Males		Females		Total	
	Baseline	Day 28	Baseline	Day 28	Baseline	Day 28
Physical health						
Physical functioning	99.33 ± 0.45	99.67 ± 0.33	95.29 ± 3.28	95.29 ± 3.25	97.19 ± 1.77	97.34 ± 1.75
Role limitations due to physical health	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	91.18 ± 6.41	100.00 ± 0.00	95.31 ± 3.45
Pain	92.00 ± 2.43	93.67 ± 3.06	88.23 ± 3.53	83.23 ± 4.87	90.00 ± 2.18	88.12 ± 3.06
General health	88.33 ± 1.99	88.00 ± 1.81	78.23 ± 3.90	79.71 ± 3.77	82.97 ± 2.42	83.59 ± 2.27
Mental health						
Social functioning	93.33 ± 3.63	92.50 ± 3.18	97.79 ± 1.60	94.12 ± 2.17	95.70 ± 1.91	93.36 ± 1.86
Role limitations due to emotional problems	88.89 ± 5.31	86.67 ± 7.83	92.16 ± 5.37	98.04 ± 1.96	90.62 ± 3.74	92.71 ± 3.88
Energy/fatigue	62.67 ± 3.54	60.17 ± 4.29	64.71 ± 3.14	67.79 ± 2.34	63.75 ± 2.32	64.22 ± 2.42
Emotional well-being	55.47 ± 3.09	58.67 ± 3.81	55.76 ± 3.44	58.12 ± 2.41	55.62 ± 2.29	58.37 ± 2.16
Global health status	58.33 ± 3.98	56.25 ± 4.27	54.41 ± 3.20	55.88 ± 3.41	56.25 ± 2.51	56.06 ± 2.67

Data represent the mean ± standard error of the mean of the scores obtained by the Short Form-36 (SF-36) health survey for each group and time point. Wilcoxon test was applied to determine difference for values obtained at each time point in all volunteers or discriminated by sex.

the study, by means of the SF-36 health survey. Although there are several clinical studies that assess the beneficial effects of lupine proteins,[23–27] no data about health status perception was previously reported. The unchanging effects on general, hepatic, and renal biochemical markers as well as on hemogram and the health survey (SF-36) allow us to conclude that the 28-day ingestion of the LPHb was safe as no side effects on their metabolism (biochemical markers) or discomfort (SF-36) were observed in the volunteers enrolled in the study.

Beside the safety, the efficacy of LPHb was explored. Regarding the cellular immune response, the LPHb consumption reduced the PHA-induced Th1 pro-inflammatory response in PBMCs

Table 8. Lipid biochemical parameters.

	Range	Males			Females			Total		
		Baseline	Day 14	Day 28	Baseline	Day 14	Day 28	Baseline	Day 14	Day 28
TC (mg dL^{-1})	150-200	159.33 ± 6.66	154.87 ± 7.76*	157.12 ± 6.84	168.06 ± 7.18	165.31 ± 6.05	162.62 ± 5.63	163.97 ± 4.91	163.15 ± 5.67	159.87 ± 4.39
HDL-C (mg dL^{-1})	39-60	53.25 ± 3.70	49.19 ± 2.98	52.81 ± 3.43	66.87 ± 1.97	69.53 ± 2.10	65.71 ± 2.45	60.06 ± 2.40	59.67 ± 2.52	59.45 ± 2.35
LDL-C (mg dL^{-1})	80-140	96.62 ± 8.00	91.37 ± 6.40	88.87 ± 5.44	84.27 ± 5.82	90.53 ± 7.32	89.73 ± 8.21	92.62 ± 5.27	90.94 ± 4.81	89.29 ± 4.78
TG (mg dL^{-1})	70-170	69.40 ± 7.91	71.44 ± 6.99	77.19 ± 6.40	55.88 ± 4.53	54.53 ± 4.11	73.76 ± 8.27	62.22 ± 4.51	62.73 ± 4.20	72.56 ± 4.48
LDL-C/HDL-C ratio	0-3	1.99 ± 0.23	1.94 ± 0.17	1.68 ± 0.14**	1.34 ± 0.10	1.32 ± 0.11	1.39 ± 0.14	1.67 ± 0.14	1.62 ± 0.11	1.60 ± 0.12*

Data present the mean ± standard error of the mean of lipid biochemical parameters values for each group and time point. Friedman test was applied to determine intergroup differences for the values obtained at each time point in all volunteers and discriminated by sex. Statistical significance with respect to day 0 (baseline). HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, triglycerides. *$p \leq 0.05$ and **$p \leq 0.01$.

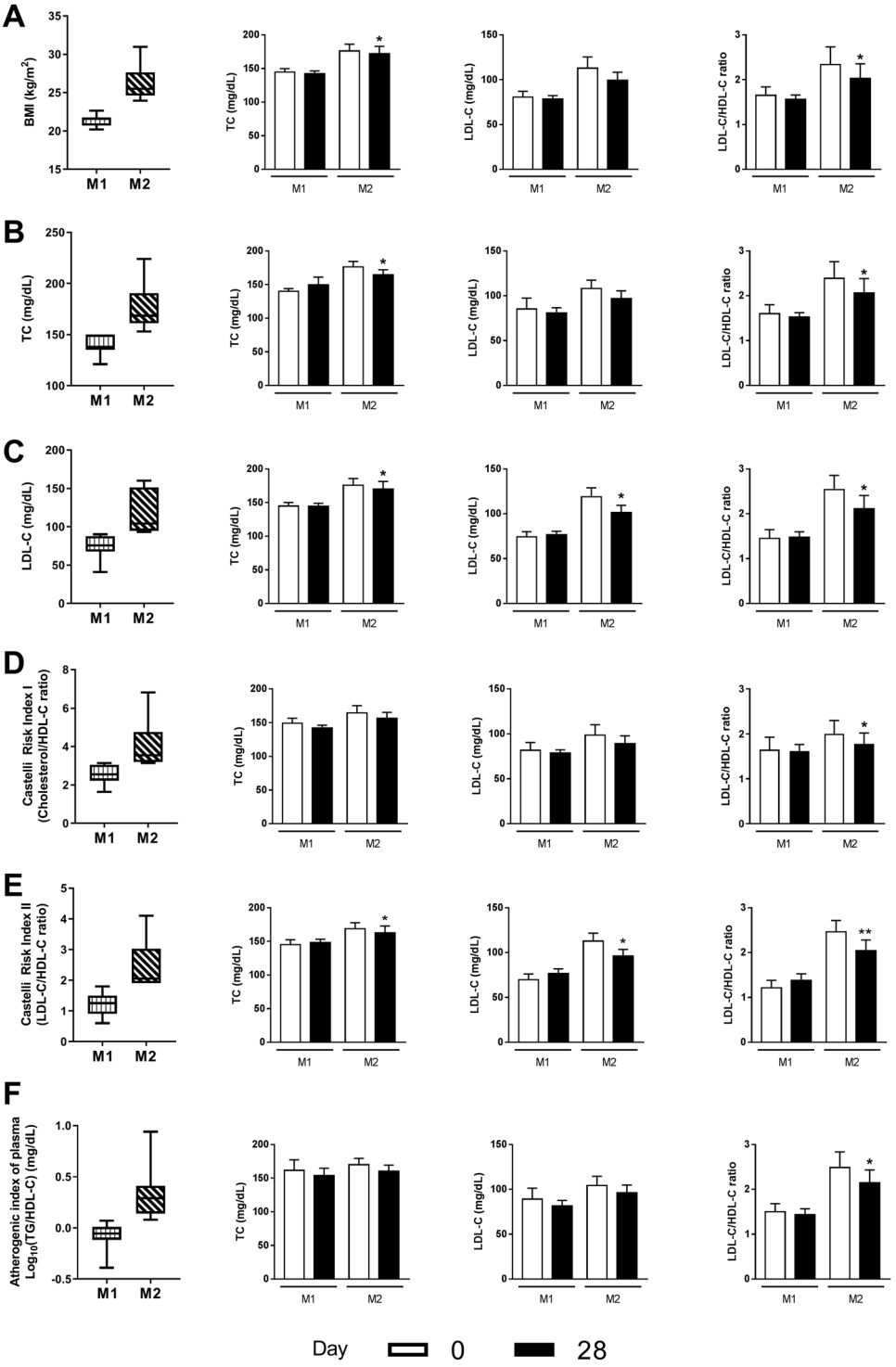

Figure 2. Lipid profile analysis of male volunteers with a possible risk of suffering cardiovascular diseases. Mean ± standard error of the mean of lipid biochemical parameters (TC, LDL-C, and LDL-C/HDL-C ratio) quantified at the start (day 0) and end of the study (day 28), calculated according to whether they were below (M1) or above (M2) the median of baseline values for each parameter considered as a risk factor for cardiovascular diseases. Risk factors considered were: BMI (A), total cholesterol (B), LDL-C (C), Castelli risk I (D) and II (E), and atherogenic index of plasma (F). Only the male population was considered (n = 15). *$p \leq 0.05$; **$p \leq 0.01$ between day 28 and the control condition (day 0, before starting the study). BMI, body mass index; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol, TC, total cholesterol; TG, triglycerides.

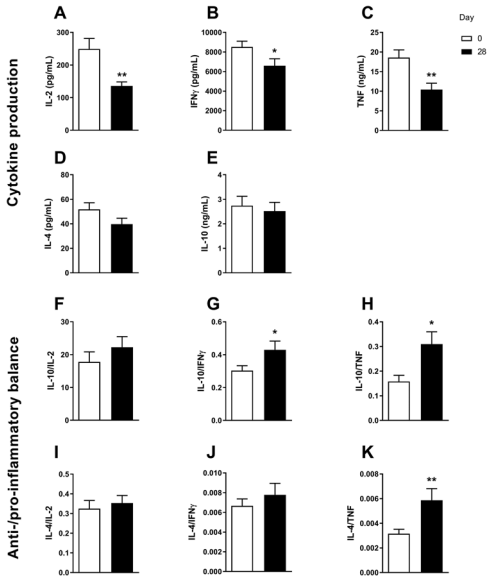

Figure 3. Cytokine production in PHA-stimulated PBMCs after 48 h of culture. Pro-inflammatory (A–C) and anti-inflammatory cytokines (D,E), ratios between IL-10 and pro-inflammatory cytokines (F–H) and between IL-4 and pro-inflammatory cytokines (I–K) were shown. Data presented as mean ± standard error of the mean of each group. Statistical significance with respect to the control group, $*p \leq 0.05$; $**p \leq 0.01$. IFN-γ, interferon-γ; IL, interleukin; TNF, tumor necrosis factor.

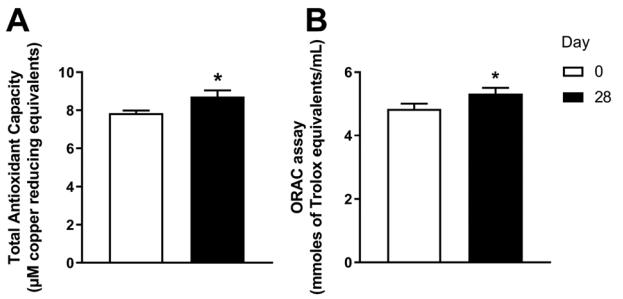

Figure 4. Antioxidant capacity. Total antioxidant capacity in μM copper reducing equivalents A) and oxygen radical absorbance capacity (ORAC) in mmoles of Trolox equivalents/mL B) were quantified at days 0 and 28 time points. Data presented as mean ± standard error of the mean of each group values obtained at each time point. Statistical significance with respect to the control group (day 0, baseline), $*p \leq 0.05$.

from participants whereas the Th2 response was not modified, skewing the Th1/Th2 balance toward an anti-inflammatory microenvironment. This finding agrees with our previous results that showed the anti-inflammatory bias of the T response on human PBMCs in vitro treated with LPHs.[14] However, this is the first report to show this effect on the T cell response after in vivo ingestion of LPHb. In this way, clinical evidence of the effect of vegetable proteins hydrolysates on the human immune status are very limited. A previous study showed that the intake of 3 g gluten hydrolysates for 6 days was able to increase natural killer (NK) cells activity without reporting side effects by the five enrolled healthy volunteers.[28] Moreover, a single oral dose of 8 g soybean protein hydrolysate modulated the frequency of blood leukocytes in 10 healthy volunteers by a transient increase of NK cells and macrophages and a decrease of B cells.[29] Therefore, to our knowledge, this is the first study to address the immunomodulatory role of LPHb in human leukocytes in a longitudinal trial.

In addition to cytokines, oxidative molecules such as reactive oxygen species (ROS) can be released from inflammatory cells at the site of inflammation.[30] Although the ROS generation is a physiological process, it is widely known that oxidative stress is generated by an imbalance between ROS production and the antioxidant capacity of the system that leads to an exacerbated accumulation of free radicals that can result in cellular damage.[31] Several enzymatic and non-enzymatic antioxidant mechanisms are key elements involved in the control of the intracellular reduction-oxidation balance by ROS detoxification.[32] The antioxidant status of PBMCs from the volunteers was significantly higher after 28 days LPHb intake, quantified both by TAC, an assay based on the determination of the copper reduction by a biological sample,[33] and by ORAC, an assay that quantify the neutralizing capacity against oxidation induced by the peroxyl radical.[34] These results agree with our previous studies,[14] showed the improvement of cell antioxidant capacity of volunteers after the LPHb consumption. Lupine or pea protein hydrolysates obtained with commercial proteases (alcalase, neutrase, and flavourzyme) have recently been shown not to increase TAC in respect to the control group (raw flour of lupine or pea, respectively).[35] However, after chromatographic separation, a sequence with antioxidant capacity was found, suggesting that the generation of the bio-functional peptides depends on the type of hydrolysis (chemical or biological hydrolysis), type of proteases, and the reaction duration.[35] No specific studies about the lupine effects on ORAC have been reported. To our knowledge, there is no study on the antioxidant status of PBMCs after a treatment with a vegetable functional food. This is the first work that describes the increased antioxidant status of human circulating leukocytes after the in vivo ingestion of a lupine-based functional food.

In addition to the immunomodulatory and anti-oxidant roles of the LPHb in the PBMCs of volunteers, the intake of the beverage showed a significant reduction in the LDL-C/HDL-C ratio, which is considered as one of the most accurate predictors of cardiovascular diseases (CVDs),[36] supporting a possible cardiovascular protective effect of the lupine hydrolysate. This effect was specifically due to the male population, which is noteworthy, giving that age-adjusted CVDs mortality rates are higher in males compared to females.[37]

In vitro studies have reported the positive influence of lupine peptides on cholesterol metabolism.[38,39] Moreover, several studies have reported the regulation of the lipid profile by lupine proteins in hypercholesterolemic adults. Different lupine protein enriched-foods (25–30g in bars, beverages, etc.), consumed for 4 weeks, reduce TC and LDL-C.[23,25,40] These positive effects have also been observed with *L. angustifolius* proteins.[24,41,42] On the contrary, consumption of lupine flour-enriched foods (bread, pasta or biscuits) during a 1 year controlled trial was reported not to affect the lipid parameters.[43] Other vegetable compounds have also demonstrated a cholesterol-lowering effect.[26,44]

Interestingly, the present study is the first to show that the daily ingestion of a LPHb containing 1 g of Alcalase 2.4 L-generated *L. angustifolius* hydrolysates for 4 weeks is enough

to significantly reduce the LDL-C/HDL-C ratio in normocholesterolemic male subjects, which supports the idea that protein hydrolysates are more bioactive than intact protein or free amino acids [45] due to the fact that the protein hydrolysis generates short peptides that are easily digestible.[46] In this regard, *L. albus* hydrolysates obtained by pepsin and trypsin digestion were reported to interfere with de novo cholesterol synthesis and increase LDL-C uptake in a human hepatocyte cell line (HepG2 cell line).[39]

Hypertension, together with increased values of BMI, TC, LDL-C, and LDL-C/HDL-C and TC/HDL-C ratios are defined as risk factors of endothelial dysfunction and, consequently, of cardiovascular risk.[47,48] In addition to lipid parameters, lupine-enriched food consumption has also shown antihypertensive activities.[43,49] The present trial revealed that males who presented high baseline CVDs risk factors underwent a remarkable decrease in cholesterol-related parameters after 28 days of LPHb consumption, supporting further approaches in pathological conditions related to hypercholesterolemia. Therefore, based on previous considerations, LPHb could be a suitable candidate to develop plant-based functional foods.

The main contribution of this study is that this is the first to show that the daily ingestion for 28 days of lupine bioactive peptides-based functional food is not only safe but also effective controlling key steps of several pathologies such as inflammation, oxidative stress and cholesterol metabolism, supporting the further evaluation of LPHs-based products in clinical conditions. However, our study has certain limitations, the most important being the absence of a control group as it is a before-and-after intervention where the baseline values of each volunteer were considered self-controlled. The placebo effect could influence our results; however, we speculate that the efficacy outcomes of this study such as inflammatory and antioxidant responses of peripheral leukocytes are difficult to be modified by placebo effect. It would even be less likely that the placebo effect is responsible for the beneficial effects of LPHb on reducing cholesterol levels and LDL-C/HDL-C index in people who present a higher baseline level of biochemical markers of cardiovascular risk. However, although blood markers are less likely to be affected by the placebo effect than more subjective measures (such as a survey), we cannot absolutely rule out the placebo effect. Although this experimental design is weaker than a randomized controlled trial with respect to establishing a cause-effect relationship, before-and-after approaches have been widely used.[50–54] Moreover, the present single-group before-after design could be biased by the modification of the subjects' lifestyle (Hawthorne effect). According to previous studies protocols,[55–59] participants were instructed not to modify their dietary and physical habits. Thus, they followed their typical eating and drinking habits and physical training throughout the trial. In fact, it is important to note that no impact in the weight of volunteers was observed after 28 days of beverage intake, which could indirectly suggest no change in eating habits or physical activity.

Furthermore, no changes in the physical or mental self-perception (SF-36 health survey) were observed by volunteers by means after LPHb ingestion. Furthermore, although Hawthorne and placebo effects are a limitation of this type of experimental design, the short period of the trial (28 days) also minimizes the probability that changes in the parameters studied are due to the subjective perception of the volunteers. Another fact that could support the causal effects of LPHb is that our results agree with previous in vitro studies showing the improvement of immune and antioxidant status as well as the hypocholesterolaemic effects driven by lupine peptides.[12–14,38,39] On the other hand, a single-group before-after design allows us to double the number of participants in the study compared to a placebo-controlled design.

In conclusion, this study reports that a 4-weeks daily intake of a functional beverage based on Alcalase 2.4 L-generated lupine protein hydrolysates is safe and effective in both reducing the production of Th1 pro-inflammatory cytokines and increasing the anti-inflammatory/pro-inflammatory microenvironment of the PHA-stimulated PBMCs, as well as increasing the cellular antioxidant capacity in healthy subjects. In addition, the beverage intake reduces the LDL-C/HDL-C atherogenic index and, noteworthy, its particularly beneficial capacity in decreasing TC, LDL-C, and LDL-C/HDL-C index in the male cohort that showed high baseline levels of well-known cardiovascular risk factors such as BMI, TC, LDL-C, Castelli risk index I and II and the atherogenic index of plasma. Therefore, this is the first study of the pleiotropic actions of a lupine bioactive peptides-based functional food on key steps of atherosclerosis including inflammation, oxidative stress and cholesterol metabolism.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

A.C.-V., P.J.L., M.C.M.-L., J.M.G., J.P., F.M. and C.C.-S: designed the experiments. I.C.-C., N.A.-S., A.I.A.-R., and A.M.-L: conducted research. I.C.-C., N.A.-S., A.I.A.-R., G.S.-S., M.S.F.-P., and A.M.-L: performed the experiments. I.C.-C., N.A.-S., A.I.A.-R., G.S.-S., M.S.F.-P., and A.C.-V: analyzed the data. I.C.-C., A.C.-V., I.-B., and N.A.-S: wrote the manuscript. All authors read and approved the final version of the manuscript.

Data Availability Statement

Data available on request from the authors.

Keywords

cholesterol, hydrolysate, inflammation, lupine, oxidative stress

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[1] B. Bigliardi, F. Galati, *Trends Food Sci. Technol.* **2013**, *31*, 118
[2] F. Rivero-Pino, F. J. Espejo-Carpio, E. M. Guadix, *Foods* **2020**, *9*, 983.
[3] L. Fu, B. J. Cherayil, H. Shi, Y. Wang, Y. Zhu, *Food Allergy: From Molecular Mechanisms to Control Strategies*, Springer Singapore, Singapore **2019**, pp. 123.
[4] A. Clemente, *Trends Food Sci. Technol.* **2000**, *11*, 254.
[5] D. W. Bruckner, *The Routledge Handbook of Animal Ethics*, Routledge **2019**, pp. 198.
[6] I. Cruz-Chamorro, N. Alvarez-Sanchez, G. Santos-Sanchez, J. Pedroche, M. S. Fernandez-Pachon, F. Millan, M. C. Millan-Linares, P. J. Lardone, I. Bejarano, J. M. Guerrero, A. Carrillo-Vico, *Nutrients* **2020**, *12*, 1673.
[7] M. Gonzalez-Montoya, B. Hernandez-Ledesma, J. M. Silvan, R. Mora-Escobedo, C. Martinez-Villaluenga, *Food Chem.* **2018**, *242*, 75.
[8] K. Sarabandi, S. M. Jafari, *Food Chem.* **2020**, *310*, 125951.
[9] C. Lammi, G. Aiello, L. Dellafiora, C. Bollati, G. Boschin, G. Ranaldi, S. Ferruzza, Y. Sambuy, G. Galaverna, A. Arnoldi, *J. Agric. Food Chem.* **2020**, 13179.
[10] Y. Li, G. Aiello, C. Bollati, M. Bartolomei, A. Arnoldi, C. Lammi, *Nutrients* **2020**, *12*, 794.
[11] M. J. C. Gomes, S. L. S. Lima, N. E. G. Alves, A. Assis, M. E. C. Moreira, R. C. L. Toledo, C. O. B. Rosa, O. R. Teixeira, P. Z. Bassinello, E. G. De Mejia, H. S. D. Martino, *Nutr. Metab. Cardiovasc. Dis.* **2020**, *30*, 141.
[12] M. C. Millan-Linares, M. M. Yust, J. M. Alcaide-Hidalgo, F. Millan, J. Pedroche, *Food Chem.* **2014**, *151*, 141.
[13] M. C. Millan-Linares, B. Bermudez, M. M. Yust, F. Millan, J. Pedroche, *J. Funct. Foods* **2014**, *8*, 224.
[14] I. Cruz-Chamorro, N. Álvarez-Sánchez, M. del Carmen Millán-Linares, M. del Mar Yust, J. Pedroche, F. Millán, P. J. Lardone, C. Carrera-Sánchez, J. M. Guerrero, A. Carrillo-Vico, *Food Res. Int.* **2019**, *126*, 108585.
[15] N. Alvarez-Sanchez, A. I. Alvarez-Rios, J. M. Guerrero, F. J. Garcia-Garcia, L. Rodriguez-Manas, I. Cruz-Chamorro, P. J. Lardone, A. Carrillo-Vico, *Exp. Gerontol.* **2018**, *108*, 201.
[16] N. Alvarez-Sanchez, A. I. Alvarez-Rios, J. M. Guerrero, F. J. Garcia-Garcia, L. Rodriguez-Manas, I. Cruz-Chamorro, P. J. Lardone, A. Carrillo-Vico, *J. Gerontol. A Biol. Sci. Med. Sci.* **2019**, 1488.
[17] J. E. Ware, Jr., C. D. Sherbourne, *Medical care* **1992**, *30*, 473.
[18] B. Ou, M. Hampsch-Woodill, R. L. Prior, *J. Agric. Food Chem.* **2001**, *49*, 4619.
[19] Z. Zhong, J. Hou, Q. Zhang, W. Zhong, B. Li, C. Li, Z. Liu, M. Yang, P. Zhao, *Lipids Health Dis.* **2019**, *18*, 1.
[20] L. Liu, P. Yin, C. Lu, J. Li, Z. Zang, Y. Liu, S. Liu, Y. Wei, *Front. Neurol.* **2020**, *11*, 408.
[21] U. Jappe, S. Vieths, *Mol. Nutr. Food Res.* **2010**, *54*, 113.
[22] L. Tordesillas, M. C. Berin, H. A. Sampson, *Immunity* **2017**, *47*, 32.
[23] M. Bahr, A. Fechner, M. Kiehntopf, G. Jahreis, *Clin. Nutr.* **2015**, *34*, 7.
[24] M. Bahr, A. Fechner, J. Kramer, M. Kiehntopf, G. Jahreis, *Nutr. J.* **2013**, *12*, 107.
[25] A. Fechner, M. Kiehntopf, G. Jahreis, *J. Nutr.* **2014**, *144*, 599.
[26] M. Busnelli, S. Manzini, C. R. Sirtori, G. Chiesa, C. Parolini, *Nutrients* **2018**, *10*, 1249.
[27] A. Cam, E. G. de Mejia, *Mol. Nutr. Food Res.* **2012**, *56*, 53.
[28] N. Horiguchi, H. Horiguchi, Y. Suzuki, *Biosci. Biotechnol. Biochem.* **2005**, *69*, 2445.
[29] D. Yimit, P. Hoxur, N. Amat, K. Uchikawa, N. Yamaguchi, *Nutrition* **2012**, *28*, 154.
[30] M. A. Torres, J. D. Jones, J. L. Dangl, *Plant Physiol.* **2006**, *141*, 373.
[31] Y. Yang, A. V. Bazhin, J. Werner, S. Karakhanova, *Int. Rev. Immunol.* **2013**, *32*, 249.
[32] H. Sies, D. P. Jones, *Nat. Rev. Mol. Cell Biol.* **2020**, 1.
[33] R. Apak, K. Güçlü, M. Özyürek, S. E. Karademir, M. Altun, *Free Radic. Res.* **2005**, *39*, 949.
[34] G. Cao, H. M. Alessio, R. G. Cutler, *Biol. Med.* **1993**, *14*, 303.
[35] E. Babini, D. Tagliazucchi, S. Martini, L. Dei Piu, A. Gianotti, *Food Chem.* **2017**, *228*, 186.
[36] M. L. Fernandez, D. Webb, *J. Am. Coll. Nutr.* **2008**, *27*, 1.
[37] T. Kuznetsova, *Sex-Specific Analysis of Cardiovascular Function*, Springer **2018**, pp. 61.
[38] C. Lammi, C. Zanoni, S. Ferruzza, G. Ranaldi, Y. Sambuy, A. Arnoldi, *Nutrients* **2016**, *8*, 437.
[39] C. Lammi, C. Zanoni, G. M. Scigliuolo, A. D'Amato, A. Arnoldi, *J. Agric. Food Chem.* **2014**, *62*, 7151.
[40] M. Naruszewicz, G. Nowicka, L. Klosiewicz-Latoszek, A. Arnoldi, C. Sirtori, *Circulation* **2006**, *114*.
[41] C. R. Sirtori, M. Triolo, R. Bosisio, A. Bondioli, L. Calabresi, V. De Vergori, M. Gomaraschi, G. Mombelli, F. Pazzucconi, C. Zacherl, A. Arnoldi, *Br. J. Nutr.* **2012**, *107*, 1176.
[42] K. Weisse, C. Brandsch, B. Zernsdorf, G. S. Nkengfack Nembongwe, K. Hofmann, K. Eder, G. I. Stangl, *Eur. J. Nutr.* **2010**, *49*, 65.
[43] R. Belski, T. A. Mori, I. B. Puddey, S. Sipsas, R. J. Woodman, T. R. Ackland, L. J. Beilin, E. R. Dove, N. B. Carlyon, V. Jayaseena, J. M. Hodgson, *Int. J. Obes.* **2011**, *35*, 810.
[44] J. Cameron, T. Ranheim, M. A. Kulseth, T. P. Leren, K. E. Berge, *Atherosclerosis* **2008**, *201*, 266.
[45] X. Wang, X. Zhang, *Bioresour. Technol.* **2012**, *126*, 307.
[46] P. R. Roberts, J. D. Burney, K. W. Black, G. P. Zaloga, *Digestion* **1999**, *60*, 332.
[47] I. Lemieux, B. Lamarche, C. Couillard, A. Pascot, B. Cantin, J. Bergeron, G. R. Dagenais, J. P. Despres, *Arch. Intern. Med.* **2001**, *161*, 2685.
[48] A. Dudina, M. T. Cooney, D. D. Bacquer, G. D. Backer, P. Ducimetiere, P. Jousilahti, U. Keil, A. Menotti, I. Njolstad, R. Oganov, S. Sans, T. Thomsen, A. Tverdal, H. Wedel, P. Whincup, L. Wilhelmsen, R. Conroy, A. Fitzgerald, I. Graham, S. investigators, *Eur. J. Cardiovasc. Prev. Rehabil.* **2011**, *18*, 731.
[49] Y. P. Lee, T. A. Mori, I. B. Puddey, S. Sipsas, Ackland, Beilin, L. J., Hodgson, J. M., *Am. J. Clin. Nutr.* **2009**, *89*, 766.
[50] M. Graff, T. D. Thacher, P. R. Fischer, D. Stadler, S. D. Pam, J. M. Pettifor, C. O. Isichei, S. A. Abrams, *Am. J. Clin. Nutr.* **2004**, *80*, 1415.
[51] R. P. Heaney, M. S. Dowell, C. A. Hale, A. Bendich, *J. Am. Coll. Nutr.* **2003**, *22*, 142.
[52] K. M. Seamans, K. D. Cashman, *Am. J. Clin. Nutr.* **2009**, *89*, 1997S.
[53] E. F. S. Authority, Wiley Online Library **2016**.
[54] C. J. Etheridge, E. Derbyshire, *Nutr. Food Sci.* **2019**, 969.
[55] D. Dicker, A. Beck, A. Markel, D. Marcovicu, S. Mazzawi, M. Sarid, E. Greenberg, R. L. Atkinson, *Obes. Facts* **2020**, *13*, 473.
[56] A. Nouvenne, A. Ticinesi, F. Allegri, A. Guerra, L. Guida, I. Morelli, L. Borghi, T. Meschi, *Clin. Chem. Lab. Med.* **2014**, *52*, 337.
[57] G. E. Crichton, P. R. Howe, J. D. Buckley, A. M. Coates, K. J. Murphy, J. Bryan, *Trials* **2012**, *13*, 1.
[58] V. Teixeira, S. M. Voci, R. S. Mendes-Netto, D. G. da Silva, *Nutr Diet* **2018**, *75*, 219.
[59] K. L. Weston, L. B. Azevedo, S. Bock, M. Weston, K. P. George, A. M. Batterham, *PLoS One* **2016**, *11*, e0159116.