Journal of Functional Foods 67 (2020) 103856

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

A polyphenol-rich Calafate (*Berberis microphylla*) extract rescues glucose tolerance in mice fed with cafeteria diet

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ARTICLE INFO

Keywords:
Obesity
Adipose tissue
Inflammation
Glucose tolerance
Natural product

ABSTRACT

The establishment of a chronic inflammatory state in the adipose tissue contributes to obesity-associated insulin resistance. Hence, disrupting the inflammatory response elicited by obesity remains a relevant target to tackle the modern-world pandemic. We evaluated the anti-inflammatory and insulin-sensitizing effect of Calafate (*Berberis microphylla*) by producing and characterizing a polyphenol-pure Calafate extract (PPCE). C57BL/6 mice fed with cafeteria diet for 14 weeks were administered PPCE (50 mg/Kg/day) for 4 weeks. PPCE administration rescued glucose tolerance and insulin-elicited AKT phosphorylation in white adipose tissue of diet-induced insulin-resistant mice. Furthermore, the cafeteria diet-induced expression of TNF-alpha and F4/80 was attenuated by PPCE administration, suggesting that PPCE rescues insulin sensibility by ameliorating the obesity-associated inflammatory state. Altogether, our data shows that Calafate represents a natural source of polyphenols with glucose tolerance-improving properties *in vivo*, suggesting a potential use of PPCE as a complementary tool against insulin resistance.

1. Introduction

Processed food are the major source of energy intake in high-income countries and processed food consumption is associated to the development of obesity (Hall et al., 2019; Poti, Braga, & Qin, 2017). In rodents, diets consisting of processed foods are known as Cafeteria Diet (CD). CD have been used to study diet-induced obesity and associated disorders, including insulin resistance, cardiovascular disease and diabetes. Representing a relevant model to study obesity-associated comorbidities (Gomez-Smith et al., 2016).

Obesity is a worldwide health burden that caused around 3.4 million deaths in 2010 (Lim et al., 2012). Obesity is characterized by an excessive accumulation of fat in adipose tissue contributing to the generation of a low-grade chronic inflammatory state with concomitant macrophage infiltration (Gustafson, Hedjazifar, Gogg, Hammarstedt, & Smith, 2015). CD consumption induces an inflammatory state in rodents (Toledo et al., 2019; Zeeni, Dagher-Hamalian, Dimassi, & Faour, 2015), that is associated to robust macrophage infiltration to the adipose tissue (Sampey et al., 2011). The interaction between macrophages and adipocytes causes an increase in pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNF-alpha), Monocyte chemoattractant protein-1 (MCP-1) and nitric oxide (NO). The increase in adipokines positively correlates with body adiposity (Ferrante, 2007), contributing to the generation and maintenance of a chronic inflammatory state associated to obesity (Shoelson, Lee, & Goldfine, 2006).

Adipokines interferes with the pivotal PI3K/AKT pathway, rendering adipose tissue inflammation as a link between obesity and insulin resistance (Huang, Liu, Guo, & Su, 2018). Ultimately, obesity-induced alterations in insulin signaling and impaired glucose homeostasis

Abbreviations: BAT, Brown adipose tissue; CD, Cafeteria diet; C-3-GE, Cyanidin-3-glucoside equivalents; Chow, Chow diet; FRAP, Ferric reducing antioxidant power; GAE, Gallic acid equivalents; LC-MS, Liquid chromatography coupled to mass spectrometry; MCP-1, Monocyte chemoattractant protein-1; NO, Nitric oxide; PPCE, Polyphenol-pure Calafate extract; T2D, Type 2 diabetes; TNF-alpha, Tumor Necrosis Factor alpha; WAT, White adipose tissue

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https://doi.org/10.1016/j.jff.2020.103856
Received 26 June 2019; Received in revised form 11 February 2020; Accepted 13 February 2020
Available online 28 February 2020

are the major contributors to the generation of type 2 diabetes (T2D) (Dandona, Aljada, & Bandyopadhyay, 2004). Evidence suggest that disrupting the inflammatory response elicited by obesity allows a recovery of insulin sensitivity (Chen, Lee, Leu, & Wang, 2019; Ruiz & Haller, 2006).

The Calafate (*Berberis microphylla*) is a native shrub that grows in Chilean and Argentinian Patagonia (Ruiz et al., 2010). Calafate fruits are dark purple, black, or bluish berries that show a high polyphenol content and a high antioxidant capacity (Ramirez, Zambrano, Sepulveda, Kennelly, & Simirgiotis, 2015). Polyphenols have been shown to ameliorate the inflammatory state associated to obesity and to improve insulin sensitivity (Anhe et al., 2017). Interestingly, we previously showed that a Calafate extract prevented the inflammation-elicited alteration of insulin-induced glucose uptake using an *in vitro* model of inflammation, suggesting an insulin-sensitizing role for Calafate (Reyes-Farias et al., 2015, 2016). However, this has not been evaluated *in vivo*. In this work, we aim to evaluate the effects of Calafate extract treatment on body weight, inflammation, glucose tolerance and insulin signaling pathway in a preclinical model of insulin-resistance.

2. Material and methods

2.1. Mice

Male C57BL/6 mice of 6–7 weeks of age and weighing 22 ± 1 g were used throughout the study. Mice were kept in an animal facility in a 12/12 h light/dark cycle, room temperature was kept constant at 20 ± 2 °C. 3–4 mice were co-housed with free access to food and water. All the experiments were approved by the Animal Ethics Committee of the University of Chile (FMUCH, Chile, protocol N° CBA #0787) (Santiago, Chile).

2.2. Experimental design

Mice were randomly assigned to one of the following experimental groups: i) A control group was always kept on a chow diet (Chow) ii) A group was kept on cafeteria diet throughout the study (CD) iii) A third group was obtained by randomly dividing the CD group after 10 weeks of cafeteria diet feeding, this group of mice received PPCE in the drinking water during 4 weeks (CD + PPCE). This group continued the cafeteria diet regime during the time PPCE was administered. Mice body weight was registered weekly. For collecting tissues, mice were intraperitoneally injected with a ketamine-xylazine mixture (91 and 9 mg/kg respectively) and euthanized by cervical dislocation. Cardiac puncture was used to collect the blood used for lipid profiling. Tissues were kept at −80° until used.

The cafeteria diet (62.9% kcal fat, 20.2% kcal carbohydrates and 16.9% kcal protein) was prepared as previously described (Garcia-Diaz et al., 2007; Paternain et al., 2011). The chow used for control feeding was Champion® Animal nutrition, Santiago, Chile (25.3% kcal fat, 54.9% kcal carbohydrates and 19.8% kcal proteins).

2.3. Calafate extract

Calafate fresh fruits were donated by the Institute of Agricultural Research of Chile (INIA). The raw extract was prepared as follows: 535 g of fresh Calafate fruit were dried for 72 h at 40 °C and pulverized in a mortar. A 24 h extraction was performed using methanol:water (1:1 v/v) and followed by methanol evaporation at 40 °C using a rotating evaporator. The raw extract was resuspended in 450 mL ultrapure water and loaded onto an Amberlite (R) XADH7 column (Sigma-Aldrich, San Luis, MO, USA). The PPCE was obtained by eluting the phenolic compounds with methanol acidified with 0.1% HCl. Methanol was evaporated as described above. Finally, the PPCE was resuspended in 250 mL ultrapure water and maintained at −20 °C until required. The PPCE was administered in the drinking water at a dose of 50 mg polyphenol/Kg daily. Polyphenol stability was evaluated in PPCE frozen samples and in the PPCE beverage using Folin ciocalteu phenol method. The PPCE beverage was replaced each second day to avoid polyphenol degradation.

2.4. Extract characterization

Total polyphenolic content was determined by the Folin ciocalteu phenol method measured at 765 nm (Singleton, 1985). Results were expressed as gallic acid equivalents (GAE). Total anthocyanins were measured by the differential pH method (Wrolstad, 1993). The absorbances were measured at 515 and 700 nm using buffers pH 1.0 and 4.5, respectively. Results were expressed as mg cyanidin-3-glucoside equivalents (C-3-GE)/100 g dry weight (DW). The antioxidant activity was measured by the ferric reducing antioxidant power (FRAP) method measured at 593 nm (Benzie & Strain, 1996). Results were expressed as mmol Fe^{+2}/100 g DW. The anthrone method was used to determine the total carbohydrate content. Measures were taken at 630 nm (Somani, Khanade, & Sinha, 1987).

2.4.1. Liquid chromatography coupled to mass spectrometry (LC-MS)

The analyses were conducted using an Agilent 1100 HPLC (Agilent Technologies Inc., CA, USA) system coupled with a Esquire 4000 ion trap LC/MS system (Bruker Daltoniks, Germany), using a C18 column (5 μm, 4.6 mm i.d × 150 cm, Luna, Phenomenex Inc, CA,USA) as previously described (Ruiz et al., 2010). Briefly, The mobile phase was water: acetonitrile: formic acid (87:3:10 v/v/v, solvent A) and water: acetonitrile: formic acid (40:50:10 v/v/v, solvent B) at a flow rate of 0.8 mL/min, using the following elution gradient: 0–15 min, 6% B; 15–30 min, 30% B; 30–35 min, 60% B; 35–41 min, 6% B; 41–50 min, 6% B. The mass spectral data were acquired in positive mode; ionization (nebulization) was performed with nitrogen as drying gas at 50 psi, 365 °C and at a flow rate of 10 L/min and capillary voltage 3000 V. All scans were performed in the range 50–1400 *m/z*. The trap parameters were set in ion charge control using manufacturer default parameters. Collision induced dissociation was performed by collisions with the helium background gas present in the trap. Fragmentation was set with Smart Frag.

2.5. Glucose tolerance test

Mice from all experimental conditions, including controls, were subjected to the glucose tolerance test. Mice were fasted for 12 h before evaluation took place. The glucose load (1.0 g/kg body weight) was injected intraperitoneally and tail blood samples were collected 0, 15, 30, 45, 60, 120 and 150 min after glucose load. Glucose concentration was determined using a glucometer (FreeStyle Optium, Abbott, Chicago, USA) and area under the curve was estimated using the baseline. Tests were performed after 9 and 13 weeks of cafeteria diet feeding.

2.6. Histological analysis

Epididymal adipose tissue samples were fixed in 4% para-formaldehyde for 14 h at 4 °C and processed for paraffin sections. Adipose tissue sections (7 μm) were stained with hematoxylin and eosin (H&E) and images were taken using a Canon EOS Rebel T3 camera mounted on a microscope (Zeiss Axioscope microscope, Zeiss, Oberkochen, Germany). The images were processed as previously described and imageJ software was used to measure adipocyte size (Parlee, Lentz, Mori, & MacDougald, 2014).

2.7. Quantitative real-time PCR

Total RNA from epididymal tissue was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). Purified RNA was treated with DNase

(DNAfree kit; Ambion, Austin, TX, USA) and quantified by measuring the absorbance at 260 and 280 nm using NanoDrop (NanoDrop Technologies, Wilmington, USA). 5 μg of total RNA was used to generate single-stranded cDNA, using kit ImProm-II™ Reverse Transcription System (Promega Corporation, Madison, WI, USA). The cDNA was amplified using the TaqMan® probes (Applied Biosystems, Foster city, CA, USA) specific for the genes F4/80 and TNF-alpha. Real-time PCR was performed in a Stratagene Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Expression levels of the target genes studied were normalized by the expression of cyclophilin A (Ppia) as internal control (also supplied by Applied Biosystems). Fold change between groups was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.8. Western blot

For insulin signaling analysis, three mice per group were fasted overnight and intraperitoneally injected with insulin (5U/g body weight) (Sigma-Aldrich, San Luis, MO, USA). After 10 min, epididymal adipose tissue was collected and frozen.

Proteins from epididymal tissue were extracted using lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Tritón x-100, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, pH 7.5) supplemented with protease-phosphatase inhibitor cocktail diluted 1:500 (Sigma-Aldrich, San Luis, MO, USA). Protein were quantified using the BCA protein assay (Pierce Biotechnology, Waltham, MA, USA). Forty micrograms of protein extract prepared in loading buffer were used for gel electrophoresis in denaturing polyacrylamide 8–12% gels (SDS-PAGE) in reducing conditions and transferred to PVDF membranes. The membranes were blocked in TBS, 0.1% Tween20 and 5% BSA. The membranes were incubated at 4 °C overnight with Rabbit anti phospho-Akt (1:2000) and Rabbit anti-Akt (1:2000) (both cell signaling Technology, Danvers, MA, USA). Later, the membrane was washed (TBS 1×, 0.05% Tween20) and incubated with Anti-Rabbit HRP secondary antibody (1:2500, cell signaling Technology, Danvers, MA, USA) for 1 h at room temperature. After washing the membranes, the bands showing the protein of interest were visualized using an enhanced chemiluminescence system (Super Signal West Femto Maximum Sensitivity, Pierce Biotechnology, Waltham, MA, USA). Films were digitalized and the intensity of the bands was quantified using the ImageJ software.

2.9. Statistical analysis

Data are expressed as mean ± SEM. Differences were assessed using *t*-test and one-way ANOVA followed by Tukey post-hoc test. All analyses were performed with the GraphPad Prism 6.0 statistical package (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Characterization of extracts

We previously produced and characterized a Calafate extract that showed anti-inflammatory properties *in vitro* (Reyes-Farias et al., 2015, 2016). However, to pursue an *in vivo* evaluation we required an extract lacking the compounds that could interfere with a diet-induced obesity mouse model, such as carbohydrates. To this end we prepared a polyphenol-purified Calafate extract (PPCE) and evaluated its antioxidant activity as well as polyphenol and carbohydrate content (results are summarized in Table 1). The PPCE showed a reduction in total polyphenols and anthocyanins when compared to the raw extract (2233 ± 6.39 and 3879 ± 7.97 mg GAE/100 g respectively for total polyphenols and 66 ± 0.30 and 74 ± 0.30 mg C-3-GE/100 g respectively for total anthocyanins). Interestingly, our PPCE showed a higher antioxidant activity when compared to the raw extract (38.44 ± 0.08 and 30.73 ± 0.04 mmol Fe^{2+}/100 g for PPCE and raw extract, respectively), suggesting successful purification of calafate polyphenols. In contrast to the raw extract, we did not detect carbohydrate content in our PPCE (130.3 ± 2.44 mg/mL for the raw extract and 0 for our PPCE). We evaluated 2 month old frozen PPCE for total polyphenols, without observing differences to the fresh samples (data not shown).

We next sought to determine the polyphenol composition of the PPCE. To this end, we performed liquid chromatography coupled to mass spectrometry (LC-MS) to identify the main anthocyanins present on the PPCE. The anthocyanin identification was performed by comparing the *m/z* signals and fragment ions of the anthocyanin pattern obtained for the calafate extract. The PPCE most abundant compounds were identified as delphinidin-3-galactoside and delphinidin-3-glucoside (Fig. 1 *m/z*: 487.3 and 465.3, peak 6), followed by petunidin-3-galactoside (Fig. 1 *m/z*: 479.1, peak 10) and malvidin-O-coumaroyl-glucoside (Fig. 1 *m/z*: 639.3, peak 7).

3.2. CD increases body weight and impairs glucose tolerance in mice

Mice were feed with CD for 10 weeks before starting the PPCE supplementation. As expected, when compared to the chow group, the CD group showed a significant increase in body weight starting from the 8th week of CD feeding (Fig. 2A, 28.9 ± 0.47 g CD and 27.3 ± 0.37 g for the chow group). A glucose tolerance test performed after 9 weeks of CD feeding revealed impaired glucose tolerance in the CD group evidenced by increased blood glucose levels at 30 and 60 min after glucose loading (Fig. 2B, 347.2 ± 13.2, 267.9 ± 12.7 and 248.7 ± 11.2, 196.6 ± 17.4, respectively) and by increased area under the curve relative to chow feed controls (Fig. 2C, 33,024 ± 1208 and 28,070 ± 1427, respectively). Taken together, these results show that before starting the PPCE supplementation, the mice fed with CD showed increased body weight and impaired glucose tolerance.

3.3. Calafate extract prevents the increase of body weight in animals fed with cafeteria diet

Mice feed with cafeteria diet for 10 weeks were randomly divided into 2 groups. A group remained on cafeteria diet without any further intervention (CD), and a group received our PPCE in the drinking water for 4 weeks without interrupting the access to cafeteria diet (CD + PPCE group). An increase in caloric intake was observed in the

Table 1
Total Polyphenolic, anthocyanin content and antioxidant capacity of Calafate.

	Raw calafate extract	Pure calafate extract
Total polyphenols (mg GAE/100 g DW)	3879 ± 7.97	2233 ± 6.39[a]
Total anthocyanins (mg C-3-GE/100 g DW)	74 ± 0.30	66 ± 0.30[a]
Antioxidant activity (mmol Fe^{+2}/100 g DW)	30.73 ± 0.04	38.44 ± 0.08[a]
Carbohydrates (mg/ml)	130.3 ± 2.44	0

Samples were analyzed in triplicates. Values are expressed as average ± SEM. Humidity percentage for calafate was 4%. GAE: gallic acid equivalents, C-3-GE: Cyanidin-3-glucoside equivalents, DW: dry weight. T-test was used to determine statistical significance, a = $p < 0.001$.

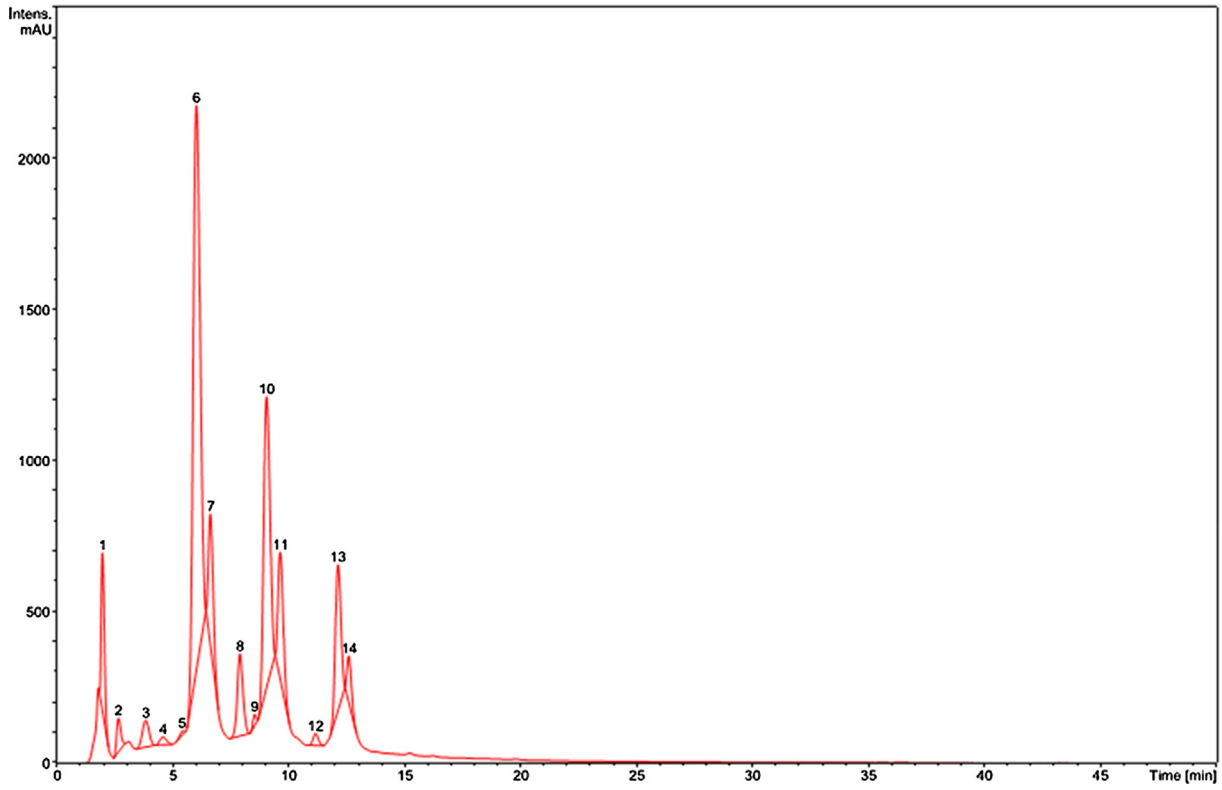

Fig. 1. HPLC UV chromatogram of PPCE detected at 520 nm. The anthocyanins identified in the PPCE are: peak 1: petunidin-*O*-p-coumaroyl-*O*-glucoside or malvidin-3-*O*-galactoside, peak 2: delphinidin-3,5-*O*-diglucoside, peak 3: petunidin-3-*O*-galactoside-5-*O*-glucoside or cyanidin-3-rutinoside, peak 4: petunidin-3-*O*-glucoside, peak 5: malvidin-3-galactoside or peonidin-3-glucoside, peak 6: delphinidin-3- glucoside or delphinidin-3- galactoside, peak 7: malvidin-*O*-coumaroyl-glucoside, peak 8: cyanidin-3-glucoside, peak 9: cyanidin-3-rutinoside, peak 10: petunidin-3-glucoside or petunidin-3-galactoside, peak 11: petunidin-3-*O*-rutinoside, peak 12: peonidin-3-glucoside, peak 13: malvidin-3-glucoside or malvidin-3-galactoside, peak 14: malvidin-3-rutinoside.

CD group relative to chow feed controls, whereas water consumption was reduced (Fig. 3A and B, CD = 3.8 ± 0.2 kcal; Chow = 3.3 ± 0.2 kcal and CD = 3.6 ± 0.2 mL; Chow = 4.3 ± 0.6 mL respectively). Similarly, the CD + PPCE group showed increased the caloric intake and reduced water consumption relative to both CD and chow feed control groups (Fig. 3A and B, CD + PPCE = 4.3 ± 0.3 kcal; CD = 3.8 ± 0.2 kcal and CD + PPCE = 3.4 ± 0.4 mL; CD = 3.6 ± 0.2 mL respectively). Interestingly, despite increased caloric intake, the CD + PPCE showed a stagnation of body weight (Fig. 3C) and a reduction in weight gain during the 4 weeks of PPCE treatment relative to the weight gain observed in the CD group, the weight gain observed for the CD + PPCE was similar to the weight gain observed for the chow-fed group (Fig. 3D, 0.3 ± 0.3 g for the chow, 0.4 ± 0.6 g for the CD + PPCE, and 3.0 ± 0.5 g for the CD group).

To understand the differences in weight gain observed for the CD and CD + PPCE groups, tissues were dissected and weighted. Although the epididymal, retroperitoneal and subcutaneous white adipose tissues (WATs) weight increased in the CD and CD + PPCE groups relative to chow feed controls (Fig. 4A), the weight increase for epididymal and retroperitoneal WATs was significantly diminished by the 4 week PPCE treatment relative to the tissue weights observed in the CD group (Fig. 4A, CD + PPCE = 27.3 ± 2.2 mg/g; CD = 43.5 ± 3.4 mg/g and CD + PPCE = 7.9 ± 0.9 mg/g; CD = 13.2 ± 0.8 mg/g respectively). Interestingly, an increase in interscapular brown adipose tissue weight was observed in the CD + PPCE group relative to both CD and chow groups (Fig. 4A, CD + PPCE = 4.7 ± 0.3 mg/g; CD = 3.4 ± 0.2 mg/g and Chow = 3.4 ± 0.2 mg/g). A histological analysis of epididymal white adipocytes revealed an increase in adipocytes size in the CD and CD + PPCE groups relative to chow feed controls, however the adipocytes size increase was significantly

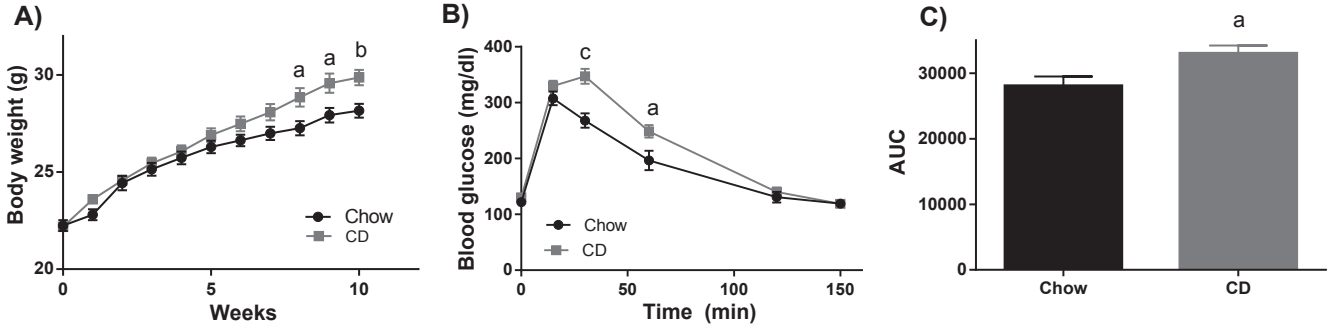

Fig. 2. CD increases body weight and impairs glucose tolerance in mice. (A) Body weights registered for mice fed with chow (n = 16) or CD (n = 27). (B) Glucose tolerance test performed after 9 weeks of CD feeding and (C) area under the curve for the glucose tolerance test (Chow: n = 13; CD: n = 27). Data are presented as mean ± SEM. T-test was used to determine statistical significance, a $p < 0.05$, b $p < 0.01$, c $p < 0.001$.

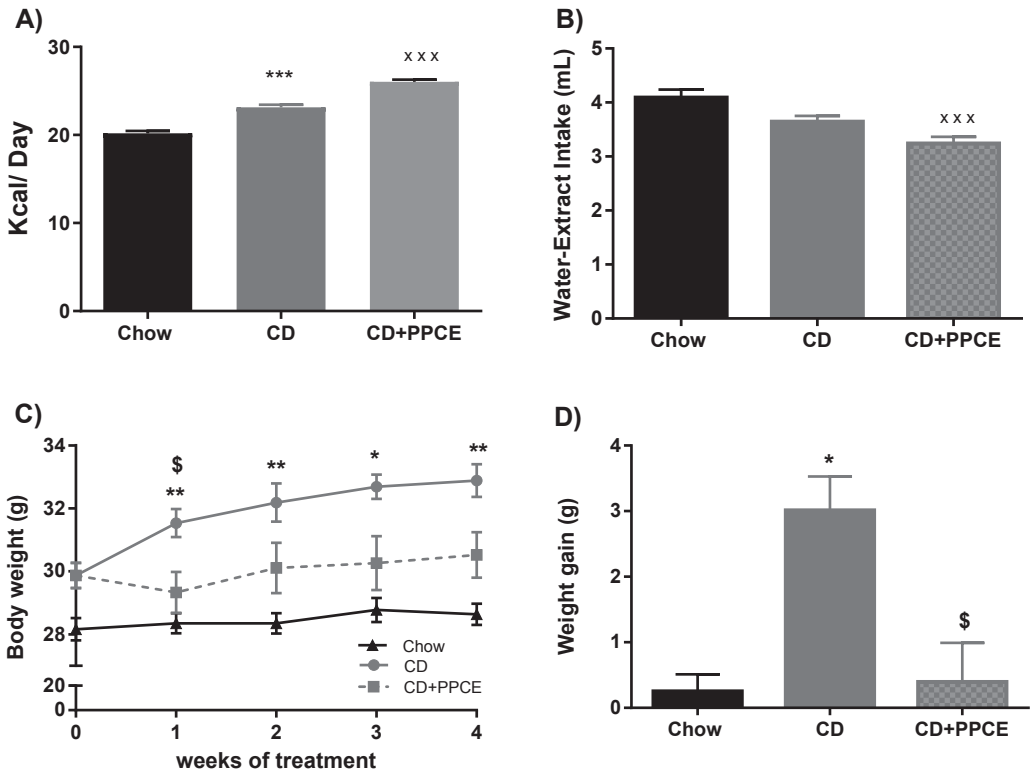

Fig. 3. PPCE attenuates the body weight increase induced by CD. (A) Food and (B) water-extract intake for each experimental group. (C) Body weights and (D) cumulative weight gain for mice fed with CD and treated with PPCE for 4 weeks. Data are presented as mean ± SEM. Chow (n = 16), CD (n = 10) y CD + PPCE (n = 13). Significances were obtained by one way-ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ CD versus Chow; $ $p < 0.01$ CD versus CD + PPCE and xxx $p < 0.001$ CD + PPCE versus Chow).

diminished by PPCE treatment (Fig. 4C and D, CD + PPCE = 17878,1 ± 669 μm^2; CD = 19784,1 ± 723 μm^2 and chow = 10407,7 ± 592 μm^2). These data suggest that PPCE contributes to body weight balance by diminishing the fat reservoir.

3.4. Calafate extract rescues glucose tolerance and inhibits the expression of inflammatory markers in mice fed a diet of cafeteria

In order to determine a possible role for our PPCE in restoring glucose homeostasis, we performed a glucose tolerance test after 3 weeks of PPCE treatment (13 weeks of cafeteria diet feeding). The glucose tolerance test revealed increased blood glucose levels after 30 and 60 min post glucose loading for the CD group relative to the chow group, similarly the area under the curve was significantly increased in the CD group. Interestingly, the CD + PPCE group showed a glucose tolerance curve similar to that of chow feed controls and the area under the curve was recovered to the control level (Fig. 5A and B, CD + PPCE = 31190,3 ± 1207 mg/dL*min; CD = 37850,2 ± 3096 mg/dL*min and chow = 28954,6 ± 799 mg/dL*min). Interestingly, in a parallel experiment in which mice received the PPCE supplementation continuously in conjunction with CD for 12 weeks did not show a recovery of glucose tolerance (supplementary Fig. 1), suggesting that the use of PPCE does not prevent the CD-induced alteration of glucose tolerance.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jff.2020.103856.

To gain a further insight into the mechanism by which PPCE contributes to the recovery of glucose tolerance, we evaluated insulin-induced AKT phosphorylation. The insulin-induced AKT phosphorylation observed in the control group was abolished in the CD group and completely restored by PPCE treatment (Fig. 5C and D). Taken together, our data suggest an insulin sensitizing role for the PPCE that might contribute to the recovery of glucose tolerance observed in the CD + PPCE group.

Finally, to explore a possible mechanism by which our PPCE contributes to insulin signaling and glucose homeostasis, we evaluated the inflammatory marker TNF-alpha and the macrophage marker F4/80 in WAT. As expected, both markers were augmented in the CD group relative to controls (Fig. 6A and B). Interestingly, PPCE treatment attenuated the increase in TNF-alpha and decreased significantly the F4/80 levels relative to the CD group (Fig. 6B, CD + PPCE = 1.7 ± 0.3 AU and CD = 2.4 ± 0.4 AU; $p < 0.05$).

4. Discussion

Natural compounds with the potential to act as palliatives for high-prevalence diseases are of great interest due to reduced side effects (Leong et al., 2014). Here we evaluated a natural source of polyphenols, the calafate, as a complementary tool against diet-induced insulin resistance in a preclinical model. Our PPCE represents a simple polyphenol purification that lacks complex processing. Despite showing diminished level of polyphenols in comparison to the raw extract, the PPCE showed higher antioxidant capacity, this might indicate that some polyphenols were lost during the filtering process. However, its efficacy represented in antioxidant capacity, was enhanced. The polyphenol composition of the PPCE is similar to that observed elsewhere for Calafate, corroborating the successful polyphenol purification (Ramirez et al., 2015; Ruiz et al., 2014). The most abundant polyphenols found in Calafate extracts are anthocyanins (Reyes-Farias et al., 2015; Ruiz et al., 2014). Our group previously characterized the anthocyanin content of a raw Calafate extract finding delphinidins and cyanidins as the principal anthocyanins families (Reyes-Farias et al., 2015). Here we identified delphinidin-3-galactoside and delphinidin-3-glucoside as the most abundant compounds of the PPCE, these observations are in accordance to previous published work (Reyes-Farias et al., 2015).

Carbohydrates have been shown to predispose to anxiety and increase adiposity and serum leptin levels (Santos et al., 2018). Therefore, our major objective when preparing the PPCE was to avoid the metabolic and behavioral interferences that could arise from the high carbohydrate content detected in the raw extract. This should be kept in mind when understanding and/or comparing polyphenol-rich fruits extracts.

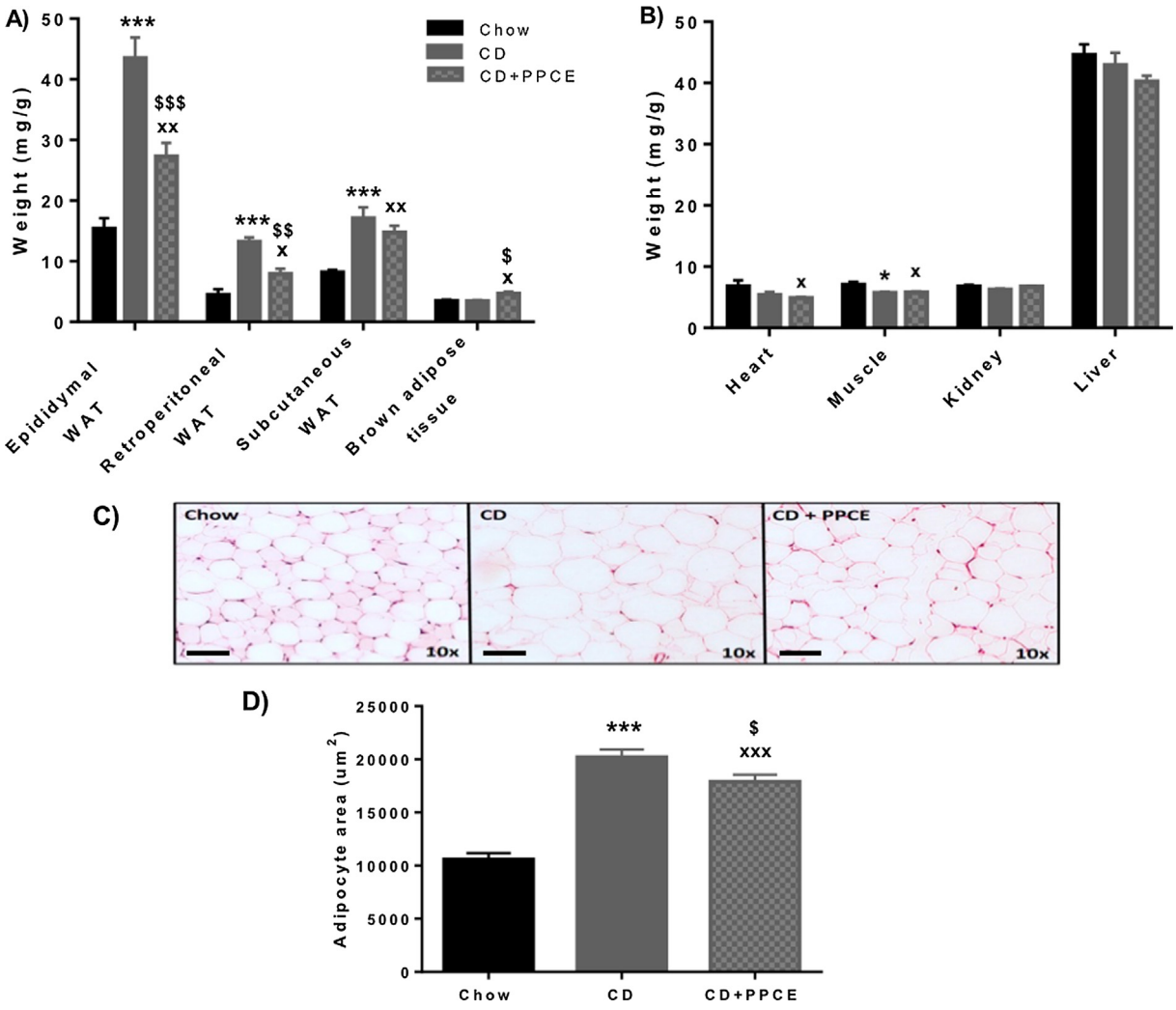

Fig. 4. Tissue weights and adipocyte size. (A) Weights for different types of fats and (B) tissues for each experimental group. The weights of tissues were normalized by total body weight for each mouse. (C) Hematoxylin and eosin staining of paraffin-embedded epididymal white adipose tissue. (D) Quantification of epididymal adipocytes size. Data are presented as mean ± SEM. Chow (n = 8), CD (n = 6) y CD + PPCE (n = 13). Photomicrographs were obtained at 10× magnification. A 100 μm calibration bar is included. Significance was obtained by one way-ANOVA (*p < 0.05, ***p < 0.001CD versus chow; x p < 0.05, xx p < 0.01 and xxx p < 0.001CD + PPCE versus chow; $ p < 0.05, $$ p < 0.01 and $$$ p < 0.001CD + PPCE versus CD).

The cafeteria diet has been previously used to study obesity and obesity-associated metabolic alterations (Higa, Spinola, Fonseca-Alaniz, & Evangelista, 2014). Our diet-induced obesity model showed physiological parameters that represent this modern pandemic. First we showed that, as previously reported (Sampey et al., 2011), CD-feed mice became hyperphagic, leading to an increase in body weight. Moreover, mice feed with our CD showed altered glucose tolerance and impaired insulin-induced AKT phosphorylation. All these parameters validate our model of diet-induced obesity associated to altered glucose homeostasis.

It has been reported that cafeteria diet and high fat diet feeding induces leptin resistance, driving an increase in food intake (Illesca et al., 2019). We observed that the CD and CD + PPCE groups presented increased food intake relative to chow controls, suggesting that our diet-induced obesity model developed leptin resistance and that PPCE treatment was unable to revert this. Further experimental work must be performed to unveil the role of the PPCE in leptin resistance. Despite increased food intake and continuous cafeteria diet feeding, the CD + PPCE group showed a stagnation of the increase in body weight induced by the cafeteria diet, suggesting a role for PPCE in weight balance. The reduction in adipose tissue weight and adipocyte size suggests that the effect of PPCE over weight balance is achieved by reducing the fat storage. However, it has been shown that polyphenols induce β-oxidation enzymes in the liver and WAT, suggesting that PPCE could also induce the mobilization and use of adipose tissue fatty acids, a point that remains to be evaluated (Fukuchi et al., 2008; Shimoda et al., 2009). It is also remarkable that the PPCE administration induced an increase in interscapular brown adipose tissue (BAT). BAT tissue has been shown to contribute to metabolic health (Silvester, Aseer, & Yun, 2019). Moreover, it has been shown that resveratrol exerts positive effects on BAT metabolism in mice, increasing BAT Ucp1 and Sirt1 gene expression (Andrade et al., 2014). Also, a green tea extract with high content in catechin-polyphenols and caffeine stimulates BAT thermogenesis (Dulloo, Seydoux, Girardier, Chantre, & Vandermander, 2000); suggesting that PPCE administration could act not only by reducing adiposity but also by increasing the BAT-mediated energy expenditure. It is worth to mention that we have now working on a model treated with high fat diet for a longer time (4 month), concomitantly with a Calafate treatment from the beginning of that treatment. In this setting, we were able to observe modifications in UCP-1 expression, as well in other thermogenic markers (data not shown). On the other hand, it has been shown that polyphenol administration ameliorates the high fat diet-induced alterations of glucose homeostasis (Anhe et al., 2017). Similarly, 4-week PPCE administration restored glucose tolerance of the

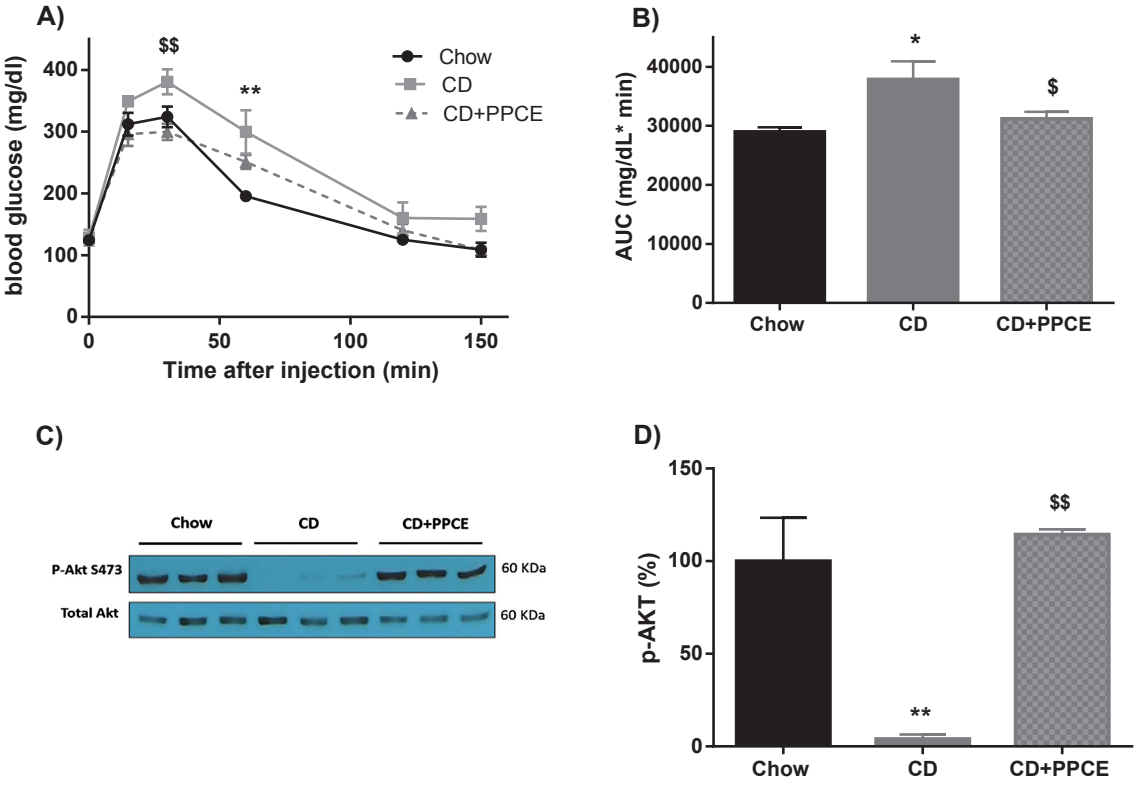

Fig. 5. Calafate extract rescues glucose tolerance and AKT phosphorylation in animals fed with CD. (A) Glucose tolerance test performed at 13 weeks of CD feeding for each experimental group (B) Area under the curve for the glucose tolerance test. Chow (n = 5), CD (n = 6) y CD + PPCE (n = 13). (C) Western blot bands for total and phosphorylated AKT (Ser 473) (D) Quantification for phosphorylated AKT/total AKT for each condition. Significance was obtained by one way-ANOVA (*p < 0.05, **p < 0.01CD versus Chow; $p < 0.05, $$p < 0.01CD versus CD + PPCE).

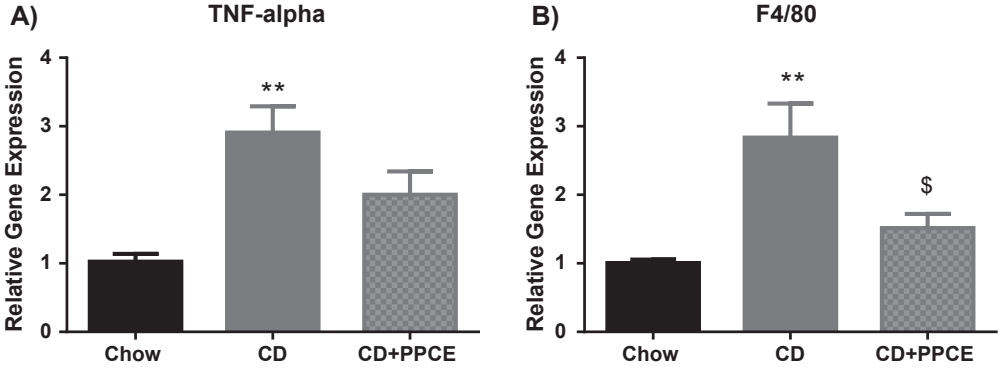

Fig. 6. Calafate extract decreases the expression of inflammatory genes. Relative gene expression for (A) TNF-alpha and (B) F4/80 for the CD and CD + PPCE experimental groups in relation to that of chow. Data are presented as mean ± SEM. chow (n = 5), CD (n = 6) y CD + PPCE (n = 10). Significant difference was obtained by one way-ANOVA (**p < 0.01CD versus chow; $p < 0.05CD versus CD + PPCE).

mice feed with cafeteria diet to levels similar to that of chow feed controls. On the other hand, mice that received PPCE continuously during the time that cafeteria diet was provided, in spite of a reduction in body weight did not recovered glucose tolerance. This dissimilarity suggests that the PPCE effect over body weight and glucose tolerance are not coupled and that the PPCE does not prevent the alterations leading to impaired glucose tolerance. It is also relevant to consider that a saturating effect of the PPCE over metabolic substrates arises in time. This might be relevant when assessing polyphenols as treatment for extended periods.

Polyphenols have been shown to counteract some hallmarks of insulin resistance. For instance, it has been reported that an apple polyphenol extract activates the translocation of the GLUT4 glucose transporter (Manzano et al., 2016). Moreover, previous works have suggested an insulin-sensitizating effect of calafate polyphenols *in vitro* (Reyes-Farias et al., 2016). To asses this possibility *in vivo*, we evaluated insulin-induced AKT phosphorylation in WAT of PPCE treated mice. The cafeteria diet reduced AKT phosphorylation to minimal levels, such effect has been reported previously for different tissues (Castro et al., 2013; Wu et al., 2016). Remarkably, PPCE administration fully recovered insulin-induced AKT phosphorylation, suggesting that Calafate polyphenols restore insulin sensibility in an *in vivo* model of diet-induced obesity.

PPCE attenuated the increase in the expression of the inflammatory markers TNF-alpha and F4/80 in WAT, suggesting the anti-inflammatory properties of polyphenols constitute a potential mechanism by through which the PPCE contributes to the restoration of insulin-elicited AKT phosphorylation and glucose tolerance recovery. Similar findings have been reported with hydroxytyrosol supplementation (Illesca et al., 2019), corroborating the WAT inflammatory state as a relevant metabolic target for polyphenols.

Polyphenols found in our PPCE, such as delphinidin-3-glucoside, have been shown to suppress lipid accumulation and to have an anti-adipogenic effect in cell cultures (Harada, Onoue, Inoue, Hanada, &

Katakura, 2018; Park, Sharma, & Lee, 2019), which is consistent with our *in vivo* observations. However, it has also been reported that polyphenols show a synergistic effect, attenuating the secretion of adipokines by hypertrophied 3T3-L1 adipocytes (Herranz-Lopez et al., 2012). Therefore, it remains a relevant challenge to determine whether the effects observed for the PPCE can be obtained by administering isolated compounds. Notwithstanding, our results show a remarkable effect of PPCE in a diet-induced obesity model, highlighting this berry as a valuable source of compounds with therapeutic potential.

5. Conclusion

Altogether, our results show that a simple polyphenol-pure extract from the Calafate berry attenuates the expression of pro-inflamatory markers and restores insulin-elicited AKT phosphorylation and glucose tolerance in a diet-induced obesity model; placing Calafate as a natural source of polyphenols that could be used for the treatment of insulin-resistance.

6. Ethics statement

All the experiments were approved by the Animal Ethics Committee of the University of Chile (Santiago, Chile), according to international guidelines (ARRIVE and EU Directive 2010/63/EU for animal experiments guidelines).

CRediT authorship contribution statement

Jessica Soto-Covasich: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Marjorie Reyes-Farias:** Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. **Rodrigo Torres:** Investigation, Writing - original draft, Writing - review & editing. **Karla Vasquez:** Methodology, Writing - review & editing. **Lissette Duarte:** Methodology, Writing - review & editing. **Javier Quezada:** Methodology, Writing - review & editing. **Paula Jimenez:** Conceptualization, Data curation, Writing - review & editing. **Maria Teresa Pino:** Conceptualization, Writing - review & editing. **Lorena Garcia-Nannig:** Conceptualization, Writing - review & editing. **Luis Mercado:** Conceptualization, Funding acquisition, Writing - review & editing. **Diego F Garcia-Diaz:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

Authors have no conflict of interest to declare

Acknowledgements

Soto Covasich J has a CONICYT Scholarship (Chile, 21120219) for his graduate studies at the PhD program in Biotechnology, Universidad Tecnica Federico Santa Maria-Pontificia Universidad Catolica de Valparaiso. This work was supported by FONDECYT Project 11110219 (CONICYT, Chile). Finally, authors thank the technical assistance of Francisca Echeverria (Department of Nutrition, University of Chile).

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