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TÍTULO: Efectos de hidrolizados proteicos vegetales procedentes de leguminosas sobre el metabolismo lipídico en un modelo experimental de rata obesa. Interacción con el ejercicio físico aeróbico

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DEPARTAMENTO DE FISIOLOGIA
UNIVERSIDAD DE GRANADA

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INFORMA:

Que la Tesis Doctoral Internacional titulada: "Effects of legume protein hydrolizates on lipid metabolism in an obese rat experimental model. Interaction with aerobic physical exercise/Efectos de hidrolizados proteicos vegetales procedentes de leguminosas sobre el metabolismo lipídico en un modelo experimental de rata obesa. Interacción con el ejercicio físico aeróbico", que presenta Dña Garyfallia Kapravelou al superior juicio del Tribunal que designe la Universidad de Granada, ha sido realizada bajo mi dirección durante los años 2010-2014, siendo expresión de la capacidad técnica e interpretativa de su autora en condiciones tan aventajadas que le hacen merecedora al Titulo de Doctora, siempre y cuando así lo considere el citado Tribunal.

Fdo. Jesús María Porres Foulquie

En Granada, 08 de enero de 2015

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Fdo. Pilar Aranda Ramírez

En Granada, 08 de enero de 2015

El trabajo de investigación que constituye esta Tesis Doctoral, titulada: "Effects of legume protein hydrolizates on lipid metabolism in an obese rat experimental model. Interaction with aerobic physical exercise/Efectos de hidrolizados proteicos vegetales procedentes de leguminosas sobre el metabolismo lipídico en un modelo experimental de rata obesa. Interacción con el ejercicio físico aeróbico", se ha financiado gracias a la ayuda recibida por el proyecto de excelencia de la Junta de Andalucia P09-AGR-4658, cuyo investigador principal es Jesús María Porres Foulquie y fue realizado en el Dpto. de Fisiologia, Facultad de Farmacia y en Instituto de Nutrición y Tecnología de los alimentos de la Universidad de Granada.

A los protagonistas de estos experimentos que al no tener voz corren el riesgo de pasar desapercibidos…

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Aunque pensaba que sería sencillo, empezar a escribir este apartado me ha costado bastante ya que no sabía por dónde ni cómo empezar. Es que como no hay normas a seguir, bibliografía que podría servir ni tampoco estilo de escritura concreto, me he encontrado entre dilemas y dudas sobre la forma que mejor quedaría. Por mucho que lo haya intentado no pude hacer otra cosa sino recurrir al uso de mi propio estilo-que tampoco está del todo definido- e intentar hacer un recorrido de todos estos años, destacando memorias que, como pinceladas de color, terminaron coloreando esta experiencia. El mayor problema que le encuentro a todo eso, es que veo imposible poder incluirlo todo, pero me da tranquilidad el hecho que ya nos conocemos y todos estos años han servido para que podamos permitirnos ese margen de error. Aparte, creo que todos quienes han llegado a conocerme de verdad, comprenden que aunque las palabras sirven para expresar lo que uno siente, más sirve el día-día para demostrarlo. Será necesario utilizar otros idiomas, aparte de español, en algunos párrafos para que las personas a las que se hace referencia puedan entenderlo.

Todo empezó cuando decidí salir de Grecia y empezar la búsqueda de algo nuevo para seguir aprendiendo en ambos niveles, profesional y personal. Así que en primer lugar me gustaría agradecerles a mis padres que me criaron entre otros, con valores como la independencia personal y el intentar enfrentarse sin miedo a situaciones nuevas, sobre todo cuando van de acuerdo con los objetivos que uno se propone y de alguna forma es necesario pasar por ello para cumplirlos. Sin embargo, aparte de la ilusión que tenía de empezar algo nuevo, el miedo también estaba allí pero, allí también estaba mi hermana quien me apoyó no solo psicológicamente sino que me acompañó físicamente a este viaje y aguantó el estrés y a veces el pánico que me llegaron a envolver y a la vez intentaba que yo viera el lado positivo de esa vida nueva que había elegido. En todo esto, lo más importante es que sé que os sentís orgullosos de mí y de mi trayecto y eso me da mucha fuerza para seguir.

Όλα ξεκίνησαν όταν αποφάσισα να φύγω απ'την Ελλάδα αναζητώντας κάτι καινούργιο ώστε να συνεχίσω να εξελίσσομαι τόσο στον προσωπικό όσο και στον επαγγελματικό τομέα. Γι' αυτό το λόγο θα ήθελα να ευχαριστήσω τους γονείς μου που εκτός των άλλων με μεγάλωσαν με αξίες όπως η ανεξαρτησία και η προσπάθεια του να αντιμετωπίζω άγνωστες και καινούργιες καταστάσεις κυρίως όταν κατα κάποιο τρόπο είναι απαραίτητο να τις ζήσω για να πετύχω τους στόχους μου. Βέβαια, εκτός απο τον ενθουσιασμό, εκεί υπήρχε και ο φόβος τον οποίο η αδερφή μου με βοήθησε να αντιμετωπίσω όχι μόνο με ψυχολογική στήριξη αλλά συνοδεύοντας με σ'αυτό το ταξίδι υπομένοντας τα αγχη και τις φοβίες που με κυρίευαν και παράλληλα προσπαθώντας να με κάνει να δω όλα τα θετικά στοιχεία που περιείχε η καινούργια ζωή που είχα διαλέξει. Γι' αυτό που κυρίως σας ευχαριστώ είναι που μου δείχνετε ποσο περύφανοι ειστε για μένα κι αυτό με κάνει να αισθάνομαι κι εγω πιο δυνατή.

De esta forma empezó todo, pero no habría sido una experiencia tan fructífera sino fuera por mis directores que me han ayudado y apoyado desde que empecé a trabajar con ellos. En primer lugar quería darle las gracias al Dr. Jesús María Porres Foulquie que me ha enseñado como trabajar en el laboratorio, y con paciencia y dedicación me ha guiado en mis primeros pasos. Desde el primer momento me había sorprendido su disponibilidad, cercanía, amabilidad y me resultaba difícil creer cómo una persona tan inteligente puede ser tan respetuosa y humilde al

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Menos mal que el día-día en el laboratorio no era solamente trabajo ya que el contacto diario con mis compañeros lo enriquecía. No voy a negar que por características de la personalidad de cada uno la afinidad no es la misma, aun así creo que el contacto diario nos ha afectado a todos y aprendimos a respetar las peculiaridades de cada uno. Por eso les agradezco a todos su contribución en estos años. No me queda otra que limitarme a mencionar solamente una frase para cada uno ya que tantos momentos no caben aquí por mucho que lo intente.

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1. Introduction, objectives, and main findings

Metabolic syndrome (MetS) is a cluster of interrelated metabolic conditions which increase the risk of developing cardiovascular disease (Kaur, 2014). MetS is characterized by central obesity, dyslipidemia, elevated blood pressure, and elevated plasma glucose (Gonçalves et al., 2014). Patients with MetS are also more susceptible to develop type 2 diabetes mellitus (Reaven, 2004), and their hepatic morphology and function can be adversely affected leading to the development of Non Alcoholic Fatty Liver Disease (NAFLD) (Marchesini et al., 2003). This specific pathology is characterized by steatosis, lobular and portal inflammation, hepatocyte ballooning, and fibrosis (Brunt & Tiniakos, 2010). Furthermore, NAFLD pathology is now considered as the liver manifestation of MetS (Angelico et al., 2005). Although the exact mechanisms leading to it are not yet completely understood, insulin resistance and chronic oxidative stress have been reported to play a major role in liver damage and development of NAFLD (Polyzos, Kountouras, & Zavos, 2009; Rolo, Teodoro, & Palmeira, 2012).

The study of MetS and the development of strategies for its prevention and treatment has attracted increasing attention in recent years due to its growing prevalence and associated comorbidities exemplified by cardiovascular disease and NAFLD (Kaur, 2014; Marchesini et al., 2003). Changes in lifestyle, i.e. caloric restriction, low fat and low glycemic index diets, consumption of foods rich in beneficial bioactive ingredients, and regular physical activity, are the primary interventions chosen to improve the alterations associated with the specific pathology.

Legumes represent an excellent source of essential nutrients and exhibit a variety of health effects related to their antioxidant (Doblado, Frías, & Vidal-Valverde, 2007) and hypolipidemic properties (Hermsdorff, Zulet, Abete, & Martínez, 2011). Such important properties derive from the specific characteristics of legume proteins, carbohydrates and lipids, and also from several non-nutritional compounds like polyphenols, phytic acid or α-galactoside oligosaccharides. Biotechnological treatments applied to legumes not only improve their nutritional value (Porres, Aranda, López-Jurado, & Urbano, 2003), but may also enhance their health-promoting potential (Doblado et al., 2007).

The nutritional relevance of lupin (*Lupinus* spp.) has gained increased attention due to its high content of protein, minerals, dietary fibre, and fat (Jesús M. Porres, Aranda, López-Jurado, & Urbano, 2006), as well as to its low levels of non-nutritional components such as trypsin

inhibitors, lectins, or alkaloids in the sweet varieties. In addition to these nutritional properties, lupin also features beneficial functional properties such as antioxidant or hypocholesterolemic effects (Martínez-Villaluenga et al., 2009; Parolini et al., 2012). *Vigna radiata* is another widely used legume for human diets. Its interesting nutrient and bioactive compound composition (Dahiya et al., 2013; Tang, Dong, Guo, Li, & Ren, 2014) can be significantly improved by technological processing like sprouting (Fernandez-Orozco et al., 2008; Silva et al., 2013). In fact, *V. radiata* sprouts are usually commercialized to take part in numerous healthy dishes that are becoming increasingly popular for the general consumer.

In recent years, new processing conditions are continuously emerging in order to improve both the nutritional and health-related properties of legume-derived foodstuffs. One good example of such new products is protein hydrolyzates, which can be produced using different chemical and biological techniques. Among the most widely used methodologies to prepare protein hydrolyzates is protein extraction under alkaline conditions followed by hydrolysis process using different exoproteases like alcalase or flavourzyme (Megías et al., 2007a). However, other alternative biotechnological treatments like germination or fermentation also render protein preparations with important degree of hydrolysis and the presence of bioactive compounds with beneficial health actions (Porres et al. 2003; Urbano et al. 2003; Kapravelou et al. 2013). Via such treatments and in particular the germination process, enhancements of protein contents and antioxidant activity of the legumes can be achieved (Doblado et al., 2007; Ghavidel & Prakash, 2007). As a result, the resulting bioactive peptides may act as potential physiological modulators of metabolism, given that they inhibit the activity of angiotensin converting enzyme and exhibit antioxidant and bile acid-binding properties (Yoshie-Stark & Wäsche, 2004), thus showing promising potential as functional ingredients.

Changes in lifestyle habits have been suggested as a valuable strategy for the combined treatment of MetS. In this context, the effects of different types of exercise have been further studied. High-intensity aerobic interval training (HIIT) has been reported to be more effective at reducing cardiovascular disease risk in rats with metabolic syndrome than moderate-intensity continuous training (Haram et al., 2009). With regard to liver metabolism, several authors have studied the effect of moderate or vigorous intensity exercise on different aspects of NAFLD. Moderate intensity cycling exercise during four weeks significantly reduced visceral adipose tissue, hepatic triglyceride concentration, and plasma free fatty acids without altering body weight (Johnson et al., 2009). Moreover, moderate intensity exercise training showed beneficial effects

on intrahepatic triglyceride content, although it did not improve hepatic lipoprotein kinetics in obese individuals with NAFLD (Sullivan, Kirk, Mittendorfer, Patterson, & Klein, 2012).

Different *in vivo* experimental models have been used to test the beneficial effects of diet and exercise on several parameters of MetS. One of the most extended methods to test the hypolipidemic properties of foodstuffs is the consumption by rats of a diet rich in cholesterol and/or saturated fat with strong atherogenic index, based on that originally formulated and assayed by (Nath, Wiener, Harper, & Elvehjem, 1959). In such an experimental model, the potential hypolipidemic effect of the tested compound or mixture can be studied at both the digestive or metabolic level. Another interesting animal experimental model that is widely accepted as effective tool to study the multifactorial effects of diet and exercise on MetS associated conditions is the obese Zucker rat, which is known to present a genetic defect in leptin receptor that causes the development of hyperphagia leading to obese phenotype (Galisteo, Duarte, & Zarzuelo, 2008). In addition to obesity, the obese Zucker rat model shares many similarities with humans affected by MetS, including dyslipidaemia, insulin resistance, hepatomegalia, altered antioxidant status, and inflammatory process (Galisteo et al., 2010; Hey-Mogensen et al., 2012).

The main objective of this Doctoral research work was to test the beneficial effects of different healthy lifestyle strategies, including nutrition and physical exercise, on several parameters of metabolic syndrome. In order to do so, the present Doctoral Thesis was structured in two different and complementary phases with their corresponding objectives:

Phase 1: Effects of *Lupinus albus* protein hydrolyzate and insluble dietary fiber residue in an experimental model of diet-induced hypercholesterolemia

In the first set of experiments, we aimed to study the potential of lupin protein hydrolyzate, combined or not with lupin insoluble fiber, as functional food ingredients with beneficial effects on different metabolic parameters using an *in vivo* experimental model of rats fed a diet rich in cholesterol and coconut oil to test: 1) their hypolipidemic action, and 2) their influence on certain parameters of hepatic, renal and large intestine functionality.

Phase 2: Effects of raw and 4d-germinated *Vigna radiata* flours combined with a HIIT protocol in an experimental model of genetically obese Zucker rat

In the second set of experiments, we aimed to assess the effects of a high intensity aerobic intervallic training protocol combined with the consumption of raw and 4-day-germinated *V. radiata* on glucose and lipid metabolism parameters, liver histology and functionality, and hepatic

antioxidant status in an animal experimental model of metabolic syndrome, the obese Zucker rat, that presents hepatic alteration related to NAFLD.

In phase 1 experiments, rats were fed hypercholesterolemic high-fat diets in which different protein (casein *vs* lupin protein hydrolyzate) or insoluble dietary fiber (cellulose *vs* lupin insoluble fiber residue) sources were included to test the potential benefits of lupin products on the experimental animal model of diet-induced hypercholesterolemia/ hyperlipidemia. The experimental model developed exhibited significant alterations in plasmatic total-cholesterol and triglycerides, morphological and compositional changes in the liver, and alterations in urinary parameters of renal function. Dietary inclusion of lupin protein hydrolyzate and insoluble fiber residue caused a significant decrease in plasma and hepatic triglyceride content, and appeared to improve glucose metabolism. Furthermore, lupin functional ingredients induced diverse potentially beneficial changes in kidney, liver, and large intestine functionality, although they could not reverse the histological alterations found in liver.

In phase 2, experiments were designed to test the influence of a HIIT protocol consisting of successive 4 min periods at 65-80% of VO_2max, followed by 3 min recovery periods at 50-65% of VO_2max, combined with the consumption of diets containing 70% of their total protein content and nearly 100% of their total dietary fiber content supplied as raw or 4-day-germinated *V. radiata*, on plasma and liver biochemical parameters of obese and lean Zucker rats. Obese rats exhibited higher food intake and body weight, and suffered significant alterations in plasma lipid profile, Area under the Curve after oral glucose overload, liver histology and functionality, and antioxidant status. Exercise increased the aerobic capacity of both rat phenotypes and diminished the severity of metabolic syndrome alterations, especially those related to glucose and lipid metabolism, affecting the levels and activity of proteins involved in metabolic pathways and the gene expression of GPX4, a key antioxidant enzyme, in the liver. Consumption of germinated *V.radiata* was efficient at reducing body weight, hepatomegalia and hepatic triglyceride content of obese Zucker rats, as well as improving their liver functionality and antioxidant status. The combination of both lifestyle strategies (plant protein hydrolyzates and physical exercise) had superior effects when compared to the combination of animal protein and physical exercise and could represent an efficient and feasible complement to the more severe pharmacological treatments that are currently in use. Therefore, such lifestyle strategies could contribute to lowering the dosage of such treatments in patients that suffer alterations caused by the MetS.

1. Introducción, objetivos y resultados principales

El Síndrome metabólico (MetS) incluye un complejo de alteraciones metabólicas que aumentan el riesgo de desarrollar enfermedades cardiovasculares (Kaur, 2014). Se caracteriza por obesidad central, dyslipidemia, hipertensión y elevados niveles de glucosa plasmática (Gonçalves et al., 2014). Los pacientes con MetS tienen alto riesgo de desarrollar diabetes tipo 2 (G. Reaven, 2004) y su morfología y funcionalidad hepática puede verse afectada, dando lugar a la enfermedad hepática no alcohólica (NAFLD) (Marchesini et al., 2003). Dicha patología está caracterizada de una serie de alteraciones hepáticas como esteatosis hepática, inflamación lobular y periportal, acumulación de triglicéridos en el citoplasma de los hepatocitos y fibrosis (Brunt & Tiniakos, 2010). Adicionalmente, se considera como la manifestación hepática del MetS (Angelico et al., 2005). Aunque no se conocen exactamente los mecanismos implicados para dicha patología, estudios recientes destacan la resistencia a la insulina y el estrés oxidativo crónico como factores principales responsables para el daño hepático el desarrollo de NAFLD (Polyzos et al., 2009; Rolo et al., 2012).

Muchos estudios se han centrado en investigar la etiología y desarrollar estrategias para la prevención y el tratamiento del MetS, debido a que la prevalencia de dicha patología y la de sus alteraciones asociadas (enfermedades cardiovasculares y la NAFLD), se han visto aumentadas en los últimos años (Kaur, 2014; Marchesini et al., 2003). Como primera línea de estrategias más utilizadas son las que engloban cambios de estilo de vida como por ejemplo, restricción calórica, dieta baja en grasa y de bajo índice glucémico, el consumo de alimentos ricos en componentes bioactivos y beneficiosos para la salud y la actividad física.

Las legumbres representan una fuente rica en nutrientes esenciales y aportan una amplia variedad de beneficios para la salud debido a sus propiedades antioxidantes (Doblado et al., 2007) y hipolipidémicas (Hermsdorff et al., 2011). Estas propiedades se atribuyen a en las características específicas de los macronutrientes (proteina, hidratos de carbono y lípidos) y a los componentes no nutricionales (polifenoles, α-galactosidases, ácido fítico) de las legumbres. Los tratamientos biotecnológicos que se aplican a las legumbres no solamente ayudan a mejorar su valor nutritivo (Porres et al. 2003), sino que además aumentan su potencial para la salud (Doblado et al., 2007).

La importancia nutricional de lupinus (*Lupinus* spp.)se basa en su alto contenido proteico, mineral, lipídico y en fibra dietética (Porres et al. 2006). Además las variedades dulces de esta legumbre contienen bajos niveles de componentes no nutricionales como inhibidores de tripsina, lectinas y alcaloides. Otro aspecto importante de lupino es que posee propiedades propiedades

funcionales como actividades antioxidantes e hypocolesterolemiantes (Martínez-Villaluenga et al., 2009; Parolini et al., 2012). *Vigna radiata* es otra legumbre altamente consumida como parte de la dieta de varios países. Su composición nutricional y su contenido en ingredientes bioactivos (Dahiya et al., 2013; Tang et al., 2014) se puede mejorar mediante varios procesos tecnológicos como la germinación (Fernandez-Orozco et al., 2008; Silva et al., 2013). Actualmente, los germinados de *V. radiata* se suelen comercializar y de esta manera, forman parte de numerosos platos que se utilizan cada vez más por los consumidores.

En la actualidad, sigue aumentando el número de tratamientos nuevos que entre sus objetivos se encuentran la mejora del valor nutricional y de las propiedades beneficiosas de alimentos derivados de las legumbres. Un buen ejemplo a destacar son los hidrolizados proteicos, los cuales se pueden elaborar utilizando diferentes procesos químicos y biológicos. Entre los procesos más utilizados para la preparación de los hidrolizados proteicos es la extracción de la proteína en condiciones alcalinas seguida por una hidrólisis mediante diferentes exoproteasas procedentes de *Bacillus licheniformis* o *Aspergillus oryzae* (Megías et al., 2007a). Además, otros procesos biotecnológicos como la germinación o la fermentación pueden resultar adecuados para la preparación de estos productos consiguendo un grado significante de hidrólisis y de la presencia de componentes bioactivos con propiedades altamente beneficiosas para la salud (Porres et al. 2003; Urbano et al. 2003; Kapravelou et al. 2013). Estos tratamientos pueden aumentar el contenido proteico y la capacidad antioxidante de las legumbres (Doblado et al., 2007; Ghavidel & Prakash, 2007). Como resultado, los componentes bioactivos del producto final pueden actuar como moduladores del metabolismo ya que entre otras propiedades inhiben la actividad de la enzima convertidora de angiotensina, tienen la capacidad de unirse con los ácidos biliares y presentan propiedades antioxidantes (Yoshie-Stark & Wäsche, 2004). Todo ello hace que tengan un potencial como alimentos funcionales.

Está demostrado que los cambios de estilo de vida se pueden considerar como una estrategia adecuada para la prevención y el tratamiento de las alteraciones que acompañan el MetS. La práctica del ejercicio físico tiene un papel fundamental. Algunos estudios han demostrado los efectos de distintos tipos de ejercicio. Se ha demostrado que un protocolo de entrenamiento aeróbico interválico de alta intensidad (HIIT) es más eficaz para la reducción de riesgo de alteraciones cardiovasculares en ratas con síndrome metabólico en comparación con un protocolo aeróbico, continuo y de intensidad moderada (Haram et al., 2009). En relación con el metabolismo hepático, muchos autores han estudiado el efecto de la intensidad de los protocolos de entrenamiento en los diferentes aspectos de NAFLD. Un protocolo de ciclismo de intensidad moderada durante cuatro semanas ha reducido significativamente el tejido adiposo visceral y la

concentración de los triglicéridos hepáticos y de los ácidos libres sin provocar alteraciones en el peso corporal (Johnson et al., 2009). Otro estudio que utilizó un protocolo de entrenamiento con intensidad similar, ha mostrado efectos beneficiosos de dicho entrenamiento en relación al contenido intrahepático de los triglicéridos aunque no hubo mejora a los niveles de lipoproteínas hepáticas en obesos pacientes con NAFLD (Sullivan et al., 2012).

Para estudiar los posibles efectos de factores como la dieta y el ejercicio sobre los distintos parámetros del MetS se han utilizado diferentes modelos experimentales *in vivo*. Uno de los modelo más utilizados para investigar las propiedades hipolipidemiantes de productos alimentarios derivados de legumbres es la administración de una dieta alta en grasa saturada y/o colesterol a los animales. Dicha dieta, de alto índice aterogénico, está basada en la originalmente formulada y utilizada por Nath et al. (1959). Este modelo experimental ofrece la posibilidad de investigar el potencial hipolipidemiante de una mezcla de componentes o incluso, de uno aislado a nivel digestivo y/o metabólico. Otro modelo animal experimental que está aceptado que se puede utilizar para el estudio de los adversos efectos de la dieta y el ejercicio sobre las alteraciones asociadas con el MetS, es el modelo experimental de la rata obesa Zucker. Dicho modelo presenta una deficiencia genética a los receptores de leptina que a su vez da lugar al desarrollo de hiperfagia y de fenotipo obeso (Galisteo et al., 2008). Además de la obesidad, el modelo experimental de la rata Zucker, comparte muchas similitudes con las alteraciones que afectan los humanos con MetS, como dyslipidemia, resistencia a la insulina, hepatomegalia, alteraciones del estatus antioxidante e inflamación (Galisteo et al., 2010; Hey-Mogensen et al., 2012).

El objetivo principal de esa tesis es el estudio de los efectos beneficiosos de diferentes estrategias basadas en la nutrición y el ejercicio físico sobre los diferentes parametros del MetS y NAFLD. Dichas estrategias que sirven para la promoción de salud, incluyen nutrición y ejercicio. Para desarrollarlo, la presente Tesis Doctoral está estructurada en dos fases diferentes y complementarias con sus sub-objetivos correspondientes:

Fase 1: Efectos de hidrolizado proteico y fibra insoluble de *Lupinus albus* en un modelo experimental de hipercodlesterolemia inducida por la dieta.

El objetivo de los experimentos de la fase 1, fue estudiar el potencial del hidrolizado proteico de *Lupinus albus*, combinado o no con su fibra insoluble, como ingredientes de alimentos funcionales con efectos beneficiosos en diferentes parámetros metabólicos. Para ello, se utilizó un modelo experimental *in vivo* de ratas alimentadas de una dieta alta en colesterol y aceite de

coco para investigar: 1) la acción hipolipidemiante de estos ingredientes y 2) la influencia de su consumo en varios parámetros de la funcionalidad hepática, renal y del intestino grueso.

Fase 2: Efectos de las harinas de *Vigna Radiata* cruda y germinada durante 4 días combinadas con ejercicio aeróbico intervalico y de alta intensidad de un modelo experimental de rata genéticamente obesa

El objetivo de los experimentos de la fase 2 fue el estudio de los efectos de un protocolo de entrenamiento aeróbico, intervalico y de alta intensidad (HIIT), combinado con el consumo de una dieta de *V. radiata* cruda y germinada durante 4 días, sobre parámetros relacionados con la histología y funcionalidad hepática, el metabolismo glucidico y lipídico y el estatus antioxidante de hígado. Para ello, utlizó el modelo experimental de la rata genéticamente obesa, la rata Zucker, que desarrolla MetS y presenta alteraciones hepáticas relacionadas con la NAFLD.

En los experimentos de la fase 1, los animales consumieron dietas altas en grasa en las cuales se incluyeron diferentes fuentes de proteína (caseina *vs* proteína de hidrolizado proteico de Lupinus) y diferentes fuentes de fibra insoluble (celulosa *vs* residuo de fibra insoluble de Lupinus) para examinar los posibles beneficios que los productos derivados de Lupinus pudiesen tener sobre un modelo animal de hipercolesterolemia/ hiperlipidemia inducida por la dieta. Los animales presentaron importantes alteraciones en los niveles de colesterol total y de los triglicéridos plasmáticos, en la morfología y la composiciónlipídica hepática, alteraciones urinarias y relacionadas con la funcionalidad renal. La inclusión de hidrolizado proteico de lupinus y del residuo de fibra insoluble en la dieta de los animales, disminuyó de forma significativa los triglicéridos plasmáticos y el contenido hepático lipídico, mejorando a la vez el metabolismo glucídico. Addicionalmente, los ingredientes funcionales de lupinus indujeron diversos cambios en el rinón, el hígado y el intestino delgado con posibles efectos beneficiosos aunque no pudieron mejorar las alteraciones histológicas que se observaron en el hígado.

Los experimentos de la fase 2, se diseñaron para investigar la influencia de un protocolo de HIIT, que consistió en periodos sucesivos de 4 min a 50-65% de VO_2max seguidos por periodos de 3min de recuperación a 50-65% of VO_2max, combinado con la administración de dietas de *V. radiata* cruda y germinada, de cuales el 70% de su contenido proteico total y casi 100% de su contenido total de fibra procedía de dicha legumbre, sobre los parámetros plasmáticos y hepáticos, de ratas Zucker obesas y delgadas. Las ratas obesas se caracterizaron por una mayor ingesta y peso corporal y alteraciones negativas en el perfil plasmático y lipídico, el area debajo de la curva tras una carga oral de glucosa, la histología y funcionalidad hepática y el estatus antioxidante. El ejercicio, aumentó la capacidad aeróbica de ambos fenotipos de animales y

redujo la severidad de las alteraciones del MetS, especialmente las del metabolismo glucidico y lipídico, afectando los niveles y la actividad de proteínas que están involucradas en estas vías metabólicas y la expresión génica de GPX4, un enzima antioxidante clave en el hígado. Por el otro lado, el consumo de la *V.radiata* germinada, ha producido ingesta y peso corporal final. Además, redujo la hepatomegalia y el contenido de triglicéridos hepáticos y mejoró la funcionalidad hepática y el estatus antioxidante de los animales obesos. La combinación de las dos estrategias (nutrición y ejercicio físico) se ha mostrado como un complemento eficaz y de fácil aplicación junto con los tratamientos farmacológicos que hoy en día se están utilizando. Esta combinación de estrategias de estilo de vida podría contribuir a disminuir la dosis de estos tratamientos en los pacientes que padecen alteraciones causadas por el MetS.

2. Bibliographic Review

2.1 Metabolic Syndrome

2.1.1 Definition

There are multiple definitions for the Metabolic Syndrome (MetS) provided that it represents a clustering of different risk factors and its pathology is not yet fully understood. It includes several metabolic disorders among which central obesity and insulin resistance are considered as causative factors (Anderson et al., 2001; Indulekha, Surendar, & Mohan, 2011). Furthermore, several other aspects, such as lack of physical activity, ageing, and hormonal imbalance -including polycystic ovary syndrome or testosterone insufficiency- are implicated in the development of this pathology (Golbidi, Mesdaghinia, & Laher, 2012; Gombet et al., 2012; Malik & Traub, 2012; Saad & Gooren, 2011). The MetS started as a concept rather than a diagnosis (Shaw & Chisholm, 2003). However, the first important step towards its definition/diagnosis was made in 1988 by(G. M. Reaven, 1988), who named it "Syndrome X" and introduced the concept of clustering risk factors and insulin resistance. This study pointed out the influence that the changes on free fatty acids (FFA) concentration exhibit on insulin resistance as well as insulin and plasmatic glucose intolerance. In addition, it suggested the possible implication of hyperinsulinemia in the aetiology and development of non-insulin induced diabetes mellitus (NIIDM), hypertension and cardiovascular diseases (CAD). As the factor of obesity or visceral obesity was not included in the definition given by Reaven, (Kaplan, 1989) included it and so the MetS was renamed into "The deadly Quartet". From this moment, several groups have attempted to develop the diagnostic criteria of this pathology. The World Health Organization (WHO) in 1998 (K. g. m. m. Alberti & Zimmet, 1998) and the European Group for the Study of Insulin Resistance criteria (EGIR); in 1999 (Balkau & Charles, 1999), both considered insulin resistance as a required factor for the definition of the MetS. In contrast, the diagnostic criteria established by the National Cholesterol Education Programme (NCEP) Adult Treatment Panel Ⅲ (ATP Ⅲ) in 2001 (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001) and the American Association of Clinical Endocrinologists (AACE) (Einhorn, 2003) were more focused in the other parameters involved in MetS rather than insulin resistance as it is more difficult to be determined. Finally, in 2005, the International Diabetes Federation (IDF) proposed a new definition with the intention to unify all the previously suggested ones (K. G. M. M. Alberti, Zimmet, & Shaw, 2006).

As below indicated (Table 1), the diagnostic criteria used for the definition of the MetS are quite similar among the different organizations and include the presence of insulin resistance, obesity, dyslipidemia, hypertension and alterations of glucose metabolism. WHO in its definition, includes as well the presence of microalbuminuria whereas AACE, takes into account other factors with possible association to insulin resistance, such as family history of type 2 diabetes mellitus, polycystic ovary syndrome, sedentary lifestyle, age end ethnic group. Even if there are obvious similarities in the diagnostic criteria used, the limits established by every organization or the way to define them varies; thus contributing to great variation of the prevalence of this pathology as observed by the different studies. The first definition established by WHO, EGIR and AACE considers the presence of insulin resistance as a required factor for the definition of the MetS. However, ATPIII and IDF do not consider it as an essential factor. In particular, AACE considers equally important the rest of the factors whereas IDF's definition focuses on the presence of obesity, calculated by the waist circumference. Furthermore, the presence of obesity is also calculated differently since some of the organizations use the BMI , others the waist to hip ratio and the most recent definition makes use of the waist circumference alone as an indicator of obesity. Finally, over the years, the limits of blood pressure indicating hypertension have lowered from 140/90 to 135/85mmHg as established by IDF in 2005.

Table 1. Diagnostic criteria proposed for the clinical diagnosis of the Metabolic Syndrome. Taken from Kaur J. (2014)

Clinical measures	WHO (1998)	EGIR (1999)	ATPIII (2001)	AACE (2003)	IDF (2005)
Insulin resistance	IGT, IFG, T2DM or lowered insulin sensitivity [a] **plus any 2 of the following**	Plasma insulin >75th percentile **plus any 2 of the following**	**None but any 3 of the following 5 features**	IGT or IGF **plus any of the following based on the clinical judgement**	None
Body weight	Men: Waist-to- hip ratio>0.90; women: waist- to- hip ratio>0.85 and/or BMI>30kg/m^2	WC≥94cm in men or ≥ 80cm in women	WC≥120cm in men or ≥ 88cm in women	BMI>25kg/m^2	Increased WC (population specific) **plus any two of the following**

Lipids	TGs ≥ 150mg/dL and/or HDL-C <35mg/dL in men or <39mg/dL in women	TGs ≥ 150mg/dL and/or HDL-C <39mg/dL in men or women	TGs ≥ 150mg/dL HDL-C <40mg/dL in men or <50mg/dL in women	TGs ≥ 150mg/dL and HDL-C <40mg/dL in men or <50mg/dL in women	TGs ≥ 150mg/dL on TGs Rx. HDL-C <40mg/dL in men or <50mg/dL in women or on HDL-C Rx
Blood pressure	≥140/90mmHg	≥140/90mmHg or on hypertension Rx	≥130/85mmHg	≥130/85mmHg	≥130mmHg systolic or ≥85mmHg diastolic or on hypertension Rx
Glucose	IGT, IFG or T2DM	IGT or IFG (but not diabetes)	>110mg/dL (includes diabetes)	IGT or IFG (but not diabetes)	>100mg/dL (includes diabetes) [b]
Other	Microalbuminuria: Urinary excretion rate of >20mg/min or albumin:creatinine ratio of >30mg/g			Other features of insulin resistance[c]	

[a] Insulin sensitivity measured under hyperinsulinemic euglucemic conditions, glucose uptake below lowest quartile for background population under investigation

[b] In 2003 the American Diabetes Association (ADA) changes the criteria for IFG tolerance from >100mg/dL

[c] Includes family history of type 2 diabetes mellitus, polycystic ovary syndrome, sedentary lifestyle, advancing age end ethnic groups susceptible to type 2 diabetes mellitus

BMI: body mass index; HDL-C: high density lipoprotein cholesterol; IFG: impaired fasting glucose; IGT: impaired glucose tolerance; Rx: receiving treatment; TGs: Triglycerides; T2DM: type 2 diabetes mellitus; WC: waist circumference

2.1.2 Prevalence

There are many factors that influence the prevalence of MetS such as the region, urban or rural environment, composition (sex, age, race and ethnicity) of the studied population and, mostly, the diagnostic criteria used. The existence of all these factors results in a great variation of the prevalence of this pathology that ranges from 10-84% (Desroches & Lamarche, 2007; Kolovou, Anagnostopoulou, Salpea, & Mikhailidis, 2007). According to IDF (2014), it is

estimated that one-quarter of the world's adult population is suffering from MetS. However, the prevalence of MetS (based on NCEP-ATP III criteria, 2001) varied from 8% to 43% in men and from 7% to 56% in women around the world. A study carried out in an adult Spanish population showed that according to IDF criteria the prevalence was around 34.2%. This percentage was lower when NCEP criteria (17.7%) or WHO criteria were taken into account (15.5%) (A. J. Cameron, Shaw, & Zimmet, 2004).

The National Health and Nutrition Examination Survey (NHANES), indicated that the prevalence varied due to the individual's weight. As a result, among the normal weight individuals the prevalence was calculated at 5%, among the overweight individuals raised up to 22% and reached 60% among the obese individuals; thus indicating that it is also highly correlated to the presence of obesity (Park Y et al., 2003). Another factor that contributes to an increase of the prevalence of this pathology is age, as it is estimated higher among the elderly populations, and in particular in postmenopausal women (Ford ES, Giles WH, & Dietz WH, 2002; Ponholzer, Temml, Rauchenwald, Marszalek, & Madersbacher, 2007). Additionally, factors such as higher genetic background, socioeconomic status, diet, levels of physical activity, smoking, family history of diabetes and education all influence the prevalence of the MetS (A. J. Cameron et al., 2004).

The different existing definitions, not only affect the prevalence of the MetS but that of the non-alcoholic fatty liver disease (NAFLD) as well, a disease strongly correlated to the presence of MetS. Among the patients of MetS and following the definition of IDF, the prevalence of NAFLD was estimated around 43%, which was increased up to 53% according to NCEP and 64% according to the diagnostic criteria established by WHO. This fact suggests that the definition established by WHO could be used as a tool for the determination of NAFLD, the hepatic manifestation of this pathology (Caballería et al., 2012).

Recent publications and reviews have suggested NAFLD to be included in the criteria for the definition of MetS due to the strong correlation that exists between them even in children population (El-Karaksy et al., 2012). However, it still remains uncertain if NAFLD can be considered as an independent factor for MetS as contradictory data exist that suggest the need of further investigation regarding this issue (Machado et al., 2012; Smits et al., 2013).

2.1.3 Prevention and treatment

In order to prevent the appearance of MetS it is important to be able to clinically identify the patients with high risk of developing this pathology. The prevention of MetS requires a

multidisciplinary approach from a team of various specialists, mostly focusing in lifestyle modifications such as weight loss, diet and exercise. In case the risk factors included in MetS are not adequately attenuated by lifestyle changes, then the administration of appropriate pharmacological agents should be considered (Deen, 2004). Up to date, a specific therapeutic approach for the whole syndrome has not been established, making difficult the clinical management of MetS. In general, most physicians treat each component of the MetS separately, focusing particularly in the ones that can be treated by drug administration. However, nowadays, for the treatment or prevention of the different risk factors involved in MetS, there are established guidelines by the National Cholesterol Education programme (NCEP), the seventh Joint National Commission (JNC-VII) for blood pressure treatment (Chobanian et al., 2003), the American Diabetes Association (ADA) (American Diabetes Association, 2005), the American Heart Association (AHA) (Grundy, 2005), and (The Evidence Report, 1998) that could be helpful not only for the physicians but for the patients as well.

Overall, a desirable and realistic goal for overweight/obese persons is to reduce the body weight by >7% to 10% over a period of 6 to 12 months. It is suggested that weight reduction should be combined with daily exercise, including at least 30 minutes of moderate-intensity physical activity. With regards to the nutritional guidelines, these consist in a low intake of saturated and total fat intake, reduced consumption of simple sugars, and high glycemic index foods; thus indicating an essential increase in the intake of fruits, vegetables, legumes, and whole grains. In case pharmacological treatment is necessary for the dyslipidemia to be adjusted to normal levels, statins can be combined with fibrates and niacin to achieve the target levels of LDL-C, triglycerides, and HDL-C. Furthermore, the treatment of hypertension is more probable to require a combination of pharmacological agents, among which angiotensin-converting-enzyme inhibitor (ACEI)/ angiotensin II receptor antagonists (ARBs) and β-blockers/Thiazides/Calcium-channel blockers (CCBs) are the first and second line agents, respectively. Finally, metformin, thiazolidinediones, and acarbose may be used in order to lower the risk for type 2 diabetes mellitus in people with impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) (Kaur, 2014).

2.2 Non-alcoholic fatty liver disease (NAFLD)

2.2.1 Definition

The non-alcoholic fatty liver disease (NAFLD) is characterized by fat infiltration of the liver (triglycerides accumulation that exceeds 5% of the liver weight) on radiological exams or biopsy,

without alcohol intake, viral infection, medication intake causing fatty liver or any other known cause. However, the fatty liver is rather a sign than a diagnosis since in some cases the accumulation of fat may not be present until late stage of the disease (Sanal, 2008). NAFLD is a generic term that includes comprises a spectrum of non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), and NASH cirrhosis (The Korean Association for the Study of the Liver (KASL), 2013). The beginning of NAFLD results from insulin resistance and frequently co-exists with other metabolic disorders that accompany obesity, type 2 diabetes and dyslipidemia (Angulo, 2007). Thus, it has been described as the hepatic manifestation of MetS (Angelico et al., 2005).

Table 2. Definition of non-alcoholic fatty liver disease related items (Taken from the Korean Association for the study of the Liver, 2013

Term	Definition
Non-alcoholic Fatty liver disease (NAFLD)	NAFLD is a generic term including a spectrum of several liver disorders, including non-alcoholic fatty liver, NASH and NASH cirrhosis. Fat accumulation of more than 5% of the liver weight on biopsy is defined as fat infiltration.
Non-alcoholic Fatty liver (NAFL)	This condition is characterized by fat infiltration of the liver without the findings of ballooning degeneration or fibrosis.
Non-alcoholic steatohepatitis (NASH)	This condition is characterized by fat infiltration of the liver with findings of inflammation associated with ballooning degeneration. It is occasionally associated with fibrosis
NASH cirrhosis	This condition is characterized by cirrhosis, associated with the histological findings of NAFL or NASH, or cirrhosis taking place in patients with NAFL and NASH proven by past histology

2.2.2 Prevalence

There are few prospective studies of NAFLD, and that makes it difficult to determine its prevalence. However, it is estimated around 20-33% in adults in the general population (J. G. Fan et al., 2011). The Dallas Heart Study (a population-based cohort study performed in an ethnically diverse community in the USA) using proton MR spectroscopy; reported that one out of three adult Americans has steatosis (Powell, Jonsson, & Clouston, 2005). A large-scale cohort study from the Framingham Heart Study; using computed tomography (CT) revealed that the prevalence of NAFLD was 17% (Speliotes et al., 2010). Furthermore, in a demographic study of ordinary people participating in the National Health and Nutrition Examination Survey III,

determined the prevalence of NAFLD and advanced fibrosis at 34% and 3.2%, respectively (D. Kim, Kim, Kim, & Therneau, 2013).

In Europe, it is estimated that NAFLD affects 22% of the general population (Szalman, Bancu, & Sin, 2013). By performing liver ultrasonography, a population-based cohort study performed in Italy found that one out of four or five adults in that country suffer from NAFLD (Bedogni et al., 2005), while in China it has been estimated that the prevalence reaches up to 15% of the population (Fan et al., 2011; Fan & Farrell, 2009). The prevalence of NAFLD among children remains still unknown, but some data indicate that 2.6–9.6% of children have NAFLD, increasing up to 38–53% among the obese children.

2.2.3 Pathogenesis

The etiopathogenesis of nonalcoholic fatty liver disease (NAFLD) has been disputed loud and long (Figure 1). It seems that genetic factors, macronutrient intake, and lifestyle factors contribute in the lipid homeostasis alteration. The resultant disequilibrium in lipid homeostasis causes triglycerides to accumulate in the liver. An increase in oxidative stress, due to the generation of reactive oxygen species as a result of mitochondrial abnormalities and induction of the cytochrome P-450 system could be one mechanism by which the nonalcoholic fatty liver develops into nonalcoholic steatohepatitis (NASH) (Sanal, 2008).

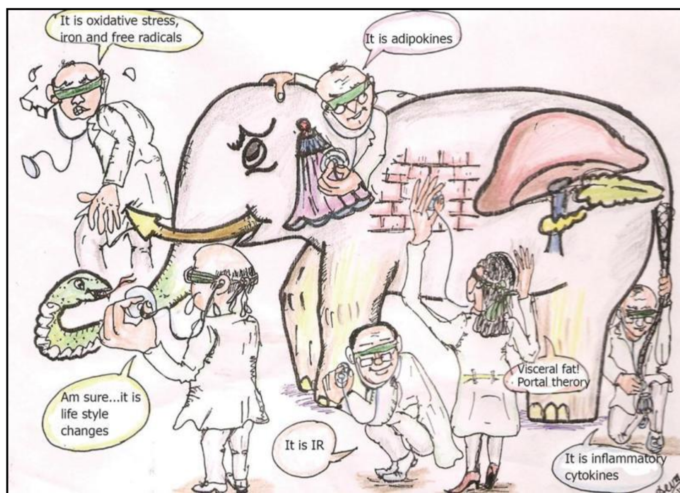

Figure 1. The term "nonalcoholic fatty liver disease" is imprecisely defined; there is uncertainty regarding its pathogenesis. The elephant (NAFLD) has been interpreted by many, and several possible aetiologies and pathogenesis models have been assigned to it like 'insulin resistance', mitochondrial defects, adipokine imbalance, visceral fat, inflammatory cytokines and oxidative stress (Sanal 2008).

The "Overflow hypothesis" was first used to describe the sequence of the events taking place in the development of NAFLD. According to this, the consumption of energy dense caloric food is increased whereas at the same time the activity level is inadequate to spend the excessive calories; the fat is then accumulated in muscle and liver. The consequent triglycerides accumulation leads to the development of insulin resistance which under normal conditions forms part of a defence mechanism against limited food availability or increased

energy needs. This mechanism promotes energy accumulation as fat and reduces energy expenditure (Manco et al., 2008).

Nowadays, the explanation of the pathology of NAFLD is based on the "two hit" hypothesis that suggests that insulin resistance is induced by the inability of adipose tissue to accommodate the excess of dietary fat. As a result, the release of several adipocytokines is enhanced; this in turn converts insulin resistance in a vicious cycle. Insulin receptors aiming to mediate signalling, use docking proteins and activate three major pathways which finally lead to increased levels of gluconeogenesis and *de novo* lipogenesis. By such mechanism the levels of free fatty acids are increased. Some are stored in the liver and muscle and the rest are exported to the plasma, incorporated into circulating lipoproteins and thus contributing to the hyperlipidemia appearing in the plasma of NAFLD patients. As a result of fat accumulation in the liver and muscle, the mitochondrial fatty acid beta oxidation is increased. Due to the high input of electrons which have restricted access to the respiratory chain, some of them can react with oxygen and form reactive oxygen species (ROS). In turn, ROS, reactive nitrogen species (RNS) and reactive aldehyde lipid peroxidation products can directly damage mitochondrial DNA and polypeptides of the respiratory chain. Antioxidant enzymes are rapidly consumed and become insufficient to combat the increasing concentration of ROS which can directly induce cell apoptosis through the activation of NF-kB factor (Manco et al., 2008). In addition to this mechanism, it has been pointed out the additional hepatic oxidative stress caused by an intestinal bacterial overgrowth. This can occur by two mechanisms: increase of endogenous ethanol production and release of bacterial lipopolysaccharides (Szalman et al., 2013).

Overall, hepatic steatosis can be induced by several possible sources such as: (1) increased free fatty acid (FFA) delivery to the liver as a result of dietary fat intake and increased lipolysis within insulin-resistant adipose tissue; (2) increased hepatic *de novo* lipogenesis (DNL) mediated by increased expression of lipogenic protiens (fatty acid synthesis, FAS); (3) decreased FFA oxidation mediated by PPAR (peroxisome proliferator receptor activity-alpha) and (4) decreased triacylglycerol export from the liver in the form of very low-density lipoprotein (Finelli & Tarantino, 2012; Postic & Girard, 2008). Indeed, the altered metabolism in adults with MetS and, in particular, in adults with NAFLD, has been observed by the increased synthesis of fatty acids during night and higher insulin during daytime (Lambert, Ramos–Roman, Browning, & Parks, 2014).

2.2.4 Diagnosis

Patients suffering NAFLD may complain of fatigue and sense of fullness or discomfort in the right upper abdomen. In patients with NAFLD a mild to moderate elevation of serum aminotransferases is the most common abnormality. With the development of fibrosis, aspartate transaminase/alanine transaminase (AST / ALT) ratio can increase above 1, while it is normally found below this value (Angulo, 2007; Manco et al., 2008). The elevated concentrations of one or both enzymes have been so frequent that nowadays is used as a surrogate for suspected fatty liver (Purcell, Flores, Zhang, Denova-Gutiérrez, & Salmeron, 2013). Other methods used for the diagnosis of NAFLD include abdominal ultrasonography and determination of serum ferritin and uric acid. However, the main disadvantage of the first method is its high cost and further investigation is needed to improve the efficacy of the other two tests to be evaluated (Kim et al., 2012; Lee et al., 2009).

Indeed, whereas NAFLD can be diagnosed by determination of aminotranferases and ultrasound evaluation of the liver brightness, the grade and stage of NASH requires liver biopsy (Angulo, 2007; Manco et al., 2008). Using liver biopsy the histological appearance of the liver can be evaluated. It can range from small and large droplet macrovescicular steatosis, with or without lobular and portal inflammation and cell injury. It has been demonstrated that 10-15% and 15-25% of NASH patients develop fibrosis and cirrhosis, respectively. Cirrhosis in turn, can lead to hepatocellular carcinoma (HCC) (Brunt & Tiniakos, 2010; Bugianesi, 2007).

The accumulation of fat in the form of triglycerides within hepatocytes is the main histological characteristic of NAFLD. It is a lesion originally termed "steatosis" after the ancient Greek word for fat, "stear". Steatosis in NAFLD is usually macrovesicular, referring to hepatocytes with a single large intracytoplasmic fat droplet or smaller well defined droplets displacing the nucleus to the cell periphery. Mixed steatosis might also occur, when, in addition to macrovesicular steatosis, groups of hepatocytes with centrally placed nuclei and numerous minute lipid droplets in the cytoplasm are observed. In simple NAFLD, in addition to steatosis, foci of lobular inflammation, mild portal inflammation, and lipogranulomas may be seen (Brunt & Tiniakos, 2010).

2.2.5 Association of NAFLD with other pathologies

Aiming to determine the independent factors of NAFLD, a multicentre, observational study including patients and healthy individuals (327 cases and 377 controls) observed that the independent variables associated with this pathology were obesity, insulin resistance and elevated ALT and GGT. The factors that resulted as best indicators were hyperglycemia and high triglyceride values (Caballería et al., 2013). In elderly populations as well, the presence of NAFLD was positively associated with higher BMI, serum levels of glucose and insulin, triglycerides, cholesterol, visceral adipose tissue and lower serum levels of HDL-cholesterol (Gianotti et al., 2014). In addition, in menopausal women, NAFLD is proved increased and negatively associated with the hormone replacement therapy treatment although more studies are required in order to confirm this finding (Florentino et al., 2013).

However, the importance of BMI is not completely clear since a study aiming to investigate the correlation between anthropometric and serologic factors with the histological changes in liver associated with NAFLD, found no correlation between BMI and this pathology. In contrast, significant though weak correlation was found with waist circumference. As for the plasmatic parameters, glucose concentration was proportional to the severity of the histological changes and ALT concentration had a positive correlation, whereas cholesterol and triglyceride concentrations showed positive relationships but not significant correlations with the presence of NAFLD (Szalman et al., 2013).

Even though the relationship between anthropometric parameters and NAFLD still remains unclear, with no doubt, the presence of obesity is linked to the presence of NAFLD. In addition, insulin resistance has demonstrated a significant association with the development of this pathology. In this regard, a study carried out in Indian patients with NAFLD, demonstrated that 80% of the patients were obese, 30% presented MetS, and 97.5% suffered from insulin resistance (Bhat & Baba, 2013). Moreover, in animal studies the strong correlation between hyperinsulinemia and NAFLD has been observed. In obese diabetic mice, hyperinsulinemia enhanced the nuclear transcriptional regulators of cholesterol homeostasis such as low density lipoprotein receptor, which in turn led to hepatic accumulation of free cholesterol. The resulting cytotoxicity may induce an easier transition from steatosis to NASH (Van Rooyen et al., 2011).

The association of altered hepatic enzymes as markers of liver function, and the development of NAFLD have also been studied. Both ALT and GGT are sensitive indicators of liver damage. On one hand, serum ALT is associated with liver fat accumulation, while in the other hand,

GGT, located in the external surface of most cells, mediates the uptake of glutathione (Oh et al., 2006). The study of (Oh et al., 2011), demonstrated that the activities of both enzymes were correlated with the presence of NAFLD in an adult healthy Korean population, also showing that ALT was a more sensitive indicator compared with GGT. This is possibly related to the direct relationship of this enzyme and the NAFLD clinical manifestations. Furthermore, elevated levels of ALT have been observed among obese and overweight when compared with normal BMI boys and girls (El-Karaksy et al., 2012; Purcell et al., 2013), and related to high waist circumference, hypertension and elevated triglyceride content in a child population in Chile (Arancibia et al., 2012). Bilirubin, the end product of heme catabolism in mammals is generally regarded as a waste product that needs to be excreted. However, it has been demonstrated the *in vitro* antioxidant capacity of this compound by supressing the oxidation of tocopherol (Stocker et al., 1987). In regard, the mean total bilirubin has a negative correlation with NAFLD in children which indicates that high bilirubin levels could be associated with a decreased likelihood of histological diagnosis of NASH (Puri et al., 2013).

2.2.6 Prevention and treatment

Up to date, there is no evidence on how the progression of NAFLD can be slowed down. However, it appears that preventing the development of insulin resistance and/or the rest of clinical manifestations linked to this pathology may help to prevent its development.. Strategies achieving maintenance of body weight may help the treatment of glucose and lipid disorders associated with NAFLD (Farrell & Larter, 2006). In general, lifestyle interventions that include exercise enrolment and diet modification can attenuate and even reverse the progression of NAFLD as shown in follow up biopsies of non-obese patients (Jin et al., 2012).

In the study of (Orchard et al., 2005), it was shown that drug administration (metformin) combined with a lifestyle intervention programme reduced the severity of MetS complications and subsequently attenuated the development of NAFLD. Another study showed that the combination of ezetimibe (5mg/kg/day) and/atorvastatin (20mg/kg/day) treatment lowered the levels of hepatic cholesterol, reduced serum insulin levels and ALT levels in female foz/foz mice, thus inversing the histological damage associated with NASH in 80% of the animals (Van Rooyen et al., 2013).

2.2 Dietary Treatment

Up to date, strategies targeting the treatment of the metabolic disorders involved in the NAFLD seem to be an adequate therapeutic alternative for this disease. Advices mainly focusing to lifestyle changes, consist in low-fat/low-glycaemic index diets and regular physical exercise (Manco et al., 2008). For this reason, legumes have gained increasing interest given that their frequent consumption can help in the control of lipid homeostasis and, consequently, reduce the risk of cardiovascular diseases. Moreover, their low glycaemic index and high content of indigestible fibre are associated with better glycaemic control of diabetic individuals. It is also demonstrated that legumes accelerate the transit of digested food, which can be effective at decreasing re-absorption of cholesterol, increasing the amounts of undigested starch and enhancing the hind gut fermentation process; all beneficial factors for the prevention of colon cancer. Finally, due to their effect on appetite–regulating hormones and satiety, legumes are claimed to help in the maintenance or reduction of body weight and improvement of metabolic risk factors (Duranti, 2006; König, Muser, Berg, & Deibert, 2012).

2.3.1 Legume protein hydrolizates

The production of legume protein hydrolizates is a particularly encouraging option to improve non-pharmacologic health strategies in addition to lifestyle changes, the use of dietary supplements and other functional foods. Specific dietary ingredients such as the ACE-inhibitory peptides are produced by hydrolyzing the proteins from different animal or plant foods, in particular, milk, soy, pea, and other legumes (Boschin, Scigliuolo, Resta, & Arnoldi, 2014)

It has been demonstrated that protein content of legumes range from 17-40%. Nevertheless, legumes are not only a protein-rich foodstuff. They also represent valuable dietary sources of complex carbohydrates, dietary fibre, minerals and vitamins (Butt & Batool, 2010). Unfortunately, legumes are reported to have low nutritive value due to the low amounts of sulphur-containing amino acids, low protein digestibility and the presence of anti-nutritional factors. In this regard, several physical and biotechnological processes such as soaking, cooking, dehulling, fermentation, germination, irradiation and enzymatic hydrolysis have been applied to legumes in order to improve their nutritional value (Mubarak, 2005; Porres et al., 2003) and enhance their health-promoting potential (Doblado et al., 2007).

The rising interest on developing new processing conditions that may improve both nutritional and health-related properties of legume foodstuffs arises from the continuous

shortage of protein in the diets of people mostly living in developing countries as well as the increasing prevalence of alterations in lipid metabolism appearing in people living in developed countries. According to the definition of World Health Organization, protein energy malnutrition (PEM) refers to "an imbalance between the supply of protein and energy and the body's demand for them to ensure optimal growth and function" (WHO/NUT/97.4). Some severe health problems are related to the lack of protein in the diet, such as physical and cognitive impairments or the kwashiorkor nutritional disorder, more often described in developing countries but still happening in developed countries as well (Bhutia, 2014; Rogers, Shaughnessy, & Davis, 2014). Protein malnutrition is also associated with anemia, leukopenia and general low immune response, affecting the expression of cytokines that are implicated in both immunity and inflammation pathways (Mello et al., 2014). Finally, more oxidant damage products (MDA and PC) and less antioxidant levels (Cu,Zn-SOD, Cp, GSH, and ascorbic acid) were described in protein malnourished children (Khare et al., 2014). In general people with protein malnutrition are more susceptible to infections (Calder, 2013; Mello et al., 2014).

On the other hand, sedentary lifestyle and high dietary fat intake in the industrialized countries have led to increased prevalence of obesity and coronary heart disease (Pereira & Gibson, 2002). The increased risk for cardiovascular disease as well as diabetes 2 are associated with MetS which in turn is linked to atherogenic dyslipidemia, elevated blood pressure, plasmatic glucose and other complications. In this regard, the ATP III update placed primary emphasis on lifestyle therapies including an antiatherogenic diet which may result in reducing the concentration of blood cholesterol and triglycerides (Grundy, 2005).

The legume-protein hydrolyzates are a good example for the preparation of new legume-based foodstuffs that could be included in the diet and act as potential health-promoting agents with antiatherogenic or antihypertensive effects. They are usually produced by protein extraction under alkaline conditions followed by hydrolysis process using different exoproteases like alcalase or flavourzyme (Megías et al., 2007a). Besides their high protein content, legume protein hydrolysates contain bioactive compounds that may act as potential physiological modulators of metabolism, given that they inhibit the activity of angiotensin converting enzyme and exhibit antioxidant and bile acid-binding properties (Segura Campos, Chel Guerrero, & Betancur Ancona, 2010; Yoshie-Stark & Wäsche, 2004), thus showing promising potential as functional ingredients. Furthermore, the beneficial effects of a pea protein hydrolysate on blood pressure were also demonstrated in both spontaneously hypertensive rats and human subjects (Li et al., 2011). In experimental models of diet-induced hypercholesterolemia, protein hydrolysates of

Lupinus albus and soybean were effective at reducing plasma and hepatic triglycerides (Choi et al., 2011; Kapravelou et al., 2013).

2.3.2 *Lupinus albus*

Lupin is a non-starch leguminous seed that belongs to the Leguminosae (or Fabaceae) family. It is an economically and agriculturally valuable plant able to grow in different soils and climates (Sujak, Kotlarz, & Strobel, 2006). Two of the *Lupinus* species, *Lupinus albus* (white lupin) and *Lupinus luteus* (yellow lupin), are mostly cultivated in Europe whereas *Lupinus angustifolius* (narrow-leafed lupin) is in Australia and *Lupinus mutabilis* (Andean Lupin) is essentially grown in South America (Annicchiarico, Manunza, Arnoldi, & Boschin, 2014). Lupin seed is characterized for its high protein content that makes it suitable for producing traditional human foods or used as animal feed. Its strong capability for nitrogen fixation and organic phosphorus release from soil makes it appropriate for crop rotation during intensive grain production (X. H. Fan, Tang, & Rengel, 2002; Honeycutt, 1998). In Western societies, it is used as an effective alternative by individuals wishing to substitute animal with plant protein in their diet to prevent cardiovascular diseases. It is also used as part of diabetic diets or celiac diets owing to the low starch content and the composition of the proteins that contain (Sirtori et al., 2004); (Annicchiarico et al., 2014). The study of (Cerezal Mezquita, Urtuvia Gatica, Ramírez Quintanilla, & Arcos Zavala, 2011) showed that the development of a sweet flour mixture containing quinua (*Chenopodium quinua* Willd) and lupin~~o~~ (*Lupinus albus* L) mixed with two traditional cereals like maize (*Zea mays* L.) and rice (*Oryza sativa* L.) was a good alternative as food supplement for celiac children aged 6-12 months. Among the advantages of the product was the improvement in protein quality of the foodstuff after the essential amino acid compensation provided by the combination of the four ingredients.

The utilization of this plant in food industry is nowadays extended to the production of protein concentrates that can be added to other food products and contribute to the enhancement of their nutritional and health promoting value, thus resulting in functional foods (Linnemann & Dijkstra, 2002).

2.3.2.1 Macronutrient, micronutrient content and non-nutritional compounds

Lupin seeds contain high concentrations of proteins (30-35%) which according to Osborne's classification in 1924, belong to the albumin and globulin fractions (ratio 1:9). Albumins are considered as the functional proteins whereas globulins the main storage proteins of the seeds.

The globulin fraction of lupin protein consists of two major globulins. The first is called α-coglutin (11S) and accounts for the 33% of total protein content. The second is β-conglutin (7S), which accounts for another 45%. There are two additional globulins of minor quantity called γ- and δ-conglutin, which account for 5 and 12%, respectively. Some reports have further sub-classified δ-conglutin into δ1- and δ2- conglutin (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008; Sirtori et al., 2004). The proteins above mentioned constitute 85-88% of the seed proteins whereas the rest is constituted by minor proteins, such as a Bowman–Birk serine proteinase inhibitor (BBI) and trypsin inhibitory activity (Duranti et al., 2008; Scarafoni, Magni, & Duranti, 2007).

In the study of (Sujak et al., 2006), in which the four varieties of lupinus species were compared, it has found that the protein content of the lupin seeds were higher than those of a most legume seeds and that *L. albus* had the second highest protein content after *L. luteus*. Similar results were also found for the Nitrogen-free extract in *L.albus*. This fraction is mainly consisted of starch, sugars and pectins, water-soluble non starch polysaccharides (NSP) as well as oligosaccharides. The oil content of most lupinus species is found to be relatively low, and white lupin exhibited the highest values. All the lupin extracts had medium/high amounts of crude fibre. With regard to the amino acid composition of lupin seed, a methionine and sulphur-containing deficiency is manifested. Additionally, poor levels of lysine, tryptophan and valine were detected whereas the level of leucine was satisfactory. Indeed, the essential amino acid content (EAA) calculated on the basis of mature human or whole egg standards, was below the 36g/16gN recommended by (Favier, Ireland-Ripert, Toque, & Feinberg, 1995). However, the variety of white lupin was proved to have greater amounts of total amino acids and higher chemical score as revealed from the comparison of concentrations of less abundant amino acid(s) to a standard.

Table 3. Chemical and amino acid composition of *Lupinus albus* seeds (g/kg Dry matter). Taken from Sujak et al. (2006)

Crude Protein	363
Oil	115
Fibre	144
Ash	39
N-free extract	339
Alkaloids	0.39

Essential amino acids (g/16gN)		Non-essential amino acids (g/16gN)	
Lys	4.9	Arg	11.1
Met+Cys	2.5	Asp	9.9
Cys	1.9	Ser	4.1
Thr	3.5	Glu	24.2
Ile	4.3	Pro	3.8
Trp	0.6	Gly	4.3
Val	4.1	Ala	3.1
Leu	7.8		
His	3.3		
Phe+Tyr	5.6		
Tyr	1.7		

In the newly developed sweet varieties the presence of anti-nutritional compounds, such as alkaloids and oligosaccharides is relatively low when compared to other lupin species. However, the quinolizidine alkaloids (QA), main antinutritional factors in lupin seeds, are responsible for their bitter taste, the unpalatability of the seeds, and sometimes their toxicity. The toxicity effects include neurological effects that can lead to the loss of motor coordination and muscular control. They are synthesized by lupin plants as a defense mechanism against pathogens and herbivores.

The main QA reported for *L. albus* are lupanine, albine, multiflorine, and 13α-hydroxylupanine, with minor amounts of α-isolupanine, angustifoline, 11,12-seco-12,13-didehydromultiflorine (formerly N-methylalbine), and some esters of 13α-hydroxylupanine. Regarding the oligosaccharide content of lupin seeds, it has been reported that although they are also considered as anti-nutritional factors since they negatively affect the digestibility and nutrient absorption, they add up great advantages in the production of different fermented products such as bread and pastry when lupin flour is used (Annicchiarico et al., 2014; El-Adawy et al., 2001; Erbaş, Certel, & Uslu, 2005; Michael, 2003).

It has been also reported that Lupin proteins can elicit food allergy responses in lupin-sensitised patients and in patients sensitised to other legume seeds, especially peanut, as a result of the sequence/structure similarities of the storage proteins. Although data are still insufficient to identify the major lupin allergens, β-conglutin, the basic subunits of α-conglutin and γ-conglutin appear to be frequently detected by specific IgEs (Duranti et al., 2008).

2.3.2.2 *In vitro* and *in vivo* studies including *L.albus* seeds and its protein hydrolizates

The hypolipidaemic effects of the white lupin seeds have been reported by various authors. In a model of rats fed with hypercholesterolemic diet, the administration of 50mg/d of total protein extract of white lupin managed to decrease total cholesterol (21%), VLDL cholesterol (30%) and triglycerides levels (17%). Moreover, the HDL-cholesterol tended to be 20% higher in lupin-treated rats whereas the concentration of glucose in plasma did not seem to be affected (Sirtori et al., 2004). In a similar experimental model, the inclusion of Lupin protein extract in a hypercholaesterolemic diet, beneficially affected the hepatic triglycerides of rats, suggesting that a possible alteration in mRNA transcript levels of genes involved in the de novo synthesis of fatty acids, as well as genes associated with TAG hydrolysis may be responsible for such improvement. The observed down regulation of SREBP-1c, and its regulated genes such as G6PDH, FAS, SCD1 and GPAT strongly suggests a diminished lipogenesis via SREBP-1c pathway (Bettzieche et al., 2008). In addition, lupin seed isolate had beneficial effects in the development of atherosclerosis in hyperlipidaemic rabbits, by reducing the volume, macrophage number, and lipid accumulation of the atherosclerotic plaques (Marchesi et al., 2008).

Similar beneficial effects on the cholesterol levels were also confirmed in human subjects with mild hypercholesterolemia, who were willing to undergo a non-drug therapeutic approach. The study aiming to develop new strategies for the prevention and control of hypercholesterolaemia,

showed a decrease in total cholesterol levels of the subjects who consumed the energy bars that included lupin as a protein source and cellulose as a source of dietary fibre (lupin/cellulose) (Sirtori et al., 2012).

Regarding the prevention of hypertension, lupin has demonstrated beneficial effects in animal and human studies. The study of (Pilvi et al., 2006), used the Goto-Kakizaki (GK) experimental model administrating a high-NaCl diet and showed that the administration of a lupin protein diet managed to reverse this pathology, lowering the values of blood pressure to normal levels and improving the impaired vascular function of the animals. The authors suggested that relatively high arginine content of lupin protein which acts as a physiological substrate for endothelial nitric oxide synthase could be on the basis of its beneficial action. Similar effects on blood pressure have been demonstrated in human studies after the consumption of protein and lupin fibre-enriched diet or lupin-enriched food products such as bread (Belski et al., 2011; Lee et al., 2009).

The antidiabetic properties of lupin seeds have also been confirmed due to the protein content of γ-coglutin, a glucoprotein that constitutes 5% of total seed proteins. The decrease in glycaemia was demonstrated by the study of (Capraro et al., 2014). In this study, supplemented pasta with lupin protein fractions was administered to Sprague Dawley rats and the results revealed lower levels of plasmatic glucose and AUC, together with a reduction on food intake and body weight of the animals.

The above discussed beneficial effects have also been demonstrated by *in vitro* studies. In HepG2 cell line, lupin protein proved to increase the activity of LDL receptors suggesting that this could be the explanation of the hypocholesterolemic effects demonstrated *in vivo* by this legume (Sirtori et al., 2004). In the same cell line, the study of (Capraro et al., 2013) described how γ-coglutin can enter in an intact form and accumulate in the cytosol of these cells, thus explaining the hypoglycaemic effects of lupin.

2.3.3 *Vigna radiata L.*

Vigna Radiata L., also known as *Phaseolus aureus*, mung beans and green gram is a popular food legume in Southern and Eastern Asian countries (China, Korea, Japan) Central Africa, South and North America and Australia (Dahiya et al., 2013; Huang, Cai, & Xu, 2014). It is believed to be a native crop from to the northeastern India-Burma (Myanmar) region of Asia (Dahiya et al., 2013; Yao et al., 2013). The grains are green or brown coloured and globose in shape with a flat hilum. The crop's main advantage is that, as a legume, does not require

fertilization from nitrogen (Murakami et al, 1991) since it can develop root nodules and fix the nitrogen from the ground in symbiosis with compatible rhizobia. It is one of the most important summer- growing legumes and has a short growth cycle (75 - 90 days). It requires little water and grows well under adverse arid or semiarid conditions. That is why it fits to different cropping systems and can be seeded after cereal crop and harvest (Dahiya et al., 2013; Graham & Vance, 2003).

It is consumed in many different forms depending on the cooking culture of every country. It can be consumed as a main dish boiled or cooked, mixed with vegetables or meat. Also as a desert or incorporated in bread or cake. It can be used to make sprouts for egg rolls or used as fresh vegetable in salads (Graham & Vance, 2003; Tang et al., 2014). In China, it is used as an ingredient of a cold soup which is prepared by boiling and then filtering the water in which the food is prepared (Cao et al., 2011).

2.3.3.1 Macronutrient content

The macronutrient composition of *Vigna radiata* varies with the different environmental conditions. It is estimated to provide 45-62% carbohydrates, 20-28% protein content and 1% fat. Mung bean protein is showed to be rich in essential amino acids such as leucine, isoleucine, and valine but slightly deficient in threonine, sulphur amino acids, lysine and thryptophan as compared with the Food and Agriculture Organization (FAO)/World Health Organization (WHO) (1973) reference (Tang et al., 2014). Among the carbohydrates, starch is the most predominant in this food. The fat composition of *Vigna radiata* is predominantly unsaturated, and among the lipid fractions; phospholipids are the most abundant followed by triglycerides (32.26% and 30.10%), whereas 1,3 diglycerides constitute the least percentage (2.8%) (Abdel-Rahman et al, 2007). Although fatty acid profile varies due to the origin of the seeds, it is demonstrated that the main fatty acid of *Vigna Radiata* is linolenic acid and it is followed by oleic and palmitic (Tang et al., 2014). Fibre content was determined to range from 8.89% to 12.85%, and insoluble fibre was the most predominant fraction (Anwar et al, 2007).

Table 4. Macronutrient composition of mung bean. Taken from Dahiya et al. 2013

Macronutrient	Average value of collected data
Protein (g/100g)	23.8
Carbohydrate (g/100g)	61.0
Lipid (g/100g)	1.22
Moisture (g/100g)	9.80
Fibre (g/100g)	4.57
Ash (g/100g)	3.51
Energy (kcal/100g)	344

2.3.3.2 Micronutrient content

Total mineral composition of *Vigna radiata* seeds varies from 2.96% to 3.39% and is rich in calcium, phosphorous, magnesium and potassium (Dahiya et al., 2013; Graham & Vance, 2003). Organic acids have also been found in mung beans and sprouts. Twenty-one organic acids, including phosphoric and citric acid, and 16 lipids, including *γ*-tocopherol, were reported to be the major components of mung beans as revealed by gas chromatography/mass spectrometry (GC/MS) (Tang et al., 2014). The content on tocopherols of *Vigna Radiata L* is also influenced by the different varieties. *γ* –tocopherol ranged from 60.7 to 80.9 mg/kg of dry seeds whereas *a*-tocopherol, demonstrated by Vitamin E activity, and *δ*-tocopherol varied from 3.1 to 10.1 and 4.6 to 11.2 mg/kg of dry seeds, respectively. The most predominant vitamin of this legume is ascorbic acid, followed by niacin and pantothenic acid (Anwar et al., 2007; Dahiya et al., 2013).

It is well known that mung beans contain high levels of secondary bioactive compounds such as flavonoids and phenolic acids. The most important flavonoids of mung beans are flavone, isoflavone, flavonoids and isoflavonoids which seem to have beneficial effects on stress condition, protection from insects, antimicrobial activity and early development of the plant

(Dénarié, Debellé, & Promé, 1996; Veitch, 2007). Flavonoids usually contain polyhydroxy substitutions and are considered as polyphenols. Extracts of *Phaseolus vulgaris L.*, rich in flavonoids and saponins, have reported to exhibit cholesterol homeostasis modulatory effects via the transcription factors sterol regulatory element-bindig proteins (SREBP) and liver X receptor (LXR) in rat hepatocytes (Chavez-Santoscoy et al., 2014).

Still, phenolics are considered as the most important bioactive phytochemicals of mung bean due to the high anti proliferative activity they present. Twelve of them have been identified in mung bean seeds and sprouts and among them p-coumaric, ferulic and synaptic acid are encountered; ferulic acid being the most predominant among them (Kim et al., 2012; Yao et al., 2011). The content of phenolic acids is affected by the colour of the seeds; the darker colour they have, the higher phenolic content they contain. In this regard, the study of (Anwar et al., 2007) revealed that total phenolic contents of four different varieties of *Vigna radiata* L. (2 to 3 mg 100 per gram of flour) was lower compared with other legumes that have darker seed coat colour such as pigeon pea (Kanatt, Arjun, & Sharma, 2011). Higher contents of phenolic acids (81.4 and 87.1 mg GAE/g dry extract) were reported by the studies of (Yao et al., 2011) and (Kruawan, Tongyonk, & Kangsadalampai, 2012), the latter reporting highest values in mung bean seed coat extract.

Beneficial effects such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities, tyrosinase inhibition, and antiproliferative and alcohol dehydrogenase activities have been attributed to the high levels of these secondary metabolites contained in mung bean. This explains why *Vigna radiata* can be used as a substitution for drugs and as a preventive or therapeutic agent for the treatment of human diseases (Kim et al., 2012). The antioxidant capacity of *Vigna radiata L.* seems to de associated to its phenolic content and affected by the medium on which the extraction process is carried out. A strong correlation between total phenolic content (TPC) and antioxidant activity was revealed in mung bean seeds as well as high tyrosinase inhibition activity (Kanatt et al., 2011; Yao et al., 2011). On the other hand, different fractions of extracts have shown different values of phenolic content, lipid peroxidation and protection of antioxidants against oxidative damage (Chung et al., 2011).

2.3.3.3 Non nutritional compounds

The term non nutritional factors refers to the chemical compounds that when present in food, affect negatively its digestion and the bioavailability of nutrients, thus restricting the whole nutritional potential of the food (Dahiya et al., 2013). Nevertheless, some of the so called non

nutritional factors are also responsible for the health benefits usually related to legume consumption. They may occur naturally, such as glucosinolates in mustard and rapeseed protein products, trypsin inhibitors and hemagglutinins in legumes, tannins in legumes and cereals, phytates in legumes, cereals and oilseeds, and gossypol in cottonseed protein products. However, they can also be formed during heat/alkaline processing of protein products, yielding Maillard compounds, oxidized forms of sulphur amino acids, D-amino acids, and lysinoalanine (LAL, an unnatural amino acid derivative). High levels of these factors in legumes and cereals have been associated to reduced protein and amino acid digestibility (Gilani, Cockell, & Sepehr, 2005). The non-nutritional factors that have been reported in *Vigna radiata* are tannins, phytic acid, haemagglutinins, flatulence factors and trypsin and proteinase inhibitors (Dahiya et al., 2013).

Phytic acid (*myo*-inositol-1, 2, 3, 4, 5, 6- hexaphosphate) is the storage form of phosphorus in seeds of cereals and legumes. It is found inside the protein bodies of cotyledons and endosperm, respectively, and considered essential for seed development and germination. Phytates are considered antinutritional factors given that they have high affinity for minerals and can bind to them in the digestive tract, inhibiting their absorption. In addition, they are not efficiently digested by human and nonruminant animals and their excretion can lead to accumulation of phosphorus in soil and water (Urbano et al. 2000; Lott et al. 2000; Sompong et al. 2012; Dahiya et al. 2013).

Saponins are considered as part of the defence system of the plants and are characterized by their bitter taste, their foaming capacity in aqueous solutions, and their ability to hemolyse red blood cells. Saponins have both deleterious and beneficial effects; they are associated with weight loss in animals and hypochoresterolemic properties in humans. The majority of their properties are attributed to their capacity to affect the membrane integrity (Francis, Kerem, Makkar, & Becker, 2002).

Oligosaccarhides, including raffinose, stachyose, and verbascose are responsible for causing flatulence in the human diet (Martínez-Villaluenga, Frias, & Vidal-Valverde, 2008). Though, their solubility in water makes easy their elimination by processes such as soaking, germination or fermentation (Tang et al., 2014). Haemagglutinins are sugar binding proteins and induce agglutination of red blood cells. They lead to abnormal absorption of nutrients due to their ability for binding to specific receptors at the epithelial cells that line the digestive tract (Dahiya et al., 2013).

Tanins are polyphenolic compounds that affect negatively protein digestibility by binding strongly to this nutrient and interfering with the action of the proteolytic enzymes. It has been demonstrated that the highest percentage of tanins is present in the seed coat and not in the cotyledon, thus indicating that dehulling is an efficient method for their reduction.

Table 5. Macro and micronutrient composition of *Vigna radiata* as reported by Dahiya et al., 2013.

Carbohydrates (%)

Monosaccharides	Total soluble sugars 5.6	Reducing sugars 1.8	Non reducing sugars 6.3	Glucose 0.3		
Oligosaccharides	Sucrose 1.3	Raffinose 1.1	Stachyose 1.6	Verbascose 2.7		
Fibres	Total dietary fibre 18.8	Insoluble fibre 15.3	Soluble fibre 2.3	Lignin 3.9	Cellulose 3.9	Hemicellulose 4.7
Starch	Amylose 24	Starch 47				

Amino acids (g/16g of Nitrogen)

Alanine 4.1	Arginine 5.8	Aspartic acid 13	Cysteic acid 13.5	Glutamic acid 18.3	Glycine 3.6	Histidine 3.2	Isoleucine 4.3	Leucine 7.6
Lysine 6.5	Methionine 1.2	Phenylalanine 5.4	Proline 4.5	Serine 4.9	Threonine 3.2	Tryptophan 1.2	Tyrosine 2.7	Valine 5.1

Lipids (% of total fat content)

Total saturated fat	Total unsaturated fat	C16:0 (Palmitic)	C18:0 (Stearic)	C18:1 (Oleic)	C18:2 (Linoleic)	C18:3 (Linolenic)	C21 (Docosanoic acid)
27.7	72.8	14.1	4.3	20.8	16.3	35.7	9.3

Vitamins (mg/100g dw)

Thiamine	Riboflavin	Niacin	Vitamin C	Pantothenic acid	Nicotinic acid
0.5	0.3	2.2	3.1	1.9	1.6

Minerals (mg/100g dw)

Ca	Cu	Fe	K	Mg	Mn	Na	P	Phytin	Zn
113.4	1.0	5.9	326	162.4	1.05	16.7	384.4	171.3	2.7

Non nutritional factors

Tannins (mg/100g)	Phytic acid (mg/100g)	Haemagglutin activity (HU/g)	Polyphenols (mg/100g)	Trypsin inhibitor activity (TIU/mg of protein
366.6	441.5	2615	462.5	17.3

2.3.4 Germination

Figure 2. Mung bean seeds (A) and germinated seeds for 24h (B), 36h (C), 48h (D), 60h (E) and 75h (F). Taken from Jom, Frank and Engel (2010).

Germination is the process by which a plant grows from a seed, using as the most common example the sprouting of a seedling from a seed of an angiosperm or gymnosperm. During this process, the proteolytic activity is increased in the cotyledons of the seeds. Germination is a biotechnological process widely used in legumes as it is proved to be an easy, economic and effective technique through which digestibility and nutritional quality of these foods are enhanced in a short period of time (Fernandez-Orozco et al., 2008; Silva et al., 2013). Briefly, germination consists in soaking the seeds in water for some hours and afterwards maintain them wet in light or darkness conditions during some days. During this period the physical changes of the seed include the seed break and the elongation of the radicle. Nowadays the consumption of sprouts has been growing due to their consideration as part of a safe and healthy diet pattern (Silva et al., 2013).

Several changes in macro and micro nutrient content are required to take place in order to carry through the germination process. For example, an increase of protein content is attributed to the use of the seed components into the growing sprout or in other words in the biosynthesis occurring during germination while fat and carbohydrates are probably used as an energy source to start this process (Mubarak, 2005). Nevertheless, there are many factors that determine the extent of nutrient changes and affect the effectiveness of the process such as the quality of the seeds and the duration of germination.

Different studies showed different changes in the macro and micro nutrient content of legumes. The studies of (Gujral et al, 2011) and (El-Adawy et al, 2003), showed that after a two-day germination, protein and starch content decreased whereas the opposite effect was observed in fat and mineral content. In contrast, (Ghavidel & Prakash, 2007) showed that after a 2 day germination period, protein and thiamine contents increased significantly whereas moisture and fat content decreased. When the duration of the germination process increased from two days to

three, protein and moisture content increased while fat, mineral and total carbohydrate content were reduced. The content of α-galactoside oligosaccharides such as stachyose and raffinose was reduced by germination but no changes were observed regarding the amino acid content. On the other hand, soluble and total dietary fibre increased and insoluble fibre was reduced (Mubarak, 2005).

The process of germination, caused a decrease in the phospholipids and triglycerides content of *Vigna radiata* while it increased that of monoglycerides1,2-(2,3)-diglycerides, sterols and free fatty acids (Abdel-Rahman et al., 2007a). However, controversial effects of germination were found in the mineral content of this legume. (Gujral et al., 2011) found no significant differences in K, Ca, P, Mg, Fe and Mn levels whereas (Ghavidel & Prakash, 2007) observed a significant decrease in Fe, Ca, and P content due to germination.

Antioxidant activity, is usually enhanced by the germination process (Doblado et al., 2007) although it seems affected by the duration of the whole process. Two-day germination decreased antioxidant activity, measured as DPPH values by (Gujral et al., 2011). However, (Fernandez-Orozco et al., 2008) showed that total phenolic content declines the first two days of germination and then increases up to the fourth day. Furthermore, the study of (X. Guo, Li, Tang, & Liu, 2012), showed that germination induced a time dependent increase in flavonoid, total phenolic and free phenolic content, all strongly linked to antioxidant activity. Vitamin C content of *Vigna radiata* L. sprouts appeared to be higher when compared with the raw seeds of this legume (Doblado et al., 2007; X. Guo et al., 2012).

Kim et al., (2012) showed that germination affected positively the content of total phenolics (TP), total flavonoids (TF), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity activity when compared with the raw seeds. In addition, sprouts also showed higher tyrosinase inhibition and ADH activities. Another factor that seems to affect the DPHH scavenging activity and ADH activity of the extracts of this legume is the medium in which the extraction of mung bean sprouts is carried out. For instance, ethanol extracts showed higher DPHH scavenging activity and higher antiproliferative effect on two human cancer cell lines, Calu-6 and SNU-601, when compared to the other solvents used to extract raw or germinated mung bean seeds. However, ADH activity was determined higher in water extracts of mung bean. This can lead to the conclusion that the extracts of the germinated seeds are more effective than the extracts of the raw seeds and are characterized by higher antioxidant activity, although this parameter is affected by several factors (variety, extract medium used).

Germination has a positive effect upon non-nutritional components that are negatively correlated with the bioavailabily of essential minerals such as iron and calcium (Ghavidel & Prakash, 2007). It seems that germination decreases the levels of trypsin inhibitors, tannins, phytic acid and hemagglutinin activities (El-Adawy et al., 2003; Ghavidel & Prakash, 2007; Mubarak, 2005). The increases of *in vitro* digestibility of protein and starch that are observed in germinated mung beans are attributed to reduction of phytic acid by increasing phytase activity (Ghavidel & Prakash, 2007) as well as the decrease of tannins, tripsin inhibitor and the pre-digestion of protein (Mubarak, 2005).

There has been a systemic assessment with the aim to determine the optimal germination time of *Vigna radiata L.* It was found that the most effective elongation of the radicle as well as the higher amount of phenolics of this specific legume is the early stage of germination (1-3days). In addition, during this period, there was an increase of ascorbic acid content and DPPH values; the first in a time dependent manner. The results of this study, suggest that a 3-4-day germination might be the optimal time period for the majority of the bioactive compounds of *Vigna radiata L* to reach their maximum content. It needs to be mentioned that despite the enhancement of total phenolic content that usually takes place due to the germination, this is not always correlated to the higher antioxidant capacity values (Huang et al., 2014). A possible explanation as suggested by (Randhir & Shetty, 2007) indicates that the quality of the phenolic content and not the concentration values of phenolic compounds might be responsible for the determination of the antioxidant capacity. Moreover, focusing on the edible characteristics of the sprouts, the level of luster and the morphology suggest that after the 4th day, they are appropriate for eating (Tang et al., 2014).

2.3.5 *In vitro* and *in vivo* studies including *Vigna radiata* L seeds and sprouts

Due to its wide use in human diet and its high levels of bioactive components, *Vigna radiata* L. has received much attention and has been assayed using *in vivo and in vitro* studies that demonstrated interesting properties of this legume for human health. The production of possible toxic compounds during the germination process was tested by *in vitro* studies performing cell proliferation and cytotoxic (MTT) experiments. Using HL60 cell line, (Martínez-Villaluenga et al., 2008) concluded that extracts of sprouts of broccoli and radish seeds induced no decrease either to cell proliferation or the metabolic status of the cells when compared to the treatment of the cells with distilled water. In agreement to this, Ali et al., (2014), also reported the safety of raw and germinated mung bean extracts on macrophage cells (RAW264.7).

The antidiabetic properties of *Vigna radiata* have been described in various animal models. Specifically, the extract of sprouts and its coats improved glucose tolerance and insulin response in diabetic KK-Ay mice by reducing blood glucose, plasma C-peptide and plasma urea levels (Yao et al, 2008). Furthermore, continuous administration of 1000mg/kg BW of fermented and non-fermented mung bean extracts to glucose-induced hyperglycaemic and alloxan-induced diabetic rats significantly reduced the elevated blood glucose level. In addition, significant improvements were also observed in the lipid profile and antioxidant activity of these animals that exhibited lower serum triglycerides, LDL, malonaldeyde (MDA) and nitric oxide (NO) levels (Yeap et al., 2012). These properties were also confirmed *in vitro* by inhibiting the activity of glycation end product~~s~~ formation (74.84% inhibition of BSA-glucose and 72.67% inhibition of BSA-MGO) (Yao et al., 2011). With regard to blood pressure regulation, the isolate protein of this specific legume has proved to contain peptides that inhibit the Angiotensin I Converting Enzyme (ACE) (Li et al, 2006).

Recently, the aqueous extract of mung bean coat (MBC) was found to be adequate for the treatment of sepsis in mice when a dose of 0.2 mL/mouse, containing 1.0 mg lyophilized MBC extract was orally administrated to them for two weeks. This study suggested that aqueous extract of MBC and, in particular, its two major flavonoids, vitexin and isovitexin, inhibited the release of endotoxin-induced HMGB1, a late mediator of lethal systemic inflammation in sepsis, and several chemokines. Taking into consideration that the doses administrated to septic mice were comparable to those used in humans and that the active compounds can be easily extracted in hot water, this treatment can be applied to clinical use (Zhu et al., 2012). In agreement with the above mentioned antiseptic properties of *Vigna radiata* L. extracts, Lee, Yoon, & Lee, (2012), confirmed that chlorogenic acid, a phenolic compound of plants and pulses, also suppressed HMGB1 release and lowered TNF and IL-1 levels after induction of sepsis in both *in vivo* and *in vitro* models. Additionally, the potent anti-inflamatory and analgesic properties of the extracts suggest its potential to be commercially marketed as supplement for inflammatory diseases treatment (Ali et al., 2014).

Due to the high antioxidant capacity of mung bean coat extract (MBCE), its administration to male Wistar rats enhanced their antioxidant capacity in plasma. In the same study, it was shown that heat exposure of the animals decreased total antioxidant activity and GSH content of plasma and increased plasmatic MDA, LDH and NOS levels, whereas pre and post heat exposure administration of MBCE ameliorated these changes. Since vitexin and isovitexin levels were higher in comparison with the rest of flavonoids as detected by HPLC technique, this leads to

the conclusion that both vitexin and isovitexin can be absorbed in vivo and MBCE could be helpful in the prevention or reduction of heat stress (Cao et al., 2011).

Besides the antioxidant properties of *Vigna radiata* L., its antimutagenic and anti-inflammatory properties have also been confirmed. Kruawan et al., (2012) showed that co-administration of mung bean acetonic extract and urethane 20mM reduced the *in vitro* induction of mitotic recombination by urethane, and determined the percentage of such inhibition to range 40-50%. On the other hand, a clear decrease of pro- inflammatory cytokines including IL-1β, IL-6, IL-12β, TNF-α, and iNOS was achieved in cells treated with ethanol extracts of *Vigna radiata L.* These benefits were attributed to the phenolic compounds such as gallic acid, vitexin and isovitexin that are present in this legume and do not produce cytotoxicity (Lee et al., 2011). Moreover, the capacity to stimulate the immune system by stimulating the proliferation of human peripheral blood mononuclear cells was also confirmed (Cherng, Chiang, & Chiang, 2007).

Finally, aqueous extract of mung beans hulls (0.05-0.2%) was proved to have antimicrobial properties against the pathogenic bacteria *Bacillus cereus* and decreased the peroxidation of lipids in irradiated chicken meat throughout the storage period under chilling conditions. These findings, in addition with the scavenging properties against superoxide radicals of the extract found in the same study make even clearer its antioxidant properties (Kanatt et al., 2011).

Due to the changes that germination brings about on the bioactive compounds of legumes, there has been an increased interest in studying the effects that germinated legumes may have on several human diseases. The study of (Hsu et al, 2011) compared the effects of a single dose and a long term administration of mung bean sprout extract on hypertensive rats. The oral administration of a single dose of 300 mg/kg body weight (BW) of raw sprout extract (RSE), dried sprout extract (DSE) and enzyme hydrolysate extract (EHE), obtained from dried sprout powder, showed no significant effect on systolic blood pressure (SBP) of spontaneously hypertensive rats. Nevertheless, when the dose was doubled a decrease of SBP was observed. In this study, the long term intervention was carried out by feeding the animals with freeze-dried mung bean sprouts and oven dried sprout powder at a dosage of 10.5 g dried sprout/day for four weeks. When this dosage was used, no differences in SBP were observed. However, using a 100× concentrated extract, the SBP was significantly decreased during the intervention period, although the antihypertensive effects of both extracts disappeared after the end of the treatment

period. This finding suggests that continuous intake is necessary in order to maintain blood pressure in the desired levels.

The hepatoprotective effects of mung bean were also confirmed in an experimental model of an ethanol-induced liver injury, by lowering the serum ALT, AST activities and cholesterol and triglycerides levels. Beneficial effects were also observed in the histology examination of mice liver. These results were associated with higher antioxidant capacity in plasma of the raw and germinated mung bean extract-treated animals (Mohd Ali et al., 2012).

2.4 Treatment with physical exercise

Taking into account that lower levels of physical activity are linked to the presence of MetS and NAFLD in adults and children (Gerber et al., 2012; Hattar et al, 2011), many lifestyle programmes have been designed in order to study the effect of different exercise trainings on the clinical manifestations of this pathology. A cross sectional study in Japanese healthy adults determined the association between the prevalence of MetS and physical activity, and concluded that a moderate activity of >26.5 Metabolic Equivalents (METs) hour/week could decrease the risk of MetS in Japanese men and women (Kim et al, 2011). Moreover, in a healthy Korean population, physical activity was inversely associated with the risk of NAFLD and directly associated with decreased levels of plasma hepatic enzymes in individuals that already suffered from this pathology (Bae et al., 2012).

These programmes can include pharmacological, diet and exercise interventions alone or combined. Moreover, the exercise protocols can differ between one and other and can consist in moderate or high intensity aerobic or anaerobic resistance training, during short or long term trial periods.

In OLEFT rats, the combination of a metformin dose (150mg/kg/day) and a moderate-intensity exercise training provided improvements regarding weekly food intake, glucose metabolism exemplified by HbA1c, fasting glucose and insulin concentrations, as well as several hepatic parameters. The specific training alone improved lipid accumulation, hepatic triglyceride content and serum ALT concentrations and the combination of both treatments resulted in more pronounced improvements (Linden et al., 2014). In type 2 diabetic patients, the combination of caloric restriction (15% protein, 30-35% fat, 50-55% carbohydrates, 1200kcal) and exercise training (65%-75% of maximum heart rate) provided a decrease of body mass index

(BMI) and ameliorated metabolic alterations, linked to this disease. In particular, the serum concentration of the hepatic enzymes, markers of hepatic functionality, (ALP, ALT, AST, GGT) and the HOMAIR index decreased due to the weight reduction as a result of the combination of diet and exercise interventions (Al-Jiffri et al, 2013).

Exercise alone (15 m/min, for 40 minutes, 5 days per week for 12 weeks) has also proved beneficial for the decrease of cardiovascular risk factors in Zucker diabetic rats. The specific experimental model develops diabetes which is accompanied by signs of cardiovascular disease as demonstrated by electrocardiographic alterations (ECG). Furthermore, the alterations include prolongation of the QTc interval, high R wave amplitude and tachycardia, which are important electrophysiologic alterations in this animal model of diabetes. However, the specific exercise did affect positively the R wave amplitude by lowering the levels of this parameter down to the reference values of control animals(VanHoose et al., 2010).

In general, the beneficial effects of regular exercise are well established even among elderly populations. Aging is associated with an increase of ROS production and a consequent reduced ability of the antioxidant buffering systems to cope with the increased oxidative stress associated with the natural process of aging. (Radák et al., 2004) proved that regular exercise attenuates the age related ROS production demonstrating higher levels of GSH and hence a higher antioxidant capacity in 30 month old rats that performed 8-week aerobic moderate intensity training.

Regarding the different types of exercise, both resistance and aerobic training have shown improvements in several metabolic parameters. (Bacchi et al., 2013) compared the possible differences between an aerobic (60-65% of heart rate, 60min daily) and an anaerobic (nine different exercises involving major muscle groups on weight machines) training on sedentary, type 2 diabetes patients with NAFLD. They concluded that both interventions are considered equally effective at improving the hepatic fat content, hepatic steatosis, insulin sensitivity, triglyceride content and changes in the adipose tissue of the individuals. A similar conclusion was achieved by (Lee et al., 2012) in obese adolescents regarding the beneficial effects on these parameters by both aerobic and resistance training. In addition, endurance training that consisted in treadmill training for 20-30min/day, 5 days/week during a total period of 8 weeks improved plasma lipid profile, body mass and abdominal fat, blood glucose concentration and systolic blood pressure in rats fed a high carbohydrate/high fat diet (HCHF) (Cameron et al, 2012).

Regarding the intrahepatic parameters, the study carried out by (Hallsworth et al., 2011) proved that resistance training in patients with NAFLD exhibited a 13% reduction in intrahepatic lipids and a decrease in glucose area under the curve. However, fasting insulin and ALT activity remained unchanged after the training programme followed during 8 weeks. The improvements observed in this study were not accompanied by further weight reduction. On the other hand, implementation of a moderate training protocol (45%-55% VO_{2max}, 30-60 minutes × 5 days/week) caused a 10% reduction of intrahepatic triglyceride content (IHTG) that was linked to ALT activity, without any change in body weight or body fat mass; in obese subjects with NAFLD (IHTG content >10 %). In addition, the VO2 max was increased after the training while no changes were observed in triglyceride content in plasma (Sullivan et al., 2012).

It appears that even a short term high intensity aerobic training has beneficial effects on parameters of lipid and glucose metabolism in obese individuals. In the study of (Haus, Solomon, Kelly, Fealy, Kullman, Scelsi, Lu, Pagadala, McCullough, Flask, & Kirwan, 2013), exercise affected favourably the levels of the hepatic enzymes, VO_{2max}, HOMA-IR index and AUC of glucose and insulin concentrations. In addition, reduced levels of glucose were correlated to a decreased ROS production, thus indicating that this training protocol could be used as a prevention strategy for NAFLD. Regarding this specific type of exercise, there is evidence that only one week at 85% of heart rate and during 60min, is enough to favourably affect the ALT activity, body fat oxidation as well as the plasma levels of the apoptotic marker, caspase-cleaved CK18 fragments, in sedentary obese individuals with NAFLD (Fealy et al., 2012).

Overall, aerobic exercise is accepted to be beneficial for the management of diabetes, obesity and cardiovascular diseases, all related to oxidative stress. The generation of oxidative stress arises from an imbalance between pro-oxidant and anti-oxidant forces as a consequence of increments in free radical formation in the body and/or the loss of antioxidant defences. The mechanism by which aerobic exercise interferes with the appearance of oxidative stress is mainly through increasing the muscle capacity for glucose oxidation and decreasing; insulin resistance in animal and human studies. There are two major classes of endogenous protective mechanisms (enzymatic and non-enzymatic) able to cope with the increase of oxidative stress. Primary anti-oxidant enzymes, including superoxide dismutase (SOD) and glutathione peroxidase (GPx), are responsible for removing superoxide radicals and H_2O_2 (Suzuki, Taniguchi, & Ookawara, 1999; Arthur, 2001). Among the non-enzymatic anti-oxidants, glutathione (GSH) is effective in

protecting tissues against lipid peroxidation initiated by ROS as a result of exercise training (Meister & Anderson, 1983; Deneke & Fanburg, 1989).

The study of (Chang et al, 2004) aimed to investigate the effects of a specific aerobic training (70% of the VO2max), on the antioxidant metabolism of Zucker obese and lean rats. The animals trained at 20m/min for 60min during 8 weeks without inclination and the hepatic activities and expression of the antioxidant profile of the animals were determined. The results clearly demonstrated that the obese phenotype is inversely associated with Mn-SOD and GPx activities, as well as with GSH content. Exercise favourably affected all of the antioxidant enzymes except CuZn-SOD activity, which remained unchanged. Similar changes were produced by phenotype and exercise in the hepatic protein expression of these enzymes, suggesting the improvement of the antioxidant capacity of the animals by this specific exercise.

It is clear enough that the mechanism through which exercise ameliorates hepatic steatosis involves AMP-activated protein kinase (AMPK), which in turn is involved in the delivery and oxidation of free acids (FA) by the tissues (Lavoie & Gauthier, 2006). It is well known that AMPK is activated under conditions of energy requirements, such as exercise or starvation (Takekoshi et al., 2006), and has a critical role in the regulation of acetyl-CoA carboxylase (ACC) and synthesis of fatty acids (Ruderman & Prentki, 2004). Activation of the phosphorylated AMPK was proved in both liver and visceral, but not subcutaneous adipose tissue in male Wistar rats after a medium intensity training (15m/min, 5d/week,12 weeks) that was estimated to elicit about 60% $VO2_{max}$ (Takekoshi et al., 2006).

However, little is known about the mediators of the activation of this enzyme. In this regard, (Moon et al, 2013), investigated the effect of exercise on AMPK and macrophage migration inhibitory factor (MIF) and how the relationship between them could affect certain parameters related to the appearance of NAFLD in male C57/BL6 (B6) mice. After carrying out a moderate exercise training (50 min/day at a speed of 18 m/min), the expression of MIF and the activation of phosphorylated AMPK in liver were higher in the exercise vs sedentary groups. Furthermore, in the same study, a dose-dependent relationship was observed between the administration of MIF and the activation of AMPK in the hepatocytes of the animals.

Other enzymes linked to NAFLD and its clinical manifestations are Fatty Acid Synthase (FAS) and PPAR-alpha. Their increased expression is associated with increased sensitivity of NAFLD and high activity of mitochondrial control and peroxisomal beta- oxidation. The process of beta-oxidation provides fatty acids that can enter the mitochondria and participate in

the synthesis of membrane and detoxification of endogenous and exogenous active molecules some of which may be PPAR ligands (Pyper et al., 2010). The enzyme FAS is considered responsible for the *de novo* lipogenesis in liver and adipose tissue of mammalian species and it is mainly regulated by insulin (Hillgartner, Salati, & Goodridge, 1995). Increased hepatic activity and protein activation of FAS have been demonstrated in obese Zucker rats when compared with their lean littermates. However, aerobic exercise (progressively increased up to 18-20m/min, 100m, 5d/week for 4 weeks) induced a down regulation of this enzyme in the obese animals, leaving it unchanged in the lean phenotype (Fiebig et al., 2002).

All three members of the PPAR subfamily function as sensors for fatty acids and fatty acid derivatives as they are involved in lipid and energy metabolism (Chawla et al., 2001). Fatty acids can directly bind to the PPAR protein and activate DNA transcription (Krey et al., 1997). In the liver, PPARα is activated by both saturated and polyunsaturated fatty acids and it plays a critical role in the adaptation under fasting conditions (Sanderson et al., 2009). The absence of PPAR-alpha has been associated with hypoglucemia, elevated FFA content and histological signs of hepatic steatosis in mice under starvation (Hashimoto et al., 2000). PPARβ/δ is shown to play a protective role in the liver by down-regulating inflammatory signals in circumstances of liver damage (Shan et al., 2008). PPARγ with its two isomorfs PPARγ1 and PPARγ2, is an essential regulator for adipocyte differentiation and promotes lipid storage in mature adipocytes by increasing the expression of several key genes in this pathway (Rosen & Spiegelman, 2001). PPARδ promotes cholesterol accumulation in human macrophages. This nuclear activation factor also leads to increased plasma HDL cholesterol levels and decreased triglyceride content in obese animal models(Leibowitz et al., 2000; Oliver et al., 2001; Vosper et al., 2001).

Male C57BL/6 mice after fed high fat diets finally develop the clinical manifestations of MetS and NAFLD. (Schultz et al., 2012), used this experimental model in order to investigate the effects of a moderate intensity swimming training (40-60%VO_{2max}, for 8 weeks) on the parameters linked to NAFLD. Indeed, after the administration of this high fat diet, the sedentary groups showed hypertrophied adipocytes and several focuses of inflammatory infiltrations in adipose tissues. The exercise induced a 10% reduction in the size of adipocytes from animals fed the two different kinds of diet when compared with their sedentary counterparts. In addition, the high fat diet caused an increased hepatic expression of FAS protein, which was reduced by the application of the exercise. However, opposite effects were observed in PPAR-alpha protein expression, which was reduced by the administration of high fat diet and enhanced by the swimming exercise.

2.5 Animal experimental models of Metabolic Syndrome

An experimental model, which closely resembles the MetS alterations in humans, is the Zucker rat. Zucker rats (*fa/fa*) have inactivating mutation in the leptin receptor that causes hyperfagia, obesity and fatty liver. Liver specific correction of leptin receptor deficiency results in reduced TG accumulation in the liver but not in other non-adipose tissues. Insulin resistance begins to appear after 2 weeks in the animals with the obese phenotype and is accompanied with obesity, serum dyslipidemia, and alterations in the antioxidant status. In contrast, the lean littermates are insulin-sensitive, normo-insulinemic and present a normal lipid profile (Galisteo et al., 2010; Hey-Mogensen et al., 2012; Zucker & Antoniades, 1972). Haram et al., (2009) used the Low intrincic aerobic treadmill running capacity (LCR) experimental model in an attempt to compare the effects of high intensity aerobic interval training (AIT) and moderate continuous exercise (CME). The AIT resulted more effective at reducing cardiovascular disease risk in rats with MetS. Compared to the CME, AIT had a more pronounced effect on improving the maximal oxygen uptake, lowering the blood pressure and increasing the concentration of HDL cholesterol.

The Zucker rat experimental model has proved adequate for the study of type 2 diabetes mellitus and obesity-associated nephropathy since its renal histological appearance resembles the classic findings of diabetic nephropathy. The implementation of a regular moderate aerobic exercise protocol improved the histological alterations of kidney in Zucker rats by decreasing the number of glomeruli with mesanglial expansion and the extension of fibrosis in tubulointerstitium, which are major pathologic findings in kidney injury. Furthermore, exercise decreased the generation of advanced glycation end products (AGEs) which is induced by hyperglycemia, oxidative stress, and inflammation (Boor et al., 2009).

3. Plant material: Processing and antioxidant capacity

3.1 Legume seeds

3.1.1 *Lupinus albus*

Lupinus albus seeds (var. multolupa) were provided by the Agrarian Research and Technology Development Service from the agriculture and Commerce council of the Junta de Extremadura (Spain). They were cleaned and stored at 4 °C until used.

3.1.2 *Vigna Radiata L.*

Vigna Radiata L. (La Asturiana, León) seeds were obtained from a commercial establishment and ground to a fine powder (0.18 mm sieve) for sample analysis and diet preparation.

3.2 Legume processing

3.2.1 Preparation of lupin protein hydrolyzates and insoluble dietary fiber residue

Lupin protein hydrolyzates were prepared by alkaline water extraction according to Martínez-Villaluenga et al. (2007), and protein hydrolysis according to Megías et al. (2007). Briefly, 100 g of lupin flour was suspended in 1 L of distilled water containing 0.25% (w/v) Na_2SO_3 and then adjusted to pH=9.0. The suspension was stirred for 50 min at room temperature, and then was centrifuged at 3000 rpm for 30 min. In order to obtain higher yields, the extraction and centrifugation were repeated on the residue. The final insoluble residue after protein extraction was collected and freeze-dried prior to storage at −20 °C until analysis and experimental diet preparation. Proteins in the final suspension were sequentially hydrolyzed with alcalase (protease from Bacillus licheniformis, Sigma, St. Louis MO) and flavourzyme (protease from Aspergillus oryzae, Sigma, St. Louis MO) using a hydrolysis reactor vessel equipped with a stirrer, thermometer, and pH electrode. Hydrolysis was performed as follows: $CaCl_2$ and $MgCl_2$ were added in enough quantities to make a 1 mM concentration in the protein suspension. Alcalase was added at time 0, and flavourzyme was added after 30 min. For alcalase hydrolysis, the enzyme/substrate ratio was 0.3 Anson units (AU)/g protein, pH=9, and temperature 50 °C. For flavourzyme hydrolysis, the enzyme/substrate ratio was 100 Leucin amino peptidase units (LAPU)/g protein, pH=8.5, and temperature 50°C. The pH was maintained throughout the

digestion process by adding 1M NaOH. Upon finishing the lupin protein hydrolysis, the pH of hydrolyzates was adjusted to 7.0 prior to freeze-drying and storage at −20°C.

3.2.2 Germination process

In order to carry out the germination process, *Vigna radiata* L. seeds were previously sterilized by immersion in sodium hypochlorite for 3 minutes. After completing this process, the seeds were washed out with sterilized, type-2 water with the aim to eliminate any traces of sodium hypochlorite, and then left soaking in water during 8 hours. Afterwards, they were distributed in various trays over sheets of filter paper where they were left covered in darkness, at 30º C, during 24, 48, 72, 96, or 120 hours. After this period, sprouted *Vigna radiata* seeds were collected, milled and lyophilized. The lyophilization aimed to evaporate the water contained in the seeds and at the same time prevent the loss of the nutritional compounds they contain. Once lyophilized, the sprouts were milled again to a fine powder (0.18 mm sieve) for sample analysis and diet preparation.

3.3. *In vitro* antioxidant capacity

3.3.1 Acetonic extraction and preparation of the extracts

Raw and germinated *V. radiata* flours were extracted twice at pH 2 during 45 min with a mixture of acetone:water:HCl (70:30:0.1) according to the methodology described by Troszyńska et al., (2002) to allow for an adequate solubility of polyphenols. After the extraction, samples were centrifuged at 3500 rpm for 15 min and the supernatant collected. The supernatants from a single extraction process were pooled to obtain a final extraction ratio of 40 mg/mL and kept at -20˚C for total extract, total polyphenol content and antioxidant capacity assays. Prior to each of the extraction and centrifugation processes, N_2 was bubbled through the samples to prevent any potential oxidation. To assess the total content of soluble extracted, samples were left to evaporate overnight under the hood and the evaporated samples left to dry at 50^0C for 48 hours until constant weight.

3.3.2 Determination of total polyphenols in *Vigna radiata* extracts

Total polyphenols were determined in raw and germinated *Vigna radiata* extracts using the Folin-Ciocalteau method as described by Dewanto et al. (2002). Calibration curve was designed based in different concentrations of gallic acid (ranging from 0 to 600µg/mL). The results were expressed as gallic acid equivalents (mg gallic acid/g of the sample). The development of this

process consisted in mixing 125 μL of the sample with 500μL of ddH_2O and 125 μL of Folin-Ciocalteau reagent. The samples were maintained in darkness during 6 minutes and afterwards they were mixed with 1.25mL of 10% Na_2CO_3/1M NaOH. After adjusting the volume to 3mL using 1 mL of ddH_2O, the samples were kept in darkness at room temperature during 90 min. Finally, the absorbance was spectrophotometrically measured at λ=760nm.

3.3.3 Inhibition of lipid peroxidation by *Vigna radiata* extracts

Thiobarbituric acid reactive substances (TBARS) in brain homogenate as a marker of lipid peroxidation after oxidative treatment were measured by the method of Ohkawa, Ohishi, & Yagi, (1979) with slight modifications. Wistar albino rats used to prepare the brain homogenates were obtained from the animal facility of the University of Granada, Spain. The brain was isolated from anesthetized rats and homogenized according to Singh et al. (2002) and Oboh & Rocha (2007), with slight modifications. Brain tissue was homogenized (1:10 w/v) in cold 1.15% KCl/0.1% Triton X-100. The homogenate was centrifuged at 7000 rpm, 4˚C for 25 min and the clear supernatant collected and stored at -20˚C for lipid peroxidation assays. The effect of anti-$FeCl_3/H_2O_2$-estimulated lipid peroxidation was determined as follows: 150 μL of the appropriately diluted extract in acetone or acetone extracting solution serving as control, were mixed with 150 μL of brain homogenate, 1250 μL of 0.15% KCl, 100μL of 5 mM $FeCl_3$, and 100 μL of 1 mM H_2O_2. The mixture was incubated at 37˚C for 60 min, after which 1750 μL of the oxidation reaction were mixed with 1500 μL of cold 0.25N HCl/15%TCA/1.34mM DETAPAC/0.5% BHT, 300 μL of 8.1% SDS, and 300 μL of 3%TBA. The mixture was incubated at 75˚C for 60 min, after which samples were left to cool before being centrifuged at 4000 rpm for 30 min. The supernatant was collected and absorbance was measured at 532 nm to detect TBARs formation. The percentage of inhibition in TBARs formation caused by the different extracts was calculated using the following equation: % inhibition = $[100-(100*(A_1/A_0)]$, where A_0 is the absorbance of the control oxidative reaction (in that case the acetone solution used for legume extraction) and A_1 for the absorbance of the chemical reaction including each acetonic legume extract. One unit of antioxidant capacity is defined as the amount of sample capable of inhibiting 50% TBARS formation compared to the control. The results were expressed as UAC/mL.

3.4 Cell Cultrure experiments: Effect of the different acetonic extracts of raw and germinated *Vigna radiata* on viability and metabolic status of HT-29 cell line

Cell culture experiments aimed to study the influence of the acetonic extracts of raw and germinated (48h, 72h and 96h) *Vigna Radiata* on viability and metabolic status of HT-29 cell line (Centro Instrumentación científica, University of Granada), alone or subjected to oxidative stress challenge. HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology and grows attached on the surface of the culture flask forming a monolayer. The cells were maintained in a sterile incubator at 37º C and 5% CO_2 using RPMI 1640 (Sigma, Life Science) supplemented with 10% of Fetal Bovine Serum (FBS) as the culture medium. Before starting the experiments, the cells were washed with PBS, treated with Trypsin solution for 3 minutes (0.25% Trypsin solution-EDTA, Sigma, Life Science) and counted with Trypan blue (0.4%, prepared in PBS), using a Neubauer chamber. The cells were seeded in two 96-well plates, one aimed to test the cell viability using Crystal Violet assay and the other in order to study the changes in the metabolic status of the cells using the3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide MTT assay (Mosmann, 1983). The cells were seeded in a concentration of 5×10^4 cells/well in 150μL RPMI, 10% FBS, and the coincubation or preincubation assays were performed using the adequately treated acetonic extracts of raw and germinated *Vigna Radiata L.* Cells were incubated for 16h with Paraquat dichroride hydrate (PQ)/S-Nitroso-N-acetyl-DL-penicillamine (SNAP) in a final concentration of 1mM PQ/ 0.7mM SNAP, that acted as peroxynitrite free-radical generator. In particular, PQ catalyzes the formation of Reactive Oxygen Species (ROS) whereas SNAP releases nitric oxide (NO). Each experiment was performed in triplicate.

REAGENTS

- 12mM Thiazolyl Blue Tetrazolium (MTT) (Sigma, Life Science) dissolved in RPMI 1640.

- 11.4 mM Crystal Violet solution (Panreac) (diluted in 50% EtOH)

- Sörensen's Glycine buffer (1M Glycine, 1M NaCl, pH=10.5)

3.4.1 Preparation of the extracts for *in vitro* experiments

The extracts of raw and germinated legume that were finally used to perform the *in vitro* experiments were prepared using the acetonic extracts previously described (section 3.2.1). Acetone was eliminated from the extracts (10 mL) under a N_2 flux and evaporated samples were re-suspended to original volume using PBS solution. Prior to the oxidative stress conditions, different dilutions of legume extracts were tested to find the lowest possible dilution that would maintain cell integrity and viability (data not shown) which was selected and used for subsequent cellular assays. For cell metabolic status and viability assays, 200 μL of extract were mixed with 1800 μL of RPMI-1460 medium, 20 μL of EtOH (0.1% final concentration) to improve extract solubility, and 2 μL of 3N NaOH to compensate the acidic pH imparted by the extracts. A blank control was prepared mixing 2000 μL of RPMI-1460 medium, 20 μL of EtOH, and 2 μL of 3N NaOH.

3.4.2 Co-Incubation experiments

1st Day: The number of the cells plated/seeded in each well was 5×10^4 cells in 150 μL of RPMI+10%FBS until next day when a 96% of confluence is achieved.

2nd Day: Discard the medium of each well and replace it with 150 μL of RPMI without BFS. Incubation during 24 h.

3rd Day: Beginning of the treatment. Remove the medium from each well and then add the corresponding treatment. The legume extracts were diluted 10× (1:10 dilution) in RPMI-1640/1 mM Paraquat/0.7 mM SNAP. The volume of each well is 150 μL. Leave them incubating during 16h

4th Day: Remove the treatments of each well and then perform Crystal Violet or MMT assay

Experimental protocol for a 96-well plate

1. The first and the last column are used as CT (only cells and RPMI-1460 medium)
2. For each one of the different treatments, a whole column is used (8 wells with V_{final}=150μL × 8wells=1200μL ~1500 μL, so as to be sure that there will be enough volume).
3. In a final volume of 1500μL the volume that corresponds to the extract is 150μL, to paraquat is 15μL (100 mM PQ in RPMI-1640) and to SNAP is 117 μL (9 mM SNAP in PBS)

4. According to these:

   - **RVR,GVR2D, GVR3D, GVR4D**: 150 μL of extract and 1350 μL PRMI without FBS
   - **CT&PQ/SNAP**: 15 μL PQ , 117 μL SNAP and 1370 μL PRMI without FBS
   - **RVR&PQ/SNAP,GVR2D&PQ/SNAP,GVR3D&PQ/SNAP, GVR4D&PQ/SNAP**: 150 μL of extract, 15 μL PQ , 117 μL SNAP and 1220μL PRMI without FBS

3.4.3 Pre-Incubation experiments

1st Day: The number of the cells plated/seeded in each well was 5×10^4 cells in 150 μL of RPMI+10%FBS until next day when a 96% of confluence is achieved.

2nd Day: Discard the medium of each well and replace it with 150μL of RPMI without FBS. Incubation during 24 hours.

3rd Day: Beginning of the treatment using only the extracts in the same concentration as used for the co-incubation. Remove the medium and add the treatment that corresponds on each well. The legume extracts were diluted 10× (1:10 dilution) in RPMI-1640. Notice that in this case there are 2 columns of every extract treatment.

4th Day: Treating the cells with PQ (1mM) & SNAP (0.7mM) in one half of the plate. For the other half of the plate, just remove the medium and replace with 150μL PRMI without FBS. Leave them incubating during 16 hours.

5th Day: Remove the treatments of each well and then perform Crystal Violet or MMT assay.

Experimental plan for one plate

1. The first and the last column of the plate are used as CT (only cells and RPMI-1460 medium)
2. For the different extract treatments the final volume is V_{final}=150μL ×8wells×2columns=2400 μL ~2500μL. In a final volume of 2500 μL the volume that corresponds to each extract is 250 μL
3. For the PQ (1mM) & SNAP (0.7mM) treatment the final volume is 5 columns × 8 wells × 150 μL=6000μL~6500μL. In a final volume of 6500 μL the volume that corresponds to PQ is 65 μL (100 mM PQ), and to SNAP is 505 μL (9 mM SNAP)

4. According to these:

- **RVR,GVR2D, GVR3D, GVR4D (3rd day)**: 250µL of each extract and 2250 µL RPMI-1640 without FBS (added to two columns of each extract treatment)

- **RVR&PQ/SNAP,GVR2D&PQ/SNAP,GVR3D&PQ/SNAP, GVR4D&PQ/SNAP (4th day, half of the plate)**: 65 µL PQ, 505 µL SNAP and 5930 µL RPMI-1640 without FBS. For the other half replace the extract medium with RPMI-1640.

Table 6. 96 well plate design of the experiments of viability and metabolic status of HT-29 cell line.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Control**	**RVR**	**GVR 2D**	**GVR 3D**	**GVR 4D**	**EtOH**	**EtoH& PQ& SNAP**	**RVR & PQ& SNAP**	**GVR 2D& PQ& SNAP**	**GVR 3D& PQ& SNAP**	**GVR 4D& PQ& SNAP**	**Control**
B												
C												
D												
E												
F												
G												
H												

3.4.4 Determination of cell viability (Crystal Violet) and metabolic status (MTT)

3.4.4.1 Crystal Violet assay

- Remove the medium of the wells
- Add 100µl of methanol which helps fixating the cells. Incubate during 30min.
- Remove methanol and add 50 µL of Crystal Violet solution. Leave it for 20min
- Wash by immersion in recipients containing tap water and remove the excess of water over pieces of paper
- Evaporate at 50º C during 1h
- Add 100 µL of 1% Sodium Dodecyl Sulfate (SDS)/well
- Read at 570nm

3.4.4.2 MTT assay

Prepare the solution of MTT at a final concentration of 0,5mg/ml: To do so, mix 18ml of culture medium without FBS with 2ml of MTT stock solution (5mg/ml)

- Remove the medium of the wells
- Add 200 µl of MTT solution in each well
- Incubate during 3h into the cell culture incubator
- After 3h, remove the medium of the wells
- Add 200 µl of DMSO and 25 µl of Sörensen's Glycine buffer
- Shake gently until total dissolution
- Read at 492nm

4. *In vivo* experiments

4.1 PHASE 1: Efects of *Lupinus albus* protein hydrolyzate and insluble dietary fiber residue in an experimental model of diet-induced hypercholesterolemia

4.1.1 Animals and experimental design

All experiments were undertaken according to Directional Guides Related to Animal Housing and Care (Council, 2010) and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada.

A total of thirty male Wistar albino rats were used in order to carry out the preliminary experiments of this study. The animals, with an initial mean weight of 170±2 g, were allocated to 5 experimental groups (n=6) according to the experimental diet administered: Casein–Cellulose normolipidemic control group (CT), Casein/Cellulose hypercholesterolemic high-fat group (HC), Casein/Lupin insoluble fiber residue hypercholesterolemic high-fat experimental group (HCF), Lupin protein hydrolyzate/Cellulose hypercholesterolemic high-fat experimental group (HID), and Lupin protein hydrolyzate/ Lupin insoluble fiber residue hypercholesterolemic high-fat experimental group (HIDF). The duration of the experiments was 30 days during which the animals were placed in a well-ventilated, thermostatically controlled (21 ± 2 °C) room with

relative humidity ranging from 40 to 60%, and 12h light/dark periods (lights on at 09:00). Throughout the trial, all rats had free access to double distilled water and consumed the experimental diets ad libitum. Food intake was recorded daily by weighing the amounts of diet given, refused and spilled, and body weight was measured every week. At the end of the experiment, rats were deprived of food for 12h, anesthetized with pentobarbital, and sacrificed. Blood was collected by puncture of the abdominal aorta (with heparin as anticoagulant) and taken for quantification of blood parameters (KX-21 Automated Hematology Analyzer, Sysmex Corporation, Kobe, Japan) prior to centrifugation at 3000 rpm for 15 min to separate plasma that was frozen in liquid N and stored at -80 °C. Liver, spleen, kidneys, colon, caecum, and caecal content, were extracted, weighed and divided in various portions and processed for histological analysis or snap-frozen in liquid nitrogen and maintained at -80ºC for their posterior use.

4.1.2 Experimental diets

AIN–93M diets were formulated following the recommendations of the American Institute of Nutrition (Reeves et al., 1993) to meet the nutrient recommendations of adult rats (NRC, 1995), with casein or lupin protein hydrolyzate as the sole sources of protein. Cholesterol (1%), and cholic acid (0.5%) (Sigma, St. Louis MO) were added following the recommendations by (Nath et al., 1959) for the preparation of hypercholesterolemic high-fat diets, to which dietary fat was added to provide a 12% content partitioned into coconut oil (8%) as saturated fat source, and sunflower seed oil (4%) as polyunsaturated fat source. For normolipidemic diet, 4% sunflower seed oil was added. 1.5% methionine was added to the hypercholesterolemic high-fat diets in order to increase their atherogenic potential through the formation of homocysteine. Dietary fiber was added as cellulose or lupin-derived insoluble fiber residue to provide a dietary level of 10%. The total fat and fiber content of lupin protein hydrolyzate and insoluble fiber residue was taken into consideration for the final concentration present in the diet; the additional amount of Ca needed to bring the dietary levels up to target requirements of the rat was supplied as Ca-citrate added to the diet. Caloric content of the diets was calculated using the Atwater General Factors.

In Table 7 the formulation and chemical composition of experimental diets is included.

Table 7. Composition of lupin protein hydrolyzate, lupin insoluble dietary fiber residue, and experimental diets*

Lupin products composition	Protein hydrolyzate	Insoluble Dietary Fiber Residue			
N × 6.25 (g/kg)	471.3	85.1			
Fat (g/kg)	111.0	67.2			
TDF (g/kg)	51.9	798.0			
Ash (g/kg)	128.6	30.0			
P (mg/kg)	4977.3	1004.6			
Ca (mg/kg)	2689.5	2246.2			
Mg (mg/kg)	2304.6	706.8			
K (g/kg)	13.5	5.0			
Diet Formulation (g/kg)	**CT**	**HC**	**HCF**	**HID**	**HIDF**
Casein	205	170	170	-	-
Lupin protein hydrolyzate	-	-	-	280	280
Methionine	5	15	15	15	15
Sucrose	100	100	100	100	100
Starch	500	410	390	330	310
Cellulose	100	100	-	100	-
Insoluble fiber residue	-	-	130	-	130
Coconut oil	-	80	80	80	80
Sunflower seed oil	40	40	30	10	-
Cholesterol	-	10	10	10	10
Cholic acid	-	5	5	5	5
Mineral mix	35	35	35	35	35
Vitamin mix	10	10	10	10	10
Calcium citrate	24	24	24	24	24

Choline bitartrate	2.5	2.5	2.5	2.5	2.5
Diet Composition	**CT**	**HC**	**HCF**	**HID**	**HIDF**
Total N (g/kg)	28.4	27.8	27.4	22.0	23.5
Insoluble N (%)	1.77	0.77	1.73	2.69	3.55
Soluble Protein N (%)	92.8	92.3	90.3	2.04	1.77
Soluble Non-Protein N (%)	5.82	6.98	8.01	95.3	94.7
Total Fat (g/kg)	46.0	118.7	112.2	120.4	122.8
TDF (g/kg)	88.4	92.2	102.7	92.1	101.0
Ash (g/kg)	32.8	30.9	29.6	58.5	55.9
P (mg/kg)	3073.3	2927.9	2637.0	2494.4	2565.6
Ca (mg/kg)	4411.0	4801.7	5015.3	5484.3	5654.6
Mg (mg/kg)	601.0	468.3	533.8	1108.6	1132.2
K (mg/kg)	3575.1	3770.0	3867.6	6777.5	7335.9

*** Results are expressed on a Dry Matter basis. Values are means of four independent replicates. TDF, Total Dietary Fiber.**

CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group.

4.2 PHASE 2: Efects of raw and 4d-germinated *Vigna radiata* flours combined with a HIIT protocol in an experimental model of genetically obese Zucker rat

4.2.1 Experimental animals

All the experiments were undertaken according to Directional Guides Related to Animal Housing and Care (Council, 2010) and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada, Spain.

For the concluding experiments, the obese Zucker rat was chosen as adequate experimental model since this model is considered appropriate for the study of metabolic syndrome. For this reason, male obese homozygous (fa/fa) and lean heterozygous (fa/+) rats of 179±2.8 and 148±3.4 g of initial weight, respectively, were used. The animals were provided by Charles River laboratories and were individually housed in a well-ventilated, thermostatically controlled (21±2ºC) room, 40-60% relative humidity and a reversed cycle of light-darkness of 12 hours. The animals were distributed in two groups, a) exercise and b) sedentary, receiving *ad libitum* distilled water and the corresponding experimental diet. Since the rat is a nocturnal animal and presents a higher activity during night, it was established that the beginning of the dark period would be at 12:00 pm so that animals would perform the training protocol during this period. Furthermore, during the dark period red light was used in order to prevent any alteration on the natural period of light of the animals or provoke them any additional stress.

4.2.2 Experimental design and development

In order to carry out all the experiments, 120 animals were used, 60 with the obese phenotype (fa/fa) and the rest with lean phenotype (fa/+). The animals were subdivided in three different subgroups according to the experimental diet consumed (n = 40, 20 lean and 20 obese). Finally, in every experimental group, 20 animals (10 lean and 10 obese) performed the aerobic interval training protocol and 20 (10 lean and 10 obese) remained sedentary during the experimental period.

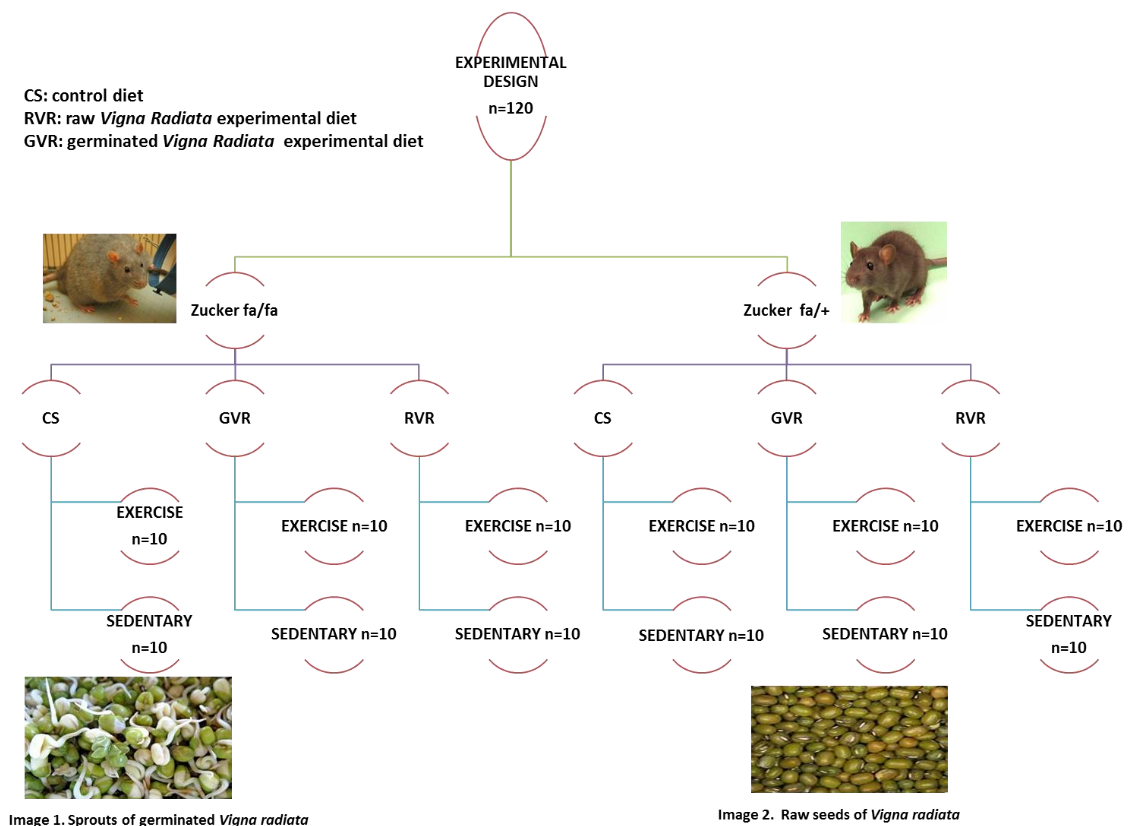

Figure 3. Experimental design of the project

Each experiment lasted for 8 weeks. Prior to the start of experiment, an initial 7-day period was used for the animals to adapt to the experimental diet and training conditions. During this period an incremental test of maximum oxygen consumption was performed to establish the intensity conditions of the training protocol. This test was also performed right before the end of the experimental period in order to study possible changes in the physical performance of the animals as a result of the training protocol. Dietary intake was registered daily and the body weight was determined once a week, after a fasting period of 12 hours. At the beginning and at the end of the experimental period, a 24h oxygen consumption test was performed in all the animals to assess the resting metabolic rate. In addition, the area under the curve after an oral glucose overload was determined during the last week of experimental period at least 24 h after the last bout of exercise.

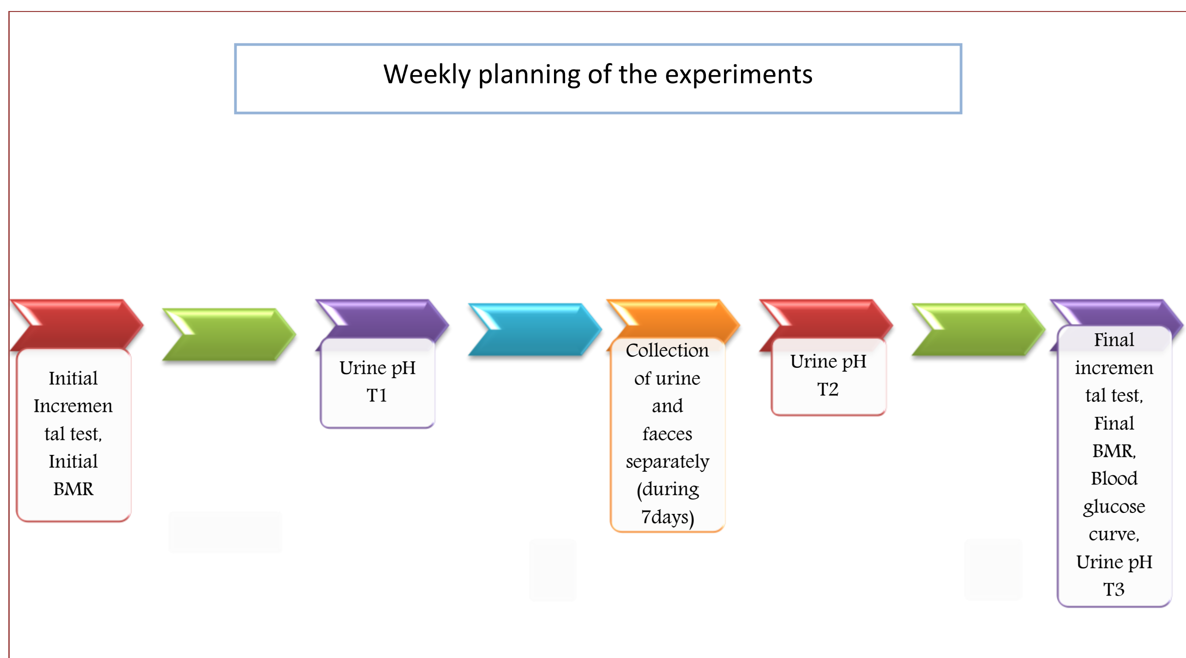

Figure 4. Weekly planning of the experimental period

At the end of the experimental period, 12h-fasted animals were anesthetized with a mix of two anaesthetics: ketamine and xylazine. Blood was extracted from the abdominal aorta and collected using heparin as an anticoagulant agent. Plasma was obtained after centrifugation of the blood samples, at 3000rpm during 15 minutes, at 4° C. Plasma samples were aliquoted, snap-frozen in liquid nitrogen and kept stored at -80ºC until further analysis. The liver, heart, aorta, kidney, colon, spleen, lung, caecum, brain, quadriceps and fat tissue were extracted rapidly and after being rinsed with 9‰ NaCl, they were weighed and divided in various portions and processed for histological analysis or snap-frozen in liquid nitrogen and maintained at -80ºC for their posterior use.

4.2.3 Experimental diets

The experimental AIN-93M diets were formulated based on the instructions published by the American Institute of Nutrition (Reeves et al., 1993) in order to meet the nutrient requirements of an adult experimental animal (*Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, 1995*). Regarding the diet consumed, the animals, both obese and lean were divided in three different groups. One of them consumed the casein control diet in which casein and whey protein provided the protein source in a proportion of 70%:30% respectively. In the other two experimental diets, the protein was provided by whey (30%) and raw or 4-day germinated *Vigna radiata* L (70%). The formulation of the raw *Vigna radiata* diet consisted in mixing the flour obtained after grinding *Vigna radiata* seeds with the rest of the ingredients of

the experimental diet. As for the germinated *Vigna radiata* experimental diet of the, the vegetable protein source was provided from the legume sprouts, that had been previously freeze-dried and grounded as described in section 3.2.2.

The protein level of the experimental diets was established at 12% adding 0.5% methionine with the objective to fulfil the specific needs that the laboratory rats have respecting this specific amino acid. The percentage of dietary fibre added in the diet was established at 10% as cellulose (insoluble and not fermentable) for the control diet, while for the leguminous diets the fibre was provided mainly from raw or germinated *Vigna radiata* flour while a small amount of cellulose was added in order to achieve the percentage of fibre established in this study. Finally, the percentage of fat was established at 4%, using sunflower oil without any addition of saturated fat or other cholesterol source.

Table 8. Formulation of experimental diets

	grams in 100g DM of experimental diet		
Components	Casein	Raw *Vigna radiata*	Germinated *Vigna radiata*
Casein	11.1	-	-
Raw *Vigna radiata*	-	36.7	-
Germinated *Vigna radiata*	-		33.3
Whey Protein	5.1	5.6	5.6
Sucrose	10	10	10
Cellulose	10	1.1	2.1
Methionine	0.5	0.5	0.5
Sunflower Oil	4	4	4
Mineral premix	3.5	3.5	3.5
Vitamin premix	1	1	1
Choline bitartrate	0.25	0.25	0.25
Starch	54.6	42.7	41.1

Composition of raw and 4day-germinated *Vigna radiata* flours. Protein=3.79 and 4.39, respectively; insoluble dietary fibre=20.9 and 16.2, respectively; Ash=3.71 and 4.34, respectively; Grasa=1.54 and 1.33, respectively.

4.2.3.1 Nitrogen and dietary fibre content detection in the experimental flours.

Nitrogen determination in casein, whey, raw and germinated *Vigna radiata* flours, and the different experimental diets was performed according to Kjeldahl's method, calculating the protein content as N×6.25. For the determination of fibre content of raw and germinated *Vigna radiata* flours, 1g of each flour previously homogenated and lyophilized, was dissolved in a phosphate solution (pH=6), maintaining the pH at this specific level. Before proceeding to the digestion of the samples, a specific crucible previously weighted containing a determined amount of silicium oxide was incorporated in the digestion equipment. The digestion process began with the addition of 100μL of α-amylase and then the samples were incubated in a hot bath at 90 ^{0}C for 30 minutes, shaking every 5 minutes. After the α-amylase digestion process, the samples were left to cool until they reached room temperature. Then, 10mL of type-2 H_2O, 100 μL of protease and 10mL of 0.275N NaOH to maintain the pH at 7.5, were added to the samples, and the mixture was incubated at 60^{0}C, for 30 min and again left to cool until reaching room temperature. The pH was then corrected to 4.0-4.6 by the addition of 10 mL 0.325N HCl. Once again incubation at 60 ^{0}C, for 30 min was performed. Afterwards, 10mL of EtOH (60%) were added to every sample and they were left to precipitate during an hour, at room temperature. Once the digestion was finished, the crucibles with the precipitate were washed out using EtOH (78%), EtOH (95%) and then acetone solution. They were left overnight at 105 ^{0}C and the next day they were weighed. The amount of fibre contained was calculated by weight difference of the crucibles prior and after the finalization of the digestion process (Prosky et al., 1992).

4.2.4 High intensity aerobic interval training protocol

The training protocol was carried out using a treadmill of 5 lanes, especially designed for experimental rats (Panlab/Harvard Apparatus, Inc. Cornellá, Barcelona). One week before the start of the experimental period, the animals were familiarized with the treadmill during 3-4 days. This adaptation period consisted in performing a low intensity training of 15 cm/min during 15 min, without inclination.

The animals belonging to the exercise group performed the training protocol 5 days a week, 60 min/day during the whole experimental period. This specific protocol is characterized as an aerobic training protocol given that the intensity used was below the 100% of the maximum oxygen consumption (VO_2 max), determined as the anaerobic threshold. At the same time it was considered intervallic, as high intensity sessions of 4 minutes were alternated with lower intensity sessions of 3 minutes, during the 60 minutes of the exercise protocol. The specific

training started at 65 and 50% of VO_2 max in the first week, for the high intensity and low intensity session, respectively, and kept increasing up to 80 and 65% of VO_2 max during the final two weeks. Below, there is a representation of the changes in the training protocol that was performed during the dark period.

Warming up	w/o	10´	10´	10´	10´	10´
Duration	45´	50´	50´	55´	60´	60´
Week	1st	2nd	3rd	4th	5-6th	7-8th
4- min session						
% $VO_{2\ max}$	65%	70%	75%	75%	75%	80%
3-min session						
% $VO_{2\ max}$	50%	55%	60%	60%	60%	65%

Figure 5. Training protocol

The sedentary groups performed a smooth training protocol which consisted in 15 minutes of 15m/min, without inclination, 2 days a week, aiming to reflect a sedentary human lifestyle.

4.2.5 Incremental test of oxygen maximum consumption

The incremental test of maximum oxygen consumption was carried out using a gas analyser, Panlab Oxylet System, which was connected to an individual treadmill. The method used by this system was based on the determination of the values of O_2 consumed and CO_2 produced through an open circuit indirect calorimetric device. The rate of gas flow inside the treadmill was established at 0.7-1.2 l/minute during the test. At the same time, gas concentration of air samples was detected by O_2 and CO_2 sensors and these values were registered in the connected computer. The protocol used was based in the one proposed by (Wisløff et al., 2001) with slight modifications (Clemente et al., 2011).

After the registration of the values of O_2 y and CO_2 of the ambient air during the first 5 min, the sensors begin to register the values of the gases inside the treadmill for the following 3 min. Once the values are stabilized, the animal is introduced inside the cage of the treadmill. For the following 5min, the sensors keep detecting the gases inside the treadmill and then the incremental test begins. During the 5 first minutes the velocity increases from 25-30cm/min and after this period, the velocity keeps increasing by a pace of 3cm/min every minute. The test

ends when the animal is visibly exhausted and rested on the shock bar during >5 seconds.

The animals were weighted prior to the performance of the incremental test. The blood content of lactate (mmol/L) was measured using the analyser "Lactate Pro Test Strip" before and after the test. In order to perform these two measurements rapidly the tail was immersed in hot water with the aim to provoke dilatation of the arteries of the animal. After drying it, a small incision was performed in the distal part of the tail of approximately 0.5-1mm. In order to prevent possible inconveniences for the animal, a small massage was performed and facilitated the process. A small drop of blood was placed in the corresponding strip and so, the concentration of lactate was determined. Once completed the incremental test, the measurement of lactate was repeated but this time the use of hot water was not necessary as the exercise has a vasodilator effect.

In the Figure 6, the progressive changes of the incremental test are represented.

Time (min.)	Velocity (cm/s)	Time (min.)	Velocity (cm/s)
5	25-30	19	66-69
6	30-33	20	69-71
7	33-36	21	71-74
8	36-39	22	74-77
9	39-42	23	77-80
10	42-45	24	80-83
11	45-48	25	83-86
12	48-51	26	86-89
13	51-54	27	89-92
14	54-57	28	92-95
15	57-60	29	95-98
16	60-63	30	98-101
17	63-66	31	101-104

Figure 6. Progressive increase of the intensity of the incremental test

4.2.6 Determination of resting oxygen consumption (Basic metabolic rate)

The measurement of the basic metabolic rate (BMR) was performed using the gas analyser Panlab Oxylet System, which was connected to two metabolic cages allowing performing the test simultaneously in two animals. The animals remained inside the metabolic cages during 24 hours, having ad libitum access to water and the corresponding experimental diet. The rate of gas flow inside the cages was established at 0.5-0.8 L/minute during the test. The technique is based on indirect calorimetry of open circuit in order to determine the consumed and produced values of O_2 and CO_2 from the animals. In this manner, the gas concentrations in samples of the air are detected by specific sensors, and the values are registered in the computer connected to the equipment.The energy spent (Kcal/min) was calculated as the mean of the values obtained, previously separated in those belonging to the light or dark period.

4.2.7 Oral glucose tolerance test

The animals were deprived from food 8 hours prior to performing the test of tolerance after oral glucose overload. Rats were weighted to calculate the adequate amount of glucose to be administered and the analyser "Breeze-2" was used to measure the concentration of blood glucose. As previously mentioned, to obtain the blood samples a small incision was performed in the distal part of the tail of approximately 0.5-1mm. In order to prevent possible inconveniences for the animal, a small massage was performed and facilitated the process. A small drop of blood was placed in the glucose strip and so, the basal concentration of glucose was measured. Right after, a solution of D-glucose prepared in saline solution (NaCl 0.9%) was administered to the animals, using a ratio of 6.9µmol of glucose/g body weight. This process was carried out according to (Prieto et al., 2004), by gavage. In addition to the basal levels (T0), the blood concentration of glucose was determined 15, 30, 60 and 90 min after the administration of glucose solution. Finally, the area under the curve was calculated using the trapezoid model with Microsoft Excel programme (arbitrary units) and the results were expressed as above mentioned.

5. Analytical methods

5.1 PHASE 1: Effects of *L. albus* protein hydrolyzate and insluble dietary fiber residue in an experimental model of diet-induced hypercholesterolemia

5.1.1 Biochemical analysis

Urinary pH was analyzed using a bench pH-meter (Crison, Barcelona, Spain). Plasma and urinary biochemical parameters were measured using a Shenzhen Midray BS-200 Chemistry Analyzer (Bio-Medical Electronics) at the Bioanalysis Unit of the Scientific Instrumentation Centre (Biomedical Research Park, University of Granada). Liver function parameters measured in plasma (AST, ALT, ALP, GGT, and bilirrubin) were assayed using commercial kits (Spinreact, S.A., Girona, Spain. Reference #s 1001161, 1001171, 41242, 100185, and 1001042, respectively).

5.1.2 Mineral, cholesterol and triglyceride content in liver

A portion of liver was freeze-dried for moisture content detection and easier handling in the process of total mineral and fat detection. Total ash and P content of freeze-dried liver were measured as previously described for the functional ingredients and experimental flours. Hepatic lipids were extracted with hexane using the method of (Folch et al., 1957). Briefly, freeze-dried liver aliquots (0.5 g) were carefully crushed and homogenized in 2000 µL of ethanol containing 0.01% 3,5-Di-*tert*-4-butylhydroxytoluene (BHT). The liver homogenate was saponified with 1mL of 11N KOH for 40min at 60°C, and extracted with 2000 µL hexane for analysis. The organic phase extracted from liver was dried under a stream of N_2 and re-dissolved in the appropriate volume of hexane. Total liver lipids were measured gravimetrically after solvent evaporation under N_2 stream. The concentration of cholesterol and triglyceride in the re-dissolved fat solution was measured using analytical kits (Spinreact, S.A., Girona, Spain. Reference #s 41021 and 41031, respectively).

5.1.3 Histological analysis

A portion of liver and proximal colon were stored in 10% formaldehyde and was subsequently immersed in several solutions that helped to preserve the histological morphology of the samples by using an automatic tissue processor (Tissue Processors Slee MTP), until they were finally included in solid paraffin (Thermo Scientific EC 350 Paraffin Embedding Centre).

The inclusion of samples in parafin consisted on sequential immersion of the samples in EtOH solutions of different concentrations, toluene and paraffin previously warmed up at 100^{0}C. Exact details of the protocol used are described in Figure 7. The histological sections of 3µm were performed by a microtome (Thermo Scientific Microm HM 325) and, afterwards, the samples were stained with Haematoxylin-Eosin and Masson's Tricrómic solutions. The observation and evaluation of the liver samples was partially blind (observer-blind) and was carried out by light microscopy (Nikon Eclipse 50i). Morphometric studies of colon perimeter and luminal surface area, length of plica mucosae, thickness of the mucosa tunic, and thickness of the muscular tunic were performed using the software Image Pro Plus 6.0.

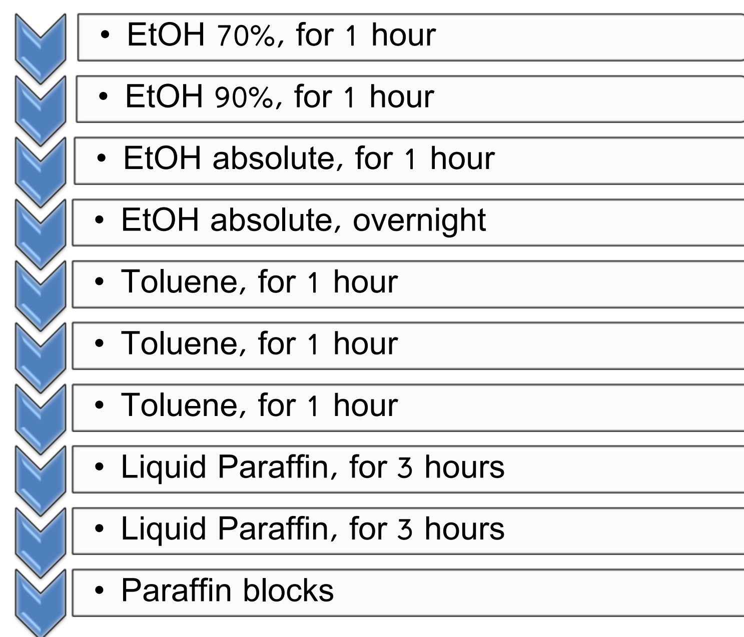

Figure 7. Histological inclusion of the samples

5.1.3.1 Hematoxylin-Eosin stain

The hematoxylin eosin stain (Figure 8) contributed to the detection and assessment of morphological changes in liver and colon due to the cholesterol content of the experimental diet.

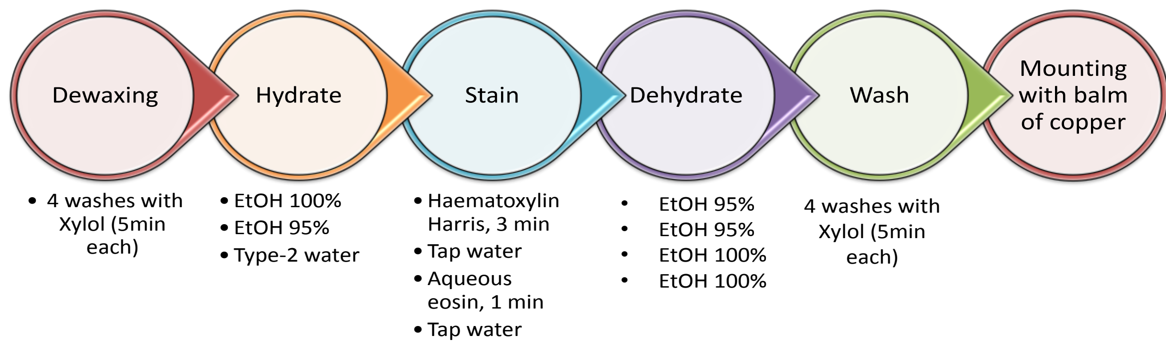

Figure 8. Hematoxylin eosin stain

REAGENTS

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1. HARRIS HAEMATOXYLIN (Harris Haematoxylin, PANREAC)

2. 1% AQUEOUS EOSIN (903006 QCA)

1mL of acetic acid was added in order to preserve the solution.

5.1.3.2 Masson's Tricrómic stain

The histological evaluation of the samples was completed with Masson's Tricromic stain as represented in Figure 9. Masson's Tricromic stain

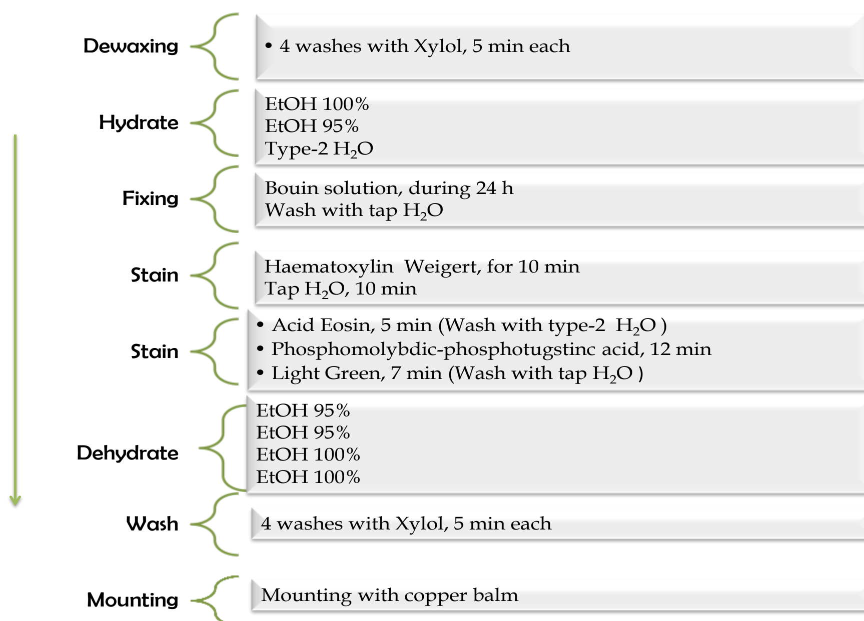

Figure 9. Masson's Tricromic stain

REAGENTS

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1. FERRIC HEMATOXYLIN OF WEIGERT´S

The preparation of this solution consisted in mixing the Solution A: Hematoxylin (Panreac) and Solución B: ferric chloride (Iron III cloride, MERK) in a 1:1 proportion. The solution remains stable for approximately 4 weeks.

Solución A: 1g of hematoxylin in 100mL of EtOH (95%)

Solución B: 4 mL of ferric chloride (29%) in type-2 water with 1% of hydrochloric acid

2. BOUIN SOLUCIÓN

75 % saturated picric acid (2g in 100mL, FLUKA)

25% Formaldehyde

5% Glacial acetic acid

3. MIX OF PHOSPHOMOLYBDIC- PHOSPHOTUNGSTIC ACID

14 mM phosphomolybdic acid (x-hydrate, Panreac)

9 mM phosphotugstinc acid (hydrate PA, Panreac)

4. SOLUTION OF ACID EOSIN

9 parts of 1% Eosin (Biebrich Scarlet-Ácid escarleta, Panreac)

1 part of 1% Acid aqueous Fuchsin (Acid fuchsin, 1% aqueous, SIGMA)

The solution is better maintained after the addition of 1% of glacial acetic acid punctually while it is used.

5. LIGHT GREEN SOLUTION

25 mM of Light green solution (Light green SF yellowish, MERCK)

The solution is better maintained with the addition of 1% of glacial acetic acid

5.2 PHASE 2: Effects of raw and 4d-germinated *Vigna radiata* flours combined with a HIIT protocol in an experimental model of genetically obese Zucker rat

5.2.1 Hematic parameters

The blood was analyzed using the hematic analyser (KX21-21 Automated Hematology Analyzer, Sysmex, Corporation, Kobe, Japan). The number of white blood cells (WBC, $\times 10^3/\mu L$), the number of red blood cells (RBC, $\times 10^6/\mu L$), hemoglobin (Hb, g/dL), hematocrit (Hct, %), mean corpuscular volume of red blood cells (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular haemoglobin concentration (MCHC, g/dL) and red blood cell distribution width (RDW, %) were determined.

5.2.2 Biochemical parameters in plasma

Biochemical parameters were measured in plasma using a Shenzhen Midray BS-200 Chemistry Analyzer (Bio-Medical Electronics) at the Bioanalysis Unit of the Scientific Instrumentation Centre (Biomedical Research Park, University of Granada). Among them: Total

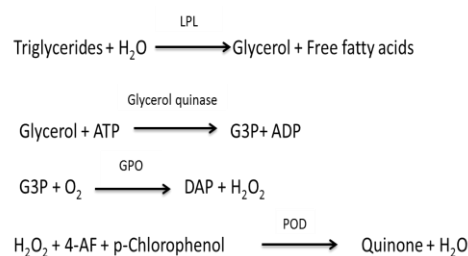
Billirubin (mg/dL), LDL cholesterol (mg/dL), HDL cholesterol (mg/dL), Total cholesterol (mg/dL), Tryglicerides (mg/dL), Alkaline phosphatase (U/L), gamma-glutamyl transpeptidase (GGT, U/L), Alanine aminotransferase (ALT, U/L), Aspartate aminotransferase (AST, U/L) and Glucose (mg/dL)were determined.

5.2.3 Liver weight, water content, total fat and triglycerides

The water content of liver was determined after lyophilisation of a small portion using the Telstar Cryodos (Terrassa, Spain, 2007) equipment. By this process, the water content of the sample is eliminated and so, the moisture content is calculated by weight difference before and after lyophilisation. The extraction of total fat and the determination of the triglyceride content were carried out as previously described in the section 3.1.2

5.2.4 Determination of liver triglyceride content

Triglyceride content of the liver lipid samples dissolved in hexane was measured using an analytical kit based on the following principle:



Where:

GPO:Glycerophosphate Dehydrogenase	G3P: Glycerol-3-phosphate
ATP: Adenosine-5-triphosphate	ADP: Adenosine-5-diphosphate
DAP: Dihydroxiacetone phosphate	H_2O_2: Hydrogen Peroxide

The amount of triglycerides is proportional to a coloured compound originated from the chemical reactions above mentioned.

5.2.5 Antioxidant activity analysis

5.2.5.1 Preparation of liver homogenates

The liver homogenates that were used to determine antioxidant status were prepared in 50 mM phosphate buffer 50mM (pH=7.4), containing 1.34mM Diethylenetriaminepentaacetic acid (DETAPAC) and 0.1% Triton X-100. The buffer was kept refrigerated during the whole homogenization process, and the established ratio between buffer and sample was 10:1 (v/w). After homogenization of the samples for 30sec (Micra D-1 homogenizer, ART moderne labortechnik) and their sonication (Sonoplus HD 2070, Bandelin) for 30 sec at 50% of maximum intensity, the samples were centrifuged at 15000 rpm, during 45 min, at 4º C. Finally, the upper layer was removed and stored at -80^{0}C until posterior determination of the different antioxidant enzyme activity.

5.2.5.2 Antioxidant status

5.2.5.2.1 Determination of total-SOD, Mn-SOD and CuZn-SOD activity

The determination of Superoxide Dismutase (SOD) activity was carried out in the homogenates previously described (section 5.2.5.1). The method used, described by (Ukeda et al., 1997), was adapted to a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Ficher Scientific, Shanghai, China). The basis of the specific technique is the SOD-induced inhibition of the oxidation reaction between the superoxide anions produced by the system xanthine/xanthine oxidase and the receptor of superoxide 2,3-bis-(2-methoxyl-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT). Briefly, the reaction mix contained 740 μL of phosphate buffer (50 mM pH=7.8), 38 μL of DETAPAC (3 mM), 55 μL of XTT (0.0007 g/mL of 50 mM phosphate buffer) and 167 μL of xanthine oxidase (70 mU/mL) in a final volume of 1mL. The samples were replicated 4 times, and the volumes added to every plate well were 150 μL of the reaction mix, 35 μL of the diluted sample and/or 35 μL of 50 mM phosphate buffer, pH 7.4 in the case of controls and, finally, 55 μL of 3 mM xanthine. The measurements were taken every minute, during a total duration of 15min, at 25 ^{0}C and λ=492nm.

The same protocol was followed for the determination of manganese (Mn) dependent SOD, with the only difference that the samples were previously treated with 120mM KCN for 30 min (final concentration of KCN 4.8mM). Subtracting the results obtained from both measurements, copper zinc (CuZn) dependent SOD was calculated. One activity unit is defined as the amount of enzyme needed to produce a 50% inhibition of XTT reduction. The % of inhibition = ((A_0-

$A_i)/A_i)*100$, where A_0 refers to the absorbance of the control and A_i is the absorbance of the sample. The results were expressed as antioxidant activity units (AAU)/mg of protein.

5.2.5.2.2 Determination of catalase activity

The determination of catalase activity was carried out according to (Aebi 1984) by monitoring the disappearance of H_2O_2 in the presence of brain homogenate. The absorbance of H_2O_2 was measured spectrophotometrically at λ=240nm using a Thermo Scientific™ BioMate 3S UV-Visible spectrophotometer (Shanghai, China). The liver homogenates of each sample were mixed with H_2O_2 (10.6 mM) prepared in 50mM phosphate buffer, pH 7, and the absorbance was determined during 1.5 min at 15 s intervals. The antioxidant unit was defined as μmol H_2O_2 consumed per minute per mg protein.

5.2.5.2.3 Determination of glutathione peroxidase activity (GPx)

This method is based in the oxidation of nicotine adenine dinucleotide phosphate (NADPH) induced by glutathione reductase, coupled with the reduction of glutathione previously oxidized by glutathione peroxidase. The activities were spectrophotometrically determined at λ=340nm, 37ºC during 4min, repeating the measurements every 15seg according to the protocol previously described by Lawrence et al., (1974) with slight modifications. Catalysed and non-catalysed reactions were simultaneously performed. Regarding the non-catalysed reactions, 240μL of 2 mM NADPH/1 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM phosphate buffer, pH 7.4, were mixed with 15 μL of liver homogenate and 10 μL of 22 mM cumene hydroperoxide in each well. As for the catalysed reaction, 240 μL 2mM NADPH/1 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM phosphate buffer,pH 7.4, were mixed with 15 μL liver homogenate, 4,5 μL glutathione reductase (0.04 mU/mL), and 10 μL 22 mM cumene hydroperoxide.

The results were expressed as nmol NADPH/min/mg of protein according to the following equation:

nmol NADPH/min/mg protein= [((ΔAbsorbance of the catalysed reaction - ΔAbsorbance of the non-catalysed reaction)/0.0062) × (total volume of the well /sample volume)]/mg of protein content of each sample.

5.2.6 Determination of liver homogenate protein content

The protein content in liver homogenates was determined according to Lowry et al., (1951), a method based on the reaction of specific amino acids (phenolics) with copper in basic pH conditions and posterior reaction of the containing protein with Folin-Ciocalteau reagent. First, a

calibration curve of bovine serum albumin (1000 μg/ml) following serial dilutions was performed. The next step consisted in preparing the Cu alkaline reagent by mixing the solutions Cu_2SO_4 (1%), Sodium-Potassium-Tartrate (2%) and Na_2CO_3 (2%)/NaOH (0.1N), maintaining a proportion of 0.1:0.1:10 up to the required volume. After adding 200μL of this reagent to 40 μL of the different dilutions of the calibration curve or samples, the mixture was incubated during 10min, in darkness. Then, 50 μL of Folin Ciocalteau reagent (1;1 dilution with distilled water) were added and the samples were mixed and incubated once again during 30min in darkness. The protein content was assessed after measuring the absorbance spectrophotometrically at 750nm using a microplate reader (ThermoFisher Scientific,Multiscan FC, Instruments Co, Ltd. Shanghai,). The corresponding absorbance values were extrapolated from the calibration curve in order to obtain the concentration of the protein of each sample expressed as μg/ml.

5.2.7 Determination of the expression of pAMPKα/AMPKα, pPARγ and FAS in liver

The expression of pAMPKα/AMPKα, pPARγ and FAS was determined by Western Blot. Samples were homogenized with a specific buffer in pH=8. The entire process was carried out under refrigerated conditions and the proportion of buffer/sample was established at 1:10 (w/v). The samples were homogenized (Micra D-1 homogenizer, ART moderne labortechnik) during 30sec and then sonicated (Sonoplus HD 2070, Bandelin) for the same time period at 50% of the maximum instrument intensity. The supernatant obtained after the centrifugation of the samples at 15000rpm, 4ºC, during 45 min was stored at -80^{0}C. In this supernatant the expression of pAMPKα/AMPKα, pPARγ and FAS were determined as well as the protein content as previously described (section 5.2.6).

REAGENTS

1. HOMOGENIZATION BUFFER OF WESTERN BLOT

- 20mM Tris HCl, pH 8
- 1% octylphenoxypolyethoxyethanol (IGEPAL)
- 1mM ethylene glycol tetraacetic acid (EGTA)
- 1mM sodium orthovanadate
- 1mM dichlorodiphenyltrichloroethane (DDT)
- tablet of ¨cocktail¨ for inhibition of proteases containing when dissolved in a final volume of 100 mL: 1mM Ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethanesulfonyl fluoride (PMSF), aprotinine(2μg/ml), leupeptine (2μg/ml)

2. LOADING BUFFER

- 2 mL 1M Tris HCl, pH 6.8
- 2.5 mL 20% SDS
- 3.75 mL Glycerol
- 875 µL β-mercaptoethanol
- 0.6 mg bromophenol blue
- make up to 10mL with type II H_2O

5.2.7.1 Determination of the expression of Protein kinase Activated of 5´-AMP (AMPKa) and its phosphorylated form in liver by Western Blot

The corresponding volume of tissue homogenate equivalent to 100µg of protein was diluted with loading buffer, heat-denaturated (90°C, 5 min) and loaded in the different wells of 12% SDS-PAGE. The different proteins were separated at 80V during 15 min and 150V during 60 min, using a Mini Trans-Blot cell system (Bio-Rad Laboratories, Hercules, CA). After completing the migration of the samples, the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), using a constant voltage of 100V for 1hour, at 4^0C. Afterwards, the membranes were immersed in blocking buffer during 1.5 hours at room temperature, prior to their staining with a solution of Ponceau Red, with the aim of verifying whether the transference had been performed correctly.

Once completed the Ponceau Red staining, the membranes were washed out with Tris-Buffered saline Tween-20 (TBST) during 30min, replacing the solution every 10min. Then, the membranes were incubated overnight with the primary antibodies anti- AMPKα and anti-pAMPKα (1:1000 in blocking solution; consisting in 5% powdered skim milk solution in TBST), at 4^0C and continuous shaking. The next day, after washing out the membranes, they were incubated with the secondary antibody (1:2000 in blocking solution), for 1.5 hours. The bound antibodies were visualized by an ECL Pro system (PerkinElmer, Boston, USA) using a Fujifilm Luminescent Analyzer LAS-4000 mini System (Fujifilm, Tokyo, Japan). The intensity of the bands obtained was quantified using the programme Image J and the results were expressed as arbitrary units of pAMPKα/AMPKα activity.

5.2.7.2 Determination of the expression of Peroxisome Proliferator-activated receptor gamma (pPARγ) in liver by Western Blot

The same protocol (section 5.2.7.1) was followed for the determination of pPARγ, using a polyclonal antibody of rabbit, anti-pPARγ in a dilution of 1:1000 prepared in blocking solution. In order to carry out the determination of the expression of this enzyme, the amount of liver homogenate used was equivalent to 80mg of protein in a 10% polyacrylamide gel. After the membranes were revealed, they were incubated overnight in TBST at room temperature. The day after, they were incubated overnight with the monoclonal antibody anti-β-actin (1:1000 in blocking solution), at 4^{0}C and continuous shaking. Once completed, and after washing out the membranes during 30min in TBST solution, they were incubated with the secondary antibody (1:2000 in blocking solution), during 1.5 hours. Finally, the membranes were washed out once again and then were revealed, determining this way the expression of β-actin. The obtained bands were quantified Using ImageJ software and were used to standardize the activity of pPARγ.

5.2.7.3 Determination of the expression of Fatty Acid Synthase (FAS) in liver by Western Blot

In a similar manner (section 5.2.7.2), the activity of FAS was assessed by using the corresponding antibody anti-FAS (1:1000 in blocking solution). The amount of liver homogenate used corresponded to 30mg of protein and the percentage of the polyacrylamide gel was set at 8%. Finally, the activity levels were standardized with the activity of β-actin in the same way that was done for the pPARγ enzyme.

REAGENTS USED FOR THE PERFORMANCE OF WESTERN BLOT

1. GEL PREPARATION:

-Mix of acrylamide/bisacrylamide: 29.2g /0.8g in100 mL of type II water. The mix is preserved at 4 ^{0}C, in darkness.

- 1.5M Tris·HCl buffer, pH 8.8. Maintained at 4^{0}C.

- 0.5M Tris·HCl buffer, pH 6.8. Maintained at 4^{0}C.

- 10% SDS (Sodium Dodecyl Sulphate)

2. ELECTROFORESIS BUFFER (SDS-PAGE) 10×. FINAL VOLUME: 1L

-Tris-Base: 30.2g

-Glycine: 144g

-SDS: 10g

3. TRANSFERENCE BUFFER 10×. FINAL VOLUME: 1L

- Tris-Base: 30.2g
- Glycine: 144g

4. TRANSFERENCE BUFFER 1×. FINAL VOLUME: 1L

- 100mL buffer 10x
- 200mL methanol
- 700mL distilled water

5. WASHING BUFFER 10×. FINAL VOLUME: 1L

-TBS 10×. pH 7.4

-Tris-Base: 60.6g

-NaCl: 87.6g

WASHING BUFFER (TBST).

TBS-T= TBS 1×+0.1% Tween 20 (Polyoxyethylene sorbitan-monolaurate)

6. PONCEAU RED SOLUTION

0.1% Ponceau Red

5% Glacial Acetic Acid

7. BLOCKING SOLUTION: 5% Powdered skim milk solution prepared in TBST.

The antibodies used were prepared in blocking solution:

1. Polyclonal antibody of rabbit, anti-AMPK-α (Cell Signaling Technology. Inc)
2. Polyclonal antibody of rabbit, anti-phospho-AMPK-α (Cell Signaling Technology. Inc)
3. Polyclonal antibody of rabbit anti-Fas (Cell Signaling Technology. *Inc)*
4. Polyclonal antibody of rabbit anti- pPARγ (Abcam, Cambridge, USA)
5. Monoclonal antibody β-actin (Abcam, Cambridge,USA)
6. Secondary Polyclonal antibody of rabbit (Cell Signaling Technology. Inc)

5.2.8 Analysis of the genetic expression of GPX1 and GPX4

Prior to the determination of genetic expression of GPx1 and GPx4, total RNA was extracted after the homogenization (Fisher Scientifics, Power Gen 125) of 10-20mg frozen liver tissue using 1ml of Trizol™ reagent (Invitrogen, UK), a solution which helps the isolation of RNA.

The next step involved the addition of 0.2 mL of chloroform followed by agitation during 15 sec and incubation at room temperature for 3 min. The samples were then centrifuged at 13000 rpm, 4^0C, during 15min, and the supernatant was transferred in an RNA-free eppendorf. The samples were spinned down once more prior to the addition of 0.5 mL of isopropanol and their incubation as previously described. Supernatants were removed and the RNA that was precipitated at the bottom of the eppendorf was washed with 0.5mL of EtOH (70%). After the final centrifugation of the samples, they were left to dry. Once dried, they were dissolved in 50μL of H_2O DEPC to prevent possible contamination. RNA purity was determined by the A=260/A=280 ratio, using a UV/VIS spectrophotometer (Thermo Spectronic, Helios γ).

The final concentration of total RNA from the liver samples was 100ng/μL in which the expression of GPX1 and 4 was determined by semi-quantitative RT PCR. Briefly, the RNA was reverse transcribed in a final volume of 15μl, which included: a) the supertranscriptase kit (Superscript Reverse transcriptase kit, Invitrogen, UK), b) a mix of deoxynucleotides triphosphate (10mM, dNTPs, Promega, UK), c) 10-20U RNaOUT (Invitrogen), d) 1mg/mL bovine serum albumin (BSA, BioLabs, UK), and e) 500μg/mL of random hexamers (Random Hexamers, Promega, UK). cDNA was retrotranscribed by the use of a thermocyclator (iCycler, Biorad) and the programme followed included: 1) 10min at 25°C, 2) 52min at 42°C, and 3) 15min at 72°C.

Once retrotrascription was completed, cDNA was amplified by adding 10× PCR buffer (w/o $MgCl_2$), 1.75mM $MgCl_2$, 1U TaqDNA polymerase and 1 μM of each specific primer for GPX1, GPX4 and GAPDH that was used as a housekeeping gene. The final volume of the samples was 15μl. After a hot start (95°C) and 4 min at 94°C, 25 cycles of 1 min at 94°C, 2 min at 59°C and 2 min at 72°C, were performed. Samples were further incubated at 72°C for 8 min to complete any elongation reaction. PCR products were then separated by gel electrophoresis on a 1.7% agarose gel containing GelRed™ (1:10,000, Biotium, UK). PRC Amplified gene products were visualized under UV light and, images were captured using Fusion Fx7 imaging system (PEQLAB Biotechnologies, UK). Optical density of the obtained products was quantified by Image J software. Expression of GPX1 and GPX4 was related to expression of GADPH.

RT-PCR reaction		PCR reaction	
Reagents (1 reaction)	**Volume (µL)**	**Reagents (1 reaction)**	**Volume (µL)**
5× first strand buffer	3	10× PCR buffer (No $MgCl_2$)	1.2
100 mM DTT	1.5	1.75 mM $MgCl_2$	1.05
10 mM dNTP's	1.5	Forward primer 1 □ M	1.5
RNaOUT (10-20 U)	0.75	Reverse primer 1 □ M	1.5
1 mg/mL BSA	1.5	DEPC-H2O	6.6
250 ng/□ L Random Hexamers	1.5	Taq	0.15
RNA	1	RT mix	3.0
Superscript III RNAse H RT	0.3		
DEPC H_2O	4.2		

Table 9. Reagents used for RT-PCR and PCR (1 reaction=15µL)

5.2.9 Histological analysis of the morphological changes in the liver

A portion of liver was stored in 10% formaldehyde and was subsequently immersed in several fixing solutions solutions as previously described in section 3.1.3. The Hematoxylin-Eosin and Masson's Tricromic stain was performed as represented in Figure 8, respectively. The observation and evaluation of the samples was partially blind (observer-blind) and were carried out by light microscopy (Nikon Eclipse 50i).

The hematoxylin eosin stain contributed to the detection and assessment of liver damage. In particular, micro and macrovesicular steatosis, lipogranulomas, portal inflammation and the

presence of multinucleic cells were evaluated by this techique. These parameters are indicators of the development of non-alcoholic fatty liver disease (NAFLD). The evaluation of liver damage was complemented by the determination of fibrosis and necrosis performed by Masson's Tricromic stain.

6. Statistical Analysis

The results of the Phase 1 study are expressed as a mean (n=6)±Standard Error of the Mean (SEM). One-way ANOVA was applied to the data with the use of SAS version 8.02 (SAS Institute, Cary, NC, USA). Differences between means were compared with Tukey's test. The level of significance was set at $P<0.05$.

The statistical analysis of Phase 2 experiments was performed by SAS, version 9.0 and IBM SPSS, version 20. Time-repeated measurement analysis was applied to weekly food intake and body weight data in order to analyze within subject effects (time) or within group effects (phenotype, diet or aerobic interval training protocol) on the above parameters. The effect of phenotype and aerobic interval training protocol on final body weight, aerobic capacity and physical performance, hematic, plasma and liver biochemical parameters, hepatic antioxidant enzyme activity, protein and gene expression was analyzed by 2×2 factorial ANOVA with phenotype and aerobic interval training protocol as main treatments. After the inclusion of the diet treatment, the effect of phenotype, diet and aerobic interval training protocol on final body weight, aerobic capacity and physical performance, hematic, plasma and liver biochemical parameters, hepatic antioxidant enzyme activity and protein expression was analyzed by 2×2×2 factorial ANOVA with phenotype, diet (RVR *vs* GVR) and aerobic interval training protocol as main treatments. Finally, comparisons between animal protein and plant protein were performed using 2×2×2 factorial ANOVA with phenotype, diet (CS *vs* RVR or CS *vs* GVR) and aerobic interval training protocol as main treatments. Results of phase 1 study are given as mean values and individual standard error of the mean whereas results of phase 2 are given as mean values and pooled standard error of the mean. The contribution to total variance (%) of each ANOVA component in phase 2 data is expressed below its P-value. Tukey's test was used to detect differences between treatment means in both experiments. The level of significance was set at $P < 0.05$.

7. Results

7.1 PHASE 1: Effects of *L. albus* protein hydrolyzate and insluble dietary fiber residue in an experimental model of diet-induced hypercholesterolemia

7.1.1 Food Intake, final body weight and digestive utilization of fat

Throughout most of the experimental period, daily food intake was higher in the experimental group of animals that consumed the normolipidemic diet when compared to the experimental groups that consumed high-fat diets, among which no significant differences were observed (Table 10). The lower dietary intake of rats that consumed the high-fat diets can be attributed to their specific composition with higher energy (1826 to 1868 KJ/100 g dry matter (DM) in high fat diets *vs* 1713 KJ/100 g DM in control normolipidemic diet) and methionine content. No significant differences in final body weight were found among the different experimental groups.

Table 10. Influence of dietary treatments on digestive utilization of fat (mean ± SEM).

	CT	HC	HCF	HID	HIDF
Food Intake (g DM/day)	22.1±0.47^{A}	16.8±0.52^{B}	19.2±0.61^{C}	16.3±0.44^{B}	17.9±0.68^{BC}
Final body weight (g)	307.2±5.63^{A}	274.7±4.37^{A}	302.1±10.6^{A}	268.1±12.4^{A}	270.8±13.9^{A}
Fat Intake (mg/day)	1101.4±23.3^{A}	2136.0±65.4^{B}	2320.7±73.7^{B}	2145.6±58.3^{B}	2396.7±91.6^{B}
Fecal weight (g DM/day)	3.05±0.12^{A}	2.96±0.10^{A}	3.08±0.20^{A}	3.08±0.16^{A}	3.79±0.27^{B}
Fecal fat content (%)	1.47±0.23^{A}	5.94±0.11^{B}	7.35±0.30^{C}	5.64±0.16^{B}	6.73±0.19^{C}
Fecal fat excretion (mg/day)	45.4±8.20^{A}	175.0±3.36^{B}	223.8±7.96^{C}	174.1±12.1^{B}	254.4±18.2^{C}
ADC (%)*	96.5±0.27^{A}	91.8±0.15^{B}	90.4±0.20^{C}	91.9±0.62^{B}	89.0±0.40^{C}

^{A,B,C} Results are means of 6 Wistar rats. Means within the same line with different superscripts differ significantly (*P<0.05*). DM, Dry Matter, ADC, Apparent Digestibility Coefficient for total fat. ADC = $[(I - F) / I] \times 100$, where I = total fat intake, and F = fecal fat excretion. CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group.

Daily fat intake was significantly higher for the groups of animals that consumed hypercholesterolemic high-fat diets compared to the control normolipidemic group (Table 10). Fecal fat content and excretion was significantly higher in the hypercholesterolemic high-fat diet-fed groups when compared to the normolipidemic control, and among the former experimental groups, the amount of fat excreted in feces was higher ($P<0.05$) in the groups of animals that consumed lupin insoluble fiber residue (HCF, HIDF). The above described results were reflected in significantly reduced fat digestibilities exhibited by the hypercholesterolemic high-fat diet-fed groups when compared to the normolipidemic control, and the lowest fat digestibility ($P<0.05$) in HCF and HIDF groups.

7.1.2 Biochemical and hematic parameters

Consumption of a Casein/Cellulose-based high-fat diet (HC) led to a considerable increase ($P< 0.05$) in the levels of plasma total-, and LDL-cholesterol, without significantly affecting the glycemia or the content of plasma triglycerides and HDL-cholesterol when compared to the normolipidemic diet (CT) (Table 11). The inclusion of lupin insoluble fiber residue in the casein-based high-fat diet at the expense of cellulose (HCF) did not cause any significant change in the above mentioned biochemical parameters when compared to the HC group, whereas substitution of casein by the lupin protein hydrolyzate (HID, HIDF) significantly decreased triglyceridemia and glycemia when compared to HC and HCF groups. On the other hand, no significant differences in total-, LDL-, or HDL-cholesterol when compared to Casein/Cellulose-based high-fat diet-fed group (HC) were observed derived from the dietary inclusion of lupin protein hydrolyzate (HID, HIDF).

Table 11. Influence of dietary treatments on plasmatic parameters (mean ± SEM).

	CT	HC	HCF	HID	HIDF
Triglycerides (mg/dL)	85.3±5.0^{AB}	109.3±9.2^{A}	119.8±5.9^{A}	73.5±6.3^{B}	67.2±4.4^{B}
Total-cholesterol (mg/dL)	51.7±3.2^{A}	119.1±10.6^{B}	116.7±8.8^{B}	124.0±4.4^{B}	124.6±4.7^{B}
LDL-cholesterol (mg/dL)	20.6±8.5^{A}	80.5±10.5^{B}	66.2±9.7^{B}	90.0±4.7^{B}	93.0±4.8^{B}
HDL-cholesterol (mg/dL)	29.3±1.56^{A}	22.7±2.17^{ABC}	26.7±2.57^{AC}	16.8±1.19^{B}	21.2±0.79^{BC}
Glucose (mg/dL)	196.0±5.8^{A}	180.0±6.0^{A}	170.6±5.7^{A}	137.6±5.3^{B}	155.6±5.9^{B}

^{A,B} Results are means of 6 Wistar rats. Means within the same line with different superscripts differ significantly (*$P<0.05$*). CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group.

Consumption of hypercholesterolemic high-fat diets (HC, HCF, HID, HIDF) caused a reduction in erythrocyte count, hematocrit, and plasma Fe content when compared to the animals fed the normolipidemic diet (Table 12). No appreciable effect derived from the protein or dietary fiber source administered could be observed in hematocrit or plasma Fe content, whereas erythrocyte count was significantly improved by the inclusion of the lupin protein hydrolyzate in the diet (HID) when compared to the HC group.

Table 12. Influence of dietary treatments on hematic parameters (mean ± SEM).

	CT	HC	HCF	HID	HIDF
WBC (×10^3/ µl)	4.60±0.59^A	4.89±0.29^A	5.12±0.37^A	5.22±0.56^A	4.22±0.39^A
RBC (×10^6/ µL)	8.93±0.15^A	7.96±0.11^B	8.23±0.07^{BC}	8.47±0.09^{AC}	8.21±0.14^{BC}
HGB (g/dL)	15.4±0.19^A	14.4±0.19^{AB}	14.4±0.28^{AB}	13.6±0.15^B	13.8±0.48^B
HCT (%)	48.1±0.62^A	45.2±0.51^B	45.3±0.66^B	44.1±0.31^B	44.1±1.25^B
MCV (fL)	53.9±0.50^{AC}	56.7±0.30^B	55.1±0.99^{AB}	52.1±0.62^C	53.7±0.76^{AC}
MCH (pg)	17.3±0.20^{AB}	18.0±0.18^A	17.5±0.04^{AC}	16.1±0.29^B	16.8±0.39^{BC}
MCHC (g/dL)	32.1±0.13^A	31.8±0.23^A	31.7±0.19^A	30.8±0.21^B	31.3±0.40^{AB}
Fe (µg/dL)	148.6±6.27^A	119.0±8.45^B	126.8±3.06^B	117.5±9.16^B	113.0±11.29^B

^{A,B,C} Results are means of 6 Wistar rats. Means within the same line with different superscripts differ significantly (*$P<0.05$*). WBC, White blood cells, RBC, Red blood cells, HGB, Hemoglobin, HCT, Hematocrit, MCV, Mean corpuscular volume, MCH, Mean corpuscular hemoglobin, MCHC, Mean corpuscular hemoglobin concentration, CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group.

7.1.3 Liver function

Liver weight and fat content were significantly affected by the dietary treatments assayed, with higher values found for all the experimental groups that consumed the hypercholesterolemic high-fat diets (HC, HCF, HID, HIDF) (Table 13). Such higher liver fat content caused a concomitant reduction in hepatic moisture, protein, ash and total-P content. However, no significant differences in the above mentioned parameters could be observed derived from the inclusion of lupin protein hydrolyzate or insoluble fiber residue. In contrast, there was a significant diet effect on the content of hepatic triglycerides that were lower in the animals fed the lupin protein hydrolyzate-based diets (HID, HIDF) when compared to those fed the casein-based high-fat diets (HC, HCF).

Plasmatic levels of AST and ALT rose significantly in the group of animals fed the Casein/Cellulose-based high-fat diet (HC) when compared to the normolipidemic control. The inclusion of lupin protein hydrolyzate or insoluble fiber residue in diets HCF, HID or HIDF caused a considerable reduction ($P < 0.05$) in ALT, whereas AST activity was only significantly improved in the HIDF group. Plasmatic GGT activity was not different among the different casein-based experimental groups (CT, HC, HCF), whereas it was significantly reduced by lupin protein hydrolyzate consumption. No significant effect of the different diets tested could be observed for plasma bilirubin or ALP activity.

Table 13. Influence of dietary treatments on weight, chemical composition, and functionality of liver (mean ± SEM).

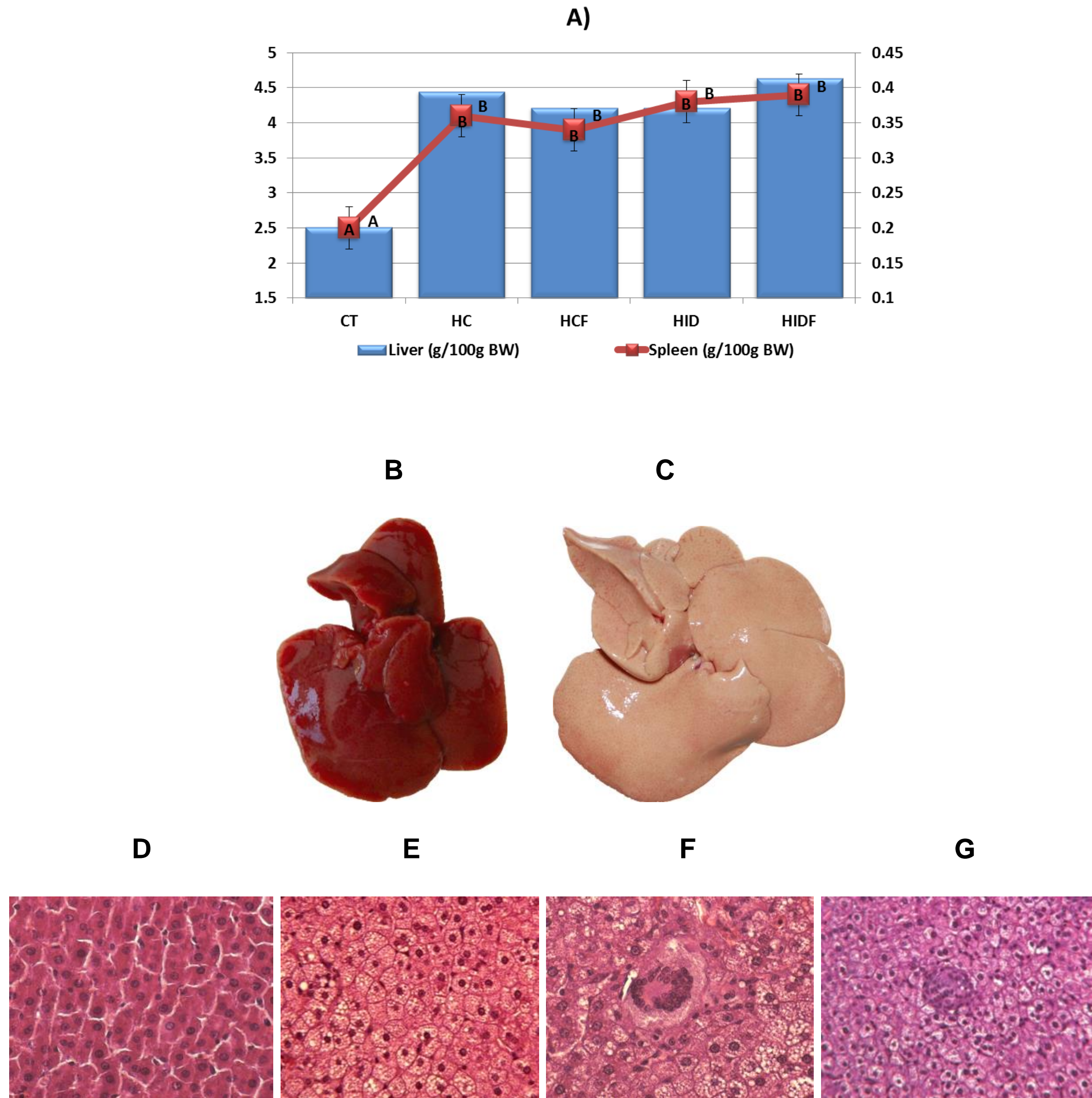
	CT	HC	HCF	HID	HIDF
Weight (g fresh sample)	7.7±0.21^{A}	12.2±0.31^{B}	12.7±0.51^{B}	11.3±0.66^{B}	12.6±0.83^{B}
Relative weight (g/100g BW)	2.51±0.03^{A}	4.45±0.11^{B}	4.22±0.19^{B}	4.22±0.22^{B}	4.64±0.12^{B}
Moisture (%)	71.9±0.20^{A}	61.1±0.97^{B}	61.9±1.14^{B}	61.8±1.21^{B}	60.0±1.05^{B}
Protein (g/100g DM)	70.9±3.04^{A}	42.0±1.32^{B}	41.7±2.74^{B}	35.8±1.24^{B}	36.0±1.94^{B}
Ash (g/100g DM)	4.56±0.17^{A}	2.59±0.16^{B}	2.78±0.24^{B}	2.73±0.15^{B}	2.87±0.18^{B}
P (mg/100g DM)	1220.1±31.6^{A}	718.0±27.0^{B}	795.4±29.5^{B}	766.4±23.9^{B}	724.3±23.4^{B}
Fat (g/100g DM)	12.6±1.38^{A}	47.8±2.65^{B}	44.6±2.43^{B}	47.2±1.59^{B}	51.4±2.24^{B}
Hepatic Triglycerides (mg/g DM)	5.8±0.53^{A}	28.8±5.80^{B}	28.8±5.05^{B}	15.4±1.94^{C}	16.0±1.92^{C}
Liver function plasmatic markers					
AST (U/L)	204.8±25.9^{A}	421.7±32.5^{B}	348.8±15.9^{BC}	333.0±12.8^{BC}	269.7±35.6^{AC}
ALT (U/L)	45.3±9.2^{A}	206.0±26.8^{B}	106.7±12.2^{C}	75.0±4.5^{A}	108.4±19.0^{C}
ALP (U/L)	261.0±20.8^{A}	338.9±20.2^{A}	330.5±25.5^{A}	349.4±27.7^{A}	320.7±20.8^{A}
GGT (U/L)	13.6±0.80^{A}	10.8±0.34^{A}	12.7±0.99^{A}	6.33±0.95^{B}	5.44±0.89^{B}
Bilirrubin (mg/dL)	0.26±0.06^{A}	0.27±0.03^{A}	0.25±0.02^{A}	0.28±0.03^{A}	0.32±0.03^{A}

^{A,B,C} Results are means of 6 Wistar rats. Means within the same line with different superscripts differ significantly (*P<0.05*). BW, Body weight; DM, Dry matter;AST, aspartate aminotransferase; ALT, alanine transaminase; ALP, Alkaline Phosphatase; GGT, Gamma-glutamyl transpeptidase; CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group

The influence of a hipercholesteolemic high-fat diet on liver morphology and different hepatic histological features is presented in Figure 10. The dietary treatment caused a significant

increase in liver size that was matched by changes in liver appearance and histology such as microvesicular change, multinucleic cells and microgranulomas. No apparent improvement in liver status was achieved by the inclusion of lupin protein hydrolyzate or insoluble fiber residue in the diet.

Figure 10. Influence of dietary treatments on liver weight, morphology and histology.



A, liver and spleen weight. Results are means of 6 Wistar rats with SEM represented as vertical bars. Means not sharing common letters differ significantly (*P<0.05*). CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group. B-E, liver macroscopic morphology and histology: B, control healthy liver; C, fatty liver after hypercholesterolemic high-fat diet treatment; D, control healthy liver histology; E,

microvesicular fat deposition after hypercholesterolemic high fat diet treatment; F, multinucleated cell after hypercholesterolemic high fat diet treatment; G, microgranuloma after hypercholesterolemic high fat diet treatment.

7.1.4 Renal function

Urinary volume was highest (*P<0.05*) in the experimental group that consumed the Lupin protein hydrolyzate/Cellulose-based high-fat diet (HID), whereas no significant difference was observed among the rest of experimental groups (Table 14). With regard to urinary pH, it significantly decreased in all the hypercholesterolemic high-fat diet-fed animals (HC, HCF, HID, HIDF) when compared to the normolipidemic controls.

The urinary content of urea was significantly lower in animals fed HID and HIDF diets when compared to the rest of experimental groups tested (CT, HC, HCF) among which no significant differences were observed. A similar trend was observed for uric acid, although no significant differences were found between the CT and HIDF groups. Glucose content was highest in HC group (*P<0.05*) followed by CT and HCF, and finally by HID and HIDF groups that exhibited the lowest values (*P<0.05*). The urinary content of albumin and creatinine increased significantly in casein-based hypercholesterolemic high-fat diet-fed animals (HC, HCF) when compared to the normolipidemic control, and returned to normal values in the HID and HIDF groups.

Calciuria increased significantly in response to lupin protein hydrolyzate and insoluble fiber residue, and the highest value was obtained for the HCF group followed by HID and HIDF groups. On the other hand, phosphaturia increased significantly in the HC and HCF groups when compared to the normolipidemic control, HID and HIDF groups. Urinary citrate was lowest (*P<0.05*) in casein-based high-fat diets when compared to their lupin protein hydrolyzate counterparts that were similar to the normolipidemic control.

Plasma markers of renal function such as urea, uric acid or creatinine were not affected by any of the dietary treatments assayed.

Table 14. Influence of dietary treatments on parameters of renal function (mean ± SEM).

	CT	HC	HCF	HID	HIDF
Kidney (g/100g BW)	0.30 ± 0.01^{A}	0.36 ± 0.01^{B}	0.35 ± 0.01^{AB}	0.35 ± 0.01^{AB}	0.35 ± 0.01^{AB}
Urine					
Urinary pH	6.82 ± 0.09^{A}	5.68 ± 0.04^{B}	5.93 ± 0.07^{B}	6.05 ± 0.17^{B}	6.09 ± 0.17^{B}
Urinary volume (mL)	2.85 ± 0.25^{A}	1.26 ± 0.15^{A}	2.09 ± 0.42^{A}	5.02 ± 0.83^{B}	3.05 ± 0.60^{A}
Urea (g/dL)	6.73 ± 0.43^{A}	8.56 ± 0.35^{A}	7.79 ± 0.76^{A}	2.40 ± 0.48^{B}	4.02 ± 0.67^{B}
Uric acid (mg/dL)	21.3 ± 2.87^{AB}	27.1 ± 5.55^{A}	27.4 ± 5.71^{A}	10.2 ± 1.44^{B}	16.1 ± 1.48^{AB}
Glucose (mg/dL)	20.6 ± 1.04^{A}	41.6 ± 4.18^{B}	26.6 ± 3.70^{A}	6.26 ± 2.33^{C}	6.75 ± 2.52^{C}
Albumin (g/dL)	0.115 ± 0.02^{A}	0.241 ± 0.03^{B}	0.209 ± 0.05^{B}	0.065 ± 0.01^{A}	0.080 ± 0.02^{A}
Creatinine (mg/dL)	146.7 ± 13.9^{A}	317.9 ± 25.0^{B}	270.0 ± 28.8^{B}	102.1 ± 21.0^{A}	143.8 ± 28.4^{A}
Citrate (mg/dL)	1082.7 ± 174.2^{A}	535.9 ± 36.3^{B}	492.6 ± 79.9^{B}	988.3 ± 118.3^{A}	1207.8 ± 254.5^{A}
Ca (mg/L)	38.7 ± 7.65^{A}	52.8 ± 7.78^{A}	214.2 ± 16.6^{B}	140.1 ± 17.9^{C}	113.7 ± 17.1^{C}
P (mg/dL)	326.6 ± 30.9^{A}	649.3 ± 76.8^{B}	625.8 ± 47.6^{B}	151.0 ± 35.2^{A}	167.6 ± 38.4^{A}
Plasma					
Urea (mg/dL)	33.5 ± 2.1^{A}	31.0 ± 4.6^{A}	32.8 ± 1.4^{A}	37.0 ± 3.8^{A}	36.7 ± 2.8^{A}
Uric acid (mg/dL)	1.95 ± 0.13^{A}	1.60 ± 0.07^{A}	1.57 ± 0.08^{A}	1.88 ± 0.14^{A}	1.60 ± 0.08^{A}
Creatinine (mg/dL)	0.47 ± 0.02^{A}	0.47 ± 0.02^{A}	0.48 ± 0.03^{A}	0.45 ± 0.02^{A}	0.42 ± 0.02^{A}

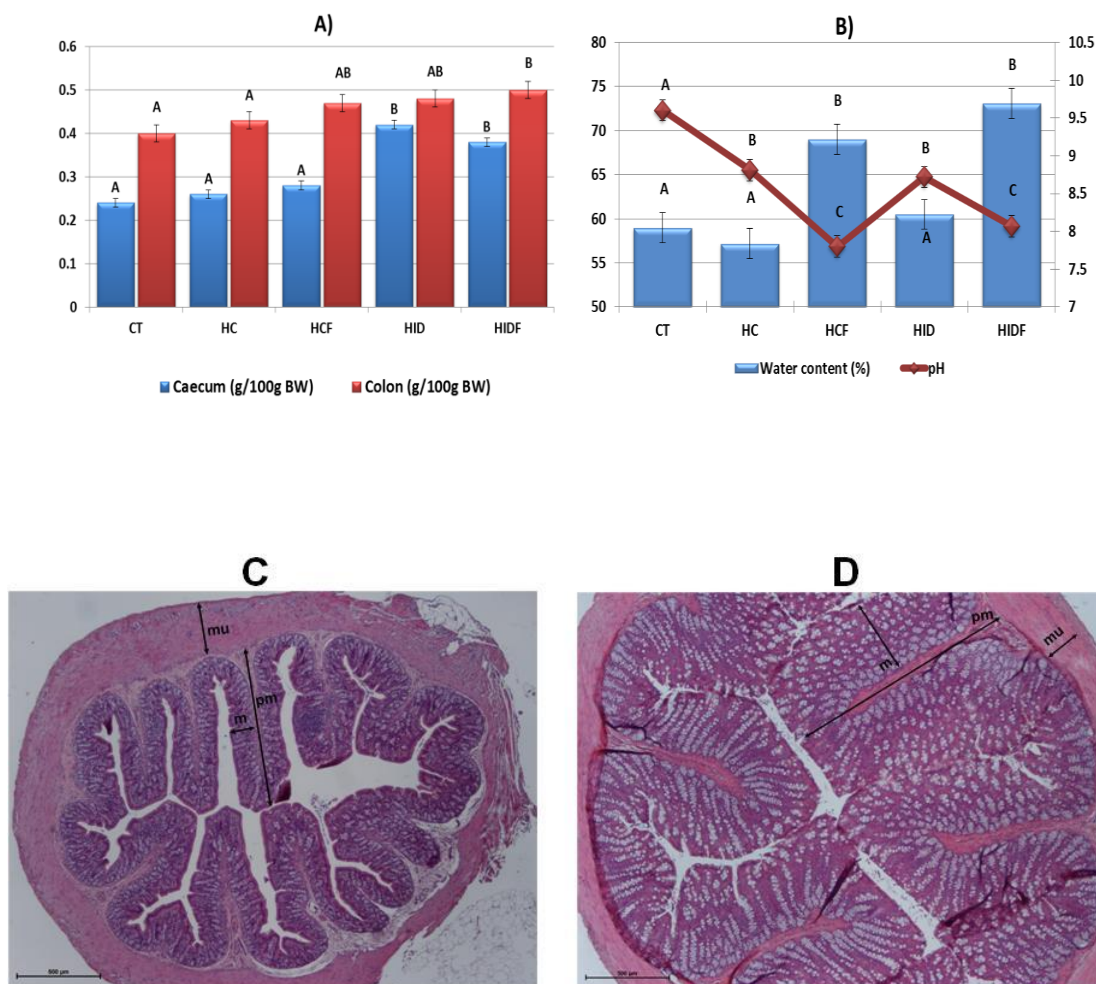
^{A,B,C} Results are means of 6 Wistar rats. Means within the same line with different superscripts differ significantly (*$P<0.05$*). BW, Body weight; CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group.

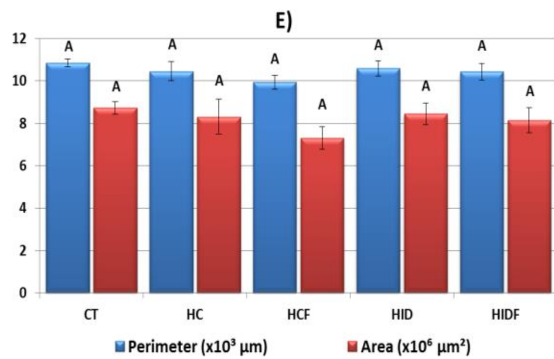
7.1.5 Large intestine histology and functionality

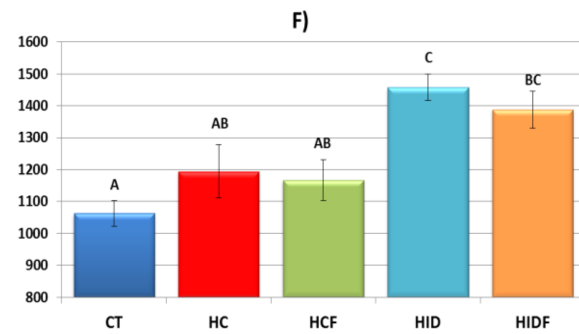
Significant diet-induced changes in large intestine weight, functionality, and morphometry were observed under our experimental conditions (Figure 11). A higher fresh weight of caecum and colon was mainly related to the inclusion of lupin protein hydrolyzate in the hypercholesterolemic high-fat diets (Figure A), whereas the degree of pH reduction, and the increased moisture levels of caecum content

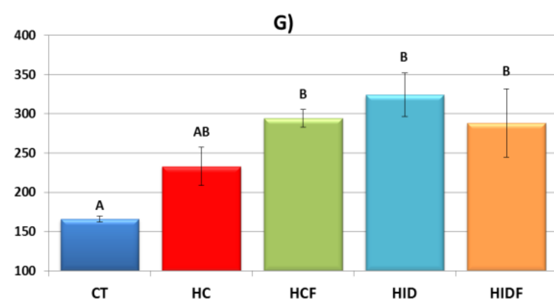
appeared to be more closely related to the dietary inclusion of lupin insoluble fiber residue (Figure B). With regard to proximal colon morphometry, the dietary inclusion of both lupin protein hydrolyzate and lupin insoluble fiber residue had a significant effect on the different parameters studied (Figures C-H). The length of *plica mucosae* was significantly increased while the thickness of muscular tunic was significantly reduced in response to lupin protein hydrolyzate (Figures D, F, H) when compared to a control normal colon (Figures C, F, H). Furthermore, the thickness of mucosal tunic was increased by both the dietary inclusion of lupin protein hydrolyzate and insoluble dietary fiber residue (Figures D, G). All such changes took place without any significant effect of the dietary treatments assayed in neither the proximal colon perimeter nor the luminal surface area (Figure E).

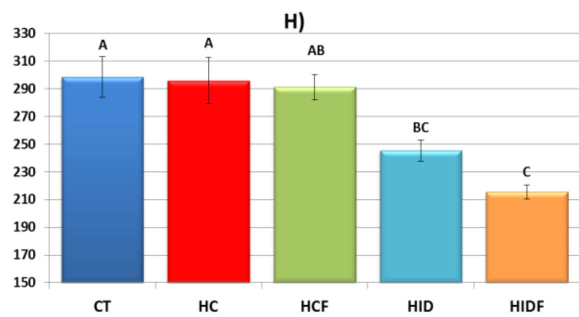
Figure 11. Influence of dietary treatments on large intestine weight, functionality, and morphometric studies.











(A) Caecum and colon weight, (B) Water content and pH of caecum content, (C) Histological view of control (CT) proximal colon, (D) Histological view of hyperplastic proximal colon (pm, plica mucosae; m, mucosa tunic; mu, external muscular tunic), (E) Perimeter (µm) and luminal surface area of the proximal colon (µm2), (F) Length of proximal colon plica mucosae (µm), (G) Thickness of proximal colon mucosa tunic (µm), (H) Thickness of proximal colon muscular tunic (µm). Values are means with Standard Error of the Mean represented as vertical bars (n = 6). Means in the same group not sharing common letters differ significantly (P<0.05). BW, Body Weight, CT, Casein/Cellulose-based normolipidemic control group ; HC, Casein/Cellulose-based hypercholesterolemic high-fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high-fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high-fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high-fat experimental group.

7.2 PHASE 2: Effects of raw and 4d-germinated *Vigna radiata* flours combined with a HIIT protocol in an experimental model of genetically obese Zucker rat

7.2.1 Cell culture experiments

7.2.1.1 Changes on HT-29 viability and metabolic status after pre-incubation with *Vigna radiata* extracts

The results of the pre-incubation assay of the cells with the *Vigna radiata* extracts and Paraquat and SNAP are shown in Figure 12. As demonstrated by the Crystal Violet assay, the viability of the cells was significantly enhanced after the addition of the *Vigna radiata* extracts, especially the one after 4-day germination, which showed the highest values. After the addition of Paraquat and SNAP, a 35% decrease in the cell viability was observed, a situation that was reversed after the addition of the extracts. In a similar manner, the *Vigna radiata* extracts did not cause any decrease in the metabolic status of the cells after the pre-incubation assay. Peroxynitrite generation caused by the action of Paraquat and SNAP caused a 7.5-fold decrease in the metabolic status of the cells that was partially reversed by the different lgume extracts. In this case, raw extract of *Vigna radiata* demonstrated the highest potential to reverse the negative effects on cell metabolic status induced by peroxynitrite, while the 4 day germinated *Vigna radiata* extract the lowest. However, there were no statistical differences between the 4 different extracts.

7.2.1.2. Changes on HT-29 viability and metabolic status after co-incubation with *Vigna radiata* extracts

The results of the co-incubation of the cells with the *Vigna radiata* extracts, Paraquat and SNAP are shown in Figure 12. An increase in the viability of the cells measured by the crystal violet assay was observed after exposure to the different extracts. Such increase was rated around 10% and no statistically significant differences were observed between the different extracts. As expected, the addition of Paraquat and SNAP caused a significant reduction on the viability of the cells and the *Vigna radiata* extracts managed to increase it up by 30-50%. In this assay the 4-day germination extract demonstrated the highest values, when compared to the rest of the extracts. The co-incubation of the cells with the *Vigna radiata* extracts revealed minor reductions in the metabolic status of the cells as demonstrated by the MTT assay. However, the addition of Paraquat and SNAP reduced the metabolic status of the cells by 80%. The *Vigna radiata* extracts induced more than 2 fold increase on the metabolic status of the cells, especially for the raw

extract which demonstrated the highest increase. The extract after 2-day germination showed the lowest improvement on metabolic status of the cells.

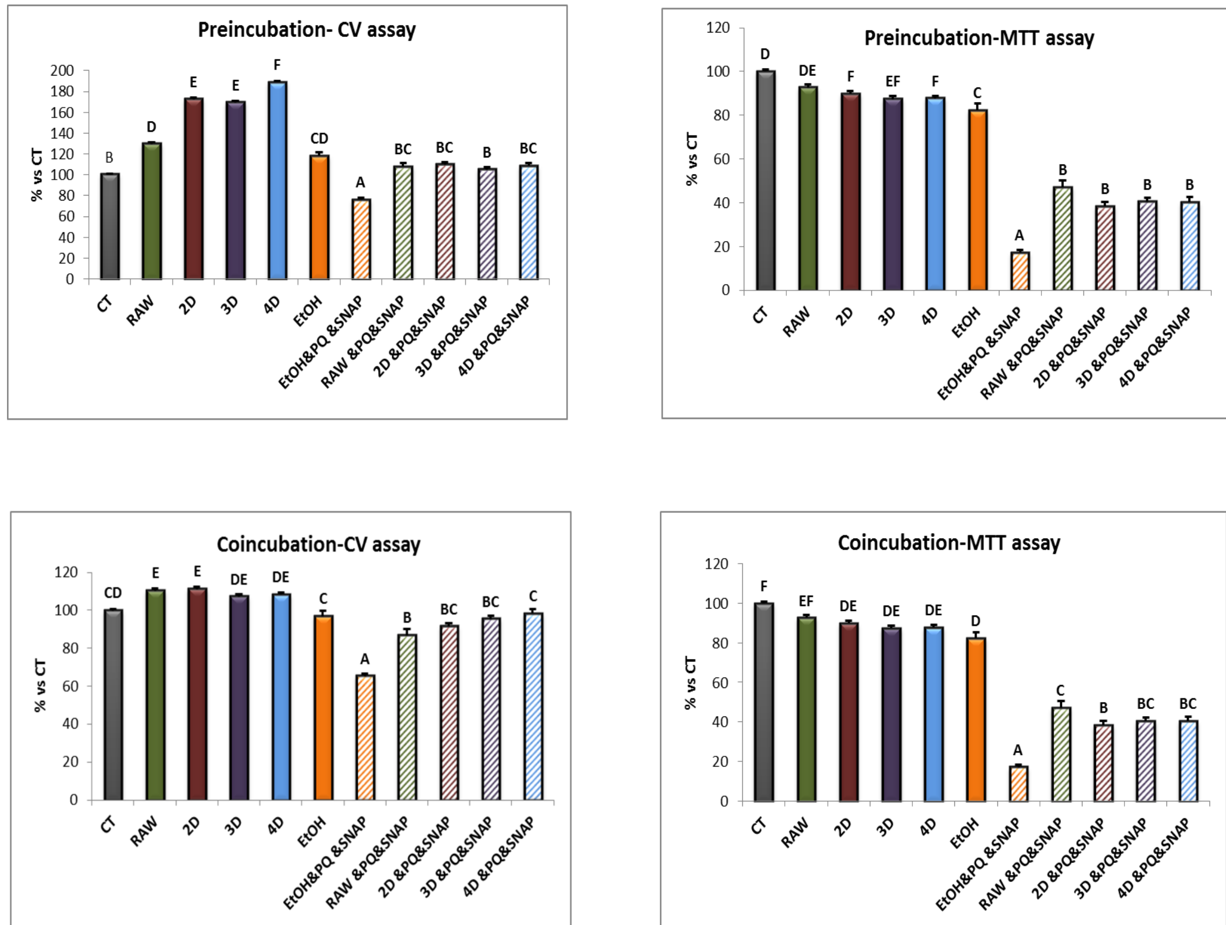


Figure 12. Effects of preincubation and coincubation of Vigna radiata extracts on HT-29 cell viability and metabolic status.

7.2.2 *In vivo* experiments: Effects of Zucker rat phenotype, diet and a protocol of interval aerobic exercise

In order to describe the results of phase 2 experiments, the following order will be applied for all the major findings. First the effects of phenotype and exercise will be reported in the experiments in which lean or obese rats with a sedentary lifestyle or following a HIIT protocol were fed the experimental diet with casein as main protein source (CS). Following that, the effects of diet will be assessed comparing the results obtained in lean or obese rats with a sedentary lifestyle or following a HIIT protocol that were fed diets with raw (RVR) or 4d-germinated *Vigna radiata* (GVR) as main protein source. Finally, the results of statistical analysis comparing the dietary treatments with casein or 4d-germinated *Vigna radiata* in lean or obese rats with a sedentary lifestyle or following a HIIT protocol, will be presented.

7.2.2.1 Weekly intake and body weight

The effects of phenotype and aerobic training protocol on weekly food intake and body weight are presented in Figures 13 and 14, respectively. Both parameters were affected by time, phenotype and exercise. There was a significant effect of phenotype on weekly food intake and body weight as the obese groups demonstrated higher values for both parameters when compared with the lean ones. Also, a significant effect due to the HIT was observed, leading to decreased values of food intake and body weight in the obese groups that underwent the exercise training protocol when they were compared with their sedentary counterparts. Moreover there was a significant effect of diet on weekly food intake that tended to be higher in the animals fed the diets of raw and 4d-germinated *Vigna radiata* when compared to those fed the casein diets. Finally, the 4d-germinated *Vigna radiata* diet led to lower weekly body weight as compared with the other two experimental diets tested.

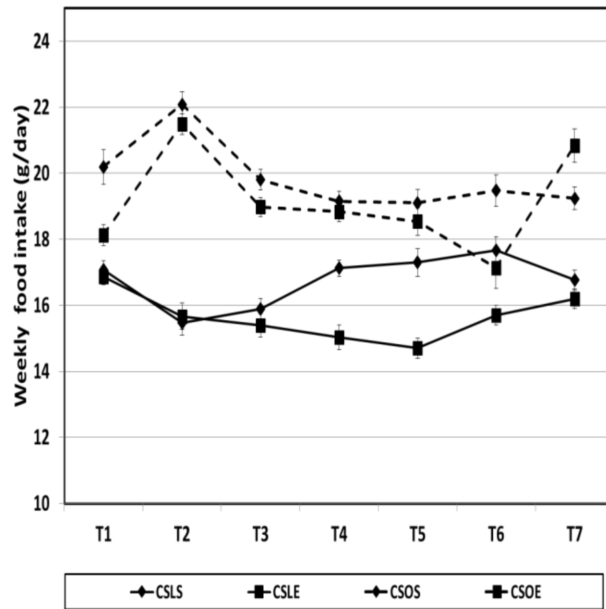
7.2.2.2 Aerobic capacity and physical performance

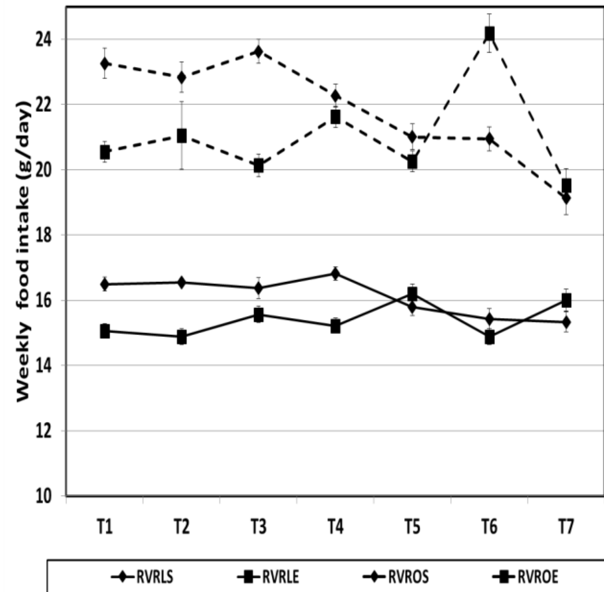
The effects of phenotype and a protocol of aerobic interval training on aerobic capacity and physical performance of Zucker rats undergoing a maximal oxygen consumption incremental test are shown in Table 15. The effects of the two *Vigna radiata* experimental diets on aerobic capacity and physical performance of Zucker rats are shown in Table 16. The comparison of the 4d-germinated *Vigna radiata* with the casein experimental diet are shown in Table 17.

Almost all parameters related to aerobic capacity and physical performance were significantly affected by phenotype in the experimental groups fed animal protein diet. Basal and exercise

lactate as well as respiratory quotient were higher in the obese compared to the lean groups, whereas opposite results were observed in running time, maximal speed and distance run. There was a significant effect of the HIIT protocol on the majority of aerobic capacity and physical performance parameters studied. Exercise caused a decrease in the levels of lactate of obese animals as measured at the end of the incremental test. However, no differences were observed among the lean phenotype animals. Furthermore, a significant effect of exercise was observed on running time, maximal speed and distance, increasing the three of them in both phenotypes. In contrast there were no statistical differences on VO_2 max and basal lactate due to exercise.

Regarding the inclusion of raw or 4d-germinated *Vigna radiata* in the experimental diets, aerobic capacity measured by maximal oxygen consumption was enhanced by the HIIT protocol at greater degree when compared to casein inclusion, and more pronounced effects were found in lean compared to obese animals. All these, gave rise to a significant exercise effect and phenotype × exercise interactions (Table16). In addition, significant exercise and diet effects, as well as exercise × diet, exercise × phenotype, and exercise × phenotype × diet interactions were observed after comparing 4d-germinated *Vigna radiata vs* casein dietary treatment (Table 17). Other parameters related to physical performance of the rats such as running time, maximal speed and distance run were also differentially affected by exercise depending on the type of diet consumed. In this regard, the enhancing effect of exercise in both lean and obese animals was greatest in rats fed 4d-germinated *Vigna radiata*, followed by rats fed raw *Vigna radiata* and casein. Such effect of the HIIT protocol assayed was also affected by rat phenotype, and all the above mentioned gave rise to significant exercise and diet effects, as well as significant exercise × diet, phenotype × exercise, and phenotype × diet interactions. Respiratory quotient as a parameter of metabolic adaptation of the animals, was mostly affected by phenotype and consumption of 4d-germinated *Vigna radiata*, which caused a significant reduction of this index in obese but not in lean animals when compared to rats fed either raw *Vigna radiata* or casein. The above mentioned gave rise to significant phenotype effect, diet effect and phenotype × diet interaction.





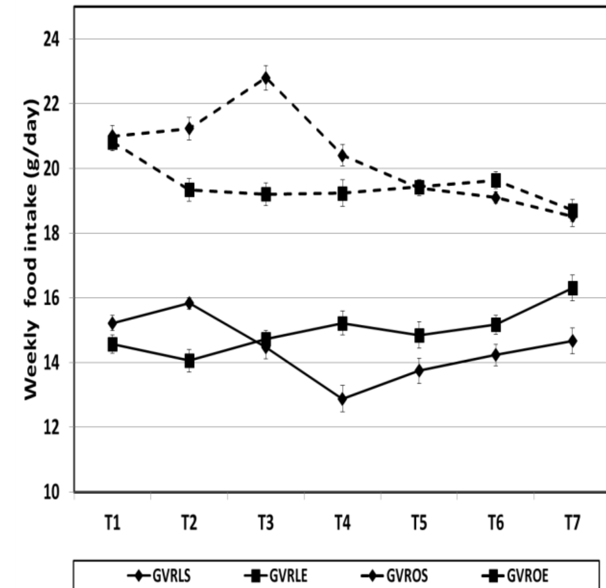
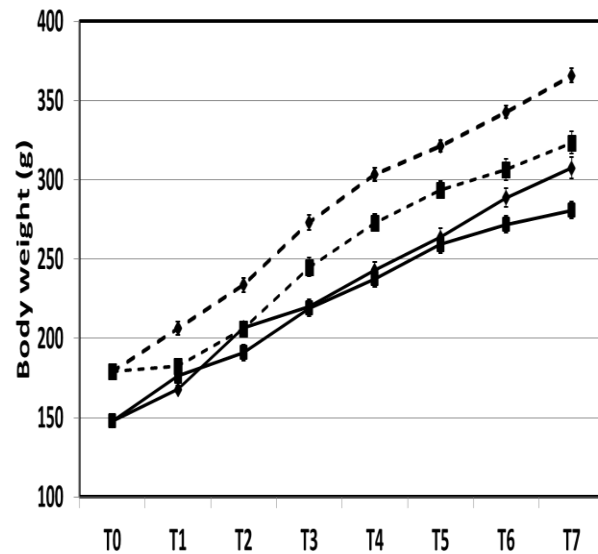
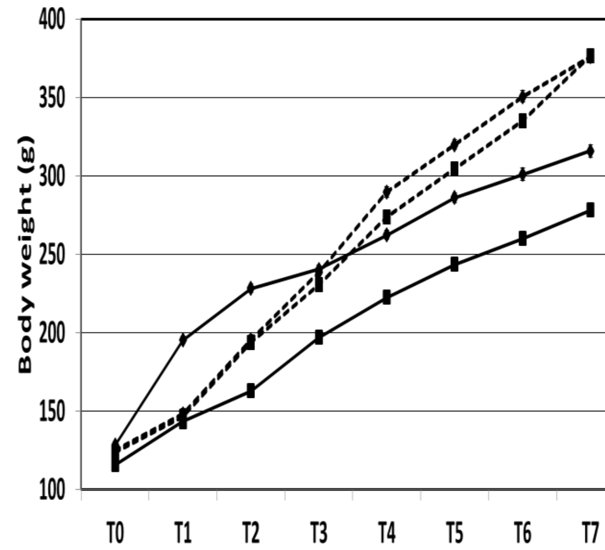


Figure 13. Effect of phenotype, diet, and a protocol of interval aerobic exercise on weekly food intake (grams Dry Matter/day). Diets: CS, casein diet, RVR, raw *Vigna radiata* diet, GVR, germinated *Vigna radiata* diet, Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing a protocol of aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing a protocol of aerobic interval exercise. Values are means ± SEM depicted by vertical bars (n = 10).



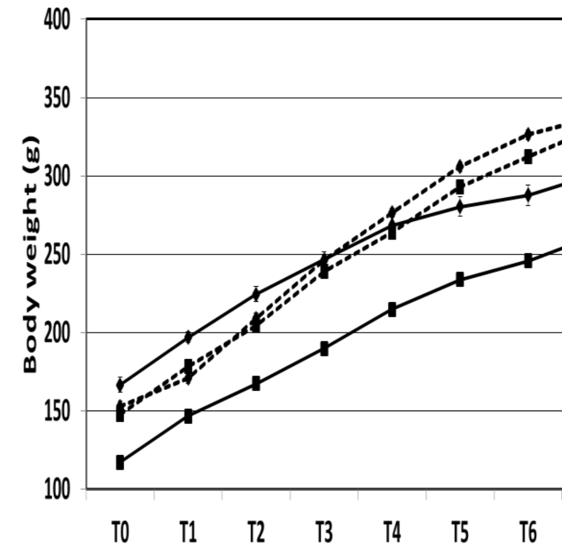
CSLS CSLE CSOS CSOE

Time Effect: P < 0.0001
Exercise Effect: P < 0.0001
Phenotype Effect: P < 0.0001
Phenotype × Exercise: P = 0.072



RVRLS RVRLE RVROS RVROE

Time Effect: P<0.001
Exercise Effect: P<0.001
Phenotype × Exercise: P<0.001
Phenotype Effect: P<0.001
Diet Effect: P=0.404



GVRLS GVRLE GVROS GVROE

Time Effect: P<0.001
Exercise Effect: P<0.001
Phenotype × Exercise: P<0.001
Phenotype Effect: P<0.0
Diet Effect: P=0.404

Figure 14. Effect of phenotype, diet, and a protocol of interval aerobic exercise weekly body weight of Zucker rats (grams). Diets: CS, casein diet, RVR, raw *Vigna radiata* diet, GVR, germinated *Vigna radiata* diet, Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing a protocol of aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing a protocol of aerobic interval exercise. Values are means ± SEM depicted by vertical bars (n = 10).

Table 15. Effect of phenotype and a protocol of interval aerobic exercise on aerobic capacity and physical performance of Zucker rats.

	Lean		Obese						
	Sedentary	Exercise	Sedentary	Exercise	SEM	R^2	Phenotype Effect	Exercise Effect	Phenotype × Exercise
Basal Lactate (mmol/L)	2.08[A]	2.15[A]	4.63[B]	3.95[B]	0.38	0.6390	P < 0.0001 (61.3%)	P = 0.2740	P = 0.1780
Exercise Lactate (mmol/L)	6.78[A]	6.17[A]	15.7[C]	10.4[B]	0.79	0.7822	P < 0.0001 (59.5%)	P = 0.0003 (12.5%)	P = 0.0076 (6.2%)
VO_2max (mL/min/kg$^{0.75}$)	18.7[A]	19.9[A]	17.7[A]	19.5[A]	1.01	0.1049	P = 0.3318	P = 0.1380	P = 0.7525
RQ	0.67[A]	0.90[B]	1.12[C]	1.21[C]	0.05	0.7052	P < 0.0001 (57.9%)	P = 0.0031 (10.5%)	P = 0.1610
Running Time (min)	13.3[B]	23.6[C]	7.6[A]	11.2[B]	0.72	0.9204	P < 0.0001 (64.8%)	P < 0.0001 (21.5%)	P < 0.0001 (5.6%)
Maximal Speed (cm/sc)	55.8[B]	85.6[C]	38.9[A]	49.8[B]	2.2	0.9161	P < 0.0001 (64.6%)	P < 0.0001 (21.7%)	P = 0.0002 (5.3%)
Distance (cm)	30456[B]	46743[C]	14538[A]	23847[B]	2066	0.8865	P < 0.0001 (65.0%)	P < 0.0001 (18.2%)	P = 0.1102

[A,B,C] Results are mean of 10 rats. Means within the same line with different superscripts differ significantly ($P < 0.05$). RQ, Respiratory quotient, VO_2 max, Maximum Oxygen Volume. SEM, pooled standard error of the mean. Data in parentheses represent the contribution to total variance of the specific ANOVA component.

Table 16. Effect of phenotype, diet and a protocol of interval aerobic exercise on aerobic capacity and physical performance of Zucker rats

	RVR				GVR												
	Lean		Obese		Lean		Obese		SEM	R^2	Phenotype Effect	Exercise Effect	Diet Effect	Phenotype × Exercise	Phenotype × Diet	Exercise × Diet	Phenotype × Exercise × Diet
	SED	EXC	EXC	SED	SED	EXC	SED	EXC									
Basal Lactate (mmol/L)	2.33^{BC}	1.54^{A}	2.76^{CD}	2.87^{CD}	2.03^{AB}	2.27^{BC}	3.38^{D}	3.20^{D}	0.154	0.616	P<0.001 (46.9%)	P=0.169	P=0.002 (5.48%)	P=0.280	P=0.210	P=0.084	P=0.003 (5.01%)
Exercise Lactate (mmol/L)	6.65^{A}	5.07^{A}	13.62^{C}	10.21^{B}	7.53^{A}	6.91^{A}	13.94^{C}	10.08^{B}	0.569	0.769	P<0.001 (60.3%)	P<0.001 (11.16%)	P=0.077	P=0.002 (3.30%)	P=0.121	P=0.744	P=0.380
VO_2max (mL/min/kg$^{0.75}$)	18.5^{A}	25.4^{E}	21.4^{BCD}	22.2^{CD}	19.1^{AB}	25.9^{E}	20.6^{ABC}	23.4^{CD}	0.604	0.668	P=0.440	P<0.001 (48.77%)	P=0.469	P<0.001 (16.21%)	P=0.654	P=0.277	P=0.266
RQ	0.68^{A}	0.98^{B}	1.26^{C}	1.25^{C}	0.95^{B}	0.88^{B}	0.88^{B}	0.84^{B}	0.032	0.794	P<0.001 (18.94%)	P=0.072	P<0.001 (13.60%)	P=0.002 (2.90%)	P<0.001 (32.66%)	P<0.001 (6.07%)	P<0.001 (4.31%)
Running Time (min)	12.5^{A}	21.3^{D}	9.51^{A}	13.9^{BC}	14.2^{BC}	27.1^{E}	8.75^{A}	16.3^{C}	0.592	0.915	P<0.001 (31.2%)	P<0.001 (49.1%)	P<0.001 (3.41%)	P<0.001 (4.07%)	P=0.001 (1.54%)	P<0.001 (2.22%)	P=0.547
Maximal Speed (cm/sec)	53.8^{B}	81.6^{D}	41.5^{A}	51.4^{B}	57.8^{BC}	96.0^{E}	41.2^{A}	63.4^{C}	1.930	0.908	P<0.001 (36.74%)	P<0.001 (42.88%)	P<0.001 (3.78%)	P<0.001 (4.92%)	P=0.195	P<0.001 (2.23%)	P=0.738
Distance (cm)	28434^{B}	65985^{D}	17260^{A}	29931^{BC}	32675^{BC}	94789^{E}	16593^{A}	39616^{C}	2304	0.931	P<0.001 (32.89%)	P<0.001 (42.79%)	P<0.001 (3.90%)	P<0.001 (9.38%)	P<0.001 (1.35%)	P<0.001 (2.82%)	P=0.033 (0.46%)

$^{A, B, C, D}$ Results are mean of 10 rats. Means within the same line with different superscripts differ significantly (P<0.05), RVR, Raw *Vigna radiata*, GVR, Germinated *Vigna radiata*, SED, sedentary rats, EXC, rats performing a protocol of aerobic interval exercise, RQ, Respiratory quotient, SEM, pooled standard error of the mean, Data in parentheses represent the contribution to total variance of the specific ANOVA component.

Table 17. Effect of phenotype, diet and a protocol of interval aerobic exercise on aerobic capacity and physical performance of Zucker rats.

	CS				GVR				SEM	R^2	Phenotype Effect	Exercise Effect	Diet Effect	Phenotype × Exercise	Phenotype × Diet	Exercise × Diet	Phenot × Exerci Diet
	Lean		Obese		Lean		Obese										
	SED	EXC	SED	EXC	SED	EXC	SED	EXC									
Basal Lactate (mmol/L)	2.08^{AB}	2.15^{AB}	4.63^{D}	3.95^{CD}	2.03^{A}	2.27^{AB}	3.38^{C}	3.20^{BC}	0.236	0.657	P<0.001 (51.9%)	P=0.249	P=0.001 (6.3%)	P=0.159	P=0.003 (4.88%)	P=0.218	P=0.7
Exercise Lactate (mmol/L)	6.78^{A}	6.17^{A}	15.65^{C}	10.40^{B}	7.53^{AB}	6.91^{A}	13.94^{C}	10.08^{B}	0.650	0.774	P<0.001 (56.7%)	P<0.001 (1.7%)	P=0.357	P<0.001 (6.0%)	P=0.054	P=0.424	P=0.4
VO_2max (mL/min/kg$^{0.75}$)	18.7^{A}	19.9^{A}	17.7^{A}	19.5^{A}	19.1^{A}	25.9^{C}	20.6^{AB}	23.4^{BC}	0.742	0.614	P=0.098	P<0.001 (0.6%)	P<0.001 (26.4%)	P=0.036 (2.7%)	P=0.695	P=0.005 (5.1%)	P=0.0 (3.0%
RQ	0.67^{A}	0.90^{B}	1.12^{C}	1.21^{C}	0.95^{B}	0.88^{B}	0.88^{B}	0.84^{B}	0.035	0.683	P<0.001 (14.2%)	P=0.481	P<0.001 (10.6%)	P=0.948	P<0.001 (33.1%)	P<0.001 (8.7%)	P=0.0
Running Time (min)	13.26^{CD}	23.56^{F}	7.63^{A}	11.18^{BC}	14.24^{DE}	27.14^{G}	8.75^{AB}	16.27^{E}	0.596	0.942	P<0.001 (47.3%)	P<0.001 (6.2%)	P<0.001 (3.1%)	P<0.001 (5.0%)	P=0.601	P<0.001 (1.4%)	P=0.4
Maximal Speed(cm/sec)	55.8^{CD}	85.6^{E}	38.90^{A}	49.75^{BC}	57.80^{CD}	96.00^{F}	41.20^{AB}	63.40^{D}	1.919	0.931	P<0.001 (47.3%)	P<0.001 (3.7%)	P<0.001 (2.4%)	P<0.001 (4.7%)	P=0.825	P=0.001 (1.4%)	P=0.5
Distance (cm)	30456^{BC}	46743^{D}	14538^{A}	23847^{AB}	32675^{BC}	94789^{E}	16593^{A}	39616^{CD}	2207	0.942	P<0.001 (35.2%)	P<0.001 (8.7%)	P<0.001 (10.8%)	P<0.001 (6.2%)	P<0.001 (4.1%)	P<0.001 (7.3%)	P<0.0 (2.3%

$^{A, B, C, D, E, F}$ Results are mean of 10 rats. Means within the same line with different superscripts differ significantly ($P<0.05$), CS, Casein control diet, GVR, Germinated *Vigna radiata*, SED, sedentary rats, EXC, rats performing a protocol of aerobic interval exercise, RQ, Respiratory quotient, VO_2max, Maximum Oxygen Volume, SEM, pooled standard error of the mean, Data in parentheses represent the contribution to total variance of the specific ANOVA component.

7.2.2.3 Plasma and hematic parameters

The effects of phenotype and a protocol of aerobic interval training on plasma parameters of glucose and lipid metabolism, and hematic parameters of Zucker rats are presented in Table 18 and Table 21. The effects of the two *Vigna radiata* experimental diets on the above mentioned parameters are shown in Table 19 and Table 22. The comparison of 4d-germinated *Vigna radiata* with the casein experimental diet is shown in Table 20 and Table 23 .

Regarding the plasma parameters related to glucose metabolism (glucose, insulin and AUC), phenotype had a significant effect on them, leading to higher values in the obese *vs* lean animals (Table 18). A similar effect of phenotype was observed on lipid metabolism parameters as measured by the concentrations of total-, LDL-, and HDL- cholesterol and triglycerides (TG) levels. In particular, due to phenotype effect, a 4.8-fold increase in Total Cholesterol and TG and a 4.1- and 1.9-fold increase in HDL- and LDL-cholesterol levels were observed, respectively.

The concentration of glucose tended to decrease due to the HIIT protocol among the obese groups although no significant differences were revealed after the multiple comparison statistical test. However, exercise did induce a significant decrease on AUC levels, only among the obese animals returning this index to values similar to those found in the lean animals. Furthermore, the HIIT protocol induced a 20 and 40% decrease of Total and LDL- cholesterol among the obese groups. No statistical effects were observed in lean animals attributed to the exercise.

When the two *Vigna radiata* diets were studied, a significant diet effect was found on plasma glucose and AUC reflected in lower values for these parameters in GVR when compared to RVR (Table 19). Such dietary effect was affected by phenotype, thus resulting in significant diet × phenotype interaction. Likewise, exercise caused a significant decrease in plasma glucose and AUC values, but these effects were more pronounced in obese than in lean rats, resulting in significant exercise × phenotype interaction. In contrast, the effect of exercise on plasma AUC was less evident in GVR compared to RVR, resulting in a significant exercise × diet interaction. Total-, LDL, and HDL-cholesterol were also significantly affected by diet and exercise, with significantly lower values found in trained vs untrained rats, and in GVR vs RVR-fed animals. In addition, these effects were more pronounced in obese when compared to lean animals, thus giving rise to significant diet × phenotype and exercise × phenotype interactions. With regard to plasma triglyceride content, consumption of GVR tended to increase such parameter in obese animals when compared to the RVR treatment. This increment was ameliorated by exercise, thus resulting in significant diet effect and phenotype × diet × exercise interaction.

When results of GVR and CS dietary treatments were compared (Table 20), the significant effects of phenotype and exercise on plasma parameters were evident as previously described. The diet effect related to the inclusion of 4d-germinated *Vigna radiata* was especially evident in AUC after glucose overload, whereas the effect of exercise on Total-, and LDL-cholesterol was enhanced by GVR when compared to the use of animal protein. Regarding triglyceride content, consumption of GVR caused a significant decrease of this parameter in lean sedentary animals, and a significant increase in the obese sedentary ones when compared to CS. The effect of exercise in lean animals were opposed depending on the type of dietary treatment, whereas in obese rats, exercise was more effective at reducing plasma triglyceride content in the GVR when compared to the CS diet.

The HIIT protocol assayed tended to increase all the hematic parameters assayed with the exception of white blood cell count (Table 21). Such enhancing effect was more clearly observed in lean than in obese rats, and gave rise to significant effect of exercise and exercise × phenotype interaction. Diet effect was not as strong or uniform as that of exercise, and affected mainly to haemoglobin content and haematocrit (Table 22).

The significant effects of phenotype and exercise on hematic parameters, as well as significant phenotype × exercise interactions, were also evident when CS results were compared to those of GVR, whereas a lower degree of effects and interactions was exhibited by the dietary intervention (Table 23).

Table 18. Effect of phenotype and a protocol of interval aerobic exercise on plasmatic parameters of Zucker rats.

	Lean		Obese						
	Sedentary	**Exercise**	**Sedentary**	**Exercise**	**SEM**	**R^2**	**Phenotype Effect**	**Exercise Effect**	**Phenotype × Exercise**
Glucose (mg/dL)	177.6^{A}	238.3^{A}	400.2^{B}	341.9^{B}	30.5	0.5114	P<0.0001 (42.0%)	P=0.9679	P=0.0607 (5.6%)
Insulin (ng/mL)	0.062^{A}	0.126^{A}	0.685^{B}	0.558^{B}	0.081	0.6929	P < 0.0001 (67.2%)	P = 0.6687	P = 0.1992
AUC (arbitrary units)	2417^{A}	2516^{A}	7054^{B}	2599^{A}	301	0.8470	P < 0.0001 (34.5%)	P < 0.0001 (18.0%)	P < 0.0001 (32.0%)
T-Cholesterol (mg/dL)	74.9^{A}	74.2^{A}	209.2^{C}	167.5^{B}	6.12	0.9224	P<0.0001 (80.6%)	P=0.0016 (2.78%)	P=0.0021 (2.61%)
LDL-Cholesterol (mg/dL)	4.88^{A}	3.40^{A}	19.9^{C}	11.8^{B}	1.27	0.7772	P<0.0001 (56.8%)	P=0.0007 (9.42%)	P=0.0148 (4.47%)
HDL-Cholesterol (mg/dL)	27.5^{A}	29.4^{A}	51.0^{B}	45.3^{B}	3.88	0.4619	P<0.0001 (41.5%)	P=0.6331	P=0.3395
Triglycerides (mg/dL)	100.2^{A}	26.5^{A}	279.3^{B}	246.2^{B}	29.9	0.6025	P<0.0001 (53.1%)	P=0.0845 (3.81%)	P=0.5051

^{A,B,C} Results are mean of 10 rats. Means within the same line with different superscripts differ significantly (P < 0.05). AUC, Area under the curve. SEM, pooled standard error of the mean. Data in parentheses represent the contribution to total variance of the specific ANOVA component.

Table 19. Effect of phenotype, diet and a protocol of interval aerobic exercise on plasmatic parameters of Zucker rats.

	RVR				GVR				SEM	R^2	Phenotype Effect	Exercise Effect	Diet Effect	Phenotype × Exercise	Phenotype × Diet	Exercise × Diet	Phenotype × Exercise × Diet
	Lean		Obese		Lean		Obese										
	SED	EXC	SED	EXC	SED	EXC	SED	EXC									
Glucose (mg/dL)	307.9^{AB}	308.7^{ABC}	509.3^{E}	391.5^{BCD}	248.2^{A}	245.6^{A}	443.6^{DE}	393.8^{CD}	19.4	0.702	P<0.001 (55.8%)	P=0.003 (3.89%)	P=0.001 (5.08%)	P=0.004 (3.72%)	P=0.296	P=0.249	P=0.198
AUC (arbitrary units)	3611^{BC}	2433^{AB}	7226^{D}	5878^{D}	1965^{A}	2722^{AB}	4312^{C}	3445^{BC}	308.1	0.770	P<0.001 (43.1%)	P=0.002 (3.19%)	P<0.001 (19.05%)	P=0.039 (1.43%)	P<0.001 (6.82%)	P=0.007 (2.53%)	P=0.100
T-Cholesterol (mg/dL)	72.5^{A}	83.0^{A}	223.9^{D}	172.3^{C}	79.4^{A}	88.9^{A}	207.3^{D}	120.1^{B}	5.1	0.936	P<0.001 (70.7%)	P<0.001 (6.21%)	P<0.001 (13.35%)	P<0.001 (11.30%)	P<0.001 (2.80%)	P=0.011 (0.61%)	P=0.015 (0.56%)
LDL-Cholesterol (mg/dL)	3.2^{A}	12.8^{B}	39.5^{D}	27.1^{C}	3.4^{A}	7.9^{AB}	36.6^{D}	16.0^{B}	1.9	0.849	P<0.001 (61.4%)	P=0.001 (2.56%)	P=0.001 (2.61%)	P<0.001 (16.35%)	P=0.099	P=0.018 (1.25%)	P=0.570
HDL-Cholesterol (mg/dL)	26.0^{A}	29.1^{AB}	59.9^{D}	46.1^{C}	27.6^{AB}	28.1^{AB}	44.3^{C}	35.1^{B}	1.9	0.803	P<0.001 (54.8%)	P<0.001 (3.81%)	P<0.001 (6.70%)	P<0.001 (7.09%)	P<0.001 (7.37%)	P=0.701	P=0.185
Triglycerides (mg/dL)	31.2^{A}	22.2^{A}	350.6^{B}	353.4^{B}	28.8^{A}	45.1^{A}	470.1^{C}	358.3^{BC}	25.8	0.846	P<0.001 (81.10%)	P=0.175	P=0.050	P=0.110	P=0.147	P=0.236	P=0.059

$^{A, B, C, D}$ Results are mean of 10 rats. Means within the same line with different superscripts differ significantly (P<0.05), RVR, Raw *Vigna radiata*, GVR, Germinated *Vigna radiata*, SED, sedentary rats, EXC, rats performing a protocol of aerobic interval exercise, AUC, Area under the curve, SEM, pooled standard error of the mean, Data in parentheses represent the contribution to total variance of the specific ANOVA component.

Table 20. Effect of phenotype, diet and a protocol of interval aerobic exercise on plasmatic parameters of Zucker rats

	CS				GVR				SEM	R^2	Phenotype Effect	Exercise Effect	Diet Effect	Phenotype × Exercise	Phenotype × Diet	Exercise × Diet	Phenotype × Exercise × Diet
	Lean		Obese		Lean		Obese										
	SED	EXC	SED	EXC	SED	EXC	SED	EXC									
Glucose (mg/dL)	177.6^{A}	238.3^{AB}	400.2^{C}	341.9^{BC}	248.2^{AB}	245.6^{AB}	443.6^{C}	393.8^{C}	25.148	0.611	P<0.001 (50.48%)	P=0.487	P=0.018 (3.38%)	P=0.023 (3.11%)	P=0.807	P=0.445	P=0.318
AUC (arbitrary units)	2417^{AB}	2516^{AB}	7054^{D}	2599^{AB}	1965^{A}	2722^{AB}	4312^{C}	3445^{BC}	307.1	0.722	P<0.001 (32.2%)	P=0.001 (5.59%)	P=0.034 (2.1%)	P<0.001 (17%)	P=0.067	P<0.001 (8.8%)	P=0.001 (5%)
T-Cholesterol (mg/dL)	74.9^{A}	74.2^{A}	209.2^{D}	167.5^{C}	79.4^{A}	88.9^{A}	207.3^{D}	120.1^{B}	5.073	0.935	P<0.001 (69.34%)	P<0.001 (6.67%)	P=0.041 (0.4%)	P<0.001 (8.79%)	P<0.001 (2.18%)	P=0.016 (0.58%)	P=0.001 (1.44%)
LDL-Cholesterol (mg/dL)	4.9^{A}	3.4^{A}	20^{D}	11.8^{BC}	3.4^{A}	7.9^{AB}	36.6^{E}	16.0^{CD}	1.305	0.891	P<0.001 (49.15%)	P<0.001 (7.68%)	P<0.001 (6.67%)	P<0.001 (11.8%)	P<0.001 (3.72%)	P=0.085	P<0.001 (4.04%)
HDL-Cholesterol (mg/dL)	27.5^{A}	24.4^{A}	51.0^{c}	45.3^{BC}	27.6^{A}	28.1^{A}	44.3^{BC}	35.1^{AB}	3.054	0.502	P<0.001 (38.8%)	P=0.156	P=0.040 (3.21%)	P=0.050 (2.92%)	P=0.074	P=0.574	P=0.801
Triglycerides (mg/dL)	100.2^{A}	26.5^{A}	279.3^{B}	246.2^{B}	28.8^{A}	45.1^{A}	470.1^{C}	358.3^{C}	28.364	0.792	P<0.001 (62.96%)	P=0.014 (1.94%)	P=0.003 (2.96%)	P=0.279	P<0.001 (5.99%)	P=0.887	P=0.040 (1.35%)

$^{A, B, C, D}$ Results are mean of 10 rats. Means within the same line with different superscripts differ significantly (P<0.05), CS, Casein control diet, GVR, Germinated *Vigna radiata*, SED, sedentary rats, EXC, rats performing a protocol of aerobic interval exercise, AUC, Area under the curve, SEM, pooled standard error of the mean, Data in parentheses represent the contribution to total variance of the specific ANOVA component.

Table 21. Effect of phenotype and a protocol of interval aerobic exercise on hematic parameters of Zucker rats.

	Lean		Obese						
	Sedentary	Exercise	Sedentary	Exercise	SEM	R^2	Phenotype Effect	Exercise Effect	Phenotype × Exercise
WBC (×10³/µL)	6.48^{A}	6.94^{A}	6.51^{A}	6.87^{A}	0.83	0.0077	0.9749	0.6186	0.9490
RBC (×10⁶/µL)	8.74^{BC}	9.21^{C}	8.11^{B}	7.44^{A}	0.26	0.4336	P < 0.0001 (35%)	P = 0.8342	P = 0.0352 (8.2%)
HGB (g/dL)	14.1^{B}	13.7^{B}	12.0^{A}	12.1^{A}	0.42	0.3679	P = 0.0001 (35.7%)	P = 0.6827	P = 0.5462
HCT (%)	45.9^{B}	46.0^{B}	41.7^{A}	39.6^{A}	0.76	0.6090	P < 0.0001 (56.6%)	P = 0.2144	P = 0.1653
MCV (fL)	51.9^{BC}	49.9^{AB}	49.2^{A}	53.5^{C}	0.60	0.798	0.5278	0.1372	P < 0.0001 (43.6%)
MCH (pg)	16.1^{B}	14.8^{A}	14.8^{A}	16.4^{B}	0.28	0.4331	0.6833	0.9433	P < 0.0001 (43.0%)
MCHC (g/dL)	31.0^{A}	29.7^{A}	29.9^{A}	30.6^{A}	0.47	0.1497	0.8148	0.4057	0.0315 (13.0%)
RDW (fL)	30.4^{A}	31.1^{A}	30.6^{A}	39.1^{B}	1.09	0.5587	0.0011 (17.2%)	0.0004 (21.0%)	0.0009 (17.6%)

^{A,B,C} Results are mean of 10 rats. Means within the same line with different superscripts differ significantly (P < 0.05). WBC, White blood cells, RBC, Red blood cells, HGB, Hemoglobin, HCT, Hematocrit, MCV, Mean corpuscular volume, MCH, Mean corpuscular hemoglobin, MCHC, Mean corpuscular hemoglobin concentration, RDW, Red cell distribution width. SEM, pooled standard error of the mean. Data in parentheses represent the contribution to total variance of the specific ANOVA component.

Table 22. Effect of phenotype, diet and a protocol of interval aerobic exercise on hematic parameters of Zucker rats.

	RVR				GVR				SEM	R^2	Phenotype Effect	Exercise Effect	Diet Effect	Phenotype × Exercise	Phenotype × Diet	Exercise × Diet	Phenotype × Exercise × Diet
	Lean		Obese		Lean		Obese										
	SED	EXC	SED	EXC	SED	EXC	SED	EXC									
WBC ($\times10^3/\mu L$)	6.06^{ABC}	3.29^{A}	5.77^{AB}	4.81^{AB}	6.81^{BC}	3.25^{A}	6.82^{BC}	9.01^{C}	0.669	0.447	P<0.001 (10.9%)	P=0.009 (5.6%)	P=0.003 (7.4%)	P<0.001 (12.5%)	P=0.025 (4.1%)	P=0.251	P=0.047
RBC ($\times10^6/\mu L$)	8.77^{A}	9.57^{AB}	9.29^{AB}	9.42^{AB}	9.16^{AB}	9.98^{B}	8.92^{AB}	8.57^{A}	0.269	0.243	P=0.086	P=0.075	P=0.583	P=0.009 (7.6%)	P=0.006 (8.5%)	P=0.443	P=0.503
HGB (g/dL)	13.2^{AB}	16.0^{CD}	13.8^{ABC}	16.1^{D}	13.8^{ABC}	15.3^{CD}	11.8^{A}	14.5^{BCD}	0.462	0.475	P=0.125	P<0.001 (32.9%)	P=0.015 (4.6%)	P=0.462	P=0.003 (6.8%)	P=0.509	P=0.344
HCT (%)	43.3^{A}	49.6^{BC}	46.2^{AB}	50.5^{BC}	45.5^{AB}	51.4^{C}	42.8^{A}	42.4^{A}	1.155	0.489	P=0.012 (4.8%)	P<0.001 (17%)	P=0.29 (3.6%)	P=0.012 (4.8%)	P<0.001 (15.8%)	P=0.131	P=0.196
MCV (fL)	49.6^{A}	51.8^{B}	49.7^{A}	52.0^{B}	49.7^{A}	51.5^{B}	49.8^{A}	51.1^{B}	0.231	0.670	P=0.693	P<0.001 (61.9%)	P=0.101	P=0.427	P=0.367	P=0.017 (2.8%)	P=0.381
MCH (pg)	15.1^{A}	17.1^{C}	14.8^{A}	16.1^{B}	15.1^{A}	15.3^{AB}	14.8^{A}	17.0^{C}	0.202	0.681	P=0.866	P<0.001 (43.5%)	P=0.165	P=0.019 (2.6%)	P<0.001 (9.7%)	P=0.117	P<0.001 (10.3%)
MCHC (g/dL)	30.4^{AB}	32.9^{B}	29.8^{AB}	30.9^{AB}	30.4^{AB}	29.7^{AB}	27.4^{A}	33.3^{B}	0.866	0.318	P=0.424	P=0.001 (12.6%)	P=0.190	P=0.028 (4.8%)	P=0.288	P=0.535	P=0.002 (10.3%)
RDW (fL)	28.5^{A}	30.4^{B}	30.9^{B}	34.6^{C}	29.8^{AB}	29.8^{AB}	29.8^{AB}	32.9^{C}	0.388	0.714	P<0.001 (30.5%)	P<0.001 (24.7%)	P=0.087	P<0.001 (8.2%)	P=0.002 (4.1%)	P=0.024 (2.1%)	P=0.245

$^{A, B, C, D}$ Results are mean of 10 rats. Means within the same line with different superscripts differ significantly (P<0.05), RVR, Raw *Vigna radiata*, GVR, Germinated *Vigna radiata*, SED,sedentary rats, EXC, rats performing a protocol of aerobic interval exercise, WBC, White blood cells, RBC, Red blood cells, HGB, Hemoglobin, HCT, Hematocrit, MCV, Mean corpuscular volume, MCH, Mean corpuscular hemoglobin, MCHC, Mean corpuscular hemoglobin concentration, RDW, Red cell distribution width, SEM, pooled standard error of the mean, Data in parentheses represent the contribution to total variance of the specific ANOVA component.

Table 23. Effect of phenotype, diet and a protocol of interval aerobic exercise on hematic parameters of Zucker rats.

	CS				GVR				SEM	R^2	Phenotype Effect	Exercise Effect	Diet Effect	Phenotype × Exercise	Phenotype × Diet	Exercise × Diet	Phenotype × Exercise × Diet
	Lean		Obese		Lean		Obese										
	SED	EXC	SED	EXC	SED	EXC	SED	EXC									
WBC ($\times10^3/\mu L$)	6.48^{AB}	6.94^{B}	6.52^{AB}	6.87^{B}	6.81^{B}	3.25^{A}	6.82^{B}	9.01^{B}	0.800	0.289	P=0.013 (6.73%)	P=0.739	P=0.561	P=0.013 (6.78%)	P=0.014 (6.64%)	P=0.271	P=0.012 (7%)
RBC ($\times10^6/\mu L$)	8.74^{B}	9.21^{BC}	8.11^{AB}	7.44^{A}	9.16^{BC}	9.98^{C}	8.92^{BC}	8.57^{AB}	0.257	0.462	P<0.001 (22.51%)	P=0.593	P<0.001 (14.04%)	P=0.002 (7.90%)	P=0.318	P=0.350	P=0.972
HGB (g/dL)	14.1^{BC}	13.7^{ABC}	12.0^{AB}	12.1^{AB}	13.8^{ABC}	15.27^{C}	11.8^{A}	14.5^{C}	0.459	0.442	P<0.001 (19.70%)	P=0.004 (7.28%)	P=0.010 (5.74%)	P=0.148	P=0.624	P=0.001 (9.30%)	P=0.566
HCT (%)	45.9^{C}	46.0^{C}	41.7^{AB}	39.6^{A}	45.5^{BC}	51.4^{D}	42.8^{ABC}	42.4^{ABC}	0.860	0.637	P<0.001 (42.4%)	P=0.099	P=0.001 (6.8%)	P=0.001 (6.01%)	P=0.631	P=0.002 (5.44%)	P=0.100
MCV (fL)	51.9^{CD}	49.9^{ABC}	49.2^{A}	53.5^{D}	49.7^{AB}	51.5^{BC}	49.8^{AB}	51.1^{ABC}	0.444	0.502	P=0.824	P<0.001 (11.13%)	P=0.073	P<0.001 (14.54%)	P=0.361	P=0.384	P<0.001 (20.89%)
MCH (pg)	16.1^{BC}	14.8^{A}	14.8^{A}	16.4^{C}	15.1^{AB}	15.3^{AB}	14.8^{A}	17.0^{C}	0.231	0.573	P=0.012 (4.17%)	P<0.001 (9.14%)	P=0.844	P<0.001 (34.87%)	P=0.109	P=0.002 (6.5%)	P=0.227
MCHC (g/dL)	31.0^{AB}	29.7^{AB}	29.9^{AB}	30.6^{AB}	30.4^{AB}	29.7^{AB}	27.4^{A}	33.3^{B}	0.915	0.250	P=0.996	P=0.095	P=0.796	P=0.001 (13.36%)	P=0.866	P=0.035 (5.08%)	P=0.087
RDW (fL)	30.4^{A}	31.1^{A}	30.6^{A}	39.1^{B}	29.8^{A}	29.8^{A}	29.8^{A}	32.9^{A}	0.800	0.591	P<0.001 (12.62%)	P<0.001 (15.29%)	P<0.001 (8.35%)	P<0.001 (13.41%)	P=0.036 (2.74%)	P=0.012 (4.02%)	P=0.041 (2.63%)

$^{A, B, C, D}$ **Results are mean of 10 rats. Means within the same line with different superscripts differ significantly ($P<0.05$), CS, Casein control diet, GVR, Germinated *Vigna radiata*, SED, sedentary rats, EXC, rats performing a protocol of aerobic interval exercise, WBC, White blood cells, RBC, Red blood cells, HGB, Hemoglobin, HCT, Hematocrit, MCV, Mean corpuscular volume, MCH, Mean corpuscular hemoglobin, MCHC, Mean corpuscular hemoglobin concentration, RDW, Red cell distribution width, SEM, pooled standard error of the mean, Data in parentheses represent the contribution to total variance of the specific ANOVA component.**

7.2.2.4 Liver morphometry, lipid composition, functionality and antioxidant status

The effects of phenotype and a protocol of aerobic interval training on liver weight, surface area, lipid composition, and functionality are presented in Table 24 and Figure 15. The effects of the two *Vigna radiata* experimental diets on the above mentioned parameters are shown in Table 25 and Figure 16. The comparison of 4d-germinated *Vigna radiata* with the casein experimental diet is shown in Table 26.

There was a significant effect of phenotype on liver weight expressed as g of FW or g/100g of BW, total fat, and triglyceride hepatic content that were higher in the obese when compared to the lean groups. The hepatic area was also higher in the obese animals and phenotype contributed in the 82.2% of the total variance for this parameter. Additionally, total water content of liver was lower in the obese *vs* lean groups. A significant effect of HIIT protocol was observed in the hepatic total fat and triglyceride contents, leading to reduced values for both parameters only among the obese groups. Opposite results were observed for the total water content of the liver, which was increased due to the HIIT protocol in the obese groups. A significant diet effect was observed for the hepatic fat and triglyceride content (Table 25). In general, the consumption of *Vigna radiata* experimental diets induced a significant decrease of hepatic triglycerides content that was more pronounced in the GVR groups. In fact, the comparison of the two *Vigna radiata* diets revealed a significant diet effect on liver weight and area, leading to lower values on the obese groups that consumed germinated *Vigna radiata* diet.

AST, ALT, ALP and GGT activities as well as plasma bilirubin levels were used as markers of liver function. A significant phenotype effect was observed on these parameters leading to higher values in the obese groups when compared to the lean ones (Table 24). Exercise managed to decrease the activities of AST and ALP among the obese groups, whereas no differences due to the exercise were observed among the lean groups. The consumption of the GVR experimental diet, resulted in lower activities of the above mentioned liver markers when compared to the levels in the groups that consumed the CS diet, thus resulting in a significant diet effect (Table 26).

With regard to the hepatic antioxidant enzyme activities, there was a significant phenotype effect on SOD activity that resulted in lower values for Cu/Zn-SOD and higher values for Mn-SOD in obese when compared to lean rats (Table 24). Furthermore a decrease in GPx activity was found in the obese sedentary rats, due to the phenotype effect on this parameter. The

training protocol induced a 40% increase in Cu/Zn-SOD in the obese animals with no significant differences in the lean groups. In contrast, due to the HIIT protocol, the activity of Mn-SOD in the lean groups increased by 43% and decreased in the obese counterparts by 20%. Such differential effects of exercise depending on rat phenotype gave rise to significant phenotype × exercise interactions ($P=0.049$ and $P<0.0001$, respectively). Additionally, HIIT protocol differentially affected GPX activity in the lean *vs* obese groups, resulting in a 17% decrease in GPX activity of lean compared to a 100% increase in obese rats. Such differential effects of the training protocol gave rise to a strong phenotype × exercise interaction ($P < 0.0001$). Finally, no significant effects due to the phenotype or HIIT protocol were observed for catalase activity. In addition to phenotype and exercise, diet also affected the activities of the liver enzymes with the exception of catalase (Table 25). Specifically, due to the effect of diet, the activity of CuZn-SOD in the lean groups that consumed RVR diet was higher when compared to the same groups that consumed the GVR diet, whereas opposite results were observed in the obese groups. Furthermore, the activity of GPx was found to increase in the groups that consumed 4d-germinated *Vigna radiata* when compared to those fed CS diet (Table 26).

Table 24. Effect of phenotype and a protocol of interval aerobic exercise on weight, chemical composition, and functionality of liver.

	Lean		Obese						
	Sedentary	Exercise	Sedentary	Exercise	SEM	R^2	Phenotype Effect	Exercise Effect	Phenotype × Exercise
Weight (g FW)	8.2^{A}	9.3^{A}	17.5^{B}	16.0^{B}	0.52	0.8738	P < 0.0001 (85.3%)	P = 0.7508	P = 0.0255 (2.0%)
Weight (g/100g FW)	2.43^{A}	2.96^{A}	4.64^{B}	4.61^{B}	0.162	0.815	P < 0.0001 (78.3%)	P = 0.085	P = 0.115
Area (cm^2)	12.5^{A}	13.2^{A}	22.6^{B}	20.5^{B}	0.526	0.8450	P < 0.0001 (82.2%)	P = 0.293	P = 0.048 (1.9%)
Moisture (%)	67.5^{BC}	69.8^{C}	61.4^{A}	65.9^{B}	0.79	0.6413	P < 0.0001 (44.0%)	P = 0.0002 (18.0%)	P = 0.1731
Fat (g/100 g DM)	7.1^{A}	4.29^{A}	19.6^{C}	12.7^{B}	1.18	0.7412	P < 0.0001 (60.6%)	P = 0.0005 (11.4%)	P = 0.1040
Triglycerides (mg/g DM)	5.29^{A}	4.14^{A}	26.9^{B}	13.4^{A}	2.47	0.6139	P < 0.0001 (46.1%)	P = 0.0096 (8.6%)	P = 0.0204 (6.7%)
Liver function plasma markers									
AST (U/L)	98.4^{A}	66.9^{A}	182.3^{B}	107.3^{A}	14.7	0.5226	P<0.0001 (25.6%)	P=0.0010 (18.8%)	P=0.1494
ALT (U/L)	25.7^{A}	31.8^{A}	61.0^{B}	59.7^{B}	4.66	0.5917	P<0.0001 (56.5%)	P=0.6134	P=0.4287
ALP (U/L)	98.2^{A}	100.7^{A}	202.6^{B}	137.6^{A}	10.5	0.6823	P<0.0001 (43.3%)	P=0.0055 (8.48%)	P=0.0030 (9.88%)
GGT (U/L)	0.10^{A}	0.70^{A}	13.9^{B}	9.33^{B}	1.56	0.6440	P<0.0001 (55.1%)	P=0.2162	P=0.1098

Bilirubin (mg/dL)	0.22^{A}	0.21^{A}	1.06^{B}	0.94^{B}	0.20	0.3173	P=0.0006 (31.4%)	P=0.7593	P=0.7912
Antioxidant enzymes									
Cu/Zn-SOD (Units/mg protein)	223.8^{C}	233.4^{C}	112.5^{A}	157.7^{B}	8.5	0.7987	P < 0.0001 (72.0%)	P = 0.0047 (5.4%)	P = 0.0494 (2.5%)
Mn-SOD (Units/mg protein)	26.8^{A}	38.2^{B}	85.8^{D}	68.9^{C}	2.6	0.9019	P < 0.0001 (82.2%)	P = 0.4839	P < 0.0001 (7.8%)
Catalase (µmol H_2O_2/min/mg protein)	487.1^{AB}	551.8^{B}	503.9^{AB}	461.7^{A}	19.4	0.2803	P = 0.0630 (7.8%)	P = 0.4260	P = 0.0053 (18.8%)
GPX (nmol NADPH/min/mg protein)	9.23^{B}	7.64^{AB}	6.38^{A}	12.96^{C}	0.543	0.704	P=0.03 (2.5%)	P< 0.0001 (17%)	P< 0.0001 (50.8%)

^{A,B,C,D} Results are mean of 10 rats. Means within the same line with different superscripts differ significantly (P < 0.05). FW, fresh weight, DM, dry matter, AST, aspartate aminotransferase, ALT, alanine transaminase, ALP, Alkaline Phosphatase, GGT, Gamma-glutamyl transpeptidase, GPX, Glutathione peroxidase. SEM, pooled standard error of the mean. Data in parentheses represent the contribution to total variance of the specific ANOVA component.

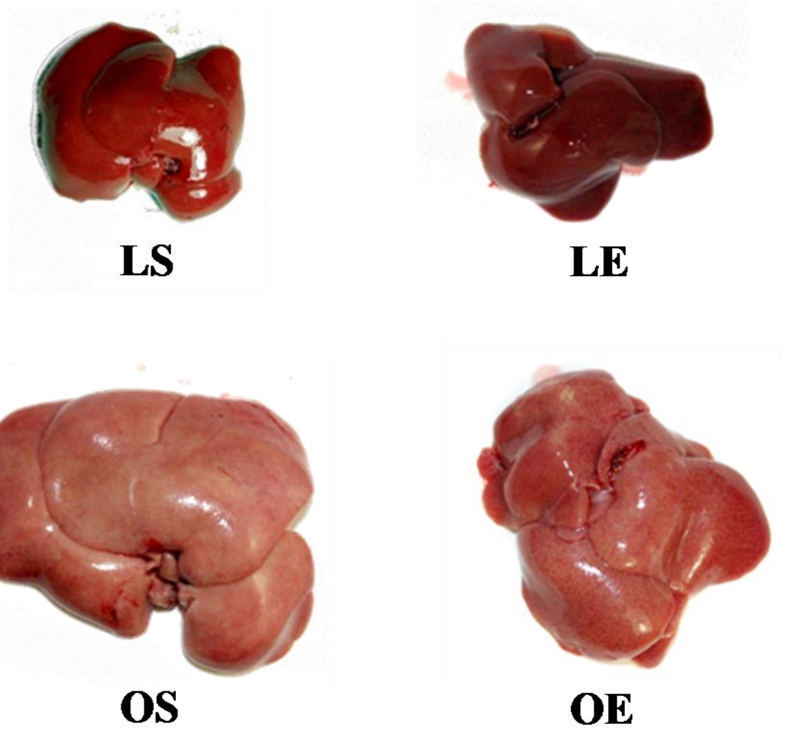


Figure 15. Effect of HIIT protocol on liver morphology of lean and obese Zucker rats.

Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing aerobic interval exercise. Photographs are representative of livers of 8-10 different rats for each experimental group.

Table 25. Effect of phenotype, diet and a protocol of interval aerobic exercise on weight, chemical composition, and functionality of liver.

	RVR				GVR				SEM	R^2	Phenotype Effect	Exercise Effect	Diet Effect	Phenotype × Exercise	Phenotype × Diet	Exercise × Diet	Phenotype × Exercise × Diet
	Lean		Obese		Lean		Obese										
	SED	EXC	SED	EXC	SED	EXC	SED	EXC									
Weight (g FW)	8.6^{A}	9.7^{A}	17.4^{D}	14.7^{C}	9.2^{A}	10.4^{A}	14.1^{BC}	12.4^{B}	0.465	0.832	P<0.001 (65.4%)	P=0.219	P=0.001 (3.2%)	P=0.001 (7.4%)	P<0.001 (6.7%)	P=0.638	P=0.361
Weight (g/100gFW)	2.75^{A}	3.37^{BC}	4.13^{D}	3.51^{C}	2.79^{AB}	3.87^{CD}	3.66^{CD}	3.40^{C}	0.139	0.585	P<0.001 (15.4%)	P<0.001 (4.3%)	P=0.857	P<0.001 (31.4%)	P=0.007 (4.8%)	P=0.049 (2.5%)	P=0.860
Area (cm²)	14.17^{A}	14.01^{A}	18.14^{C}	24.03^{D}	14.89^{AB}	15.39^{AB}	16.92^{BC}	18.21^{C}	0.597	0.755	P<0.001 (42.1%)	P<0.001 (8.4%)	P=0.002 (3.9%)	P<0.001 (5.2%)	P<0.001 (9.7%)	P=0.002 (2.8%)	P=0.002 (3.5%)
Moisture (%)	69.3^{D}	69.3^{D}	58.2^{A}	64.5^{BC}	69.4^{D}	67.7^{CD}	63.0^{B}	67.1^{CD}	0.926	0.671	P<0.001 (41.7%)	P=0.002 (5.1%)	P=0.026 (2.6%)	P<0.001 (10.8%)	P=0.001 (5.9%)	P=0.149	P=0.885
Fat (g/100 g DM)	1.52^{A}	3.05^{A}	21.9^{B}	6.09^{A}	3.58^{A}	6.93^{A}	17.9^{B}	6.15^{A}	1.571	0.714	P<0.001 (33.3%)	P<0.001 (10.5%)	P=0.512	P<0.001 (24.0%)	P=0.020 (2.5%)	P=0.151	P=0.695
Triglycerides (mg/g DM)	0.59^{A}	0.90^{A}	17.99^{C}	1.72^{A}	0.25^{A}	3.39^{A}	12.4^{B}	0.95^{A}	0.114	0.802	P<0.001 (28.0%)	P<0.001 (17.5%)	P=0.209	P<0.001 (30.1%)	P=0.008 (2.2%)	P=0.020 (1.7%)	P=0.511
Liver function plasma markers																	
AST (U/L)	95.4^{C}	61.6^{A}	178.0^{D}	96.1^{C}	67.3^{AB}	57.9^{A}	104.4^{C}	88.8^{BC}	5.561	0.826	P<0.001 (34.8%)	P<0.001 (19.6%)	P<0.001 (13.0%)	P=0.001 (2.8%)	P=0.002 (2.6%)	P<0.001 (8.1%)	P=0.010 (1.7%)
ALT (U/L)	37.3^{A}	28.6^{A}	160.8^{D}	59.0^{B}	35.5^{A}	26.6^{A}	115.4^{C}	25.6^{A}	4.625	0.922	P<0.001 (36.0%)	P<0.001 (28.1%)	P<0.001 (4.6%)	P<0.001 (19.7%)	P<0.001 (3.7%)	P=0.379	P=0.353
ALP (U/L)	99.0^{AB}	152.6^{CD}	268.2^{F}	180.2^{DE}	80.8^{A}	140.7^{C}	201.8^{E}	124.6^{BC}	6.612	0.892	P<0.001 (39.9%)	P=0.008 (1.1%)	P<0.001 (10.4%)	P<0.001 (34.0%)	P<0.001 (3.7%)	P=0.364	P=0.812
GGT (U/L)	0.80^{A}	0.21^{A}	8.81^{B}	14.6^{C}	0.80^{A}	0.10^{A}	15.8^{C}	6.63^{B}	1.129	0.767	P<0.001	P=0.114	P=0.834	P=0.429	P=0.896	P<0.001	P<0.001

											(61.4%)					(7.1%)	(7.1%)
Bilirubin (mg/dL)	0.12^{A}	0.12^{A}	0.53^{B}	0.34^{AB}	0.11^{A}	0.10^{A}	0.99^{C}	0.43^{B}	0.061	0.719	P<0.001 (46.0%)	P<0.001 (7.0%)	P=0.004 (3.6%)	P<0.001 (7.2%)	P=0.001 (4.6%)	P=0.034 (1.8%)	P=0.042 (1.7%)
Antioxidant enzymes																	
Cu/Zn-SOD (Units/mg protein)	175.0^{B}	165.7^{B}	217.9^{C}	391.4^{D}	84.9^{A}	71.1^{A}	174.9^{B}	223.6^{C}	7.596	0.954	P<0.001 (48.7%)	P<0.001 (4.6%)	P<0.001 (25.1%)	P<0.001 (12.2%)	P=0.007 (0.6%)	P<0.001 (2.0%)	P<0.001 (2.2%)
Mn-SOD (Units/mg protein)	71.3^{C}	73.5^{C}	51.8^{AB}	48.2^{A}	101.7^{D}	102.5^{D}	100.8^{D}	67.8^{BC}	4.352	0.751	P<0.001 (25.1%)	P=0.202	P<0.001 (39.7%)	P=0.004 (3.6%)	P=0.574	P=0.010 (2.8%)	P=0.008 (3.1%)
Catalase (µmol H_2O_2/min/mg protein)	731.4^{C}	729.1^{C}	569.6^{AB}	570.5^{AB}	705.5^{C}	669.2^{BC}	543.4^{A}	583.6^{AB}	25.523	0.536	P<0.001 (49.3%)	P=0.967	P=0.186	P=0.224	P=0.352	P=0.868	P=0.225
GPX (nmol NADPH/min/mg protein)	16.5^{C}	14.0^{ABC}	11.6^{AB}	12.4^{AB}	11.5^{A}	14.9^{AB}	12.3^{AB}	12.2^{AB}	0.843	0.372	P<0.001 (15.4%)	P=0.419	P=0.033 (4.8%)	P=0.450	P=0.081 (3.2%)	P=0.011 (6.9%)	P=0.021 (5.7%)

$^{A, B, C, D, E, F}$ Results are mean of 10 rats. Means within the same line with different superscripts differ significantly (P<0.05), RVR, Raw *Vigna radiata* diet, GVR, Germinated *Vigna radiata* diet, SED,sedentary rats, EXC, rats performing a protocol of aerobic interval exercise, FW, fresh weight, DM, dry matter, AST, aspartate aminotransferase, ALT, alanine transaminase, ALP, Alkaline Phosphatase, GGT, Gamma-glutamyl transpeptidase, GPX, Glutathione peroxidase, NADPH, Nicotinamide adenine dinucleotide phosphate, SEM, pooled standard error of the mean, Data in parentheses represent the contribution to total variance of the specific ANOVA component.

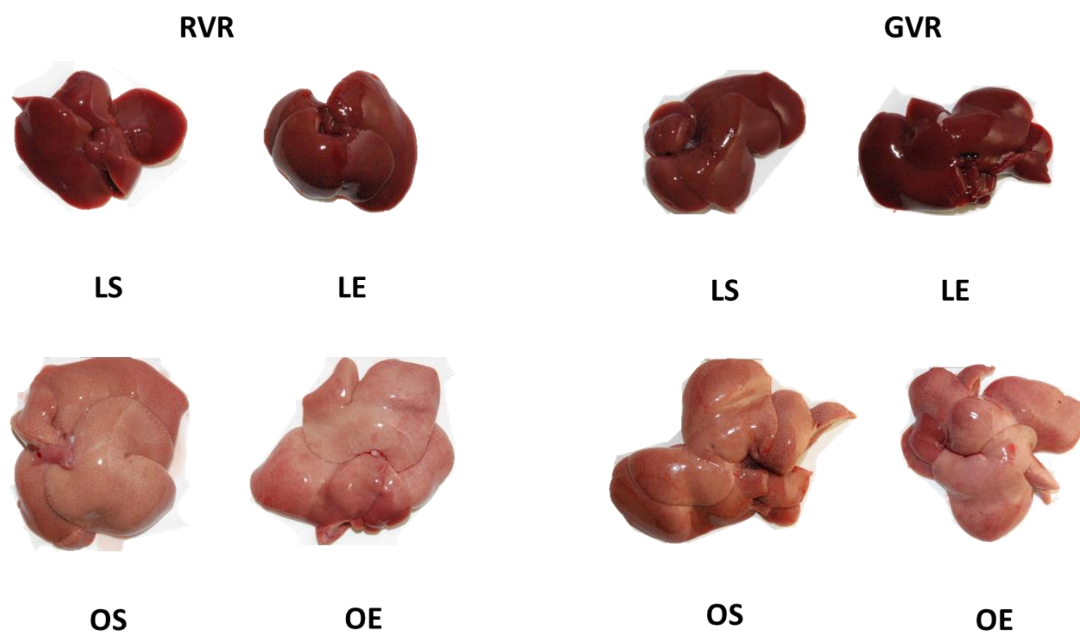


Figure 16. Effect of *V.radiata* diets and HIIT protocol on liver morphology of lean and obese Zucker rats.

Diets: RVR, Raw *V.Radiata*, GVR, 4d-germinated *V.radiata* ;Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing aerobic interval exercise. Photographs are representative of livers of 8-10 different rats for each experimental group.

Table 26. Effect of phenotype, diet and a protocol of interval aerobic exercise on weight, chemical composition, and functionality of liver.

	CS				GVR				SEM	R^2	Phenotype Effect	Exercise Effect	Diet Effect	Phenotype × Exercise	Phenotype × Diet	Exercise × Diet	Phenotype × Exercise × Diet
	Lean		Obese		Lean		Obese										
	SED	EXC	SED	EXC	SED	EXC	SED	EXC									
Weight (g FW)	8.2^{A}	9.3^{A}	17.5^{D}	16.0^{DE}	9.21^{A}	10.4^{AB}	14.1^{CD}	12.4^{BC}	0.491	0.839	P<0.001 (67.6%)	P=0.444	P=0.001 (2.6%)	P<0.001 (3.47%)	P<0.001 (10.14%)	P=0.944	P=0.757
Increment of weight (g/100g FW)	2.43^{A}	2.96^{AB}	4.64^{D}	4.61^{D}	2.79^{AB}	3.94^{C}	3.66^{C}	3.40^{BC}	0.140	0.782	P<0.001 (38.4%)	P<0.001 (4.2%)	P=0.035 (1.12%)	P<0.001 (7.5%)	P<0.001 (25.18%)	P=0.327	P=0.035 (1.51%)
Area (cm²)	12.51^{A}	13.16^{AB}	22.60^{F}	20.50^{EF}	14.89^{ABC}	15.39^{BC}	16.92^{CD}	18.21^{DE}	0.625	0.760	P<0.001 (53.51%)	P=0.941	P=0.093	P=0.176	P<0.001 (18.05%)	P=0.069	P=0.038 (1.56%)
Moisture (%)	67.5^{CD}	69.8^{D}	61.4^{A}	65.9^{BC}	69.4^{CD}	67.7^{CD}	63.0^{AB}	67.1^{CD}	0.864	0.564	P<0.001 (35.28%)	P<0.001 (8.73%)	P=0.274	P=0.001 (6.9%)	P=0.299	P=0.075	P=0.146
Fat (g/100 g DM)	7.09^{AB}	4.29^{A}	19.61^{C}	12.73^{BC}	3.58^{A}	6.93^{AB}	17.86^{C}	6.15^{AB}	1.569	0.629	P<0.001 (37.1%)	P<0.001 (7.93%)	P=0.055	P<0.001 (10.57%)	P=0.077	P=0.681	P=0.012 (3.72%)
Triglycerides (mg/g DM)	5.29^{ABC}	4.14^{ABC}	26.94^{D}	13.38^{C}	0.25^{A}	3.39^{AB}	12.42^{BC}	0.95^{A}	2.102	0.662	P<0.001 (26.9%)	P<0.001 (6.75%)	P<0.001 (15.25%)	P<0.001 (10.1%)	P=0.001 (6.5%)	P=0.288	P=0.713
AST (U/L)	98.4^{AB}	66.89^{AB}	182.25^{C}	107.3^{B}	67.30^{AB}	57.87^{A}	104.38^{AB}	88.78^{AB}	11.063	0.573	P<0.001 (24.7%)	P<0.001 (11.2%)	P<0.001 (12.7%)	P=0.139	P=0.051	P=0.013 (4%)	P=0.238
ALT (U/L)	25.67^{A}	31.80^{A}	61.03^{B}	59.67^{B}	35.50^{A}	26.56^{A}	115.41^{C}	25.58^{A}	4.009	0.863	P<0.001 (32.2%)	P<0.001 (14.3%)	P=0.015 (1.22%)	P<0.001 (14.17%)	P=0.036 (0.91%)	P<0.001 (15.3%)	P<0.001 (8.26%)
ALP (U/L)	98.22^{AB}	100.7^{AB}	202.6^{D}	137.57^{BC}	80.78^{A}	140.72^{C}	201.81^{D}	124.58^{BC}	9.015	0.733	P<0.001 (39.3%)	P=0.005 (3.23%)	P=0.924	P<0.001 (25.7%)	P=0.172	P=0.060 (1.42%)	P=0.008 (2.88%)

GGT (U/L)	0.10^{A}	0.70^{A}	13.9^{DE}	9.33^{CD}	0.80^{AB}	0.10^{A}	15.83^{E}	6.63^{BC}	1.332	0.714	P<0.001 (58.72%)	P<0.001 (5.55%)	P=0.828	P<0.001 (5.84%)	P=0.915	P=0.130	P=0.379
Bilirubin (mg/dL)	0.22^{A}	0.21^{A}	1.06^{B}	0.94^{B}	0.11^{A}	0.10^{A}	0.99^{B}	0.43^{AB}	0.149	0.443	P<0.001 (34.57%)	P=0.081	P=0.069	P=0.095	P=0.463	P=0.333	P=0.303
Antioxidant enzymes																
Cu/Zn-SOD (Units/mg protein)	223.8^{D}	233.4^{D}	112.5^{B}	157.7^{C}	84.9^{AB}	71.1^{A}	174.9^{C}	223.6^{D}	8.685	0.868	P=0.239	P<0.001 (5%)	P<0.001 (11.67%)	P<0.001 (5.96%)	P<0.001 (63.54%)	P=0.384	P=0.281
Mn-SOD (Units/mg protein)	26.77^{A}	38.15^{A}	85.81^{BC}	68.92^{B}	101.73^{C}	102.48^{C}	100.82^{C}	67.84^{B}	4.346	0.840	P<0.001 (5.95%)	P=0.006 (2.1%)	P<0.001 (39.08%)	P<0.001 (8.72%)	P<0.001 (26.98%)	P=0.036 (1.16%)	P=0.660
Catalase (µmol H_2O_2/min/mg protein)	486.4^{AB}	551.8^{AB}	503.9^{AB}	461.8^{A}	705.5^{D}	699.2^{CD}	543.4^{AB}	583.6^{BC}	23.228	0.605	P<0.001 (13.0%)	P=0.394	P<0.001 (37.7%)	P=0.372	P=0.013 (3.99%)	P=0.622	P=0.007 (4.77%)
GPX (nmol NADPH/min/mg protein)	9.23^{AB}	7.64^{A}	6.38^{A}	12.96^{CD}	11.46^{BC}	14.94^{D}	12.3^{BCD}	12.2^{BCD}	0.713	0.647	P=0.951	P<0.001 (9.62%)	P<0.001 (30.88%)	P=0.028 (2.73%)	P=0.028 (2.74%)	P=0.490	P<0.001 (18.4%)

$^{A, B, C, D, E, F}$ Results are mean of 10 rats. Means within the same line with different superscripts differ significantly (P<0.05), CS, Casein control diet, GVR, Germinated *Vigna Radiata*, SED, sedentary rats, EXC, rats performing a protocol of aerobic interval exercise, FW, fresh weight, DM, dry matter, AST, aspartate aminotransferase, ALT, alanine transaminase, ALP, Alkaline Phosphatase, GGT, Gamma-glutamyl transpeptidase, GPX, Glutathione peroxidase, NADPH, Nicotinamide adenine dinucleotide phosphate, SEM, pooled standard error of the mean, Data in parentheses represent the contribution to total variance of the specific ANOVA component.

7.2.2.5 Liver histology

There was a clear phenotype effect on the histology parameters related to liver functionality (Table 27,Figure 17) in the different experimental groups. This resulted in a manifestation of micro and macrovesicular steatosis signs as well as lipogranulomas and portal inflammation on the obese animals. Exercise could partly reverse these alterations, leading to a decrease of micro and macrovescicular steatosis, fatty droplets, lipogranulomas and portal inflammation. Despite these improvements, exercise caused the appearance of necrosis in the lean phenotype and of multinucleic cells, necrosis and fibrosis in the obese groups. Administration of *Vigna radiata* diets induced a decrease of the micro and macrovescicular steatosis as well as a decrease in the number of fatty droplets in the obese animals. Such improvements on the histology parameters were more pronounced in the groups that consumed GVR diet in which the combination of the specific diet and the HIIT protocol led to greater changes on the histology parameters related to the appearance of non-alcoholic fatty liver disease when compared to the combination of HITT protocol and casein or raw *Vigna radiata*. The signs of necrosis remained in some of the groups that consumed *Vigna radiata* experimental diets whereas no signs of fibrosis were observed. Likewise, no multinucleic cells were detected in the livers of the animals that consumed the *Vigna radiata* experimental diets (Table 28, Figure 18).

Table 27. Effect of phenotype and a protocol of interval aerobic exercise on liver histology of Zucker rats fed CS experimental diet.

	Microvescicular steatosis	Fatty droplets	Multinucleic cells	Lipogranulomas	Portal Inflammation	Necrosis	Fibrosis
LS	-	-	-	-	-/+	-	-
LE	-	-	-	+	+	-/+	-
OS	++++	++++	-	+++	+++	-	-
OE	+++	+++	++	++/+++	++/+++	++	++

LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing a protocol of aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing a protocol of aerobic interval exercise. Grading score of the histological alterations: -, non-existent; +, mild; ++, mild/moderate; +++, moderate; ++++, abundant; +++++, severe.

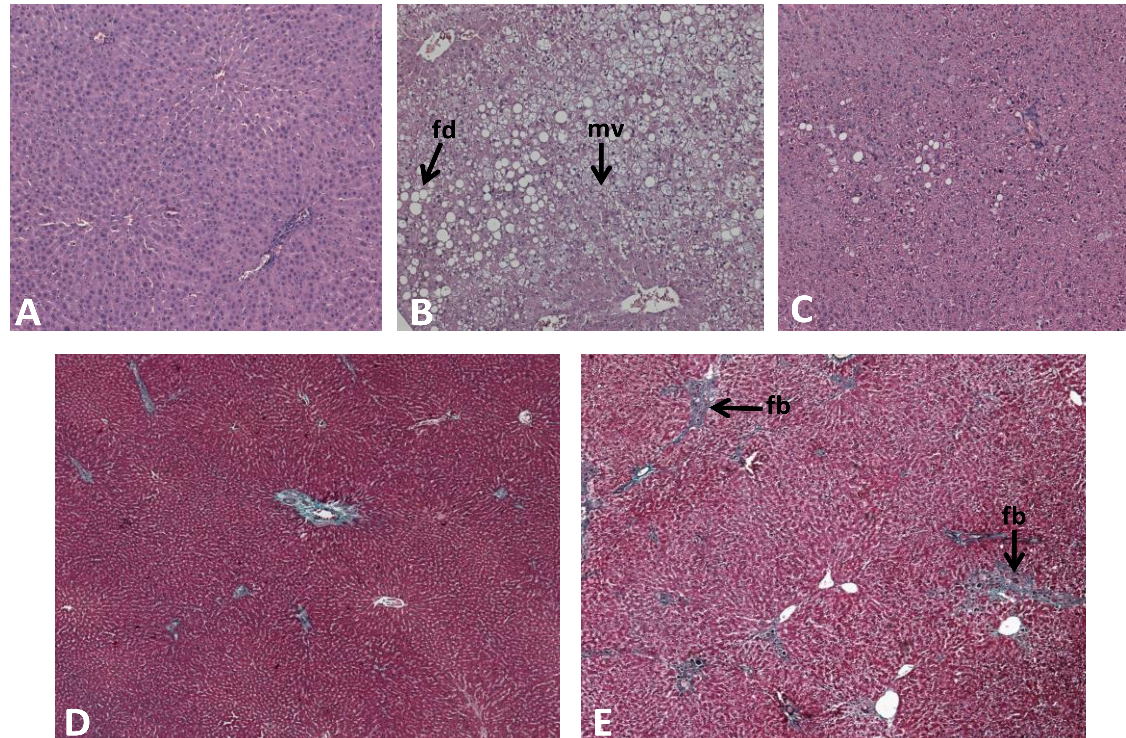


Figure 17. Effect of HIIT protocol on liver histology of lean and obese Zucker rats fed CS diet.

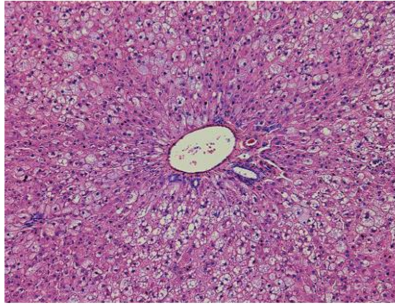
(A) Histological view of control LS liver HE stain, (B) Histological view of OS liver HE stain with clear signs of microvesicular steatosis and fatty droplet accumulation, (C) Histological view of OE liver HE stain with diminished signs of microvesicular steatosis (mv) and fatty droplet accumulation (fd), (D) Histological view of LE liver MT stain, (E) Histological view of OE liver MT stain with signs of fibrosis (fb). Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing aerobic interval exercise. Photographs are representative of livers of 8-10 different rats for each experimental group.

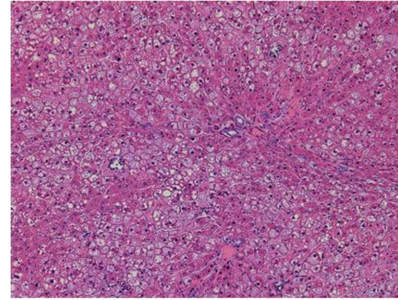
Table 28. Effect of phenotype, diet and a protocol of interval aerobic exercise on liver histology of Zucker rats fed RVR or GVR experimental diets.

	RVR				GVR			
	Lean		Obese		Lean		Obese	
	SED	EXC	SED	EXC	SED	EXC	SED	EXC
Microvescicular steatosis	-	-/+	++++/+++++	+++/++++	-	+/++	++++/+++++	+/++
Fatty droplets	-	-	++++	++/+++	-	-	++++	+
Multinucleic cells	-	-	-	-	-	-	-	-
Lipogranulomas	-	+	++/+++	++/+++	++	++	++	+/++
Portal Inflammation	-/+	+/++	+++	++	+/++	+/++	+++	++
Necrosis	-	-/+	-	-	-	+/++	-	++
Fibrosis	-	-	-	-	-	-	-	-

RVR, Raw *Vigna radiata*, GVR, Germinated *Vigna radiata*, SED,sedentary rats, EXC, rats performing a protocol of aerobic interval exercise. Grading score of the histological alterations: -, non-existent; +, mild; ++, mild/moderate; +++, moderate; ++++, abundant; +++++, severe.

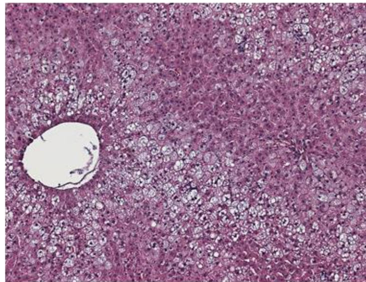
RVR

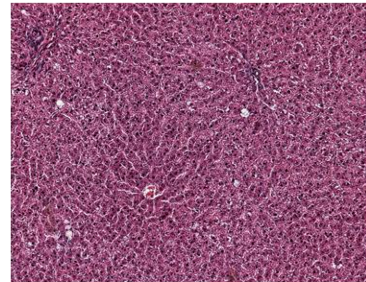




OS **OE**

GVR





OS **OE**

Figure 18. Effect of HIIT protocol on liver histology of lean and obese Zucker rats fed raw and 4d- germinated *V.radiata* diet

7.2.2.6 Liver protein expression

The effects of phenotype, aerobic interval training protocol and dietary treatments on the expression of AMPK, pAMPK, PPAR-γ, and FAS are shown in Figure 19. Western blot analysis indicated a significantly lower activation of AMPKα (shown by the ratio pAMPKα/AMPKα) in the liver of obese animals when compared to their lean counterparts. However, there were no changes attributed to phenotype as long as pPARγ and FAS activation. Exercise had no major effects on AMPK phosphorylation in the different experimental groups, but caused an increase of pPARγ and FAS expression in both phenotypes (Figure A). Dietary treatment with *Vigna radiata* affected significantly the expression of pAMKα and FAS in the liver. The groups that consumed the GVR diet had in general lower levels of these proteins in almost all the experimental groups when compared to the RVR and CS treatments. However, the expression of pPARγ was not affected by the two different *Vigna radiata* experimental diets tested. Exercise caused a significant reduction in pAMPKα expression of lean and obese rats fed GVR, but it did not affect the expression of this protein in animals fed RVR, thus giving rise to a significant diet × exercise interaction. Furthermore, exercise increased pPARγ expression of lean animals fed RVR and GVR diets, but not of their obese counterparts in which the expression was reduced, thus resulting in significant phenotype × exercise interaction.

7.2.2.7 Liver gene expression of GPx1 and GPx4

The effects of phenotype and HIIT protocol on the liver expression of GPX1 and GPX4 genes are shown in Figure 20. The expression of GPX1 and GPX4 was not significantly affected by phenotype. There was a significant enhancing effect of exercise only on the expression of liver GPX4 gene, in both phenotypes.

Figure 19. Effect of phenotype, HIIT protocol, and dietary treatment on the hepatic protein expression of AMPKα/PAMPKα, PPARγ, and FAS.

(A)

pAMPKα/AMPKα expression

Phenotype Effect: P=0.010
Exercise Effect: P=0.037
Phenotype × Exercise: P=0.052

Phenotype Effect: P=0.052
Diet Effect: P=0.197
Phenotype × Diet: P=0.478
Phenotype × Exercise × Diet: P=0.385

Exercise Effect: P=0.046
Phenotype × Exercise: P=0.512
Exercise × Diet: P=0.042

B)

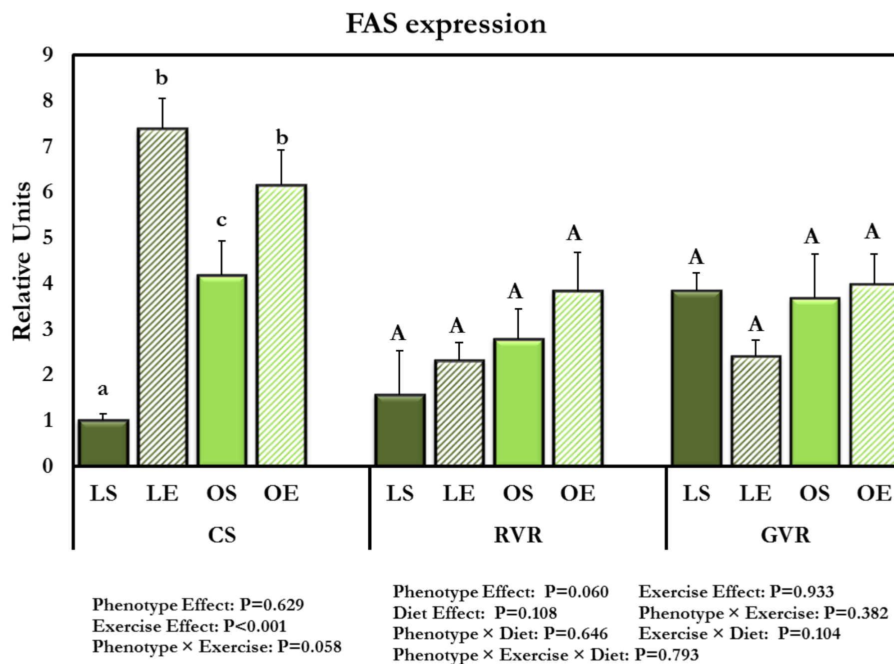
pPARγ expression

Phenotype Effect: P=0.848
Exercise Effect: P<0.001
Phenotype × Exercise: P=0.748

Phenotype Effect: P=0.525
Diet Effect: P=0.015
Phenotype × Diet: P=0.043
Phenotype × Exercise × Diet: P=0.131

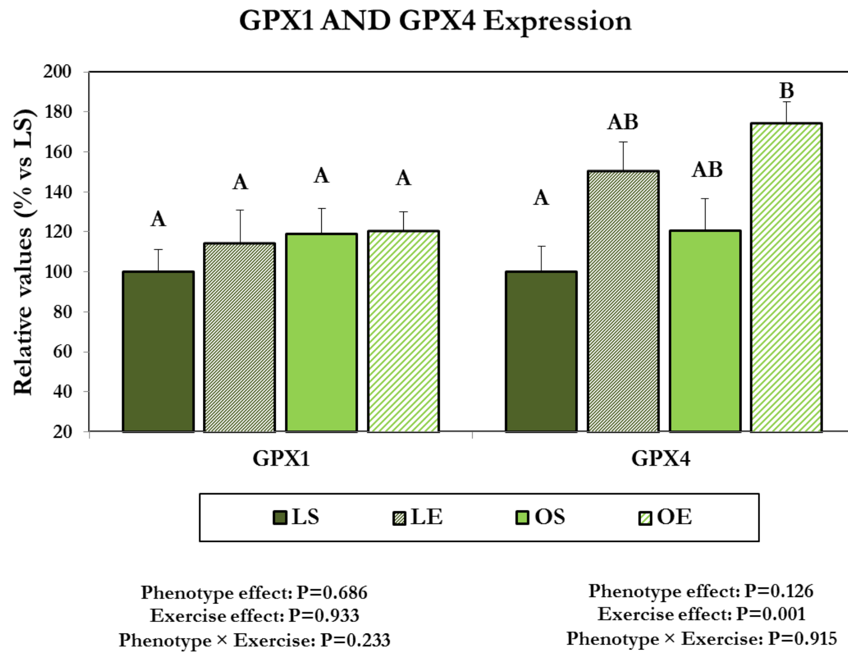
Exercise Effect: P=0.498
Phenotype × Exercise: P<0.001
Exercise × Diet: P=0.249

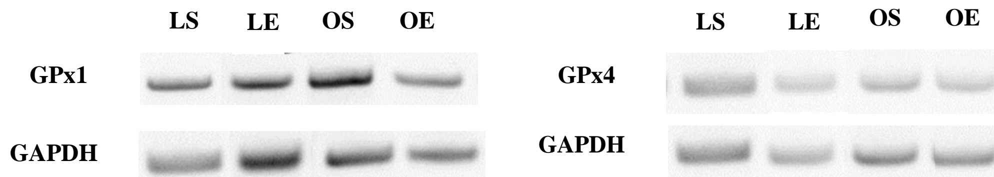
(C)



Western blot analysis of (A) AMPKα/PAMPKα, (B) PPARγ, (C) FAS expression. Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing a protocol of aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing a protocol of aerobic interval exercise. Immunoblots are representative of liver homogenates of eight different rats for each experimental group. The amount of sample loaded per lane was 100 µg of protein for AMPKα/PAMPKα, 30 µg of protein for FAS, and 80 µg of protein for PPARγ. Levels of pAMPK were normalized to the total AMPK. Levels of FAS and PPARγ were normalized against β-actin. Densitometric analysis values represented in the graphs are means ± SEM depicted by vertical bars (n = 8). Means without a common letter differ, $P < 0.05$.

Figure 20. Effect of phenotype and a protocol of interval aerobic exercise on gene expression of GPX1 and GPX4.





Groups: LS, Lean (fa/+) sedentary rats, LE, Lean (fa/+) rats performing a protocol of aerobic interval exercise, OS, Obese (fa/fa) sedentary rats, OE, Obese (fa/fa) rats performing a protocol of aerobic interval exercise. Bands are representative of liver homogenates of eight different rats for each experimental group. Densitometric analysis values represented in the graphs are means ± SEM depicted by vertical bars (n = 8). Means without a common letter differ, P < 0.05.

8. DISCUSSION

The study of MetS and the development of strategies for its prevention and treatment has attracted increasing attention in recent years due to its growing prevalence and associated comorbidities, exemplified by cardiovascular disease and NAFLD, which is considered as the hepatic manifestation of the MetS (Kaur 2014; Marchesini et al. 2003). Still, the aetiology of the development and progression of NAFLD remains uncertain,; while its prevalence keeps increasing worldwide (Fan et al. 2011; Szalman, Bancu, and Sin 2013). Non-pharmacological strategies based on lifestyle changes such as caloric restriction and increase of physical activity still remain the primary targets for the prevention of this disease. Additionally, there is an increasing interest on the development of functional products that can result helpful in the prevention and dietary treatment of alterations (hypertension, hyperlipidemia, hyperglucemia) related to MetS.

Legumes, have attracted the attention of the nutritional community due to their high protein content, the bioactive compounds they contain and the easy and economic procedures that can be used in order to achieve a high- nutritional value product. These processes can also be used in order to reduce or even eliminate the so called antinutritional compounds that can reduce nutrient bioavailability (especially protein and minerals) or cause allergenic responses and even toxicity. Germination and enzymatic protein hydrolysis are among the processes that can result useful for the development of such products. The main advantages of these techniques are their relative ease and low cost to develop and the fact that the higher nutritive value of the ending product is assured through the simultaneous enhancement of protein and bioactive compounds content and the reduction in the levels of antinutritional factors.

Besides the lifestyle changes focusing on dietary habits, the increase of physical activity has proved to be helpful towards the prevention of cardiovascular diseases and alterations related to glucose and lipid metabolism. However, the type and intensity of exercise are still a matter of debate.

This Doctoral research work aimed to study three parameters that can affect the progression of NAFLD, such as phenotype, diet, and high-itensity interval training (HIIT) protocol. First of all, Phase 1 experiments intended to investigate the induction of NAFLD through the administration of hypercholesterolemic diets in which different protein (casein *vs*

lupin protein hydrolizate) or insoluble dietary fibre (cellulose *vs* lupin insoluble fiber residue) sources were included, and to test the potential benefits of lupin products on the experimental animal model of diet-induced hypercholesterolemia/hyperlipidemia. These effects were investigated on biochemical, hepatic, renal and intestinal parameters of the animals. Taking in consideration the results of phase 1 experiments, we decided to design a second set of experiments in which a more advanced experimental model in terms of metabolic syndrome and NAFLD, the obese Zucker rat, was introduced. In addition, we combined two different interventions; a HIIT consisting of successive 4 min periods at 65-80% of VO_2max, followed by 3 min recovery periods at 50-65% of VO_2max, and the inclusion of raw and germinated *Vigna radiata* as protein and fibre source in the experimental diets. Germination was used as a natural process to obtain protein hydrolyzates, and *Vigna radiata* was chosen since it is a widely commercialized legume food product for its use in different healthy foodstuffs and meals. To develop a sprouted legume product with maximum functional capacity, different germination periods were assayed, and the potential antioxidant capacity of the sprouts obtained was assessed as model feature to establish the optimal characteristic of the selected product. The effects of the two lifestyle interventions implemented (nutrition and physical exercise) were assessed on aerobic capacity, hematic, plasma and liver biochemical parameters of obese and lean Zucker rats.

The results of the phase 1 experiments showed significant alterations in plasmatic total cholesterol and triglycerides content, morphological and compositional changes in liver and alterations in urinary parameters of renal function of the animals due to the hypercholesterolemic/high-fat diets tested. The dietary inclusion of lupin protein hydrolizate and insoluble fibre residue favourably affected plasma triglycerides and hepatic lipid composition as well as glucose metabolism.

In phase 2 experiments, a clear phenotype effect on the majority of the parameters studied was observed. Obese rats exhibited higher food intake and body weight, and suffered significant alterations in plasma lipid profile, AUC after oral glucose overload, liver histology and functionality, and antioxidant status. Exercise increased the aerobic capacity of both rat phenotypes and diminished the severity of MetS alterations, especially those related to glucose and lipid metabolism, affecting the levels of proteins involved in such metabolic pathways, the activity of enzymes related to redox status, and the gene expression of GPX4, a key antioxidant enzyme, in liver. The utilization of *Vigna radiata* experimental diets resulted in improvements of plasma glucose and lipid metabolism as well as in the aerobic capacity and

antioxidant status parameters. The resulting differences due to the diet effect were in general more pronounced in the germinated *Vigna radiata* experimental diet.

8. Discussion

8.1. Phase 1: Effects of *L. albus* protein hydrolyzate and insluble dietary fiber residue in an experimental model of diet-induced hypercholesterolemia

8.1.1. Lipid metabolism and liver function

The alteration in biochemical parameters related to lipid metabolism in response to a diet high in cholesterol and saturated fatty acids with considerable atherogenic potential was partially compensated by the dietary inclusion of lupin functional products. The consumption of lupin protein hydrolyzate and insoluble fiber residue did induce a significant reduction in plasma triglyceride content, although no effect in plasma levels of cholesterol was achieved. The hypolipidemic effects of *L. albus* protein extracts have been confirmed by other animal studies in which the cholesterol and triglyceride plasmatic levels were reduced by the inclusion of lupin protein as part of the experimental diet (Sirtori et al., 2004; Marchesi et al., 2008). Various factors are probably involved in the beneficial effects of lupin products on lipid metabolism. Namely, i) a digestive effect caused by binding of lupin protein hydrolyzate and fiber to bile acids (Yoshie-Stark & Wäsche, 2004) and their potential inhibition of micellar solubility (Alhaj et al., 2010) that was reflected in lower fat digestibility. ii) modifications in hepatic lipid metabolism caused indirectly through the action of SCFA released by the large intestine fermentation of lupin soluble and insoluble fiber residue and α-galactoside oligosaccharides(Alhaj et al., 2010), or else by a direct effect of the protein hydrolyzate. High Arg/Lys ratios have been considered good predictive markers of the hypolipidemic potential of a protein (Gudbrandsen et al., 2005), and lupin protein exhibits a considerable higher ratio when compared to casein (Bettzieche et al., 2009). Specifically, previous studies by our group (Martínez-Villaluenga et al., 2007), have described the amino acid composition of *Lupinus albus* var. *multolupa*, and the Arg/Lys ratio for this particular lupin variety is 4 and 8.2-fold higher in the seed flour and protein isolate, respectively, than the value reported for casein (Gudbrandsen et al., 2005). The elevated Arg/Lys ratio of lupin protein has been related to the altered expression of sterol regulatory element-binding protein-1c (SREBP-1c) (Spielmann et al., 2007) and the concominant down-regulation of genes responsible for fatty acid synthesis

or the up-regulation of genes involved in triglyceride hydrolysis (Bettzieche et al., 2008) or bile acid synthesis (Parolini et al., 2012). Howard and Udenigwe (2013) have reviewed the mechanism of food protein hydrolysate-induced hypolidemia, and point out to the modulation by protein hydrolyzates of the expression of genes involved in lipid metabolism at the hepatic and adipocyte level. Such modulation results in the upregulation of mRNA expression of proteins involved in fatty acid oxidation like PPARα as well as LDL-receptor and cholesterol-metabolizing enzymes, or else in the downregulation of mRNA expression of proteins involved in lipogenesis like fatty acid synthase. Such effects in lipid metabolism can be directly related to the changes in hepatic cholesterol and triglyceride profile observed under our experimental conditions, and, indirectly, to the improvement achieved in the levels of ALT, a well-known index of acute liver damage. Furthermore, in case of GGT, a marker of chronic liver damage, the plasmatic activity of this enzyme was lowered to values that were well below those of the control normolipidemic experimental group. However, despite the beneficial effects of lupin protein hydrolyzate and insoluble fiber on hepatic cholesterol and triglycerides metabolism, no effects were observed of the lupin products tested on hepatomegalia and steatosis induced by the hypercholesterolemic high-fat diets tested. This is in contrast to what has been reported by Fontanari et al., (2012), who found that *Lupinus albus* protein isolate and lupin seed flour decreased liver steatosis and histological alterations in hamsters fed a hypercholesterolemic diet. Possible reasons for such findings are differences in experimental animal species and diet formulation, with lower dietary content of cholesterol, higher levels of choline chloride, and the absence of added cholate reported by the above mentioned authors.

Lupin protein hydrolyzate and insoluble fiber residue also showed a promising potential in the management of glucose metabolism as seen by their lowering effects on glycemia, hypertriglyceridemia, a marker of insulin resistance, and urinary glucose excretion. Usually, plasma tryglicerides and glucose are elevated in type-II diabetes patients as part of the metabolic syndrome (Eckel, Grundy, & Zimmet, 2005). Lupin γ-Conglutin has been described to reduce plasma glucose concentration when orally administered to both rats and humans, thus exhibiting great potential in the control of glycaemia (Lovati et al., 2012). It is interesting to report that under our experimental conditions, the hypoglycemic properties of lupin protein still remain after hydrolysis of the above mentioned fraction during the sequential protease treatment carried out to prepare the lupin protein hydrolyzate. Such hypoglycemic activity exhibited by the lupin protein hydrolyzate can be explained based on the results by Morato et al., (2013), who found that the amino acid L-isoleucin and the peptide L-leucyl-L-isoleucin

showed the greatest efficiency among different amino acids and dipeptides derived from whey protein in traslocating the GLUT-4 glucose transporter from the cytosol to the plasma membrane, thus increasing glucose capture by skeletal muscle.

8.1.2 Renal and large intestine function

The different urinary parameters measured were altered in the Casein/Cellulose-based high-fat diet group (HC) in what could be the initial stages of renal injury. Furthermore, the acidification of urine and increased urinary P-excretion in combination with hypocitraturia that were observed under our experimental conditions in the above mentioned experimental group have been linked to kidney stone formation (Amanzadeh et al., 2003). Urinary acidification, hypercalciuria, hyperphosphaturia, and hypocitraturia have been described in response to hyperproteic diets (Aparicio et al., 2011), but less is known about the effect of hypercholesterolemic diets. Noticeably, several alterations in renal histology, antioxidant status, and lipid metabolism have been related to such diet-induced hypercholesterolemia in animal models similar to that developed in the present study (Al-Rejaie et al., 2012). In this regard, lupin protein hydrolyzate did show enormous potential to restore all the altered urinary markers with the exception of urinary calcium that was higher in the HID and HIDF when compared to the high-fat Casein/Cellulose group.

Compared to a poorly fermentable insoluble fiber like cellulose, lupin insoluble fiber residue or the amount of soluble fiber present in the protein hydrolyzate showed the typical pattern of large intestine fermentation with hypertrophy of caecum and colon and acidification of caecum content (Younes, Demigné, & Rémésy, 1996). Intestinal fermentation of complex carbohydrates is known to generate SCFA (Henningsson, Nyman, & Björck, 2001) that may act as main energy substrate for colonocytes or in the regulation of lipid metabolism (Bouhnik et al., 2004). Furthermore, lupin insoluble fiber residue exhibited enhanced water holding capacity that has the potential to increase stool volume and confers this functional product with a greater capability to drag and eliminate secondary bile acids and other toxic substances. The physiological and proliferative effects of dietary fiber on caecum and colon are largely dependent on its variable chemical nature (Folino, McIntyre, & Young, 1995) and fermentable capacity by the intestinal microbiota (McCullogh et al., 1998). An interesting finding of the present study is the differential effects on colon morphometry of the non-starch polysaccharides present in the two functional ingredients assayed. Soluble dietary fiber and α-galactoside oligosaccharides associated to lupin protein hydrolyzate exhibited a significant proliferative action due to its rapidly fermentable nature, whereas the dilution potential,

bulking capacity, and lowering the luminal pH action predominated in the lupin insoluble fiber residue.

8.2. Phase 2: Effects of raw and 4d-germinated *Vigna radiata* flours combined with a HIIT protocol in an experimental model of genetically obese Zucker rat

8.2.1 Food intake and body weight

Zucker obese rats are known to present a genetic defect in leptin receptor and the resulting development of hyperphagia as well as other metabolic disturbances in the obese phenotype have been previously reported (Galisteo et al., 2010). In this regard, the anorectic effects of exercise on Zucker rats have been described by the study of Kibenge & Chan, (2002) in which a low-moderate intensity swimming exercise was applied. Such anorectic effects were related to an increased production of corticotrophin-releasing hormone (CRH) and would in turn lead to a lower weight gain both in obese and lean animals.

The decreased food intake and increased sense of satiety in humans and animal studies has been previously reported due to legume intake or administration of legume extracts. In particular, α-galactoside oligosaccharides, lectins and α-amylase inhibitors exhibit a lowering effect on food intake in humans. These results have been attributed to gas production by large intestinal fermentation and to the interference with adequate functioning of digestive enzymes such as proteases, amylases, glycosidase and phosphatases. In addition, lower food intake was observed after prolonged germination time of peas in rodents; a fact that was attributed in the possible appearance of compounds that could cause the loss of the legume´s organoleptic properties and could result in lower food acceptability (Urbano et al., 2003). It is possible that 4-day germination of *Vigna radiata* is sufficient for these compounds to appear resulting in slightly decreased palatability, although such germination period can be considered optimal for the highest generation of potentially beneficial bioactive compounds from *Vigna Radiata* (Huang et al., 2014), and in terms of antioxidant capacity based on the *in vitro* and cell culture experiments carried out prior to feeding the animals with the *Vigna* experimental diets.

8.2.2 Aerobic capacity and physical performance

Physical performance was always lower in obese when compared to lean Zucker rats due to the severe metabolic disturbances, impaired skeletal muscle perfusion, and muscular atrophy inherent to this experimental model. Low intrinsic aerobic capacity in rats has been related to lower energy expenditure and reduced whole body and hepatic mitochondrial lipid oxidation, which in turn made the animals more susceptible to dietary-induced hepatic steatosis (Morris

et al., 2014). As a result of the aerobic interval training protocol, there was a clear improvement in the aerobic capacity of lean and obese rats that is more pronounced when combined with the consumption of GVR diet. The enhancement in aerobic capacity and parameters of physical performance derived from aerobic interval exercise observed in this study corroborates with other authors (Tjønna et al., 2008; Haram et al., 2009) that related such changes to amelioration in several risk factors of MetS associated cardiovascular disease.

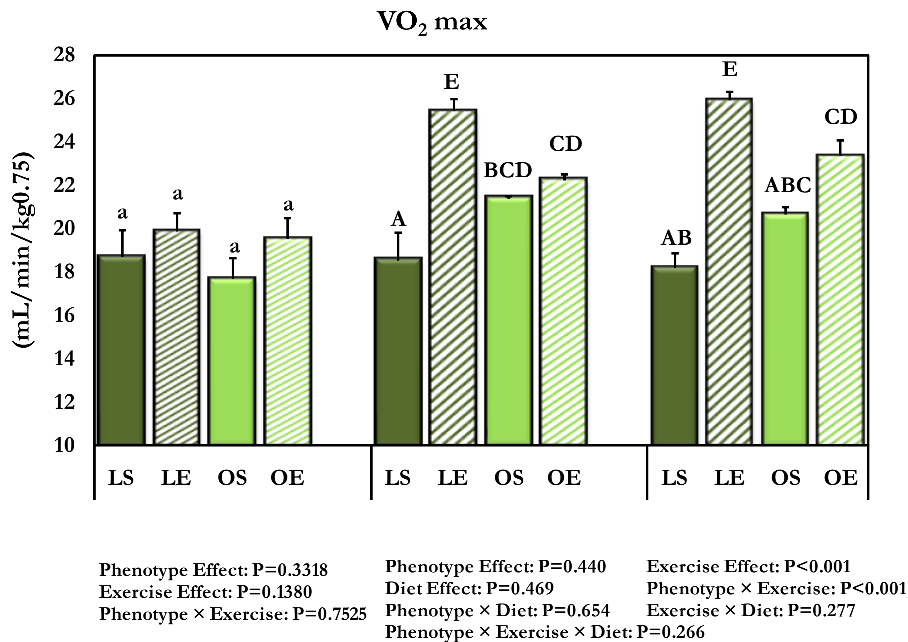


Figure 21. VO_2 max in the three different dietary treatments (CS, RVR and GVR) during maximal oxygen consumption incremental test.

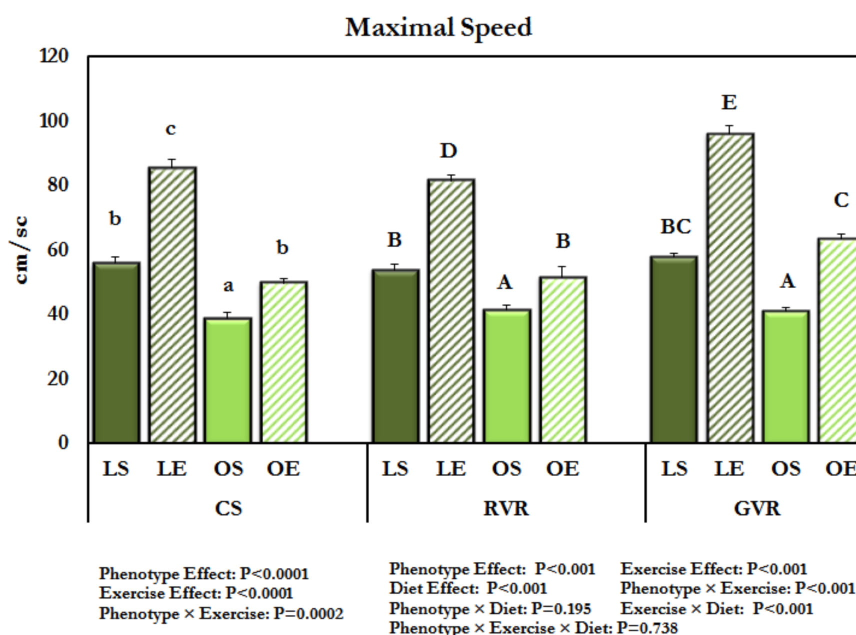


Figure 22. Maximal speed achieved during the performance of maximal oxygen consumption incremental test in the three different dietary treatments.

Regarding the physical performance parameters, a significant improvement was observed due to the HIIT protocol in both phenotypes. Such improvement was higher after the inclusion of the legume based diets.

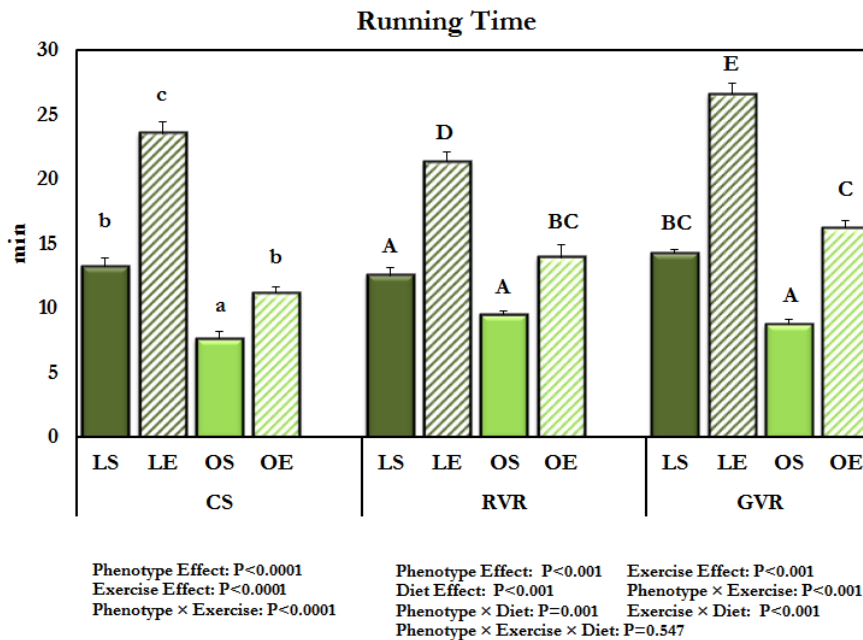


Figure 23. Duration of the maximal oxygen consumption incremental test in the three different dietary treatments.

Under our experimental conditions, the higher physical performance of trained Zucker rats was related to significant changes in glucose and lipid metabolism as well as to improved hepatic histology and function altered in NAFLD. Similar improvements in physical performance were observed after the administration of fenugreek in animals that performed a swimming protocol. The administration of fenugreek resulted in longer swimming time thus suggesting the activation of lipid rather than glucose metabolic pathways as energy source for the performance of the training protocol (Ikeuchi et al., 2006). Moreover, the combination of exercise and the administration of soy-derived isoflavones in postmenopausal women managed to favourably affect the altered liver function as well as the stage of fatty liver disease (Barsalani et al., 2012). Another factor that has to be taken under consideration is the generally improved health status of the animals due to the consumption of the GRV experimental diet. The improvement in glucose and lipid metabolism, together with the enhanced liver

morphology and functionality are also reflected in improved aerobic capacity and physical performance.

The experimental model of obese Zucker rat has been described to exhibit impaired lactate transport by the skeletal muscle that can be alleviated by endurance exercise (Metz et al., 2005). The aerobic training protocol tested in our study achieved a slight decrease in basal blood lactate content when compared to untrained animals and a more consistent reduction after the incremental oxygen consumption test. Since lactate release under exercise conditions is mostly related to skeletal muscle metabolism, our results suggest that the benefits of the HIIT protocol on lactate uptake and metabolism are clear. This improvement in lactate transport and metabolism represents an important benefit on glucose metabolism in relation to hyperlactatemia and aggravation of insulin resistance. A more pronounced improvement of these parameters was observed by the administration of RVR and GVR experimental diets that resulted in lower basal lactate level in the obese animals when compared to CS. Such improvement is in agreement with the findings of Ikeuchi et al., (2006) which linked the dietary-induced decrease in lactate levels caused by fenugreek with higher degree of lipolysis and higher utilization of lipids instead of carbohydrates during exercise.

8.2.3 Plasma parameters

The development of metabolic syndrome in obese Zucker rats was directly related to alterations in glucose and lipid metabolism as a consequence of the obese phenotype. Insulin resistance and elevated concentrations of lipoproteins have been reported by other authors as main factors taking part in the metabolic syndrome (Hwang et al., 2013; Kelly et al., 2014). Moreover, the beneficial effects of different types of aerobic exercise on glucose and lipid metabolism have been extensively reported in the literature (Haram et al., 2009; Johnson et al., 2009; Rosety-Rodriguez et al., 2012). Our results confirm such positive actions of HIIT protocol, and point out to training-induced enhanced insulin sensitivity in the obese animals as seen by changes in plasma AUC after an oral glucose overload.

Likewise, the specific action of the training protocol at decreasing total- and LDL-cholesterol, while leaving HDL-cholesterol unchanged, suggests a direct protection against well-known cardio-metabolic risk factors. Such effects on the plasma lipid profile could be explained by a lower free fatty acid uptake and lipogenesis in the adipose tissue as suggested by the reduction in FATP-1 and FAS protein levels found by Haram et al., (2009). In addition, it has been reported that physical exercise can lead to a significant improvement in the content

and functionality of mitochondria measured by increased citrate synthase activity, and palmitate oxidation (Linden et al. 2014). Furthermore, physical exercise is a successful strategy to prevent and mitigate NASH-induced mitochondrial bioenergetics impairment, thus improving lipid metabolism in liver (Gonçalves et al., 2014).

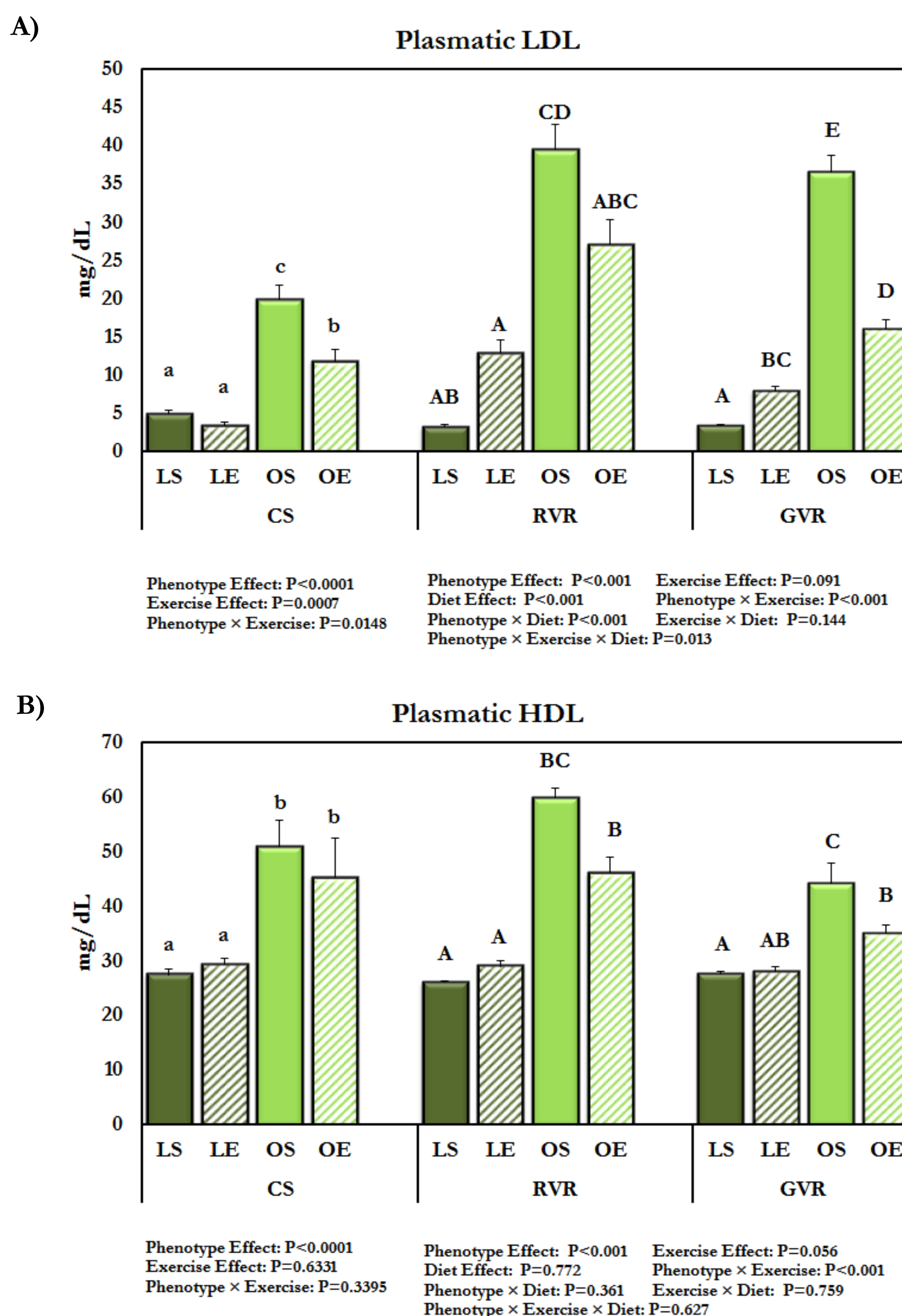


Figure 24. Plasma LDL- (A) and HDL-cholesterol (B) in the three different dietary treatments.

The improvement action exerted by the HIIT protocol on the above mentioned parameters of glucose and lipid metabolism was further enhanced in the groups that consumed the *Vigna radiata* diets, especially in 4d-germinated *Vigna* that exhibited by itself a very strong action on

plasma AUC. The antidiabetic properties of the legumes are largely attributed to specific properties of the nutrients they contain and to the activity of some of their non-nutritional components. For example, in *Lupinus albus*, the antidiabetic properties are attributed to the capacity of γ-coglutin to enter and be accumulated in an intact form in the cytosol of the hepatic cells. In the case of *Vigna radiata* L., its antidiabetic properties are mainly assigned to the synergistic effects of its two major phenolics: vitexin and isovitexin, as well as to the slow-digestible nature of its complex carbohydrates. In addition, some of the non-nutritional components present in legumes like α-glucosidase and α-amylase inhibitors, as well as the soluble dietary fibres, exhibit the ability to slow down the digestion and absorption of carbohydrates through the small intestine, thus contributing to the suppression of postprandial hyperglucemia. The hypoglycaemic properties of *Vigna radiata* have been confirmed by *in vitro* studies demonstrating the inhibition of glycation endproduct formation by extracts of mung bean coats (Yao et al., 2008, 2011; Ramadan, El-Beih, & Abd El-Kareem, 2011; Capraro et al., 2014).

The hypolipidemic effects of legumes have been widely reported in animal and human studies, suggesting as possible explanation the down-regulation of the genes involved in the *de novo* synthesis of fatty acids as well as the ones associated with TAG hydrolysis (Bettzieche et al., 2008; Sirtori et al., 2012). Such effect is also thought to arise from the phytosterol content of mung beans which is similar to blood cholesterol, facilitating the prevention of cholesterol biosynthesis and absorption (Tang et al., 2014). Moreover, it has been proposed that an activation of LDL receptors in the hepatic cells might be responsible for such improvements in the concentrations of the lipoproteins (Sirtori et al., 2004).

Under our experimental conditions, the administration of *Vigna radiata* experimental diets, in particular that of 4d-germinated *Vigna radiata*, could not reverse the elevated level of triglycerides in plasma that were even higher than those found in the CS group. Such higher content of triglycerides can be attributed to the genetic variations that characterize the specific experimental model rather to any specific effect of *Vigna* consumption.

8.2.4 Hematic parameters

The alteration in hematic parameters of obese when compared to the lean Zucker rats can be attributed to the inflammatory process usually associated to obesity and the metabolic syndrome. Such process is responsible for the detrimental storage of Fe in different tissues at the expense of blood, which can lead to iron-deficiency in overweight individuals (Tussing-

Humphreys et al., 2009). The relationship between Fe storage, oxidative stress and the inflammatory process developed in such individuals plays an important role in their compromised health status, and liver is one of the more affected organs with a higher Fe accumulation in NAFLD (Aigner et al., 2008). Our findings demonstrate an improvement of several hematic parameters by the nutritional and physical exercise interventions, thus suggesting their ameliorating effect upon these alterations.

8.2.5 Liver area, lipid composition, functionality, and antioxidant status

The aerobic interval training triggered a clear improvement in liver lipid composition (lower total fat and triglyceride content) as described by other authors in different human and animal models (Johnson et al., 2009; Linden et al. 2014). HIIT can lead to such improvements in lipid composition through increases in mitochondrial content and oxidative phosphorylation, or greater lipid and carbohydrate oxidation (Barker et al., 2014; Larsen et al., 2014).

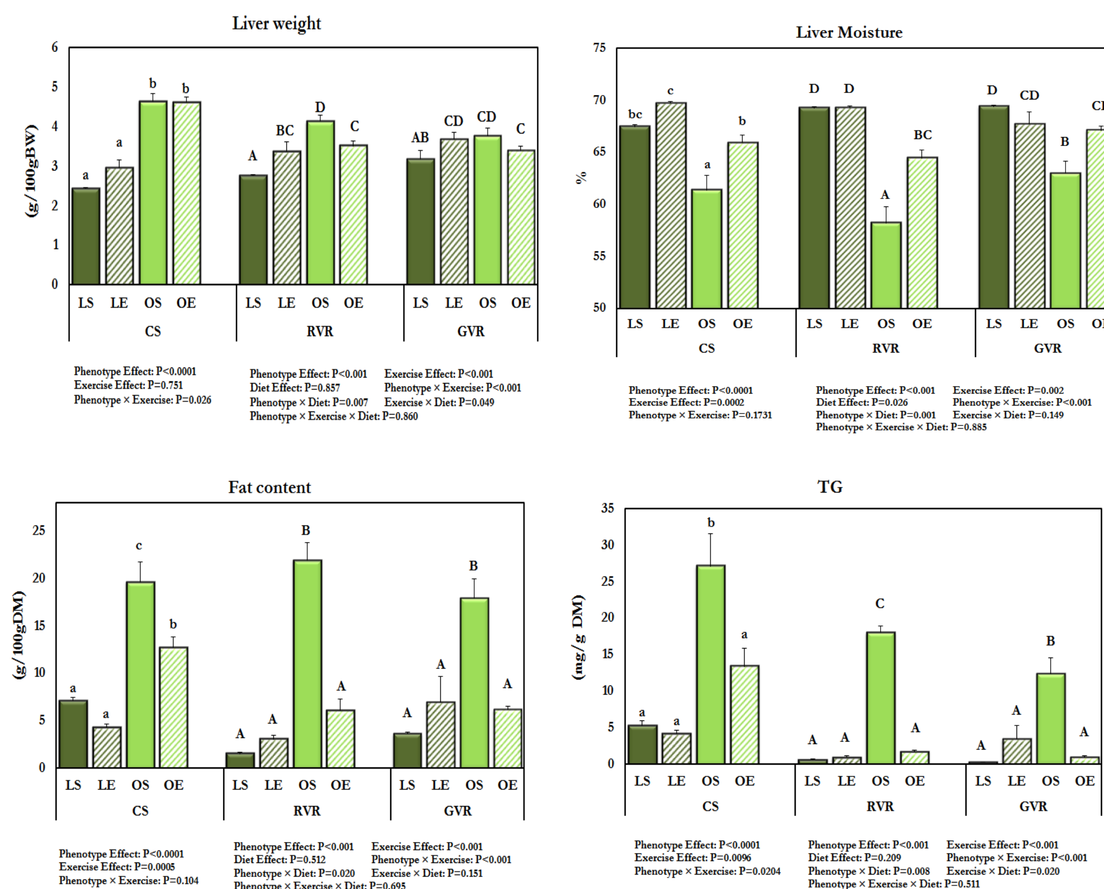


Figure 25. Liver weight, moisture, fat and triglyceride content in the three dietary treatments.

Indeed, a long term aerobic training, for 3 months, at 60-75% of VO_2max has been shown to induce a decrease in intrahepatic lipids in obese female adolescents (Lee et al., 2012), moreover a 7-day aerobic training protocol during 1 h at 80-85% of maximum heart rate in obese individuals with hepatic steatosis resulted in increased resting fat oxidation and favourable effects in hepatic lipid composition by increasing polyunsaturated lipid index (Haus, Solomon, Kelly, Fealy, Kullman, Scelsi, Lu, Pagadala, McCullough, Flask, & Kirwan, 2013). Likewise, the beneficial effect of a 12 week interval training on lipid oxidation was also proven in healthy, sedentary subjects (Astorino et al., 2013).

The lipid parameters previously mentioned were further improved after the administration of the *Vigna radiata* diets as shown by the lower levels of total hepatic fat and triglyceride content in the animals that consumed these diets. Furthermore, the combination of HIIT protocol and *Vigna* diets led to a much more pronounced decrease in the obese phenotype. Legume consumption is a feasible and well known nutritional strategy to decrease liver weight and total fat content (Kapravelou et al., 2013) 2014;). Phytosterols present in legumes such as soybeans have been reported to exert protective effects against NAFLD since they inhibit the absorption of cholesterol in the intestine and ameliorate the impaired hepatic β-oxidation that characterizes this specific disease (Laos et al., 2014). In a similar manner, the extract of *Casia tora* seeds managed to decrease the hepatic lipid accumulation and therefore regulate lipid homeostasis by the simultaneous upregulation of genes associated to lipid oxidation and the downregulation of genes linked to the lipid synthesis (Tzeng et al., 2013). Under our experimental conditions, the administration of the GVR diet led to a greater improvement of the NAFLD disease as demonstrated by the lower liver weight and liver area of the obese animals that consumed this specific diet. Such increased potential of the GVR diet may be due to the physical changes that take place during germination such as the decrease of phospholipids and the triglycerides content as well as the increase in polyphenol content, antioxidant capacity and vitamin C levels of the *Vigna radiata* sprouts as confirmed by other studies (Abdel-Rahman et al., 2007; Doblado et al., 2007; Guo et al., 2012; Kim et al., 2012).

Fatty liver has been associated to high plasma AST and ALT activities resulting from hepatic damage mediated by inflammation and oxidative stress which is reflected in higher levels of hepatic nitrate and malondialdehyde (Jung and Kim 2013; Linden et al. 2014). Furthermore, the presence of diabetes has been correlated to higher levels of these markers (Garcíacaballero et al., 2014). Significant improvements on plasma parameters of liver functionality have been observed under our experimental conditions related to the fat

composition changes in the obese Zucker rats. It is worth mentioning that our exercise training protocol has been beneficial both in acute and chronic hepatic markers (AST and ALP activities, respectively) of altered functional status, although it was not able to reverse the hepatobiliary disorder resembled by the high plasma bilirubin levels in obese rats. The administration of the GVR experimental diet when combined with the HIIT protocol led to major improvements of the hepatic plasma parameters in the obese trained groups, demonstrating a synergistic effect between those two factors. Similar protective effects against the development of liver injury induced by administration of a high fat diet have been attributed on the phytosterol, flavonoid and phenolic acid content of legumes that contribute to inhibit the increment of AST and ALT activities in plasma (Ramadan et al., 2011; Dai et al., 2013). The increase in phenolic content of *Vigna radiata* due to the germination process as reported previously (Kim et al., 2012) might be a possible explanation for such protective effect exerted by the GVR experimental diet.

Oxidative stress is suggested as one of the main factors involved in the development of NAFLD (Rolo et al., 2012; Tariq, Green, & Hodson, 2014). Indeed, the "two-hit" hypothesis on NASH development points out to oxidative stress as one of the factors directly promoting the progress from steatosis to the advanced stages of the pathology (Rolo et al., 2012). Mitochondrial dysfunction appears to be the most important factor in the development of NASH, by both enhancing pro-oxidant mechanisms such as decreased electron transfer chain or induction of cytochrome P450 2E1, and reducing anti-oxidant defence mechanisms (lower hepatic catalase, GPX or Mn-SOD activities) (Jung and Kim 2013; Tariq, Green, and Hodson 2014).

A decrease in the functionality of antioxidant defence system has been described as one major promoting factor in the development of oxidative stress in patients with NASH (Videla et al., 2004). In this regard, obese Zucker rats with fatty liver have been described to exhibit an altered antioxidant status as shown by the decrease in liver content of GSH, tocopherol, and catalase activity (Soltys, Dikdan, & Koneru, 2001). Furthermore, in this experimental model, oxidative stress promotes the activation of stress-response nuclear transcription factors, such as Stat-3 (Dikdan et al., 2004). Exercise is a useful lifestyle intervention strategy to improve oxidative stress in the muscle of type 2 diabetic rats (Qi et al. 2011; Rosety-Rodriguez et al. 2012) and plasma of obese middle-age women (Shin et al. 2008). Furthermore, in obese individuals with hepatic steatosis, short-term aerobic exercise has proved to favourably alter hepatic lipid composition, insulin resistance, and oxidative stress; risk factors that influence

the severity of NAFLD (Haus et al., 2013). However, the effects of exercise on oxidative stress may vary depending on parameters such as age, health status, severity of the pathology, and/or type and intensity of the exercise protocol applied. Under our experimental conditions the effect of exercise on SOD, GPX, and catalase activities differed between obese and lean rats, a finding that can be attributed to the compromised antioxidant status of the obese animals associated to their fatty liver condition. Exercise was an effective strategy to lower oxidative stress and revert SOD and GPX activities to levels closer to those of the lean rats.

A positive influence of dietary legume administration on the balance between oxidative and antioxidative agents through the regulation of antioxidant enzyme activities has been reported by various studies. Soybean protein supplementation seems to restore the levels of SOD, catalase and GPX in fatty liver animal models therefore concluding its ability to remove reactive oxygen species and reduce lipid peroxidation (Jung and Kim 2013; Morita et al. 2012; Yang et al. 2011). Moreover, similar protective effects against reactive oxygen and nitrogen species formation have been attributed to the phenolic components of mung bean extracts implying its use as an easily accessible source of natural antioxidants (Chung et al., 2011).

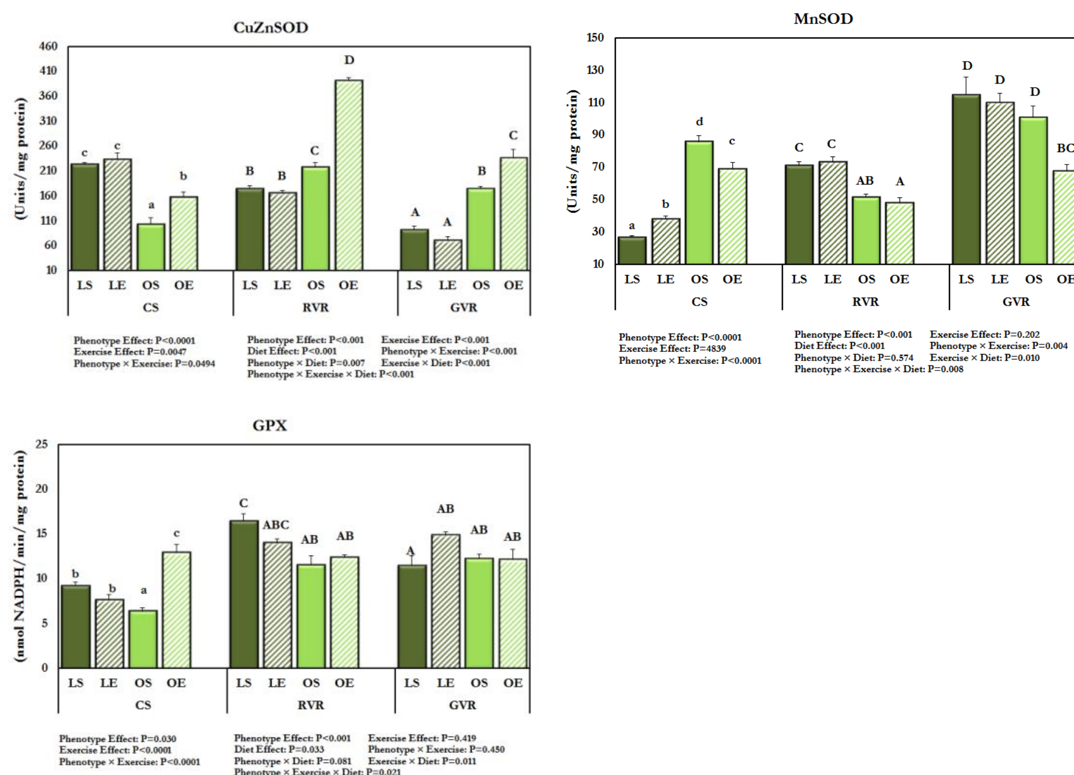


Figure 26. Effects of phenotype, exercise and diet in the activities of antioxidant enzymes

Overall, the administration of *Vigna* diets under our experimental conditions had a significant influence in the antioxidant mechanisms studied since the raw *Vigna radiata* diet led

to higher GPX and catalase levels, whereas the germinated one resulted in higher levels of Cu/Zn, MnSOD and GPX activities when compared to CS groups. Such effects on redox status are complemented by the enhanced antioxidant capacity exhibited by 4d-germinated *Vigna radiata* using different *in vitro* and cell culture techniques. Exercise maintained its effect in CuZn and MnSOD activities of obese rats, but lost its inducing influence on GPX activity due to the strong effect of *Vigna* consumption on that enzyme. It seems that the two *Vigna* experimental diets partly managed to restore the antioxidant enzyme activities competing the strong deleterious influence that phenotype exerted upon them.

8.2.6 Liver histology

It is well established that the appearance of NAFLD and NASH are accompanied by the triglyceride accumulation in liver. Furthermore, signs of fibrosis have been reported in animals with hepatic NASH induced by high fat diet administration (Yang et al. 2011). The improvement in liver histological features associated to changes in lipid composition and function exerted by the training protocol in the obese Zucker rat shows the prospective benefits of this type of exercise in ameliorating hepatic damage present in NAFLD. Nevertheless, although the training protocol has shown interesting results on glucose and lipid parameters in plasma, i.e. lower lipid content and decreased macro and microvesicular steatosis in liver of obese rats, the potentially deleterious effects of that intensive type of exercise on individuals prone to liver damage (e.g. suffering from MetS), should be considered, since necrosis and fibrosis were detected in the liver of trained rats, especially in the obese animals. The consumption of *Vigna radiata* diets managed to improve the hepatic injury of the obese animals, by decreasing the number of fatty droplets as well as the microvescicular steatosis. In a similar manner, soybean administration reversed the high-fat-diet induced NASH by decreasing the accumulation of triglycerides in liver. The amino acid profile, peptides or isoflavones of this legume seem responsible for such beneficial effect through the decrease of liver fat and oxidative stress they can induce; therefore regulating the inflammatory response of NASH (Yang et al. 2011). Moreover, soy phospholipids are linked to the decrease of hepatic accumulation of triglycerides, diglycerides, ceramides and oleates; the endproducts of fatty acid synthesis (Mori et al., 2011). Other authors suggest the role of β-sitosterol content of pigeon pea on the excretion of cholesterol by converting it into bile acids, thus suppressing fatty degeneration and cytoplasmatic vacuolization in liver (Dai et al., 2013). It is worth pointing out that under our experimental conditions, the combination of the GVR diet and the HIIT protocol induced greater changes in the micro and macro vesicular steatosis

signs when compared to the other two experimental diets tested. Such results point out on the one hand to the advantages of plant-based foodstuffs as essential components in dietary strategies for the prevention and treatment of NAFLD, and, on the other hand, to the advantages of certain biotechnological treatments like germination to potentiate the benefits of other lifestyle strategies actually in use for the treatment of the above mentioned disease.

8.2.7 Liver protein and gene expression

Activation of AMPK is reduced in the obese Zucker rat due to an excess of energetic substrates entering the liver (Galisteo et al., 2010). Although exercise has been reported to activate AMPK in the skeletal muscle (Sasaki et al., 2014), our findings do not show any effect of the aerobic interval protocol in the liver. Regarding the legume based diets used in these studies no significant changes were reported in the activation of this enzyme. In contrast, the existing data suggest that other legumes induce an activation of the phosphorylated AMPKα in a similar manner as exercise does. In particular, a herbal product of traditional Chinese medicine which consists in a mixture of refined extracts of the active components of three plants: a)the total alkaloids from the stems of *Coptis chinensis* (Ranunculaceae), b) total saponins from the roots of *Astragalus membranaceus* (Leguminosae), and c) sugar alcohols from the flower buds of *Lonicera japonica* (Caprifoliaceae), led to the activation of hepatic pAMPKα imitating the role that metformin, a well-known pharmacological agent for the diabetes treatment, exert on this enzyme. (Gao et al., 2014). Similarly, the administration of *Cassia tora* extract, a leguminous seed plant, has been reported to enhance the phosphorylation of AMPKα as well as its primary downstream targeting enzyme, acetyl-CoA carboxylase (ACC), which in turn leads to upregulation of CPT1 gene expression. According to this, the authors suggest that the extract of the specific legume enhances hepatic fatty metabolism and lipid oxidation through an AMPK-ACC-CPT1 pathway (Tzeng et al., 2013).

Activation of liver PPAR-γ has been reported to improve insulin sensitivity and NASH in human patients (Neuschwander-Tetri et al., 2003) and this correlates with the significant increase of liver PPAR-γ protein expression exerted by our exercise protocol in CS experimental groups. Increased insulin sensitivity could in term lead to a higher entrance of glucose in the liver and other organs like skeletal muscle. Higher levels of glucose in the liver of trained rats can augment its conversion in free fatty acids (Oakes et al., 1997) through FAS activation directly or indirectly induced by PPAR-γ. Under our experimental conditions, the increased expression of PPAR-γ induced by exercise was matched by increased expression of FAS in the animals fed CS.

The binding capacity of amorfrutins from legumes to PPAR-γ has been suggested to induce its activation, therefore acting as insulin sensitizers. This suggests that targeting PPAR-γ with the use of amorfrutins may help minimize the side effects of the pharmacologic agents so far used that exert the same function (Weidner et al., 2012). Furthermore, isoflavones such as genistein and daidzein demonstrate similar binding effects on PPAR-γ thus improving insulin resistance (Wagner et al., 2008; Quang et al., 2013). Consumption of *Vigna radiata* diets appeared to induce a significant increase in PPAR-γ expression of obese animals that correlated well with their positive effects on glucose metabolism. In contrast, the enhancing effect of exercise on PPAR-γ expression was only apparent in lean rats, whereas the opposite action was observed in obese animals. This differential effect of diet and exercise on PPAR-γ expression can be attributed to the strong influence of the genetic metabolic disorder suffered by obese Zucker rats in which the presence of numerous factors capable of interacting with our two interventions have modified the response of liver in terms of nuclear factor activation.

Increased expression of Fatty Acid Synthase (FAS) is associated with increased sensitivity to NAFLD and high activity of mitochondrial control and peroxisomal beta-oxidation. It is principally considered responsible for the *de novo* lipogenesis and regulated by insulin (Hillgartner et al., 1995). Increased hepatic activity and protein activation of FAS have been demonstrated in obese Zucker rats when compared with their lean littermates whereas exercise is proved as a successful strategy for its down-regulation (Fiebig et al., 2002; Schultz et al., 2012). Dietary phospholipids and isoflavones such as genistein have been shown to decrease FAS activation; the latter demonstrating a regulatory effect on the metabolic abnormalities triggered by the decline of the estradiol level induced after ovariectomization of the rats (Mori et al., 2011; Choi, Koh, & Song, 2012). Moreover, the study of Shirouchi et al., (2010) pointed out the effect of dietary soybean phospholipids in the activation of FAS attributing such finding to the acetone insoluble components they contain. Under the experimental conditions of the present study, the effects of phenotype and exercise on FAS expression were significantly attenuated by *Vigna radiata* consumption that acted as strong protecting dietary ingredient against fatty acid accumulation in the liver of Zucker rats. In this regard, the diet effect was stronger when compared to the exercise effect that was hardly noticed in contrast to what was observed in the animals fed CS diet. The effects of *Vigna radiata* in FAS expression did not appear to be influenced by PPAR-γ expression in a different way of what was observed with the CS groups. It can be hypothesized that other nuclear

activating factors like PPAR-α or SREBP can be influenced by the dietary treatment and exhibit a stronger action on FAS expression, or else that the amount of PPAR-γ expressed may not correspond to the active for of the nuclear factor.

Increased hepatic GPX1 gene expression is linked to induced toxic hepatitis and the development of NASH, both associated with high oxidative stress (Carazo et al., 2011; Iskusnykh et al., 2013). However, neither exercise nor phenotype had a significant effect on the hepatic GPX1 gene expression under our experimental conditions. Similar findings have been observed in pediatric patients with NASH that underwent liver biopsy (Desai et al., 2014). Furthermore, Jenkins et al., (2009), found no differences in GPX1 gene expression of mononuclear cells (CFU-EC) isolated from peripheral blood samples of active (>3 yr moderate- to high-intensity endurance exercise for >4 h/week) or inactive (≤20 min endurance exercise ≤2 days/week) healthy participants after completing a 30-min treadmill run at 75–80% VO_2max.

While GPX4 deficiency has been linked to disorders associated with reactive oxygen species and lipid peroxides generated in mitochondria (Imai and Nakagawa, 2003), its overexpression is associated with the inhibition of atherosclerosis development in *ApoE*$^{-/-}$ mice (Guo et al., 2008) and lipid peroxidation in endothelial cells (Sneddon et al. 2003). In our study, hepatic GPX4 gene expression was up-regulated by exercise in both lean and obese groups. Similar results were obtained by Daussin et al., (2012) in the expression of GPX4 after endurance training for 10 days.

9. Conclusions

Phase 1. Effects of *L. albus* protein hydrolyzate and insluble dietary fiber residue in an experimental model of diet-induced hypercholesterolemia.

1. To test the functional effects of vegetable protein hydrolyzates and insoluble dietary fiber residue on different parameters of metabolic syndrome and NAFLD, we have generated an experimental animal model of diet-induced hyperlipidemia/hypercholesterolemia feeding the animals with hypercholesterolemic high-fat diets in which different protein (casein *vs* lupin protein hydrolyzate) or insoluble dietary fiber (cellulose *vs* lupin insoluble fiber residue) sources were included. The experimental model exhibited significant alterations in plasmatic total-cholesterol and triglycerides, morphological and compositional changes in the liver, and alterations in urinary parameters of renal function. Thus, it represents an adequate model to test the biological actions of the different functional components tested.

2. The efficacy shown by lupin protein hydrolyzate at reducing plasma and hepatic triglycerides as well as glycemia in our experimental model makes it an interesting and relevant nutritional component in the diet of patients with type II diabetes and/or metabolic syndrome. In addition, lupin protein hydrolyzate exhibited a protective effect against the initial stages of hypercholesterolemic high fat diet-induced renal alterations. Such health benefits can be complemented by Lupin protein hydrolyzate and insoluble fiber effects on large intestine physiological status due to its fermentative and water holding capacity.

Phase 2. Effects of raw and 4d-germinated *Vigna radiata* flours combined with a HIIT protocol in an experimental model of genetically obese Zucker rat.

3. To test the individual or combined effects of raw or 4-day-germinated *V. radiata* and a HIIT protocol, the experimental animal model of obese Zucker rat was chosen. This animal model exhibits a variety of alterations in glucose and lipid metabolism that lead to NAFLD and are related to the metabolic syndrome. Such alterations could be targeted with the different lifestyle changes that were addressed in this Doctoral research.

4. The HIIT protocol used in this study is a feasible intervention strategy to improve plasma and hepatic biochemical parameters as well as hepatic histological alterations inherent to NAFLD in obese Zucker rats. The training protocol was especially efficient to improve insulin sensitivity and decrease the hepatic lipid content, as well as ameliorating the oxidative stress

conditions in this organ. Nevertheless, the high intensity exercise also induced some alteration in liver microscopic morphology that could reflect an excessive intensity of the training protocol tested.

5. Consumption of 4-day-germinated *V. radiata* reinforced the benefits of HIIT training with regard to body weight control, amelioration of hepatomegalia, reduction of hepatic lipid content and improvement of liver functionality and antioxidant status that are negatively affected by NAFLD. The combination of both lifestyle strategies has proved to be an efficient and feasible alternative to the more severe pharmacological treatments that are currently in use, and could contribute to lowering the dosage of such treatments in patients that suffer alterations caused by the MetS.

6. The general effects of the interventions carried out in this Thesis on several parameters of metabolic syndrome are underlined by changes in hepatic protein and gene expression, although such changes in the expression of key enzymes in glucose and lipid metabolism and antioxidant status were greatly influenced by the different phenotypic and lifestyle factors studied. Further research work conducted in this field by our research group is committed to unravel the different molecular mechanisms involved in the beneficial actions of high-intensity aerobic intervallic exercise and protein hydrolyzates in lipid and glucose metabolism and redox status.

10. Conclusiones

Fase 1. Efectos de hidrolizado proteico y fibra insoluble de *Lupinus albus* en un modelo experimental de hipercolesterolemia inducida por la dieta

1. Para determinar los efectos funcionales de los hidrolizados proteicos vegetales y del residuo de fibra insoluble sobre los diferentes parametros del syndrome metabólico y de Enfermedad hepática no alcoólica (NAFLD), se ha generado un modelo experimental animal de hipercolesterolemia/hiperlipidemia inducida por la dieta, tras el consumo de una dieta hipercolesterolemica y de alto contenido lipídico por los animales. En dichas dietas, se incluyeron distintas fuentes de proteína (caseína *vs* hidrolizado proteico de lupinus) o de fibra dietética insoluble (celulosa *vs* residuo de fibra insoluble de lupinus). La dieta empleada en nuestro modelo experimental produjo importantes alteraciones en los niveles de colesterol y trigliceridos en plasma, cambios en la composición y morfología hepática y modificaciones en los parámetros urinarios de la función renal. Por lo tanto, representa un modelo adecuado para estudiar el potencial biológico de los distintos componentes funcionales que se estudiaron.

2. La eficacia mostrada por *Lupinus* en la reducción de los niveles de trigliceridos plasmáticos y hepáticos, así como de la glucosa plasmática en nuestro modelo experimental hace que sea un componente nutricional interesante y relevante para su inclusión en la dieta de pacientes con diabetes tipo II y/o síndrome metabólico. Además, el hidrolizado proteico de *Lupinus* tuvo un efecto beneficioso sobre las etapas iniciales de las alteraciones renales inducidas por una dieta hipercolesterolémica y de alto contenido lipídico. Dichos beneficios para la salud se complementan por los efectos positivos que el hidrolizado proteico y la fibra insoluble de *Lupinus* tienen sobre el estatus fisiológico del intestino grueso debido a su capacidad fermentativa y de retención de agua.

Fase 2: Efectos de las harinas de *Vigna Radiata* cruda y germinada durante 4 días combinadas con ejercicio aeróbico intervalico y de alta intensidad de un modelo experimental de rata genéticamente obesa

3. Para probar el efecto aislado o combinado de las harinas de *V. radiata* cruda o germinada durante 4 días, y un protocolo de ejercicio aeróbico interválico y de alta intensidad (HIIT), se

ha elegido el modelo experimental de rata Zucker obesa. Este modelo animal manifestó varias alteraciones en el metabolismo glucídico y lipídico que condujeron al desarrollo de NAFLD y están relacionadas con el síndrome metabólico. Dichas alteraciones se podrían prevenir mediante las diferentes estrategias que incluyen cambios de estilo de vida y están mencionadas en esta Tesis Doctoral.

4. El protocolo HIIT que se utilizó en este estudio, es una intervención viable para la mejora de los parámetros bioquímicos plasmáticos y hepáticos, además de las alteraciones histológicas de hígado, asociadas con el desarrollo de NAFLD en las ratas Zucker obesas. El protocolo de ejercicio se ha demostrado especialmente eficaz hacia la mejora de la resistencia a la insulina y la reducción del contenido lipídico hepático, además de la mejora de estrés oxidativo en este órgano. Sin embargo, el protocolo de ejercicio específico también indujo algunas alteraciones en la miscroscopía hepática que podrían ser atribuidas a la intensidad excesiva del ejercicio utilizado.

5. El consumo de *V. radiata* germinada fortaleció los efectos beneficios del protocolo HIIT en relación al control de peso corporal, mejora de hepatomegalia, reducción de contenido hepático lipídico y mejora de la funcionalidad hepática y de estatus antioxidante que se ven negativamente afectados por la NAFLD. La combinación de ambas estrategias de vida se ha demostrado como una alternativa eficiente y viable frente a los tratamientos farmacológicos más intensos que se están utilizando hoy en día y podrían contribuir en reducir la dosis de dichos tratamientos en pacientes que sufren las alteraciones causadas por el MetS.

6. Los efectos generales de las intervenciones que se llevaron a cabo en esta Tesis sobre varios parámetros de síndrome metabólico se han acompañado por cambios en la expresión proteica y expresión genética en el hígado, aunque estos cambios en la expresión de enzimas claves del metabolismo lipídico y glucídico se han visto afectados en gran parte por los diferentes factores de fenotipo y de estilo de vida que se estudiaron. Para esclarecer algunos aspectos, actualmente nuestro grupo de investigación está desarrollando estudios para aclarar los distintos mecanismos moleculares involucrados en la influencia beneficiosa de este protocolo de ejercicio específico y de los hidrolizados proteicos sobre el metabolicmo lipídico, glucídico y de estatus redox.

11. References

Abdel-Rahman, E.-S. A., El-Fishawy, F. A., El-Geddawy, M. A., Kurz, T., & El-Rify, M. N. (2007a). The Changes in the Lipid Composition of Mung Bean Seeds as Affected by Processing Methods. *International Journal of Food Engineering*, *3*(5).

Aebi, H. (1984). Catalase in vitro. *Methods Enzymol.*, *105*, 121–126.

Alberti, K. G. M. M., Zimmet, P., & Shaw, J. (2006). Metabolic syndrome-a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabetic Medicine: A Journal of the British Diabetic Association*, *23*(5), 469–480.

Alberti, K. G. M. M., & Zimmet, P. (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO Consultation. *Diabetic Medicine*, *15*(7), 539–553.

Alhaj, O. A., Kanekanian, A. D., Peters, A. C., & Tatham, A. S. (2010). Hypocholesterolaemic effect of Bifidobacterium animalis subsp. lactis (Bb12) and trypsin casein hydrolysate. *Food Chemistry*, *123*(2), 430–435.

Ali, N. M., Mohd Yusof, H., Yeap, S.-K., Ho, W.-Y., Beh, B.-K., Long, K., Alitheen, N. B. (2014). Anti-Inflammatory and Antinociceptive Activities of Untreated, Germinated, and Fermented Mung Bean Aqueous Extract. *Evidence-Based Complementary and Alternative Medicine*, *2014,* Article ID 350507, 6 pages.

Al-Jiffri, O., Al-Sharif, F., Abd El-Kader, S., & Ashmawy, E. (2013). Weight reduction improves markers of hepatic function and insulin resistance in type-2 diabetic patients with non-alcoholic fatty liver. *African Health Sciences*, *13*(3), 667–672.

Al-Rejaie, S. S., Abuohashish, H. M., Alkhamees, O. A., Aleisa, A. M., & Alroujayee, A. S. (2012). Gender difference following high cholesterol diet induced renal injury and the protective role of rutin and ascorbic acid combination in Wistar albino rats. *Lipids in Health and Disease*, *11*, 41.

Amanzadeh, J., Gitomer, W. L., Zerwekh, J. E., Preisig, P. A., Moe, O. W., Pak, C. Y. C., & Levi, M. (2003). Effect of high protein diet on stone-forming propensity and bone loss in rats. *Kidney International*, *64*(6), 2142–2149.

American Diabetes Association. (2005). Clinical Practice Recommendations 2005. *Diabetes Care*, *28 Suppl 1*, S1–79.

Anderson, P. J., Critchley, J. A. J. H., Chan, J. C. N., Cockram, C. S., Lee, Z. S. K., Thomas, G. N., & Tomlinson, B. (2001). Factor analysis of the metabolic syndrome: obesity vs insulin resistance as the central abnormality. *International Journal of Obesity*, *25*(12), 1782–1788.

Angelico, F., Del Ben, M., Conti, R., Francioso, S., Feole, K., Fiorello, S., Violi, F. (2005). Insulin resistance, the metabolic syndrome, and nonalcoholic fatty liver disease. *The Journal of Clinical Endocrinology and Metabolism*, *90*(3), 1578–1582.

Angulo, P. (2007). GI Epidemiology: nonalcoholic fatty liver disease. *Alimentary Pharmacology & Therapeutics*, *25*(8), 883–889.

Annicchiarico, P., Manunza, P., Arnoldi, A., & Boschin, G. (2014). Quality of Lupinus albus L. (white lupin) seed: extent of genotypic and environmental effects. *Journal of Agricultural and Food Chemistry*, *62*(28), 6539–6545.

Anwar, F., Latif, S., Przybylski, R., Sultana, B., & Ashraf, M. (2007). Chemical Composition and Antioxidant Activity of Seeds of Different Cultivars of Mungbean. *Journal of Food Science*, *72*(7), S503–S510.

Aparicio, V. A., Nebot, E., Kapravelou, G., Sánchez, C., Porres, J. M., López Jurado, M., & Aranda, P. (2011). [Resistance training reduces the metabolic acidosis and hepatic and renal hypertrophy caused by the consumption of a high protein diet in rats]. *Nutrición Hospitalaria*, *26*(6), 1478–1486.

Arancibia, G., García, H., Jaime, F., Bancalari, R., & Harris, P. R. (2012). Association of metabolic syndrome markers with abnormal alanine aminotransferase levels in healthy children. *Revista Médica de Chile*, *140*(7), 896–901.

Arthur, J. R. (2001). The glutathione peroxidases. *Cellular and Molecular Life Sciences CMLS*, *57*(13-14), 1825–1835.

Astorino, T. A., Schubert, M. M., Palumbo, E., Stirling, D., & McMillan, D. W. (2013). Effect of two doses of interval training on maximal fat oxidation in sedentary women. *Med Sci Sports Exerc*, *45*(10), 1878–1886.

Bacchi, E., Negri, C., Targher, G., Faccioli, N., Lanza, M., Zoppini, G., Moghetti, P. (2013). Both resistance training and aerobic training reduce hepatic fat content in type 2 diabetic subjects with nonalcoholic fatty liver disease (the RAED2 randomized trial). *Hepatology*, *58*(4), 1287–1295.

Bae, J. C., Suh, S., Park, S. E., Rhee, E. J., Park, C. Y., Oh, K. W., Lee, W.-Y. (2012). Regular exercise is associated with a reduction in the risk of NAFLD and decreased liver enzymes in individuals with NAFLD independent of obesity in Korean adults. *PloS One*, *7*(10), e46819.

Balkau, B., & Charles, M. A. (1999). Comment on the provisional report from the WHO consultation. European Group for the Study of Insulin Resistance (EGIR). *Diabetic Medicine: A Journal of the British Diabetic Association*, *16*(5), 442–443.

Barker, A. R., Day, J., Smith, A., Bond, B., & Williams, C. A. (2014). The influence of 2 weeks of low-volume high-intensity interval training on health outcomes in adolescent boys. *Journal of Sports Sciences*, *32*(8), 757–765.

Barsalani, R., Riesco, E., Lavoie, J.-M., & Dionne, I. J. (2012). Effect of exercise training and isoflavones on hepatic steatosis in overweight postmenopausal women. *Climacteric*, *16*(1), 88–95.

Bedogni, G., Miglioli, L., Masutti, F., Tiribelli, C., Marchesini, G., & Bellentani, S. (2005). Prevalence of and risk factors for nonalcoholic fatty liver disease: The Dionysos nutrition and liver study. *Hepatology*, *42*(1), 44–52.

Belski, R., Mori, T. A., Puddey, I. B., Sipsas, S., Woodman, R. J., Ackland, T. R., Hodgson, J. M. (2011). Effects of lupin-enriched foods on body composition and cardiovascular disease risk factors: a 12-month randomized controlled weight loss trial. *International Journal of Obesity*, *35*(6), 810–819.

Bettzieche, A., Brandsch, C., Eder, K., & Stangl, G. I. (2009). Lupin protein acts hypocholesterolemic and increases milk fat content in lactating rats by influencing the expression of genes involved in cholesterol homeostasis and triglyceride synthesis. *Molecular Nutrition & Food Research*, *53*(9), 1134–1142.

Bettzieche, A., Brandsch, C., Weiße, K., Hirche, F., Eder, K., & Stangl, G. I. (2008). Lupin protein influences the expression of hepatic genes involved in fatty acid synthesis and triacylglycerol hydrolysis of adult rats. *British Journal of Nutrition*, *99*(05), 952–962.

Bhat, G., & Baba, C. S. (2013). Insulin resistance and metabolic syndrome in nonobese Indian patients with non-alcoholic fatty liver disease. *Tropical Gastrology*, *34*(1), 18–24.

Bhutia, D. T. (2014). Protein Energy Malnutrition in India: The Plight of Our Under Five Children. *Journal of Family Medicine and Primary Care*, *3*(1), 63–67. doi:10.4103/2249-4863.130279

Boor, P., Celec, P., Behuliak, M., Grančič, P., Kebis, A., Kukan, M., Šebeková, K. (2009). Regular moderate exercise reduces advanced glycation and ameliorates early diabetic nephropathy in obese Zucker rats. *Metabolism*, *58*(11), 1669–1677.

Boschin, G., Scigliuolo, G. M., Resta, D., & Arnoldi, A. (2014). Optimization of the Enzymatic Hydrolysis of Lupin (Lupinus) Proteins for Producing ACE-Inhibitory Peptides. *Journal of Agricultural and Food Chemistry*, *62*(8), 1846–1851.

Bouhnik, Y., Attar, A., Joly, F. A., Riottot, M., Dyard, F., & Flourié, B. (2004). Lactulose ingestion increases faecal bifidobacterial counts: a randomised double-blind study in healthy humans. *European Journal of Clinical Nutrition*, *58*(3), 462–466.

Brunt, E. M., & Tiniakos, D. G. (2010). Histopathology of nonalcoholic fatty liver disease. *World Journal of Gastroenterology : WJG*, *16*(42), 5286–5296. doi:10.3748/wjg.v16.i42.5286

Bugianesi, E. (2007). Non-alcoholic steatohepatitis and cancer. *Clinics in Liver Disease*, *11*(1), 191–207, x–xi.

Butt, M. S., & Batool, R. (2010). Nutritional and functional properties of some promising legumes protein isolates. *Pakistan Journal of Nutrition*, *9*(4), 373–379.

Caballería, L., Arteaga, I., Pera, G., Rodríguez, L., Alumà, A., Auladell, M. A., & Torán, P. (2013). [Risk factors associated with non-alcoholic fatty liver disease: a case-control study]. *Medicina Clínica*, *141*(6), 233–239.

Caballería, L., Pera, G., Rodríguez, L., Auladell, M. A., Bernad, J., Canut, S., & Torán, P. (2012). Metabolic syndrome and nonalcoholic fatty liver disease in a Spanish population: influence of the diagnostic criteria used. *European Journal of Gastroenterology & Hepatology*, *24*(9), 1007–1011.

Calder, P. C. (2013). Feeding the immune system. *Proceedings of the Nutrition Society*, *72*(03), 299–309. doi:10.1017/S0029665113001286

Cameron, A. J., Shaw, J. E., & Zimmet, P. Z. (2004). The metabolic syndrome: prevalence in worldwide populations. *Endocrinology and Metabolism Clinics of North America*, *33*(2), 351–375.

Cameron, I., Alam, M. A., Wang, J., & Brown, L. (2012). Endurance exercise in a rat model of metabolic syndrome. *Canadian Journal of Physiology and Pharmacology*, *90*(11), 1490–1497.

Cao, D., Li, H., Yi, J., Zhang, J., Che, H., Cao, J., … Jiang, W. (2011). Antioxidant Properties of the Mung Bean Flavonoids on Alleviating Heat Stress. *PLoS ONE*, *6*(6), e21071.

Capraro, J., Magni, C., Faoro, F., Maffi, D., Scarafoni, A., Tedeschi, G., … Duranti, M. (2013). Internalisation and multiple phosphorylation of γ-Conglutin, the lupin seed glycaemia-lowering protein, in HepG2 cells. *Biochemical and Biophysical Research Communications*, *437*(4), 648–652.

Capraro, J., Magni, C., Scarafoni, A., Caramanico, R., Rossi, F., Morlacchini, M., & Duranti, M. (2014). Pasta supplemented with isolated lupin protein fractions reduces body weight gain and food intake of rats and decreases plasma glucose concentration upon glucose overload trial. *Food & Function*, *5*(2), 375–380.

Carazo, A., León, J., Casado, J., Gila, A., Delgado, S., Martín, A., … Salmerón, J. (2011). Hepatic expression of adiponectin receptors increases with non-alcoholic fatty liver disease progression in morbid obesity in correlation with glutathione peroxidase 1. *Obesity Surgery*, *21*(4), 492–500.

Cerezal Mezquita, P., Urtuvia Gatica, V., Ramírez Quintanilla, V., & Arcos Zavala, R. (2011). [Product development on the basis of cereal and leguminous flours to coeliac disease in children aged 6-24 months; II: properties of the mixtures]. *Nutrición Hospitalaria*, *26*(1), 161–169.

Chang, S.-P., Chen, Y.-H., Chang, W.-C., Liu, I.-M., & Cheng, J.-T. (2004). Increase of Anti-Oxidation by Exercise in the Liver of Obese Zucker Rats. *Clinical and Experimental Pharmacology and Physiology*, *31*(8), 506–511.

Chavez-Santoscoy, R. A., Gutierrez-Uribe, J. A., Granados, O., Torre-Villalvazo, I., Serna-Saldivar, S. O., Torres, N., … Tovar, A. R. (2014). Flavonoids and saponins extracted from black bean (Phaseolus vulgaris L.) seed coats modulate lipid metabolism and biliary cholesterol secretion in C57BL/6 mice. *The British Journal of Nutrition*, *112*(6), 886–899.

Chawla, A., Repa, J. J., Evans, R. M., & Mangelsdorf, D. J. (2001). Nuclear Receptors and Lipid Physiology: Opening the X-Files. *Science*, *294*(5548), 1866–1870.

Cherng, J.-M., Chiang, W., & Chiang, L.-C. (2007). Immunomodulatory activities of edible beans and related constituents from soybean. *Food Chemistry*, *104*(2), 613–618.

Chobanian, A. V., Bakris, G. L., Black, H. R., Cushman, W. C., Green, L. A., Izzo, J. L., … National High Blood Pressure Education Program Coordinating Committee. (2003). Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension*, *42*(6), 1206–1252.

Choi, J. S., Koh, I.-U., & Song, J. (2012). Genistein reduced insulin resistance index through modulating lipid metabolism in ovariectomized rats. *Nutrition Research*, *32*(11), 844–855.

Choi, J.-Y., Jeon, J.-E., Jang, S.-Y., Jeong, Y.-J., Jeon, S.-M., Park, H.-J., & Choi, M.-S. (2011). Differential effects of powdered whole soy milk and its hydrolysate on antiobesity and antihyperlipidemic response to high-fat treatment in C57BL/6N mice. *Journal of Agricultural and Food Chemistry*, *59*(6), 2584–2591.

Chung, I.-M., Yeo, M.-A., Kim, S.-J., & Moon, H.-I. (2011). Protective effects of organic solvent fractions from the seeds of Vigna radiata L. wilczek against antioxidant mechanisms. *Human & Experimental Toxicology*, *30*(8), 904–909.

Clemente, V. J. C., Martín, S., Porres, J., Fuentes, S., & Aranda, P. (2011). Efecto de la suplemenación de vitamina en el rendimiento de una prueba incremental de consumo máximo de oxígeno en ratas wistar. *Archivos de Medicina Del Deporte: Revista de La Federación Española de Medicina Del Deporte Y de La Confederación Iberoamericana de Medicina Del Deporte*, (143), 168–173.

Council, E. U. (2010). DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union*, (276), 33–79.

Dahiya, P. K., Linnemann, A. R., S van Boekel, M. A. J., Khetarpaul, N., Grewal, R. B., & Nout, M. J. R. (2013). Mung bean: technological and nutritional potential. *Critical Reviews in Food Science and Nutrition*, 55(5), 670-688.

Dai, F.-J., Hsu, W.-H., Huang, J.-J., & Wu, S.-C. (2013). Effect of pigeon pea (Cajanus cajan L.) on high-fat diet-induced hypercholesterolemia in hamsters. *Food and Chemical Toxicology*, *53*, 384–391.

Daussin, F. N., Rasseneur, L., Bouitbir, J., Charles, A.-L., Dufour, S. P., Geny, B., … Richard, R. (2012). Different timing of changes in mitochondrial functions following endurance training. *Medicine and Science in Sports and Exercise*, *44*(2), 217–224.

Deen, D. (2004). Metabolic syndrome: time for action. *American Family Physician*, *69*(12), 2875–2882.

Dénarié, J., Debellé, F., & Promé, J.-C. (1996). Rhizobium Lipo-Chitooligosaccharide Nodulation Factors: Signaling Molecules Mediating Recognition and Morphogenesis. *Annual Review of Biochemistry*, *65*(1), 503–535.

Deneke, S. M., & Fanburg, B. L. (1989). Regulation of cellular glutathione. *The American Journal of Physiology*, *257*(4 Pt 1), L163–173.

Desai, S., Baker, S. S., Liu, W., Moya, D. A., Browne, R. W., Mastrandrea, L., Zhu, L. (2014). Paraoxonase 1 and oxidative stress in paediatric non-alcoholic steatohepatitis. *Liver International: Official Journal of the International Association for the Study of the Liver*, *34*(1), 110–117.

Desroches, S., & Lamarche, B. (2007). The evolving definitions and increasing prevalence of the metabolic syndrome. *Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquée, Nutrition Et Métabolisme*, *32*(1), 23–32.

Dewanto, V., Wu, X., Adom, K. K., & Liu, R. H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry*, *50*(10), 3010–4.

Dikdan, G. S., Saba, S. C., Dela Torre, A. N., Roth, J., Wang, S., & Koneru, B. (2004). Role of oxidative stress in the increased activation of signal transducers and activators of transcription-3 in the fatty livers of obese Zucker rats. *Surgery*, *136*(3), 677–685.

Doblado, R., Frías, J., & Vidal-Valverde, C. (2007). Changes in vitamin C content and antioxidant capacity of raw and germinated cowpea (Vigna sinensis var. carilla) seeds induced by high pressure treatment. *Food Chemistry*, *101*(3), 918–923.

Duranti, M. (2006). Grain legume proteins and nutraceutical properties. *Fitoterapia*, *77*(2), 67–82.

Duranti, M., Consonni, A., Magni, C., Sessa, F., & Scarafoni, A. (2008). The major proteins of lupin seed: Characterisation and molecular properties for use as functional and nutraceutical ingredients. *Trends in Food Science & Technology*, *19*(12), 624–633.

Eckel, R. H., Grundy, S. M., & Zimmet, P. Z. (2005). The metabolic syndrome. *The Lancet*, *365*(9468), 1415–1428.

Einhorn, D. (2003). American College of Endocrinology position statement on the insulin resistance syndrome. *Endocrine Practice*, *9*, 5–21.

El-Adawy, T. A., Rahma, E. H., El-Bedawey, A. A., & El-Beltagy, A. E. (2003). Nutritional potential and functional properties of germinated mung bean, pea and lentil seeds. *Plant Foods for Human Nutrition*, *58*(3), 1–13.

El-Adawy, T. A., Rahma, E. H., El-Bedawey, A. A., & Gafar, A. (2001). Nutritional potential and functional properties of sweet and bitter lupin seed protein isolates. *Food Chemistry*, *74*(4), 455–462.

El-Karaksy, H., Helmy, H., Anwar, G., El-Hennawy, A., El-Koofy, N., El-Raziky, M., Hassani, F. (2012). The association of metabolic syndrome, insulin resistance and non-alcoholic fatty liver disease in overweight/obese children. *Saudi Journal of Gastroenterology*, *18*(1), 44.

Erbaş, M., Certel, M., & Uslu, M. K. (2005). Some chemical properties of white lupin seeds (Lupinus albus L.). *Food Chemistry*, *89*(3), 341–345.

Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. (2001). Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA*, *285*(19), 2486–2497.

Fan, J.-G., & Farrell, G. C. (2009). Epidemiology of non-alcoholic fatty liver disease in China. *Journal of Hepatology*, *50*(1), 204–210.

Fan, J. G., Jia, J. D., Li, Y. M., Wang, B. Y., Lu, L. G., Shi, J. P., Chinese Association for the Study of Liver Disease. (2011). Guidelines for the diagnosis and management of nonalcoholic fatty liver disease: Update 2010. *Journal of Digestive Diseases*, *12*(1), 38–44.

Fan, X. H., Tang, C., & Rengel, Z. (2002). Nitrate uptake, nitrate reductase distribution and their relation to proton release in five nodulated grain legumes. *Annals of Botany*, *90*(3), 315–323.

Farrell, G. C., & Larter, C. Z. (2006). Nonalcoholic fatty liver disease: From steatosis to cirrhosis. *Hepatology*, *43*(S1), S99–S112.

Favier, J.-C., Ireland-Ripert, J., Toque, C., & Feinberg, M. (1995). *Répertoire général des aliments : table de composition = Composition tables*. Paris: Technique & Documentation. Retrieved from http://www.documentation.ird.fr/hor/fdi:010009113

Fealy, C. E., Haus, J. M., Solomon, T. P. J., Pagadala, M., Flask, C. A., McCullough, A. J., & Kirwan, J. P. (2012). Short-term exercise reduces markers of hepatocyte apoptosis in nonalcoholic fatty liver disease. *Journal of Applied Physiology*, *113*(1), 1–6.

Fernandez-Orozco, R., Frias, J., Zielinski, H., Piskula, M. K., Kozlowska, H., & Vidal-Valverde, C. (2008). Kinetic study of the antioxidant compounds and antioxidant

capacity during germination of Vigna radiata cv. emmerald, Glycine max cv. jutro and Glycine max cv. merit. *Food Chemistry*, *111*(3), 622–630.

Fiebig, R. G., Hollander, J. M., Ney, D., Boileau, R., Jeffery, E., & Ji, L. L. (2002). Training down-regulates fatty acid synthase and body fat in obese Zucker rats. *Medicine and Science in Sports and Exercise*, *34*(7), 1106–1114.

Finelli, C., & Tarantino, G. (2012). Have guidelines addressing physical activity been established in nonalcoholic fatty liver disease? *World Journal of Gastroenterology : WJG*, *18*(46), 6790–6800.

Florentino, G. S. de A., Cotrim, H. P., Vilar, C. P., Florentino, A. V. de A., Guimaraes, G. M. A., Barreto, V. S. T., Barreto, V. S. T. (2013). Non alcoholic fatty liver disease in menopausal women. *Archivos de Gastroenterologia*, *50*(3), 180–185.

Folch, J., Lees, M., Sloane-Stanley, G. H., & others. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*, *226*(1), 497–509.

Folino, M., McIntyre, A., & Young, G. P. (1995). Dietary fibers differ in their effects on large bowel epithelial proliferation and fecal fermentation-dependent events in rats. *The Journal of Nutrition*, *125*(6), 1521–1528.

Fontanari, G. G., Batistuti, J. P., Cruz, R. J. da, Saldiva, P. H. N., & Arêas, J. A. G. (2012). Cholesterol-lowering effect of whole lupin (Lupinus albus) seed and its protein isolate. *Food Chemistry*, *132*(3), 1521–1526.

Ford ES, Giles WH, & Dietz WH. (2002). Prevalence of the metabolic syndrome among us adults: Findings from the third national health and nutrition examination survey. *JAMA*, *287*(3), 356–359.

Francis, G., Kerem, Z., Makkar, H. P. S., & Becker, K. (2002). The biological action of saponins in animal systems: a review. *The British Journal of Nutrition*, *88*(6), 587–605.

Galisteo, M., Duarte, J., & Zarzuelo, A. (2008). Effects of dietary fibers on disturbances clustered in the metabolic syndrome. *The Journal of Nutritional Biochemistry*, *19*(2), 71–84.

Galisteo, M., Morón, R., Rivera, L., Romero, R., Anguera, A., & Zarzuelo, A. (2010). Plantago ovata husks-supplemented diet ameliorates metabolic alterations in obese Zucker rats through activation of AMP-activated protein kinase. Comparative study with other dietary fibers. *Clinical Nutrition*, *29*(2), 261–267.

Gao, L., Liu, Q., Liu, S., Chen, Z., Li, C., Lei, L., … Shen, Z. (2014). A refined-JinQi-JiangTang tablet ameliorates prediabetes by reducing insulin resistance and improving beta cell function in mice. *Journal of Ethnopharmacology*, *151*(1), 675–685.

Garcíacaballero, M., Reyes-Ortiz, A., Martínez-Moreno, J. M., & Toval-Mata, J. A. (2014). Metabolic disorders of liver and iron in diabetic and non-diabetic patients BMI < 35 or > 35 before gastric bypass. *Nutrición Hospitalaria*, *29*(5), 1088–1094.

Gerber, L., Otgonsuren, M., Mishra, A., Escheik, C., Birerdinc, A., Stepanova, M., & Younossi, Z. M. (2012). Non-alcoholic fatty liver disease (NAFLD) is associated with low level of physical activity: a population-based study. *Alimentary Pharmacology & Therapeutics*, *36*(8), 772–781.

Ghavidel, R. A., & Prakash, J. (2007). The impact of germination and dehulling on nutrients, antinutrients, in vitro iron and calcium bioavailability and in vitro starch and protein digestibility of some legume seeds. *LWT - Food Science and Technology*, *40*(7), 1292–1299.

Gianotti, G., Cenni, A., Bianchi, G., Masetti, M., Zappoli, P., Muscari, A., & Zoli, M. (2014). Diastolic dysfunction and cardiovascular risk in old subjects: Possible association with NAFLD? *Archives of Gerontology and Geriatrics*, *58*(2), 188–195.

Gilani, G. S., Cockell, K. A., & Sepehr, E. (2005). Effects of Antinutritional Factors on Protein Digestibility and Amino Acid Availability in Foods. *Journal of AOAC International*, *88*(3), 967–987.

Golbidi, S., Mesdaghinia, A., & Laher, I. (2012). Exercise in the Metabolic Syndrome. *Oxidative Medicine and Cellular Longevity*, *2012*, e349710.

Gombet, T., Longo-Mbenza, B., Ellenga-Mbolla, B., Ikama, M. S., Mokondjimobe, E., Kimbally-Kaky, G., & Nkoua, J.-L. (2012). Aging, female sex, migration, elevated HDL-C, and inflammation are associated with prevalence of metabolic syndrome among African bank employees. *International Journal of General Medicine*, *5*, 495–503.

Gonçalves, I. O., Passos, E., Rocha-Rodrigues, S., Diogo, C. V., Torrella, J. R., Rizo, D., … Magalhães, J. (2014). Physical exercise prevents and mitigates non-alcoholic steatohepatitis-induced liver mitochondrial structural and bioenergetics impairments. *Mitochondrion*, *15*, 40–51.

Graham, P. H., & Vance, C. P. (2003). Legumes: Importance and Constraints to Greater Use. *Plant Physiology*, *131*(3), 872–877.

Grundy, S. M. (2005). Metabolic Syndrome Scientific Statement by the American Heart Association and the National Heart, Lung, and Blood Institute. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *25*(11), 2243–2244.

Gudbrandsen, O. A., Wergedahl, H., Liaset, B., Espe, M., & Berge, R. K. (2005). Dietary proteins with high isoflavone content or low methionine-glycine and lysine-arginine ratios are hypocholesterolaemic and lower the plasma homocysteine level in male Zucker fa/fa rats. *The British Journal of Nutrition*, *94*(3), 321–330.

Gujral, H. S., Angurala, M., Sharma, P., & Singh, J. (2011). Phenolic Content and Antioxidant Activity of Germinated and Cooked Pulses. *International Journal of Food Properties*, *14*(6), 1366–1374.

Guo, X., Li, T., Tang, K., & Liu, R. H. (2012). Effect of Germination on Phytochemical Profiles and Antioxidant Activity of Mung Bean Sprouts (Vigna radiata). *Journal of Agricultural and Food Chemistry*, *60*(44), 11050–11055.

Guo, Z., Ran, Q., Roberts, L. J., Zhou, L., Richardson, A., Sharan, C., Yang, H. (2008). Suppression of atherogenesis by overexpression of glutathione peroxidase-4 in apolipoprotein E-deficient mice. *Free Radical Biology & Medicine*, *44*(3), 343–352.

Hallsworth, K., Fattakhova, G., Hollingsworth, K. G., Thoma, C., Moore, S., Taylor, R., Trenell, M. I. (2011). Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. *Gut*, 60(9), 1278-80.

Haram, P. M., Kemi, O. J., Lee, S. J., Bendheim, M. ?., Al-Share, Q. Y., Waldum, H. L., … Wisl?ff, U. (2009). Aerobic interval training vs. continuous moderate exercise in the metabolic syndrome of rats artificially selected for low aerobic capacity. *Cardiovascular Research*, *81*(4), 723–732.

Hashimoto, T., Cook, W. S., Qi, C., Yeldandi, A. V., Reddy, J. K., & Rao, M. S. (2000). Defect in peroxisome proliferator-activated receptor alpha-inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. *The Journal of Biological Chemistry*, *275*(37), 28918–28928.

Hattar, L. N., Wilson, T. A., Tabotabo, L. A., Smith, E. O., & Abrams, S. H. (2011). Physical activity and nutrition attitudes in obese Hispanic children with non-alcoholic steatohepatitis. *World Journal of Gastroenterology: WJG*, *17*(39), 4396–4403.

Haus, J. M., Solomon, T. P. J., Kelly, K. R., Fealy, C. E., Kullman, E. L., Scelsi, A. R., Kirwan, J. P. (2013). Improved hepatic lipid composition following short-term exercise in nonalcoholic fatty liver disease. *The Journal of Clinical Endocrinology and Metabolism*, *98*(7), E1181–1188.

Henningsson, A. M., Nyman, E. M., & Björck, I. M. (2001). Content of short-chain fatty acids in the hindgut of rats fed processed bean (Phaseolus vulgaris) flours varying in distribution and content of indigestible carbohydrates. *The British Journal of Nutrition*, *86*(3), 379–389.

Hermsdorff, H. H. M., Zulet, M. Á., Abete, I., & Martínez, J. A. (2011). A legume-based hypocaloric diet reduces proinflammatory status and improves metabolic features in overweight/obese subjects. *European Journal of Nutrition*, *50*(1), 61–69.

Hey-Mogensen, M., Jeppesen, J., Madsen, K., Kiens, B., & Franch, J. (2012). Obesity augments the age-induced increase in mitochondrial capacity for H2O2 release in Zucker fatty rats. *Acta Physiologica*, *204*(3), 354–361.

Hillgartner, F. B., Salati, L. M., & Goodridge, A. G. (1995). Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis. *Physiological Reviews*, *75*(1), 47–76.

Honeycutt, C. W. (1998). Crop rotation impacts on potato protein. *Plant Foods for Human Nutrition*, *52*(4), 279–291.

Howard, A., & Udenigwe, C. C. (2013). Mechanisms and prospects of food protein hydrolysates and peptide-induced hypolipidaemia. *Food & Function*, *4*(1), 40–51.

Hsu, G.-S. W., Lu, Y.-F., Chang, S.-H., & Hsu, S.-Y. (2011). Antihypertensive Effect of Mung Bean Sprout Extracts in Spontaneously Hypertensive Rats. *Journal of Food Biochemistry*, *35*(1), 278–288.

Huang, X., Cai, W., & Xu, B. (2014). Kinetic changes of nutrients and antioxidant capacities of germinated soybean (Glycine max L.) and mung bean (Vigna radiata L.) with germination time. *Food Chemistry*, *143*, 268–276.

Hwang, J. H., Kam, S., Shin, J., Kim, J.-Y., Lee, K.-E., Kwon, G.-H., … Hwang, T.-Y. (2013). Incidence of metabolic syndrome and relative importance of five components as a predictor of metabolic syndrome: 5-year follow-up study in Korea. *Journal of Korean Medical Science*, *28*(12), 1768–1773.

Ikeuchi, M., Yamaguchi, K., Koyama, T., Sono, Y., & Yazawa, K. (2006). Effects of fenugreek seeds (Trigonella foenum greaecum) extract on endurance capacity in mice. *Journal of Nutritional Science and Vitaminology*, *52*(4), 287–292.

Imai, H., & Nakagawa, Y. (2003). Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. *Free Radical Biology & Medicine*, *34*(2), 145–169.

Indulekha, K., Surendar, J., & Mohan, V. (2011). High sensitivity C-reactive protein, tumor necrosis factor-α, interleukin-6, and vascular cell adhesion molecule-1 levels in Asian Indians with metabolic syndrome and insulin resistance (CURES-105). *Journal of Diabetes Science and Technology*, *5*(4), 982–988.

Iskusnykh, I. Y., Popova, T. N., Agarkov, A. A., Pinheiro de Carvalho, M.A, A, & Rjevskiy, S. G. (2013). Expression of Glutathione Peroxidase and Glutathione Reductase and Level of Free Radical Processes under Toxic Hepatitis in Rats. *Journal of Toxicology*, *2013,* Article ID 870628, 9 pages.

Jenkins, N. T., Witkowski, S., Spangenburg, E. E., & Hagberg, J. M. (2009). Effects of acute and chronic endurance exercise on intracellular nitric oxide in putative endothelial progenitor cells: role of NAPDH oxidase. *American Journal of Physiology. Heart and Circulatory Physiology*, *297*(5), H1798–1805.

Jin, Y.-J., Kim, K. M., Hwang, S., Lee, S. G., Ha, T.-Y., Song, G.-W., … Suh, D.-J. (2012). Exercise and diet modification in non-obese non-alcoholic fatty liver disease: Analysis of biopsies of living liver donors. *Journal of Gastroenterology and Hepatology*, *27*(8), 1341–1347.

Johnson, N. A., Sachinwalla, T., Walton, D. W., Smith, K., Armstrong, A., Thompson, M. W., & George, J. (2009). Aerobic exercise training reduces hepatic and visceral lipids in obese individuals without weight loss. *Hepatology*, *50*(4), 1105–1112.

Kanatt, S. R., Arjun, K., & Sharma, A. (2011). Antioxidant and antimicrobial activity of legume hulls. *Food Research International*, *44*(10), 3182–3187.

Kaplan, N. M. (1989). The deadly quartet. Upper-body obesity, glucose intolerance, hypertriglyceridemia, and hypertension. *Archives of Internal Medicine*, *149*(7), 1514–1520.

Kapravelou, G., Martínez, R., Andrade, A. M., Sánchez, C., Chaves, C. L., López-Jurado, M., Porres, J. M. (2013). Health promoting effects of Lupin (Lupinus albus var. multolupa)

protein hydrolyzate and insoluble fiber in a diet-induced animal experimental model of hypercholesterolemia. *Food Research International*, *54*(2), 1471–1481.

Kaur, J. (2014). A Comprehensive Review on Metabolic Syndrome. *Cardiology Research and Practice*, *2014*, Article ID 943162, 21 pages.

Kelly, C. T., Mansoor, J., Dohm, G. L., Chapman, W. H. H., Pender, J. R., & Pories, W. J. (2014). Hyperinsulinemic syndrome: the metabolic syndrome is broader than you think. *Surgery*, *156*(2), 405–411.

Khare, M., Mohanty, C., Das, B. K., Jyoti, A., Mukhopadhyay, B., & Mishra, S. P. (2014). Free Radicals and Antioxidant Status in Protein Energy Malnutrition. *International Journal of Pediatrics*, *2014*, Article ID 254396, 6 pages.

Kibenge, M. T., & Chan, C. B. (2002). The effects of high-fat diet on exercise-induced changes in metabolic parameters in Zucker fa/fa rats. *Metabolism*, *51*(6), 708–715.

Kim, C.-W., Chang, Y., Sung, E., Shin, H., & Ryu, S. (2012). Serum ferritin levels predict incident non-alcoholic fatty liver disease in healthy Korean men. *Metabolism*, *61*(8), 1182–1188.

Kim, D., Kim, W. R., Kim, H. J., & Therneau, T. M. (2013). Association between noninvasive fibrosis markers and mortality among adults with nonalcoholic fatty liver disease in the United States. *Hepatology*, *57*(4), 1357–1365.

Kim, D.-K., Jeong, S. C., Gorinstein, S., & Chon, S.-U. (2012). Total Polyphenols, Antioxidant and Antiproliferative Activities of Different Extracts in Mungbean Seeds and Sprouts. *Plant Foods for Human Nutrition*, *67*(1), 71–75.

Kim, J., Tanabe, K., Yokoyama, N., Zempo, H., & Kuno, S. (2011). Association between physical activity and metabolic syndrome in middle-aged Japanese: a cross-sectional study. *BMC Public Health*, *11*(1), 624.

Kolovou, G. D., Anagnostopoulou, K. K., Salpea, K. D., & Mikhailidis, D. P. (2007). The Prevalence of Metabolic Syndrome in Various Populations: *The American Journal of the Medical Sciences*, *333*(6), 362–371.

König, D., Muser, K., Berg, A., & Deibert, P. (2012). Fuel selection and appetite-regulating hormones after intake of a soy protein-based meal replacement. *Nutrition*, *28*(1), 35–39.

Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M. G., & Wahli, W. (1997). Fatty Acids, Eicosanoids, and Hypolipidemic Agents Identified as Ligands of Peroxisome Proliferator-Activated Receptors by Coactivator-Dependent Receptor Ligand Assay. *Molecular Endocrinology*, *11*(6), 779–791.

Kruawan, K., Tongyonk, L., & Kangsadalampai, K. (2012). Antimutagenic and co-mutagenic activities of some legume seeds and their seed coats. *J Med Plants Res*, *6*(22), 3845–3851.

Lambert, J. E., Ramos–Roman, M. A., Browning, J. D., & Parks, E. J. (2014). Increased De Novo Lipogenesis Is a Distinct Characteristic of Individuals With Nonalcoholic Fatty Liver Disease. *Gastroenterology*, *146*(3), 726–735.

Laos, S., Caimari, A., Crescenti, A., Lakkis, J., Puiggròs, F., Arola, L., & del Bas, J. M. (2014). Long-term intake of soyabean phytosterols lowers serum TAG and NEFA concentrations, increases bile acid synthesis and protects against fatty liver development in dyslipidaemic hamsters. *The British Journal of Nutrition*, *112*(5), 663–673.

Larsen, S., Danielsen, J. H., Sønderg\a ard, S. D., Søgaard, D., Vigelsoe, A., Dybboe, R., … Helge, J. W. (2014). The effect of high-intensity training on mitochondrial fat oxidation in skeletal muscle and subcutaneous adipose tissue. *Scandinavian Journal of Medicine & Science in Sports*. doi/10.1111/sms.12252/full

Lavoie, J.-M., & Gauthier, M.-S. (2006). Regulation of fat metabolism in the liver: link to non-alcoholic hepatic steatosis and impact of physical exercise. *Cellular and Molecular Life Sciences CMLS*, *63*(12), 1393–1409.

Lawrence, R., Sunde, R., Schwartz, G., & Hoekstra, W. (1974). Glutathione peroxidase activity in rat lens and other tissues in relation to dietary selenium intake, *18*, 563–569.

Lee, C.-H., Yoon, S.-J., & Lee, S.-M. (2012). Chlorogenic acid attenuates high mobility group Box 1 (HMGB1) and enhances host defense mechanisms in murine sepsis. *Molecular Medicine*, *18*(1), 1437.

Lee, S., Bacha, F., Hannon, T., Kuk, J. L., Boesch, C., & Arslanian, S. (2012). Effects of aerobic versus resistance exercise without caloric restriction on abdominal fat, intrahepatic lipid, and insulin sensitivity in obese adolescent boys: a randomized, controlled trial. *Diabetes*, *61*(11), 2787–2795.

Lee, S.-J., Lee, J. H., Lee, H.-H., Lee, S., Kim, S. H., Chun, T., & Imm, J.-Y. (2011). Effect of mung bean ethanol extract on pro-inflammtory cytokines in LPS stimulated macrophages. *Food Science and Biotechnology*, *20*(2), 519–524. doi:10.1007/s10068-011-0072-z

Lee, Y.-J., Lee, H.-R., Lee, J.-H., Shin, Y.-H., & Shim, J.-Y. (2009). Association between serum uric acid and non-alcoholic fatty liver disease in Korean adults. *Clinical Chemistry and Laboratory Medicine*, *48*(2), 175–180.

Lee, Y. P., Mori, T. A., Puddey, I. B., Sipsas, S., Ackland, T. R., Beilin, L. J., & Hodgson, J. M. (2009). Effects of lupin kernel flour–enriched bread on blood pressure: a controlled intervention study. *The American Journal of Clinical Nutrition*, *89*(3), 766–772.

Leibowitz, M. D., Fiévet, C., Hennuyer, N., Peinado-Onsurbe, J., Duez, H., Berger, J., … Auwerx, J. (2000). Activation of PPARδ alters lipid metabolism in db/db mice. *FEBS Letters*, *473*(3), 333–336.

Li, G.-H., Wan, J.-Z., Le, G.-W., & Shi, Y.-H. (2006). Novel angiotensin I-converting enzyme inhibitory peptides isolated from Alcalase hydrolysate of mung bean protein. *Journal of Peptide Science*, *12*(8), 509–514.

Li, H., Prairie, N., Udenigwe, C. C., Adebiyi, A. P., Tappia, P. S., Aukema, H. M., … Aluko, R. E. (2011). Blood Pressure Lowering Effect of a Pea Protein Hydrolysate in Hypertensive Rats and Humans. *Journal of Agricultural and Food Chemistry*, *59*(18), 9854–9860.

Linden, M. A., Fletcher, J. A., Morris, E. M., Meers, G. M., Kearney, M. L., Crissey, J. M., … Rector, R. S. (2014). Combining metformin and aerobic exercise training in the treatment of type 2 diabetes and NAFLD in OLETF rats. *American Journal of Physiology - Endocrinology and Metabolism*, *306*(3), E300–E310.

Linnemann, A. R., & Dijkstra, D. S. (2002). Toward sustainable production of protein-rich foods: Appraisal of eight crops for Western Europe. Part I. Analysis of the primary links of the production chain. *Critical Reviews in Food Science and Nutrition*, *42*(4), 377–401.

Lott, J. N. A., Ockenden, I., Raboy, V., & Batten, G. D. (2000). Phytic acid and phosphorus in crop seeds and fruits: a global estimate. *Seed Science Research*, *10*(01), 11–33.

Lovati, M. R., Manzoni, C., Castiglioni, S., Parolari, A., Magni, C., & Duranti, M. (2012). Lupin seed γ-conglutin lowers blood glucose in hyperglycaemic rats and increases glucose consumption of HepG2 cells. *The British Journal of Nutrition*, *107*(1), 67–73.

Lowry, O. H., Rosebrough, N. J., Farr, L., Randall, R. J., & Randall, R. J. (1951). ARTICLE : PROTEIN MEASUREMENT WITH THE. *J.Biol. Chem.*, *193*, 265–275.

Machado, M. V., Coutinho, J., Carepa, F., Costa, A., Proença, H., & Cortez-Pinto, H. (2012). How adiponectin, leptin, and ghrelin orchestrate together and correlate with the severity of nonalcoholic fatty liver disease. *European Journal of Gastroenterology & Hepatology*, *24*(10), 1166–1172.

Malik, S. M., & Traub, M. L. (2012). Defining the role of bariatric surgery in polycystic ovarian syndrome patients. *World Journal of Diabetes*, *3*(4), 71–79. doi:10.4239/wjd.v3.i4.71

Manco, M., Bottazzo, G., DeVito, R., Marcellini, M., Mingrone, G., & Nobili, V. (2008). Nonalcoholic Fatty Liver Disease in Children. *Journal of the American College of Nutrition*, *27*(6), 667–676.

Marchesi, M., Parolini, C., Diani, E., Rigamonti, E., Cornelli, L., Arnoldi, A., … Chiesa, G. (2008). Hypolipidaemic and anti-atherosclerotic effects of lupin proteins in a rabbit model. *The British Journal of Nutrition*, 1–4.

Marchesini, G., Bugianesi, E., Forlani, G., Cerrelli, F., Lenzi, M., Manini, R., … others. (2003). Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology*, *37*(4), 917–923.

Martínez-Villaluenga, C., Frias, J., & Vidal-Valverde, C. (2008). Alpha-Galactosides: Antinutritional Factors or Functional Ingredients? *Critical Reviews in Food Science and Nutrition*, *48*(4), 301–316.

Martínez-Villaluenga, C., Urbano, G., Porres, J. M., Frias, J., & Vidal-Valverde, C. (2007). Improvement in food intake and nutritive utilization of protein from Lupinus albus var. multolupa protein isolates supplemented with ascorbic acid. *Food Chemistry*, *103*(3), 944–951.

Martínez-Villaluenga, C., Zieliński, H., Frias, J., Piskuła, M. K., Kozłowska, H., & Vidal-Valverde, C. (2009). Antioxidant capacity and polyphenolic content of high-protein lupin products. *Food Chemistry*, *112*(1), 84–88.

McCullogh, J. S., Ratcliffe, B., Mandir, N., Carr, K. E., & Goodlad, R. A. (1998). Dietary fibre and intestinal microflora: effects on intestinal morphometry and crypt branching. *Gut*, *42*(6), 799–806.

Megías, C., Pedroche, J., Yust, M. M., Girón-Calle, J., Alaiz, M., Millán, F., & Vioque, J. (2007a). Affinity Purification of Copper Chelating Peptides from Chickpea Protein Hydrolysates. *Journal of Agricultural and Food Chemistry*, *55*(10), 3949–3954.

Megías, C., Pedroche, J., Yust, M. M., Girón-Calle, J., Alaiz, M., Millán, F., & Vioque, J. (2007b). Affinity Purification of Copper Chelating Peptides from Chickpea Protein Hydrolysates. *Journal of Agricultural and Food Chemistry*, *55*(10), 3949–3954.

Meister, A., & Anderson, M. E. (1983). Glutathione. *Annual Review of Biochemistry*, *52*(1), 711–760.

Mello, A. S., Oliveira, D. C. de, Bizzarro, B., Sá-Nunes, A., Hastreiter, A. A., Beltran, J. S. de O., … Fock, R. A. (2014). Protein Malnutrition Alters Spleen Cell Proliferation and IL-2 and IL-10 Production by Affecting the STAT-1 and STAT-3 Balance. *Inflammation*, 1–14.

Metz, L., Vermaelen, M., Lambert, K., Broca, C., Sirvent, P., Raynaud, E., & Mercier, J. (2005). Endurance training increases lactate transport in male Zucker fa/fa rats. *Biochemical and Biophysical Research Communications*, *331*(4), 1338–1345.

Michael, J. P. (2003). Indolizidine and quinolizidine alkaloids. *Natural Product Reports*, *20*(5), 458–475.

Mohd Ali, N., Mohd Yusof, H., Long, K., Yeap, S. K., Ho, W. Y., Beh, B. K., … Alitheen, N. B. (2012). Antioxidant and Hepatoprotective Effect of Aqueous Extract of Germinated and Fermented Mung Bean on Ethanol-Mediated Liver Damage. *BioMed Research International*, *2013*, Article ID 693613, 9 pages.

Moon, H. Y., Song, P., Choi, C. S., Ryu, S. H., & Suh, P.-G. (2013). Involvement of exercise-induced macrophage migration inhibitory factor in the prevention of fatty liver disease. *The Journal of Endocrinology*, *218*(3), 339–348.

Morato, P. N., Lollo, P. C. B., Moura, C. S., Batista, T. M., Carneiro, E. M., & Amaya-Farfan, J. (2013). A dipeptide and an amino acid present in whey protein hydrolysate increase

translocation of GLUT-4 to the plasma membrane in Wistar rats. *Food Chemistry*, *139*(1-4), 853–859.

Mori, T., Kondo, H., Hase, T., & Murase, T. (2011). Dietary phospholipids ameliorate fructose-induced hepatic lipid and metabolic abnormalities in rats. *The Journal of Nutrition*, *141*(11), 2003–2009.

Morris, E. M., Jackman, M. R., Johnson, G. C., Liu, T.-W., Lopez, J. L., Kearney, M. L., … Thyfault, J. P. (2014). Intrinsic aerobic capacity impacts susceptibility to acute high-fat diet-induced hepatic steatosis. *American Journal of Physiology - Endocrinology and Metabolism*, *307*(4), E355–E364.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63.

Mubarak, A. E. (2005). Nutritional composition and antinutritional factors of mung bean seeds (Phaseolus aureus) as affected by some home traditional processes. *Food Chemistry*, *89*(4), 489–495.

Murakami, T., Siripin, S., Wadisirisuk, P., Boonkerd, N., Yoneyama, T., Yokoyama, T., and. (1991). The nitrogen fixing ability of mung bean (Vigna radiata) in Proceedings of the mungbean meeting, 187–198.

Nath, N., Wiener, R., Harper, A. E., & Elvehjem, C. A. (1959). Diet and Cholesteremia I. Development of a Diet for the Study of Nutritional Factors Affecting Cholesteremia in the Rat. *The Journal of Nutrition*, *67*(2), 289–307.

Neuschwander-Tetri, B. A., Brunt, E. M., Wehmeier, K. R., Oliver, D., & Bacon, B. R. (2003). Improved nonalcoholic steatohepatitis after 48 weeks of treatment with the PPAR-gamma ligand rosiglitazone. *Hepatology (Baltimore, Md.)*, *38*(4), 1008–1017.

Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, 1995. (n.d.-a). Retrieved from /catalog/4758/nutrient-requirements-of-laboratory-animals-fourth-revised-edition-1995

Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, 1995.

Oakes, N. D., Camilleri, S., Furler, S. M., Chisholm, D. J., & Kraegen, E. W. (1997). The insulin sensitizer, BRL 49653, reduces systemic fatty acid supply and utilization and tissue lipid availability in the rat. *Metabolism*, *46*(8), 935–942.

Oboh, G., & Rocha, J. B. T. (2007). Polyphenols in red pepper [Capsicum annuum var. aviculare (Tepin)] and their protective effect on some pro-oxidants induced lipid peroxidation in brain and liver. *European Food Research and Technology*, *225*(2), 239–247.

Oh, H. J., Kim, T. H., Sohn, Y. W., Kim, Y. S., Oh, Y. R., Cho, E. Y., … Kim, H. C. (2011). Association of serum alanine aminotransferase and ?-glutamyltransferase levels within the reference range with metabolic syndrome and nonalcoholic fatty liver disease. *The Korean Journal of Hepatology*, *17*(1), 27–36.

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95*(2), 351–358.

Oh, S.-Y., Cho, Y.-K., Kang, M.-S., Yoo, T.-W., Park, J.-H., Kim, H.-J., … Shin, J.-H. (2006). The association between increased alanine aminotransferase activity and metabolic factors in nonalcoholic fatty liver disease. *Metabolism*, *55*(12), 1604–1609.

Oliver, W. R., Shenk, J. L., Snaith, M. R., Russell, C. S., Plunket, K. D., Bodkin, N. L., … Willson, T. M. (2001). A selective peroxisome proliferator-activated receptor δ agonist promotes reverse cholesterol transport. *Proceedings of the National Academy of Sciences*, *98*(9), 5306–5311.

Orchard, T. J., Temprosa, M., Goldberg, R., Haffner, S., Ratner, R., Marcovina, S., & Fowler, S. (2005). The Effect of Metformin and Intensive Lifestyle Intervention on the Metabolic Syndrome: The Diabetes Prevention Program Randomized Trial. *Annals of Internal Medicine*, *142*(8), 611–619.

Park Y, Zhu S, Palaniappan L, Heshka S, Carnethon MR, & Heymsfield SB. (2003). The metabolic syndrome: Prevalence and associated risk factor findings in the us

population from the third national health and nutrition examination survey, 1988-1994. *Archives of Internal Medicine*, *163*(4), 427–436.

Parolini, C., Rigamonti, E., Marchesi, M., Busnelli, M., Cinquanta, P., Manzini, S., … Chiesa, G. (2012). Cholesterol-lowering effect of dietary Lupinus angustifolius proteins in adult rats through regulation of genes involved in cholesterol homeostasis. *Food Chemistry*, *132*(3), 1475–1479.

Pereira, D. I. A., & Gibson, G. R. (2002). Effects of Consumption of Probiotics and Prebiotics on Serum Lipid Levels in Humans. *Critical Reviews in Biochemistry and Molecular Biology*, *37*(4), 259–281.

Pilvi, T. K., Jauhiainen, T., Cheng, Z. J., Mervaala, E. M., Vapaatalo, H., & Korpela, R. (2006). Lupin protein attenuates the development of Hypertension and normalises the vascular function of NaCl-loaded Goto-Kakizaki rats. *Journal of Physiology and Pharmacology*, *57*(2), 167–176.

Polyzos, S. A., Kountouras, J., & Zavos, C. (2009). Nonalcoholic Fatty Liver Disease: The Pathogenetic Roles of Insulin Resistance and Adipocytokines. *Current Molecular Medicine*, *9*(3), 299–314.

Ponholzer, A., Temml, C., Rauchenwald, M., Marszalek, M., & Madersbacher, S. (2007). Is the metabolic syndrome a risk factor for female sexual dysfunction in sexually active women? *International Journal of Impotence Research*, *20*(1), 100–104.

Porres, J. M., Aranda, P., López-Jurado, M., & Urbano, G. (2003). Effect of Natural and Controlled Fermentation on Chemical Composition and Nutrient Dialyzability from Beans (Phaseolus vulgaris L.). *Journal of Agricultural and Food Chemistry*, *51*(17), 5144–5149.

Porres, J. M., Aranda, P., López-Jurado, M., & Urbano, G. (2006). Nutritional evaluation of protein, phosphorus, calcium and magnesium bioavailability from lupin (Lupinus albus var. multolupa)-based diets in growing rats: effect of alpha-galactoside oligosaccharide

extraction and phytase supplementation. *The British Journal of Nutrition*, *95*(6), 1102–1111.

Postic, C., & Girard, J. (2008). The role of the lipogenic pathway in the development of hepatic steatosis. *Diabetes & Metabolism*, *34*(6, Part 2), 643–648.

Powell, E. E., Jonsson, J. R., & Clouston, A. D. (2005). Steatosis: Co-factor in other liver diseases. *Hepatology*, *42*(1), 5–13.

Prieto, P. G., Cancelas, J., Villanueva-Peñacarrillo, M. L., Valverde, I., & Malaisse, W. J. (2004). Plasma D-Glucose, D-Fructose and Insulin Responses after Oral Administration of D-Glucose, D-Fructose and Sucrose to Normal Rats. *Journal of the American College of Nutrition*, *23*(5), 414–419.

Prosky, L., Asp, N.-G., Schweizer, T. F., De Vries, J. W., & Furda, I. (1992). Determination of insoluble and soluble dietary fiber in foods and food products : collaborative study. In *Journal of AOAC International* , 75, 360–367.

Purcell, M., Flores, Y. N., Zhang, Z.-F., Denova-Gutiérrez, E., & Salmeron, J. (2013). Prevalence and predictors of alanine aminotransferase elevation among normal weight, overweight and obese youth in Mexico. *Journal of Digestive Diseases*, *14*(9), 491–499.

Puri, K., Nobili, V., Melville, K., Corte, C. D., Sartorelli, M. R., Lopez, R., … Alkhouri, N. (2013). Serum bilirubin level is inversely associated with nonalcoholic steatohepatitis in children. *Journal of Pediatric Gastroenterology and Nutrition*, *57*(1), 114–118.

Pyper, S. R., Viswakarma, N., Yu, S., & Reddy, J. K. (2010). PPAR?: energy combustion, hypolipidemia, inflammation and cancer. *Nuclear Receptor Signaling*, *8*.

Quang, T. H., Ngan, N. T. T., Minh, C. V., Kiem, P. V., Tai, B. H., Nhiem, N. X., … Kim, Y. H. (2013). Anti-Inflammatory and PPAR Transactivational Properties of Flavonoids from the Roots of Sophora flavescens. *Phytotherapy Research*, *27*(9), 1300–1307.

Radák, Z., Chung, H. Y., Naito, H., Takahashi, R., Jung, K. J., Kim, H.-J., & Goto, S. (2004). Age-associated increase in oxidative stress and nuclear factor κB activation are attenuated in rat liver by regular exercise. *The FASEB Journal*, *18*(6), 749–750.

Ramadan, G., El-Beih, N. M., & Abd El-Kareem, H. F. (2011). Anti-metabolic syndrome and immunostimulant activities of Egyptian fenugreek seeds in diabetic/obese and immunosuppressive rat models. *British Journal of Nutrition*, *105*(07), 995–1004.

Randhir, R., & Shetty, K. (2007). Mung beans processed by solid-state bioconversion improves phenolic content and functionality relevant for diabetes and ulcer management. *Innovative Food Science & Emerging Technologies*, *8*(2), 197–204.

Reaven, G. (2004). The metabolic syndrome or the insulin resistance syndrome? Different names, different concepts, and different goals. *Endocrinology and Metabolism Clinics of North America*, *33*(2), 283–303.

Reaven, G. M. (1988). Role of Insulin Resistance in Human Disease. *Diabetes*, *37*(12), 1595–1607.

Reeves, P. G., Nielsen, F. H., Fahey Jr, G. C., & others. (1993). AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr*, *123*(11), 1939–1951.

Rogers, A. S., Shaughnessy, K. K., & Davis, L. S. (2014). Dermatitis and dangerous diets: a case of kwashiorkor. *JAMA Dermatology*, *150*(8), 910–911.

Rolo, A. P., Teodoro, J. S., & Palmeira, C. M. (2012). Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radical Biology and Medicine*, *52*(1), 59–69.

Rosen, E. D., & Spiegelman, B. M. (2001). PPARγ: a Nuclear Regulator of Metabolism, Differentiation, and Cell Growth. *Journal of Biological Chemistry*, *276*(41), 37731–37734.

Rosety-Rodriguez, M., Rosety, I., Fornieles-Gonzalez, G., Diaz-Ordonez, A. J., Camacho, A., Rosety, M. A., … Ordonez, F. J. (2012). A 6-week training program increased muscle antioxidant system in elderly diabetic fatty rats. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, *18*(9), BR346–BR350.

Ruderman, N., & Prentki, M. (2004). AMP kinase and malonyl-CoA: targets for therapy of the metabolic syndrome. *Nature Reviews Drug Discovery*, *3*(4), 340–351.

Saad, F., & Gooren, L. J. (2011). The role of testosterone in the etiology and treatment of obesity, the metabolic syndrome, and diabetes mellitus type 2. *Journal of Obesity*, *2011*.

Sanal, M. G. (2008). The blind men "see" the elephant-the many faces of fatty liver disease. *World Journal of Gastroenterology : WJG*, *14*(6), 831–844.

Sanderson, L. M., Degenhardt, T., Koppen, A., Kalkhoven, E., Desvergne, B., Muller, M., & Kersten, S. (2009). Peroxisome Proliferator-Activated Receptor ?/? (PPAR?/?) but Not PPAR? Serves as a Plasma Free Fatty Acid Sensor in Liver. *Molecular and Cellular Biology*, *29*(23), 6257–6267.

Sasaki, T., Nakata, R., Inoue, H., Shimizu, M., Inoue, J., & Sato, R. (2014). Role of AMPK and PPARγ1 in exercise-induced lipoprotein lipase in skeletal muscle. *American Journal of Physiology. Endocrinology and Metabolism*, *306*(9), E1085–1092.

Scarafoni, A., Magni, C., & Duranti, M. (2007). Molecular nutraceutics as a mean to investigate the positive effects of legume seed proteins on human health. *Trends in Food Science and Technology*, *18*(9), 454–463.

Schultz, A., Mendonca, L. S., Aguila, M. B., & Mandarim-de-Lacerda, C. A. (2012). Swimming training beneficial effects in a mice model of nonalcoholic fatty liver disease. *Experimental and Toxicologic Pathology*, *64*(4), 273–282.

Segura Campos, M. R., Chel Guerrero, L. A., & Betancur Ancona, D. A. (2010). Angiotensin-I converting enzyme inhibitory and antioxidant activities of peptide fractions extracted

by ultrafiltration of cowpea Vigna unguiculata hydrolysates. *Journal of the Science of Food and Agriculture*, *90*(14), 2512–2518.

Shan, W., Nicol, C. J., Ito, S., Bility, M. T., Kennett, M. J., Ward, J. M., … Peters, J. M. (2008). Peroxisome proliferator-activated receptor-β/δ protects against chemically induced liver toxicity in mice. *Hepatology*, *47*(1), 225–235.

Shaw, J. E., & Chisholm, D. J. (2003). 1: Epidemiology and prevention of type 2 diabetes and the metabolic syndrome. *Medical Journal of Australia*, *179*(7), 379–383.

Shirouchi, B., Nagao, K., Furuya, K., Shiojiri, M., Liu, X., & Yanagita, T. (2010). Physiological Effects of Dietary PIPS Soybean-Derived Phospholipid in Obese Zucker (fa/fa) Rats. *Bioscience, Biotechnology, and Biochemistry*, *74*(11), 2333–2335.

Silva, L. R., Pereira, M. J., Azevedo, J., Gonçalves, R. F., Valentão, P., de Pinho, P. G., & Andrade, P. B. (2013). Glycine max (L.) Merr., Vigna radiata L. and Medicago sativa L. sprouts: A natural source of bioactive compounds. *Food Research International*, *50*(1), 167–175.

Singh, R. P., Chidambara Murthy, K. N., & Jayaprakasha, G. K. (2002). Studies on the Antioxidant Activity of Pomegranate (Punica granatum) Peel and Seed Extracts Using in Vitro Models. *Journal of Agricultural and Food Chemistry*, *50*(1), 81–86.

Sirtori, C. R., Lovati, M. R., Manzoni, C., Castiglioni, S., Duranti, M., Magni, C., … Arnoldi, A. (2004). Proteins of White Lupin Seed, a Naturally Isoflavone-Poor Legume, Reduce Cholesterolemia in Rats and Increase LDL Receptor Activity in HepG2 Cells. *The Journal of Nutrition*, *134*(1), 18–23.

Sirtori, C. R., Triolo, M., Bosisio, R., Bondioli, A., Calabresi, L., De Vergori, V., … Arnoldi, A. (2012). Hypocholesterolaemic effects of lupin protein and pea protein/fibre combinations in moderately hypercholesterolaemic individuals. *British Journal of Nutrition*, *107*(08), 1176–1183.

Smits, M. M., Ioannou, G. N., Boyko, E. J., & Utzschneider, K. M. (2013). Non-alcoholic fatty liver disease as an independent manifestation of the metabolic syndrome: Results of a US national survey in three ethnic groups. *Journal of Gastroenterology and Hepatology*, *28*(4), 664–670.

Soltys, K., Dikdan, G., & Koneru, B. (2001). Oxidative stress in fatty livers of obese Zucker rats: Rapid amelioration and improved tolerance to warm ischemia with tocopherol. *Hepatology*, *34*(1), 13–18.

Sompong, U., Somta, P., Raboy, V., & Srinives, P. (2012). Mapping of quantitative trait loci for phytic acid and phosphorus contents in seed and seedling of mungbean (Vigna radiata (L.) Wilczek). *Breeding Science*, *62*(1), 87–92.

Speliotes, E. K., Massaro, J. M., Hoffmann, U., Vasan, R. S., Meigs, J. B., Sahani, D. V., … Fox, C. S. (2010). Fatty liver is associated with dyslipidemia and dysglycemia independent of visceral fat: The Framingham heart study. *Hepatology*, *51*(6), 1979–1987.

Spielmann, J., Shukla, A., Brandsch, C., Hirche, F., Stangl, G. I., & Eder, K. (2007). Dietary lupin protein lowers triglyceride concentrations in liver and plasma in rats by reducing hepatic gene expression of sterol regulatory element-binding protein-1c. *Annals of Nutrition and Metabolism*, *51*(4), 387–392.

Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N., & Ames, B. N. (1987). Bilirubin is an antioxidant of possible physiological importance. *Science*, *235*(4792), 1043–1046.

Sujak, A., Kotlarz, A., & Strobel, W. (2006). Compositional and nutritional evaluation of several lupin seeds. *Food Chemistry*, *98*(4), 711–719.

Sullivan, S., Kirk, E. P., Mittendorfer, B., Patterson, B. W., & Klein, S. (2012). Randomized trial of exercise effect on intrahepatic triglyceride content and lipid kinetics in nonalcoholic fatty liver disease. *Hepatology (Baltimore, Md.)*, *55*(6), 1738–1745.

Suzuki, K., Taniguchi N, & Ookawara T. (1999). Superoxide dismutase (SOD), *57*, 449–502.

Szalman, K., Bancu, L., & Sin, A. (2013). Correlations between anthropometric and serologic elements of metabolic syndrome and histopathologic features of nonalcoholic fatty liver disease. *Romanian Journal of Morphology and Embryology = Revue Roumaine De Morphologie Et Embryologie*, *54*(1), 173–178.

Takekoshi, K., Fukuhara, M., Quin, Z., Nissato, S., Isobe, K., Kawakami, Y., & Ohmori, H. (2006). Long-term exercise stimulates adenosine monophosphate–activated protein kinase activity and subunit expression in rat visceral adipose tissue and liver. *Metabolism*, *55*(8), 1122–1128.

Tang, D., Dong, Y., Guo, N., Li, L., & Ren, H. (2014). Metabolomic analysis of the polyphenols in germinating mung beans (Vigna radiata) seeds and sprouts. *Journal of the Science of Food and Agriculture*, *94*(8), 1639–1647.

Tariq, Z., Green, C. J., & Hodson, L. (2014). Are oxidative stress mechanisms the common denominator in the progression from hepatic steatosis towards non-alcoholic steatohepatitis (NASH)? 34(7), 180-190.

The Evidence Report. (1998). *Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults*. National Heart, Lung, and Blood Institute.

The Korean Association for the Study of the Liver (KASL). (2013). KASL clinical practice guidelines: Management of nonalcoholic fatty liver disease. *Clinical and Molecular Hepatology*, *19*(4), 325.

Tjønna, A. E., Lee, S. J., Rognmo, Ø., Stølen, T. O., Bye, A., Haram, P. M., … Wisløff, U. (2008). Aerobic Interval Training Versus Continuous Moderate Exercise as a Treatment for the Metabolic Syndrome A Pilot Study. *Circulation*, *118*(4), 346–354.

Troszyńska, A., Estrella, I., López-Amóres, M. L., & Hernández, T. (2002). Antioxidant Activity of Pea (Pisum sativum L.) Seed Coat Acetone Extract. *LWT - Food Science and Technology*, *35*(2), 158–164.

Tzeng, T.-F., Lu, H.-J., Liou, S.-S., Chang, C. J., & Liu, I.-M. (2013). Cassia tora (Leguminosae) seed extract alleviates high-fat diet-induced nonalcoholic fatty liver. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *51*, 194–201.

Ukeda, H., Maeda, S., Ishii, T., & Sawamura, M. (1997). Spectrophotometric Assay for Superoxide Dismutase Based on Tetrazolium Salt 3'-{1-[(Phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonic Acid Hydrate Reduction by Xanthine–Xanthine Oxidase. *Analytical Biochemistry*, *251*(2), 206–209.

Urbano, G., Aranda, P., Gómez-Villalva, E., Frejnagel, S., Porres, J. M., Frías, J., … López-Jurado, M. (2003). Nutritional evaluation of pea (Pisum sativum L.) protein diets after mild hydrothermal treatment and with and without added phytase. *Journal of Agricultural and Food Chemistry*, *51*(8), 2415–2420.

Urbano, G., López-Jurado, M., Aranda, P., Vidal-Valverde, C., Tenorio, E., & Porres, J. (2000). The role of phytic acid in legumes: antinutrient or beneficial function? *Journal of Physiology and Biochemistry*, *56*(3), 283–294.

VanHoose, L., Sawers, Y., Loganathan, R., Vacek, J. L., Stehno-Bittel, L., Novikova, L., … Smirnova, I. V. (2010). Electrocardiographic changes with the onset of diabetes and the impact of aerobic exercise training in the Zucker Diabetic Fatty (ZDF) rat. *Cardiovasc Diabetol*, *9*(56), 20.

Van Rooyen, D. M., Gan, L. T., Yeh, M. M., Haigh, W. G., Larter, C. Z., Ioannou, G., … Farrell, G. C. (2013). Pharmacological cholesterol lowering reverses fibrotic NASH in obese, diabetic mice with metabolic syndrome. *Journal of Hepatology*, *59*(1), 144–152.

Van Rooyen, D. M., Larter, C. Z., Haigh, W. G., Yeh, M. M., Ioannou, G., Kuver, R., … Farrell, G. C. (2011). Hepatic Free Cholesterol Accumulates in Obese, Diabetic Mice and Causes Nonalcoholic Steatohepatitis. *Gastroenterology*, *141*(4), 1393–1403.e5.

Veitch, N. C. (2007). Isoflavonoids of the Leguminosae. *Natural Product Reports*, *24*(2), 417–464.

Videla, L. A., Rodrigo, R., Orellana, M., Fernandez, V., Tapia, G., QuiñOnes, L., … Poniachik, J. (2004). Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients. *Clinical Science*, *106*(3), 261.

Vosper, H., Patel, L., Graham, T. L., Khoudoli, G. A., Hill, A., Macphee, C. H., … Palmer, C. N. A. (2001). The Peroxisome Proliferator-activated Receptor δ Promotes Lipid Accumulation in Human Macrophages. *Journal of Biological Chemistry*, *276*(47), 44258–44265.

Wagner, J. D., Zhang, L., Shadoan, M. K., Kavanagh, K., Chen, H., Tresnasari, K., … Adams, M. R. (2008). Effects of soy protein and isoflavones on insulin resistance and adiponectin in male monkeys. *Metabolism*, *57, Supplement 1*, S24–S31.

Weidner, C., Groot, J. C. de, Prasad, A., Freiwald, A., Quedenau, C., Kliem, M., … Sauer, S. (2012). Amorfrutins are potent antidiabetic dietary natural products. *Proceedings of the National Academy of Sciences*, *109*(19), 7257–7262.

Wisløff, U., Helgerud, J., Kemi, O. J., & Ellingsen, Ø. (2001). Intensity-controlled treadmill running in rats: V˙o 2 max and cardiac hypertrophy. *American Journal of Physiology - Heart and Circulatory Physiology*, *280*(3), H1301–H1310.

Yao, Y., Chen, F., Wang, M., Wang, J., & Ren, G. (2008). Antidiabetic Activity of Mung Bean Extracts in Diabetic KK-Ay Mice. *Journal of Agricultural and Food Chemistry*, *56*(19), 8869–8873.

Yao, Y., Cheng, X., Wang, L., Wang, S., & Ren, G. (2011). Biological potential of sixteen legumes in China. *International Journal of Molecular Sciences*, *12*(10), 7048–7058.

Yao, Y., Yang, X., Tian, J., Liu, C., Cheng, X., & Ren, G. (2013). Antioxidant and antidiabetic activities of black mung bean (Vigna radiata L.). *Journal of Agricultural and Food Chemistry*, *61*(34), 8104–8109.

Yeap, S. K., Mohd Ali, N., Mohd Yusof, H., Alitheen, N. B., Beh, B. K., Ho, W. Y., … Long, K. (2012). Antihyperglycemic Effects of Fermented and Nonfermented Mung Bean Extracts on Alloxan-Induced-Diabetic Mice. *BioMed Research International*, *2012*, e285430.

Yoshie-Stark, Y., & Wäsche, A. (2004). In vitro binding of bile acids by lupin protein isolates and their hydrolysates. *Food Chemistry*, *88*(2), 179–184. doi:10.1016/j.foodchem.2004.01.033

Younes, H., Demigné, C., & Rémésy, C. (1996). Acidic fermentation in the caecum increases absorption of calcium and magnesium in the large intestine of the rat. *The British Journal of Nutrition*, *75*(2), 301–314.

Zhu, S., Li, W., Li, J., Jundoria, A., Sama, A. E., & Wang, H. (2012). It is not just folklore: the aqueous extract of mung bean coat is protective against sepsis. *Evidence-Based Complementary and Alternative Medicine*, *2012,* Article ID 498467, 10 pages.

Zucker, L. M., & Antoniades, H. N. (1972). Insulin and Obesity in the Zucker Genetically Obese Rat "Fatty." *Endocrinology*, *90*(5), 1320–1330.

12. ANEXO

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Health promoting effects of Lupin (Lupinus albus var. multolupa) protein hydrolyzate and insoluble fiber in a diet-induced animal experimental model of hypercholesterolemia

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New functional foods are increasingly sought to improve the treatment of diseases related to glucose and lipid metabolism. Lupin (Lupinus albus) is an excellent candidate since it exhibits several health-promoting effects. Such beneficial effects can be enhanced by technological treatments like protein hydrolysis with microbial proteases. The aim of this study was to assess the effect of lupin protein hydrolyzate, combined or not with lupin insoluble fiber, on different plasmatic, hepatic, renal and large intestine parameters using an in vivo experimental model of diet-induced hypercholesterolemia. Lupin protein hydrolyzate and insoluble fiber residue were obtained by aqueous protein extraction and sequential hydrolysis with proteases from Bacillus licheniformis and Aspergillus oryzae. The protein hydrolyzate was effective at reducing plasma and hepatic triglycerides, and showed promising effects on glucose metabolism as well as protection against dietary-induced renal alterations. The insoluble fiber residue increased fecal fat excretion, and improved parameters of large intestine physiological status due to its fermentative and water holding capacity.

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1. Introduction

Legumes represent an excellent source of essential nutrients and exhibit a variety of health effects related to their antioxidant (Doblado, Frias, & Vidal-Valverde, 2007) and hypolipidemic properties (Hermsdorff, Zulet, Abete, & Martinez, 2011). Such important properties derive from the specific characteristics of legume proteins, carbohydrates and lipids, and also from several non-nutritional compounds like poly-phenols, phytic acid or α-galactoside oligosaccharides. Legume-based diet consumption has also been related to large intestine hypertrophy due to fermentation of complex carbohydrates such as dietary fiber and α-galactoside oligosaccharides by colonic microbiota, which generates significant amounts of short-chain fatty acids (SCFA) like acetate, propionate or butyrate (Henningsson, Nyman, & Björck, 2001). These fermentation products exhibit cholesterol-lowering effects (Nishimura, Tanabe, Yamamoto, & Fukushima, 2011), and stimulate the growth of bifidobacteria, thus showing a prebiotic action (Bouhnik et al., 2004). However, they also exhibit significant proliferative effects in colonic mucosa which may pose potential danger in the development of colon cancer (Lupton, 2004).

The nutritional relevance of lupin (Lupinus spp.) has increased in recent years due to its high content of protein, minerals, dietary fiber, and fat (Porres, Aranda, López-Jurado, & Urbano, 2006), as well as to its low levels of non-nutritional components such as trypsin inhibitors, lectins, or alkaloids in the sweet varieties. In addition to these nutritional properties, lupin also features beneficial functional properties such as antioxidant or hypocholesterolemic effects (Martinez-Villaluenga et al., 2009; Parolini et al., 2012).

Biotechnological treatments applied to legumes not only improve their nutritional value (Porres, Aranda, Lopez-Jurado, & Urbano, 2003), but may also enhance their health-promoting potential (Doblado et al., 2007). In recent years, new processing conditions are continuously emerging to improve both the nutritional and health-related properties of legume-derived foodstuffs. One good example of such new products is protein hydrolyzates, which are usually produced by protein extraction under alkaline conditions followed by hydrolysis process using different exoproteases like alcalase or flavourzyme (Megías et al., 2007). The

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resulting bioactive peptides may act as potential physiological mo-dulators of metabolism, given that they inhibit the activity of angiotensin converting enzyme and exhibit antioxidant and bile acid-binding proper-ties (Yoshie-Stark & Wäsche, 2004), thus showing promising potential as functional ingredients.

Among the most widely used in vivo methods to test the hypo-lipidemic properties of foodstuffs is the consumption by experimental animals of a diet rich in cholesterol and/or saturated fat with strong atherogenic index, based on that originally formulated and assayed by Nath, Wiener, Harper, and Elvehjem (1959). In such an experimental model, the potential hypolipidemic effect of the tested compound or mixture can be studied at both the digestive or metabolic level. However, the model has also some disadvantages (Dabai et al., 1996) such as the strongly deleterious effects of a hypercholesterolemic diet in the metabolism of experimental animals and specifically on liver status. On the other hand, the benefits of certain functional food ingredients are better shown under conditions of hypercholesterolemia and hypertriglyceridemia rather than under normal physiological con-ditions (Bettzieche et al., 2008), thus reinforcing the validity of such experimental model.

In the present study, we aimed to study the potential of lupin protein hydrolyzate, combined or not with lupin insoluble fiber, as functional food ingredients with beneficial effects on different metabolic parameters using an in vivo experimental model of rats fed a diet rich in cholesterol and coconut oil to test: 1) their hypolipidemic action, and 2) their influence on certain parameters of renal and gut metabolism.

2. Materials and methods

2.1. Plant material

Lupin seeds (Lupinus albus var. multolupa) were provided by the Agrarian Research and Technology Development Service from the Agriculture and Commerce Council of the Junta de Extremadura (Spain). Seeds were cleaned and stored at 4 °C until used.

2.2. Preparation of lupin protein hydrolyzates and insoluble dietary fiber residue

Lupin protein hydrolyzates were prepared by alkaline water extraction according to Martinez-Villaluenga, Urbano, Porres, Frias, and Vidal-Valverde (2007), and protein hydrolysis according to Megías et al. (2007). Briefly, 100 g of lupin flour was suspended in 1 L of distilled water containing 0.25% (w/v) Na_2SO_3 and then adjusted to pH 9.0. The suspension was stirred for 50 min at room temperature, and then was centrifuged at 3000 rpm for 30 min. In order to obtain higher yields, the extraction and centrifugation were repeated on the residue. The final insoluble residue after protein extraction was collected and freeze-dried prior to storage at −20 °C until analysis and experimental diet preparation. Proteins in the final suspension were sequentially hydrolyzed with alcalase (protease from Bacillus licheniformis, Sigma, St. Louis MO) and flavourzyme (protease from Aspergillus oryzae, Sigma, St. Louis MO) using a hydrolysis reactor vessel equipped with a stirrer, thermometer, and pH electrode. Hydrolysis was performed as follows: $CaCl_2$ and $MgCl_2$ were added in enough quantities to make a 1 mM concentration in the protein suspension. Alcalase was added at time 0, and flavourzyme was added after 30 min. For alcalase hydrolysis, the enzyme/substrate ratio was 0.3 Anson units (AU)/g protein, pH 9, and temperature 50 °C. For flavourzyme hydrolysis, the enzyme/substrate ratio was 100 Leucin amino peptidase units (LAPU)/g protein, pH 8.5, and temperature 50°C. The pH was maintained throughout the digestion process adding 1M NaOH and aliquots were taken at time 0 and every 15min to test for protein hydrolysis by SDS-PAGE separation. Upon finishing the lupin protein hydrolysis, the pH of hydrolyzates was adjusted to 7.0 prior to freeze-drying and storage at −20 °C.

2.3. Experimental diets

AIN–93M diets were formulated following the recommendations of the American Institute of Nutrition (Reeves, Nielsen, & Fahey, 1993), to meet the nutrient recommendations of adult rats (NRC, 1995), with casein or lupin protein hydrolyzate as the sole sources of protein. Cholesterol (1%), and cholic acid (0.5%) (Sigma, St. Louis MO) were added following the recommendations by Nath et al. (1959) for the preparation of hypercholesterolemic high-fat diets, to which dietary fat was added to provide a 12% content partitioned into coconut oil (8%) as saturated fat source, and sunflower seed oil (4%) as polyunsaturated fat source. For normolipidemic diet, 4% sunflower seed oil was added. 1.5% methionine was added to the hypercholesterolemic high-fat diets in order to increase their atherogenic potential through the formation of homocysteine. Dietary fiber was added as cellulose or lupin-derived insoluble fiber residue to provide a dietary level of 10%. The total fat and fiber content of lupin protein hydrolyzate and insoluble fiber residue was taken into consideration for the final concentration present in the diet; the additional amount of Ca needed to bring the dietary levels up to target requirements of the rat was supplied as Ca-citrate added to the diet. Caloric content of the diets was calculated using the Atwater General Factors.

2.4. Animals and experimental design

A total of thirty male Wistar albino rats with an initial mean live weight of 170 ± 2 g were allocated to 5 experimental groups (n = 6): Casein–Cellulose normolipidemic control group (CT), Casein/Cellulose hypercholesterolemic high-fat group (HC), Casein/Lupin insoluble fiber residue hypercholesterolemic high-fat experimental group (HCF), Lupin protein hydrolyzate/Cellulose hypercholesterolemic high-fat experimental group (HID), and Lupin protein hydrolyzate/ Lupin insoluble fiber residue hypercholesterolemic high-fat experimental group (HIDF). The experiment lasted for 30 days during which animals were placed in a well-ventilated, thermostatically controlled (21 ± 2 °C) room with relative humidity ranging from 40 to 60%, and 12 h light/dark periods (lights on at 09:00). Throughout the trial, all rats had free access to double distilled water and consumed the experimen-tal diets ad libitum. Food intake was recorded daily by weighing the amounts of diet given, refused and spilled, and body weight was measured every week. On the last week of experimental period (days 25–30) a balance experiment was carried out during which rats were housed in individual metabolic cages. Fecal output was collected daily and separately for each rat and frozen at −20 °C. The frozen rat feces were freeze-dried, weighed and ground for analysis of total fat content. On day 29 of experimental period, a 12h urine sample from each animal was collected for volume, pH, mineral, and biochemical analysis. During these 12 h, located in the dark cycle, water was removed in order to avoid interferences with urine collection. At the end of the experiment, rats were deprived of food for 12 h, anesthetized with pentobarbital, and sacrificed. Blood was collected by puncture of the abdominal aorta (with heparin as anticoagulant) and taken for quantification of blood parameters (KX-21 Automated Hematology Analyzer, Sysmex Corporation, Kobe, Japan) prior to centrifugation at 3000 rpm for 15 min to separate plasma that was frozen in liquid N and stored at −80 °C. Liver, spleen, kidneys, caecum, and caecal content, were extracted, weighed and immediately frozen in liquid N and stored at −80 °C. The colon was extracted and weighted, and its proximal portion was fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned for hematoxylin and eosin, and Masson's trichrome staining for general light microscopy histological examination. Morphometric studies of colon perimeter and luminal surface area, length of plica mucosae, thickness of the mucosa tunic, and thickness of the muscular tunic were performed using the software Image Pro Plus 6.0.

All experiments were undertaken according to Directional Guides Related to Animal Housing and Care (European Union Council, 2010)

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and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada.

2.5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Was done according to the method of Laemmli (1970). The final concentration of acrylamide in the running gel was 15%. Equal amounts of N (12.5 μg) were loaded in each lane. The gels were fixed and stained with 0.2% Coomassie brilliant blue R-250 in methanol/acetic acid/water (5:4:1 v/v/v). The mixture of molecular weight markers ranged 8– 220 kDa (ColorBurst™, Sigma, St. Louis MO).

2.6. Mass spectrophotometry

An Ultra Performance Liquid Chromatography (UPLC) (ACQUITY H CLASS. WATERS) coupled by quadrupole-time-of-flight mass spectrome-try (SYNAP G2. WATERS) was employed for all high-resolution mass spectrometry analysis. Prior to mass spectrometry analysis, all powdered samples were dissolved in distilled water, diluted in deionized water with 0.1% of acid formic, and filtered through 0.22 μm nylon disk filters (Millipore). 10 μL of the final solution was injected into the chromato-graph. Analytical separation of peptides and proteins was performed on an ACQUITY BEH 300 C4 analytical column (100 mm × 2.1 mm internal diameter, 1.7 μm). A mobile phase consisting in a gradient program combining deionized water with 0.1% of acid formic as solvent A and acetonitrile with 0.1% of acid formic as solvent B was used. The initial conditions were 95% A and 5% B. A linear gradient was then established to reach 95% (v/v) of B at 15 min. Total run time was 25 min and post-delay time 5 min. Mobile phase flow rate was 0.4mL/min.

After chromatographic separation, a high-resolution mass spectro-metry analysis was carried out in positive electro spray ionization (ESI + ve). The gas used for desolvation (800 L/h) and Cone (25 L/h) was high-purity nitrogen. Spectra were recorded over the mass/charge (m/z) range 50–1200.

2.7. Composition analysis of lupin protein hydrolyzate, lupin insoluble fiber residue, and experimental diets

Moisture content of the lupin protein hydrolyzate, insoluble fiber residue and experimental diets was determined by drying to constant weight in an oven at 105 ± 1 °C. Total N was determined according to Kjeldahl's method. Crude protein was calculated as N × 6.25. Insoluble N and soluble protein and non-protein N of the experimental diets were measured using the methodology described by Periago, Ros, Martínez, and Rincón (1996). Samples (0.5 g) were thrice extracted with 10mL of 0.02N NaOH during 45min and the three extracts pooled. Insoluble material was removed by centrifugation at 3000 rpm for 20 min. The supernatant was mixed with 20 mL of 30% trichloroacetic acid (TCA) and the mixture stirred for 15 min at 4 °C. Protein was removed by centrifugation at 3000 rpm for 15 min. Total N was measured in the insoluble material (insoluble N), protein pellet (protein N) and supernatant (non protein N). Amino acid composition of lupin protein hydrolyzate and the different experimental diets tested were measured using Waters AccQ.Tag method for pre-column derivatization and analysis of amino acids combined with a narrow-bore chromatography (2.1 mm × 150 mm) column and Waters 2475 Multi-λ fluoresce detector.

Total fat content was determined by gravimetry of the ether extract after acid hydrolysis of the sample. Total Dietary Fiber (TDF) content of the samples was quantified according to Prosky, Asp, Schweizer, De Vries, and Furda (1992). Ash content was measured by calcination at 450 °C to a constant weight. Samples of ashed material were dissolved in 6 N HCl before analyses. Ca and Mg content were determined by atomic absorption spectrophotometry, and K content was determined by atomic emission spectrophotometry using a Perkin Elmer AAnalyst 300 spectrophotometer (PerkinElmer, Wellesley, MA, USA). Lanthanum chloride was added to Mg samples in order to prevent interferences caused by phosphate ions. Total P was measured spectrophotome-trically using the technique described by Chen, Toribara, and Warner (1956). Analytical results were validated by the following standard reference materials: CRM-383 (haricot beans; Community Bureau of Reference), and CRM-709 (pig feed; Community Bureau of Reference).

2.8. Biochemical analysis

Urinary pH was analyzed using a bench pH-meter (Crison, Barcelona, Spain). Plasma and urinary biochemical parameters were measured using a Shenzhen Midray BS-200 Chemistry Analyzer (Bio-Medical Electronics) at the Bioanalysis Unit of the Scientific Instrumentation Centre (Biomedical Research Park, University of Granada). Liver function parameters measured in plasma (AST, ALT, ALP, GGT, and bilirrubin) were assayed using commercial kits (Spinreact, S.A., Girona, Spain. Reference #s 1001161, 1001171, 41242, 100185, and 1001042, respectively).

2.9. Mineral, cholesterol and triglyceride content in liver

A portion of liver was freeze-dried for moisture content detection and easier handling in the process of total mineral and fat detection. Total ash and P content of freeze-dried liver were measured as pre-viously described for the functional ingredients and experimental diets. Hepatic lipids were extracted with hexane using the method of Folch, Lees, and Stanley (1957). Briefly, freeze-dried liver aliquots (0.5 g) were carefully crushed and homogenized in 2000 μL of ethanol containing 0.01% BHT. The liver homogenate was saponified with 1mL of 11N KOH for 40min at 60°C and extracted with 2000 μL hexane for analysis. The organic phase extracted from liver was dried under a stream of N_2 and re-dissolved in the appropriate volume of hexane. Total liver lipids were measured gravimetrically after solvent evaporation under N_2 stream. The concentration of cholesterol and triglyceride in the re-dissolved fat solution was measured using analytical kits (Spinreact, S.A., Girona, Spain. Reference #s 41021 and 41031, respectively).

2.10. Statistical analysis

Results are expressed as mean (n=6)±Standard Error of the Mean (SEM). One-way ANOVA was applied to the data with the use of SAS version 8.02 (SAS Institute, Cary, NC, USA). Differences between means were compared with Tukey's test. The level of significance was set at
P b 0.05.

3. Results

3.1. Composition of lupin protein hydrolyzate, lupin insoluble fiber residue, and experimental diets

The composition in total nitrogen, amino acids, TDF, fat, ash and dietary essential minerals of lupin protein hydrolyzate and insoluble dietary fiber residue, together with the formulation and proximate composition of the different experimental diets is presented in Table 1. The lupin protein hydrolyzate obtained exhibited high protein content, and moderate amounts of fat, ash and TDF. Potassium was the mineral with highest concentration in the protein hydrolyzate followed by P, Ca, and Mg. Amino acid profile was characterized by a high content of Arg, His, Leu, Ile, and Phe + Tyr, whereas the contents of Lys and Met were lower. The insoluble dietary fiber residue was composed mainly of dietary fiber (79.8%), although it exhibited minor proportions of protein, fat and ash.

With regard to the experimental diets, 90–93% of the total N content of the casein-based diets (CT, HC, and HCF) corresponded to soluble

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Table 1
Composition of lupin protein hydrolyzate, lupin insoluble dietary fiber residue, and experimental diets.

Lupin product composition	Protein hydrolyzate	Insoluble dietary fiber residue
N × 6.25 (g/kg)	471.3	85.1
Fat (g/kg)	111.0	67.2
TDF (g/kg)	51.9	798.0
Ash (g/kg)	128.6	30.0
P (mg/kg)	4977.3	1004.6
Ca (mg/kg)	2689.5	2246.2
Mg (mg/kg)	2304.6	706.8
K (g/kg)	13.5	5.0

Diet formulation (g/kg)	CT	HC	HCF	HID	HIDF
Casein	205	170	170	–	–
Lupin protein hydrolyzate	–	–	–	280	280
Methionine	5	15	15	15	15
Sucrose	100	100	100	100	100
Starch	500	410	390	330	310
Cellulose	100	100	–	100	–
Insoluble fiber residue	–	–	130	–	130
Coconut oil	–	80	80	80	80
Sunflower seed oil	40	40	30	10	–
Cholesterol	–	10	10	10	10
Cholic acid	–	5	5	5	5
Mineral mix	35	35	35	35	35
Vitamin mix	10	10	10	10	10
Calcium citrate	24	24	24	24	24
Choline bitartrate	2.5	2.5	2.5	2.5	2.5

Diet composition	CT	HC	HCF	HID	HIDF
Total N (g/kg)	28.4	27.8	27.4	22.0	23.5
Insoluble N (%)	1.77	0.77	1.73	2.69	3.55
Soluble protein N (%)	92.8	92.3	90.3	2.04	1.77
Soluble non-protein N (%)	5.82	6.98	8.01	95.3	94.7
Total fat (g/kg)	46.0	118.7	112.2	120.4	122.8
TDF (g/kg)	88.4	92.2	102.7	92.1	101.0
Ash (g/kg)	32.8	30.9	29.6	58.5	55.9
P (mg/kg)	3073.3	2927.9	2637.0	2494.4	2565.6
Ca (mg/kg)	4411.0	4801.7	5015.3	5484.3	5654.6
Mg (mg/kg)	601.0	468.3	533.8	1108.6	1132.2
K (mg/kg)	3575.1	3770.0	3867.6	6777.5	7335.9

Amino acid composition (g/100 g)	Protein hydrolyzate	CT	HC	HCF	HID	HIDF
Asp	0.82	1.12	0.91	0.89	0.35	0.35
Ser	4.17	0.95	0.77	0.75	1.50	1.28
Glu	2.62	4.92	3.98	3.92	1.09	1.23
Gly	0.84	0.31	0.25	0.25	0.26	0.29
His	4.37	0.61	0.49	0.48	1.52	1.27
Arg	5.10	0.61	0.49	0.49	1.89	2..03
Thr	2.37	0.71	0.57	0.57	0.89	0.69
Ala	1.38	0.53	0.43	0.42	0.52	0.51
Tyr	2.90	0.77	0.62	0.61	1.09	0.93
Val	2.16	0.86	0.70	0.69	0.78	0.81
Met	0.59	1.36	3.70	3.65	4.93	6.07
Lys	1.11	1.27	1.03	1.01	0.21	0.36
Ile	2.21	0.77	0.62	0.61	0.70	0.75
Leu	5.09	1.31	1.06	1.04	1.78	1.83
Phe	2.45	0.96	0.78	0.77	0.63	0.53

Results are expressed on a dry matter basis. Values are means of four independent replicates. TDF, Total Dietary Fiber. CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group.

protein N, whereas 5.8–8% corresponded to soluble non-protein N. In contrast, the content of soluble protein N in the lupin protein hydrolyzate-based diets was very minor (1.8–2.0%), whereas the major proportion of total N content corresponded to soluble non protein N (95%). The content of insoluble N was very low in all the experimental diets tested. Amino acid composition followed the same pattern as in the lupin protein hydrolyzate, with the exception of Met content that was significantly increased due to supplementation of this amino acid to the hypercholesterolemic high-fat diets. When compared to the casein-based diets, the contents of Arg, Met, Leu and Ile were con-siderably higher in the lupin protein hydrolyzate-based diets, whereas Lys content was lower. Total fat content increased from 4.5% in the control diet (CT) to values close to 12% in all the hypercholesterolemic high-fat diets tested (HC, HCF, HID, HIDF). Total Dietary Fiber content was within the formulated value of 10% in all the experimental diets.

Fig. 1A shows the changes in the SDS-PAGE pattern of proteins extracted from the lupin flour as a result of sequential enzymatic hydrolysis by proteases from B. licheniformis and A. oryzae. The digestion process caused the disappearance of main protein bands and the subsequent appearance of a smear of low molecular weight protein bands from the first stages of protease digestion. Fig. 1B shows a chromatogram and a mass spectrum (4.11 min) of L. albus protein before hydrolysis. Consistent with the complexity of the sample, a wide range of chromatographic peaks were detected. The presence of proteins was proved by high-resolution mass spectrometry as it is indicated in the mass spectrum example at 4.11 min (major peak). A typical protein profile for mass spectrometry was clearly obtained, due to the detection of multiple-charged ions provided by electro spray ionization of proteins present in the sample. In comparison to the former results, a chromatogram and a mass spectrum (0.60 min) of L. albus protein after hydrolysis process are presented in Fig. 1C. A simpler chromatogram was achieved, indicating the disappearance of major peaks. Based on mass spectrum profile, proteins were not detected. Furthermore, the appearance of mono-charged ions involved the presence of smaller molecules such as peptides. Major peaks were detected in the mass/charge (m/z) range of 86–770.

3.2. Food Intake, final body weight, and digestive utilization of fat

Throughout most of the experimental period, daily food intake was higher in the experimental group of animals that consumed the normolipidemic diet when compared to the experimental groups that consumed high-fat diets, among which no significant differences were observed (Table 2). The lower dietary intake of rats that consumed the high-fat diets can be attributed to their specific composition with higher energy (1826 to 1868 KJ/100 g dry matter (DM) in high fat diets vs 1713 KJ/100 g DM in control normolipidemic diet) and methionine content. No significant differences in final body weight were found among the different experimental groups.

Daily fat intake was significantly higher for the groups of animals that consumed hypercholesterolemic high-fat diets compared to the control normolipidemic group (Table 2). Fecal fat content and excretion was significantly higher in the hypercholesterolemic high-fat diet-fed groups when compared to the normolipidemic control, and among the former experimental groups, the amount of fat excreted in feces was higher (P b 0.05) in the groups of animals that consumed lupin insoluble fiber residue (HCF, HIDF). The above described results were reflected in significantly reduced fat digestibilities exhibited by the hypercholesterolemic high-fat diet-fed groups when compared to the normolipidemic control, and the lowest fat digestibility (P b 0.05) in HCF and HIDF groups.

3.3. Biochemical and hematic parameters

Consumption of a Casein/Cellulose-based high-fat diet (HC) led to a considerable increase (P b 0.05) in the levels of plasma total-, and LDL-cholesterol, without significantly affecting the glycemia or the content of plasma triglycerides and HDL-cholesterol when compared to the normolipidemic diet (CT) (Table 2). The inclusion of lupin insoluble fiber residue in the casein-based high-fat diet at the expense of cellulose (HCF) did not cause any significant change in the abovementioned biochemical parameters when compared to the HC group, whereas

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substitution of casein by the lupin protein hydrolyzate (HID, HIDF) significantly decreased triglyceridemia and glycemia when compared to HC and HCF groups. On the other hand, no significant differences in total-, LDL-, or HDL-cholesterol when compared to Casein/Cellulose-based high-fat diet-fed group (HC) were observed derived from the dietary inclusion of lupin protein hydrolyzate (HID, HIDF).

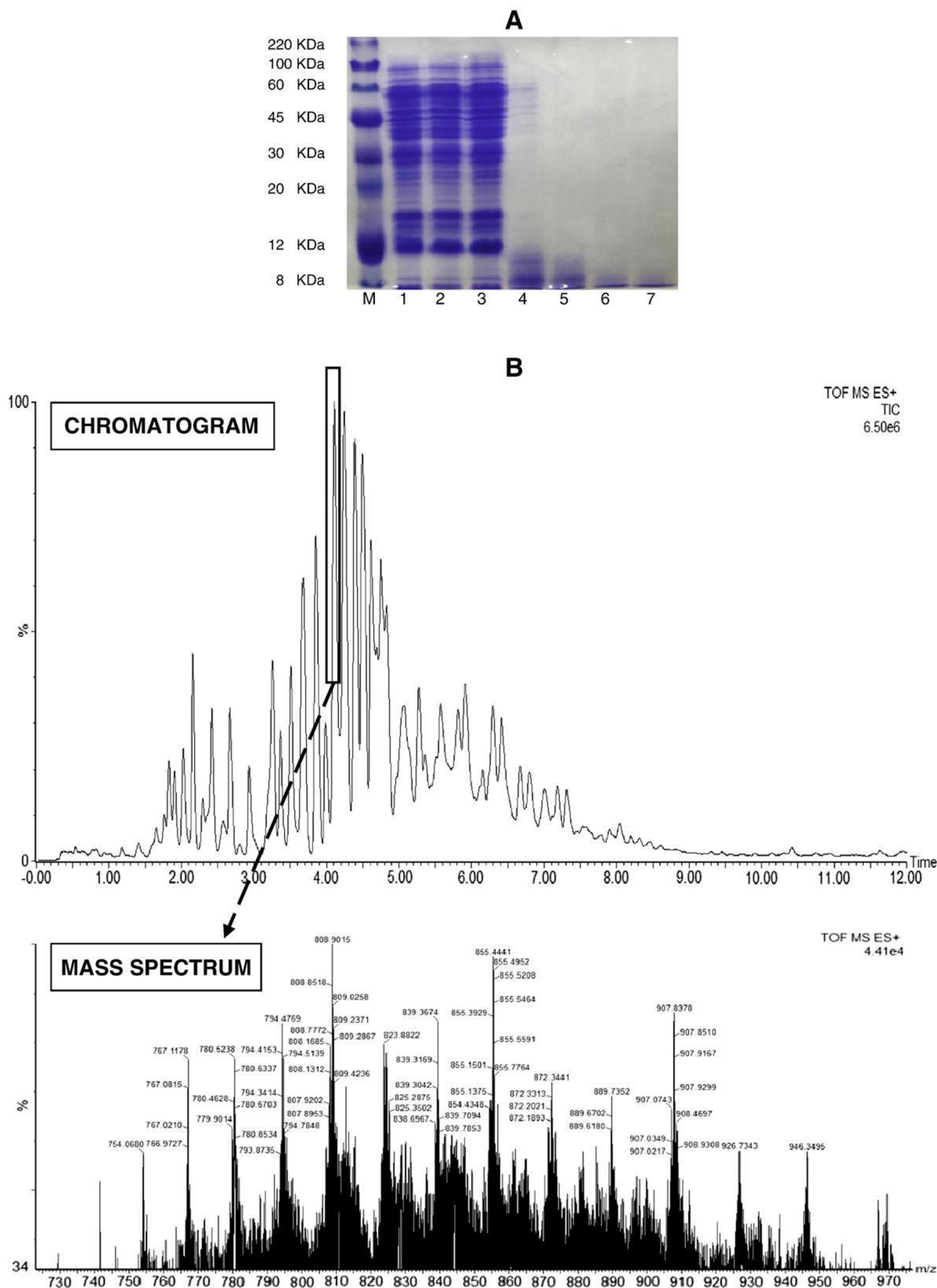

Fig. 1. Detection processes of Lupinus albus protein sequential digestion with proteases from Bacillus licheniformis and Aspergillus oryzae. (A) Band pattern in SDS-PAGE. M, molecular weight markers, 1, lupin protein extract prior to heating at 50 °C to start the sequential digestion process, 2, lupin protein extract upon reaching the temperature of 50 °C, 3, time 0′ of Bacillus licheniformis protease digestion, 4, time 15′ of Bacillus licheniformis protease digestion, 5, time 30′ of Bacillus licheniformis protease digestion, 6, time 15′ of Aspergillus oryzae protease digestion, 7, time 30′ of Aspergillus oryzae protease digestion. The amount of Kjeldahl-N loaded per lane was 12.5 μg. The figure is representative of 3 independent analyses.
(B) Chromatogram of protein before hydrolysis and Mass Spectrum of major peak (4.11 min) of chromatogram, (C) Chromatogram of protein after hydrolysis and Mass Spectrum of major peak (0.60 min) of chromatogram.

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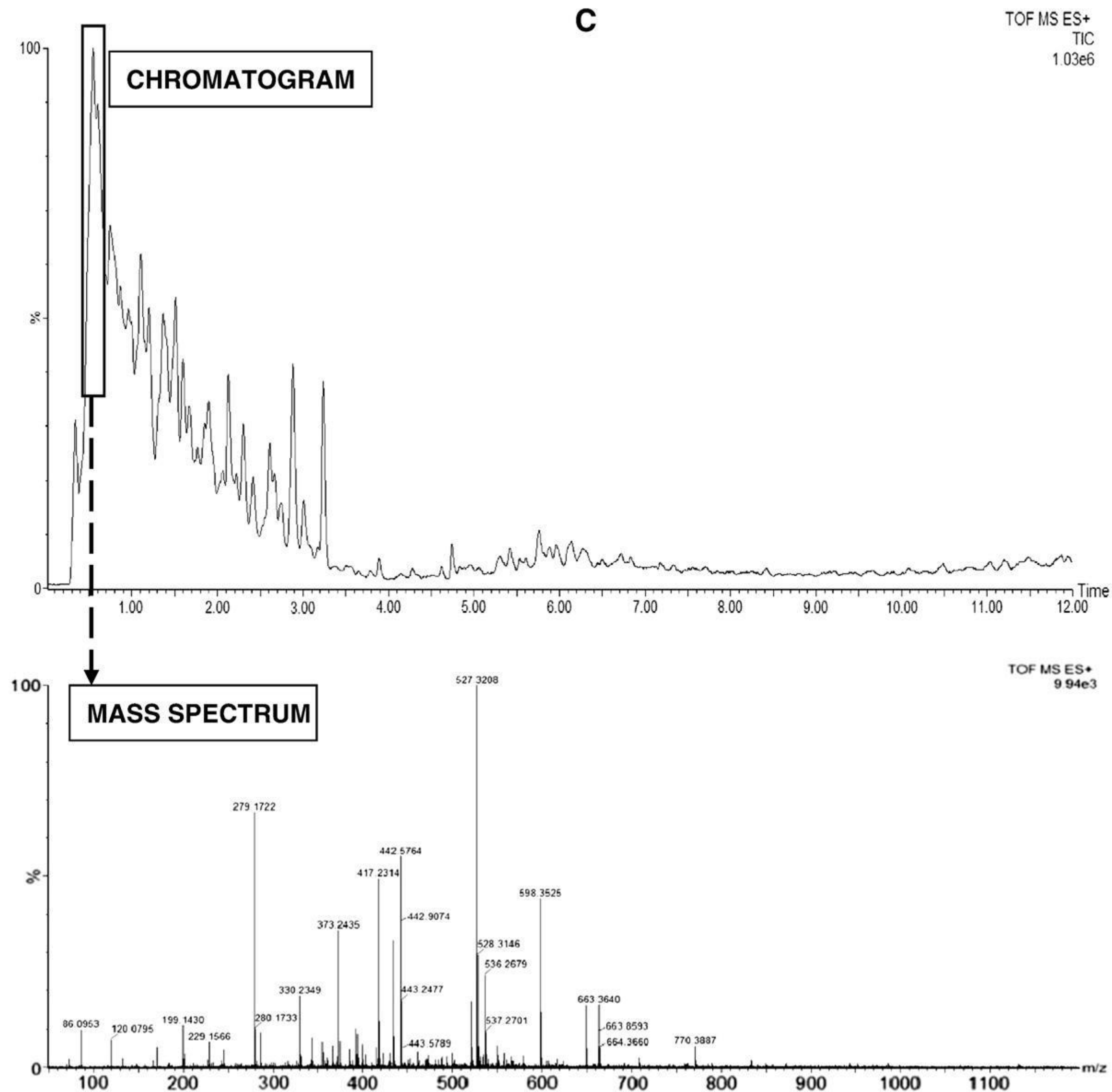

Fig. 1 (continued).

Consumption of hypercholesterolemic high-fat diets (HC, HCF, HID, and HIDF) caused a reduction in erythrocyte count, hematocrit, and plasma Fe content when compared to the animals fed the nor-molipidemic diet (Table 2). No appreciable effect derived from the protein or dietary fiber source administered could be observed in hematocrit or plasma Fe content, whereas erythrocyte count was significantly improved by the inclusion of the lupin protein hydrolyzate in the diet (HID) when compared to the HC group.

3.4. Liver function

Liver weight and fat content were significantly affected by the dietary treatment, with higher values found for all the experimental groups that consumed the hypercholesterolemic high-fat diets (HC, HCF, HID, and HIDF) (Table 3). Such higher liver fat content caused a concomitant reduction in hepatic moisture, protein, ash and total-P content. However, no significant differences in the abovementioned parameters could be observed derived from the inclusion of lupin protein hydrolyzate or insoluble fiber residue. In contrast, there was a significant diet effect on the content of hepatic cholesterol and triglycerides that were lower in the animals fed the lupin protein hydrolyzate-based diets (HID, HIDF) when compared to those fed the caseine-based high-fat diets (HC, HCF).

Plasmatic levels of AST and ALT rose significantly in the group of animals fed the Casein/Cellulose-based high-fat diet (HC) when com-pared to the normolipidemic control. The inclusion of lupin protein hydrolyzate or insoluble fiber residue in diets HCF, HID or HIDF caused a considerable reduction (P b 0.05) in ALT, whereas AST activity was only significantly improved in the HIDF group. Plasmatic GGT activity was not different among the different casein-based experimental groups (CT, HC, HCF), whereas it was significantly reduced by lupin protein hydrolyzate consumption. No significant effect of the different diets tested could be observed for plasma bilirubin or ALP activity.

3.5. Renal function

Urinary volume was highest (P b 0.05) in the experimental group that consumed the Lupin protein hydrolyzate/Cellulose-based high-fat diet (HID), whereas no significant difference was observed among the rest of experimental groups (Table 4). With regard to urinary pH, it significantly decreased in all the hypercholesterolemic high-fat diet-fed animals (HC, HCF, HID, and HIDF) when compared to the normolipidemic controls.

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Table 2
Influence of the dietary treatments on the digestive utilization of fat, plasmatic, and hematic parameters (mean ± SEM).

	CT	HC	HCF	HID	HIDF
Digestive utilization of fat					
Food intake (g DM/day)	22.1± 0.47^{A}	16.8 ± 0.52^{B}	19.2 ± 0.61^{C}	16.3± 0.44^{B}	17.9± 0.68^{BC}
Final body weight (g)	307.2± 5.63^{A}	274.7 ± 4.37^{A}	302.1 ± 10.6^{A}	268.1± 12.4^{A}	270.8± 13.9^{A}
Fat intake (mg/day)	1101.4± 23.3^{A}	2136.0± 65.4^{B}	2320.7 ± 73.7^{B}	2145.6± 58.3^{B}	2396.7± 91.6^{B}
Fecal weight (g DM/day)	3.05± 0.12^{A}	2.96 ± 0.10^{A}	3.08 ± 0.20^{A}	3.08± 0.16^{A}	3.79± 0.27^{B}
Fecal fat content (%)	1.47± 0.23^{A}	5.94 ± 0.11^{B}	7.35 ± 0.30^{C}	5.64± 0.16^{B}	6.73± 0.19^{C}
Fecal fat excretion (mg/day)	45.4± 8.20^{A}	175.0± 3.36^{B}	223.8 ± 7.96^{C}	174.1± 12.1^{B}	254.4± 18.2^{C}
ADC (%)[a]	96.5± 0.27^{A}	91.8 ± 0.15^{B}	90.4 ± 0.20^{C}	91.9± 0.62^{B}	89.0± 0.40^{C}
Plasmatic parameters					
Triglycerides (mg/dL)	85.3± 5.0^{AB}	109.3 ± 9.2^{A}	119.8 ± 5.9^{A}	73.5± 6.3^{B}	67.2± 4.4^{B}
Total-cholesterol (mg/dL)	51.7± 3.2^{A}	119.1 ± 10.6^{B}	116.7 ± 8.8^{B}	124.0± 4.4^{B}	124.6± 4.7^{B}
LDL-cholesterol (mg/dL)	20.6± 8.5^{A}	80.5 ± 10.5^{B}	66.2 ± 9.7^{B}	90.0± 4.7^{B}	93.0± 4.8^{B}
HDL-cholesterol (mg/dL)	29.3± 1.56^{A}	22.7 ± 2.17^{ABC}	26.7 ± 2.57^{AC}	16.8± 1.19^{B}	21.2± 0.79^{BC}
Glucose (mg/dL)	196.0± 5.8^{A}	180.0 ± 6.0^{A}	170.6 ± 5.7^{A}	137.6± 5.3^{B}	155.6± 5.9^{B}
Hematic parameters					
WBC ($\times10^3/\mu$L)	4.60± 0.59^{A}	4.89 ± 0.29^{A}	5.12 ± 0.37^{A}	5.22± 0.56^{A}	4.22± 0.39^{A}
RBC ($\times10^6/\mu$L)	8.93± 0.15^{A}	7.96 ± 0.11^{B}	8.23 ± 0.07^{BC}	8.47± 0.09^{AC}	8.21± 0.14^{BC}
HGB (g/dL)	15.4± 0.19^{A}	14.4 ± 0.19^{AB}	14.4 ± 0.28^{AB}	13.6± 0.15^{B}	13.8± 0.48^{B}
HCT (%)	48.1± 0.62^{A}	45.2 ± 0.51^{B}	45.3 ± 0.66^{B}	44.1± 0.31^{B}	44.1± 1.25^{B}
MCV (fL)	53.9± 0.50^{AC}	56.7 ± 0.30^{B}	55.1 ± 0.99^{AB}	52.1± 0.62^{C}	53.7± 0.76^{AC}
MCH (pg)	17.3± 0.20^{AB}	18.0 ± 0.18^{A}	17.5 ± 0.04^{AC}	16.1± 0.29^{B}	16.8± 0.39^{BC}
MCHC (g/dL)	32.1± 0.13^{A}	31.8 ± 0.23^{A}	31.7 ± 0.19^{A}	30.8± 0.21^{B}	31.3± 0.40^{AB}
Fe (μg/dL)	148.6± 6.27^{A}	119.0 ± 8.45^{B}	126.8 ± 3.06^{B}	117.5± 9.16^{B}	113.0± 11.29^{B}

[A,B,C] Results are means of 6 Wistar rats. Means within the same line with different superscripts differ significantly (P b 0.05). DM, dry matter; SEM, Standard Error of the Mean.
CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group.
[a] ADC, Apparent Digestibility Coefficient for total fat. ADC = [(I − F) / I] × 100, where I = total fat intake, and F = fecal fat excretion.

The urinary content of urea was significantly lower in animals fed HID and HIDF diets when compared to the rest of experimental groups tested (CT, HC, and HCF) among which no significant differences were observed. A similar trend was observed for uric acid, although no significant differences were found between the CT and HIDF groups. Glucose content was highest in HC group (P b 0.05) followed by CT and HCF, and finally by HID and HIDF groups that exhibited the lowest values (P b 0.05). The urinary content of albumin and creatinine in-creased significantly in casein-based hypercholesterolemic high-fat diet-fed animals (HC and HCF) when compared to the normolipidemic control, and returned to normal values in the HID and HIDF groups.

Calciuria increased significantly in response to lupin protein hydrolyzate and insoluble fiber residue, and the highest value was obtained for the HCF group followed by HID and HIDF groups. On the other hand, phosphaturia increased significantly in the HC and HCF groups when compared to the normolipidemic control, HID and HIDF groups. Urinary citrate was lowest (P b 0.05) in casein-based high-fat diets when compared to their lupin protein hydrolyzate counterparts that were similar to the normolipidemic control.

Plasma markers of renal function such as urea, uric acid or creatinine were not affected by any of the dietary treatments assayed.

3.6. Large intestine histology and functionality

Significant diet-induced changes in large intestine weight, func-tionality, and morphometry were observed under our experimental conditions (Fig. 2). A higher fresh weight of caecum and colon was mainly related to the inclusion of lupin protein hydrolyzate in the hypercholesterolemic high-fat diets (Fig. 2A), whereas the degree of pH reduction, and the increased moisture levels of caecum content

Table 3
Influence of dietary treatment on weight, chemical composition, and functionality of liver (mean ± SEM).

	CT	HC	HCF	HID	HIDF
Weight (g fresh sample)	7.7 ± 0.21^{A}	12.2± 0.31^{B}	12.7 ± 0.51^{B}	11.3 ± 0.66^{B}	12.6 ± 0.83^{B}
Relative weight (g/100 g BW)	2.51 ± 0.03^{A}	4.45± 0.11^{B}	4.22 ± 0.19^{B}	4.22 ± 0.22^{B}	4.64 ± 0.12^{B}
Moisture (%)	71.9 ± 0.20^{A}	61.1± 0.97^{B}	61.9 ± 1.14^{B}	61.8 ± 1.21^{B}	60.0 ± 1.05^{B}
Protein (g/100 g DM)	70.9 ± 3.04^{A}	42.0± 1.32^{B}	41.7 ± 2.74^{B}	35.8 ± 1.24^{B}	36.0 ± 1.94^{B}
Ash (g/100 g DM)	4.56 ± 0.17^{A}	2.59± 0.16^{B}	2.78 ± 0.24^{B}	2.73 ± 0.15^{B}	2.87 ± 0.18^{B}
P (mg/100 g DM)	1220.1 ± 31.6^{A}	718.0± 27.0^{B}	795.4 ± 29.5^{B}	766.4 ± 23.9^{B}	724.3 ± 23.4^{B}
Fat (g/100 g DM)	12.6 ± 1.38^{A}	47.8± 2.65^{B}	44.6 ± 2.43^{B}	47.2 ± 1.59^{B}	51.4 ± 2.24^{B}
Hepatic total-cholesterol (mg/g DM)	4.2 ± 0.67^{A}	11.2± 0.91^{B}	11.2 ± 0.62^{B}	8.0 ± 1.25^{C}	8.7 ± 1.11^{C}
Hepatic triglycerides (mg/g DM)	5.8 ± 0.53^{A}	28.8± 5.80^{B}	28.8 ± 5.05^{B}	15.4 ± 1.94^{C}	16.0 ± 1.92^{C}
Liver function plasmatic markers					
AST (U/L)	204.8 ± 25.9^{A}	421.7± 32.5^{B}	348.8 ± 15.9^{BC}	333.0 ± 12.8^{BC}	269.7 ± 35.6^{AC}
ALT (U/L)	45.3 ± 9.2^{A}	206.0± 26.8^{B}	106.7 ± 12.2^{CA}	75.0 ± 4.5^{A}	108.4 ± 19.0^{CA}
ALP (U/L)	261.0 ± 20.8^{A}	338.9± 20.2^{A}	330.5 ± 25.5^{A}	349.4 ± 27.7^{A}	320.7 ± 20.8^{A}
GGT (U/L)	13.6 ± 0.80^{A}	10.8± 0.34^{A}	12.7 ± 0.99^{A}	6.33 ± 0.95^{B}	5.44 ± 0.89^{B}
Bilirubin (mg/dL)	0.26 ± 0.06^{A}	0.27± 0.03^{A}	0.25 ± 0.02^{A}	0.28 ± 0.03^{A}	0.32 ± 0.03^{A}

[A,B,C] Results are means of 6 Wistar rats. Means within the same line with different superscripts differ significantly (P b 0.05). BW, body weight; DM, dry matter; SEM, Standard Error of the Mean; CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/ Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group.

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Table 4
Influence of the dietary treatments on parameters of renal function (mean ± SEM).

	CT	HC	HCF	HID	HIDF
Kidney (g/100 g BW)	0.30 ± 0.01^{A}	0.36± 0.01^{B}	0.35± 0.01^{AB}	0.35 ± 0.01^{AB}	0.35± 0.01^{AB}
Urine					
Urinary pH	6.82 ± 0.09^{A}	5.68± 0.04^{B}	5.93± 0.07^{B}	6.05 ± 0.17^{B}	6.09± 0.17^{B}
Urinary volume (mL)	2.85 ± 0.25^{A}	1.26± 0.15^{A}	2.09± 0.42^{A}	5.02 ± 0.83^{B}	3.05± 0.60^{A}
Urea (g/dL)	6.73 ± 0.43^{A}	8.56± 0.35^{A}	7.79± 0.76^{A}	2.40 ± 0.48^{B}	4.02± 0.67^{B}
Uric acid (mg/dL)	21.3 ± 2.87^{AB}	27.1± 5.55^{A}	27.4± 5.71^{A}	10.2 ± 1.44^{B}	16.1± 1.48^{AB}
Glucose (mg/dL)	20.6 ± 1.04^{A}	41.6± 4.18^{B}	26.6± 3.70^{A}	6.26 ± 2.33^{C}	6.75± 2.52^{C}
Albumin (g/dL)	0.115 ± 0.02^{A}	0.241± 0.03^{B}	0.209± 0.05^{B}	0.065 ± 0.01^{A}	0.080± 0.02^{A}
Creatinine (mg/dL)	146.7 ± 13.9^{A}	317.9± 25.0^{B}	270.0± 28.8^{B}	102.1 ± 21.0^{A}	143.8± 28.4^{A}
Citrate (mg/dL)	1082.7 ± 174.2^{A}	535.9± 36.3^{B}	492.6± 79.9^{B}	988.3 ± 118.3^{A}	1207.8± 254.5^{A}
Ca (mg/L)	38.7 ± 7.65^{A}	52.8± 7.78^{A}	214.2± 16.6^{B}	140.1 ± 17.9^{C}	113.7± 17.1^{C}
P (mg/dL)	326.6 ± 30.9^{A}	649.3± 76.8^{B}	625.8± 47.6^{B}	151.0 ± 35.2^{A}	167.6± 38.4^{A}
Plasma					
Urea (mg/dL)	33.5 ± 2.1^{A}	31.0± 4.6^{A}	32.8± 1.4^{A}	37.0 ± 3.8^{A}	36.7± 2.8^{A}
Uric acid (mg/dL)	1.95 ± 0.13^{A}	1.60± 0.07^{A}	1.57± 0.08^{A}	1.88 ± 0.14^{A}	1.60± 0.08^{A}
Creatinine (mg/dL)	0.47 ± 0.02^{A}	0.47± 0.02^{A}	0.48± 0.03^{A}	0.45 ± 0.02^{A}	0.42± 0.02^{A}

^{A,B,C}Results are means of 6 Wistar rats. Means within the same line with different superscripts differ significantly (P b 0.05). BW, body weight; SEM, Standard Error of the Mean; CT, Casein/Cellulose-based normolipidemic group; HC, Casein/Cellulose-based hypercholesterolemic high fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high fat experimental group.

appeared to be more closely related to the dietary inclusion of lupin insoluble fiber residue (Fig. 2B). With regard to proximal colon morphometry, the dietary inclusion of both lupin protein hydrolyzate and lupin insoluble fiber residue had a significant effect on the different parameters studied (Fig. 2C–H). The length of plica mucosae was significantly increased while the thickness of muscular tunic was significantly reduced in response to lupin protein hydrolyzate (Fig. 2D, F, and H) when compared to a control normal colon (Fig. 2C, F, and H). Furthermore, the thickness of mucosal tunic was increased by both the dietary inclusion of lupin protein hydrolyzate and insoluble dietary fiber residue (Fig. 2D and G). All such changes took place without any significant effect of the dietary treatments assayed in neither the proximal colon perimeter nor the luminal surface area (Fig. 2E).

4. Discussion

Protein hydrolyzates of animal or vegetal origin are strongly coming up as powerful functional ingredients which could be extremely helpful in the prevention and dietary treatment of several alterations (i.e. hypertension, hyperlipidemia, hyperglycemia) that take part in the metabolic syndrome. In the present study, rats were fed hyper-cholesterolemic high-fat diets in which different protein (casein vs lupin protein hydrolyzate) or insoluble dietary fiber (cellulose vs lupin insoluble fiber residue) sources were included to test the potential benefits of lupin products on the experimental animal model of diet-induced hypercholesterolemia /hyperlipidemia. The experimental model developed exhibited significant alterations in plasmatic total-cholesterol and triglycerides, morphological and compositional changes in the liver, and alterations in urinary parameters of renal function. Dietary inclusion of lupin protein hydrolyzate and insoluble fiber residue caused a significant decrease in plasma triglycerides and hepatic lipid composition, and appeared to improve glucose metabolism. Further-more, lupin functional ingredients induced diverse potentially beneficial changes in kidney and large intestine functionality.

4.1. Lupin protein hydrolyzates

An optimal degree of lupin protein hydrolysis achieved by sequential digestion with proteases from B. licheniformis and A. oryzae was con-firmed under our experimental conditions, by the switch in protein fractions from a high dietary content of soluble protein N in the casein-based diets to higher soluble non-protein N content that did not precipitate after treatment with TCA in the lupin protein hydrolyzate-based diets. Furthermore, SDS-PAGE separation clearly shows how the high molecular weight protein bands disappeared to form lower molecular weight peptides. In addition, a UPLC coupled to mass spectro-metry analysis of lupin protein prior to or after the hydrolysis process confirmed the chemical and electrophoretical results. Both chromato-grams and mass spectrums clearly led us to two main conclusions: First, the disappearance of proteins after hydrolysis and, second, the presence, instead of proteins, of mono-charged molecules resembling the appearance of di- to hepta-peptides, as shown by the molecular weight range of peaks in the mass spectrum. Molecular size of bioactive peptides is known to affect some of their potentially beneficial effects such as antioxidant capacity. ABTS radical scavenging activity was improved in peptides with molecular size below 1.4 kDa (Cheung, Cheung, Tan, & Li-Chan, 2012), whereas specific peptide composition seemed to have a stronger influence on effective lipid peroxidation. In addition, lower molecular weight of peptides is likely to improve their chance to be absorbed intact from the gastrointestinal tract (Roberts, Burney, Black, & Zaloga, 1999), thus ensuring their bioactive effects.

4.2. Lipid metabolism and liver function

The alteration in biochemical parameters related to lipid metabo-lism in response to a diet high in cholesterol and saturated fatty acids with considerable atherogenic potential was partially compensated by the dietary inclusion of lupin functional products. The consumption of lupin protein hydrolyzate and insoluble fiber residue did induce a significant reduction in plasma triglyceride content, although no

Fig. 2. Influence of dietary treatments on large intestine weight, functionality, and morphometric studies. (A) Caecum and colon weight, (B) water content and pH of caecum content, (C) histological view of control (CT) proximal colon, (D) histological view of hyperplastic proximal colon (pm, plica mucosae; m, mucosa tunic; mu, external muscular tunic), (E) perimeter (μm) and luminal surface area of the proximal colon (μm^2), (F) length of proximal colon plica mucosae (μm), (G) thickness of proximal colon mucosa tunic (μm), (H) thickness of proximal colon muscular tunic (μm). Values are means with Standard Error of the Mean represented as vertical bars (n =6). Means in the same group not sharing common letters differ significantly (P b 0.05). BW, body weight, CT, Casein/Cellulose-based normolipidemic control group; HC, Casein/Cellulose-based hypercholesterolemic high-fat experimental group; HCF, Casein/Lupin insoluble fiber residue-based hypercholesterolemic high-fat experimental group; HID, Lupin protein hydrolyzate/Cellulose-based hypercholesterolemic high-fat experimental group; HIDF, Lupin protein hydrolyzate/Lupin insoluble fiber residue-based hypercholesterolemic high-fat experimental group.

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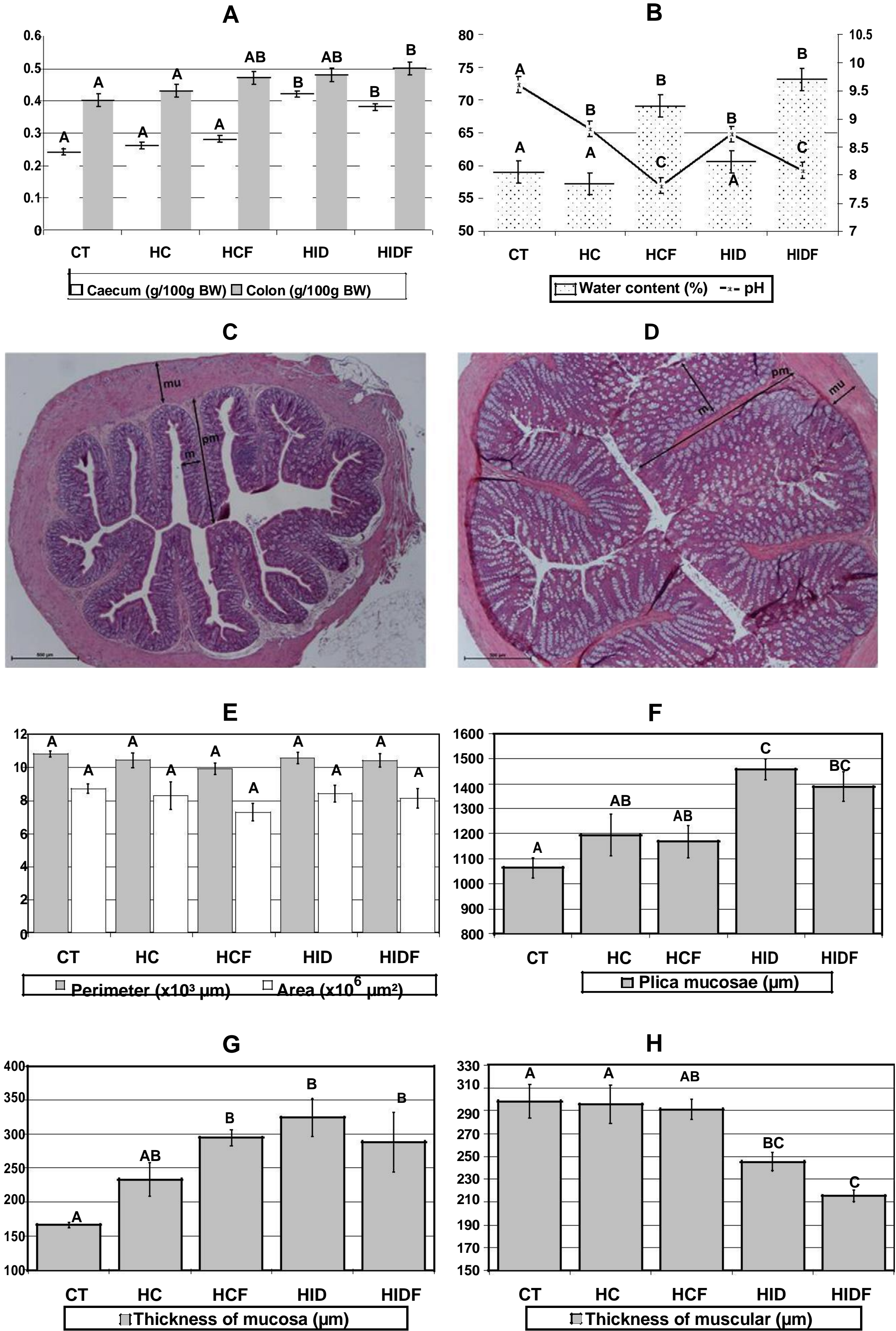

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effect in plasma levels of cholesterol was achieved. Various factors are probably involved in the beneficial effects of lupin products on lipid metabolism namely, i) a digestive effect caused by binding of lupin protein hydrolyzate and fiber to bile acids (Yoshie-Stark & Wäsche, 2004) and their potential inhibition of micellar solubility (Alhaj, Kanekanian, Peters, & Tatham, 2010) that was reflected in lower fat digestibility; and ii) modifications in hepatic lipid metabolism caused indirectly through the action of SCFA released by the large intestine fermentation of lupin soluble and insoluble fiber residue and α-galactoside oligosaccharides (Alhaj et al., 2010), or else by a direct effect of the protein hydrolyzate. High Arg/Lys ratios have been con-sidered good predictive markers of the hypolipidemic potential of a protein (Gudbrandsen, Wergedahl, Liaset, Espe, & Berge, 2005), and lupin protein exhibits a considerable higher ratio when compared to casein (Bettzieche, Brandsch, Eder, & Stangl, 2009). Specifically, previous studies by our group (Martinez-Villaluenga et al., 2007) have described the amino acid composition of L. albus var. multolupa, and the Arg/Lys ratio for this particular lupin variety is 4 and 8.2-fold higher in the seed flour and protein isolate, respectively, than the value reported for casein (Gudbrandsen et al., 2005). Such higher Arg/Lys ratio has been confirmed in the lupin protein hydrolyzate and related diets under the experimental conditions of the present study (Table 1). The elevated Arg/Lys ratio of lupin protein has been related to the altered expression of sterol regulatory element-binding protein-1c (SREBP-1c) (Spielman et al., 2007) and the concomitant down regulation of genes responsible for fatty acid synthesis or the up regulation of genes involved in triglyceride hydrolysis (Bettzieche et al., 2008) or bile acid synthesis (Parolini et al., 2012). Howard and Udenigwe (2013) have reviewed the mechanism of food protein hydrolysate-induced hypolidemia, and point out to the modulation by protein hydrolyzates of the expression of genes involved in lipid metabolism at the hepatic and adipocyte level. Such modulation results in the upregulation of mRNA expression of proteins involved in fatty acid oxidation like PPARα as well as LDL-receptor and cholesterol-metabolizing enzymes, or else in the downregulation of mRNA expression of proteins involved in lipogenesis like fatty acid synthase. Such effects in lipid metabolism can be directly related to the changes in hepatic cholesterol and triglyceride profile observed under our experimental conditions, and, indirectly, to the improvement achieved in the levels of ALT, a well-known index of acute liver damage. Furthermore, in case of GGT, a marker of chronic liver damage, the plasmatic activity of this enzyme was lowered to values that were well below those of the control normolipidemic experimental group. However, despite the beneficial effects of lupin protein hydrolyzate and insoluble fiber on hepatic cholesterol and trygliceride metabolism, no effects were observed of the lupin products tested on hepatomegalia and steatosis induced by the hypercholesterolemic high-fat diets tested. This is in contrast to what has been reported by Guadagnucci Fontanari, Batistuti, Cruz, Nascimiento Saldiva, and Gomes Arêas (2012), who found that L. albus protein isolate and lupin seed flour decreased liver steatosis and histological alterations in hamsters fed a hypercholes-terolemic diet. Possible reasons for such findings are differences in experimental animal species and diet formulation, with lower dietary content of cholesterol, higher levels of choline chloride, and the absence of added cholate reported by the abovementioned authors.

The altered hematic parameters related to a potentially low Fe status can be attributed to splenomegaly induced by hypercholesterolemic high-fat diet consumption (data not shown), given the essential role of spleen in erythrocyte metabolism. Thus, Fe status could be com-promised by altered spleen and liver functionality as a result of the high-fat dietary treatments applied.

Lupin protein hydrolyzate and insoluble fiber residue also showed a promising potential in the management of glucose metabolism as seen by their lowering effects on glycemia, hypertriglyceridemia, a marker of insulin resistance, and urinary glucose excretion. Usually, plasma tryglicerides and glucose are elevated in type-II diabetes patients as part of the metabolic syndrome (Eckel, Grundy, & Zimmet, 2005). Lupin

γ-Conglutin has been described to reduce plasma glucose concentration when orally administered to both rats and humans, thus exhibiting great potential in the control of glycaemia (Lovati et al., 2012). It is interesting to report that under our experimental conditions, the hypoglycemic properties of lupin protein still remain after hydrolysis of the abovementioned fraction during the sequential protease treatment carried out to prepare the lupin protein hydrolyzate. Such hypoglycemic activity exhibited by the lupin protein hydrolyzate can be explained based on the results by Morato et al. (2013) who found that the amino acid L-isoleucin and the peptide L-leucyl–L-isoleucin showed the greatest efficiency among different amino acids and dipeptides derived from whey protein in translocating the GLUT-4 glucose transporter from the cytosol to the plasma membrane, thus increasing glucose capture by skeletal muscle. Lupin protein hydrolyzate-based diets exhibited con-siderably higher content of Leu + Ile when compared to casein-based diets (Table 1). Furthermore, an extensive release of free amino acids and di- to penta-peptides takes place during the process of enzymatic protein digestion under our experimental conditions (Fig. 1B and C). Therefore, the process of protein hydrolysis by recombinant proteases may have induced the release of L-leucyl–L-isoleucin bioactive peptide and L-isoleucin. This bioactive substances act through mechanism that appear to be, at least in part, independent of insulin action, and could be of great relevance in the dietetic treatment of type II diabetes and related metabolic alterations like the metabolic syndrome.

4.3. Renal and large intestine function

The different urinary parameters measured were altered in the Casein/Cellulose-based high-fat diet group (HC) in what could be the initial stages of renal injury. Furthermore, the acidification of urine and increased urinary P excretion in combination with hypocitraturia that were observed under our experimental conditions in the above-mentioned experimental group have been linked to kidney stone formation (Amanzadeh et al., 2003). Urinary acidification, hypercalciuria, hyperphosphaturia, and hypocitraturia have been described in response to hyperproteic diets (Aparicio et al., 2011), but less is known about the effect of hypercholesterolemic diets. Noticeably, several alterations in renal histology, antioxidant status, and lipid metabolism have been related to such diet-induced hypercholesterolemia in animal models similar to that developed in the present study (Al-Rejaie, Abuohashish, Alkhamees, Aleisa, & Alroujayee, 2012). In this regard, lupin protein hydrolyzate did show enormous potential to restore all the altered urinary markers with the exception of urinary calcium that was higher in the HID and HIDF when compared to the high-fat Casein/Cellulose group.

Compared to a poorly fermentable insoluble fiber like cellulose, lupin insoluble fiber residue or the amount of soluble fiber present in the protein hydrolyzate showed the typical pattern of large intestine fermentation with hypertrophy of caecum and colon and acidification of caecum content (Younes, Demigné, & Rémésy, 1996). Intestinal fermentation of complex carbohydrates is known to generate SCFA (Henningsson et al., 2001) that may act as main energy substrate for colonocytes or in the regulation of lipid metabolism (Bouhnik et al., 2004). Furthermore, lupin insoluble fiber residue exhibited enhanced water holding capacity that has the potential to increase stool volume and confers this functional product with a greater capability to drag and eliminate secondary bile acids and other toxic substances. The physiological and proliferative effects of dietary fiber on caecum and colon are largely dependent on its variable chemical nature (Folino, McIntyre, & Young, 1995) and fermentable capacity by the intestinal microbiota (McCullough, Ratcliffe, Mandir, Carr, & Goodlad, 1998). An interesting finding of the present study is the differential effects on colon morphometry of the non-starch polysaccharides present in the two functional ingredients assayed. Soluble dietary fiber and α-galactoside oligosaccharides associated to lupin protein hydrolyzate exhibited a significant proliferative action due to its rapidly fermentable

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nature, whereas the dilution potential, bulking capacity, and lowering the luminal pH action predominated in the lupin insoluble fiber residue.

5. Conclusions

The efficacy shown by lupin protein hydrolyzate at reducing plasma and hepatic triglycerides as well as glycemia in our experimental model makes it an interesting and relevant nutritional component in the diet of patients with type II diabetes and/or metabolic syndrome. In addition, lupin protein hydrolyzate exhibited a protective effect against the initial stages of hypercholesterolemic high fat diet-induced renal alterations. Such health benefits can be complemented by Lupin protein hydrolyzate and insoluble fiber effects on large intestine physiological status due to its fermentative and water holding capacity. The dietary inclusion of both functional products has great potential in the prevention and treatment of lipid and glucose metabolism disorders in combination with adequate physical activity and pharmacological treatments.

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References

Alhaj, O. A., Kanekanian, A.D., Peters, A.C., & Tatham, A. S. (2010). Hypocholesterolemic effect of Bifidobacterium animalis subs. Lactis (Bb12) and trypsin casein hydrolysate. Food Chemistry, 123, 430–435.

Al-Rejaie, S. S., Abuohashish, H. M., Alkhamees, O. A., Aleisa, A.M., & Alroujayee, A. S. (2012). Gender difference following high cholesterol diet induced renal injury and the protective role of rutin and ascorbic acid combination in Wistar albino rats. Lipids in Health and Disease, 11, 41.

Amanzadeh, J., Gitomer, W. I., Zerwekh, J. E., Preisig, P. A., Moe, O. W., Pak, C., et al. (2003). Effect of high protein diet on stone-forming propensity and bone loss in rats. Kidney International, 64, 2142–2149.

Aparicio, V. A., Nebot, E., Kapravelou, G., Sánchez, C., Porres, J. M., López-Jurado, M., et al. (2011). Resistance training reduces the metabolic acidosis and hepatic and renal hypertrophy caused by the consumption of high protein diet in rats. Nutrición Hospitalaria, 26, 1478–1486.

Bettzieche, A., Brandsch, C., Eder, K., & Stangl, G. (2009). Lupin protein acts hypo-cholesterolemic and increases milk fat content in lactating rats by influencing the expression of genes involved in cholesterol homeostasis and triglyceride synthesis. Molecular Nutrition and Food Research, 53, 1134–1142.

Bettzieche, A., Brandsch, C., Weiße, K., Hirche, F., Eder, K., & Stangl, G. I. (2008). Lupin protein influences the expression of hepatic genes involved in fatty acid synthesis and triacylglycerol hydrolysis of adult rats. British Journal of Nutrition, 99, 952–962.

Bouhnik, Y., Attar, A., Joly, F. A., Riottot, M., Dyard, F., & Flourie, B. (2004). Lactulose ingestion increases faecal bifidobacterial counts: A randomised double-blind study in healthy humans. European Journal of Clinical Nutrition, 58, 462–466.

Chen, P.S., Toribara, T. Y., & Warner, H. (1956). Microdetermination of phosphorus. Analytical Chemistry, 28, 1756–1758.

Cheung, I. W. Y., Cheung, L. K. Y., Tan, N. Y., & Li-Chan, E. C. Y. (2012). The role of molecular size in antioxidant activity of peptide fractions from Pacific hake (Merluccius productus) hydrolysates. Food Chemistry, 134, 1297–1306.

Dabai, F. D., Walker, A. F., Sambrook, I. E., Welch, V. A., Owen, R. W., & Abeyasekera, S. (1996). Comparative effects on blood lipids and faecal steroids of five legume species incorporated into a semi-purified, hypercholesterolaemic rat diet. British Journal of Nutrition, 75, 557–571.

Doblado, R., Frias, J., & Vidal-Valverde, C. (2007). Changes in vitamin C content and antioxidant capacity of raw and germinated cowpea (Vigna sinensis var. carilla) seeds induced by high pressure treatment. Food Chemistry, 101, 918–923.

Eckel, R. H., Grundy, S. M., & Zimmet, P. Z. (2005). The metabolic syndrome. Lancet, 365, 1415–1428.

European Union Council (2010). Directional on the protection of animals used for scientific purposes. Official Journal of the European Union, 276, 33–79.

Folch, J., Lees, M., & Stanley, M. (1957). A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226, 497–509.

Folino, M., McIntyre, A., & Young, G. P. (1995). Dietary fibers differ in their effects on large bowel epithelial proliferation and fecal fermentation dependent events in rats. Journal of Nutrition, 125, 1521–1528.

Guadagnucci Fontanari, C., Batistuti, J. P., Cruz, R. J., Nascimiento Saldiva, P. H., & Gomes Arêas, J. A. (2012). Cholesterol lowering effect of whole lupin (Lupinus albus) seed and its protein isolate. Food Chemistry, 132, 1521–1526.

Gudbrandsen, O. A., Wergedahl, H., Liaset, B., Espe, M., & Berge, R. K. (2005). Dietary proteins with high isoflavone content or low methionine–glycine and lysine–arginine ratios are hypocholesterolaemic and lower the plasma homocysteine level in male Zucker fa/fa rats. British Journal of Nutrition, 94, 321–330.

Henningsson, A.M., Nyman, E. M. G. L., & Björck, I. M. E. (2001). Content of short-chain fatty acids in the hindgut of rats fed processed bean (Phaseolus vulgaris) flours varying in distribution and content of indigestible carbohydrates. British Journal of Nutrition, 86, 379–389.

Hermsdorff, H., Zulet, M.A., Abete, I., & Martinez, A. J. (2011). A legume-based hypocaloric diet reduces proinflammatory status and improves metabolic features in overweight/obese subjects. European Journal of Nutrition, 50, 61–69.

Howard, A., & Udenigwe, C. C. (2013). Mechanisms and prospects of food protein hydrolysates and peptide-induced hypolipidaemia. Food & Function, 4, 40–51.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685.

Lovati, M. R., Manzoni, C., Castiglioni, S., Parolari, A., Magni, C., & Duranti, M. (2012). Lupin seed gamma-conglutin lowers blood glucose in hyperglycaemic rats and increases glucose consumption of HepG2 cells. British Journal of Nutrition, 107, 67–73.

Lupton, J. R. (2004). Microbial degradation products influence colon cancer risk: The butyrate controversy. Journal of Nutrition, 134, 479–482.

Martinez-Villaluenga, C., Urbano, G., Porres, J. M., Frias, J., & Vidal-Valverde, C. (2007). Improvement in food intake and nutritive utilization of protein from Lupinus albus var. multolupa protein isolates supplemented with ascorbic acid. Food Chemistry, 103, 944–951.

Martinez-Villaluenga, C., Zielinski, H., Frias, J., Piskula, M., Kozlowska, H., & Vidal-Valverde, C. (2009). Antioxidant capacity and polyphenolic content of high-protein lupin products. Food Chemistry, 112, 84–88.

McCullough, J. S., Ratcliffe, B., Mandir, N., Carr, K. E., & Goodlad, R. A. (1998). Dietary fibre and intestinal microflora: Effects on intestinal morphometry and crypt branching. Gut, 42, 799–806.

Megías, C., Pedroche, J., Yust, M. M., Girón-Calle, J., Alaiz, M., Millán, F., et al. (2007). Affinity purification of copper chelating peptides from chickpea protein hydrolysates. Journal of Agricultural and Food Chemistry, 55, 3949–3954.

Morato, P. N., Lollo, P. C. B., Moura, C. S., Batista, T. M., Carneiro, E. M., & Amaya-Farfan, J. (2013). A dipeptide and an amino acid present in whey protein hydrolysate increase translocation of GLUT-4 to the plasma membrane in Wistar rats. Food Chemistry, 139, 853–859.

Nath, N., Wiener, R., Harper, A. E., & Elvehjem, C. A. (1959). Diet and cholesteremia: I. Development of a diet for the study of nutritional factors affecting cholesteremia in the rat. Journal of Nutrition, 67, 289–307.

National Research Council (1995). Nutrient requirements of the laboratory rat. Nutrient requirements of laboratory animals fourth revised edition. Washington, DC: National Academy Press.

Nishimura, N., Tanabe, H., Yamamoto, T., & Fukushima, M. (2011). Raw chinese yam (Dioscorea opposita) promotes cecal fermentation and reduces plasma non-HDL cholesterol concentration in rats. Journal of Nutritional Science and Vitaminology, 57, 340–347.

Parolini, C., Rigamonti, E., Marchesi, M., Busmelli, M., Cinquanta, P., Manzini, S., et al. (2012). Cholesterol-lowering effect of dietary Lupinus angustifolius proteins in adult rats through regulation of genes involved in cholesterol homeostasis. Food Chemistry, 132, 1475–1479.

Periago, M. J., Ros, G., Martínez, C., & Rincón, F. (1996). Variations of non-protein nitrogen in six Spanish legumes according to the extraction method used. Food Research International, 29, 489–494.

Porres, J. M., Aranda, P., Lopez-Jurado, M., & Urbano, G. (2003). Effect of natural and controlled fermentation on chemical composition and nutrient dialyzability from beans (Phaseolus vulgaris L.). Journal of Agricultural and Food Chemistry, 51, 5144–5149.

Porres, J. M., Aranda, P., López-Jurado, M., & Urbano, G. (2006). Nutritional evaluation of protein, phosphorus, calcium and magnesium bioavailability from lupin (Lupinus albus var. multolupa)-based diets in growing rats: Effect of α-galactoside oligosaccharide extraction and phytase supplementation. British Journal of Nutrition, 95, 1102–1111.

Prosky, L., Asp, N. G., Schweizer, T., De Vries, J. W., & Furda, I. (1992). Determination of insoluble and soluble dietary fiber in foods and food products: Collaborative study. Journal of the Association of Official Analytical Chemists, 75, 360–367.

Reeves, P. G., Nielsen, F. H., & Fahey, G. C. (1993). AIN-93G purified diets for laboratory rodents: Final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. Journal of Nutrition, 123, 1939–1951.

Roberts, P. R., Burney, J. D., Black, K. W., & Zaloga, G. P. (1999). Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. Digestion, 60, 332–337.

Spielman, J., Shukla, A., Brandsch, C., Hirche, F., Stangl, G. I., & Eder, K. (2007). Dietary lupin protein lowers triglyceride concentrations in liver and plasma in rats by reducing hepatic gene expression of sterol regulatory element-binding protein-1c. Annals of Nutrition and Metabolism, 51, 387–392.

Yoshie-Stark, Y., & Wäsche, A. (2004). In vitro binding of bile acids by lupin protein isolates and their hydrolysates. Food Chemistry, 88, 179–184.

Younes, H., Demigné, C., & Rémésy, C. (1996). Acidic fermentation in the caecum increases absorption of calcium and magnesium in the large intestine of the rat. British Journal of Nutrition, 75, 301–314.