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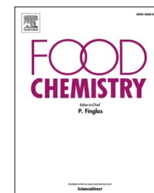

# Chemical and biological characterization of the DPP-IV inhibitory activity exerted by lupin (*Lupinus angustifolius*) peptides: From the bench to the bedside investigation

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ABSTRACT

Dipeptidyl peptidase IV (DPP-IV) is considered a key target for the diabetes treatment, since it is involved in glucose metabolism. Although lupin protein consumption shown hypoglycemic activity, there is no evidence of its effect on DPP-IV activity. This study demonstrates that a lupin protein hydrolysate (LPH), obtained by hydrolysis with Alcalase, exerts anti-diabetic activity by modulating DPP-IV activity. In fact, LPH decreased DPP-IV activity in a cell-free and cell-based system. Contextually, Caco-2 cells were employed to identify LPH peptides that can be intestinally *trans*-epithelial transported. Notably, 141 different intestinally transported LPH sequences were identified using nano- and ultra-chromatography coupled to mass spectrometry. Hence, it was demonstrated that LPH modulated the glycemic response and the glucose concentration in mice, by inhibiting the DPP-IV. Finally, a beverage containing 1 g of LPH decreased DPP-IV activity and glucose levels in humans.

## 1. Introduction

Dipeptidyl peptidase IV (DPP-IV) is a serine exopeptidase that cleaves X-proline or X-alanine dipeptides from the N-terminus of polypeptides, which is expressed by a wide range of human organs, such as the intestine, lung, and kidney (Röhrborn, Wronkowitz, & Eckel, 2015). More in details, this enzyme is expressed on the surface of many cell types as a transmembrane glycoprotein, but it is also found in blood plasma and body fluids in a soluble form (Shao, Xu, Yu, Pan, & Chen, 2020). Glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are two hormones, commonly named incretins, released after meal consumption. This release is carried out in the small intestine and the function of these incretins is to regulate the glucose homeostasis, through the increase of insulin and decrease of glucagon secretion. Among several DPP-IV substrates, there are both GLP-1 and GIP (Nauck, Vardarli, Deacon, Holst, & Meier, 2011), which are rapidly degraded (Mentlein, Gallwitz, & Schmidt, 1993). Due to this important physiological function, the DPP-IV enzyme is a key protagonist in glucose homeostasis and points out its importance in the pharmacological strategies, in which the first classes of oral antidiabetic drugs are inhibitors of DPP-IV activity (Deacon & Lebovitz, 2016). Gliptins are DPP-IV inhibitory drugs and the first to have been approved by US FDA, in 2006, was Sitagliptin (Thornberry & Weber, 2007). However, continually new gliptins are developed, approved, and

*Abbreviations:* AP, apical solution; BL, basolateral solution; DPP-IV, dipeptidyl peptidase IV; iAUC, incremental area under the curve; LPH, lupin protein hydrolysate; LPHb, LPH-based beverage; OGTT, oral glucose tolerance test; SD, standard diet; WD, western diet.

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marketed.

In this context, many components of food, herbal preparations, and secondary metabolites, including phenolic compounds from extra virgin olive oil (Lammi et al., 2021), alkaloids from the *Castanospermum australe* seed extract (Bharti et al., 2012), and procyanidins from grape seed (Gonzalez-Abuin et al., 2012), have been successfully investigated as natural DPP-IV inhibitors. Accordingly, milk, fish, wheat gluten, beans, eggs, soybeans, and microalgae have been extensively studied as valuable sources of bioactive peptides capable of modulating the DPP-IV activity (Ji, Zhang, & Ji, 2017a, 2017b; Liu, Cheng, & Wu, 2019; Nongonierma, Mazzocchi, Paolella, & FitzGerald, 2017; Song, Wang, Du, Ji, & Mao, 2017). It has been demonstrated that the inhibition activity exerted by food peptides on DPP-IV activity can be carried out through an (i) competitive, (ii) uncompetitive, (iii) non-competitive, and (iv) mixed-type inhibition (Liu et al., 2019). Thus, these food-derived peptides can act in the same active site of the DPP-IV enzyme and/or in a different area of the catalytic site (Lacroix & Li-Chan, 2016). However, most of the recent studies showing the inhibitory effects of food peptides on DPP-IV activity have been assayed using *in silico* or *in vitro* approaches (Liu et al., 2019). Therefore, *in vivo* experimental approaches are required to corroborate the *in vitro* observed effect of these food-derived peptides. Previous articles of our group have shown that a *Lupinus angustifolius* protein hydrolysate (LPH), generated by the hydrolysis with Alcalase, exerts antioxidants, anti-inflammatory, and hypocholesterolemic effects in mice and humans (Cruz-Chamorro et al., 2021; Cruz-Chamorro et al., 2019; Cruz-Chamorro et al., 2023; Santos-Sánchez, Cruz-Chamorro, Álvarez-Ríos, et al., 2022; Santos-Sánchez et al., 2021; Santos-Sánchez, Cruz-Chamorro, Bollati, et al., 2022). Although lupin proteins have shown anti-hyperglycemic activity (Grácio et al., 2021), few previous evidences regarding the action of lupin hydrolysates/peptides on DPP-IV activity are available (Lammi, Zanoni, Arnoldi, & Vistoli, 2016; Pugliese, Bollati, Gelain, Arnoldi, & Lammi, 2019; Rivero-Pino, Espejo-Carpio, & Guadix, 2021). Therefore, the aim of the present work was to evaluate the possible DPP-IV activity inhibition and the possible action on glucose concentration by LPH through a bench-to-bedside experimental design ranging from preclinical to clinical approaches. Firstly, we evaluated the LPH capability to reduce the DPP-IV activity employing a cell-free system to test the action of LPH on modulating the activity of the purified enzyme and a cell-based system in which human intestinal Caco-2 cells are employed. Contextually, this cells line was also used to identify LPH peptides that can be intestinally trans-epithelial transported. Notably, intestinally transported medium- and short-sized peptides sequences from LPH were identified by using nano- and ultra-chromatography coupled with high resolution mass spectrometry.

The second objective of the study was the *in vivo* and clinical evaluations of LPH effect on DPP-IV activity and glycemia levels on apolipoprotein (Apo) E knockout (ApoE$^{-/-}$) mice and in serum from healthy subjects after 28 days of ingestion of an experimental beverage (LPHb) containing 1 g LPH within the Lupine-1 clinical food trial (Cruz-Chamorro et al., 2021).

## 2. Materials and methods

### 2.1. LPH preparation

Seeds of lupin (*Lupinus angustifolius*) were donated from Koipesol Semillas, S.A. (Sevilla, Spain). Lupin protein isolate (LPI) was obtained through the method of Yust, Pedroche, Millán-Linares, Alcaide-Hidalgo, and Millán (Plant Proteins Group, Instituto de la Grasa) (del Mar Yust, Pedroche, del Carmen Millán-Linares, Alcaide-Hidalgo, & Millán, 2010). Briefly, defatted lupin flour was extracted with 0.25% $Na_2SO_3$ (w/v) at pH 10.5, for 1 h. After centrifuging the extract at 7500 rpm for 15 min, the supernatant was recovered, and the pellet was extracted again. Both of the supernatants were adjusted to the isoelectric point of lupin proteins (pH 4.3). The resulting precipitate was washed with distilled water adjusted to pH 4.3 and centrifuged to remove residual salts and other non-protein compounds. Finally, the precipitated proteins were lyophilised and stored at room temperature. LPH preparation, characterization, chemical composition, and degree of hydrolysis were described in detail elsewhere (Cruz-Chamorro et al., 2019). Briefly, *Lupinus angustifolius* protein isolate was hydrolyzed with Alcalase (Novozymes, Bagsvaerd, Denmark) with 15 min of incubation. Hydrolysis was performed in a bioreactor under stirring at a controlled pH and temperature. Lupin protein isolate was suspended in distilled water (10% w/v) and hydrolysed with Alcalase 2.4L (2.4L AU/g; Novozymes, Bagsvaerd, Denmark) for 15 min at pH 8, 50 °C, and E/S = 0.3 AU/g protein. Enzyme was inactivated by heating at 85 °C for 15 min. LPH has been obtained after centrifugation and then lyophilized until use. The quantification of amino acids was performed according to (Alaiz, Navarro, Girón, & Vioque, 1992). Tryptophan content was analysed according to the method of Yust et al. (Yust, Pedroche, Girón-Calle, Vioque, Millán, & Alaiz, 2004). Samples were hydrolyzed with 4 mL of HCl 6 N for 24 h at 110 °C under nitrogen atmosphere with the aim of minimizing oxidation processes, especially for those more sensitive amino acids such as sulfur-containing amino acids. Acid hydrolysis completely destroys the tryptophan contents, so a basic hydrolysis was exclusively developed to calculate the tryptophan contents of the samples. Likewise, individual calibration curves for each amino acid were developed using an amino acid standard where these amino acids were submitted to the same analytical conditions of the samples to avoid the mistakes made for the modification or loss of amino acids during acid or basic hydrolysis. The chemical and amino acid composition of LPH is described in Supporting materials Table S1 and Table S2, respectively.

### 2.2. In vitro and in situ evaluation

#### 2.2.1. Cell-free in vitro system

To evaluate the LPH capacity to modulate DPP-IV activity the DPP (IV) Inhibitor Screening Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) was employed, as previously described (Lammi, Zanoni, Arnoldi, et al., 2016). The final concentrations of LPH were 0.1, 0.5, 1.0, 2.5, or 5.0 mg/mL. More in details, 50 μL of samples (30 μL 1X assay buffer, 10 μL LPH or vehicle and 10 μL of DPP-IV enzyme) were mixed with 50 μL of substrate solution (200 μM H-Gly-Pro-7-amino-4-methylcoumarin) and incubated at 37 °C for 30 min. Then, the fluorescence intensity was read using the Synergy H1 microplate reader (ex./em. 350/450 nm). Sitagliptin (100 μM), a well-known hypoglycemic drug, was used as a positive control. For more detailed, see the Supporting materials.

#### 2.2.2. In situ assay using Caco-2 cells

A total of 3 × $10^4$ Caco-2 cells were cultured in complete growth medium (all Gibco reagents, Fisher Scientific, Milan, Italy) at 37 °C under an atmosphere of 5% $CO_2$. Two days after seeded, the spent medium was discarded and Caco-2 cells were treated with 0.5, 1.0, and 5.0 mg/mL of LPH and/or vehicle for 1, 3, and 6 h at 37 °C. Subsequently, 40 μL of H-Gly-Pro-AMC (20.0 μM) was added to each well, and the fluorescence signal was acquired for 10 min. For more detailed, see the Supporting materials.

#### 2.2.3. Assessment of LPH effects on Caco-2 cell viability

A total of Caco-2 cells (3 × $10^4$ cells/well/96-well plates) were treated with several concentrations of LPH (0.1, 0.5, and 5.0 mg/mL) or vehicle for 48. After incubation, the medium was removed and 100 μL of MTT were added and incubated for 2 h. After removing the solution, 100 μL/well of the lysis buffer were added. The absorbance at 575 nm was read in the Synergy™ HT-multimode microplate reader (BioTek Instruments, Bad Friedrichshall, Germany).

#### 2.2.4. Analysis of the LPH peptides trans-epithelial transport

Trans-epithelial passage of LPH peptides was assessed in a cell

culture model of a differentiated Caco-2 cells monolayer, according to previously described conditions (Lammi, Zanoni, Ferruzza, Ranaldi, Sambuy, & Arnoldi, 2016) and detailed informed in the Supporting materials. Briefly, peptide transportation by mature Caco-2 cells was assayed by loading the apical (AP) compartment with 500 µL of AP transport solution containing 1.0 mg/mL of LPH and the basolateral (BL) compartment with 700 µL of BL transport solution for 2 h. Finally, the AP and BL solutions were collected and stored at −80 °C prior to analysis.

#### 2.2.5. Peptide identification

The medium and short peptides from the LPH, AP, and BL samples were identified according to the detailed protocol informed elsewhere (Santos-Sánchez, Cruz-Chamorro, Bollati, et al., 2022). Briefly, medium size peptides were identified by nano HPLC on an Ultimate 3000 coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide spectra were acquired in the 300–2000 *m/z* range at 30 000 resolutions for the full scan. On the other hand, short peptides were analysed with Vanquish binary pump H coupled to a hybrid quadrupole-Orbitrap mass spectrometer Q Exactive (Thermo Fisher Scientific) using a heated ESI source operating in positive ion mode. Raw data files were acquired using Xcalibur software (version 2.2, Thermo Fisher Scientific) for medium-size peptides and Compound Discoverer (using a workflow specifically dedicated to short peptide analysis) for short-size peptides. Three experimental replicates were performed for each sample.

### 2.3. In vivo evaluation

#### 2.3.1. Animal model experimental design

Nineteen 4-week-old male ApoE$^{-/-}$ mice were initially divided into two groups: mice fed a standard diet (SD) ($n = 5$) or a western diet (WD) ($n = 14$) (Test Diet 58v8, 45% energy from fat; diet compositions in Supporting materials Table S3). When mice fed WD were 6 weeks old, they were randomly divided into two groups and daily intragastrically treated with 100 mg/kg of LPH (WD + LPH, $n = 7$) or vehicle (physiological saline solution containing 0.25% of carboxymethylcellulose) (WD, $n = 7$) for 12 weeks. SD-fed mice were also intragastrically treated with vehicle (SD). The LPH dose was equivalent to 8.12 mg/kg in humans, calculated according to (Reagan-Shaw, Nihal, & Ahmad, 2008). The mice were then sacrificed, and the blood was recovered in Minicollect EDTA tubes (Greiner Bio-one, Kremsmünster, Austria) by cardiac puncture. Subsequently, plasma was obtained by centrifugation (3,000*g*, 4 °C, 10 min) and stored at −20 °C until use. All experimental procedures were conducted under the Spanish legislation and under the EU Directive 2010/63/EU for animal experiments and were approved by the Ethics Committee of the Virgen Macarena and Virgen del Rocío University Hospitals (reference 21/06/2016/105).

#### 2.3.2. Oral glucose tolerance test (OGTT)

Evaluation of the LPH effect on glycemic response of mice SD fed was performed using an OGTT. Ten 18-week-old male ApoE$^{-/-}$ mice were so divided: control ($n = 5$) and LPH group ($n = 5$). 30 min before oral glucose load (2 g/kg), fasted mice were intragastrically treated with vehicle (control group, C) or 100 mg/kg LPH (LPH group). Immediately before oral glucose load (time 0), blood glucose concentration was quantified by vein tail puncture using the Accu-Chek® Aviva glucometer (Roche Diagnostic, Bassel, Switzerland). Blood glucose levels were determined 15, 30 and 60 min after oral glucose loading. The glycemia changes were quantified by subtracting at each measured glucose concentration (15, 30, and 60 min) the glucose concentration at time 0. The incremental area under the curve (iAUC) at time 60 was calculated for each animal using the trapezoid rule.

#### 2.3.3. Effect of treatment with an experimental beverage containing LPH (LPHb) on DPP-IV activity in healthy subjects

DPP-IV activity and glucose levels were evaluated in the serum of healthy subjects after 28 days of 200 mL LPHb ingestion (containing 1 g of LPH) within the Lupine-1 clinical food trial, an open-label longitudinal study (ClinicalTrials.gov Identifier: NCT02590887). The preparation and composition of LPHb, subjects, and study design were described in a previous report (Cruz-Chamorro et al., 2021). Briefly, 33 healthy volunteers (16 males and 17 females) with an average age of 30.27, daily consumed a fasting single dose of 200 mL of LPHb (containing 1 g LPH) for 28 days. The nutritional information and the inclusion/exclusion criteria values of LPHb are shown in the Supporting materials Table S4 and Table S5, respectively.

Fasting blood samples from volunteers were drawn in VACUETTE® Z Serum Sep Clot Activator tubes (Greiner Bio-one) at baseline (time 0) and at the end of the study (28 days), blood serum was obtained and stored at −20 °C until use. All procedures followed the Helsinki Declaration of 1975, as revised in 1983. The clinical food study was approved by the Ethics Review Board of Virgen Macarena and Virgen del Rocío University Hospital (reference number 2015/110).

#### 2.3.4. Glucose quantification

Human and mouse blood glucose concentration was quantified in serum or plasma, respectively, throughout chemiluminescence immunoassay techniques using the COBAS e601 modular analyzer (Roche Diagnostic).

#### 2.3.5. Blood-circulating DPP-IV activity

The assay was carried out in black 96-well plates mixing 30 µL human serum sample or 30 µL mouse plasma sample and 30 µL of 100 µM H-Gly-Pro-AMC (AnaSpec Inc., Fremont, CA, USA). Free AMC group was monitored by measuring its fluorescence signals every 1 min. for up to 10 min.

#### 2.3.6. Quantification of DPP-IV proteins

Quantification of DPP-IV protein levels was carried out using Dipeptidyl Peptidase IV (CD26) ELISA kit (BioVendor, Karásek, Brno, Czech Republic).

### 2.4. Statistical analysis

All the data sets were checked for normal distribution by D'Agostino and Pearson test. Since all data followed a normal distribution, a T-test student, an analysis of variance (ANOVA) followed by post-hoc tests or a Mixed-effects analysis test followed by post-hoc tests was applied (GraphPad Software v.8, La Jolla, CA, USA). The parametric Pearson's correlation was applied to analyze the correlations. The size effect of the variables studied in the mice experiments was analyzed by Cohen's test using Jeffreys's Amazing Statistics Program (JASP v. 0.16.3, Amsterdam, The Netherlands), and a d-value $>0.80$ was considered as 'large effect size'. Data were expressed as means ± standard deviation; *p*-values $\leq 0.05$ were considered significant.

## 3. Results

### 3.1. LPH decreases DPP-IV activity

As shown in Fig. 1, LPH decreased DPP-IV activity in a dose-dependent manner, with respect to the control group (C) ($p < 0.0001$), in a cell-free *in vitro* system. In particular, LPH were capable to inhibit DPP-IV activity by 18.50% ± 0.25%, 40.11% ± 4.40%, 61.29% ± 3.80%, and 79.88% ± 3.58%, at 0.5, 1.0, 2.5, and 5.0 mg/mL, respectively. The internal positive control Sitagliptin inhibited the activity of DPP-IV by 98.58% ± 0.08% ($p < 0.0001$).

To assess the enzymatic inhibition kinetic, the Caco-2 cells system was used treating them with several LPH concentrations for 1, 3, and 6 h.

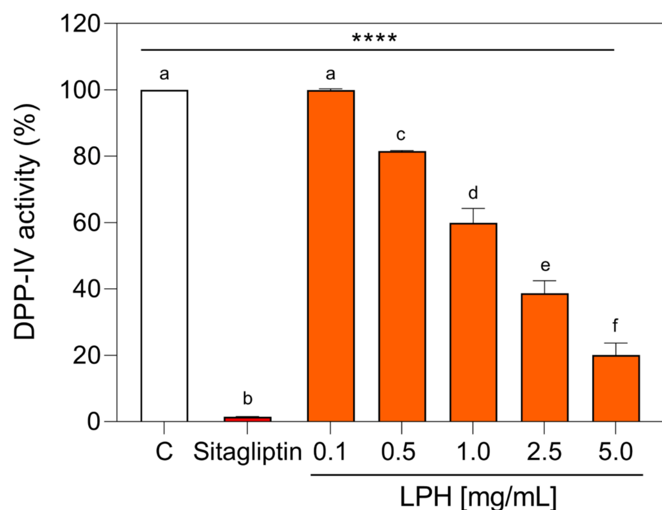


**Fig. 1.** Quantification of cell-free *in vitro* DPP-IV activity. Inhibition capacity of LPH tested at different concentrations (0.1–5.0 mg/mL) in DPP-IV activity using an *in vitro* cell-free system. Data were expressed as a percentage of the control group (C) and represented as mean ± SD of two independent experiments in triplicate. Data were analysed by One-way ANOVA followed by Tukey's post-hoc test. ****, $p < 0.0001$. Lowercase letters represent a statistical difference. C, control group; LPH, lupin protein hydrolysates.

Fig. 2A shows that LPH inhibited DPP-IV activity by 13.1 ± 5.9%, 23.2 ± 0.8%, and 38.3 ± 12.4% after 1 h of incubation, 20.6 ± 1.7%, 44.2 ± 2.6%, and 63.7 ± 5.2% after 3 h of incubation, and 15.9 ± 5.1%, 39.4 ± 0.2%, and 63.4 ± 2.7% after 6 h of incubation, at 0.5, 1.0, and 5.0 mg/mL, respectively. Maximum reductions in DPP-IV activity were observed after 3 h of treatment at 1.0 and 5.0 mg/mL (Fig. 2A). LPH did not alter the concentration of DPP-IV protein at 1.0 mg/mL (supernatant: 89.49 ± 11.41%, $p = 0.39$; lysate: 96.10 ± 4.59%, $p = 0.41$); not at 5.0 mg/mL (supernatant: 93.28 ± 2.61%, $p = 0.63$; lysate: 109.8 ± 0.09%, $p = 0.07$), in Caco-2 cell supernatants and lysates (Fig. 2B). To exclude any potential cytotoxic effects associated with LPH, MTT viability assay was performed under the same culture conditions as the DPP-IV activity study. No effects of LPH were observed at any dose tested in Caco-2 cells compared to the control group (Supporting materials Fig. S1).

### 3.2. Trans-epithelial transport of LPH

To investigate intestinal transport of LPH peptides, the trans-epithelial passage of LPH was assayed in differentiated Caco-2 cells as a model of the intestinal barrier. After 2 h of LPH incubation (1.0 mg/mL), the AP and BL medium were collected and submitted to nano-HPLC-MS/MS, further analyzed by UHPLC-HRMS and processed by Compound Discoverer for the tentative identification. Of the 1,843 different sequences identified in the whole LPH, of which 1,685 and 158 were medium- and short-size, respectively (Supporting materials Table S6 and Table S7, respectively), 206 peptides (11.17%) crossed the differentiated Caco-2 cells, being identified in the BL medium. 197 out of 206 (95.63%) presented motifs with already demonstrated DDP-IV activity, according to the BIOPEP-UWM database. Furthermore, 141 (68.45%) were considered inhibitory DPP-IV peptides (with scores > 294.00) using the iDPP-IV-SCM tool and the probability that each peptide could be bioactive was predicted using the PeptideRanker tool (Mooney, Haslam, Pollastri, & Shields, 2012) (Table 1).

### 3.3. LPH modulates the in vivo glycemia response

LPH administration (100 mg/kg) 30 min before glucose load attenuated the glycemic response. As shown in Fig. 3A, 15 min after glucose load, the animals reach maximum glycemia levels. At this point, LPH-treated mice had a glucose concentration 25.29% lower than the control group (C: 360.6 ± 36.25 mg/dL; LPH: 269.4 ± 37.46 mg/dL; $p = 0.003$). Moreover, the mean increase in glucose concentration at the peak of the curve was 46.14% higher in the mice in the control group compared to the LPH-treated mice (C: 158.2 ± 31.95 mg/dL; LPH: 85.2 ± 31.00 mg/dL; $p = 0.0001$) (Fig. 3B). Although no significant differences in glucose clearance were observed at 30 and 60 min between C and LPH groups, a significant decrease in iAUC was observed in LPH-treated mice during OGTT (C: 3,535 ± 540.6; LPH: 1,807 ± 728.1; $p = 0.0028$) (Fig. 3C).

### 3.4. LPH modulates the plasma glucose concentration in mice

Since we observed a modulation in the glycemia response by LPH treatment, the glucose plasma concentration was analyzed in mice of the three experimental groups (SD; WD, WD + LPH). As shown in Fig. 4A, WD-fed mice have a higher glucose concentration than the SD-fed mice (increase of 37.84 ± 9.53 mg/dL; $p < 0.001$). Although the glucose concentration of LPH-treated mice (WD + LPH) was slightly higher than SD-fed mice (increase of 19.74 ± 2.41 mg/dL; $p = 0.037$), a significant decrease in glucose concentration was observed compared to the WD group (decrease of 18.1 ± 7.12 mg/dL; $p = 0.038$) (Fig. 4A).

### 3.5. LPH palliates the increased DPP-IV activity WD-induced

After evaluating the *in situ* inhibition of DPP-IV activity and the reduction of glucose concentration exerted by LPH in WD-fed mice, the effect of LPH on the plasma DPP-IV activity was investigated in the mice. An increase of 95.60 ± 16.42% ($p = 0.023$) in the DPP-IV activity of WD-fed mice was observed compared to the SD-fed mice group (Fig. 4B). 12 weeks of LPH treatment (WD + LPH) reduced DPP-IV activity by 67.60 ± 26.24% compared to the WD-fed mice group ($p = 0.023$) (Fig. 4B). In fact, WD + LPH mice showed DPP-IV activity like that of the SD-fed mice group ($p = 0.667$).

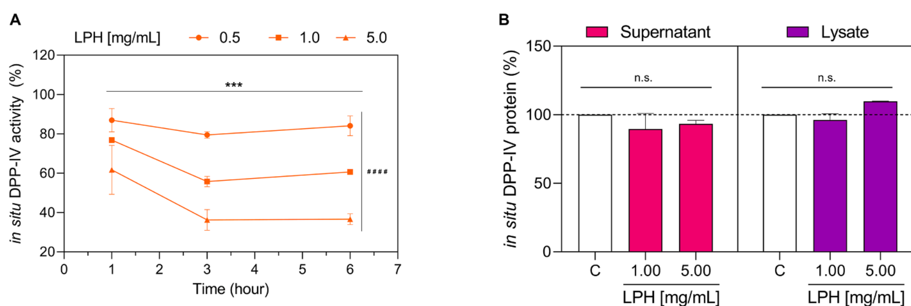


**Fig. 2.** *In situ* DPP-IV activity and protein concentration in Caco-2 cells. (A) *in situ* DPP-IV activity on Caco-2 cells treated 1, 3, and 6 h at the indicated concentration of LPH. Data were expressed as a percentage of the basal value (time 0) and represented as mean ± SD of two independent experiments in triplicate. Data were analysed by Two-way ANOVA followed by Tukey's post-hoc test. ***, $p \leq 0.001$ with respect to the time. ####, $p < 0.0001$ with respect to the concentrations. (B) DPP-IV protein in the supernatant and lysate of Caco-2 cells treated with LPH at 1 and 5 mg/mL. Data were expressed as a percentage of the untreated control group (C) and represented as mean ± SD of two independent experiments in triplicate. Data were analysed by One-way ANOVA followed by Tukey's post-hoc test. n.s., not significant differences. LPH, lupin protein hydrolysate.

**Table 1**
Sequence (1-letter code), molecular weight (MW), bioactivity score, and inhibitory DPP-IV activity score of the peptides identified in the BL solution.

| Sequence | MW | Bioactivity score[a] | iDPP-IV[b] | | | DPP-IV inhibitor[c] |
|---|---|---|---|---|---|---|
| | | | | X = I | X = L | |
| AF | 236.12 | 0.973 | 486.00 | | | yes |
| AVGF | 392.21 | 0.710 | 315.33 | | | yes |
| AW | 275.13 | 0.967 | 638.00 | | | yes |
| AX[d] | 202.13 | 0.658 | | 445.00 | 497.00 | yes |
| AXPX | 412.27 | 0.903 | | 384.67 | 419.33 | yes |
| AY | 252.11 | 0.346 | 494.00 | | | yes |
| EITPDRNPQAQ | 1267.62 | 0.111 | 318.10 | | | yes |
| EITPDRNPQVQ | 1295.65 | 0.108 | 325.60 | | | yes |
| ERQE | 560.26 | 0.038 | 318.67 | | | yes |
| ESE | 363.13 | 0.032 | 304.50 | | | yes |
| ETW | 434.18 | 0.247 | 488.50 | | | yes |
| EWE | 462.18 | 0.182 | 487.00 | | | yes |
| EY | 310.12 | 0.067 | 554.00 | | | yes |
| FA | 236.12 | 0.956 | 486.00 | | | yes |
| FAN | 350.16 | 0.714 | 391.00 | | | yes |
| FEGF | 498.21 | 0.935 | 328.33 | | | yes |
| FENX | 521.25 | 0.504 | | 357.00 | 374.33 | yes |
| FER | 450.22 | 0.529 | 315.00 | | | yes |
| FET | 395.17 | 0.237 | 412.50 | | | yes |
| FF | 312.15 | 0.999 | 540.00 | | | yes |
| FH | 302.14 | 0.953 | 559.00 | | | yes |
| FNGF | 483.21 | 0.984 | 335.00 | | | yes |
| FQ | 293.14 | 0.916 | 590.00 | | | yes |
| FR | 321.18 | 0.986 | 354.00 | | | yes |
| FVF | 411.22 | 0.970 | 415.50 | | | yes |
| FVFX | 524.30 | 0.962 | | 353.33 | 370.67 | yes |
| FVR | 420.25 | 0.672 | 322.50 | | | yes |
| FX | 278.16 | 0.995 | | 499.00 | 551.00 | yes |
| FXE | 407.21 | 0.763 | | 387.50 | 413.50 | yes |
| FXEE | 536.25 | 0.317 | | 350.33 | 367.67 | yes |
| FXQY | 569.29 | 0.884 | | 365.67 | 383.00 | yes |
| FXSQ | 493.25 | 0.679 | | 292.00 | 309.33 | no; yes |
| FXTQ | 507.27 | 0.617 | | 366.00 | 383.33 | yes |
| FY | 328.14 | 0.982 | 548.00 | | | yes |
| FYSP | 512.23 | 0.862 | 361.67 | | | yes |
| GF | 222.10 | 0.995 | 439.00 | | | yes |
| GNFY | 499.21 | 0.885 | 337.67 | | | yes |
| GNPEEEHPETQ | 1265.52 | 0.107 | 341.70 | | | yes |
| GPX | 285.17 | 0.943 | | 439.00 | 465.00 | yes |
| GW | 261.11 | 0.993 | 591.00 | | | yes |
| GX | 188.12 | 0.925 | | 398.00 | 450.00 | yes |
| GY | 238.10 | 0.742 | 447.00 | | | yes |
| HGPX | 422.23 | 0.842 | | 389.00 | 406.33 | yes |
| HHRE | 577.27 | 0.117 | 312.67 | | | yes |
| HHREREQEQQPRPQ | 1853.90 | 0.058 | 299.85 | | | yes |
| KFNW | 593.30 | 0.900 | 329.33 | | | yes |
| LNQLDPSPR | 1038.55 | 0.400 | 310.00 | | | yes |
| NALEPDNRVE | 1155.55 | 0.125 | 299.67 | | | yes |
| NF | 279.12 | 0.941 | 566.00 | | | yes |
| NTR | 389.20 | 0.094 | 329.50 | | | yes |
| NX | 245.14 | 0.503 | | 525.00 | 577.00 | yes |
| NXR | 401.24 | 0.567 | | 304.50 | 330.50 | yes |
| NY | 295.12 | 0.225 | 574.00 | | | yes |
| PS | 202.10 | 0.393 | 537.00 | | | yes |
| PY | 278.13 | 0.737 | 758.00 | | | yes |
| PYQE | 535.23 | 0.162 | 451.33 | | | yes |
| QNY | 423.18 | 0.210 | 447.00 | | | yes |
| QQXY | 550.28 | 0.492 | | 382.33 | 399.67 | yes |
| QR | 302.17 | 0.274 | 404.00 | | | yes |
| QSQY | 524.22 | 0.157 | 325.00 | | | yes |
| QTY | 410.18 | 0.120 | 438.50 | | | yes |
| QVFE | 521.25 | 0.172 | 385.67 | | | yes |
| QXST | 447.24 | 0.217 | | 295.00 | 312.33 | yes |
| QYQ | 437.19 | 0.166 | 459.00 | | | yes |
| RFQ | 449.24 | 0.794 | 337.00 | | | yes |
| RNXF | 548.31 | 0.906 | | 293.00 | 310.33 | no; yes |
| RRPF | 574.33 | 0.945 | 306.00 | | | yes |
| RWF | 507.26 | 0.994 | 388.00 | | | yes |
| RWX | 473.28 | 0.968 | | 367.50 | 393.50 | yes |
| RXN | 401.24 | 0.505 | | 304.50 | 330.50 | yes |
| RXX | 400.28 | 0.844 | | 271.00 | 323.00 | no; yes |
| RXY | 450.26 | 0.697 | | 295.50 | 321.50 | yes |
| RYE | 466.22 | 0.131 | 319.00 | | | yes |

(continued on next page)

**Table 1** (*continued*)

| Sequence | MW | Bioactivity score[a] | iDPP-IV[b] | | | DPP-IV inhibitor[c] |
|---|---|---|---|---|---|---|
| | | | | X = I | X = L | |
| SGPF | 406.19 | 0.964 | 325.33 | | | yes |
| SVXF | 464.26 | 0.816 | | 282.33 | 299.67 | no; yes |
| SW | 291.12 | 0.934 | 479.00 | | | yes |
| SX | 218.13 | 0.530 | | 286.00 | 338.00 | no; yes |
| SXY | 381.19 | 0.595 | | 282.00 | 308.00 | no; yes |
| SY | 268.11 | 0.262 | 335.0. | | | yes |
| SYE | 397.15 | 0.103 | 305.50 | | | yes |
| SYF | 415.17 | 0.896 | 302.50 | | | yes |
| TF | 266.13 | 0.827 | 549.00 | | | yes |
| TW | 305.14 | 0.814 | 701.00 | | | yes |
| TX | 232.14 | 0.278 | | 508.00 | 560.00 | yes |
| TY | 282.12 | 0.114 | 557.00 | | | yes |
| TYEEPQEQEQGQGPRPQ | 1999.89 | 0.067 | 320.19 | | | yes |
| VAF | 335.18 | 0.560 | 388.50 | | | yes |
| VF | 264.15 | 0.815 | 561.00 | | | yes |
| VFR | 420.25 | 0.710 | 322.50 | | | yes |
| VR | 273.18 | 0.115 | 375.00 | | | yes |
| VRV | 372.25 | 0.066 | 333.00 | | | yes |
| VVPQ | 441.26 | 0.083 | 460.67 | | | yes |
| VW | 303.16 | 0.802 | 713.00 | | | yes |
| VX | 230.16 | 0.254 | | 520.00 | 572.00 | yes |
| VXAP | 398.25 | 0.439 | | 405.33 | 422.67 | yes |
| VXR | 386.26 | 0.358 | | 302.00 | 328.00 | yes |
| VY | 280.14 | 0.099 | 569.00 | | | yes |
| WFQ | 479.22 | 0.983 | 506.00 | | | yes |
| WQPR | 585.30 | 0.900 | 435.33 | | | yes |
| WSHQ | 556.24 | 0.486 | 362.67 | | | yes |
| XAF | 349.20 | 0.965 | | 357.50 | 383.50 | yes |
| XEGE | 446.20 | 0.121 | | 316.67 | 334.00 | yes |
| XF | 278.16 | 0.955 | | 499.00 | 551.00 | yes |
| XFAF | 496.27 | 0.989 | | 328.33 | 345.67 | yes |
| XH | 268.15 | 0.613 | | 518.00 | 570.00 | yes |
| XNWN | 545.26 | 0.856 | | 414.33 | 431.67 | yes |
| XNWQ | 559.28 | 0.804 | | 422.33 | 439.67 | yes |
| XP | 228.15 | 0.937 | | 709.00 | 761.00 | yes |
| XR | 287.20 | 0.791 | | 313.00 | 365.00 | yes |
| XSXY | 494.27 | 0.750 | | 264.33 | 299.00 | no; yes |
| XTF | 379.21 | 0.891 | | 389.00 | 415.00 | yes |
| XV | 230.16 | 0.248 | | 520.00 | 572.00 | yes |
| XVSY | 480.26 | 0.244 | | 285.00 | 302.33 | no; yes |
| XW | 317.17 | 0.993 | | 651.00 | 703.00 | yes |
| XX | 244.18 | 0.932 | | 458.00 | 562.00 | yes |
| XXF | 391.25 | 0.991 | | 364.00 | 416.00 | yes |
| XXPH | 478.29 | 0.880 | | 409.00 | 443.67 | yes |
| XXYQ | 535.30 | 0.698 | | 352.00 | 386.67 | yes |
| XY | 294.16 | 0.782 | | 507.00 | 559.00 | yes |
| XYE | 423.20 | 0.264 | | 391.50 | 417.50 | yes |
| YEE | 439.16 | 0.042 | 415.00 | | | yes |
| YF | 328.14 | 0.982 | 548.00 | | | yes |
| YFAN | 513.22 | 0.625 | 353.33 | | | yes |
| YFR | 484.24 | 0.941 | 316.00 | | | yes |
| YFS | 415.17 | 0.743 | 302.50 | | | yes |
| YGPX | 448.23 | 0.905 | | 385.33 | 402.67 | yes |
| YH | 318.13 | 0.294 | 567.00 | | | yes |
| YKR | 465.27 | 0.251 | 181.0. | | | yes |
| YNPQ | 520.2281 | 0.335 | 458.00 | | | yes |
| YNVN | 508.23 | 0.102 | 387.00 | | | yes |
| YR | 337.18 | 0.525 | 362.00 | | | yes |
| YSFN | 529.22 | 0.635 | 300.33 | | | yes |
| YT | 282.12 | 0.113 | 557.00 | | | yes |
| YTX | 395.21 | 0.352 | | 393.00 | 419.00 | yes |
| YTYE | 574.23 | 0.083 | 370.33 | | | yes |
| YVAN | 465.22 | 0.096 | 360.33 | | | yes |
| YVX | 393.23 | 0.332 | | 399.00 | 425.00 | yes |
| YX | 294.16 | 0.771 | | 507.00 | 559.00 | yes |
| YXA | 365.20 | 0.576 | | 361.50 | 387.50 | yes |
| YXS | 381.19 | 0.426 | | 282.00 | 308.00 | no; yes |
| YYF | 491.21 | 0.939 | 413.00 | | | yes |

[a] According to PeptideRanker analysis (Mooney et al., 2012). Any peptide predicted with a bioactivity score over a 0.5 threshold is considered bioactive.
[b] According to the iDPPIV-SCM tool (Charoenkwan, Kanthawong, Nantasenamat, Hasan, & Shoombuatong, 2020).
[c] Values higher than 294 are considered as positive result.
[d] X, Ile (I) or Leu (L).

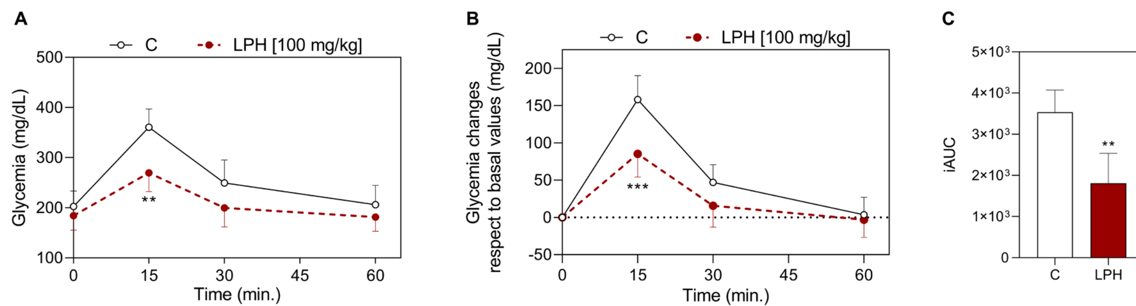


**Fig. 3.** *In vivo* glycemia response in ApoE$^{-/-}$ mice. (A) Oral glucose tolerance test. Blood glucose was measured immediately before de glucose intake (time 0) and monitored after 15, 30, and 60 min. (B) Glycemia levels with respect to the baseline values were represented after subtracting the glucose baseline concentration at each subsequent measurement. (C) The incremental area under the curve (iAUC) was calculated at the end-point time (60 min). $n = 5$/group. Data sets from (A) and (B) were analysed by Mixed-effects analysis followed by Sidak's post-hoc test, while data set from (C) was analysed by unpaired *T*-student test. Data were represented as the mean ± standard deviation. ** $p \leq 0.01$, *** $p \leq 0.001$ versus control group (C); LPH; lupin protein hydrolysates-treated mice.

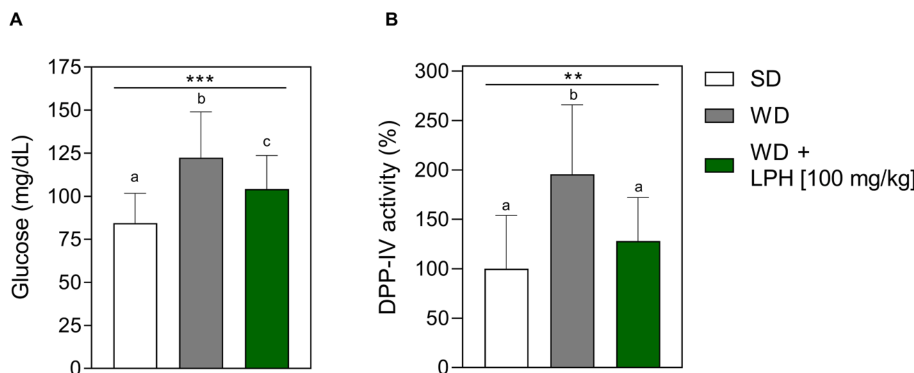


**Fig. 4.** Plasma glucose concentration and DPP-IV activity in ApoE$^{-/-}$ mice. (A) Concentration of plasma glucose from ApoE$^{-/-}$ mice fed with standard diet (SD, $n = 5$), Western diet (WD, $n = 7$), and WD plus lupin protein hydrolysates (WD + LPH, $n = 7$). (B) Percentage of plasma DPP-IV activity with respect to the SD group. All data sets were analysed by One-way ANOVA followed by Tukey's post-hoc test. Data were represented as the mean ± standard deviation. ** $p \leq 0.01$, *** $p \leq 0.001$. Lowercase letters represent a statistical difference.

### 3.6. LPHb decreases DPP-IV activity and plasma glucose concentration

Once the inhibitory effects of LPH on DPP-IV activity were described in both *in vitro* and *in vivo* mouse model, the activity of DPP-IV was evaluated in serum from healthy subjects ($n = 33$) who ingested an experimental beverage containing LPH (LPHb) within the Lupine-1 clinical trial. As shown in Fig. 5A, LPHb intake for 28 days decreased DPP-IV activity by 7.51% ($p = 0.020$), compared to baseline values. Moreover, the concentration of DPP-IV proteins was not altered during the 28 days of study (Fig. 5B).

Although in a previous study, no decrease in glucose levels concentration was observed after 28 days of LPHb ingestion (day 0: 86.06 ± 9.40 mg/dL; day 28:87.94 ± 8.21 mg/dL; $p = 0.152$) (Cruz-Chamorro et al., 2021), a positive correlation ($r = 0.402$; $p = 0.034$) between DPP-IV activity and plasma glucose concentration was observed (Fig. 5C). After stratifying subjects into two groups: i) participants who underwent no variation or increase in plasma glucose concentration (Fig. 5D) and ii) subjects who showed a decrease in glucose concentration after 28 days of LPHb ingestion (Fig. 5F), we observed a significant decrease in DPP-IV activity of 11.24 % ($p = 0.040$) only in the group that showed significantly lower plasma glucose levels after 28 days of LPHb consumption (Fig. 5G), while no differences were found in the DPP-IV activity of the group without changes or increased plasma glucose levels after LPHb ingestion (Fig. 5E).

## 4. Discussion

The present study shows the inhibitory role of LPH in DPP-IV activity and its glucose-lowering capacity through a bench-to-bedside experimental design ranging from *in vitro* to *in vivo* approaches in mice and humans. Therefore, this study provides significant evidence on the *in vivo* antidiabetic potential of an Alcalase-generated LPH. First, our results show that LPH decreases DPP-IV activity in a dose-dependent manner in a cell-free system. After this first screening, the kinetic of enzyme inhibition was carried out in Caco-2 cells, since these cells express several intestinal enzymes involved in food digestion (Darmoul et al., 1992). LPH treatment significantly inhibited DPP-IV activity, with a maximum reduction after 3 h of treatment. It is also important to underline that a soluble form of DPP-IV is present in plasma and other body fluids (Lambeir, Durinx, Scharpé, & De Meester, 2003). Emerging evidence suggests a potential role for this soluble form in the pathophysiology of metabolic and cardiovascular diseases (dos Santos et al., 2013). Our results indicate that LPH directly inhibits the enzyme activity without exerting any effect on both intracellular levels and the soluble form of DPP-IV. Similar results have previously been described on hydrolysates from *Arthrospira platensis* (spirulina) and *Chlorella pyrenoidosa* proteins (Li, Aiello, Bollati, Bartolomei, Arnoldi, & Lammi, 2020; Yuchen et al., 2021). In particular, spirulina hydrolysate obtained with pepsin inhibited DPP-IV activity by 64% in a cell-free system and 34% in Caco-2 cells, while the inhibition of hydrolysates obtained with trypsin reached 72% and 41% in a cell-free system and Caco-2 cells, at a dose of 5.0 mg/mL, respectively (Aiello, Li, Boschin, Bollati, Arnoldi, & Lammi, 2019; Yuchen et al., 2021). Other studies have also shown the capacity of hempseed and soybean protein hydrolysates to inhibit DPP-IV activity. In particular, 1.0 mg/mL of peptic hempseed hydrolysates inhibited the DPP-IV activity by 32% *in vitro* and 22% in Caco-2 cells (Lammi, Bollati, Gelain, Arnoldi, & Pugliese, 2019), while DPP-IV activity was inhibited by 2.5 mg/mL of peptic soybean hydrolysates by 31% and 11%, *in vitro* and in Caco-2 cells, respectively (Lammi, Arnoldi, & Aiello, 2019). Furthermore, flaxseed, rapeseed, sunflower, sesame, soybean, whey, and casein hydrolysates generated using Alcalase, the same enzyme used in this study, showed the capacity to reduce the DPP-IV activity using a cell-free *in vitro* system (Han, Álvarez, Maycock, Murray, & Boesch, 2021). Specifically, whey hydrolysates showed the

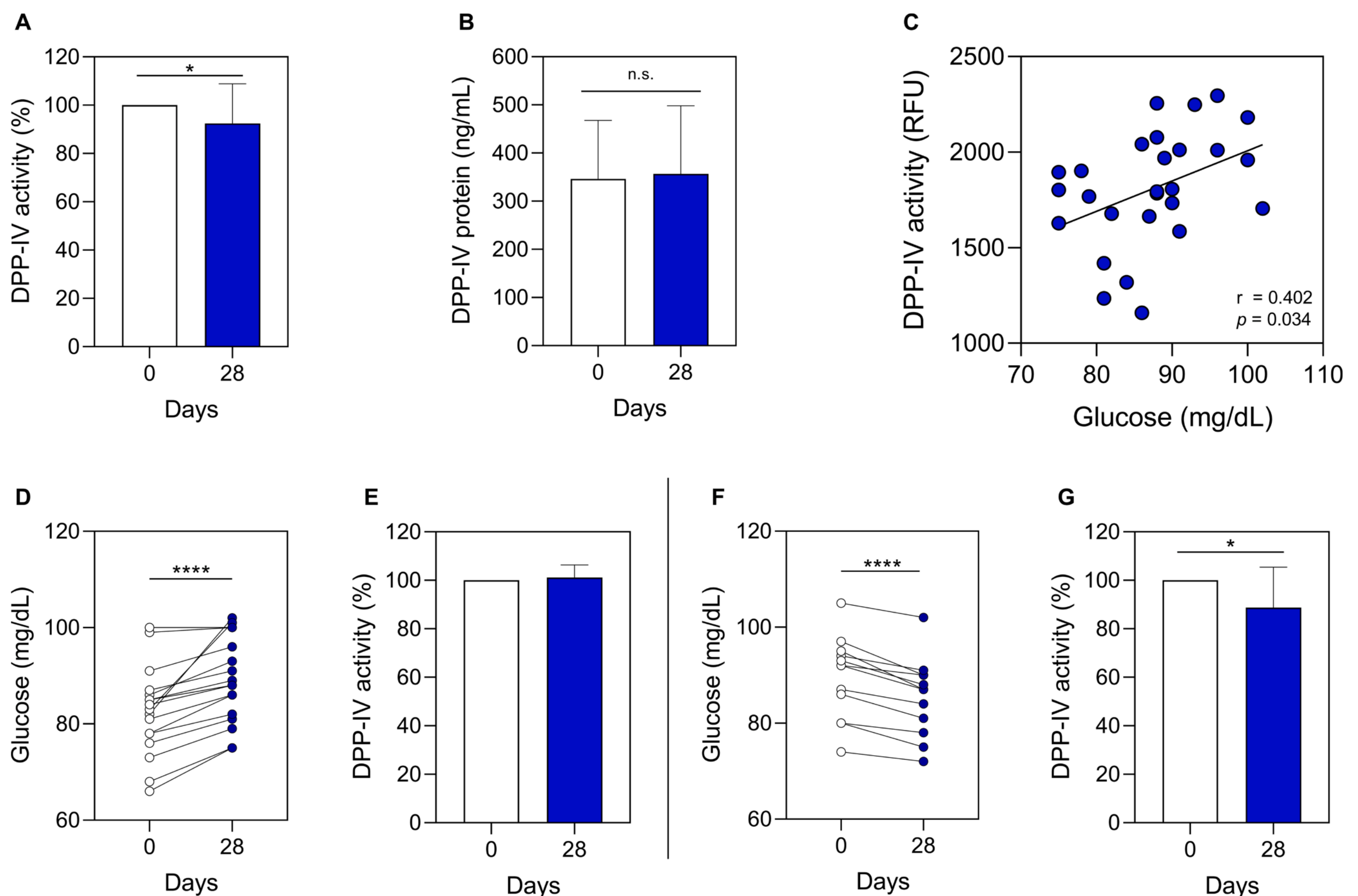


**Fig. 5.** Circulating DPP-IV activity and plasma glucose concentration in humans. (A) Percentage of serum DPP-IV activity of healthy human subjects who participated in the Lupine-1 clinical study. Day 0 corresponds to the baseline of the study, while day 28 represents the number of days the volunteers ingested the experimental beverage containing the lupin protein hydrolysates (LPH). (B) Concentration of DPP-IV proteins in serum samples. (C) Pearson's correlation between DPP-IV activity and the plasma glucose concentration. (D) Volunteers showing an increase in glucose concentration ($n = 18$) and (E) their DPP-IV activity. (F) Volunteers showing a decrease in glucose concentration ($n = 12$) and (G) their DPP-IV activity. Data were represented as the mean ± standard deviation. Data sets were analysed by paired *T*-student test. *, $p < 0.05$; *** $p \leq 0.001$; n.s. not significant.

greatest inhibition (by 42%), followed by the rapeseed hydrolysates that reach a value of 30%, while the other hydrolysates inhibited the DPP-IV activity by around 20%, at a concentration of 10.0 mg/mL. Finally, a recent study demonstrated that lupin peptide mixture (fraction 3 – 0,8 kDa) from *Lupinus albus* obtained after enzymatic treatment with subtilisin, trypsin and Flavourzyme, exerts *in vitro* DPP-IV inhibitory activity with $IC_{50}$ equal to 1.73 mg/mL (Rivero-Pino et al., 2021). Interestingly, in this study, it was observed that potato, pea, soy, chickpea, quinoa, and lentil derived peptide mixtures (fraction 3 – 0,8 kDa) dropped the DPP-IV activity with $IC_{50}$ of 1.84, 2.09, 1.16, 3.45, 1.47, and 1.81 mg/mL, respectively (Rivero-Pino et al., 2021).

It is important to underline that the bioactivity of food-derived peptides lies in their capacity to cross the intestinal barrier and maintain their biological activity. In this line, the peptidomics analysis carried out in the present study showed that 141 of the identified peptides in the LPH (11.17% of the whole peptides) were able to cross the human intestinal barrier. Interestingly, 95.63% of them contained at least one motive with already demonstrated DPP-IV activity and more than 60% are considered inhibitory DPP-IV peptides. These results support the bioavailability of these peptides showing a higher percentage of peptides crossing the intestinal barrier than a previous study subjected in Cowpea protein hydrolysate (Marques et al., 2018) and similar to studies performed in protein hydrolysates from *Lupinus albus* (Lammi, Aiello, et al., 2016). Under the operating conditions of the peptidomics characterization, it was not possible to discriminate the isobaric leucine (L) and isoleucine (I) using tandem mass spectra. In this situation, the 1-letter code X was used. However, when the bioactivity score and the DPP-IV inhibitor score was calculated, X was replaced by both L and I letter code. For this reason, there are peptides such as FXSQ, RNXF, RXX, or SVXF that do not have DPP-IV activity with I (FISQ, RNIF, RII, SVIF) but do with leucine L (FLSQ, RNLF, RLL, SVLF). Most DPP-IV inhibitors have an hydrophobic or aromatic amino acid (Ala, Val, Ile, Leu, Met, Phe, Tyr, or Trp) at their N-terminal region. However, several non-inhibitory peptides have hydrophobic or aromatic amino acids at their N-terminus, indicating that N-terminal hydrophobicity or aromaticity is a desirable characteristic, but not sufficient for inhibition *per se* (Nongonierma, Dellafiora, Paolella, Galaverna, Cozzini, & FitzGerald, 2018).

To perform a "proof of concept" on the antidiabetic effect of LPH through the modulation of DPP-IV activity, a mouse animal model and a clinical trial were realized to evaluate the *in vivo* effective capacity of LPH to modulate glucose levels targeting DPP-IV. Western diet-fed $ApoE^{-/-}$ mice are a widely used animal model of metabolic syndrome. In fact, it has been shown that the Western diet, in addition to increase hyperlipidemia, exacerbates insulin resistance and type 2 diabetes (T2DM), generating a metabolic syndrome with great similarity to the human disease (Camargo, Matos, Araujo, Carvalho, Amaral, & Camporez, 2022; Ma et al., 2022; Schierwagen et al., 2015). In this line, the altered lipid metabolism is one of the several mechanisms that cause insulin resistance in this model (Bartelt et al., 2011; Lee et al., 2014). LPH consumption improved oral glucose tolerance, as well as

overcoming the WD-induced increased in the glucose plasma concentration after 12-weeks of treatment in ApoE$^{-/-}$ mice. LPH also counteracted the increase in DPP-IV activity induced by WD. In this sense, the Cohen's test analysis shows a large size effect on all the variables studied (Supporting materials Table S8). Therefore, these findings clearly indicate that the hypoglycemic effect of LPH is mediated by decreasing the activity of the soluble form of DPP-IV. Moreover, the DPP-IV activity was evaluated in the serum of healthy subjects who ingested LPHb, an experimental beverage containing 1 g of LPH for 28 days. LPHb consumption decreased DPP-IV activity by 7.51%, compared to baseline values (before starting LPHb ingestion). In addition, no correlation was observed between the DPP-IV activity inhibition and the decrease of its protein levels, confirming the direct effect of LPH on the activity of the DPP-IV enzyme. At last, a positive correlation was observed between DPP-IV activity and plasma glucose concentration. Thus, after stratifying subjects into two groups: i) participants who underwent no variation or increase in plasma glucose concentration and ii) subjects who showed a decrease in glucose concentration after 28 days of LPHb ingestion, a significant decrease in DPP-IV activity of 11.24 % was found only in the group that showed significantly lower plasma glucose levels after 28 days of LPHb consumption. This fact reinforces the causal relationship between DPP-IV activity and plasma glucose levels in the context of the present *in vivo* study.

Many food matrices and/or by-products in combination with different proteolytic enzymes (Alcalase, Papain, Flavourzyme, pepsin, and trypsin) have been characterized as inhibitors of DPP-IV activity (Nongonierma & FitzGerald, 2019). However, there are very few studies that have addressed the *in vivo* effects of food-derived hydrolysates/ peptides on DPP-IV activity in animal models and clinical studies.

Thus, in the present study we demonstrate that the action mechanism of LPH was related to inhibition of DPP-IV activity instead of reducing the concentration of circulating DPP-IV protein.

## 5. Conclusion

For the first time, thanks to this bench-to-bedside experimental study, ranging from *in vitro* to *in vivo* approaches in mice and humans, the biological effects of an LPH on the inhibition of DPP-IV activity and glycemia was demonstrated. LPH decreases *in vitro* DPP-IV activity in a dose-dependent manner both in a cell-free system and in Caco-2 cells. In addition, the combination of trans-epithelial transport and peptidomics analysis showed hundreds of LPH peptides with the capacity to cross the intestinal barrier and inhibit DPP-IV activity. Finally, the *in vivo* inhibitory role of LPH in DPP-IV activity was confirmed as well as the glucose-lowering effects in mouse and human studies, showing that the LPH directly inhibits the enzyme activity without exerting any effect on the concentration of DPP-IV protein. To our knowledge, no previous studies have evaluated the anti-diabetic properties of DPP-IV inhibitory hydrolysates in humans.

Taking all these results into account, our study strongly supports the use of LPH as new pleiotropic ingredients for the development of functional foods and/or nutraceuticals for the prevention of type 2 diabetes and conditions associated with glycemia disorders.

## CRediT authorship contribution statement

**Ivan Cruz-Chamorro:** Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Guillermo Santos-Sánchez:** Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Justo Pedroche:** Resources, Funding acquisition. **Francisco Millán:** Resources, Funding acquisition. **María del Carmen Millán-Linares:** Resources, Funding acquisition. **Anna Arnoldi:** Supervision, Resources, Writing - original draft. **Antonio Carrillo-Vico:** Supervision, Resources, Writing - review & editing. **Carmen Lammi:** Conceptualization, Supervision, Resources, Methodology, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136458.

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