

# Gamma-conglutin peptides from Andean lupin legume (*Lupinus mutabilis* Sweet) enhanced glucose uptake and reduced gluconeogenesis *in vitro*

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## ABSTRACT

The present study aimed to analyze the mechanism of action of a hydrolyzed gamma-conglutin fraction (Cgh) extracted from Andean *L. mutabilis* legume on glucose metabolism. At 5 mg/mL, Cgh from *L. mutabilis* inhibited dipeptidyl peptidase IV activity (DPP-IV) (100%), and increased ( $p < 0.05$ ) 6.5-fold the glucose uptake in a dual-layered enterocyte/adipocyte culture system compared to untreated cells, through an enhancement of glucose transporter type-4 translocation. In this model, insulin-induced glucose uptake was potentiated by addition of *L. mutabilis* hydrolysates. Also, Cgh *L. mutabilis* hydrolysates decreased ( $p < 0.05$ ) by 50% gluconeogenesis in a dual-layered enterocyte/hepatocyte system and reduced phosphoenolpyruvate carboxykinase expression. These data showed that *L. mutabilis* hydrolysates can affect glucose metabolism inhibiting DPP-IV enzymatic activity, improving insulin receptor sensitivity and inhibiting hepatic gluconeogenesis.

## 1. Introduction

Non-communicable chronic diseases (NCDs), such as type-2 diabetes (T2D) are increasing health problems that affect all societies around the globe (Allen et al., 2018). The dramatic increase of NCDs has been associated with limited physical activity and consumption of Western diet in urban environments (Dehghan et al., 2017). Currently, more than half of the human population lives in urban cities where physical inactivity and the consumption of food rich in simple sugars and fat, at the expense of more traditional foods, are common (Dehghan et al., 2017). In contrast, active physical activity and traditional diets rich in complex carbohydrates, polyunsaturated fats, and proteins have been associated with a decreased risk for NCDs (Durstine, Gordon, Wang, & Luo, 2013; Orozco et al., 2008; World Cancer Research Fund & The NCD Alliance, 2014). Therefore, there is a great deal of interest in the study of the active principles from natural products and traditional diets to reduce NCDs, including T2D and comorbidities (Beidokhti & Jäger, 2017; Ríos, Francini, & Schinella, 2015).

Consumption of traditional Andean foods has been associated with benefits to prevent and complement current treatments for metabolic diseases such as T2D (Baldeón, Castro, Villacrés, Narváez, & Fornasini, 2012; Fornasini et al., 2012). In this regard, published data indicate

that consumption of food rich in protein present in legumes, could improve insulin and glucose homeostasis (Baldeón et al., 2012). Lupin is a legume rich in protein that has been consumed for centuries in Europe, The Middle East, and the Andean Region; traditionally, Andean lupin seeds are consumed cooked and debittered (Atchison et al., 2016).

Clinical studies with animals and humans indicate that consumption of seeds of the genus *Lupinus* or its derived alkaloids has positive effects on hyperglycemia and glucose homeostasis. We have shown that consumption of Andean *L. mutabilis* decreases blood glucose and improves insulin sensitivity in subjects with dysglycemia or T2D (Baldeón et al., 2012; Fornasini et al., 2012). The possible components of *Lupinus* seeds and their mechanism of action to improve glucose metabolism in T2D have now started to be elucidated. Bertoglio et al. (2011) have reported the hypoglycaemic effect of a *Lupinus albus* seed gamma-conglutin ( $\gamma$ -conglutin)-enriched preparation in rats and humans. That study showed a dose response hypoglycemic effect of  $\gamma$ -conglutin during a glucose challenge of 2 g/Kg in rats. In a similar manner, a progressive hypoglycemic effect was observed in 15 human volunteers that were challenged with 75 g of carbohydrates and treated with increasing doses of the  $\gamma$ -conglutin (ranging from 750 to 3000 mg (Bertoglio et al., 2011). In that study, no effects on serum insulin concentrations were observed. The chronic use of  $\gamma$ -conglutin treatment in hyperglycemic

Abbreviations: Cg, purified  $\gamma$ -conglutin; Cgh, purified  $\gamma$ -conglutin hydrolysates; SCgh, semipurified  $\gamma$ -conglutin hydrolysates; SGDh, simulated gastrointestinal digestion of lupin protein isolate hydrolysates

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induced rats also showed a positive effect on glucose and insulin concentrations. Thus, the concomitant treatment of glucose (overage intake 2–3 g/d) and  $\gamma$ -conglutin (28 mg/kg body weight) attenuated the increase in plasma glucose and insulin concentrations (Lovati et al., 2012). In an animal model of insulin resistance and diabetes, consumption of  $\gamma$ -conglutin from *L. albus* decreased serum glucose concentrations and expression of the hepatic neoglucogenic glucose-6-phosphatase (G6pc) gene; the authors of the study concluded that G6pc inhibition reduced hepatic glucose production (González-Santiago et al., 2017). Also, it has been shown that  $\gamma$ -conglutin treatment to diabetic rats increased insulin gene expression at mRNA and protein levels (Vargas-Guerrero, García-López, Martínez-Ayala, Domínguez-Rosales, & Gurrola-Díaz, 2014). Furthermore, Terruzzi et al. (2011) have shown that  $\gamma$ -conglutin stimulation of mouse C2C12 myoblastic cell line elicited the activation of intracellular kinases very similar to the effects provoked by insulin. These data indicate that components in *L. albus* beans have important effects on insulin and glucose metabolism. In this regard, studies of dietary proteins have shown that bioactive peptides can affect insulin and glucose metabolism through the inhibition of alpha-glucosidase, dipeptidyl peptidase IV (DPP-IV), glucose uptake, and glucose production (Gothai et al., 2016; Patil, Mandal, Tomar, & Anand, 2015).

In spite of this progress with *L. albus*, little is known about which components in Andean *L. mutabilis* are responsible for the observed effect of decreasing blood glucose in T2D. Therefore, the objective of the present investigation was to study systematically, the role of  $\gamma$ -conglutin hydrolysates derived from Andean *L. mutabilis* beans on glucose metabolism *in vitro*. To test the potential mechanisms of action of *L. mutabilis* hydrolysates on glucose metabolism, the effect on DPP-IV inhibition, cellular glucose uptake, Glut-4 membrane translocation, and gluconeogenesis were assessed.

## 2. Materials and methods

### 2.1. Materials

Dry mature *Lupinus mutabilis* seeds cultivar 450, was kindly provided by the Instituto Nacional de Investigaciones Agropecuarias (INIAP), Quito, Ecuador. Human intestinal Caco-2 cell line (HBT-37), murine adipocytes 3T3-L1 (CL-173) and human hepatoma HepG2 cells (HB-8065) were obtained from ATCC (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), Minimum Essential Medium (MEM), fetal bovine serum (FBS), newborn calf serum (NCS) and antibiotics were obtained from Gibco (Life Technologies, Grand Island, NY). HiTrap Q HP column, HiTrap SP HP column, PVDF Hybond-P membranes were obtained from GE Healthcare (Buckinghamshire, UK). Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), unless otherwise specified. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

### 2.2. *Lupinus mutabilis* hydrolysates preparation and characterization

To test the effects of *L. mutabilis* hydrolysates on glucose metabolism, hydrolysates were prepared from two sources using pancreatin and pepsin digestion: (a) from purified  $\gamma$ -conglutin and (b) from lupin protein isolate (see below).

#### 2.2.1. Gamma-conglutin isolation

Raw lupin seeds were ground to obtain flour ( $\approx 420 \mu\text{m}$  mesh size), which was then defatted using *n*-hexane (3:1 v/w). Defatted flour was stored at 4 °C until use within 1 month. Gamma-conglutin was extracted as previously described (Czubinski et al., 2015). Briefly, defatted flour was stirred in water for 2 h (ratio 1:15) at 4 °C and then centrifuged at 8000 rpm for 15 min at 4 °C to discard albumins. The pellet was stirred with 10% NaCl solution (pH 7.0) for 12 h at 4 °C (ratio 1:10). After centrifugation, the pellet was discarded and proteins were precipitated

by direct addition of ammonium sulfate. After centrifugation, the pellet containing the total globulin fraction was dissolved in 0.1 M phosphate buffer (pH 6.8) and then filtered through a 0.45 mm PVDF filter membrane (Millipore, Burlington, MA). The total globulin fraction was subjected to fast protein liquid chromatography (FPLC), using a HiTrap Q HP column equilibrated with 20 mM Tris buffer (pH 7.5) for anion exchange. The fraction containing  $\gamma$ -conglutin did not become attached to the column under these conditions. The obtained fraction was concentrated using centrifugal filters of 10 kDa MWCO (Millipore, Billerica, MA) and then subjected to cation exchange chromatography with a HiTrap SP HP column equilibrated with 20 mM Tris buffer (pH 7.5); proteins were eluted in a NaCl gradient (0–1 M). The  $\gamma$ -conglutin fraction was concentrated using the centrifugal filters and buffer-exchanged to water. The resulting solution was freeze-dried and kept at –20 °C until further use; this fraction was called purified  $\gamma$ -conglutin (Cg).

#### 2.2.2. Lupin protein isolation

Defatted lupin flour was dissolved in water (1:10 flour/water ratio). The pH was adjusted to 8.0 with 0.1 M NaOH and the protein extraction was carried out at 35 °C, stirring for 1 h. The mixture was centrifuged at 6500 rpm for 15 min at 25 °C. The precipitate was re-extracted under identical conditions to maximize yield and both extracts were combined. Then, the pH was adjusted to 4.3 with 0.1 M HCl to precipitate protein, followed by centrifugation at 9000 rpm for 20 min. The supernatant was discarded and the pellet freeze-dried in a Lab Conco Freeze Dryer 4.5 (Kansas, MO). Dried lupin protein isolates (LPI) were stored at –20 °C until use.

#### 2.2.3. Enzymatic digestion

To obtain  $\gamma$ -conglutin hydrolysates (Cgh), Cg was sequentially digested with pancreatin (pH 7.5) and pepsin (pH 2.0), in a ratio 1:20 w/w (enzyme/substrate) at 37 °C for 1 h each, considering the reported resistance of  $\gamma$ -conglutin to pancreatin digestion (Czubinski, Siger, and Lampart-szczapa, 2016). The resulting material was freeze-dried and stored at –20 °C until analysis. A different approach was followed to obtain semi-purified  $\gamma$ -conglutin hydrolysates (SCgh); LPI was suspended in water and digested initially with pancreatin as described above. After this first digestion, the mixture was filtered using centrifugal filters of 10 kDa MWCO to eliminate the potential resulting peptides from pancreatin digestion. The supernatant was digested with pepsin, and the material was freeze-dried and kept at –20 °C until analysis. The other portion of the LPI was digested using a simulated gastrointestinal digestion method previously reported (Megias et al., 2004). Briefly, LPI was suspended in water, and a sequential enzyme digestion with pepsin (pH 2.0) and then pancreatin (pH 7.5), in a ratio 1:20 w/w (enzyme/substrate) at 37 °C, for 1 h each. The resulting freeze-dried material was labeled as simulated gastrointestinal digestion of lupin protein isolate hydrolysates (SGDh). For all digestions, the hydrolysis was stopped by heating samples at 75 °C for 20 min.

All *L. mutabilis* hydrolysates and Cg were resuspended in sterile water or in culture media for cellular assays.

#### 2.2.4. Protein profile by SDS-polyacrylamide gel electrophoresis

The electrophoretic profile of lupin derived hydrolysates was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% Tris-HCl precast protein gels (Bio-Rad, Richmond, CA). Equal amounts of protein mixed with 2%  $\beta$ -mercaptoethanol Laemmli Sample Buffer (ratio 1:1 v/v) were loaded for SDS-PAGE analysis. A broad range prestained SDS-PAGE standard was used to verify the relative molecular masses. Gel was stained with SimplyBlue staining solution (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Images were taken using a Gel Logic 4000 Pro Imaging System (Carestream Health Inc., Rochester, NY).

#### 2.2.5. Immunodetection of gamma-conglutin

Gamma-conglutin protein isolate was separated by SDS-PAGE and

transferred to a PVDF Hybond-P membrane, blocked, washed and incubated overnight at 4 °C with anti- $\gamma$ -conglutinin rabbit polyclonal antibody (diluted 1:1000) (Agrisera, AS08 335, Sweden). The membrane was washed and incubated with donkey anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) secondary antibody (diluted 1:2500). Protein expression was detected using 1:1 chemiluminescent reagents of ECL Prime Western Blotting kit (GE Healthcare, Buckinghamshire, UK) and visualized using a Gel Logic 4000 Pro Imaging System.

#### 2.2.6. Characterization of peptides

To characterize protein hydrolysates from digested Cg and LPI, samples were diluted in deionized water (1 mg/mL) and analyzed by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC–ESI–MS) using a Q-TOF Ultima mass spectrometer (Waters, Milford, USA), equipped with an Alliance 2795 HPLC system. The separation of the components was performed using a mobile phase of solvent A (95% H<sub>2</sub>O, 5% ACN and 0.1% formic acid) and solvent B (95% ACN, 5% H<sub>2</sub>O and 0.1% formic acid) in a flow rate of 200  $\mu$ L/min. The elution was in a linear gradient (0 min, 90% A; 2 min, 90% A; 40 min, 65% A; 60 min, 10% A; 65 min, 10% A; 66 min, 90% A; 80 min, 90% A). The temperature was kept at 20 °C during the whole procedure. A splitter with a split ratio of 1:10 was used, where one part went to the mass spectrometer and ten parts to waste. The Q-TOF Ultima mass spectrometer was equipped with a Z-spray ion source. Using a positive ion electrospray mode (+ESI), the analysis on the Q-TOF was carried out in V-mode with an instrument resolution between 9000 and 10,000 based on full width at half maximum, with a flow rate of 20  $\mu$ L/min. The source temperature was set at 80 °C and desolvation temperatures were set at 250 °C, respectively. The Q-TOF was operated at capillary voltage of 3.5 kV and a cone voltage of 35 V. The final detector was a microchannel plate with high sensitivity. The MassLynx 4.1 V software (Waters, Milford, MA) was used to control the instruments and to process the data in order to get the highest probability of the peptides sequences. The obtained sequences were submitted to PepDraw tool (<http://www.tulane.edu/~biochem/WW/PepDraw/index.html>, accessed in 12/01/2017) to determine predicted physicochemical properties of the peptides. Additionally, the bioactive potential of the peptides was predicted using the BioPep tool (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). The presence of the peptides in lupin (*L. mutabilis*) was confirmed using BLAST® tool (<http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed in 12/01/2017).

#### 2.3. Effect of *Lupinus mutabilis* hydrolysates on dipeptidyl peptidase IV activity

To determine the potential inhibitory effect of Cg, Cgh, SCgh, and SGDh from *L. mutabilis* on the activity of DPP-IV, the commercial kit DPP-IV-Glo™ Protease Assay (Promega, Madison, WI) was used, following manufacturer's protocol. Two concentrations of each treatment were assayed (2 and 5 mg/mL). Sitagliptin (0.2 mM) was used as a positive inhibitor control. The signal produced, in relative light units (RLU), was measured after 30 min in an Ultra Microplate Reader (Biotek Instruments, Winooski, VT) and then compared to the blank. Enzyme inhibition was determined as follow:

$$\% \text{ Inhibition} = \left[ \frac{\text{Initial Activity} - \text{Inhibitor}}{\text{Initial Activity}} \right] \times 100$$

#### 2.4. Cellular studies

##### 2.4.1. Cell culture

Caco-2 cells were subcultured using EMEM supplemented with 20% FBS, 1% penicillin–streptomycin, and 1% sodium pyruvate at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. 3T3-L1 cells were maintained in

DMEM supplemented with 1.5 g/L sodium bicarbonate, 10% newborn calf serum (NCS), and antibiotics. To differentiate 3T3-L1 cells into adipocytes, cells were subcultured at a density of  $6 \times 10^3$  viable cells/cm<sup>2</sup>; after 48 h, maintenance medium was replaced with DMEM containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methyl-xanthine, 0.25  $\mu$ M dexamethasone, 1  $\mu$ g/mL insulin and 2  $\mu$ M rosiglitazone (Zebisch, Voigt, Wabitsch, & Brandsch, 2012). After 48 h, the medium was changed to DMEM containing 10% FBS and 1  $\mu$ g/mL insulin for 48 h. On day 7, the medium was changed to DMEM containing 10% FBS and mature adipocytes were kept in this culture media until use (Zebisch et al., 2012). HepG2 cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotics. The cells were grown at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> and were subcultured at a density of  $6 \times 10^3$  viable cells/cm<sup>2</sup> 3 days before assays.

##### 2.4.2. Cell viability

In order to assess the potential detrimental effect of *L. mutabilis* hydrolysates on cells viability, Cell Titer 96® Aqueous One Solution Proliferation Assay (Promega, Madison, WI) was used. Each cell line was seeded in 200  $\mu$ L of culture media, in 96-well plates ( $1 \times 10^4$  cells/well), and cultured under above described conditions. Cells were treated with Cgh, SCgh and SGDh (2 and 5 mg/mL) and incubated for 24 h. Media were removed and replaced with 100  $\mu$ L of fresh medium supplemented with 20  $\mu$ L of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] to each well and incubated for 2 h. Subsequently, absorbance was read at 490 nm in an Ultra Micro plate Reader. Percentage of viable cells was calculated with respect to the untreated cells as follows:

$$\% \text{ Viability} = \left[ \left( \frac{\text{Abs treatment}}{\text{Abs untreated cells}} \right) \times 100 \right]$$

##### 2.4.3. Dual-layered enterocyte/adipocyte and enterocyte/hepatocyte cell culture systems

To mimic gastrointestinal absorption and to test absorbed *L. mutabilis* hydrolysates on adipocytes and hepatocytes, a dual culture of intestinal epithelial cells (IEC) over mature adipocytes or hepatocytes were used. Treatments with *L. mutabilis* hydrolysates were applied on the apical side of Caco-2 cells and their effects were determined in underlying adipocytes, or hepatocytes culture media or cellular lysates. Hanging Caco-2 cells were seeded on 24-transwell polystyrene membrane (0.4  $\mu$ m pore) inserts, at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> to start a IEC monolayer. Integrity of Caco-2 cells monolayer was evaluated by transepithelial electrical resistance (TEER) measurements (Millipore, Millicell ERS-2 Volt ohmmeter, Billerica, MA); mean stable TEER values of approximately 1300  $\Omega$  was indicative of intact IEC monolayer. The terms apical and basolateral were used to describe the upper and lower chambers of cell inserts. Only those Caco-2 cells that successfully formed a monolayer were used.

##### 2.5. Effect of *L. mutabilis* hydrolysates on glucose uptake in mature adipocytes

Caco-2 and 3T3-L1 cells were prepared as indicated in Sections 2.4.1 and 2.4.3, respectively. Once independent cultures were ready to use, Caco-2 inserts were placed over differentiated 3T3-L1 adipocytes cultured on 24-well plates. Subsequently, mature adipocytes were cultured overnight in low-glucose serum-free DMEM (5.5 mM) supplemented with 0.25% bovine serum albumin (BSA). On the day of the experiment, Caco-2 cells were apically treated with Cgh, SCgh, and SGDh for 24; based on preliminary data, two concentrations of each treatment were assayed (2 and 5 mg/mL) with or without insulin (100 nM). After treatment, Caco-2 cells were discarded and medium from 3T3-L1 cells was changed to high-glucose DMEM (25 mM) and incubated for 12 h. Subsequently, media was collected to detect D-

glucose using the Amplex Red Glucose/Glucose Oxidase Assay Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Adipocytes treated with insulin (100 nM) and without treatment were used as positive and negative controls, respectively. Cells were lysed using RIPA lysis buffer system (Santa Cruz Biotechnology, Santa Cruz, CA) and assayed for protein concentration through the DC protein assay (Bio-Rad, Richmond, CA), with BSA as standard. Glucose consumption was determined subtracting quantified glucose concentration in each well from initial glucose concentration (25 mM) in DMEM. The results were normalized to the cells' protein concentration.

## 2.6. Effect of *Lupinus mutabilis* hydrolysates on glucose transporter type 4 translocation in mature adipocytes

3T3-L1 cells were seeded on 12-well silicone cultivation chamber for cell culture and immunofluorescence staining (Ibidi, Am Klopferstspitz, Germany) at a density of  $2 \times 10^4$  viable cells/well and differentiated as previously described in Section 2.4.1. Mature adipocytes were cultured overnight in low-glucose serum-free DMEM (5.5 mM) supplemented with 0.25% BSA. Then, 3T3-L1 cells were treated with Cgh, SCgh and SGDh (5 mg/mL) for 4 h; at the end of treatment period, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), permeabilized with 0.1% Triton X-100 and blocked with Image-iT FX Signal Enhancer (Invitrogen, Carlsbad, CA). Adipocytes were incubated with anti-GLUT4 monoclonal-antibody (sc-53566) overnight, after washing, cells were stained with Alexa Fluor 488 Goat Anti-Rabbit IgG (A-11029) (Molecular Probes, Eugene, OR) secondary antibody for 3 h avoiding light exposure. Subsequently, cells were covered with ProLong Gold Antifade Mountant with 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR) for 24 h at room temperature and avoiding light exposure. The chambers were stored at 4 °C until further use. Glucose transporter type 4 (GLUT-4) translocation was determined with a Zeiss LSM 880 laser-scanning confocal microscope (Carl Zeiss AG, Germany), images were captured with 63×/1.4 Oil DIC M27 objective. The images were obtained using a 405 (10 mW) (415–470 nm emission), and a 488 (10 mW) (500–550 nm emission) laser line. The individual channels were obtained using a sequential scanning mode to prevent bleed-through of the excitation signal. Laser power, gain, and offset were kept constant across the samples, and the samples scanned in a high-resolution format of 1024 × 1024 pixels averaging four frames. Single optical planes of the individual channels were captured and all of the optical planes were displayed as a gallery. Individual images were analyzed for mean intensities and areas at multiple regions of interest among several cells in the field of view and expressed as average relative intensity in arbitrary units (AU) per area of analysis ( $\mu\text{m}^2$ ) in the membrane area of the cells using the program AxioVision Rel 4.8 (Carl Zeiss, Jena, Germany). All of the image panels were resized and consolidated, and the brightness of the final collage displayed was increased by 20% as a whole.

## 2.7. Effect of *Lupinus mutabilis* hydrolysates on glucose production in hepatocytes

The dual enterocyte/hepatocyte system was prepared as described in Section 2.4.3. Caco-2 inserts were placed over HepG2 cells cultured on 24-well plates. HepG2 cells were cultured overnight in low-glucose serum-free DMEM (5.5 mM); then washed twice with PBS to eliminate traces of glucose. To measure glucose production in HepG2 cells, hepatocytes were cultured in glucose-free DMEM. Caco-2 cells were treated on the apical side with Cgh, SCgh, and SGDh for 6 h; two concentrations of each treatment were assayed (2 and 5 mg/mL). Cells treated with metformin (1 mM) and cells without treatment were used as positive and negative control, respectively.

After treatment, Caco-2 cells were discarded and HepG2 culture media were changed to glucose production buffer (GPB) (glucose-free

DMEM supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate) and incubated for 3 h. Subsequently, media were collected to detect D-glucose using Amplex Red Glucose/Glucose Oxidase Assay Kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The results were normalized to the cellular protein concentration as indicated in Section 2.5.

## 2.8. Effect of *Lupinus mutabilis* hydrolysates on hepatic phosphoenolpyruvate carboxykinase expression

The effect of *L. mutabilis* hydrolysates on phosphoenolpyruvate carboxykinase (PEPCK) expression was assessed by western blot (WB). HepG2 cells were cultured overnight in low-glucose serum-free DMEM (5.5 mM); after that, cells were washed twice with PBS and treated directly with Cgh, SCgh, and SGDh for 6 h. Two concentrations of each treatment were assayed (2 and 5 mg/mL) in duplicate. Cells treated with metformin (1 mM) or without treatments were used as positive and negative control, respectively. After treatment, culture media were changed to GPB and incubated for additional 3 h. Subsequently, HepG2 cells were lysed with RIPA buffer; lysates were separated through SDS-PAGE and transferred to PVDF Hybond-P membranes; primary (sc-271029) and secondary (GE Healthcare, LNXA931, Buckinghamshire, UK) antibodies were used following manufacturer's recommended dilutions for WB. Protein expression was detected using 1:1 chemiluminescent reagents of ECL Prime Western Blotting kit (GE Healthcare, Buckinghamshire, UK) and visualized using a Gel Logic 4000 Pro Imaging System. The intensity of each band was normalized to GAPDH (sc-47724), and the results were expressed as expression level relative to a control.

## 2.9. Statistical analysis

The results were expressed as the mean  $\pm$  SD of at least two independent experiments with three repetitions each and analyzed through ANOVA. Statistical significance was determined using Tukey's test for multiple mean comparisons in the software JMP version 7.0 (SAS Institute, Cary, NC).

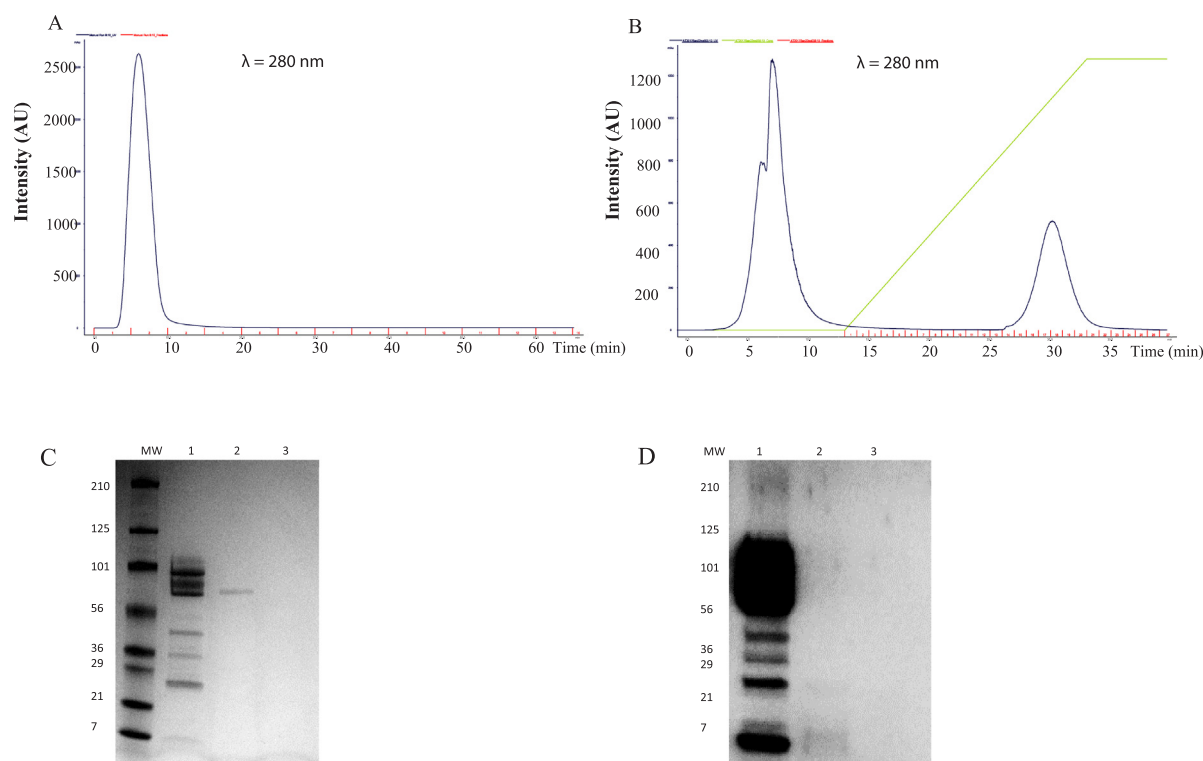
# 3. Results

## 3.1. Purification and characterization of lupin protein isolate and gamma-conglutin from *Lupinus mutabilis*

Fig. 1 shows the sequential anion (A) and cation (B) FLPC analysis for  $\gamma$ -conglutin purification. During cation exchange chromatography, a single peak was collected at the end of the elution phase for SDS-PAGE,  $\gamma$ -conglutin WB analysis, and further digestion. Fig. 1C shows SDS-PAGE for purified  $\gamma$ -conglutin and the corresponding hydrolysates after pancreatin and pepsin digestion. There were bands compatible with dimers of  $\gamma$ -conglutin of approximately 100 kDa and monomer of 47 kDa (Fig. 1C). After pancreatin and pepsin digestion, the majority of bands were not observed. Specific staining with anti- $\gamma$ -conglutin antibody also showed monomers and dimers of  $\gamma$ -conglutin and other bands corresponding to reported subunits of the molecule (Fig. 1D).

Sequences and predicted physicochemical characteristic of peptides from Cgh are shown in Table 1. An example of the peptide sequence analysis is shown in Fig. 2. A total of 13 peptides were sequenced with a length between 4 and 9 amino acid residues. The molecular weight of the peptides ranged from 458.2 to 881.4 Da. According to the predicted physicochemical properties, the peptides had different isoelectric points, with negative and positive net charges. BioPep tool indicated that the peptides had potential to inhibit the activities of angiotensin converting-enzyme, and DPP-IV. Since the proteome of *L. mutabilis* has not been completely sequenced, the peptide sequences were compared with *Lupinus* spp proteome using BLAST®. Comparison of peptides found in Cgh with sequences from  $\beta$ -,  $\gamma$ -, and  $\delta$ -conglutin from *Lupinus*





**Fig. 1.**  $\gamma$ -conglutinin purification and characterization. (A) FPLC anion exchange chromatogram. (B) FPLC cation exchange chromatogram. (C, D) SDS-PAGE and western blot membrane, respectively, of separations profiles of purified  $\gamma$ -conglutinin digestions: 1 purified  $\gamma$ -conglutinin (Cg), 2 Cg after pancreatin digestion, 3 Cg after pancreatin and pepsin digestion (Cgh).

*albus* and *Lupinus angustifolius*, confirmed the homology with proteins from the genus *Lupinus*.

### 3.2. Inhibition of dipeptidyl peptidase IV catalytic activity by *Lupinus mutabilis* hydrolysates

As indicated in Fig. 3, sitagliptin completely inhibited DPP-IV

activity; except for purified undigested Cg, *L. mutabilis*, all treatments (Cgh, SCgh, and SGDh) also inhibited DPP-IV activity. The highest dose of Cgh was more effective than the other *L. mutabilis* hydrolysates treatments and was similar to incretin sitagliptin inhibitory effect. Absence of Cg inhibition of DPP-IV activity indicated the need for hydrolysis of this large protein to generate enzyme inhibitory peptides.

**Table 1**

Sequence and predicted physicochemical properties of peptides present in the purified fraction of conglutinin-gamma from *Lupinus mutabilis*.

| Peptide sequence | Molecular mass (Da) | Isoelectric point | Net charge | Hydrophobicity (Kcal/mol) | Predicted bioactivity                                       | Presence in <i>Lupinus</i> spp <sup>a</sup>                             |
|------------------|---------------------|-------------------|------------|---------------------------|---|---|
| RLGN             | 458.2               | 10.6              | +1         | +10.4                     | ACE inhibitor, DPPIV inhibitor                              | Hypothetical protein TanjilG_00936 [ <i>Lupinus angustifolius</i> ]     |
| VNEGA            | 488.2               | 3.2               | −1         | +13.5                     | ACE inhibitor, DPPIV inhibitor                              | conglutin beta [ <i>Lupinus albus</i> ]                                 |
| SEIGGA           | 532.2               | 3.1               | −1         | +13.6                     | ACE inhibitor, DPPIV inhibitor                              | conglutin gamma [ <i>Lupinus albus</i> ]                                |
| NPDDC            | 562.1               | 2.8               | −2         | +16.1                     | DPPIV inhibitor   | conglutin beta [ <i>Lupinus albus</i> ]                                 |
| SAPRST           | 617.7               | 10.7              | +1         | +11.5                     | ACE inhibitor, DPPIV inhibitor                              | conglutin delta seed storage protein precursor [ <i>Lupinus albus</i> ] |
| GALGLGH          | 623.3               | 7.6               | 0          | +11.6                     | ACE inhibitor, DPPIV inhibitor                              | conglutin beta [ <i>Lupinus albus</i> ]                                 |
| VVVVDE           | 658.3               | 3.0               | −2         | +13.3                     | Anticancer, DPPIV inhibitor                                 | conglutin beta [ <i>Lupinus albus</i> ]                                 |
| NVLSQL           | 672.3               | 5.4               | 0          | +7.0                      | DPPIV inhibitor   | conglutin beta [ <i>Lupinus angustifolius</i> ]                         |
| PQNLDL           | 698.3               | 2.9               | −1         | +10.8                     | ACE inhibitor, DPPIV inhibitor                              | conglutin beta [ <i>Lupinus albus</i> ]                                 |
| AGGPQQR          | 712.3               | 10.7              | +1         | +14.1                     | Antiamnesic, ACE inhibitor, antithrombotic, DPPIV inhibitor | hypothetical protein TanjilG_30295 [ <i>Lupinus angustifolius</i> ]     |
| LTFPGSAD         | 806.3               | 3.1               | −1         | +11.0                     | Antiamnesic, ACE inhibitor, antithrombotic, DPPIV inhibitor | conglutin beta, partial [ <i>Lupinus angustifolius</i> ]                |
| PELSGAAH         | 867.4               | 5.0               | −1         | +15.8                     | ACE inhibitor, DPPIV inhibitor, antioxidative               | conglutin gamma [ <i>Lupinus albus</i> ]                                |
| LPKHSAD          | 881.4               | 5.1               | −1         | +20.1                     | ACE inhibitor, DPPIV inhibitor                              | conglutin beta [ <i>Lupinus angustifolius</i> ]                         |

ACE: Angiotensin converting enzyme, DPPIV: dipeptidyl peptidase IV.

<sup>a</sup> According to BLAST®.

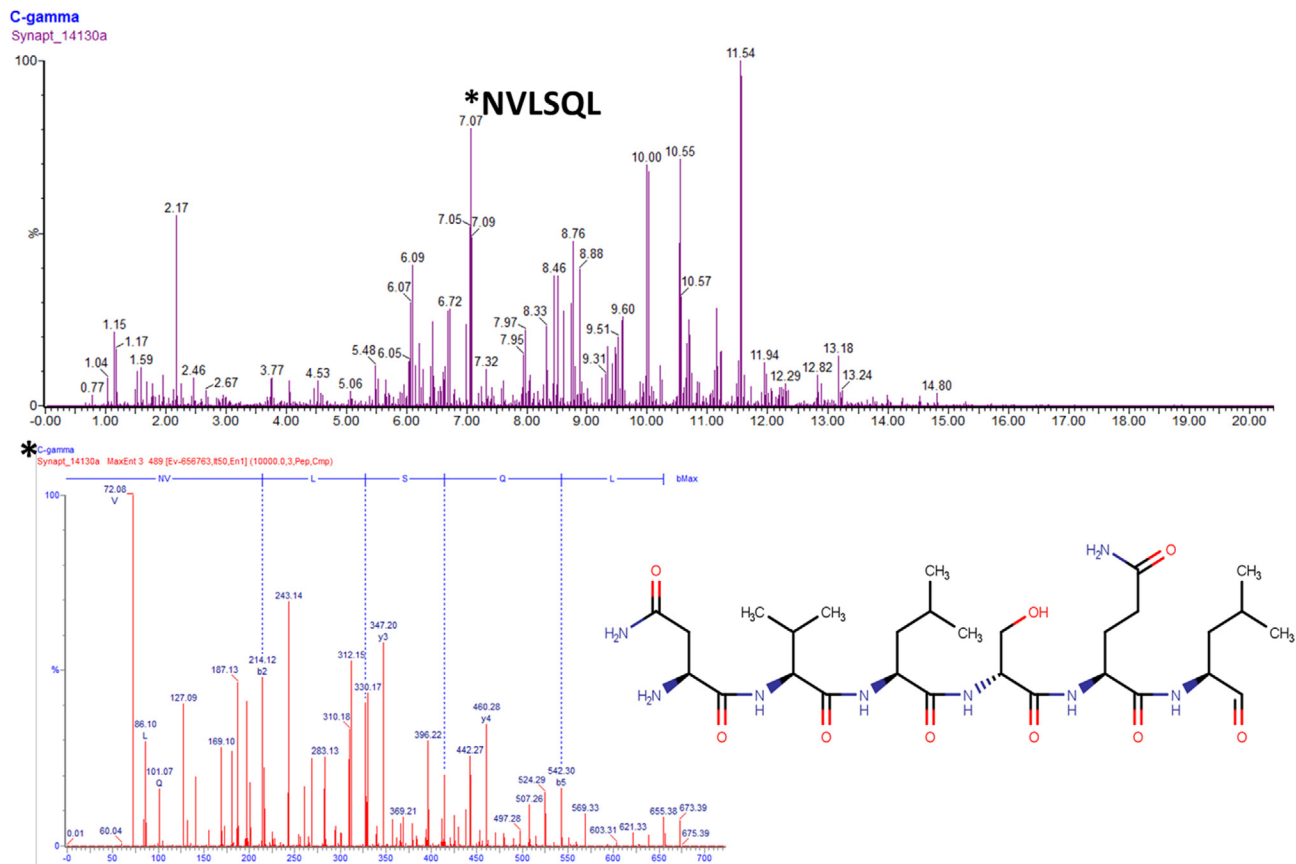


Fig. 2. Representative spectra showing the peptide sequencing process of the  $\gamma$ -conglutin hydrolysates by tandem mass spectrometry. The peptide NVLSQL is shown as an example.

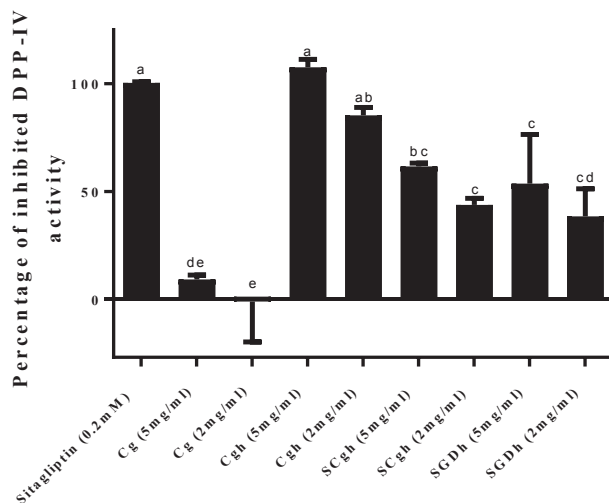


Fig. 3. Inhibitory effect of  $\gamma$ -conglutin and of lupin hydrolysates on enzymatic activity of dipeptidyl peptidase-IV (DPP-IV). Two concentrations (2 and 5 mg/ml) of Cg, Cgh, SCgh and SGDh were evaluated. Experiments were carried out in triplicates, results are expressed as the mean  $\pm$  SD of a representative experiment; bars with different letters indicate statistical difference ( $p < 0.05$ ) relative to positive control sitagliptin, according to Tukey's test.

### 3.3. Cell viability

To determine a potential effect of *L. mutabilis* hydrolysates on Caco-2, 3T3-L1, and HepG2 cells, cellular viability was evaluated upon hydrolysates treatments. Results indicated that after 24 h of high and low doses of hydrolysates treatments (2 and 5 mg/ml), there were not

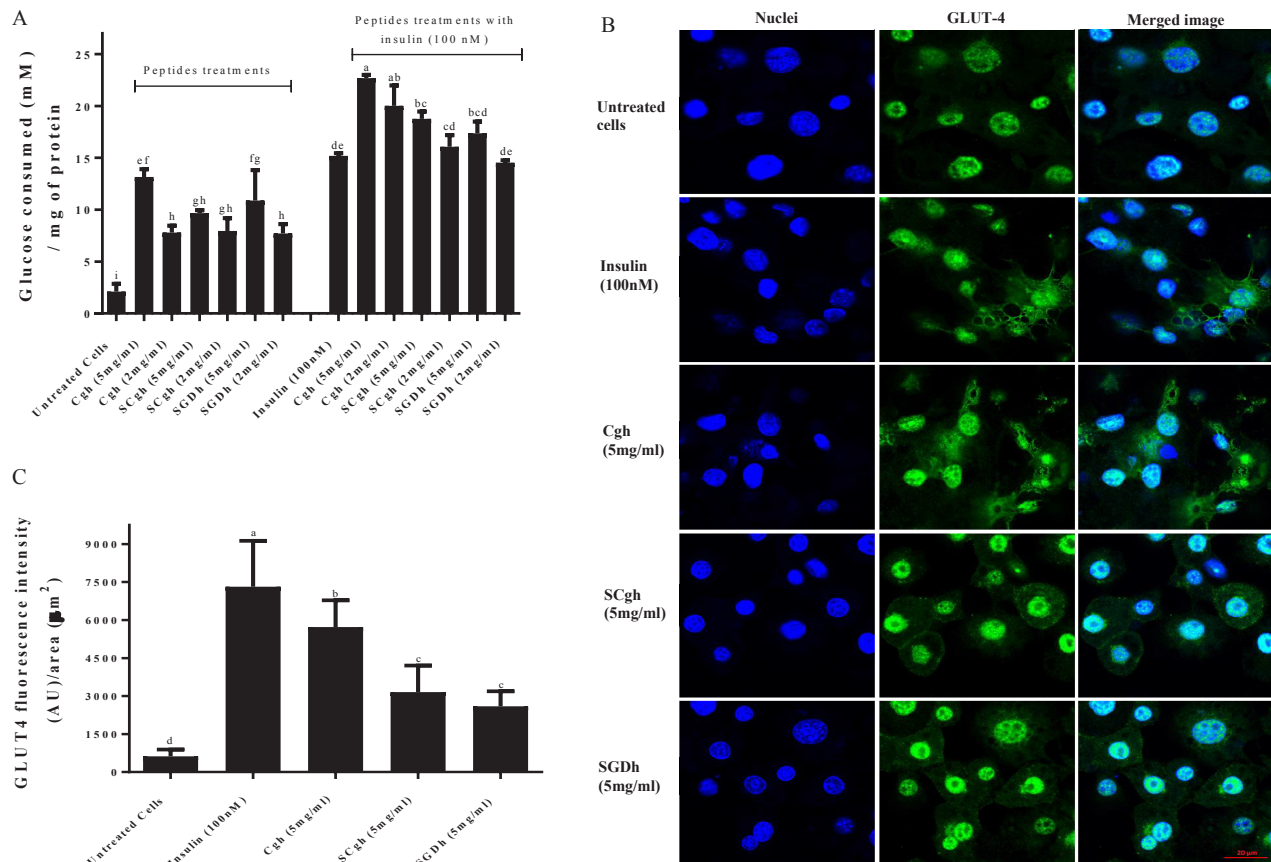
negative effects on the viability of cells (data not shown). We used these doses for all subsequent experiments.

### 3.4. Cellular glucose uptake

To mimic gastrointestinal absorption of *L. mutabilis* hydrolysates and their effect on glucose uptake by adipocytes, a Caco-2 cells/3T3-L1 cells dual culture system was used. As expected, insulin treatment significantly increased cellular glucose uptake (Fig. 4A). Purified  $\gamma$ -conglutin hydrolysates, SCgh, and SGDh significantly ( $p < 0.05$ ) increased adipocytes-glucose intracellular transport. Glucose uptake was greater upon SGDh treatment at 5 mg/ml. In order to test a potential interaction between *L. mutabilis* hydrolysates and insulin, cells were treated with Cgh, SCgh, or SGDh in the presence or absence of 100 nM insulin. Combined treatments further increased cellular glucose uptake by 3T3-L1 adipocytes. Insulin/Cgh treatments induced the greatest glucose uptake by adipocytes. Similarly, insulin-induced glucose uptake was potentiated by the simultaneous treatment with SCgh at 5 mg/ml (Fig. 4A).

### 3.5. Glucose transporter type 4 membrane translocation

The increased glucose uptake was stimulated by *L. mutabilis* hydrolysates, alone or in combination with insulin. This indicated that glucose entrance to the cells could be mediated by insulin dependent GLUT-4. Accordingly, the effects of Cgh, SCgh, and SGDh on GLUT-4 translocation in mature adipocytes were evaluated. Insulin treatment significantly increased GLUT-4 translocation from the cytosol to the cell surface membrane, Fig. 4B, C. Treatments with Cgh, SCgh, or SGDh significantly increased GLUT-4 translocation to the plasma membrane compared with untreated control cells. Stimulation with 5 mg/ml Cgh



**Fig. 4.** Effect of lupin hydrolysates on glucose uptake and glucose transporter type 4 (GLUT-4) translocation in mature 3T3-L1 adipocytes. (A) Concentration of glucose (mM) consumed by 3T3-L1 cells after Cgh, SCgh, and SGDh treatments. Two concentrations (2 and 5 mg/ml) of each treatment were evaluated, with or without Insulin (100 nM). Results were normalized to the cellular protein concentration. Results are expressed as the mean  $\pm$  SD; bars with different letters indicate statistical difference relative to untreated cells ( $p < 0.05$ ), according to Tukey's test. (B) Confocal laser scanning microscopy depicting two-dimensional fluorescence detection and quantification, determined by the intensity (AU) over area ( $\mu\text{m}^2$ ) of GLUT-4 in adipocytes after Cgh, SCgh and SGDh treatments. (C) Fluorescence intensity was quantified ( $\text{AU}/\mu\text{m}^2$ ) in the membrane region of the adipocytes and normalized using the same laser gain for all the samples to evaluate GLUT-4 membrane translocation. Bars with different letters are significantly different ( $P < 0.05$ ) according to Tukey's test.

elicited greater translocation than SCgh, or SGDh treatments.

### 3.6. Effect of *Lupinus mutabilis* hydrolysates on gluconeogenesis in hepatocytes

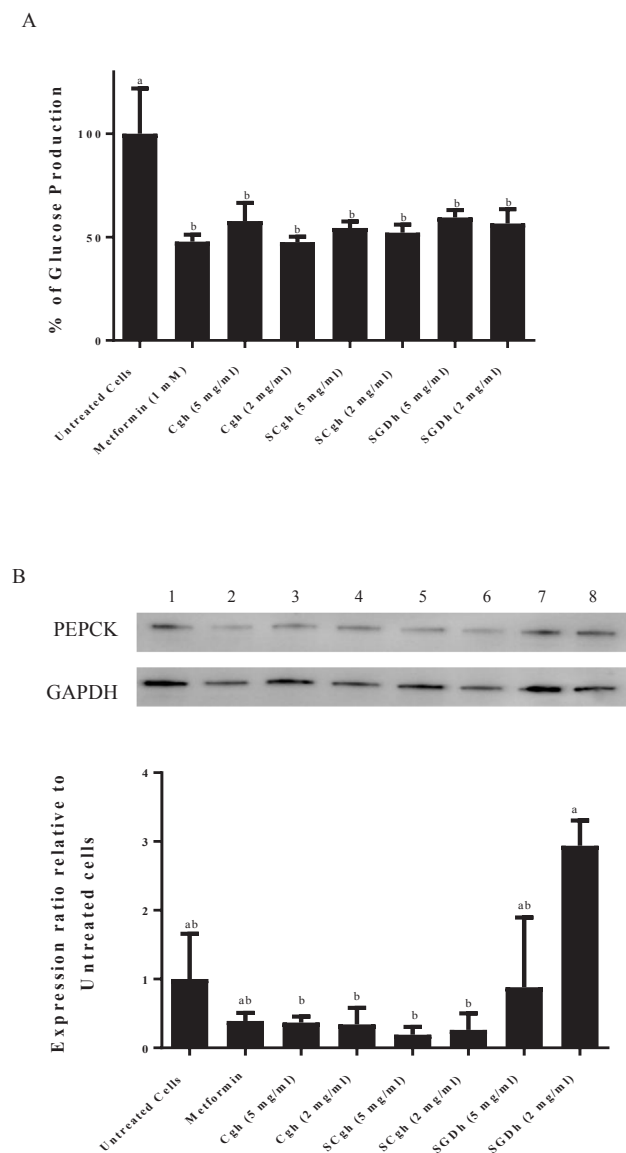
Fig. 5A shows glucose production by HepG2 in the presence of *L. mutabilis* hydrolysates using the dual Caco2/HepG2 system. The anti-diabetic drug metformin significantly ( $p < 0.05$ ) reduced glucose concentration in culture buffer of HepG2 cells, Fig. 5A. In addition, Cgh, SCgh and SGDh treatments decreased HepG2 glucose production in a similar manner than metformin. There were not significant differences in the level of inhibition of glucose production among different *L. mutabilis* hydrolysates. Moreover, *L. mutabilis* hydrolysates that inhibited gluconeogenesis in HepG2 cells, also affected the expression of PEPCK (Fig. 5B). Basal expression of PEPCK was decreased by metformin, Cgh and SCgh treatments, although the differences were not statistically different, Fig. 5B.

## 4. Discussion

Our *in vitro* results showed that the potential cellular and molecular mechanisms responsible for the hypoglycemic effect of *L. mutabilis* observed before in individuals with dysglycemia and T2D could be related to: (a) inhibition of DPP-IV enzymatic activity, (b) stimulation of glucose uptake due to GLUT-4 cell surface translocation, and (c) inhibition of hepatic gluconeogenesis.

Most common current anti-diabetic treatments include: I. inhibitors of DPP-IV enzymatic activities; II. drugs that improve insulin receptor sensitivity; and III. drugs that decrease gluconeogenesis (American Diabetes Association, 2016). Consumption of lupin seeds or their derivatives decrease abnormal serum glucose concentrations (Baldeón et al., 2012; Fornasini et al., 2012).

Here, a systematic approach to study the potential molecular and cellular mechanism of *L. mutabilis* hydrolysates to improve glucose metabolism was performed. *L. mutabilis* derived Cgh, SCgh, and SGDh inhibited DPP-4 activity with Cgh been the most inhibitory. Our previous work with protein isolates from black beans (*Phaseolus vulgaris* L.) digested with pepsin/pancreatin also generated several peptides with angiotensin converting enzyme (ACE) and DPP-IV inhibitory properties (Mojica & de Mejía, 2015). Studies to optimize the generation of anti-diabetic peptides from black beans indicate that the use of alcalase for 2 h with a ratio of enzyme substrate (E/S) of 1:20 produces hydrolysates that inhibit 96.7% of DPP-IV enzymatic activity (Mojica & de Mejía, 2016). In the present study, Cgh were generated after pancreatin/pepsin digestion with a ratio of E/S 1:20 w/w; Cgh inhibitory activity was similar in magnitude than the anti-diabetic drug sitagliptin that was used as a positive control. The absence of undigested  $\gamma$ -conglutinin direct inhibition of DPP-IV activity indicated that this protein had to be digested, as it happens within the gastrointestinal tract, to generate enzyme inhibitory hydrolysates. It will be important to further characterize *L. mutabilis* hydrolysates and study the specific molecular inhibitory DPP-IV enzymatic activities.



**Fig. 5.** Effect of lupin hydrolysates on glucose production and phosphoenolpyruvate carboxykinase expression levels in HepG2 hepatocytes. (A) Percentage of glucose produced by HepG2 cells relative to untreated cells, after Cgh, SCgh and SGDh treatments. The results were normalized to the cellular protein concentration. Results are expressed as the mean  $\pm$  SD; bars with different letters indicate statistical difference relative to untreated cells ( $p < 0.05$ ), according to Tukey's test. (B) A representative image of a western blot membrane for Phosphoenolpyruvate carboxykinase (PEPCK) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression; lanes: 1 untreated cells, 2 Metformin (1 mM), 3 Cgh (5 mg/ml), 4 Cgh (2 mg/ml), 5 SCgh (5 mg/ml), 6 SCgh (2 mg/ml), 7 SGDh (5 mg/ml), and 8 SGDh (2 mg/ml).

The putative effect of *L. mutabilis* hydrolysates in insulin receptor sensitivity was subsequently evaluated. Previous work showed that  $\gamma$ -conglutin from lupin seeds modulated insulin-signaling pathways regulating cellular energy metabolism in myoblastic C2C12 cells (Terruzzi et al., 2011). In that study, myoblast direct treatment with  $\gamma$ -conglutin activated IRS-1/PI-3K/Akt-1/CBL proteins, causing translocation of GLUT-4 to the cell surface membrane increasing glucose uptake. Under resting conditions with low insulin concentrations, GLUT-4 resides in the membrane of intracellular vesicles in adipocytes; upon insulin stimulation GLUT-4 containing vesicles fuse with cell surface plasma membrane to facilitate glucose uptake into the cell (Saltiel & Kahn, 2001). We reasoned that absorption of intact  $\gamma$ -conglutin ( $\sim 47$  kDa)

through intestinal epithelium could be limited due to its high molecular weight. Accordingly, a dual cell culture system Caco-2 cells/differentiated 3T3-L1 adipocytes was used to mimic gastrointestinal absorption of *in vitro* generated *L. mutabilis* hydrolysates, similar to those produced by the digestion of proteins in the gastrointestinal tract. The observed biological effects on mature 3T3-L1 adipocytes after apical treatment of  $\gamma$ -conglutin hydrolysates (Cgh, SCgh, and SGDh) on Caco-2 cells, increasing glucose uptake, demonstrated that active hydrolysates were absorbed through IEC to affect adipocytes biology.

Regarding glucose uptake, previous studies show that direct  $\gamma$ -conglutin treatment (10  $\mu$ mol/L) for 24 or 48 h to human HepG2 cells significantly increased glucose consumption when cells were cultured in high glucose medium but not when cells were cultured in normal glucose concentrations (Lovati et al., 2012). Glucose uptake was further increased when HepG2 cells were exposed to  $\gamma$ -conglutin plus insulin (100 nmol/L) or metformin (10 mmol/L) (Lovati et al., 2012). It is important to note that glucose uptake by hepatocytes does not depend on insulin stimulation. In the present study, Cgh, SCgh, and SGDh treatments to insulin dependent 3T3-L1 adipocytes cultured in high glucose media also increased glucose uptake; similar to Lovati's study, in our investigation, addition of insulin to *L. mutabilis* hydrolysates further increased glucose uptake. In clinical studies, *L. mutabilis* consumption decreased serum glucose only in patients with dysglycemia but not in subjects with normal serum glucose concentrations (Fornasini et al., 2012). Also, *L. mutabilis* hypoglycemic effects were greater in patients taking metformin (Baldeón et al., 2012). Together, these studies indicate that *L. mutabilis* and its derivate protein do not affect glucose metabolism when glucose concentrations are within the normal range. However, when glucose concentrations are abnormally high in cell culture media or in subjects with T2D or dysglycemia, *L. mutabilis* hypoglycemic effects are evident and are further increased with insulin or metformin treatments.

Lastly, the effect of *L. mutabilis* hydrolysates on internal glucose production, was also assessed. A characteristic pathological feature in T2D is the abnormal hepatic production of glucose (American Diabetes Association, 2016). Under normal conditions, hepatic gluconeogenesis contributes to maintain normo-glycemia during fasting periods to preserve brain and other tissues functions. Hepatic glucose-6-phosphatase (G6Pase) and PEPCK enzymatic activities control the gluconeogenic pathway; these enzymes are activated in hepatic HepG2 cells and consequently glucose synthesis and release (American Diabetes Association, 2016). In a recent publication (González-Santiago et al., 2017), demonstrated that daily  $\gamma$ -conglutin treatment (150 mg/kg) to insulin resistant rats for one week decreased serum glucose and insulin concentrations. In that study, the expression of the neoglucogenic genes fructose-bisphosphatase (*Fbp1*), glucose-6-phosphatase (*G6pc*), and phosphoenolpyruvate carboxykinase (*Pck1*) was decreased in insulin resistant rats treated with  $\gamma$ -conglutin. Authors indicated that  $\gamma$ -conglutin could decrease hepatic glucose production through inhibition of *G6pc* (González-Santiago et al., 2017). In the present study, absorbed Cgh, SCgh and SGDh through a Caco-2 cell monolayer decreased HepG2 cells glucose production, inhibition of glucose production was similar to metformin treatment. It is important to consider that inhibition of glucose production by *L. mutabilis* hydrolysates and metformin in HepG2 cells was accompanied by a decrease in PEPCK protein expression although the decrease was not statistically different. In this regard, it is well known that one of the main mechanisms of metformin to decrease serum glucose concentrations is the blockage of hepatic gluconeogenesis through inhibition of gluconeogenic enzymes expression (Bailey & Turner, 1996; Kim et al., 2008). These studies demonstrate that inhibition of hepatic glucose production by lupinus derived peptides could be due to their effect on the expression of gluconeogenic enzymes at the mRNA and protein levels.



## 5. Conclusions

The present study expands previous work on lupin derived peptides on glucose metabolism. Data showed that *L. mutabilis* hydrolysates can affect glucose metabolism inhibiting DPP-IV enzymatic activity, improving insulin receptor sensitivity, and inhibiting hepatic gluconeogenesis. It will be important to characterize the structure and function of *L. mutabilis* hydrolysates to improve their effects on glucose metabolism in T2D. These results strengthen the recommendation for the consumption of legumes, part of traditional diets, for the general population.

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## Declarations of interest

None.

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