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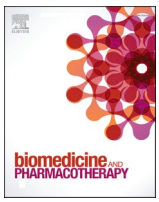

Anti-obesogenic effect of lupin-derived protein hydrolysate through modulation of adiposopathy, insulin resistance and gut dysbiosis in a diet-induced obese mouse

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ABSTRACT

The prevalence of obesity is increasingly widespread, resembling a global epidemic. Lifestyle changes, such as consumption of high-energy-dense diets and physical inactivity, are major contributors to obesity. Common features of this metabolic pathology involve an imbalance in lipid and glucose homeostasis including dyslipidemia, insulin resistance and adipose tissue dysfunction. Moreover, the importance of the gut microbiota in the development and susceptibility to obesity has recently been highlighted. In recent years, new strategies based on the use of functional foods, in particular bioactive peptides, have been proposed to counteract obesity outcomes. In this context, the present study examines the effects of a lupin protein hydrolysate (LPH) on obesity, dyslipidemia and gut dysbiosis in mice fed a high-fat diet (HFD). After 12 weeks of LPH treatment, mice gained less weight and showed decreased adipose dysfunction compared to the HFD-fed group. HFD-induced dyslipidemia (increased triglycerides, cholesterol and LDL concentration) and insulin resistance were both counteracted by LPH consumption. Discriminant analysis differentially distributed LPH-treated mice compared to non-treated mice. HFD reduced gut ecological parameters, promoted the blooming of deleterious taxa and reduced the abundance of commensal members. Some of these changes were corrected in the LPH group. Finally, correlation analysis suggested that changes in this microbial population could be responsible for the improvement in obesity outcomes. In conclusion, this is the first study to show the effect of LPH on improving weight gain, adiposopathy and gut dysbiosis in the context of diet-induced obesity, pointing to the therapeutic potential of bioactive peptides in metabolic diseases.

1. Introduction

Obesity is one of the most prevalent diseases worldwide, affecting more than 650 million people according to the World Health Organization. Its prevalence has tripled in the last 50 years and given that genetic variations over this period of time are not sufficient to explain this fact [1], changes in lifestyle have been proposed to be responsible for this increase [2]. Consumption of high-fat and

Abbreviations: AMOVA, Analysis of Molecular Variance; CLS, Crown-like structure; DIO, Diet-induced obesity; EpiWAT, epididymal adipose tissue; FAO, Food and Agriculture Organisation; HDL, High-density lipoprotein; HFD, High-fat diet; HOMA-IR, homeostatic model assessment for insulin resistance; LDL, Low-density lipoprotein; LEfSe, Linear discriminant analysis Effect Size; LPH, Lupin protein hydrolysate; NMDS, Non-metric Multidimensional Scaling; PBS, Phosphate buffered saline; PCoA, Principal Coordinate Analysis; PFA, Paraformaldehyde; rRNA, ribosomic ribonucleic acid; SCFA, Short-chain fatty acid; SD, Standard diet.

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high-sugar diets with increased palatability and reduced nutritional value, combined with physical inactivity, sleep disturbances, and drugs with weight gain as a side effect are the key environmental factors for the development of obesity [2,3].

This metabolic pathology usually leads to adipose tissue dysfunction, which is characterized by adipocyte hypertrophy, inflammation and changes in the profile of substances secreted by the tissue (i.e., adipokines) [4]. Obesity is also related to higher concentrations of serum lipids, such as triglycerides and free cholesterol, and induces changes in lipoproteins proportion with patients showing increased levels of low-density lipoproteins (LDL) and decreased concentrations of high-density lipoproteins (HDL) [5]. Furthermore, this condition has been associated with elevated levels of serum glucose and insulin due to the development of insulin resistance in different tissues [6]. All these features are important risk factors for other comorbidities, such as cardiovascular diseases [7], type II diabetes [8], fatty liver diseases [9] and some cancers [10].

In recent years, the relevance of the gut microbiota in the development and susceptibility to obesity and other metabolic pathologies has been highlighted. Given the importance of gut microbes in the digestive process, dietary variations are expected to result in modifications in the composition of the gut microbiota [11,12]. In this context, intestinal dysbiosis, an abnormal state of intestinal microbial populations, occurs after chronic changes in diet and environmental factors [13]. A reduction in ecological parameters such as richness and α-diversity has been reported in both obese humans and rodents [14]. In terms of population profile, obesity induces blooming of deleterious groups, including *Oscillibacter* and *Lachnoclostridium* [15,16], both belonging to the phylum *Bacillota*, as well as the reduction of beneficial microbial members such as *Akkermansia* or the *Lachnospiraceae NK4A136* group [17,18]. Interestingly, these alterations are not only a consequence of obesity but seem to play a causal role in its pathophysiology. In this line, gut microbial transplantation from obese to germ-free mice results in increased body weight gain in the recipients compared to those receiving microbiota from lean donors [19,20].

One of the approaches proposed to counteract the harmful effects of obesity is the use of functional foods that have several health benefits beyond their nutritional value [21]. In this regard, bioactive peptides from various sources (animal and vegetal) have been raised as functional ingredients due to their wide range of effects in a variety of biological processes. These encrypted peptides appear after the hydrolysis of a protein substrate and exert novel biological activities that are not present in the original proteins [22]. Although this process can be carried out by fermentation or using chemical compounds, enzymatic hydrolysis has been shown to be more efficient, reproductible, and less contaminant compared to other methods [23]. Some of these biological effects exhibited by bioactive peptides are anti-inflammatory, hypoglycaemic or lipid-lowering activities. Although some of the molecular pathways involved in these effects remain unclear, various mechanisms have been clearly defined such as the inhibitory capacity of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity [24]. In particular, peptides generated after enzymatic hydrolysis of protein isolates from lupin seed (*Lupinus angustifolius*) have been widely studied over the last years showing beneficial effects in several pathological processes [25]. Lupin is a member of the *Fabaceae* family, which is characterized by a high proportion of protein and fibre as well as a high concentration of micronutrients in its seeds [26]. This plant can be grown in a wide variety of environments, which allows its production at a global scale, with Oceania and Europe being the two main producers according to the Food and Agriculture Organization of the United Nations (FAO). These considerations combined with the previous knowledge of the therapeutic potential of bioactive peptides make the study of lupin hydrolysate a topic of great biomedical interest.

In this context, our group has previously described the lipid-lowering, anti-inflammatory, and antioxidant effects of a lupin protein hydrolysate (LPH) obtained after enzymatic digestion using Alcalase 2.4 L under controlled conditions both *in vitro* [27] and *in vivo* [28,29]. To the best of our knowledge, no lupin hydrolysate has shown beneficial effects in obesity or dysbiosis. In light of these considerations, the main objective of this work was to evaluate the effects of LPH consumption on the development of obesity, as well as the evaluation of the role of gut microbiota in a murine preclinical model of obesity induced by high-fat diet consumption.

2. Materials and methods

2.1. LPH synthesis and characterization

LPH was generated in the facilities of the Instituto de la Grasa (Consejo Superior de Investigaciones Científicas-CSIC, Seville, Spain) as previously described [27]. Briefly, lupin protein isolate was dissolved in distilled water containing Alcalase 2.4 L (2.4 AU/g; Novozymes, Bagsvaerd, Denmark) in a balance enzyme/substrate equal to 0.3 AU per gram of protein. Enzymatic hydrolysis was performed under controlled conditions (pH 8.0 at 50ºC for 15 min) followed by the enzyme inactivation by heating at 85ºC. The supernatant containing LPH was obtained by centrifugation at 10,437 g for 15 min and it was lyophilized and stored at room temperature.

In this dry matter, protein concentration, ashes, moisture, fiber, fats, soluble sugars and phenol content were measured as described elsewhere [27]. Amino acid composition was determined using the method developed by Alaiz *et al.* [30] using standard amino acid mix solution from Sigma (St. Louis, MO, EEUU). The molecular weight profile was obtained by molecular exclusion chromatography using a Superose 12HR 10/30 column (exclusion limit 2×10^6 Da and optimal separation range 1000–3×10^5 Da).

2.2. Animal and experimental design

Eight-week-old male C57BL/6N mice were housed under standard conditions (12/12 h light/dark cycles, temperature 22 ± 2°C and humidity <55 %) in the animal facilities of the Instituto de Biomedicina de Sevilla (IBiS) with free access to food and water. After two weeks of acclimatisation, mice were randomly assigned to three groups: mice fed a standard diet (SD), a high-fat diet (HFD) or a HFD and treated intragastrically with LPH (HFD+LPH) at a dose of 100 mg/kg, according to previous studies [28,29], five days a week for 12 weeks. The SD and HFD groups were treated with vehicle under the same conditions as the HFD+LPH group. In this study, 4 independent experiments with 5–6 mice per group were carried out. The composition and references of both diets are summarized in Supplementary Table 1. Food consumption and individual body weight were measured weekly. After 16 hours of fasting, the animals were sacrificed with an intraperitoneal injection of sodium thiopental (50 mg/Kg, B. Braun Medical SA, Barcelona, Spain) and blood was collected by cardiac puncture with heparin-treated syringes. The animals were then perfused with phosphate buffered saline (PBS) for 5 minutes using an FH100 peristaltic pump (Thermo Scientific, Vantaa, Finland). Epididymal adipose tissue (EpiWAT) was dissected, snap-frozen and stored at −80ºC until use or fixed in a 4 % paraformaldehyde (PFA) solution. All experimental procedures were approved by the Ethics Committee of the Virgen Macarena-Virgen del Rocío University Hospital and were conducted under Spanish legislation and the EU Directive 2010/63/EU for animal experiments (reference number 16/03/2023/004).

2.3. Serum biochemical profile

Blood from cardiac puncture was centrifuged at 3000 g for 15 min at 4ºC to collect serum samples that were aliquoted and stored at −80ºC until use. Cholesterol, HDL, LDL, triglycerides and glucose concentrations were measured by chemoluminiscence in the Cobas Integra 400 (Roche Diagnostics, Indianapolis, IN, USA) at the Estación Biológica de

Doñana (EBD-CSIC, Seville, Spain).

2.4. Adipokines and insulin quantification

The serum concentrations of leptin, adiponectin and insulin were quantified using enzyme-linked immunosorbent assays (ELISA) kits from Biovendor (RD291001200R; RD293023100R) and Mercodia (10–1247–01), respectively, following the manufacturer's instructions.

2.5. Histological analysis

Adipose tissue samples fixed in 4 % PFA were dehydrated, embedded in paraffin blocks and sliced 4 μm thick. Slides were stained with hematoxylin-eosin (PanReac AppliChem, Barcelona, Spain) and cover mounted for posterior observation. Photomicrographs were obtained using Leica THUNDER microscope (Leica) and Leica Application Suite X software (Leica Microsystems). Adipocyte morphology (size and number) and number of crown-like structures (CLS) was blinded analyzed using ImageJ v1.53 h public software (NIH, Bethesda, MD, USA). Data were collected from an average of 100 adipocytes for morphology and ~60 mm^2 of adipose tissue for CLS quantification and two slices per mouse and tissue.

2.6. High-throughput 16 S rRNA gene amplicon analysis

Before euthanasia, stool samples were collected and stored at −80ºC until use. Total DNA was extracted using the E.Z.N.A.® Stool DNA Kit (Omega BIO-TEK) following the manufacturer's instructions. DNA quantity and quality were evaluated using the Qubit Fluorometer (Life Technologies). Amplicon-sequencing targeting of the 16 S rRNA gene was performed at the Genomics Core Facility of the IBiS. The amplicon libraries were prepared using the primer of the V3–V4 region according to the 16 S metagenomic sequencing library preparation protocol (Part # 15044223 Rev. B). The libraries were pooled at 8 pM concentrations before loading onto the MiSeq (Illumina, San Diego, CA, USA) using the Reagent Kit v3 (300 cycles) in paired-end mode, with 25 % PhiX added for clear cluster separation.

The raw sequences were processed using mothur [31]. The reads were trimmed based on quality scores and aligned with the SILVA 138.1 database [32]. Chimeric sequences were removed using the VSEARCH algorithm [33] and reads classified as "eukaryote", "mitochondria", "chloroplast" or "unknown" were removed. Operational taxonomic units (OTUs) were clustered at 97 % of sequence similarity and the final taxonomic identification was assigned with the SILVA 138.1 database. Ecological index (Shannon and Sobs), and dissimilarity between communities (Jaccard index) were calculated using mothur subroutines. Differential abundance analysis was performed using the Linear discriminant analysis Effect Size (LEfSe) algorithm [34].

2.7. Statistical analysis

Statistical analysis was performed using SPSS® Stat Software v26 (IBM Corporation, Armonk, NY, USA). Data were represented as the mean and standard error of the mean (SEM) or with box-and-whiskers plot showing median, interquartile range and maximum and minimum values. Graphs were designed using GraphPad Prism v8 software (GraphPad Software, Boston, MA, USA). For discriminant analysis, morphometrical parameters (body weight, adipose tissue weight) and serum lipids (cholesterol, triglycerides, LDL, HDL) were used to create canonical discriminant functions. Differences among groups were evaluated using parametric one-way analysis of variance (ANOVA) and Tukey's multiple comparison test was used to compare each experimental group. Spearman's rank correlation coefficients were calculated to create a correlation matrix between the gut microbiota profile and obesity markers. Differences with values of $p \leq 0.05$ were considered statistically significant.

3. Results

3.1. LPH has peptides with potential bioactivity and shows optimal amino acid composition

Chemical characterization of LPH showed that protein was the mayor compound (79.56 ± 0.03 %), followed by ashes (11.42 ± 0.13 %), moisture (6.63 ± 0.18 %), fiber (4,46 ± 0.62 %), fats (1.93 ± 0.01 %) and residual quantities of soluble sugars and phenols (less than 1.0 %) (Fig. 1A). Molecular weight distribution of peptides revealed high proportion of small peptides (1–5 kDa) representing 45.56 % of total peptides. Medium-size (5–50 kDa) and long peptides (50 to >100 kDa) composed 30.43 % of LPH (Fig. 1B and C).

Quantification of amino acid composition is shown in Table 1. LPH presented all essential amino acids i.e., leucine (8.54 ± 0.55 %), phenylalanine (5.59 ± 0.45) and its non-essential derivate tyrosine (5.77 ± 0.18), lysine (5.17 ± 0.07), threonine (4.53 ± 0.32), valine (4.18 ± 0.12), isoleucine (4.12 ± 0.46), histidine (1.68 ± 0.12), methionine (0.89 ± 0.02) and tryptophan (0.81 ± 0.01).

3.2. LPH treatment reduces body weight gain and adiposopathy without affecting energy intake in diet-induced obese mice

After 12 weeks of HFD consumption, mice showed a significant increase in final body weight and body weight gain compared to SD-fed mice (Fig. 2A–C). LPH supplementation significantly reduced the obesogenic effect of HFD from week seven of intervention onward (Fig. 2A), without affecting food or energy consumption (Fig. 2E and F).

Compared to SD mice, high-fat diet consumption significantly increased EpiWAT weight depots and adiposity index (referred to as the ratio of EpiWAT weight to body weight) (Fig. 3A–C). This increase was ameliorated after LPH treatment. Histological analysis revealed important dysfunction of adipose tissue with presence of hypertrophic adipocytes and high frequency of CLS, an important inflammatory trait, in the HFD group (Fig. 3E–I). Animals supplemented with LPH showed a decrease in both the adipocyte diameter and frequency of larger adipocytes and CLS, suggesting a protective role of this hydrolysate in adiposopathy.

3.3. LPH supplementation ameliorates dyslipidemia, insulin resistance and restores adipokine profile

Serum analysis revealed an important dyslipidemia in HFD-induced obese mice, with significant increase in plasma cholesterol, triglycerides, LDL, HDL and LDL to HDL ratio compared to SD-fed mice (Fig. 4A–E). Treatment with LPH significantly counteracted the diet-induced increase in cholesterol, triglycerides, LDL, HDL and the LDL/HDL ratio, improving in lipid homeostasis.

Regarding glucose homeostasis, animals fed a high-fat diet had a higher glucose and insulin concentration in serum (Fig. 5A and B). Although the HFD+LPH group showed similar glucose levels, the insulin concentration was significantly reduced compared to the HFD mice. Furthermore, higher values of homeostatic model assessment for insulin resistance (HOMA-IR), associated with a worsening of insulin resistance, were found in the HFD group, while LPH-treated animals showed a 2-fold reduction in this index (Fig. 5C). These differences suggest an improvement in insulin sensitization after LPH treatment.

Systemic levels of adiponectin and leptin, two of the most biologically relevant adipokines, were also measured. Adiponectin concentration showed no significant differences among groups, being slightly lower in the HFD group (Fig. 5D). Nevertheless, leptin level and leptin/adiponectin ratio were dramatically increased in HFD-fed animals when compared to SD group (Fig. 5E and F). LPH supplementation counteracted both values, which indicates an improvement in adipose tissue function.

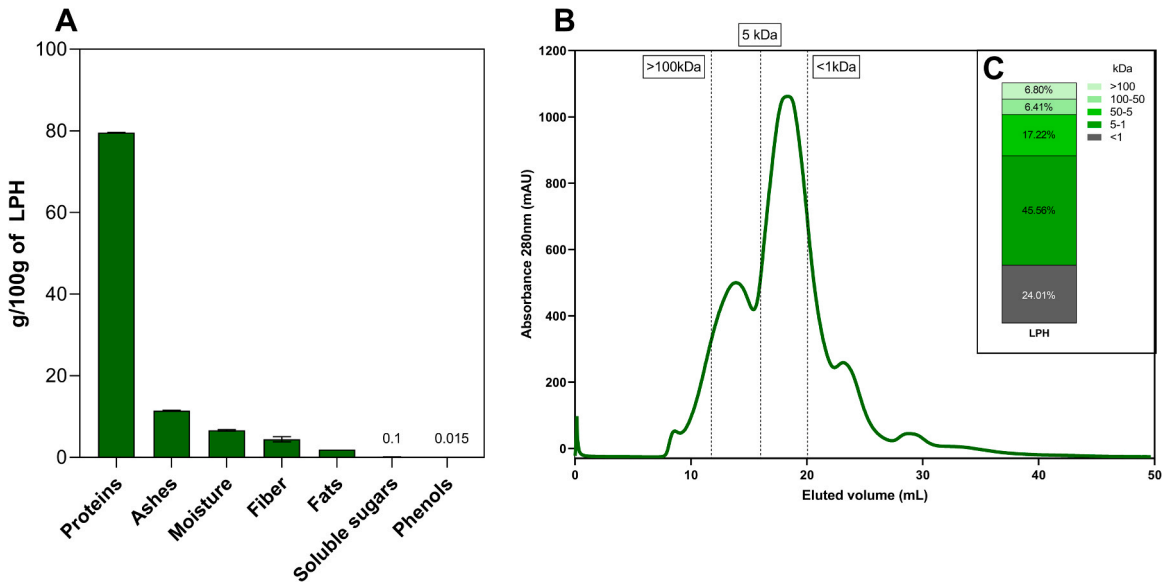


Fig. 1. Chemical composition and molecular weight distribution of LPH. Chemical composition of LPH (A). Molecular Weight distribution of LPH peptides (B). Percentage of peptides according to their molecular weight (C). All values are shown as the mean of three independent measurements and the standard deviation.

Table 1
Amino acid composition of LPH. Each amino acid is labelled with 3-letters and 1-letter code (between brackets). Measurements showed mean and standard deviation of grams of each amino acid per 100 g of LPH. [1]Recommended proportion of essential amino acids per 100 g of proteins according to the Food and Agriculture Organization of the United Nation (FAO) guidelines.[2] Tyr is derived from phenyalanine.

Amino acid	Amount (g/100 g)	FAO[1]
Essential amino acids		
Leu (L)	8.54 ± 0.55	5.9
Phe (F)	5.59 ± 0.45	3.8[2]
Lys (K)	5.17 ± 0.07	4.5
Thr (T)	4.53 ± 0.32	2.3
Val (V)	4.18 ± 0.12	3.9
Ile (I)	4.12 ± 0.46	3.0
His (H)	1.68 ± 0.12	1.5
Met (M)	0.89 ± 0.02	1.6
Trp (W)	0.81 ± 0.01	0.6
Non-Essential amino acids		
Glu (E) + Gln (Q)	21.74 ± 0.90	
Arg (R)	12.50 ± 0.37	
Asp (D) + Asn (N)	7.43 ± 1.77	
Ser (S)	6.92 ± 0.62	
Tyr (Y)[2]	5.77 ± 0.18	
Gly (G)	4.74 ± 0.45	
Ala (A)	4.44 ± 0.09	
Pro (P)	0.56 ± 0.19	
Cys (C)	0.38 ± 0.04	0.6

3.4. Discriminant analysis differentially distributed LPH-treated mice compared to HFD-fed mice

Due to the variability described in obesity mouse models, a discriminant analysis was performed to check the segregation between the three experimental groups. A differential clustering of the three groups was shown (Fig. 6A). This analysis revealed that 100.0 % of animals in the SD group were predicted to belong to this group and 83.3 % of the HFD mice were clustered in its experimental group (Fig. 6B). Regarding LPH-treated animals, 65.2 % of animals were predicted to be part of the LPH group, 21.7 % were found in the HFD group and 13.0 % in the SD group.

3.5. LPH consumption alters the microbiota composition in mice fed an obesity inducing diet

The number of observed species (Sobs) showed a significant reduction of microbial richness in those mice fed an HFD, while LPH supplementation tended to increase this value (Fig. 7A). α-Diversity, represented as Shannon's Index, did not show changes among the three experimental groups (Fig. 7B). Regarding the composition of the microbiota, PCoA showed three different groups with significantly different molecular variance (AMOVA p-value<0.001) (Fig. 7C). NMDS analysis also showed the differential clustering of the three experimental groups (Fig. 7D).

Phyla analysis demonstrated significant changes in HFD-fed mice compared to the SD-fed group, some of which were recovered in the HFD+LPH group (Fig. 7E). Phyla are shown as follows the abundance in the SD group: *Bacillota* (formerly known as *Firmicutes*), *Bacteroidota, Bacillota to Bacteroidota* ratio, *Verrucomicrobiota, Pseudomonadota* (formerly known as *Proteobacteria*), *Cyanobacteriota, Patescibacteria, Actinomycetota* (formerly known as *Actinobacteria*), *Thermodesulfobacteriota* and *Deferribacterota* (Fig. 7F–O). In particular, HFD consumption increased the abundance of *Patescibacteria* and *Thermodesulfobacteriota,* reduced *Cyanobacteria,* and tended to reduce *Verrucomicrobiota* levels (p=0.071). LPH supplementation restored abundances of *Patescibacteria* and *Thermodesulfobacteriota* and significantly increased *Verrucomicrobiota* when compared to the HFD group. LPH-treated mice also showed a tendency to increase *Pseudomonadota*. In addition, HFD diet induced a non-significant increase in the abundance of *Bacillota* and the *Bacillota* to *Bacteroidota* ratio compared to SD, which was partially restored after LPH treatment. No differences in *Actinomycetota* and *Deferribacterota* were observed.

The Linear discriminant analysis Effect Size (LEfSe), which determines the taxa that explain differences between groups, showed 26 different taxa with a LDA score >3 among the three experimental conditions (Fig. 8). The analysis, summarized in the table within Fig. 8, revealed that HFD-induced changes in the abundance of *Oscillospiraceae_unclassified, Oscillibacter, Candidatus_*Saccharimonas*, Ruminococcaceae_unclassified, Desulfovibrio,* and *Akkermansia* were reversed by LPH treatment. An increased abundance in *Dubosiella*, *Clostridium_sensu_stricto_1*, *Ileibacterium* and *Turicibacter* was also observed in LPH-treated mice compared to the HFD group.

With the aim of study the relationship between the gut microbiota

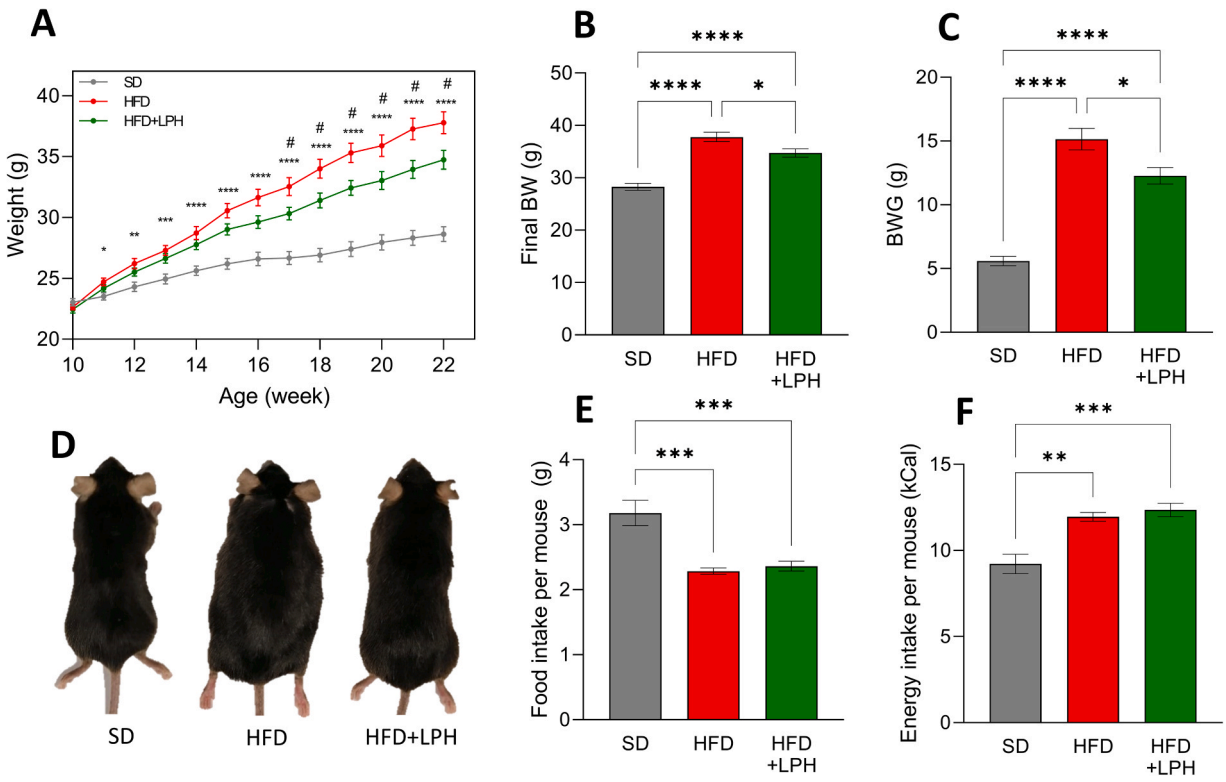


Fig. 2. Body weight and food intake analysis. Body weight recorded during the study (A). Final body weight (B). Body weight gain (BWG) after 12 weeks of LPH treatment (C). Representative image of a mouse in each group (D). Food intake per mouse (E). Energy intake per mouse calculated based on the kCal content of each diet (F).(n = 18 in SD group; n = 26 HFD in group; n = 24 in HFD+LPH group, from four independent experiments). All values are shown as the mean and the standard error of the mean of each group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. In A, * represents differences between SD and HFD groups, and # between HFD and HFD+LPH groups.

profile and obesity markers, the abundance of the 26 different taxa was correlated with the EpiWAT weight, the final body weight and the levels of plasma lipids, glucose, insulin and adipokines. A significant correlation was observed between EpiWAT weight, final body weight, serum lipid markers and leptin in most of the differentially distributed microbial groups (Fig. 9). Interestingly, those over-represented in HFD mice (marked in red) showed a positive correlation with these obesity features, while genus more abundant in SD and LPH groups (grey and green-labelled, respectively) negatively correlated with these markers.

4. Discussion

This work shows the beneficial effects of LPH on obesity. After 12 weeks of LPH treatment, mice gained less weight and had less adiposity compared to the HFD-fed group. A reduction in dyslipidemia, insulin resistance and improvement in adipokines profile associated with significant changes in the composition of the microbiota was also observed in the LPH group.

The molecular characterization of LPH revealed a high proportion of small peptides (1–5 kDa), which could potentially be responsible for the beneficial effects, as small-sized peptides have been associated with higher bioactivity and bioavailability [35]. In this context, the use of external enzymatic hydrolysis, which allows the generation of neo-peptides different from those generated by physiological digestion, has been suggested as a strategy to enhance the beneficial effects of lupin-derived products [22]. In fact, some studies using whole, non-hydrolysated, lupin protein or flour in hypercholesterolemic or overweight patients have not shown effects on body weight loss, waist circumference or body fat reduction [36,37]. Finally, according to FAO guidelines, LPH has optimal levels of essential amino acids, characteristic of high-quality proteins, with the exception of sulphur-containing amino acids (Met and Cys), which are inherently less abundant in legume species [38].

The present results in body weight gain demonstrated the obesogenic effect of HFD consumption. Interestingly, HFD-fed animals supplemented with LPH significantly reduced body weight gain. Moreover, no changes in food intake were observed between the HFD and LPH groups, suggesting physiological effects of lupin peptides independently of feeding control or appetite. This result is consistent with other studies using different protein hydrolysates derived from *Clupea harengus* [39], *Pelteobagrus fulvidraco* [40], *Gadus chalcogrammus* skin [41], *Spirulina platensis* [42] or synthetic dipeptides [43] in mouse models of diet-induced obesity (DIO).

Adipose dysfunction or adiposopathy is a common feature in obese subjects and is characterized by the presence of hypertrophic adipocytes, impaired adipose tissue expandability, altered lipid and glucose metabolism, and inflammation [4]. This study showed larger EpiWAT depots in HFD-fed mice, as well as bigger adipocytes and increase number of CLS compared to the SD group, which were counteracted after LPH supplementation. Although the role of LPH in adiposity has previously been observed in a transgenic mouse model of hypercholesterolemia without changes in body weight [29], this is the first study to show anti-obesogenic effects of a *Lupinus angustifolius* hydrolysate in a DIO mouse model associated with an improvement in adipocyte hypertrophy and inflammation, both important hallmark of obesity.

Dyslipidemia is a key outcome of metabolic diseases. In fact, imbalance in the LDL/HDL ratio is a major risk factor for cardiovascular diseases, one of the most important comorbidities in the obese population [44–46]. In this line, bioactive peptides with lipid-lowering properties have been proposed due to their activity on key enzymes of lipid metabolism, such as HMG-CoA reductase [24], without significant side effects [47]. Previously, we have described the lipid-lowering effects of

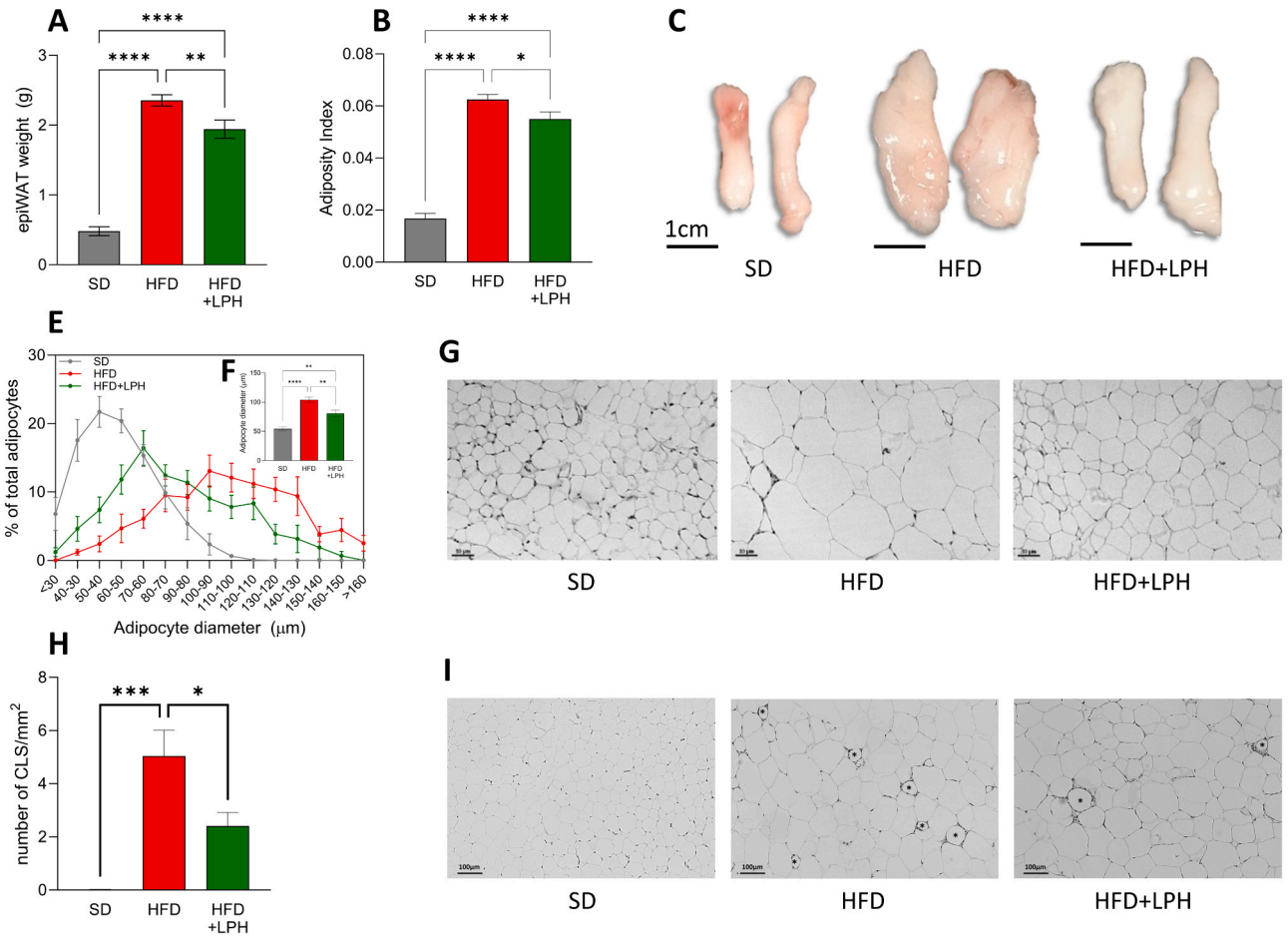


Fig. 3. Analysis of adipose tissue dysfunction. Epididymal white adipose tissue (EpiWAT) weight (A). Adiposity index defined as the coefficient between EpiWAT weight and body weight (B). (n = 18 in SD group; n = 26 HFD in group; n = 24 in HFD+LPH group, from four independent experiments). Representative images of both epididymal adipose depots (C). Distribution of total adipocytes based on the diameter (E). Mean adipocyte diameter (F). Representative microphotographs of hematoxylin and eosin of epiWAT (G). Number of Crown-like structures (CLS) per mm2 (H). Representative microfotographs showing CLS (I). (n = 6–10 per group, from two independent experiments). All values are shown as the mean and the standard error of the mean of each group. ns non statistical differences, * p<0.05; **p<0.01; ***p<0.001;****p<0.0001.

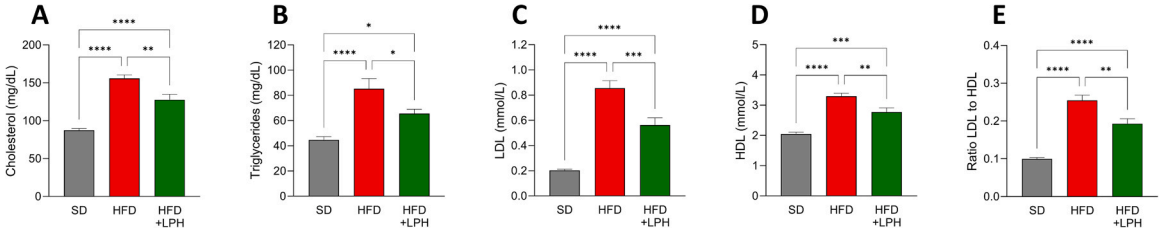


Fig. 4. Serum markers of dyslipidemia. Serum concentration of cholesterol (A), triglycerides (B), low-density lipoprotein (LDL) (C), high-density lipoprotein (HDL) (D) and the ratio between LDL and HDL (E). (n = 18 SD group; n = 24 HFD group; n = 23 HFD+LPH group, from four independent experiments). Data are shown as the mean and the standard error of the mean of each group. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

LPH in a mutant mouse model of atherosclerosis [28], with significant reductions in total cholesterol, LDL and triglycerides (-11.90 %, −10.06 %, −15.04 %, respectively). However, the magnitude of this reductions is greater in the present study (total cholesterol: −18.11 %, LDL: −33,26 % and triglycerides: −23.15 %). In addition, the data shown in the present manuscript are of special interest because they are associated with a reduction in body weight gain in a specific model of obesity in wild-type mice.

Insulin resistance is a failure to coordinate insulin signaling in tissues characterized by increased systemic glucose and insulin concentrations [48]. This pathological condition is a common feature of the obese population and leads to the development of different comorbidities [6]. In this work, no significant changes were detected in glucose concentration between treated and untreated HFD-fed animals. Considering that glucose levels are very dependent on the fed state, a possible explanation for this could be the fasted state of the animals when serum samples were collected. However, an increased value of HOMA-IR was detected in HFD group and counteracted after LPH supplementation. In this context, other bioactive peptides have shown insulin sensitization capacity leading to a reduction of serum insulin levels [49]. Although in previous study we and others have demonstrated the hypoglycemic capacity of lupin bioactive peptides through DDP-IV inhibition [50,51],

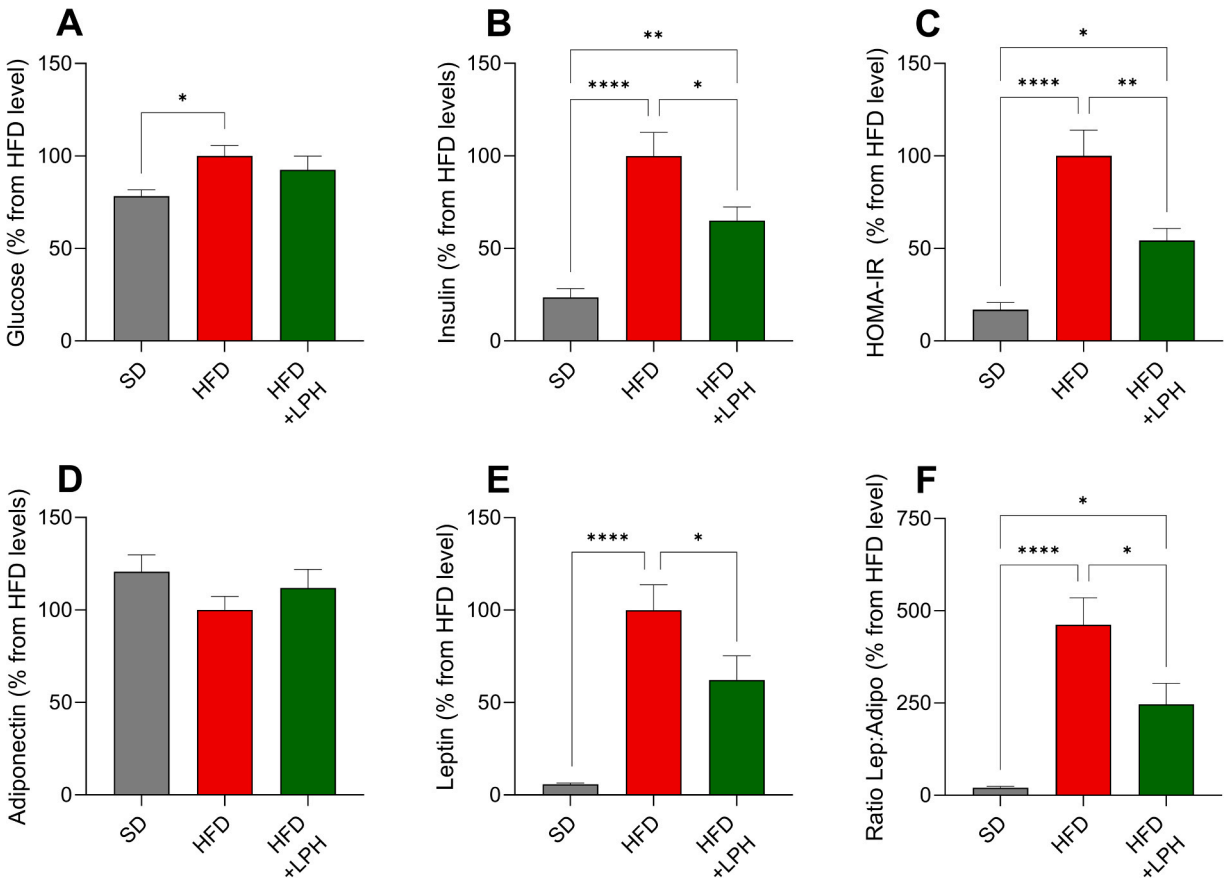


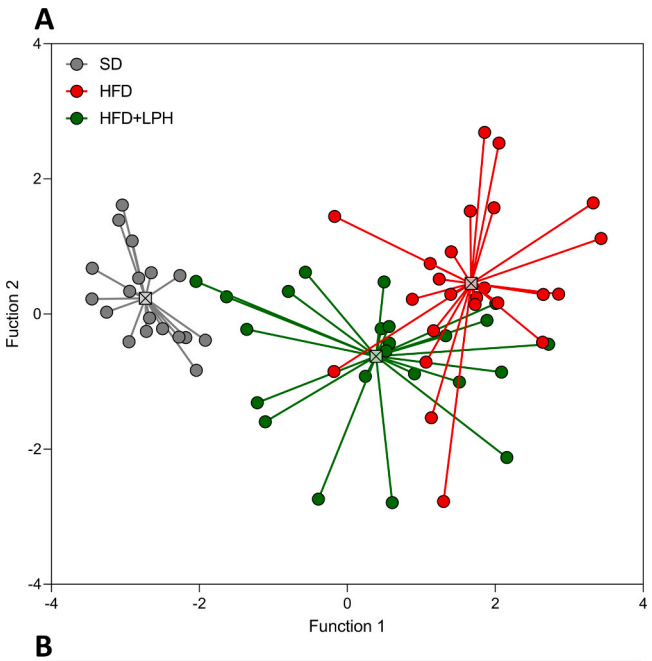
Fig. 5. Glucose homeostasis and adipokines levels in serum. Serum levels of glucose (A), insulin (B) and calculated homeostatic model assessment for insulin resistance (HOMA-IR) (C). Serum concentration of adiponectin (D), leptin (E) and ratio leptin (Lep) to adiponectin (Adipo) (F). (n = 12 per group, from two independent experiments). Data are normalized to HFD group. * p<0.05; **p<0.01; ***p<0.001; ****p<0.0001* p<0.05; **p<0.01; ***p<0.001;****p<0.0001.

in the present work we show for the first time a lupin-derived hydrolysate with beneficial effects on insulin resistance in the context of obesity.

Adipokines are molecules secreted by different cell types within adipose tissues that exhibit autocrine, paracrine and endocrine functions [52]. Most of them have important effects on energetic homeostasis, regulating different processes such as satiety (e.g. leptin and vaspin) or insulin sensitization (e.g. adiponectin and apelin). In the context of adipose tissue dysfunction produced during obesity development, important changes in the adipose secretome are produced and increased proportion of leptin with reduction in adiponectin (i.e., ratio leptin to adiponectin) are detected [53,54]. Overproduction of leptin also leads to leptin resistance, causing a vicious cycle of leptin production due to impaired signaling, which could also impact in the progression of obesity complications [55]. Thus, reduction of leptin and leptin/adiponectin ratio observed in LPH supplemented group could be related to an improvement of adipose tissue homeostasis or due to a direct action of some bioactive peptides on the molecular pathway of leptin signaling that improve sensitization. Although more studies should be conducted to elucidate the underlying process, to the best of our knowledge, this is the first study that demonstrates the beneficial effects of LPH on adipokine homeostasis.

The variety in the inter-individual responses to obesogenic stimulus (e.g., high-caloric diet consumption, sedentary lifestyle) is well known. In this context, some authors have proposed a new paradigm that defines 'obesities' rather than obesity [56]. This is not an exclusive human feature, rodents also show high heterogeneity when exposed to high-fat diets [57,58]. Taking this into account, a discriminant analysis was performed to assess the individual distribution among groups and to detect obesity-resistant mice. Interestingly, the success of the DIO model was 100 %, as all HFD-fed mice were predicted to belong to a group other than SD. Regarding the LPH-treated group, although most of them were in a group other than HFD (65.3 %), some showed an obese phenotype (21.7 %), indicating the existence of non-responders individuals. It is also important to highlight that 13.0 % of the LPH-treated mice (which had consumed a high-fat diet for 12 weeks) had a SD phenotype, suggesting a complete reversal of the obesity outcomes. Further studies are needed to examine the molecular variations that could be responsible for these divergences.

In addition to the features mentioned above, the microbiota plays a key role in obesity. Thus, in the early 2000s Gordon's lab published some of the most relevant microbiota-related papers describing the role of gut microbes in fat storage and energy harvesting [20,59]. They also demonstrated that germ-free mice, i.e., mice lacking the gut microbiota, are resistant to diet-induced obesity, highlighting the relevance of this symbiotic population in the development of this disease [60]. Over the last two decades, great efforts have been made to elucidate key points in the relationship between metabolic diseases and the microbiota [61]. In particular, the diversity and richness of the microbial population have been identified as important parameters involved in the proper functioning and resilience of ecosystems [62]. Therefore, considering the intestinal niche as a highly evolved and complex environment, it is not surprising that a reduction of these parameters has a negative impact on host health [63–65]. In the present study, no significant changes in diversity among groups were detected. However, a significant reduction in the number of different bacterial groups detected was observed in the HFD group compared to SD mice. Interestingly, LPH showed a clear tendency to reverse this decrease, suggesting the beneficial role of these peptides against loss of richness. Furthermore, the β-Diversity measurements showed a differential clustering among three experimental



Experimental Group	Predicted Group		
	SD	HFD	HFD+LPH
SD	100,0% (18)	0,0% (0)	0,0% (0)
HFD	0,0% (0)	83,3% (20)	16,7% (4)
HFD+LPH	13,0% (3)	21,7% (5)	65,3% (15)

Fig. 6. Discriminant analysis. Plot of every mouse according to the canonical discriminant functions (A). Table showing the distribution of animals according to Discriminant Analysis. Numbers in brackets refers to the number of animals in each group (B).

groups, indicating profound and significant changes in this microbial population. At the phylum level, the most common dysbiosis marker in obesity is the ratio of *Bacillota* to *Bacteroidota*. Although some researchers debate the relevance of this feature due to the high variability between cohorts, an increase in this ratio has been associated with a worsening of metabolic health status [66–68]. In the present study, no significant changes in this parameter were observed. Nevertheless, a clear tendency to increase was detected in the HFD-fed group, while LPH-treated mice showed a lower ratio.

According to the statistical analyses performed with the LEfSe algorithm, 26 microbial groups showed a different relative abundance, of which we will focus on the discussion of the groups altered after HFD consumption and recovered with LPH treatment. First, unclassified members of the *Oscillospiraceae* family and the genus *Oscillibacter* were increased in HFD-fed mice and restored in the LPH-treated group. Previous studies using high-fat diets have shown an increase in *Oscillibater spp*, which was reduced in mice treated with anti-obesity compounds [69–71]. Although the molecular processes involved in the relationship between this genus and host health remain unclear, some authors have shown a strong correlation with inflammation [71] and increased gut permeability [72]. In the present work, a significant positive correlation was observed between *Oscillibacter* abundance and LDL and the LDL/HDL ratio, suggesting a novel role for this genus in lipid homeostasis.

Moreover, an increase in the abundance of the *Candidatus Saccharimonas* genus was observed within the HFD group, while a significant reduction was detected in LPH-treated mice. The *Candidatus Saccharimonas* genus has been associated with intestinal inflammation [73,74] and its abundance increases in obese individuals [75]. Furthermore, a study involving patients with obstructive sleep apnoea showed a higher concentration of this bacteria in the oral microbiota and a positive correlation between its abundance and lipid serum, suggesting that these patients may be more prone to obesity [76]. In line with these

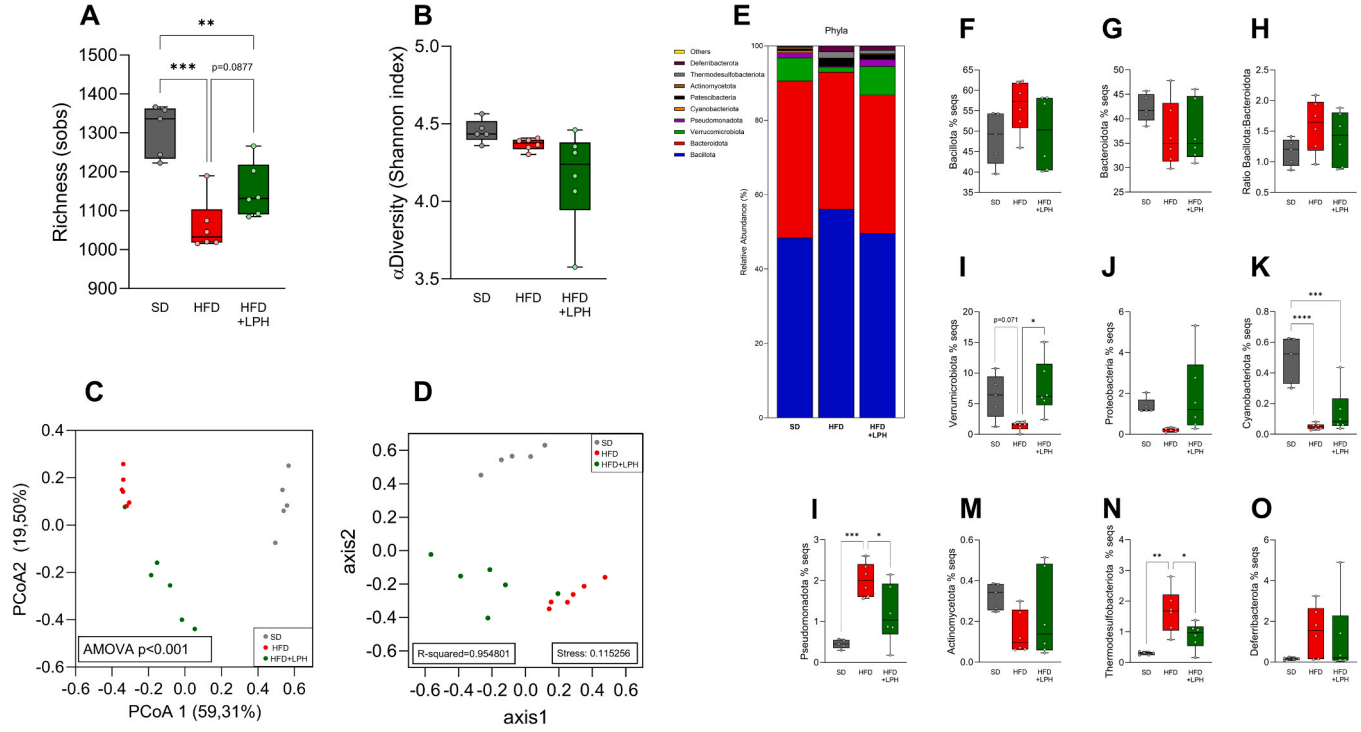


Fig. 7. General analysis of gut microbiota population. Indexes of richness (A) and α-diversity (B). β-diversity data represented as Principal Coordinate Analysis (PCoA) (C) and nonmetric multidimensional scaling analysis (NMDS) (D). Distribution of microbiota at phyla level (E) and % of sequences identified as phylum *Bacillota* (F), *Bacteroidota* (G), Ratio *Bacillota:Bacteroidota* (H), *Verrucomicrobiota* (I), *Pseudomonadota* (J) *Cyanobacteriota* (K), *Patescibacteria* (L), *Actinomycetota* (M), *Thermodesulfobacteriota* (N), *Deferribacterota* (O). Data are represented using box-and-whisker plot showing median, interquartile range and maximum and minimum values (n = 5–6 per group). Non statistical differences (ns). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

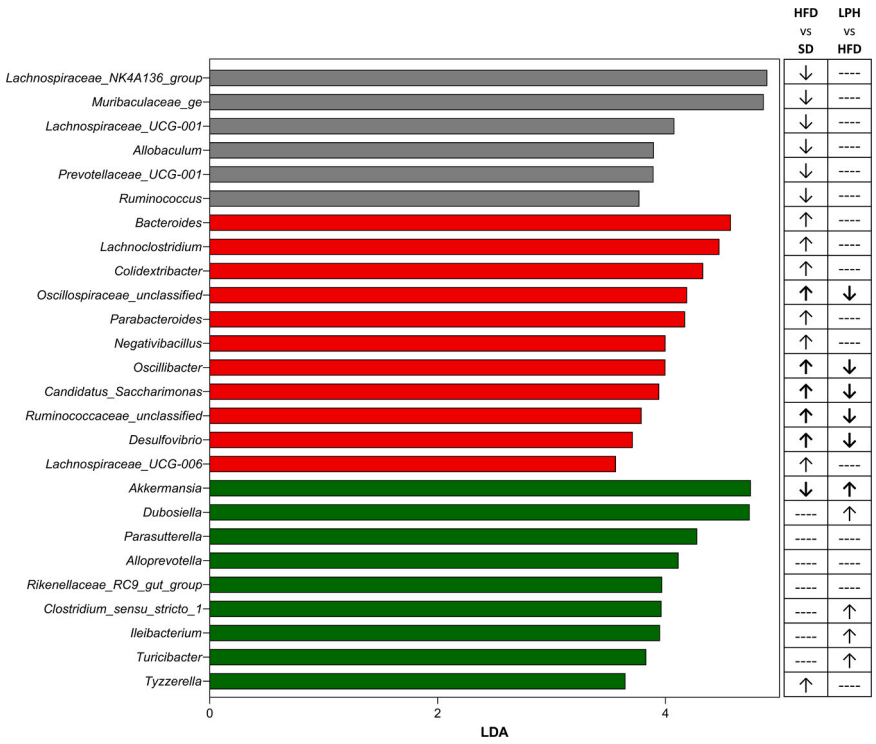


Fig. 8. Differential microbial features. Linear discriminant effect size analysis (LefSe), cutting in LDA score >3. Table in the right summarizes results of ANOVA's multiple comparison, being arrows significant changes ($p<0.05$) in the comparison indicated above. Direction of the arrow represent increase (↑) or decrease (↓) while vertical line (—) correspond to non-significant difference.

works, our results show a positive correlation between the proportion of *Candidatus Saccharimanos* and most markers of dyslipidemia (LDL, HDL, cholesterol and the LDL/HDL ratio).

Regarding the family *Rumminococcaceae*, some members have been shown to exert protective effects in obese rodents and humans [77,78]. Consistent with this, *Ruminoccocus*, one of the main genus representing this family, decreased in the HFD group. On the contrary, the unclassified members of this family, *Ruminococcaceae_unclassified*, increased in this group. According to the SILVA 138.1 database [32], these members can be *Anaerofilum*, *Angelakisella*, *CAG-352*, *Caproiciproducens*, *DTU089*, *Fournierella*, *Pygmaiobacter*, *UCG-001,* and [*Eubacterium*] *siraeum* group. Among all these genera, *Anaerofilum* and *Angelakisella* have been strongly associated with obesity [79,80]. Therefore, the observed reduction in their abundances after LPH ingestion could be associated with a better metabolic state.

Desulfovibrio is a member of sulphate-reducing bacteria that produces significant amounts of H_2S, a genotoxic compound [81]. The present results show a significant increase in the abundance of this genus after a high-fat diet intervention. Interestingly, this population was depleted in LPH-supplemented animals. Exposure of *Desulfovibrio* strains to germ-free mice increased the expression of CD36, an important importer of fatty acids, in the intestine [82]. Remarkably, our data show a positive correlation between the abundance of *Desulfovibrio* and serum triglyceride levels. This is of particular interest and further studies should be conducted to determine the association between *Desulfovibrio* and dyslipidemia and the role of LPH in this process.

Akkermansia is a genus belonging to the phylum *Verrucomicrobiota*, of which the better-known specie is *Akkermansia muciniphila*. This bacteria produces beneficial health effects, such as reduction of intestinal permeability [83] and production of short-chain fatty acids (SCFAs) [84], which help to reduce inflammation and control lipid homeostasis [18]. For this reason, the role of this specie in obesity has been extensively studied, with numerous works describing a significant reduction in abundance in both obese patients [67,85] and rodents [86,87]. Here, the abundance of *Akkermansia* was reduced in the gut of HFD-fed mice compared to the SD group. Interestingly, LPH supplementation was sufficient to fully restore healthy levels. Furthermore, an inverse correlation of *Akkermansia* with body weight and EpiWAT weight was detected. Consistent with these results, a randomized controlled trial in obese patients demonstrated the beneficial effect of 3 months supplementation with the probiotic *Akkermansia muciniphila* on metabolic status, showing a decrease in body weight and fat mass compared to the placebo group [88]. Although some authors have reported an increase in *Akkermansia* abundance after treatment with Walley Pollock skin hydrolysates [41] and *Arthrospira platensis* phycobiliprotein [89], to our knowledge, this is the first study to show the capacity of a lupin hydrolysate to protect against *Akkermansia* depletion induced by a high-fat diet.

We also found some microbial members that were more abundant in the LPH group compared to the HFD group, without changes between SD-fed and obese mice. First, LPH-treated mice showed an increase in *Dubosiella* and *Ileibacterium,* two novel members of the *Erysipelotrichaceae* family described by Cox *et al.* in 2017 [90]. Wan *et al.* have shown the beneficial effects of *Dubosiella* on a mouse model of ulcerative colitis by controlling inflammation and SCFAs production [91]. Furthermore, a study comparing the beneficial effects on oxidative stress and vascular function between resveratrol, a potent antioxidant, and *Dubosiella newyorkensis*, showed a similar or even greater capacity of this probiotic in both biological processes during aging [92]. Regarding *Ileibacterium*, some researchers have found a significant reduction in this genus after a high-fat diet [93,94]. Different treatments with alkaloid compounds and other plant-derived products increase the abundance of *Ileibacterium* [93,95]. Another significantly increased population in the LPH group is *Clostridium sensu stricto*. This microbial group has been shown to be reduced in DIO mice and to exert beneficial health effects as SCFA-producer [96]. Interestingly, a recent study has described a significant increase in this population in obesity-resistant wild-type mice compared to obesity-prone animals [97]. For these reasons, some *Clostridium* members have been proposed as a probiotic for the prevention of obesity. Finally, *Turicibacter* increases in LPH-treated mice compared to

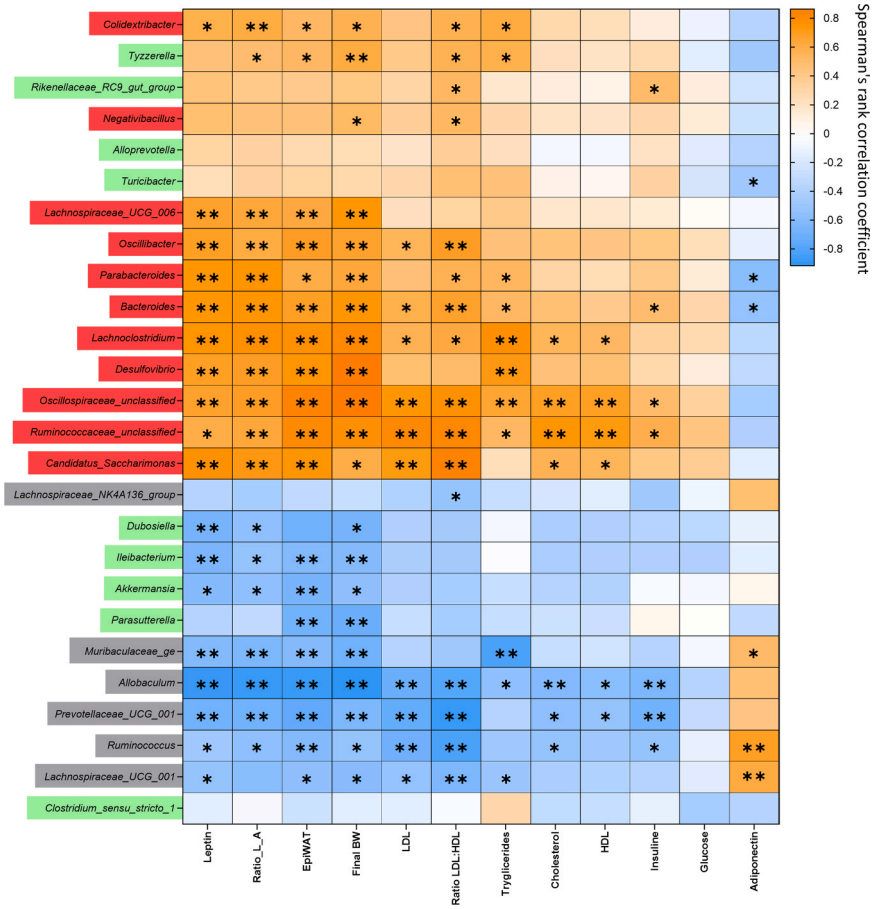


Fig. 9. Correlation between microbial taxa and obesity markers. Spearman's Rank correlation coefficient was used to create the matrix, with orange showing positive and blue negative correlation. *p<0.05; **p<0.01.

obese mice. Other groups have observed a significant reduction in this genus in mice fed a high-fat diet [98,99]. Moreover, a recent study has described the role of different *Turicibacter* strains in the metabolism of lipids and bile acids, concluding that some of them have genes with beneficial effects on this process [100]. These results could partly explain the improvement in lipid homeostasis in the present work.

5. Conclusions

In the present study, we conclude that consumption of LPH reduces body weight gain and adiposopathy in a mouse model of diet-induced obesity. LPH also alleviates the dyslipidemia and insulin resistance associated with the consumption of high-fat diet and promotes a healthy gut microbiota, reducing the dysbiosis, allowing the recovery of the richness of the gut ecosystem, controlling the overgrowth of harmful taxa and promoting the blooming of beneficial bacteria.

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CRediT authorship contribution statement

Eduardo Ponce-España: Conceptualization, Investigation, Formal Analysis, Data Curation, Writing – original draft, Writing – review & editing. **Ivan Cruz-Chamorro:** Investigation, Formal Analysis. **Guillermo Santos-Sánchez:** Investigation, Formal Analysis. **Ana Isabel Álvarez-López:** Investigation, Formal Analysis. **José María Fernández-Santos:** Resources. **Justo Pedroche:** Resources. **María Carmen Millán-Linares:** Resources. **Ignacio Bejarano:** Resources, Supervision, Funding acquisition. **Patricia Judith Lardone:** Resources, Supervision, Funding acquisition. **Antonio Carrillo-Vico:** Conceptualization, Writing – original draft, Writing – review & editing, Resources, Supervision, Funding acquisition.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117198.

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