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Satiating potential and anti-obesogenic effect of
Mediterranean foodstuffs. *In vitro* and
in vivo assessments

Capacidad saciante y efecto anti-obesogénico de
alimentos mediterráneos. Evaluación *in vitro* e
in vivo

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**Satiating potential and anti-obesogenic effect of
Mediterranean foodstuffs. *In vitro* and
in vivo assessments**



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SUMMARY

Recently, obesity has increased dramatically, and it has now reached epidemic proportions in both, developed and developing countries. In fact, obesity could become the most common health problem of the 21st century. In 2016, more than 1.9 billion adults were overweight and 650 million were obese, corresponding to 39% and 13% of world's adult population, respectively. The global importance of overweight and obesity is worrying, in part, due to their public health significance because they are associated with a higher risk of type 2 diabetes, coronary heart disease, certain cancers, and a shorten life expectancy. Although obesity is due to a multifactorial causation, an imbalance between calories ingested and expended for an extended period, together with a sedentary lifestyle, are the major causes. Regulation of food intake by enhancing satiety and the promotion of a healthy life style shape the key to mitigate this epidemic. Moreover, the adherence to a healthy diet together with the inclusion into the diet of ingredients capable to promote satiety are the two key strategies to prevent and treat overweight and obesity.

Regarding with the first point of the equation, an example quite representative of healthy diet is the Mediterranean diet. Several studies have proved the effect of this diet on body weight reduction, especially when it is energy-restricted and associated with physical exercise. Recently, it has showed that the adherence to a Mediterranean diet was associated to a reduced risk to develop overweight and obesity, being related to reduced body weight and related chronic diseases risk.

However, and focusing in satiety, the mechanisms involved in the regulation of food intake are very complex and not-dependent of a single action mechanism. In fact, several mechanisms such as internal and external factors regulate satiety and hence food intake. Some of them are macronutrient related and others are related to hormones and afferent signals, such as insulin, glucagon-like peptide 1 (GLP-1) and leptin, among others. Nowadays, most weight lost diets fail, mainly because of hunger feeling, so

enhancing satiety could be a promising tool to help decrease food intake and therefore prevent and treat obesity and overweight. Certain ingredients are able to induce satiety and can be considered as functional agents. These could produce a decrease of appetite and food intake leading to a negative energy balance and obesity control. It has been extensively studied that several ingredients can modulate the appetite and enhance satiety by releasing several hormones and gut peptides with anorexigenic activity such as leptin, insulin, GLP-1, PYY, CCK, etcetera, or through suppression of orexigenic hormones as ghrelin.

On the basis of this background, this manuscript has been developed with the objective to identify and to evaluate the satiety potential of several ingredients consumed usually in the Mediterranean diet. As well, this doctoral dissertation has been divided into three parts, attending to the methodology used (*in vitro*, lab-scale and *in vivo*) and classified into seven chapters with different goals but sharing the same main aim, satiety.

In the **first chapter**, it has been described the state of the art of satiety and satiation, as well as the satiety potential of each macronutrients (proteins, carbohydrates and fats) and different Mediterranean foodstuffs, because their satiety effectiveness. Main conclusion of this chapter revealed that satiety and satiation are consequence of a complex system not controlled by a single actor. They are regulated by meal quantity and quality, sensations and experiences, central nervous system signals, hormone satiety signals, etc. Other conclusion of this chapter demonstrated that protein is the most satiating macronutrient due to its composition in peptides and amino acids that led to enhance satiety signals and thermogenesis. Furthermore, revised literature exerted that whey protein can be an excellent strategy to enhance satiety because its protein quality and digestion, which triggers satiating peptides, among other features. In addition, it has been studied the anti-obesogenic and satiating features of many Mediterranean active compounds or ingredients (hesperetin, *capsicum*, pistachio, carob pulp...), being eventually selected to further studies whey, egg white, tiger nut and nopal.

Next chapters (2, 3, 4 and 5) have been designed to know the composition of different types of whey milk (from cow, sheep, goat and a mixture of them) through a simulated digestion, as well as to study the *in vitro* potential of them as anti-inflammatory, secretagogue (satiating hormone release) and modulator of gut microbiota.

Particularly, main goal of **chapter 2** has been to perform an initial characterisation of nutritional components of whey and its peptidomic profile, as start point to understand how this nutritional composition could affect different physiological effects studied in next chapters. Furthermore, the degree of protein hydrolysis and the potential effects of biopeptides on human health have been evaluated after performing a whole simulated *in vitro* digestion inspired in that proposed by COST Action INFOGEST, followed by a colonic fermentation for 24 hours. Results and conclusions of this chapter have shown that exist differences in the nutritional composition of several types of whey. Concretely, sheep whey was the richest one in protein content, meanwhile goat milk whey yielded the highest concentration of lactose. In addition, and after a simulated digestion and 24-h of fermentation, whey proteins from goat showed a high digestibility, meanwhile, proteins from cow whey were more resistant to gastro intestinal digestion. After a whole digestion, several biopeptides was found showing interesting biological effects such as antihypertensive, satiating, antidiabetic and antioxidant/anti-inflammatory. Sheep whey elicited the highest amount of biopeptides with antihypertensive potential. Moreover, sheep followed by goat whey generated a high variety of satiating biopeptides, which demonstrated potential effectiveness for managing food intake and preventing obesity.

It is well-known that obesity is closely linked to gut health and immune system. In particular, a low-grade of systemic inflammation, with implications in the development of metabolic syndrome and insulin resistance, has been related to obesity. Moreover, several studies have demonstrated that the low-grade of gut inflammation would precede the low-grade of systemic inflammation related to diet induced obesity, since pro-inflammatory markers such as interleukins and TNF- α have been identified after high-fat diets in the gut. With the aim to assay the anti-inflammatory capacity of several types of whey, **chapter 3** was developed by using an *in vitro* co-culture model

of gut inflammation. For this purpose, the co-culture Caco-2 and RAW 264.7 cell lines and LPS/ TNF- α induced inflammation through a whole *in vitro* digestion (from mouth to colon) was designed. After 4 h of incubation with several digested or fermented whey, the secretion of IL-6, IL-8 and TNF- α of stimulated Caco-2 and RAW 264.7 cell was investigated as biomarkers of low-grade of inflammation related to metabolic disease. Additionally, the integrity of tight junction cell barrier was assessed by measuring the transepithelial electrical resistance (TEER). Main results and conclusions of this chapter have demonstrated the potential of digested and fermented whey from cow, sheep, goat and mixture to minimize the *in vitro* inflammation. All digested and fermented samples showed a protective effect in cell barrier permeability, being lesser the effect in the case of fermented samples of goat and mixture whey. Fermented whey elicited the highest anti-inflammatory potential by reducing dramatically the secretion of IL-8 and TNF- α , mainly, due to their protein breakdown products as peptides and amino acids, and short chain fatty acids (SCFA) produced by gut microbiota. Digested samples from whey and even more, fermented, mostly from cow and sheep whey milk, could be used as a nutritional strategy to preserve the gut barrier integrity and to mitigate the low-grade of local and systemic inflammation related to obesity and metabolic disorders.

Chapter 4 has been designed to know the role as secretagogue of several types of raw whey and after a whole digestion (from mouth to 24h of colon) on two satiating peptides (CCK and GLP-1) releasing. The intestinal tumour cell line, STC-1, was used to know the grade in which whey, and its metabolites (derived from digestion and fermentation), triggers the secretion of satiating gut peptides such as CCK and GLP-1. After 2 h of incubation with raw, digests and fermented samples of whey milk, differences in the hormone release according to the phase of digestion was found. Whey, after small intestine digestion showed the highest satiating potential due to its protein degradation, followed by fermented samples, which caused the satiating effect through the synergistic effect of SCFA and protein breakdown products. The least satiating effect was found in undigested whey, suggesting that intact whey protein is not a good satiating candidate, compared to digested proteins. Focusing on the potential of each type of whey according to stage of digestion, undigested sheep whey showed the highest GLP-1 releasing. After digestion, goat whey could be the best stimulator for

GLP- 1 secretion, maybe due to its peptide profile. Under fermentation stage, mixture whey was the most secretagogue of CCK, although in this case it cannot be attributed the satiating effect to specific component of whey. In general terms, the additive effect of protein, lactose and SCFA could potentiate the global satiating effect of whey through *in vitro* gastro intestinal digestion.

The interactions between genetic and environment factors such as diet, style of life and physical activity are the main cause to develop overweight and obesity, but it is not totally explained by these factors. Recently, it has been proposed gut microbiota, the bacteria community that reside in gastrointestinal tract, as a key point in this equation. An altered gut microbiota composition could be previous status to develop metabolic disorders such as obesity and diabetes, inflammatory bowel diseases and colon cancer. In fact, it has been described a gut microbiota associated to obesity and normal-weight stages. Microbiota related to obesity is characterised by a low diversity of bacteria population with increases of *Firmicutes* and decreases in *Bacteroidetes*, meanwhile normal-whey microbiota profile is rather than linked to increases in *Bifidobacterium animalis*, *Methanobrevibacter smithii* and phylum *Bacteroidetes*. Moreover, SCFAs derived from bacterial fermentation has been associated to multiple biological activities, being the most interesting metabolites produced by gut microbiota such as regulation of energy intake and energy metabolism. Taking into account those relevant findings, in **chapter 5** it has been assessed the qualitative and quantitative differences of the gut microbiota, from normal-weight and obese human donors, in response to different types of whey milk. The composition of the different bacterial genus including SCFA and BCFA production were investigated by using an *in vitro* digestion followed by a stirred and non-pH controlled faecal batch culture fermentation for 48 hours. Results and main conclusions of that chapter demonstrated that whey milk from cow and sheep, and also mixture, had a prebiotic effect on the gut microbiota of normal-weight and obese donors during batch culture fermentation. These types of whey stimulated the growth of probiotic bacteria, demonstrating a marked bifidogenic effect in both groups of donors. Moreover, whey fermentation led to the production of a wide variety of SCFAs and BCFAs, especially in obese donors, which could be beneficial for colonic health. Supplementing diet with these types of whey can be a promising strategy to modulate the gut microbiota and to stimulate selectively the growth of probiotic bacteria. It is

worth mentioning that chapter 5 has been published in an indexed journal (Food&Function) (doi: 10.1039/c7fo00197e).

Chapter 6, differently previous chapters developed using *in vitro* methodology, was conducted as a lab-scale experiment. It was designed and carried out with the collaboration of Dr. M^a Asunción Hidalgo Montesinos belonging to the Department of Chemical Engineering of the Faculty of Chemistry of University of Murcia. Ultrafiltration technology is a suitable and ecological alternative to revalorise whey by separating main components of whey milk: lactose and protein. After that, protein could be concentrated to obtain a satiating ingredient based on high protein concentration. Sheep whey has a protein fraction richer than other types of whey, such as cow and goat, and it could be an excellent ingredient to enhance satiety, both because its protein fraction and the revalorisation of a by-product usually wasted in the dairy industry. Because of that, chapter 6 has the objective of optimising the ultrafiltration technology (varying pressure, lactose concentration and ultrafiltration membranes) on lactose separation and protein rejection in sheep whey. For this purpose, three ultrafiltration membranes with different composition and molecular weight cut-off were used (GR60PP, GR80PP and GR90PP). Moreover, the viability of the Spiegler-Kedem-Kachalsky model to predict the rejection of lactose with the different membranes was assayed. Main results revealed that lactose separation was not affected by pressure in GR60PP and GR90PP, removing 85% and 80% of mentioned disaccharide, respectively. In addition, when feeding concentration increased, lactose removing was stable in three membranes, being GR60PP the most efficient one, with a 90% of disaccharide removal. In all cases 100% of protein recovery was obtained. Finally, the Spiegler-Kedem-Katchalsky model could be used to predict accurately the effect of different experimental conditions on the separation of lactose and protein from sheep whey using ultrafiltration being a suitable tool to predict the permeation pattern for a given membrane and whey.

Finally, **chapter 7** had the main objective of evaluating the satiating and anti-obesogenic effect of several ingredients from Mediterranean regions: goat whey protein, egg white protein, tiger nut and nopal. The experiment was conducted by using obese C57BL/6J mice, fed under 11 weeks with different types of diet based on that

ingredients. Particularly, two high-protein diets were designed, based on whey or egg protein and two high-fibre diets formulated with tiger nut and nopal. Several satiety markers, such as nocturnal feeding pattern, cumulative food and energy intake, and circulating satiating peptides, have been determined to know deeply the effectiveness suppressing hunger of each ingredient. In addition, it was investigated whether the consumption of selected ingredients could revert the “obese phenotype” previously induced to animals. With this purpose several parameters related to energy homeostasis and obesity has been analysed, such as energy expenditure, blood lipids, organs morphology and body composition. Main conclusions derived from this chapter demonstrated the satiating potential and the ability to revert an obese condition of several Mediterranean ingredients (whey, egg white, tiger nut and nopal). Obese mice fed with high-protein diets (goat whey and egg white) and nopal exhibited decreases in food and energy intake after over 11 weeks of treatment. Body weight and total fat storage were notably reduced after whey, egg and nopal consumption and maintained over the time. Food intake also may be related to promote satiety sensations, as well as diuresis could be had an additive effect in the exacerbate body weight reduction of nopal group. However, the satiating and anti-obesogenic effect of tiger nut was only maintained in a short-term, showing final BW reductions lower than in the case of other Mediterranean ingredients. All these findings have been supported by a complex mechanism that included increases in energy expenditure, the analysis of feeding pattern after a meal and the circulating satiating or orexigenic hormones in each group. In general terms, two high-protein diets have showed satiety effectiveness, the feeding pattern and hormone secretion revealed that whey induced more satiety than satiation and the contrary in case of egg. Moreover, high-fibre diet based on nopal presented a similar satiating and anti-obesogenic features than goat whey proteins. Finally, all experimental diets provoked improvements of blood lipids related to obese stage demonstrating the capacity to alleviating cholesterol levels, among other blood parameters. Although all of Mediterranean diets tested in obese mice over 11 weeks have demonstrated beneficial effect on satiety and body weight management, whey, egg white and nopal exhibited promising effect on regulating markers related to obesity and satiety signals.

RESUMEN

Recientemente la obesidad se ha incrementado dramáticamente, alcanzando proporciones epidémicas, tanto en países desarrollados como en vías de desarrollo. Ciertamente, la obesidad puede llegar a ser el problema de salud pública más común del siglo XXI. En 2016 más de 1.9 mil millones de adultos tenían sobrepeso y 650 eran obesos, correspondiendo al 39% y 13% de la población mundial adulta, respectivamente. La importancia global de sobrepeso y obesidad es preocupante, en parte debido a su magnitud en salud pública, ya que están asociados con un alto riesgo de padecer diabetes tipo 2, enfermedad coronaria, ciertos tipos de cánceres y con una corta esperanza de vida. Aunque la obesidad es debida a causas multifactoriales, un desequilibrio entre las calorías ingeridas y las gastadas, durante un largo periodo de tiempo, junto con un estilo de vida sedentario, son sus mayores causas. La regulación de la ingesta de alimentos, aumentando la saciedad y promoviendo un estilo de vida saludable podrían ser claves para mitigar esta epidemia. Además, la adherencia a una dieta saludable, junto con la introducción en la dieta de ingredientes capaces de aumentar la saciedad son dos estrategias clave para prevenir y tratar sobrepeso y obesidad.

En relación con el primer punto de esta ecuación, un ejemplo bastante representativo de dieta saludable es la dieta mediterránea. Varios estudios han demostrado el efecto de esta dieta sobre la reducción del peso corporal, especialmente cuando la restricción de energía se asociaba con ejercicio físico. Recientemente ha sido puesto de manifiesto que la adherencia a la dieta mediterránea se relacionó con una reducción del riesgo de desarrollar sobrepeso y obesidad, estando asociada con una reducción del peso corporal y con un menor riesgo de padecer enfermedades crónicas relacionadas.

Sin embargo, y centrándonos en saciedad, los mecanismos involucrados en la regulación de la ingesta de alimentos son muy complejos y no solamente se explican por un solo mecanismo de acción. De hecho, varios mecanismos como factores externos e internos regulan la saciedad, y por lo tanto la ingesta de alimentos. Algunos de ellos están relacionados con los macronutrientes, mientras que otros se relacionan con hormonas y

señales aferentes como son insulina, péptido similar a glucagón-1 (GLP-1) y leptina, entre otros. Hoy en día, muchas dietas para perder peso fracasan, principalmente por la sensación de hambre, por lo que el aumento de la saciedad podría ser una herramienta prometedora que podría ayudar a disminuir la ingesta, prevenir y tratar el sobrepeso y la obesidad. Ciertos ingredientes son capaces de inducir saciedad, pudiendo ser considerados como agentes funcionales. Estos ingredientes podrían disminuir el apetito y la ingesta de alimentos, llevando a un balance energético negativo, lo que controlaría la obesidad. Se ha estudiado extensamente que ciertos ingredientes pueden modular el apetito y aumentar la saciedad, induciendo la secreción de hormonas saciantes y péptidos intestinales con actividad anorexígena como son la leptina, insulina, GLP-1, PYY, CCK, etcétera, o bien a través de la supresión de hormonas orexígenas como es grelina.

En base a estos antecedentes, este manuscrito ha sido desarrollado con el objetivo de identificar y evaluar el poder saciante de varios ingredientes consumidos normalmente en la dieta mediterránea. Además, esta tesis doctoral ha sido dividida en tres partes, según la metodología empleada (*in vitro*, en planta piloto e *in vivo*), y clasificada en siete capítulos con diferentes objetivos, pero compartiendo la misma finalidad, la saciedad.

En el **primer capítulo** ha sido descrito el estado del arte de saciedad y saciación, así como el potencial saciante de cada macronutriente (proteínas, carbohidratos y grasas), y de diferentes ingredientes mediterráneos dada su eficacia saciante. La principal conclusión de este capítulo fue que saciedad y saciación son consecuencia de un complejo sistema que no es únicamente controlado por un actor. Ambas están reguladas por la calidad y cantidad del alimento, sensaciones y experiencias, señales en el sistema nervioso central, señales hormonales, etc. Otra conclusión de este capítulo demostró que la proteína es el macronutriente más saciante debido a su composición en péptidos y aminoácidos, los cuales dan lugar al aumento de señales saciantes y termogénesis. Además, la bibliografía revisada mostró que la proteína de suero de leche puede ser una excelente estrategia para aumentar la saciedad, por su calidad proteica y digestión, lo que pueden dar lugar a desencadenar la liberación de péptidos saciantes, entre otras características. Adicionalmente, se han estudiado el efecto anti-obesogénico y saciante de varios ingredientes o compuestos activos de origen mediterráneo (hesperetina, *capsicum*,

pistacho, algarroba...), siendo finalmente seleccionados para futuros estudios el suero, la clara de huevo, la chufa y el nopal.

Los siguientes capítulos (2, 3, 4 y 5) han sido diseñados para conocer la composición de diferentes tipos de suero de leche (de vaca, oveja, cabra y una mezcla de ellos), mediante una digestión simulada, así como el estudio *in vitro* del potencial antiinflamatorio, estimulador de hormonas saciantes y modulador de la microbiota intestinal.

Concretamente, en el **capítulo 2** el principal objetivo ha sido realizar una caracterización inicial del suero de leche y su perfil peptidómico, como puntos de partida para entender cómo la composición nutricional del suero de leche puede afectar a los diferentes efectos fisiológicos estudiados en los siguientes capítulos. Además, el grado de hidrólisis proteica y el potencial efecto para la salud de los biopéptidos liberados del suero han sido evaluados tras llevar a cabo una completa digestión simulada, inspirada en la acción COST de INFOGEST, seguida de una fermentación colónica durante 24 horas. Los resultados y conclusiones de este capítulo mostraron que existían diferencias en la composición nutricional de los diferentes tipos de suero de leche. Concretamente, el suero de oveja mostró ser el más rico en proteína, mientras que el suero de cabra presentó la mayor concentración en lactosa. Además de ello, y tras la simulación de una digestión y fermentación de 24 h, las proteínas de suero de leche de cabra resultaron ser más digestibles, mientras que las de vaca fueron más resistentes a la digestión gastrointestinal. Tras una digestión completa, varios biopéptidos fueron identificados mostrando interesantes efectos como son antihipertensivos, saciantes, antidiabéticos, antioxidantes y antiinflamatorios. El suero de oveja produjo la mayor cantidad de biopéptidos con potencial antihipertensivo. Además, el suero de oveja, seguido del de cabra, generaron una gran variedad de biopéptidos saciantes, los cuales han demostrado tener una potencial eficacia para en control de la ingesta de alimentos y prevención de obesidad.

Es bien conocido el hecho de que la obesidad está estrechamente relacionada con la salud intestinal y el sistema inmune. En particular, un bajo grado de inflamación sistémica con implicaciones en el desarrollo de síndrome metabólico y resistencia a la insulina, han sido relacionados con la obesidad. Además, varios estudios han demostrado

que ese bajo grado de inflamación está relacionado con una inducción a obesidad por la dieta, ya que marcadores proinflamatorios como interleucinas y TNF- α han sido identificados en el intestino después del consumo de dietas altas en grasa. Con el objetivo de determinar la capacidad antiinflamatoria de distintos tipos de suero de leche, el **capítulo 3** fue desarrollado mediante el uso de un modelo de inflamación intestinal *in vitro*. Con ese propósito, fueron empleados el co-cultivo de líneas celulares Caco-2 y RAW 264.7, y una inflamación inducida con LPS/ TNF- α , a través de una digestión *in vitro* (de boca hasta colon). Tras 4 horas de incubación con distintos sueros digeridos o fermentados, la secreción de IL-6, IL-8 and TNF- α provocados por la estimulación de las líneas celulares Caco-2 y RAW 264.7 fueron investigadas como biomarcadores de ese bajo grado de inflamación asociado con enfermedades metabólicas. Además, la integridad de la barrera celular fue evaluada mediante la medida de la resistencia eléctrica transepitelial (TEER). Los principales resultados y conclusiones de este capítulo demostraron el potencial de los sueros digeridos y fermentados de vaca, oveja, cabra y mezcla, para minimizar la inflamación *in vitro*. Todos los digeridos y fermentados mostraron un efecto protector de la permeabilidad de la barrera celular, siendo menor el efecto protector en el caso de los sueros fermentados de cabra y los de mezcla. Los sueros fermentados mostraron el mayor efecto antiinflamatorio, reduciendo dramáticamente la secreción de IL-8 y TNF- α , debido principalmente a los productos derivados de la ruptura proteica como son los péptidos, los aminoácidos y los ácidos grasos de cadena corta (AGCC) producidos por la microbiota intestinal. Los digeridos de suero y más concretamente, los fermentados de vaca y oveja, podrían ser usados como estrategia nutricional para preservar la integridad de la barrera celular intestinal y mitigar ese bajo grado de inflamación, local o sistémica, relacionada con obesidad y desórdenes metabólicos.

El **capítulo 4** ha sido diseñado para conocer el papel estimulante en la secreción de dos péptidos saciantes (CCK y GLP-1) de diferentes tipos de sueros crudos, así como tras una completa digestión (desde la boca hasta 24 h de colon). La línea celular proveniente de tumores intestinales, STC-1, fue empleada para conocer en qué grado el suero de leche y sus metabolitos (derivados tras una digestión y una fermentación), desencadenan la secreción de péptidos intestinales saciantes como son CCK y GLP-1. Tras 2 horas de incubación con diferentes sueros de leche crudos, digeridos y

fermentados, se encontraron diferencias en la secreción de hormonas, dependiendo de la fase de digestión. El suero de leche tras una digestión de intestino delgado mostró la mayor capacidad saciante debido a su degradación proteica, seguido de las muestras de suero fermentadas, las cuales debieron su capacidad saciante al efecto sinérgico de los AGCC y a los productos derivados de la ruptura proteica. El menor efecto saciante fue encontrado en las muestras de suero crudas, lo que sugiere que la proteína intacta no es un buen candidato saciante, comparado con la proteína digerida. Centrándonos en el potencial saciante de cada uno de los tipos de suero y según el estadio de la digestión, las muestras de suero de oveja crudo provocaron más liberación de GLP-1. Tras la digestión, el suero de cabra fue el mayor estimulador de GLP-1, debido a su perfil peptídico. Bajo condiciones de fermentación, el suero de leche de mezcla fue el mayor estimulante de CCK, aunque ese efecto no pudo ser atribuido a ningún componente específico del suero. En términos generales, el efecto aditivo de las proteínas, de la lactosa y de los AGCC podrían haber sido los responsables de potenciar el efecto saciante global del suero de leche a través de una digestión gastrointestinal *in vitro*.

Las interacciones entre factores genéticos y ambientales como son la dieta, el estilo de vida y la actividad física, son las principales causas de sobrepeso y obesidad, pero no son totalmente explicadas por esos factores. Recientemente ha sido propuesto que la microbiota intestinal, la comunidad bacteriana que reside en el tracto gastrointestinal, es el principal punto clave en esta ecuación. Una microbiota alterada en su composición puede ser el estadio previo al desarrollo de desórdenes metabólicos como son obesidad, diabetes, enfermedad inflamatoria intestinal y cáncer de colon. De hecho, ha sido descrita una microbiota intestinal asociada a obesidad así como a estados de normo peso. Microbiota correspondiente a estadios de obesidad es caracterizada por un bajo grado de diversidad, con aumentos en *Firmicutes* y reducciones en *Bacteroidetes*, mientras que un perfil de microbiota propia de estadios normo peso está asociada con incrementos en poblaciones bacterianas como son *Bifidobacterium animalis*, *Methanobrevibacter smithii* y el filo *Bacteroidetes*. Además, los AGCC derivados de la fermentación bacteriana han sido relacionados con múltiples actividades biológicas, siendo una de las más interesantes la relacionadas con la regulación de la ingesta energética y el metabolismo energético. Teniendo en cuenta estos hallazgos, en el **capítulo 5** han sido evaluadas las diferencias cualitativas y cuantitativas en la microbiota de donantes humanos con normo peso y con

obesidad, en respuesta a los distintos tipos de sueros de leche. La composición de diferentes géneros bacterianos, así como la producción de AGCC y ácidos grasos de cadena ramificada (AGCR), fueron investigados mediante el empleo de una digestión *in vitro* seguida de una fermentación fecal (en agitación y con pH no controlado) durante 48 h. Los resultados y principales conclusiones de este capítulo demostraron que el suero de leche de vaca, oveja y de mezcla tuvieron un efecto prebiótico sobre la microbiota de los donantes con normo peso y con obesidad durante la fermentación fecal. Estos tipos de suero estimularon el crecimiento de bacterias probióticas, demostrando el marcado efecto bifidogénico de éstos en ambos grupos de donantes. Además, la fermentación del suero llevó a la producción de gran variedad de AGCC y AGCR, especialmente en el caso de los donantes obesos, lo cual podría tener efectos beneficiosos para la salud intestinal. La suplementación con estos tipos de suero puede ser una prometedora estrategia para modular la microbiota intestinal y para estimular de forma selectiva el crecimiento de bacterias probióticas. Merece la pena destacar que este capítulo ha sido publicado en una revista indexada (Food&Function) (doi: 10.1039/c7fo00197e).

El **capítulo 6**, de forma diferente a los capítulos anteriores desarrollados siguiendo una metodología *in vitro*, ha sido llevado a cabo en planta piloto. Fue diseñado y llevado a cabo con la colaboración de la Dra. M^a Asunción Hidalgo Montesinos, perteneciente al departamento de Ingeniería Química de la facultad de Química de la Universidad de Murcia. La tecnología de ultrafiltración es una alternativa útil y ecológica para revalorizar el suero, mediante la separación de sus principales componentes: lactosa y proteína. Tras el uso de esta tecnología, la proteína puede ser concentrada para obtener un ingrediente saciante basado en una alta concentración proteica. El suero de leche de oveja presenta una fracción proteica superior a otros tipos de suero como es el caso del suero de vaca y de cabra, y podría ser un excelente ingrediente que aumentase la saciedad, debido a su fracción proteica, además de la revalorización de un subproducto usualmente desechado en la industria láctea. Por todo ello, el capítulo 6 ha tenido el objetivo de optimizar la tecnología de ultrafiltración (variando la presión, concentración de lactosa y membranas de ultrafiltración) en la separación de la lactosa y en el rechazo de proteína de suero de leche de oveja. Con este propósito, tres membranas de ultrafiltración, siendo diferentes en su composición y en corte de peso molecular fueron evaluadas (GR60PP, GR80PP y GR90PP). Además, la viabilidad del modelo de predicción de rechazo de lactosa,

Spiegler-Kedem-Kachalsky, con las diferentes membranas fue evaluado. Los principales resultados revelaron que la separación de lactosa no se vio afectada por la presión en las membranas GR60PP y GR90PP, extrayéndose el 85% y el 80% del mencionado disacárido, respectivamente. Además, cuando la concentración en la fuente de alimentación se aumentaba, la separación de lactosa se mantuvo estable en el caso de las tres membranas, siendo la membrana GR60PP la más eficiente, con un 90% de separación del disacárido. En todos los casos el 100% de proteína fue separada. Finalmente, el modelo Spiegler–Kedem–Katchalsky pudo ser empleado para predecir, de forma muy precisa, el efecto de las diferentes condiciones experimentales en la separación de lactosa y proteína del suero de leche de oveja mediante el empleo de ultrafiltración, mostrando ser una herramienta útil para predecir el patrón de permeado para una membrana y suero dados.

Finalmente, el **capítulo 7** tuvo como principal objetivo el de evaluar el efecto saciante y anti-obesogénico de diferentes ingredientes típicos de regiones mediterráneas como son la proteína de suero de cabra, la proteína de clara de huevo, la chufa y el nopal. El experimento fue llevado a cabo mediante el empleo de ratones C57BL/6J obesos, alimentados durante 11 semanas con diferentes dietas experimentales basadas en esos ingredientes. Concretamente fueron diseñadas dos dietas altas en proteínas, basadas en proteína de suero y en proteína de huevo, y dos dietas altas en fibra, formuladas con chufa y nopal. Diversos marcadores saciantes como son el comportamiento alimentario nocturno, la ingesta de alimentos e ingesta energética y los péptidos saciantes circulantes fueron determinados, para poder conocer en profundidad la eficiencia como supresores del hambre de cada uno de los ingredientes. Además de ello, también fue investigado si el consumo de esos ingredientes seleccionados podría revertir el “fenotipo obeso” inducido previamente a los animales. Con ese propósito, varios parámetros relacionados con la homeostasia energética y la obesidad fueron analizados, como son el gasto energético, los lípidos sanguíneos, la morfología de los órganos y la composición corporal. Las principales conclusiones obtenidas de este capítulo demostraron el potencial saciante y la habilidad para revertir la condición obesa de los diferentes ingredientes mediterráneos (suero, clara de huevo, chufa y nopal). Los ratones obesos alimentados con dietas elevadas en proteínas (de suero de cabra y de huevo) así como los alimentados con nopal, mostraron reducciones en la ingesta alimenticia y energética tras 11 semanas de

tratamiento. La composición corporal y la grasa acumulada fue notablemente menor tras el consumo de suero, clara de huevo y nopal y mantenida durante el tiempo. Además, la reducción de la ingesta estuvo relacionada con sensaciones saciantes, así como con un efecto diurético, lo cual puede tener un efecto aditivo en la exacerbada reducción de peso mostrada por el grupo alimentado con nopal. Sin embargo, el efecto saciante y anti-obesogénico de la chufa sólo fue mantenido a corto plazo, mostrando reducciones en el peso corporal final menores que en el caso de otros ingredientes. Todos estos hallazgos fueron debidos a un complejo mecanismo que incluyó incrementos en el gasto energético, el análisis del comportamiento alimentario y el estudio de las hormonas circulantes saciantes u orexígenas dependiendo de cada grupo. En general, las dos dietas altas en proteína mostraron eficacia saciante, el comportamiento alimentario y la secreción hormonal revelaron que el suero indujo más saciedad que saciación, y lo contrario para el caso de la proteína de huevo. Además de ello, la dieta alta en fibra basada en nopal presentó similares características saciantes y anti-obesogénicas que la proteína de suero de cabra. Finalmente, todas las dietas experimentales provocaron mejoras en los lípidos sanguíneos relacionados con estadios obesos, demostrando la capacidad para atenuar los niveles de colesterol, de entre varios parámetros sanguíneos determinados. Aunque todos los ingredientes mediterráneos evaluados durante 11 semanas en ratones obesos han demostrado tener efectos beneficiosos en saciedad y control de peso, el suero, la clara de huevo y el nopal mostraron prometedores efectos en la regulación de marcadores relacionados con la obesidad y señales saciantes.

LIST OF ABBREVIATIONS

AA: amino acids

ACE: angiotensin-converting-enzyme

Aw: solvent permeability (s/m)

Awf: final solvent permeability (s/m)

Awo: initial solvent permeability (s/m)

BAT: brown adipose tissue

BCAA: branched-chain amino acids

BCFA: branched-chain fatty acid

BMI: body mass index

BSA: bovine serum albumin

CCK: cholecystokinin

Cf: concentration in the feed stream (kg/m^3)

Cp: concentration in the permeate (kg/m^3)

CT: computerised tomography

CV: coefficient of variation

DIO: diet-induced obesity

DIT: diet-induced thermogenesis

DMEM: dulbecco's modified eagles medium

DPP4: dipeptidyl-peptidase 4

EE: energy expenditure

EEC: enteroendocrine cell

EIA: enzyme immunoassay

Fw: fouling parameter (%)

GC: gas chromatography

GIP: glucose-dependent insulintropic polypeptide

GLP-1: glucagon-like peptide-1

GMP: glycomacropeptide

GOS: galacto-oligosaccharide

HDL-C: high density lipoproteins cholesterol

HPLC: high-performance liquid chromatography

HPLC-MS: high-performance liquid chromatography coupled to mass spectrometry

HU: hounsfield units

ICP-MS: inductively coupled plasma mass spectrometry

Ig: immunoglobulin

IL: interleukin

IMI: inter-meal interval duration (min)

Jp: permeate flux ($\text{kg}/\text{m}^2\text{s}$)

Js: solute flux ($\text{kg}/\text{m}^2\text{s}$)

Jw: solvent flux ($\text{kg}/\text{m}^2\text{s}$)

kDa: kilodalton

LDL-C: low density lipoproteins cholesterol

LNAA: large neutral amino acids

LP: lactoperoxidase

LPS: lipopolysaccharide

LZ: lysozyme

MBM: minimum basal medium

MFI: median fluorescent intensity

MWCO: molecular weight cut-off (kDa)

NCBI: national center for biotechnology information

NF: nanofiltration

OD: optical density

P: pressure

PBS: phosphate buffered saline

PCR: polymerase chain reaction

Ps: solute permeability (s/m)

R_{exp}: experimental rejection (%)

R_{obs}: observed rejection (%)

R: membrane rejection (%)

r²: statistical correlation coefficient

RID: refractive index detector

RO: reverse osmosis

RP-HPLC: reversed-phase high-performance liquid chromatography

RQ: respiratory quotient

SCFA: short-chain fatty acid

SG: specific gravity

SKK: Spiegler-Kedem-Kachalsky

SPI: scored peak intensity

STC-1: intestinal secretin tumour cell line 1

T^a: temperature (°C)

TC: total cholesterol

TEER: transepithelial electrical resistance

TNF- α : tumour necrosis factor-alpha

UF: ultrafiltration

VAS: visual analogue scale

VOI: volume of interest

WAT: white adipose tissue

WPC: whey protein concentrate

WPI: whey protein isolated

α -LA: alpha-lactalbumin

β -LG: beta-lactoglobulin

ΔP : hydraulic pressure across the membrane (Nw/m²)

$\Delta \Pi$: osmotic pressure (Nw/m²)

σ : reflection coefficient (dimensionless)

*General introduction. Satiating potential of macronutrients
and Mediterranean foodstuffs. CHAPTER 1*



1. INTRODUCTION

Obesity has increased dramatically during the past decades and it has now reached epidemic proportions in both, developed and developing countries. In fact, obesity could become the most common health problem of the 21st century. In 2016, more than 1.9 billion adults were overweight and 650 million were obese, corresponding to 39% and 13% of world's adult population, respectively (WHO, 2018).

In Europe, the prevalence of obesity in men ranges from 4.0% to 28.3% and in women from 6.2% to 36.5% with considerable geographic variation. Prevalence rates in Central, Eastern, and Southern Europe are higher than those in Western and Northern Europe (Berghoefer *et al.*, 2008). If the observed trends persist, by 2030 the absolute number of obese could rise to a total of 1.12 billion, reaching 20% of the world's adult population (Kelly *et al.*, 2008).

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. The World Health Organization (WHO) defined overweight as having a body mass index >25 kg/m², meanwhile obesity starts with ≥ 30 kg/m². Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify overweight and obesity in adults. Obesity prevalence is increasing and its importance to public health is considerable because it is associated with a higher risk of type 2 diabetes, coronary heart disease, certain cancers (Allison *et al.*, 1999), and a shorter life expectancy.

Obesity is thought to be a multifactorial disorder. Although genetic factors may predispose to overweight and obesity, two major reasons are implied: reduction in physical activity and an increase in energy intake, primarily from fat-rich and energy-dense foods. In the energy balance equation, energy intake is probably much more sensitive to dietary changes than is energy expenditure (EE) (Prentice *et al.*, 1989, Stubbs, 1999). The mechanisms involved in the regulation of food intake are, however, complex and imply several different mechanisms, involving internal as well as external

factors. Some of them are macronutrient related (eg, glucostatic and lipostatic mechanisms) and others are related to hormones and afferent signals, such as insulin, glucagon-like peptide 1 (GLP-1) and leptin, among others. In addition, body weight maintenance requires a multifactorial management because many complex mechanisms are involved in this regulation (food intake regulation, energy homeostasis, etcetera).

Nowadays, most weight lost diets fail, mainly because of hunger feeling, so enhancing satiety could be an expectant tool to help decrease food intake and therefore prevent and treat obesity and overweight. Certain ingredients are capable to induce satiety and can be considered as functional agents. These could produce a decrease of appetite and food intake leading to a negative energy balance and obesity control. It has been extensively studied that several ingredients can modulate the appetite and enhance satiety by releasing several hormones and gut peptides with anorexigenic activity such as leptin, insulin, GLP-1, GIP, PYY, CCK, etcetera, or through suppression of orexigenic hormones as ghrelin.

2. HUNGER AND SATIETY MECHANISMS

The answer is no easy since the control of appetite is affected by several factors such as hedonic, psychophysiological and homeostatic. Feed pattern is considered as a sequence of eating, a meal, and non-eating episodes. Hunger, appetite, satiation and satiety could be analysed from several points of view.

Blundell *et al.*, (2001) described the complex mechanism of regulation of appetite based in three steps or levels corresponding to *i*) physiological events and cultural/behaviour events; *ii*) biological or metabolic events and *iii*) metabolic interactions in central nervous system (CNS). The first one included human routine with set times for eating and sleeping, hunger feelings, craving and desire to eat. Other factors were based on food availability and their organoleptic properties such as taste, smell, texture, meal macronutrients and energy intake (Blundell *et al.*, 2001a). Regarding biological or metabolic levels, several researchers have postulated different theories under these biological aspects. For instance, Cannon and Washburn (1993)

proposed the theory of the *contraction of the stomach* that postulates we feel hungry when the stomach contracts (Cannon and Washburn, 1993). This was rejected because people without stomach also felt hungry. Mayer (1955) proposed the *glucostatic theory*, suggesting hungry appears when the blood glucose level falls slightly (Mayer, 1955). Linked to this, the *insulin theory* says that hungry feelings arise when circulating insulin levels suffer a sudden increase (Holt and Miller, 1995). Kennedy (1953) postulated the *lipostatic theory*. He proposed the existence of hypothalamic receptors that detected elevated plasma level of fatty acids, which triggered hungry. The hypothalamus may detect the amount of body fat leading to generate signals of hunger inhibition (Kennedy, 1953). Furthermore, Mellinkoff *et al.*, (1997) proposed one of the most accepted theories, suggesting a relationship between amino acid concentration and appetite, the *amino acid theory*. Specifically, they proposed that an elevated concentration of blood or plasma amino acids, which cannot be used in the synthesis of protein, may serve as a satiety signal that is connected in a "satiety" centre in the brain. (Mellinkoff *et al.*, 1997). Finally, in the last decade scientists have generated a wealth of information about the mechanisms involved in the regulation of food intake, postulating the existence of a neuroendocrine system regulating hunger and satiety (neurotransmitter and metabolic events). The mechanisms that regulate homeostasis and balance energy include variety of signals, such as gastrointestinal hormones, cytokines, metabolic intermediates and nutrients (Schwartz *et al.*, 2000, Fisler *et al.*, 1995).

Satiation and satiety are two terms related to the inhibition of food intake. However, several definitions such as hunger, appetite, "liking" and "wanting" must be explained to understand basic differences among them. In general terms, satiation is the process that brings eating to an end. Therefore, it controls meal size and it is also known as intra-meal satiety. Satiation leads to inhibit the motivation to eat during, and within an eating event (Tremblay and Bellisle, 2015). This can be conditioned by a complex formed by stimulatory influences related to nature of food (texture, taste, etcetera), physiological factors (hunger) and influences from environment as well as social situation (de Castro, 2010).

Unlike satiety, which is the inhibitory mechanism that leads to inhibition of further eating and declines hunger (and increase fullness) after a meal has finished. This

is also known as post-ingestive satiety or inter-meal satiety (Blundell *et al.*, 2001a). Hence, satiation refers to meal size and satiety is more related to the inter-meal interval. Satiety and satiation intensity and duration may vary according the potential of the physiological signals generated by the action of food and the nature of food *per se* (Blundell *et al.*, 1987). Both terms are interlinked since they share many inhibitory influences, but they are certainly different. Meanwhile satiation could inhibit the ongoing intake and happens during the ingestion episode, satiety prevents the ingestion and suppresses the ingestive responses during an specific time (Tremblay and Bellisle, 2015).

Hunger can be due to changes in physical sensations in specific parts of the body (stomach, limbs or head), including feelings as weakness or emptiness in stomach (Blundell *et al.*, 2010). It is not directly measurable, but it can be inferred from objective conditions. On the contrary, appetite is the interval driving force to search, choose and intake of food. This can be measured with subjective ratings (conscious drive or motivation to eat). These subjective measures have shown to be very reproducible, sensitive and predictive of food intake (Flint *et al.*, 2000, Degraaf, 1993, Stubbs *et al.*, 2000). In relation to hunger and appetite is important to differentiate two concepts related to food reward such as “liking” and “wanting”. The first one could refer to the delight or pleasant of a given food, its palatability. Contrarily, “wanting” is associated to the disposition to eat or appetite (Berridge, 1996). However, two food reward terms are dissociated in animals and in humans, too. For instance, food “liking” and food “wanting” were determined after a meal consumption in human volunteers, and when subjects wanted high-fat savoury foods, liking was not different. In addition, when volunteers liked high-fat sweet foods, no differences in wanting were observed (Finlayson *et al.*, 2007). However, these terms were different regarding food pattern since differences in the neural substrates could be taken place. Meanwhile food wanting was related to dopaminergic neurotransmitter, food liking was associated to GABAergic and opioid neurotransmitter (Havermans *et al.*, 2009, Berridge and Robinson, 1998).

The satiety cascade was proposed more than 30 years ago by Blundell *et al.*, (1987) and it has been widely used as a conceptual framework to examine the impact of food on satiation and satiety (Blundell *et al.*, 1987). In figure 1.1. it can be observed an

adaptation of the proposed explanation of satiety and satiation mechanism of Blundell *et al.*, (1987).

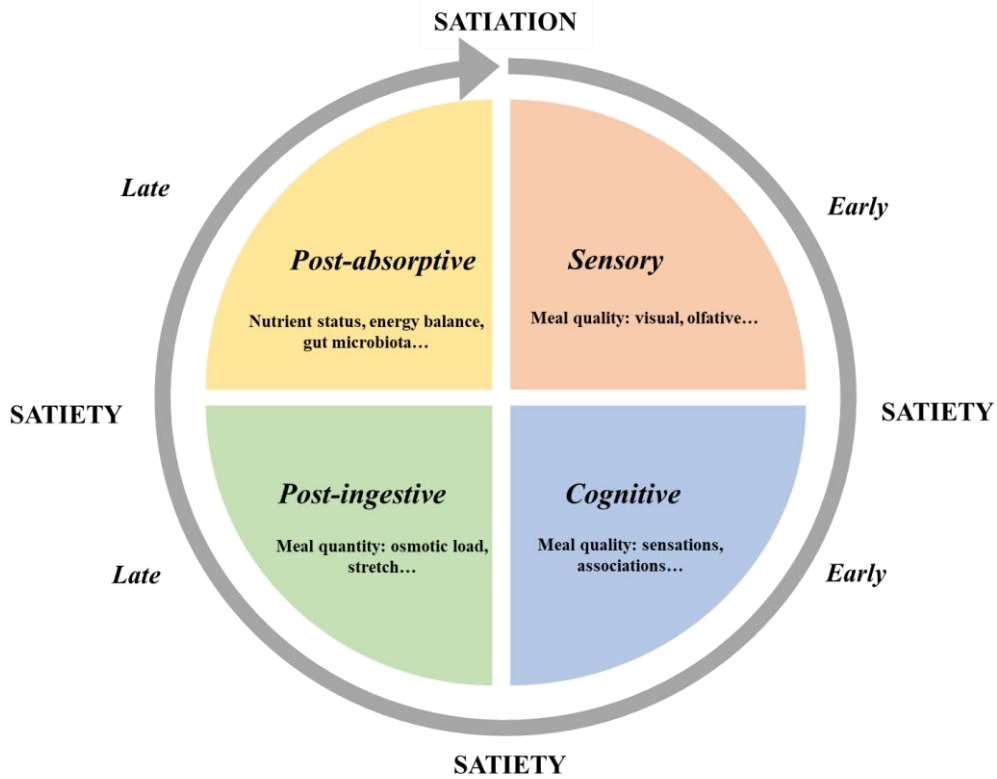


Figure 1.1. Satiety mechanism and phases of satiation and satiety depending on sensorial and cognitive influences, post-ingestive (or pre-absorptive) and post-absorptive stage.

The figure combines the biological systems implied in the control of appetite, the behavioural events and psychological experiences, including central nervous system (CNS), which finally determines meal- by- meal appetite control. Related to control mechanism or satiety there is to consider the short-term control or short-term satiety and chronic or medium-long term control. The first one is controlled by physical signals and release of digestive peptides in response to food; whereas the second one is affected by signals such as leptin and insulin.

The onset of an eating episode can be influenced by sensory factors and meal features such as meal size, energy density, palatability, taste, variety, etcetera (Blundell *et al.*, 1987), and thus, influencing satiation. Furthermore, cognitive factors such as food reward, expectations, memory, pleasantness, etcetera; could influence the eating events. These sensory and cognitive factors lead to define meal quality and quantity (Amin and Mercer, 2016) and subsequently both of them, contribute to satiation events. However, cognitions and beliefs about the satiety value of a meal could influence not only satiation but also satiety. For instance, a liquid preload lead to a greater postprandial hunger and lower fullness sensations compared to a solid preload suggesting that the sensory and cognitive effect of food influences satiety by physiological and endocrine mechanism (Cassady *et al.*, 2012). Although satiation and satiety seem to be often overlapping, the cessation of eating during inter-meal events (satiety) and the inhibition of an ongoing intake behaviour (satiation) could be different. For instance, strategies to enhance satiation are related to ingredients that provide reduced energy density. Contrarily, “satiety strategy” comprises a reinforcing “satiety efficiency” by using ingredients with a high satiating potential as fibres and/or proteins (Blundell *et al.*, 2010).

The stretch or distension of the stomach detected by mechanoreceptors in the gut wall, together with early indications of osmotic load, provide an early physical indication of meal size and energy load, leading to a feedback of meal quantity (Amin and Mercer, 2016) and reinforcing satiation. When the nutrient chemoreceptors and stretch receptors in the gastro intestinal tract are stimulated by the presence of nutrient-specific meal (protein, carbohydrates and fat) (meal quality) or a change in volume (meal quantity), “satiety peptides” and “satiety hormones” are led to be released. These post-ingestive (pre-absorptive) signals within the gut trigger the release of hormones into gastro-intestinal tract such as ghrelin, cholecystokinin (CCK), peptide YY (PYY) and GLP-1. They act as potent cues for satiation and medium-term satiety through their impact on stomach emptying, by stimulating afferent signals to the CNS or directly influencing CNS function. The post-absorptive (long-term satiety) impact of circulating nutrients, as amino acids and hormones designed to control their circulating levels, also induce or reduce appetite. The oxidation and metabolism of nutrients and the storage of energy also produces potent post-absorptive effects on energy regulation mediated

mainly by leptin and insulin (Blundell *et al.*, 2001a). At this level it is important to consider the key role of gut microbiota to promote satiety signals since satiety is not only affected by macronutrients *per se*. Nutrients as proteins and non-digestible carbohydrates that reach the colon could serve as substrate for gut microbiota. This fermentation produces several metabolites, such as short-chain fatty acids (SCFA), that could trigger the releasing of satiating gut peptides like GLP-1 and PYY (Cani *et al.*, 2009). Finally, the brain integrates all satiating signals from the periphery of organism, and they are the reflect of energy input (energy intake) and energy output (EE) balance which drives to suppress hunger or contrarily allows continuing eating.

3. SATIETY SIGNALS

Satiety signals or biomarkers of satiety could be used as a tool to evaluate the “satiating efficiency” of foods. These can be classified depending on their effect on satiety being anorexigenic or orexigenic signals; or based in the site of releasing: CNS and peripheral signals. When the food reaches the stomach or intestine, it induces the release of many peripheral hormones related with satiety. Additionally, satiety signals could lead to sustain a short-term or long-term satiety.

Centrally satiety signals could be related to inhibition and suppression of hunger or the opposite. For instance, neuropeptide Y (NPY), which promotes hunger, is secreted in the arcuate nucleus of hypothalamus and promotes the ingestion, specially of carbohydrates. Agouti-related protein (AgRP), is one of most potent orexigenic molecules, concretely for fat and sucrose intake. Galanin, orexins A and B and ghrelin are other example of orexigenic compounds. Moreover, several anorexigenic neuropeptides have been described such as proopiomelanocortin (POMC) expressed in hypophysis, nucleus of solitarius tract and arcuate nucleus of hypothalamus. This has two receptors involved into central hunger suppression (MC3R and MC4R) as well as cocaine-and amphetamine-regulated transcript peptide (CART) that could inhibit NPY, among others (Hita *et al.*, 2006).

The peripheral peptides or hormones include CCK, GLP-1, PYY, bombesin, gastrin-releasing peptide (GRP), enterostatin, amylin, glucose-dependent insulinotropic polypeptide (GIP), pancreatic polypeptide, somatostatin and “hunger” hormones as ghrelin. Many of these hormones released in the gastro intestinal tract or pancreas act as a satiating signal that can be transmitted via afferent fibres of the vagus nerve towards the nucleus of solitarius tract in the brain, where the signal will be integrated (Blundell *et al.*, 2001). Additionally, long-term satiating signals include leptin and insulin.

CCK is released in the I-cells of the proximal small intestine in response to dietary protein and fat, and their degraded products, being lower stimulated by carbohydrates. This peptide promotes digestion through bile and enzyme released and also slows gastric emptying, the so-called “*duodenal brake*” mechanism (eg, adjustments of stomach and gut motility after food ingestion) (Crawley and Corwin, 1994, Moran, 2000). This produces the contraction of pylorus and gastric distention, which subsequently activates the satiating centre in CNS (nucleus tractus solitarius) via afferent signals by the vagus nerve. CCK suppresses hunger after a meal and reduces the meal size, therefore it can be used as a suitable satiating marker (de Graaf *et al.*, 2004, Hita *et al.*, 2006).

Another important gut hormone is GLP-1. Its major function, as incretin, is the augment of insulin secretion after oral intake of glucose, being essential in the regulation of insulin secretion and glucose homeostasis. This incretin is released from the L-cells of the distal small intestine and the large intestine in response to carbohydrates, proteins and fats (Gribble and Reimann, 2016). Its stimulation can be mediated directly by the contact in the distal intestine or indirectly by neurohumoral mechanism (Brubaker and Anini, 2003, Cummings and Overduin, 2007). Interestingly, GLP-1 is a key mediator in the intestinal negative feedback phenomenon called “*ileal brake*”, inhibiting proximal motility and delaying gastric emptying. The activation of “*ileal brake*” results in a reduction of food intake, suppression of hunger caused by the inhibition of gastric emptying and distally, inhibition of the motility and secretion of bile acid, gastric acid and pancreatic enzyme secretion (Maljaars *et al.*, 2008a). In addition, GLP-1 reduces food intake in humans and animals, increasing satiety and inducing anorexia, possibly through the role of vagal and direct central pathways

(Cummings and Overduin, 2007, Turton *et al.*, 1996). Determinations of GLP-1 also can be used as a biomarker of satiety since it is a causal factor in the satiation process (de Graaf *et al.*, 2004). Moreover, it could be stimulated by the action of GIP and GRP and inhibited by galanin and somatostatin (Hita *et al.*, 2006).

Similarly, GIP is released from K cells in the duodenum after fat and glucose ingestion (de Graaf *et al.*, 2004). GIP has not been reported to have an acute influence on food intake. However, GIP-receptor-knockout mice are resistant to obesity when fed a high-fat diet. The reason for this resistance is unclear and there has been speculation that it might reflect a direct effect on adipocytes rather than on central appetite-regulating circuits (Miyawaki *et al.*, 2002). Additionally, it has been proved that GIP may stimulate fat deposition in adipocytes via enhancement of lipoprotein lipase (Moran-Ramos *et al.*, 2012). There is a wide evidence of GIP role on obesity since hyper-GIP-emia secretion is showed in obese subjects and in individuals fed under high-fat diets (Salera *et al.*, 1982, Morgan *et al.*, 1988, Ross and Dupre, 1978). Furthermore, GIP could stimulate the secretion of GLP-1 (Hita *et al.*, 2006).

PYY is released in the same place than GLP-1 in response of dietary fat, protein and carbohydrate. It seems that its release depends on caloric content of foods (Hita *et al.*, 2006). PYY reduces gastric motility, slowing caecal-oral transit time (Batterham *et al.*, 2002). Fermentation of dietary fibre in the colon produces SCFA, which triggers GLP-1 and PYY release (Wanders *et al.*, 2011). Moreover, the satiating effect of PYY may be related to the inhibition of electric activity of NPY-producing neurons and by activating POMC-producing neurons (Hita *et al.*, 2006).

Amylin is secreted by β -cells in pancreas. This has a complementary action to insulin effects over glucose homeostasis. It seems that amylin has a synergic effect together CCK to suppress appetite by a central mechanism. Additionally, intrahypothalamic increases of amylin have been related with increases in serotonin, suggesting the important role of neurotransmitter in the satiating effect of amylin (Gutzwiller *et al.*, 1994, Hita *et al.*, 2006).

Furthermore, bombesin and GRP are neurotransmitters involved in several gastrointestinal functions such as stimulation of CCK release and antrum and pyloric concentration (Walsh *et al.*, 1981, de Graaf *et al.*, 2004). The role of somatostatin in hunger regulation is limited, existing few studies linking somatostatin and appetite regulation in humans (de Graaf *et al.*, 2004). Furthermore, enterostatin could be involved in the selective inhibition of fat intake and preferences for foods containing high-fat proportions. The effect of enterostatin on food intake regulation is not still clear (de Graaf *et al.*, 2004). Pancreatic polypeptide is synthesised and released from the endocrine pancreas. It is then released after a meal and reduces appetite (Batterham *et al.*, 2003).

Stomach secretes ghrelin, or growth hormone (GH)- releasing peptide. It is the only known circulating orexigenic, or appetite stimulant, therefore it is known as “hunger hormone” (Kojima and Kangawa, 2008). It is mainly produced by the endocrine cells of the gastric mucosa of the fundus but is also found in much smaller amounts in other tissues (small intestine, pituitary gland, pancreas, etcetera.). The acetylation of ghrelin is needed to its activation by binding to its receptor, GHS-R (Murphy and Bloom, 2006). Ghrelin levels rise prior to meals, then fall quickly after ingestion of nutrients (Cummings *et al.*, 2001). Thus, it is postulated that one primary role of ghrelin is to act as a meal initiator. New strategies based on administration of ghrelin agonist are being covered, with special attention in the treatment of anorexia and cancer (Murphy and Bloom, 2006).

Other satiety signals derived from energy balance and adipose tissue could act as post-ingestive influences that inform the brain about the stores of adipose tissue (Blundell *et al.*, 2001). These are hormones with anorexigenic effect, such as leptin and insulin. Both of them are involved in the long-term regulation of satiety and energy balance. In fact, leptin is closely and directly related to adipose tissue, as evidenced by increased adipose *ob* mRNA and serum leptin levels in obese humans and other mammals (Ahima and Flier, 2000). It is a regulator of energy balance, changes in the energy reserves are communicated to the CNS by leptin and another signal. It is well-known that the suppressive effects of leptin in food intake could be additionally mediated by reductions in the expression of NPY and increases in anorexigenic

neuropeptides (CART) (Hita *et al.*, 2006). Leptin determines changes in feeding behaviour, with suppression of appetite, and an increase in metabolic activity and EE (thermogenesis). Despite of leptin not being the major cause of obese people weight gain, it seems clear that its deficiencies or lower levels, because of leptin gene mutations, can lead to a great risk (Blundell *et al.*, 2001). Obese subjects could exert a leptin resistance showing high concentration of leptin. These resistances can be related with deficiencies in the leptin-carrying protein, due to the hematoencephalic barrier or because faults in the signal pathways (Hita *et al.*, 2006).

Insulin is produced in the β cells of the pancreatic islets and secreted in blood in response to small increases in blood glucose concentrations (Schwartz *et al.*, 2000, Mayer, 1955). The insulin response to a meal is mediated, in part, by the insulintropic incretin hormones GLP-1 and GIP. Anorexigenic effect may be determined by decreasing the expression of NPY (potent orexigenic agent) in the arcuate nucleus, similar to the effect of leptin. Insulin also activates thermogenesis by stimulating the activity of the sympathetic nervous system. Thus, it stimulates other satiety signals as CCK, which is a long-term connection between appetite regulation and energy metabolism (Pliquett *et al.*, 2006). Secretion of these hormones depends on the level of energy reserves, including food intake, but leptin has a more important role than insulin in the central control of energy homeostasis (Schwartz *et al.*, 2000).

4. MACRONUTRIENTS AND SATIETY

There is an extensive literature concerning the satiating macronutrient capacity. Numerous studies have determined and assessed its satiating capacity in order to formulate new satiating foods that can claim and help in the prevention and treatment of overweight and obesity. Regarding these claims on satiety, The European Food Safety Agency (EFSA) demands new ingredients based in scientific researches that show sustainable changes in appetite and reductions in food intake that are sustained for a long time. In 2006, the European Commission adopted regulation 1924/2006 on the use of nutrition and health claims made on foods and non-alcoholic beverages. Claims were based on the nutritional profiles of products, harmonising the use of nutrition and health

claims, ensuring critically any claim is demonstrated by scientific evidence (Regulation EC N° 1924/2006).

The following explanation of satiating capacity of macronutrients: proteins, carbohydrates and fats try to focus in the main role of macronutrient due to their potential to suppress appetite by enhancing satiety signals. Finally, a brief explanation about the important role on satiety of a micronutrient, calcium, will be take into consideration.

4.1. Proteins: the most satiating macronutrient

Considering different satiating efficacies of the three macronutrients, either over short-term over 24h (Lejeune *et al.*, 2006, Veldhorst *et al.*, 2008, Smeets *et al.*, 2008), a hierarchy has been observed with protein being the most satiating and fat the least satiating one (Eisenstein *et al.*, 2002, Latner and Schwartz, 1999, Westerterp-Plantenga *et al.*, 1999). Additionally, foods rich in protein suppress food intake at a later meal, more than carbohydrate or fat, and provide stronger feelings of satiety immediately after their consumption (Latner and Schwartz, 1999, Porrini *et al.*, 1997, Hill, 1986, Rolls *et al.*, 1988, Stubbs *et al.*, 1999).

Protein satiating effects and body-weight regulation can be explained through a multifactorial approach such as by stimulating gut hormone secretion, digestion effects, postprandial amino acids levels, EE and probably gluconeogenesis (Drummen *et al.*, 2018). In addition, a wide range of studies have reported that an increase of protein intake can influence both short-and long-term body weight regulation by several mechanisms (Westerterp-Plantenga, 2008, Westerterp-Plantenga and Lejeune, 2005, Halton and Hu, 2004, Paddon-Jones *et al.*, 2008, Veldhorst *et al.*, 2008, Westerterp-Plantenga *et al.*, 1999).

1. Increasing protein intake can lead to enhance satiating hormones in similar or lower energy intake due to amino acids composition.
2. Dietary protein-induced thermogenesis and EE.

3. High-protein diet lead to lowering energy efficiency in overfeeding conditions preserving and contributing to storage of fat free mass.

In rat studies, protein was also more potent than carbohydrates in reducing appetite dose-dependently. It seems that protein has the highest satiating effect when compared with other macronutrients in human subjects and animals (Porrini *et al.*, 1997, Reid and Hetherington, 1997, Trigazis *et al.*, 1997, Bensaid *et al.*, 2002). However, there are discrepancies related to the duration of eating, the way of administration (oral, intra-gastric or intra-venous) (Burton-Freeman *et al.*, 1997, Reid and Hetherington, 1997, Trigazis *et al.*, 1997, Eisenstein *et al.*, 2002, Raben *et al.*, 2003), and the source of protein which can influence the satiating effect, although it is unlikely that it originates from the presence of some specific amino acid as a precursor of neurotransmitters, i.e. tryptophan and serotonin or tyrosine and dopamine (Burton-Freeman *et al.*, 1997).

High-protein diets affect both short and long-term satiety. In short-term, high protein diet induces a negative fat-balance with an increase of fat oxidation, and a positive protein-balance which can explain larger weight loss and subsequent body-weight maintenance (Westerterp-Plantenga, 2008). In long-term, many studies have analysed the protein effect on body-weight loss (Skov *et al.*, 1999) comparing high-protein diet with a control one to evaluate weight loss over 6 months in obese human. These authors investigated the satiating effect of two diets: one with 25% energy intake from protein (25% protein, 45% carbohydrates and 30% fat) and another with 12% energy intake from protein (12% protein, 58% carbohydrates and 30% fat). It was found that weight loss and fat loss were significantly higher in the high protein group, due to a lower energy intake. In a follow-up study, it was observed that after 12 months the weight loss was not significantly greater among the subjects in the high protein group, but they had a greater reduction in intra-abdominal adipose tissue (Due *et al.*, 2004). Other research has reached the same conclusion, that long-term diets with relative high-protein intake lead to a greater reduction in fat free mass (FFM) (Layman *et al.*, 2009, Brinkworth *et al.*, 2004a, Brinkworth *et al.*, 2004b). However, some studies have reported different results in long-term, maybe because dosage of protein. Furthermore, an extensive systematic review and meta-analysis have concluded that the short-term

benefits of high-proteins diets could persist minimally over the long-time obtaining more benefits when more diet compliance has taken place (Clifton *et al.*, 2014).

Protein is usually consumed from different sources: meat, fish, plants or dairy products, and protein could induce satiety with a single meal (with contents 28% to 81% of energy from proteins) reducing the subsequent energy intake (Barkeling *et al.*, 1990, Hill, 1986, Smeets *et al.*, 2008, Stubbs *et al.*, 1996, Johnson and Vickers, 1993). In addition, the effect of protein in satiety could be dose-dependently. However, a chronic ingestion of high protein diet, mainly with high sulphur-containing amino acids, (cysteine, homocysteine, methionine, taurine) could have an indirect effect on blood pressure by an induction of renal structure damage (Frassetto *et al.*, 2006, Remer, 2001), as well as adverse effect on calcium balance, progression of cardiovascular disease and liver function (Anderson *et al.*, 2000, Jeor *et al.*, 2001, Eisenstein *et al.*, 2002). Regarding this adverse event, WHO recommends that dietary protein should be adjust at ~10–15% of energy intake, for an energy balance diet and stable weight (FAO and WHO, 2000).

The source of protein could affect satiety, maybe through satiating hormone stimulation. Digestibility can be translated in terms of postprandial levels of amino acids and diet-induced thermogenesis (DIT) pathways. Animal protein showed a higher digestibility and thermogenesis than vegetable protein, which in longer term, it contributes to the low energy-efficiency of protein. For instance, pork meat protein has been shown to produce 2% higher EE than soy protein (Hall *et al.*, 2003). In lean men, the satiating effect of fish protein was reported to be higher than that of beef or chicken (Uhe *et al.*, 1992). However, under longer term conditions, a variety of the sources of protein intake is always present.

Anderson *et al.*, (2004) demonstrated, in short-term, the higher satiating potential of whey proteins than soy protein or egg albumen. They showed that next meal intake after these protein preloads was higher in the case of soy and egg proteins than whey (Anderson *et al.*, 2004). When the satiating potential of whey relative to soy and casein were compared results showed that diets containing 10% of energy from whey protein decreased hunger, estimated by appetite rankings, more than studied proteins

coinciding with the levels of leucine, lysine, tryptophan, isoleucine, and threonine yielded by whey proteins. However, this difference in appetite was not found in diets with 25% of energy from protein, although at these concentrations of whey the levels of satiating hormones were enhanced in respect to soy and casein (Veldhorst *et al.*, 2009a). Other research studied several types of breakfast containing different sources of proteins, concluding that the source of protein affected satiety greatly (Veldhorst *et al.*, 2009b). Nevertheless, other research have showed that the source of protein did not affect satiety (Lang *et al.*, 1998). They compared the effect of egg albumin, casein, gelatine, soy protein, pea protein and wheat gluten, and no differences in 24-h macronutrient intake were attributed to any source of protein more than other.

Another important factor that determines postprandial protein metabolism is its digestion and absorption rate. Authors suggest differences in two types of proteins according with this digestion and absorption rate: “fast” and “slow” proteins. Thus, the first ones are rapidly digested, such as whey, producing a strong increase in postprandial protein metabolism synthesis and oxidation of amino acids resulting in a higher peak of plasma amino acids, which is closely related with higher peaks in satiating hormones such as GLP-1 and CCK (Hall *et al.*, 2003). These effects are more important than slowly digested protein (“slow” protein), such as casein (Boirie *et al.*, 1997, Dangin *et al.*, 2001, Dangin *et al.*, 2002), which coagulates in the stomach due to gastric acid (Billeaud *et al.*, 1990). As a result, overall gastric emptying time for casein appeared to be longer and a smaller postprandial increase in plasma amino acids compared with the non-coagulating whey protein was observed. Also, postprandial satiety appeared to be larger after a whey preload than after a casein-preload, related to more elevated concentrations of amino acids in the blood together with stronger elevation of both CCK and GLP-1 (Hall *et al.*, 2003).

Furthermore, the mechanism to enhance satiety is considered under different perspectives: *i*) in concentrations of “satiety” hormones, *ii*) in EE and thermogenesis, *iii*) in concentrations of metabolites, i.e. amino acids and gluconeogenesis.

i) Protein-induced satiety and "satiety hormones"

Many authors have hypothesised that protein intake is closely related with a high increase in concentration of anorexigenic gut hormones (GLP-1, CCK and PYY) or a larger decrease in the concentration of orexigenic hormones such as ghrelin (Smeets *et al.*, 2008, Lejeune *et al.*, 2006, Bowen *et al.*, 2006, Hall *et al.*, 2003, van Loon *et al.*, 2000, Batterham *et al.*, 2006). Hall *et al.*, (2003) showed that an acute intake of protein was closely related with changes in these hormones (Hall *et al.*, 2003). Nevertheless, Smeets *et al.*, (2008) had the evidence that there is not a relationship between a high intake of protein and changes in ghrelin, PYY and GLP-1 between a high protein and adequate protein intake (Smeets *et al.*, 2008). For this reason, the concentration of hormones may underscore nutrient-induced satiety, but there is not a mathematical relationship between high protein intake and high concentration of GLP-1. Some author suggested that high protein intake in presence of carbohydrates could produce a high release of GLP-1, maybe due to carbohydrate stimulating protein metabolism (van Loon *et al.*, 2000). With regard to PYY, Batterham *et al.*, (2006) observed a relationship between high protein intake and high PYY concentration in lean and obese subjects (Batterham *et al.*, 2006). Additionally, Hall *et al.*, (2003) showed that after consumption of proteins from whey, CCK, GIP and GLP-1 levels increased, resulting in a great satiating effect (Hall *et al.*, 2003). Other research showed that protein increased as dose-dependently are involved in the releasing of satiating peptides as GLP-1, PYY and CCK, among others, which mediated in the satiating effect of proteins (Belza *et al.*, 2013).

Furthermore, it has been shown that under physiological conditions, undigested nutrients can reach the ileum and activate "ileal brake": negative feedback mechanisms that influence the function of more proximal parts of the gastrointestinal tract. This mechanism results in reduction of hunger and food intake, partly by inhibition of gastric emptying (that enhanced and prolonged gastric distension) (Clarkston *et al.*, 1997, Hveem *et al.*, 1996, Jones *et al.*, 1997, Geliebter, 1988, Goetze *et al.*, 2007). Chemoreceptors can detect nutrients in the intestinal lumen located in the small intestine and it could trigger the release of hormones, such as CCK (by enteroendocrine cells in response to protein) (Phillips and Powley, 1996, Eastwood *et al.*, 1998, Mathis

et al., 1998, Phifer and Berthoud, 1998, Schwartz, 2000). CCK could act on gastric kinetic through a vagal loop and stimulates satiety through low affinity vagal CCK-receptors.

There is controversy about evidences in the satiety stimulation through “*ileal brake*” after protein infusion in the ileum (Read *et al.*, 1984, Spiller *et al.*, 1988, Welch *et al.*, 1988a, Layer *et al.*, 1995). However, the available evidence indicates that ileal protein infusions in both, humans (Spiller *et al.*, 1988) and animals (Siegle *et al.*, 1990), activate the ileal brake, although differences may exist between different types of protein or amino acids (Meyer *et al.*, 1998). The exposition of intestinal tissue to intact pea and wheat protein induced an increase in the release of CCK and GLP-1, whereas other tested proteins did not affect these satiety hormones (Geraedts *et al.*, 2010).

Another aspect to consider after protein digestion is the production of peptides. These peptides exert effect on food intake via the gut, slowing stomach emptying (Blundell *et al.*, 2001b), and direct or indirect stimulation of gut hormone receptors, including CCK (Schwartz *et al.*, 2000, Ahima and Flier, 2000) and GLP-1 (Aziz and Anderson, 2003). The type and amount of these “biopeptides” will depend on the source of protein. For instance, milk protein proteolysis includes casomorphins, CMP (also called glycomacropeptide) and leucine, a branched-chain amino acid (BCAA). Casomorphins is the first product of the digestion of casein (Daniel *et al.*, 1990) and in rats and humans studies, it induces the CCK release (Pedersen *et al.*, 2000, Yvon *et al.*, 1994). The CMP is a strong suppressant of appetite, but there are little published study trials of its effect on food intake (Gustafson *et al.*, 2001). Leucine has been proposed to be a key amino acid in the food intake regulation and lean mass maintenance on energy reduced diets, probably because its metabolic roles in maintaining glucose homeostasis and stimulating protein synthesis, particularly when subjects are on a weight reducing diet with exercise (Layman, 2003).

ii) Protein intake and diet-induced thermogenesis (DIT)

Energy expenditure and thermogenesis have been suggested to explain protein-induced satiety. They are associated with an increase in body temperature, metabolic rate and hepatic ATP production. A relationship between augmentations in 24-h DIT and satiety has been reported using energy-balanced high-protein diets (Drummen *et al.*, 2018, Lejeune *et al.*, 2006). The theoretical basis of this relationship between satiety and DIT may be that increased resting EE implies an increased oxygen consumption and an increase in body temperature, which may be led to satiety feelings (Westerterp-Plantenga *et al.*, 1999).

When thermogenic effect of the 3 macronutrients is compared, proteins have the highest being 20-30%, thermic effect for carbohydrates is 5-10% and finally thermogenic effect for fat correspond to 0-3% (Tappy, 1996). Hence, a high protein diet induces a greater thermic response in healthy subjects compared with a high fat diet (Westerterp *et al.*, 1999). This suggested that protein is the macronutrient that produce more thermogenic effect participating in protein-induced satiety (Crovetti *et al.*, 1998), but this effect is not always observed (Eisenstein *et al.*, 2002, Raben *et al.*, 2003).

Recent findings suggest that this elevated thermogenesis and increases in several satiating hormones, such as GLP-1, could be the reason why protein induced satiety (Westerterp-Plantenga, 2008). The lower energy efficiency of protein may be due to increased DIT and because of storing of fat free mass results more costly than disposition of fat mass (Pullar and Webster, 1977). Westerterp-Plantenga *et al.*, (1999) showed that when subjects (lean women) consumed identical amounts of energy and volume, in identical meal patterns and comparable foods with respect to organoleptic characteristics, a difference in the satiety level due mainly to a high protein-high carbohydrate diet vs a high fat diet was related to a difference in the 24 h DIT component of EE. Also, Crovetti *et al.*, (1998) demonstrated that offering a mixed high protein meal with 68 vs 10% of energy from protein had a relationship between satiety and EE.

iii) Amino acids and gluconeogenesis.

Amino acids can contribute to the perception of postprandial satiety. Mellinkoff *et al.*, (1956) suggested a relationship between amino acid concentration and appetite (*aminostatic theory*). Specifically, they suggested that an elevated concentration of blood or plasma amino acids, which cannot be used in the synthesis of protein, may serve as a satiety signal that it is connected in a "satiety" centre in the brain (Mellinkoff *et al.*, 1956).

The variation in free amino acids concentrations could be involved in a central nutrient chemosensory system for essential amino acids (Gietzen and Magrum, 2001). This could also involve other specific mechanisms associated with the central availability of specific amino acid precursors (histidine, tryptophan and tyrosine) of the neurotransmitter's histamine, serotonin and the catecholamines (dopamine, adrenaline, noradrenaline). Tryptophan has been related with appetite regulation since it can be act as precursor of serotonin. This neurotransmitter is involved in eating behaviour appetite because of its anorexigenic effects in the brain (Leibowitz and Alexander, 1998).

Increasing central tryptophan availability enhances brain serotonin, which has been studied in several dietary intervention (Markus *et al.*, 2002). It is well-known that the absorption of tryptophan in the brain depends on its concentrations, as well as the plasma ratio tryptophan: LNAA (large neutral amino acids) (Beulens *et al.*, 2004). The source of protein can also affect these aspects, for instance: the ingestion of α -lactalbumin alone, or as a part of a meal, can increase the level of tryptophan and tryptophan: LNAA ratio since it contains high levels of this amino acid compared with other protein sources such as collagen hydrolysate or gelatine (Markus *et al.*, 2002).

Furthermore, the satiating properties of protein are mediated by a complex mechanism being each protein specific (Veldhorst *et al.*, 2008), maybe due to the individual amino acid profile. Leucine has showed to decrease food intake in rats and body weight (Cota *et al.*, 2006). In addition, very high-protein with excess of specific amino acids (methionine, tryptophan and histidine) can produce a toxic effect leading to an aversive anorexic response.

Other example was lysine ingestion, which increased postprandial glucose (Kalogeropoulou *et al.*, 2009) and stimulated the secretion of CCK and GLP-1 (Tome *et al.*, 2009). Threonine was reported to improve food intake and weight gain (Ciftci and Ceylan, 2004). Ayaso *et al.*, (2014) studied the individual effect of amino acids and satiety in rats. They showed that lysine and tryptophan reduced food intake mainly due to an increase in satiety, meanwhile proline and threonine affect minimally and arginine supplementation decreased satiety (Ayaso *et al.*, 2014).

Moreover, amino acids induced gluconeogenesis and decrease in glycaemia, which contributes to satiety. High protein diet satiety could be due to the improvement of glucose homeostasis by the modulation of hepatic gluconeogenesis and glucose metabolism. This mechanism may affect the intake regulation more than satiety because this has only been proved in animal models (Westerterp-Plantenga *et al.*, 2006). There is a controversy whether these effects of protein are related with concentration of indispensable amino acids (IAA). For instance, casein is a complete protein and it contains all IAA, while gelatine (incomplete protein) has a lack (tryptophan) or deficiency (methionine and histidine) in essential amino acids.

In summary, protein is the most satiating macronutrient, by acting on the relevant metabolic targets of satiety and EE in negative energy balance, thereby preventing a weight cycling effect. The source of protein can play an important role in satiety and food intake as well. Furthermore, it is necessary to determine the optimal dose of proteins that lead to achieve a balance between satiety and minimal adverse effects.

4.2. Carbohydrates: the role of fibres and glycaemic index on satiety

Fibre is a complex and varied macronutrient with a wide range of non-starch polysaccharides (carbohydrates) and lignin (a non-carbohydrate alcohol derivative), which are either soluble or insoluble and fermentable or non-fermentable (Burton-Freeman, 2000). Dietary fibre consists of non-digestible carbohydrates and lignin. However functional fibre consists of isolated, non-digestible carbohydrates that have

beneficial physiological effects in humans. Total fibre is the sum of dietary fibre and functional fibre. The mechanism by which carbohydrates inhibit appetite are very complex and not fully understood but is likely to be multifactorial. In fact, fibres produce several mechanisms underpinning appetite control (Wanders *et al.*, 2011), for instance the role of hyperglycaemia like a postabsorptive factor involved in appetite, effect of bulking of fibre in the stomach (delay emptying gastric), low energy density of fibres or pre-absorptive signalling from the digestive tract after a meal with carbohydrates. Clearly, there are functional fibres that have little or no effect on satiety, while other fibre types bind to water and swell causing bulking and increase viscosity. Heaton *et al.*, (1973) proposed that fibre could interfere with energy intake through three mechanisms: displacing available calories and nutrient from the diet; increasing chewing and secretion of saliva resulting in an expansion of the stomach and increased satiety and reducing the absorption of the small intestine (Heaton, 1973).

One of the most important mechanisms by which carbohydrate regulates food intake and satiety is its effect on blood glucose. Jenkins *et al.* (2002) used the term “glycaemic index” to define carbohydrate quality. The glycaemic index (GI) of each food is defined as the ability of that food to increase blood glucose 2 hours after eating it (Niwano *et al.*, 2009). Originally, it was expressed as a percentage of the area under a 2h blood glucose curve attributable to the food of interest divided by that for the same amount of standard carbohydrates such as glucose. A food with a higher GI is assumed to result in a high glycaemic response, which induces several hormonal and metabolic changes that can negatively affect health conditions. Food with low glycaemic responses should generally induce important benefits (Aston, 2006, Jenkins *et al.*, 2002). The concept that glucose regulates satiety and food intake is the basis for the *glucostatic theory* of food intake regulation, which proposes that lower blood glucose concentrations are closely linked with intake of food. Conversely, satiety and the termination of eating will occur after an increase in blood glucose (Mayer, 1955).

Regarding the role of GI as a predictor of appetite control, hunger and satiety, is very controversial. An inverse relationship between the glycaemic response to mixed meals and satiety within 2–6 h has been reported (Woodend and Anderson, 2001). But the associations among satiety, high- and low-glycaemic-index food, and blood glucose

concentrations have not been delineated. Thus, the rapid increase in blood glucose after the ingestion of rapidly digestible (high-glycaemic-index carbohydrates) may increase satiety, at least in the short term. On the contrary, the consumption of slowly digestible (low-glycaemic-index carbohydrates) results in slow and prolonged glucose disposal, which may be more effective in sustaining satiety in the long term. Ingestion of high GI food increased hunger and lowered satiety in short-term human intervention studies. This short-term effect may be attributed to the rapid decline in blood glucose level following a hyperinsulinemic response (*glucostatic theory*), being the long-term effect inconsistent (Niwano *et al.*, 2009). It is difficult to use the GI as a predictor of appetite control, hunger and satiety in long-term, because there is a large variance in GI values, mainly due to inter- and intra-individual variance. Reliable studies *in vivo*, *ex vivo*, even *in vitro* method to predict the rate of glucose entry from food into the body are necessary to clarify the relationship between GI and satiety in long-term. (Niwano *et al.*, 2009).

Soluble forms and carbohydrate form resistant to digestion generally decrease gastric emptying and/or slow energy and nutrient absorption, leading to lower postprandial glucose and lipid level. In addition, dietary fibres may influence fat oxidation and fat storage, and promote the release of several satiating peptides like CCK, GLP-1 and PYY. The release of GLP-1 and PYY may be mediated by the release of SCFA as a result of colonic microbial fermentation.

Howarth *et al.*, (2003) reviewed the effects of dietary fibre on hunger, satiety and energy intake. Most studies with controlled energy intake reported an increase in post-meal satiety and a decrease in subsequent hunger. Researches with *ad libitum* energy intake showed that increasing dietary fibre, an additional 14 g of fibre per day, resulted in a 10% decrease energy intake and weight loss 1.9 Kg through 3.8 months of intervention (Howarth *et al.*, 2003).

Certain cereals such as wholegrain rye, which is rich in source of fermentable dietary fibres like arabinoxylans, β -glucan, fructan and resistant starch (Frolich *et al.*, 2013, Isaksson *et al.*, 2011) could be a saccharolytic bacteria fermentable ingredient. These bacteria, such as *Bifidobacterium* and *Bacteroides* (Duncan *et al.*, 2009) utilize

these components with a resulting production of SCFA, gasses and other metabolites (Wong *et al.*, 2006). This fermentation could be one of the mechanisms why fibre consumption influences appetite, mainly due to SCFA production, which has been reported to enhance the production of satiety-inducing hormones such as GLP-1 and PYY (Piche *et al.*, 2003). Other authors confirmed the impact of gut microbiota fermentation of inulin-type fructan or oligofructose on appetite, correlated with the enhance of GLP-1 and PYY (Cani *et al.*, 2009), and decrease of ghrelin level (Parnell and Reimer, 2009).

In general, carbohydrates could have a great impact on satiety, at least in the short term, mainly because blood glucose and insulin levels. Furthermore, many fibres provide increased viscosity and gastric filling, which could result in increased satiety (short-term). In addition, the effect of non-digestible fibre on colonic microbiota is of great interest for its SCFA production, which can lead to the production of intestinal peptides resulting in satisfying long-term satiety.

4.3. Fats: the key role of saturation and chain length degree

The role of different macronutrient in appetite suppression has been highly studied. Certainly, protein is the most satiating macronutrient, followed by carbohydrates, while fats seem to have a weak satiating effect providing more energy per gram ingested. In fact, a weakened satiety response to fatty meals has been associated with a susceptibility to weight gain and obesity (Blundell *et al.*, 2005). Furthermore, this macronutrient provides more kilocalories per gram (9 Kcal/gram) *versus* 4 Kcal/gram for both protein and carbohydrates. Despite this, dietary fat could have an effect on the release of satiety gut signal, like CCK and GLP-1.

When dietary fats enter the gastrointestinal tract, they reduce hunger by eliciting satiety signals (Maljaars *et al.*, 2007b). Specifically, dietary fat (triacylglycerols or fatty acids) has been demonstrate to trigger the release of CCK in the proximal small intestine (duodenum) and GLP-1 in the distal small intestine (ileum) (Maljaars *et al.*, 2007b). This release of endogenous CCK and GLP-1 may suggest that dietary fat could

have a strong effect on the development of satiation and post-meal satiety. In fact, infusion of fat into the ileum activates the *ileal brake* mechanism (Van Citters and Lin, 2006), through the digestive tract to optimise nutrient digestion and absorption (Lawton *et al.*, 2000).

The physicochemical property of fats may affect satiety, specifically the degree of saturation and chain length of fatty acids. In general terms, the lesser saturation degree and more chain length the more release of satiety signals. These aspects affect the release of satiety signals, but the mechanism is not yet understood. The health benefits of long-chain omega-3 polyunsaturated fatty acids (LC 3-3 PUFA) have been extensively studied, notably eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Their benefits have been associated to improving cardiovascular health and reducing cardiovascular disease (Krebs *et al.*, 2006). In addition, it is being currently investigated its role in fat metabolism, satiety and its potential to reduce obesity. Regarding chain length of fatty acids, Feinle *et al.*, (2001) demonstrated that duodenal infusions of long chain triglycerides produced greater effects on hunger and CCK release than medium chain triglycerides (Feinle *et al.*, 2001). Other researches also showed that duodenal infusion of free fatty acids of a chain length of C12 produced great effect on food intake, appetite and CCK and GLP-1 release, compared with free fatty acids with chain length of C10 (Feltrin *et al.*, 2004). The saturation degree of fatty acids is additionally a key point in satiety. Emulsions enriched with linoleic acid (18:2) reduced food intake more than oleic (18:1) or stearic (18:0) acids without affecting satiety (Lawton *et al.*, 2000). Maljaars *et al.*, (2007) demonstrated that after infused into the ileum, triacylglycerols with unsaturated fatty acids increase satiety, and not in the case of saturated ones (Maljaars *et al.*, 2007a). However, other studies reported no effect on energy intake after 2 weeks of consumption of PUFAs (linoleic acid and linoleic acid) or MUFAs (oleic acid) (Kamphuis *et al.*, 2001). A weaker effect of monounsaturated fatty acids (MUFAs) on prospective consumption ratings than polyunsaturated fatty acids (PUFAs) were observed comparing post-ingestive satiety. Differences between ratings of hunger, desire to eat, and fullness were not found (Lawton *et al.*, 2000). Alfenas *et al.*, (2003) compared the appetite effects of two fat sources rich in monounsaturated fatty acids (peanut oil and canola oil) with one rich in

saturated fatty acids (butter), obtaining no different satiety effect (Alfenas and Mattes, 2003).

Many authors have studied the role of polyunsaturated acids like a dietary strategy to modify the perception of satiety. PUFA can interact with neuroendocrine factors involved with energy metabolism such as ghrelin (Cummings and Overduin, 2007, Murphy *et al.*, 2006), insulin (Haugaard *et al.*, 2006, Nettleton and Katz, 2005) or leptin (Mori *et al.*, 2004, Perez-Matute *et al.*, 2007, Winnicki *et al.*, 2002). The mechanism that explains why PUFA: saturated fatty acids (SFA) ratio may influence satiety, could be due to whole-body macronutrient oxidation (Jones and Schoeller, 1988). In fact, oxidation studies suggest that dietary SFA are not oxidised as fuel sources as rapidly as PUFA and that they may favour fat deposition (Jones *et al.*, 1992). Shimomura *et al.* (1990) found that rats fed with safflower oil diet (PUFA) accumulated less body fat than those fed with beef-tallow diet (SFA) (Shimomura *et al.*, 1990). The main reason of this fact is explained because the degree of oxidative metabolism of free fatty acids (and glucose) in the liver constitutes a source of information useful for the appetite control (Friedman and Tordoff, 1986, Friedman *et al.*, 1986, Langhans and Scharrer, 1987, Stubbs *et al.*, 1995). The effect of dietary fat on food intake mainly depends on whether the fatty acids are oxidised or stored. The majority of studies suggest that oxidised fat enhances satiety, whereas fat that is stored shows the opposite effect (Friedman, 1998).

Furthermore, Mori *et al.* (2007) examined the effects of supplementing the diet with fish oils rich in n-3 PUFA in C57BL/6J mice compared to a diet based in triacylglycerol without fish oil. Animals supplemented with 8% fish high-fat (30%) diet for 5 months exhibited an attenuation of body weight compared with the 30% triglyceride diet. This weight loss was not accounted for differences in energy intake, but it was associated with increased intestinal expression of genes involved in fat oxidation. The authors concluded that the anti-obesity effect of fish oil was associated with an up-regulation of intestinal lipid oxidation (Mori *et al.*, 2007).

The ileal brake is a feedback mechanism activated by nutrients, especially fat, with marked effects on satiety. This is primary inhibitory distal-to-proximal feedback

mechanism that controls meal transit through the gastrointestinal tract. Many studies have demonstrated that direct delivery of lipids, specially long chain fatty acids, into the ileum delays gastric emptying (Welch *et al.*, 1988a), which may prolong small intestinal transit time (Read *et al.*, 1984) and induces satiety signals (Welch *et al.*, 1985). But the evidence for direct effect of ileal brake and food intake and satiety in human is limited due to very few researches (Welch *et al.*, 1985, Welch *et al.*, 1988b). Maljaars *et al.*, (2008) studied the effect of ileal fat perfusion on satiety and hormone release in healthy volunteers (Maljaars *et al.*, 2008b). The results of this study showed that ileal perfusion of 3 and 9 grams of fats increased satiety during and after fat perfusion (satiety was measured with VAS), and during ileal perfusion CCK increased dose dependently, being correlated with satiety.

In summary, although fats have been associated to weight gain, overweight and obesity, there are current researches that establish a relationship between fats intake, specifically PUFA, and weight control and satiety signals. Due to this, fats could be an acceptable strategy capable to increase satiety. The review of fats presented here, suggests the role, mainly of PUFA, to enhance satiety. There is a wide body of literature relating PUFA intake and hunger, satiety and reduction of body-fat, in short and long-term, although most of them are performed in animal models. Thus, more studies are needed, especially human long-term trials to establish a clear and certain relationship between fats intake and satiety. If results are positive, fats could be used as either supplementation or added in foods as a strategy to enhance satiety and to improve body composition in overweight and obese population.

4.4. Calcium and satiety: management of lipolysis and lipogenesis

Some micronutrients have an especial interest in terms of satiety and body-weight management. In this regard, it has been proved that calcium has a relevant role in the regulation of adipose tissue and body weight. Augmentation of adipocyte intracellular calcium promotes lipogenesis, triglycerides storage and inhibition of lipolysis. The mechanism proposed by which calcium could lead to reduce lipogenesis is explained by the fact that increments in dietary calcium provoke a suppression in

calciotropic hormones and, thereby a reduction in intracellular calcium into adipocyte, leading to a reduction in the lipid storage (Al-Mana *et al.*, 2012, Kovacs and Mela, 2006, Zemel, 2004). Several interventional and epidemiological clinical trials have suggested that increasing calcium from diet lead to the stimulation of lipolysis and lipogenesis inhibition (Garcia-Lorda *et al.*, 2005). Al-Mana *et al.*, (2012) reported that a consumption of standard breakfast containing 500 mg of calcium reduced energy intake in obese and overweight women. Other researches showed that increasing dietary calcium promoted a modulation in energy attenuating obesity risk (Zemel, 2004). Additionally, the role of calcium in fat storage is promoted by a second interesting mechanism. Dietary calcium forms complex with non-absorbed fat, similar to indigestible calcium soaps, producing the fat excretion. Hence, high concentration of calcium from diet could inhibit the absorption of dietary fat. In this sense, it has been demonstrated that rats fed with high-calcium diet gained less weight and fat content than controls due to a lower digestible energy intake and increased faecal fat excretion (Papakonstantinou *et al.*, 2003).

5. MEDITERRANEAN FOODSTUFFS AND SATIETY

The present manuscript has been inspired in the search of ingredients that provides “satiating power” being capable of having longer lasting satiating effect. Bearing this in mind, this study has been developed in the context of the Satiety Innovation (SATIN) project financed by The European Union (EU) (project number 289800, FP7-KBBE) which has been conceived to investigate and reformulate new enhance-satiety ingredients which conduct to a long-term appetite regulation (Amin and Mercer, 2016). Additionally, since 2016, the Ministry of Economy and Competitiveness of the Government of Spain is funding a second project named “Innovations in satiety based ingredients and food characteristic of the Mediterranean region and its diet” (MEDSATIN) which allowed to research Mediterranean ingredients with satiety potential.

In line with the need to explore new Mediterranean ingredients capable to enhance satiety, certain extracts or ingredients typically consumed in Mediterranean

countries have been selected. Some of them have been selected because of its biological features, such as egg white, carob pulp, hesperetin, certain species of the genus *Capsicum*, pistachio, nopal and tiger nut. Furthermore, proteins from milk whey have been selected, specially milk whey belonging to animal species typical of Mediterranean regions such as milk whey from cow, sheep and goat. These proteinaceous ingredients have been the one of the main objectives of this Doctoral Thesis and they have been amply explained and studied on its satiating properties in next chapters (chapter 2, 3, 4, 5 and 6).

All the mentioned natural ingredients will be an essential tool for the development of new food through its handling and incorporation into functional foods to reduce appetite and food intake, being a key tool for the prevention and treatment of overweight and obesity.

5.1. Egg white

Egg white has been described as an excellent quality source of protein. As whey protein, egg white protein provides all nine essential amino acids and it has considered as a “complete protein” (Gilbert *et al.*, 2011). Its proteinaceous fraction are composed mainly by ovalbumin, ovotransferrin, ovomucoid, lysozyme and ovomucin (Chang *et al.*, 2018). Many biological effects has been attributed to egg protein because the physiological effect of its protein fraction and biopeptides (antihypertensive, antimicrobial, immunomodulatory, antilipidemic, etcetera) (Jahandideh *et al.*, 2016, Mine, 2007, Matsuoka *et al.*, 2014). Furthermore, the effect of egg protein in increasing satiety and promoting weight loss has been previously demonstrated (Vander *et al.*, 2005, Vander Wal *et al.*, 2008, Semon *et al.*, 1987). The satiating effect of egg white has been related to its protein composition, biopeptides and the generation of postprandial plasma amino acids. For instance, when Sprague-Dawley rats were fed with different concentrations (20% or 35%) and several sources of proteins (wheat gluten or egg white protein), a greatest satiety was observed in animals treated to egg protein compared to wheat gluten. The mechanism was explained because the higher

plasma amino acid concentration observed after ingestion of egg protein (Du *et al.*, 2017). More satiating features of egg white have been explained in chapter 7.

5.2. Carob Pulp (*Ceratonia siliqua*)

Fruits from *Ceratonia siliqua* (carob) belongs *Leguminosae* family, widely cultivated in the Mediterranean region usually in mild and dry places with poor soils. It contains a high amount in fibre, and it is a source of polyphenols (tannins). Its potential has been reported as antihypertensive and anti-inflammatory because of its phenolic compounds proportions, which may vary depending on the portion of the fruit (germ, peel, seed and pod) (Rico *et al.*, 2019). Many researches have proved that insoluble dietary fibre from carob exert an antioxidant potential (Kumazawa *et al.*, 2002, Ydjedd *et al.*, 2017). In general, fibre increases time of gastric emptying and nutrient absorption, leading to a sensation of fullness and lowering hunger. In this sense, locust bean gum was related to the regulation of glucose and insulin homeostasis (Tsai and Peng, 1981). The effect of this undigestible gel-forming was proved in rats reducing the time-gastric emptying and improving the levels of postprandial glucose (Tsai and Peng, 1981). Furthermore, carob pulp had the ability to low cholesterol by increasing excretion of cholesterol and bile salts in faeces using animal models (Wursch, 1979). The lower effect on cholesterol and triglycerides has also been proved in human with hypercholesterolemia showing a reduction in LDL cholesterol and triglycerides after carob consumption (Zunft *et al.*, 2003, Ruiz-Roso *et al.*, 2010). Additionally, carob pulp was involved in the regulation of an orexigenic hormone, ghrelin (Gruendel *et al.*, 2007) and lipid oxidation. The main conclusion of that study was that after carob consumption the levels of ghrelin, triglycerides, and non-esterified fatty acids were lowered. Moreover, carob intake decreased energy expenditure and increased the respiratory quotient suggesting that carob was involved not only in satiety but also in increases of fat oxidation (Gruendel *et al.*, 2006). Recently it has been proved that snacks made of carob with a low GI produced a diminished glycaemic response followed by increases of satiety (VAS) and subsequently lower energy intake (Papakonstantinou *et al.*, 2017). Finally, seed, peel and pod extracts from carob attenuated the fat accumulation of mature adipocytes by using 3T3-L1 pre-adipocytes which suggest that carob may have a

key role in alleviating metabolic syndrome (Rico *et al.*, 2019). On the basis of the above, carob pulp may be a suitable candidate to promote satiety effects apart from the beneficial effects on glucose homeostasis and the lowering-lipidemic effects.

5.3. Hesperetin

Citric fruits are rich in C vitamin, but they additionally contain flavonoids, such as hesperetin, showing interesting biological effects (Wilcox *et al.*, 1999). Hesperetin, (3,5,7-trihydroxy 4'-methoxyflavanone) is mainly present in grapefruit and oranges and it possess antioxidant, anti-inflammatory, hypolipidemic and cholesterol-lowering effects (Erlund *et al.*, 2001). Concretely, Kim *et al.*, (2003) showed the key role of hesperetin metabolites on the lowering effect of plasmatic lipids (Kim *et al.*, 2003). Furthermore, the hypoglycaemic effect of hesperetin has been demonstrated in diabetic mice (Jung *et al.*, 2006). These results revealed the key role of hesperetin in the regulation of cholesterol and glucose homeostasis. The satiating potential of this flavonoid has been carried out in enteroendocrine cells (STC-1). This study showed that hesperetin induced the CCK release in a dose-dependent manner via increasing intracellular calcium concentrations and subsequent activation of transient receptor potential channels (TRPA1) (Kim *et al.*, 2013). In consequence, hesperetin could exert qualities to contribute suppressing hunger and eventually, be taken into consideration as a candidate for obesity therapy.

5.4. Active compound of pepper (*Capsicum sp.*)

Red peppers are usually consumed in Mediterranean countries being a common ingredient of the Mediterranean gastronomy. Hot red peppers contain active components, named capsaicinoids that confer pungency. These compounds include capsaicin, dihydrocapsaicin, nordihydrocapsaicin, among other compounds, being the first one the most active (Ludy *et al.*, 2012). Capsaicin shows a satiating potential and an effect of energy balance being interesting in terms of regulation of food intake and body weight. However, the strong pungent effect of these compounds could provoke

nociceptive, sensory burn and side effects on the oral mucosa and gastrointestinal effects (Lejeune *et al.*, 2003). Nevertheless, a potential alternative to use capsaicin preserving the beneficial effects on energy balance could be a non-pungent capsaicin analogue, named capsinoids, which the most bred correspond to CH-19 sweet. Capsinoids includes capsiate, dihydrocapsiate and nordihydrocapsiate, being capsiate the most representative (Ludy *et al.*, 2012). Regarding the effect of capsiate and capsaicin in energy balance, it may be related to their stimulation of thermogenesis leading to increase EE to CNS activation, in addition to increase substrate oxidation (Ludy *et al.*, 2012, Ludy and Mattes, 2011). Several publications have focused in the potential of capsaicin as a satiating ingredient (Westerterp-Plantenga *et al.*, 2005, Ludy and Mattes, 2011, Reinbach *et al.*, 2010), and minor in the case of capsiate (Reinbach *et al.*, 2010).

Furthermore, it has been reported that capsaicin activates the transient receptor potential vanilloid-1 (TRPV1) present through the gastro intestinal tract, which provokes an inhibition of adipogenesis and apoptosis of pre-adipocyte and adipocytes (Leung, 2014). Several studies have demonstrated the effects of capsaicin and encapsulated capsaicin (Yoshioka *et al.*, 2004, Westerterp-Plantenga *et al.*, 2005). Similar results were obtained to capsiate, enhancing fat oxidation, CNS activation, reductions in body weight and fat (total and visceral fat) (Kawabata *et al.*, 2006, Ohnuki *et al.*, 2001).

A recent metanalysis concluded that capsaicin or capsinoids increased EE and reduced respiratory quotient (RQ), which suggested rise in fat oxidation in overweight and obese people. Different results were shown when the BMI were minor 25 Kg/m², indicating that capsaicin or capsiate could be a new therapy to treat obesity by producing an negative energy balance and fat oxidation (Zsiboras *et al.*, 2018).

All of these studies demonstrated the effect of capsiate and capsaicin in a short-term satiety. There is scarce of long-term studies that demonstrate the satiating potential of these compounds. One study showed that the diet supplementation with red pepper for 3 months resulted in a modest weight-loss and increases of fat oxidation and rising in EE (Lejeune *et al.*, 2003). Other research proved the effect of two dose of

capsaicinoid supplements (high and low dose) after 12 weeks. They showed that high dose of capsaicinoid reduced self-reported energy intake and lower dose producing decreases of waist: hip ratio (Urbina *et al.*, 2017).

Finally, and together with the satiating potential of red pepper, the production and the consumption of these type of products have a great impact in Region of Murcia, since there are products protected under a Protected Designation of Origin “Pimentón de Murcia”.

5.5. Pistachio (*Pistacia vera L.*)

In general, nuts are rich in unsaturated fatty acids, proteins, dietetic fibre and minerals such as magnesium and potassium. Several epidemiological and interventional studies have demonstrated the biological effects of nut consumption being important particularly in reducing risk of coronary diseases and diabetes (Jiang *et al.*, 2002, Hu *et al.*, 1998). Furthermore, pistachio contains carotenoids (xanthophyll) and phytosterols showing a great antioxidant potential due its phenolic compound profile (Tomaino *et al.*, 2010). Additionally, pistachios exert beneficial effects on inflammatory processes and disorders related to the production of free radicals (Gulati *et al.*, 2014).

It has been demonstrated that the consumption of pistachio, together with food rich in carbohydrates, for 5-10 weeks diminished the postprandial glucose, reduced blood pressure and improved the peripheral vascular response in patients suffering dyslipidaemia. In that study it was also evaluated the satiating effect of pistachios, showing an increment in GLP-1 secretion and insulin-sparing properties (Kendall *et al.*, 2014). Consumption of pistachios with shell reduced the energy intake around 40% compared to the consumption without peel, maybe due to the time used to remove shell, or the great volume of eaten food.

5.6. Nopal (*Opuntia ficus indica*)

Nopal cactus (*Opuntia ficus indica*) belong to *Cactaceae* family. It is commonly referred to nopal cactus (cladodes) or prickly pear (fruit). *Cactaceae* are well adapted to arid climate typical of Mediterranean countries. Its exploitation as a food is usually based on the consumption of the prickly pear, but the consumption of cactus leaves used as vegetables in the cuisine is increasing. Nopal is rich in amino acids (glutamine, leucine, phenylalanine, valine, etc.), polyphenols (phenols, flavonoids, betaxhantin and betayanin), sterols, vitamins A, B, C and E, minerals (Ca, Na and Fe), polyunsaturated fatty acids and fibres (more than 18% w/w in dry basis) (El-Mostafa *et al.*, 2014, Stintzing and Carle, 2005). It is worth mentioning the high content in fibre and calcium playing both of them important roles in satiety. Many biological properties are attributed to nopal, for instance, potent antioxidant, hypoglycaemic, antidiarrheal, anti-inflammatory, anti-arthritis, ulcer-inhibitory, anti-cancerigen, antilipidemic, cholesterol-lowering, hepatoprotector, antimicrobial and neuroprotective properties (El-Mostafa *et al.*, 2014, Stintzing and Carle, 2005).

The effect of isorhamnetin glycosides, a methylated flavonol contained in nopal, has been investigated after 12 weeks of supplementation to diet-induced-obesity C57BL/6 mice model. Results showed that nopal extract improved glucose tolerance, increased EE, reduced body weight gain, adipocyte size, as well as lower fatty acid synthesis and a highest fatty acid oxidation (Rodriguez-Rodriguez *et al.*, 2015). These results suggested that obesity could be preventable with dietary administration of *Opuntia*. A systematic review and a metanalysis of randomised clinical trials have showed that supplementation with *Opuntia ficus indica* reduced the percentage of body fat and total cholesterol due to fat excretion with faeces. However, non significant differences on body weight were observed (Chong *et al.*, 2014).

The high content of fibre from nopal was assayed in rats to determine wheter it could influenciate the expression of the SCFA receptor GPR43. This specific receptors bind directly to butyrate which lead to different beneficial effects on colonocytes. The results of that study concluded that using 5% of nopal fibre, the expression of SCFA receptor was increased more than 90% (Osorio *et al.*, 2011). Furthermore, the effect of

mucilages and pectins from nopal as prebiotic was determined. The treatment of cultures with mucilage from nopal increased the populations of lactobacilli and bifidobacteria 23.8% and 25%, respectively, apart from decreasing pathogen bacteria such as enterobacteria and staphylococci, among others. Additionally, the production of SCFA after fermentation was measured showing great increases in propionic and butyric acid (Guevara-Arauza *et al.*, 2012).

The features of nopal on food intake and weight-loss regulation makes it an excellent candidate to develop foodstuff based on *Opuntia*. That is why this ingredient has been selected as satiating ingredient to ameliorate food intake and reduce body fat mass in obese mice (chapter 7).

5.7. Tiger nut (*Cyperus esculentus L.*)

Tiger nut (*Cyperus esculentus L.*) belongs *Cyperaceae* family. Despite its name, it is not a real nut because is a tuber but sharing chemical composition with nuts and tubers (Sanchez-Zapata *et al.*, 2012). It has been consumed since ancient times and currently amplily consumed in Mediterranean regions, specially in Valencia, where the milk from tigernut “horchata de chufa” is recognised under the Protected Designation of Origin “Chufa de Valencia”. Tiger nut is especially rich in fats (24% w/w), carbohydrates (43% w/w), fibres (9% w/w), a moderate content in proteins (5% w/w), minerals as P and K and vitamins C and E (Sanchez-Zapata *et al.*, 2012). The benefits of dietary fibre from tiger nuts have been reported, having special interest the properties against obesity, diabetes, coronary heart diseases, gastro intestinal disorders and colon cancer (Sanchez-Zapata *et al.*, 2012). The high percentage of fibre presented in tiger nut posseses several health benefits as satiating effects since fibre absorbed water in the stomach after ingestion, increasing the volume and reducing the intake of foods (Sanchez-Zapata *et al.*, 2010).

Moon *et al.*, (2012) showed that a supplementation (10%) of defatted chufa in mice fed with a high-fat diet, produced a significant reduction of body-weight gain, decreases in serum total cholesterol, tryglycerides, serum insuline and leptin levels and

reductions in adipocyte size. These results suggested that tiger nut could be useful for the prevention of hyperlipidemia and diet-induced obesity (Moon *et al.*, 2012). Furthermore, the hepatoprotector effect of tiger nut milk was demonstrated in rats where the dietary supplementation with this ingredient led to preventing acetaminophen-induced liver injury, maybe due to the action of antioxidant related to increases in the synthesis of glutathione (Onuoha *et al.*, 2017).

Although few studies have demonstrated clearly the satiating potential of tiger nut *per se*, its improved effect on metabolic syndrome makes it a candidate to study in what extent it may promote satiety using as a model DIO mice (chapter 7).

6. MAIN CONCLUSIONS: IMPACT IN THE FOLLOWING RESEARCHES

Looking for ingredients with satiating properties and particularly, Mediterranean ingredients, are the main goal that structure the present manuscript. Among all literature revised, protein has shown to be the most satiating of all macronutrients. Furthermore, evidences suggest that from all protein source reviewed, animal proteins are most effective than vegetable ones. Considering proteins from animals, dairy protein and particularly whey protein have exerted a promising effect on food intake and body-weight regulation. Taking into account these considerations, milk whey, has been selected because its effectiveness in satiety and its higher content in protein and calcium.

In line with the promotion of Mediterranean food in which the present manuscript is based on, milk whey from cow, sheep and goat have been selected to evaluate through *in vitro* and *in vivo* its potential effects on satiety. Firstly, a characterisation of all types of whey milk has been developed to know the differences in the nutritional composition for each of them (figure 1.2). Subsequently, the behaviour and features of milk whey after a simulated digestion from mouth to colon have been driven to understand how degraded proteins may affect inflammation and satiety taken as two key points in this manuscript (chapter 2). Modulation of gut microbiota, inflammation and satiety by milk whey have been the three key points considered in view in the next chapters of this research. Concretely, milk whey from different

ruminant species has been evaluated as anti-inflammatory agent, under the premise that occurrence a low-grade systemic inflammation in obesity stages (chapter 3). Regarding satiety, these selected types of whey have also assayed to evaluate their potential to stimulate peptide hormone secretion using enteroendocrine cell cultures (chapter 4). Obesity is characterised by low gut microbiota's diversity, existing an "obese bacteria profile". Under this hypothesis, whey has been subjected to digestion and colonic fermentation to research its potential as modulators of gut microbiota typically observed in normal-weight status (chapter 5).

It is well-known that, comparing whey from several species, sheep whey is the richest in proteins of all of them. However, the industrial separation of protein fraction from lactose to obtain protein concentrates of this type of whey is not common and extended in the dairy industry. Consequently, nowadays the commercialisation of protein concentrates from sheep whey is not available. The absence of protein concentrates from sheep whey may be due to minor interest on these by-products compared to typically used whey concentrates from cow whey, or maybe because the hard-working separation. In consequence, chapter 6 has been dedicated to performing the protein separation by using ultrafiltration techniques at laboratory scales in order to understand the yield of protein concentration using sweet whey from sheep animals.

Finally, after an in-depth satiety's review, selected Mediterranean ingredients such as whey, egg white, tiger nut and nopal have been used to determine their potential as suppressant of food intake in diet-induced satiety C57BL/6J mice. Moreover, obesity-related parameters were investigated such as, feeding behaviour, EE, satiating hormones, fat composition, etcetera. All these aspects have allowed a full loop to largely understand satiety mechanism.

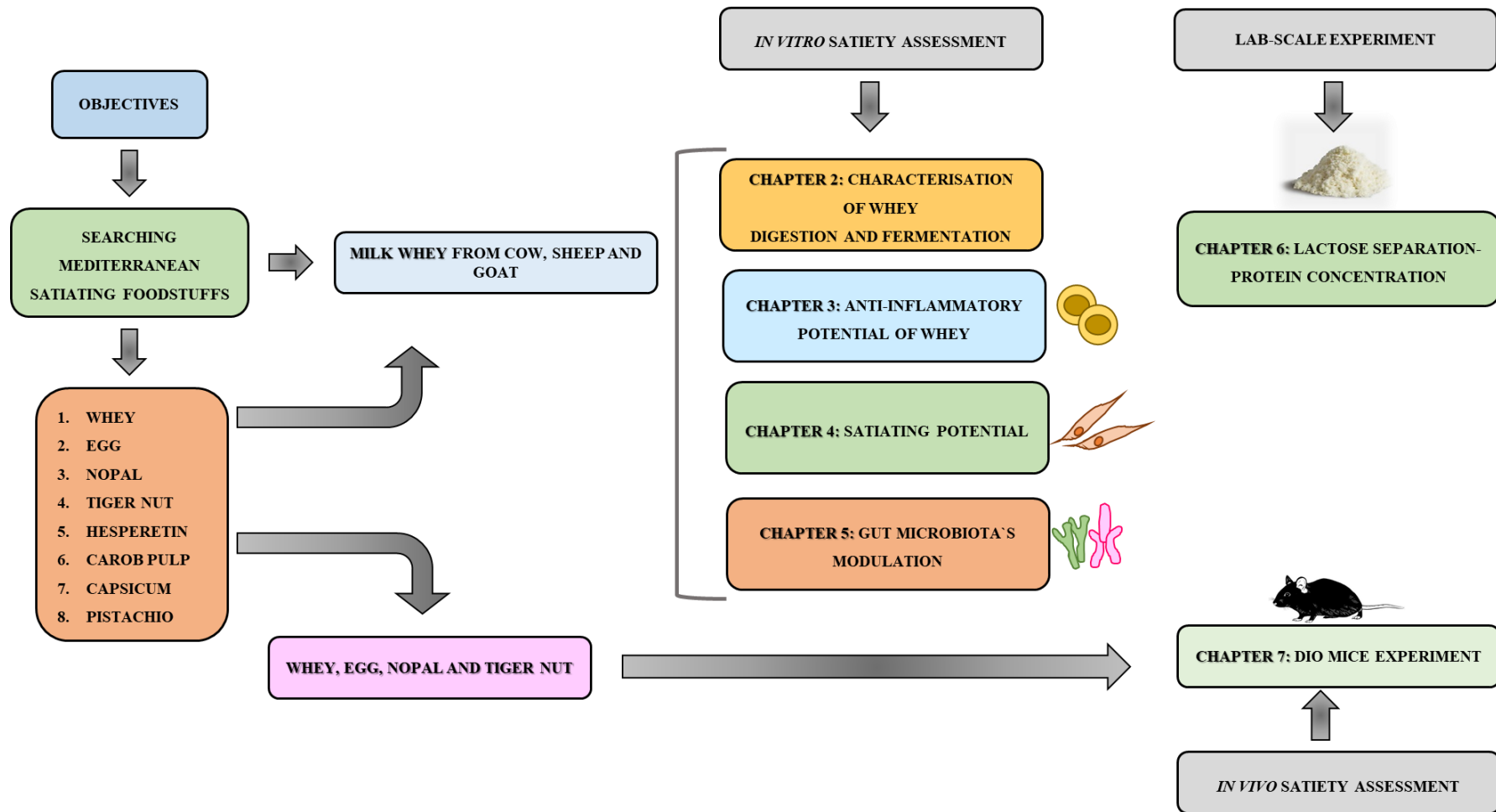


Figure 1.2. Flowchart of the experiments carried out in the present manuscript.

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Nutritional characterisation of milk whey. Protein and peptide profile through whole digestion: from mouth to colon. CHAPTER 2



1. INTRODUCTION

1.1. Milk whey: current state and general composition

Milk whey is a by-product of the dairy industry resulted from cheese or casein production, but its nutritional composition make it a high quality food complement for human nutrition (Madureira *et al.*, 2007). Whey is a greenish yellow liquid produced from the cheese-manufacture (85%) and the production of caseinates (15%) (Figure 2.1). The European Union (EU) is the first producer of cheese worldwide and consequently the number one in whey production.



Figure 2.1. General appearance of milk whey.

In Spain, as well as in Southern European countries, the production of quality cheeses from sheep and specially, from goat is increasing, in part, due to the promotion of Mediterranean foodstuffs with added value. Furthermore, the production of whey leads to a high interest due to: *i*) the large volume of production (more than 1700 tons in

Spain in 2016 (MAGRAMA, 2016)), *ii*) the very high nutritional value of whey (Madureira *et al.*, 2007) and *iii*) its efficient use as a byproduct since it has been destined as feed for farm animals.

Whey is a soluble fraction of milk rich in proteins, lactose and minerals (Hernandez-Ledesma *et al.*, 2011). It contains approximately the half of solids from milk, corresponding 20% of protein and most of the lactose from the origin milk, a little amount of fat, minerals and water-soluble vitamins (Fox, 2015, Wit, 2001) (Figure 2.2). Considering a dry basis, whey contains about 70-80% of lactose, 9% of proteins and 8-20% of minerals (Daufin., 1998). Major compounds of whey are water, lactose, major proteins such as beta-lactoglobulin (β -LG), alpha-lactalbumin (α -LA), bovine serum albumin (BSA), immunoglobulins (Ig) and other proteins as glycomacropeptide (GMP). Major mineral fractions correspond to calcium, magnesium, phosphorous, potassium, chloride and sodium. Fat fractions of whey corresponds mainly to triglycerides, diglycerides, fatty acids and phospholipids. Furthermore, minority components of milk whey are minor proteins such as Ig A, lactoferrin, lactoperoxidase (LP) and lysozyme (LZ); trace elements (zinc, iron, copper and iodine); vitamins and non-protein nitrogen (NPN) as amino acids (AA), urea, cholin and orotic acid (Wit, 2001). Ultra-trace elements, growth factors, hormones, other enzymes and protein and amino acids (AA) can be found in lower concentrations.

Two types of whey could be found depending on the type of cheese production: sweet whey with pH values of 5.2-6.7, obtained after cheese rennet or enzyme-coagulation (Madureira *et al.*, 2007, Pintado *et al.*, 2001); or acid with pH values lesser than 5 or 5.1. This whey is generated by using an acid precipitation of casein, which produces low pH values (Tamime, 2009).

Milk whey can be subjected to several treatments to produce different types of derived products. Several categories of whey can be produced concentrating or removing part of its valuable compounds. Thus, whey can be presented as without lactose, demineralised whey, whey powder, whey protein concentrate (WPC) and/or whey protein isolated (WPI). Several food technologies to originate whey products are based on separation of proteins from lactose leading to protein concentration.

Concentration and separation techniques include membrane technologies such as ultrafiltration, diafiltration, nanofiltration, microfiltration and reverse osmosis to separate water from milk (Wit, 2001, Walzem *et al.*, 2002).

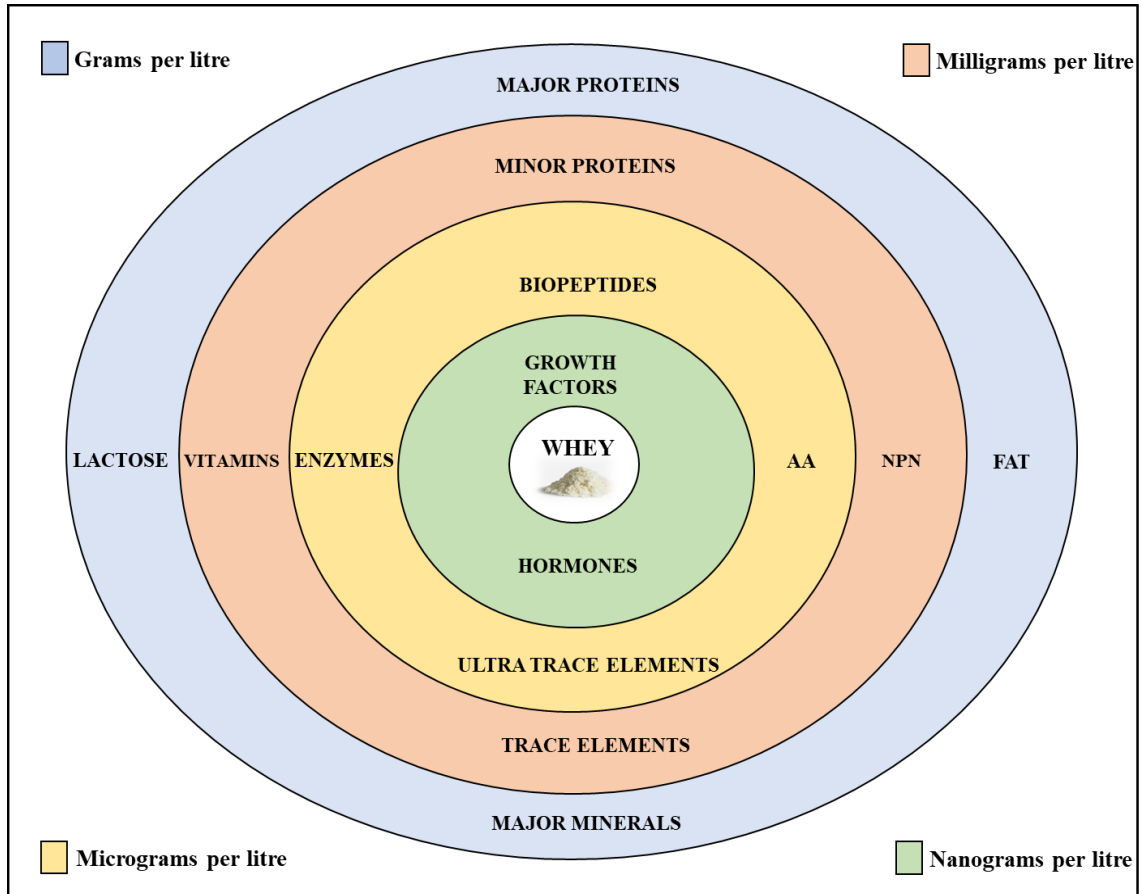


Figure 2.2. Ring diagram of nutrient compound on milk whey based in the figure proposed by Wit, (2001) with slight modifications. External rings represent the nutritional components in high concentrations, and the opposite progressing into small rings. AA: amino acids, NPN: non-protein nitrogen.

Milk whey offers multiple healthy effects on human such as antimicrobial and antiviral activities, modulation of immune system and anticarcinogenic effects, among others (Madureira *et al.*, 2007, Madureira *et al.*, 2010). Furthermore, the effect of whey on nutritional health has focused the main attention of the present study, specifically the role of milk whey on satiety, which will be explained hereafter. The biological effects

are due to the protein fraction *per se*, or by the action of breakdown proteins generated after protein hydrolysis such as peptides and amino acids.

1.2. Satiating effect of milk whey as an ingredient: protein and lactose

In the last years overweight and obesity are being considered as a public health problem with a much-needed consideration. In 2016, around 1.9 billion of adults were overweight and 650 million were obese, tripling the worldwide prevalence since 1975 (WHO, 2016, WHO, 2018). Moreover, in Spain prevalence of overweight and obesity in adults reached 52.7% in 2014, despite the fact that the Mediterranean diet is usually consumed (EUROSTAT, 2014) This so-called, 21st century epidemic, comprises a significant negative impact in public health due to its association with an increased risk of type 2 diabetes, cardiovascular diseases, certain cancers and a shorter life expectancy (Turnbaugh *et al.*, 2009). Regulation of food intake and a healthy life style shape the key to mitigate and prevent overweight and obesity. In this regard, satiety and satiation are two key concepts involved in food intake regulation (Cummings and Overduin, 2007).

Proteins are the most satiating macronutrient in reducing food intake and enhancing satiety (Bendtsen *et al.*, 2013). Nevertheless, the effect of proteins in satiation seems to be dependent on the protein source and even its absorption after digestion (Anderson *et al.*, 2004, Veldhorst *et al.*, 2009, Hall *et al.*, 2003).

The protein fraction of milk whey has been deeply studied by many researches. Although it may depend on many factors, in general, it is composed of a complex mixture of globular proteins. Some authors have determined that the concentrations of β -LG accounted 48-58% and 13-19% for α -LA, being both in the majority proteins from whey (Bonnaillie *et al.*, 2014). However, the concentration of proteins and other components of whey, are dependent on several factors such as those that vary the composition of milk at source (animal species, breed, lactation period and feeding) and/or other factors related with the cheese processing (Sanz Ceballos *et al.*, 2009, Hejtmankova *et al.*, 2012, de Wit, 1998, Madureira *et al.*, 2007). Ruprichova *et al.*,

(2014) showed that α -LA was higher in goat compared to sheep and cow whey protein, whereas β -LG was more abundant in the case of sheep whey, moderate for cow and minor in the case of goat milk. Moreover, differences could be observed according to the lactation period.

Satiating properties of milk whey has been widely studied. The satiating effect of whey is attributed to its protein fraction *per se*, β -LG, α -LA and GMP, among others (Madureira *et al.*, 2007). As well as, its peptides and amino acids derived from protein digestion also have a key role in satiety (Veldhorst *et al.*, 2009). Other potential satiety whey components are galactooligosaccharide (GOS) (Keenan *et al.*, 2006, Overduin *et al.*, 2013) and calcium (Garcia-Lorda *et al.*, 2005, Zemel, 2004, Al-Mana *et al.*, 2012). One of the satiating mechanism from which milk whey reduces food intake is the stimulation of satiating hormones as cholecystokinin (CCK) (Schwartz *et al.*, 2000) and glucagon-like-peptide-1 (GLP-1), more than other milk's proteins as casein (Aziz and Anderson, 2003, Hall *et al.*, 2003, Veldhorst *et al.*, 2009).

β -LG represents the major part of whey protein. Its hydrolysis along gastrointestinal digestion can lead to produce biopeptides as lactokinins (YL and ALPMHIR) with angiotensin-converting-enzyme (ACE) inhibitory effects (Maes *et al.*, 2004). The biopeptide β -lactorphin has been described as ACE inhibitory and as agonist opioid leading to regulate the intestinal absorption (Sipola *et al.*, 2002). This peptide released from β -LG, named lactostatin, may have implications decreasing cholesterol levels (Morikawa *et al.*, 2007). Additionally, β -LG is rich in amino acids as cysteine, with interesting implications on glutathione synthesis (de Wit, 1998).

α -LA is present in a minor extent to β -LG although, it is majority compared to BSA, and Ig, being approximately 20% of the total whey proteins (Madureira *et al.*, 2007, Walzem *et al.*, 2002). The beneficial effect of α -LA can be considered at three stages, as an intact protein, according to its derived peptides and considered as the total amino acids (Chatterton *et al.*, 2006). Peptides generated after digestion of α -LA may have antimicrobial and opioid effects (α -lactorphin) (Pellegrini *et al.*, 1999, Teschemacher *et al.*, 1997). In addition, α -LA possess antitumoral effects due to its antiproliferative action in cell lines from adenocarcinoma (Sternhagen and Allen, 2001).

The anticarcinogenic effect of this protein has been demonstrated when α -LA was joined to oleic acid giving the complex HAMLET or BAMLET (“human- α -LA made lethal to tumour cells” and “bovine- α -LA made lethal to tumour cells”, respectively (Pepe *et al.*, 2013, Hsieh *et al.*, 2015). α -LA contains high amount of AA as tryptophan, cysteine (which are precursors of serotonin and glutathione), *i*-leucine, leucine, and lysine (Lien, 2003) which could have implications on satiety. Additionally, levels of serotonin (5-hydroxytryptamine) could affect food intake apart from modulating mood. Increases in serotonin levels are associated with more preference for proteins and a decreased food intake (Ashley *et al.*, 1979, Shorposner *et al.*, 1986). The neurotransmitter is synthesised from tryptophan and other large neutral amino acids (LNAA) such as valine, leucine, *i*-leucine, tyrosine and phenylalanine. Increased levels of tryptophan-LNAA ratio could lead to enhance serotonin levels and promote satiety (Beulens *et al.*, 2004). High-calcium and α -LA diet have proved to be related to weight loss and reduced fat adiposity in mice (Pilvi *et al.*, 2008). Furthermore, (Hursel *et al.*, 2010) it has been demonstrated the potential satiety of a yogurt fortified with α -LA.

Despite de fact, the previous proteins are the major part in milk whey taking an important role in satiety, the whole satiating effect of whey may be produced by whey protein as a whole. In this sense, Chungchunlam *et al.*, (2017) have demonstrated that the satiating effect of whey was attributable to all whey proteins (Chungchunlam *et al.*, 2017).

GMP is a glycoprotein composed by a rich fraction of branched-chain amino acids (BCAA) such as tryptophan, phenylalanine and tyrosine. Several researches have described the relationship between GMP and satiety by several pathways such as by increasing CCK and decreasing weight gain and fat cumulation when ingested with isolated whey proteins (Burton-Freeman, 2008, Royle *et al.*, 2008).

In minor proportion, milk whey also includes other proteins (BSA, Ig, LF and LP) with opioid and ACE inhibitory activity (Meisel and Schlimme, 1996), anticarcinogenic effects (Laursen *et al.*, 1990), modulation of immune system, antimicrobial and antiviral activities (Madureira *et al.*, 2007, Wit, 2001), antibacterial and immune system modulator (Madureira *et al.*, 2007, Lonnerdal and Iyer, 1995,

Legrand *et al.*, 2005) and the ability to make iron from diet more available for absorption (Wit, 2001).

1.3. Potential satiating effect of whey proteins along digestion

Hydrolysis of proteins starts in the stomach by the proteolytic action of pepsin along with a low pH (HCl). Subsequently, a large proportion of proteins are degraded into peptides and some amino acids at this stage but not all protein fraction since some proteins are resistant to gastric degradation. Hereafter, intact proteins, partially hydrolysed ones, peptides and amino acids continue moving onto the first portions of the small intestine, the duodenum. At this stage, the pancreatic juice (trypsin, chymotrypsin, carboxypeptidases, etcetera) and bile salts, at higher pH respect to the stomach, follow the protein degradation, so little intact protein and abundant peptides and amino acids will be generated after intestinal digestion. Finally, in the epithelial brush-border peptidases breakdown peptides into amino acids and later, they can be absorbed (Caira *et al.*, 2016).

However, the digestion of whey protein results in a complex process since many factors such as the composition of whey, the enzyme-to-substrate ratio, temperature, pH affect the protease activity of pepsin (Ekmekcioglu, 2002, Eriksen *et al.*, 2010). Additionally, under simulated conditions, several physiological aspects cannot be considered such as absorption processes. Nevertheless, the digestion protocol proposed in this study has demonstrated its validity and reproducibility since the digestion of milk (skim milk powder) *in vitro* and *in vivo* has been compared. The main conclusion from this study was that milk proteins detected after gastric *in vitro* digestion was comparable with milk proteins derived from duodenal *in vivo* digestion using pigs as an animal model. Moreover, hydrolysed proteins found in the intestinal *in vitro* digestion were similar to those obtained from distal jejunal *in vivo* digestion (Egger *et al.*, 2017b).

Furthermore, whey protein acts as “fast protein” under stomach conditions being more satiating in “short-term” since it does not coagulate in the acidic medium from the stomach, unlike casein (“slow protein”). This resistance to precipitation in the acid

medium leads to whey protein to enter quickly in jejunum, raising higher postprandial concentrations of amino acids in plasma than casein.

The nutritional value and biological functions of whey protein varies during digestion since a high variety of peptides and amino acids can be released from native protein. Biopeptides can be defined as small fragments of proteins which exert a positive impact on health (Korhonen and Pihlanto, 2006). These peptides may interact with hormone receptors acting as inhibitors or agonist, depending on the sequence of amino acids. Thus, the biological activity of biopeptides will depend on their specific sequence (Hernandez-Ledesma *et al.*, 2014). The main biological activities of biopeptides derived from milk has been described as ACE inhibitory, dipeptidyl peptidase 4 (DPP4) inhibitory, antimicrobial, antioxidant and anti-inflammatory (Hernandez-Ledesma *et al.*, 2002, Hernandez-Ledesma *et al.*, 2005, Nongonierma and FitzGerald, 2016).

Hypertension, hyperglycaemia, obesity and a low-grade of systemic inflammation has been related with the development of metabolic syndrome being important questions to consider because of their impact on human health. Thus, biopeptides could regulate hypertension inhibiting ACE (Egger and Menard, 2017). Moreover, ACE inhibitory peptides from milk could be released under fermentation by the proteolytic action of microorganism (*Enterococcus faecalis*, *Lactobacillus helveticus*, etcetera) (Hernandez-Ledesma *et al.*, 2014).

Biopeptides also showed effects on lipid metabolism since several peptides decreased cholesterol levels (Yoshikawa, 2015). Recently, the relationship between cancer and biopeptides has been described. Although a clear effect of antitumour peptides *in vivo* studies has not been stablish yet, it is worth to mention that some peptides form in milk have induced apoptosis in tumour cells (Blanco-Miguez *et al.*, 2016). Another effect of biopeptides has been described as antimicrobial due to its effect against Gram negative and Gram positive bacteria (Wakabayashi *et al.*, 2003) in part by the action of peptides derived from lactoferrin (Puknun *et al.*, 2013).

Furthermore, biopeptides may be involved on the inflammation regulation, which could be a suitable strategy in the treatment of chronic inflammation related to overweight and obesity (Hotamisligil, 2006). Tavares *et al.*, (2013) showed the anti-inflammatory effect of hydrolysates (peptides) of whey protein (Tavares *et al.*, 2013). It has even been demonstrated that whey protein fermented by *Lactobacillus paracasei* decreased pro-inflammatory cytokines (IL-4 and INF- γ) and increased the production of anti-inflammatory ones (IL-10) (Prioult *et al.*, 2004).

Apart from the previous biological effects, it has been demonstrated the satiety effects of peptides. Satiating hormones, such as glucagon-like-peptide-1 (GLP-1), are released by intestinal cells induced by a nutrient-sensing mechanism. It has been demonstrated that GLP-1 has effect on satiety and in diabetes due to its incretin effect. However, incretins could be degraded by the action of DPP4, a protease from intestinal brush-border. Many biopeptides derived from milk and milk whey had effect on the reduction or inhibition of DPP4 leading to preserving or activating the incretin activity (Horner *et al.*, 2016, Nongonierma and FitzGerald, 2016). Aside from GLP-1, hydrolysates of proteins, peptones and peptides have showed a strong effect on the satiating hormone CCK (Nemoz-Gaillard *et al.*, 1998, Foltz *et al.*, 2008, Choi *et al.*, 2007). In addition, peptides that contained BCAA, specially leucine, may regulate the secretion of anorexigenic or orexigenic hormones as CCK, GLP-1, glucose-dependent insulinotropic peptide (GIP), peptide YY and ghrelin (Hernandez-Ledesma *et al.*, 2014). In line with this satiating effect, there are the biopeptides exert opioid effects too. They could be involved in the inhibition of intestinal peristalsis and motility which prolonged the gastrointestinal transient time, showing antisecretory activities and stimulating endocrine responses such as insulin and somatostatin (Clare and Swaisgood, 2000).

Nonetheless, the hydrolysis of whey does not finalise after gastric or intestinal digestion. In the large intestine, certain gut bacterias have saccharolytic or proteolytic activity to finish breaking and fermenting the end-products of digestion, carbohydrates and proteins, respectively (Macfarlane *et al.*, 1986, Macfarlane *et al.*, 1988, Schaafsma, 2008). At this stage, it is worthy of note lactose content, the major sugar in milk whey. Although the effect on satiety of lactose is not directly considered, it can serve as a substrate for bacteria fermentation leading to the production of short-chain fatty acids

(SCFA). These SCFA could regulate the release of gut satiating peptides and subsequently controlling appetite (Tolhurst *et al.*, 2012). Besides, lactose could act as prebiotic, since it served as substrate for probiotic bacteria to produce galacto-oligosaccharides (GOS) (Aehle, 2007). Additionally, GOS could be involved in the upregulation of satiety signals (Hong *et al.*, 2016).

Taking all this into account, the main objective of the present chapter was to perform an initial characterisation of whey nutritional components as start point to understand how this nutritional composition could affect physiological effects studied in next chapters. Furthermore, the degree of protein hydrolysis and the potential effects of biopeptides on human health have been evaluated after performing a whole simulated *in vitro* digestion inspired in that proposed by COST Action INFOGEST followed by a colonic fermentation.

2. MATERIALS AND METHODS

Whey samples of different ruminant species were subjected to an *in vitro* digestion followed by a fermentation, simulating all phases of gastro intestinal digestion, from the mouth to the colon. Crude samples, as well samples derived from several stages of the *in vitro* digestion were analysed to determine their concentration in macronutrients and minerals. The flow chart of the present study can be observed in figure 2.3. The initial characterisation of milk whey, as a complete food, was carried out focusing on the role of proteins as a satiating ingredient.

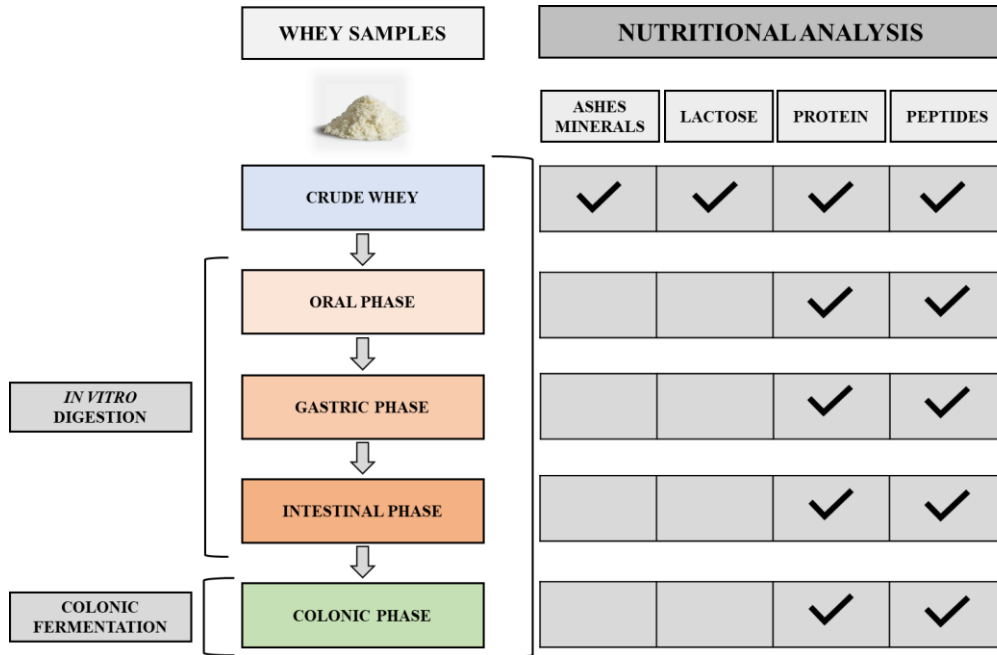


Figure 2.3. Flow chart of the present study.

2.1. Milk whey samples

Four types of sweet milk whey were used in the present study corresponding to Friesian cow whey, and ruminant's species typical from Southern of Spain as Segureña sheep whey and Murciano-Granadina goat whey. Additionally, a mixture of the above milk wheys was studied (60% from cow, 20% from sheep and 20% from goat). All these milk wheys were provided by Palancares Alimentación S.L., a local cheese factory (Murcia, Spain). Samples were defatted by centrifuging at 3.000 g for 15 min and lyophilised (Lyophilizer Telstar Lyoquest. Spain). Finally, samples were stored at room temperature away from light and humidity.

2.2. *In vitro* digestion of milk whey: performing digestion from mouth to small intestine

Simulated gastrointestinal digestions were performed using *in vitro* method, conducted in three phases to simulate oral, gastric and intestinal digestion. We used a standardised and static *in vitro* method proposed by Minekus *et al.*, (2014) and using as initial substrate 1 g of each lyophilised sample of milk whey (Precision balance Ohaus Discovery. USA) (Minekus *et al.*, 2014). This digestion method has demonstrated to be useful for digestion of dairy products (Egger *et al.*, 2016).

To start with the *in vitro* experiment, several salty solutions were prepared in milli Q water and stored refrigerated at 4 °C until assays (Table 2.1). Digestion was performed in duplicate.

2.2.1. Oral phase

To prepare simulated salivary fluid (SSF), 20 mL of solution were used, and pH was adjusted to 7 using NaOH (1 M). To simulate the salivary conditions, α -amylase was added at a concentration of 150 U/mL (75 U/mL in the final mixture). $\text{CaCl}_2(\text{H}_2\text{O})_2$ was added to achieve a final concentration in the mixture of 0.75 mM. Afterwards, 1 g of each sample of skimmed whey were mixed with 1 mL of distilled water, being the final volume of 2 mL. Then the solution was mixed in a glass beaker with 2 mL of SSF (50:50 w/v). The mixture was maintained for 2 minutes in a shaking water bath at 37 °C and 60 strokes per minute (Thermostatic bath Heater Unitronic 320 PR. Spain) in order to simulate mastication.

Table 2.1. Stock solutions of simulated digestion fluids. Concentrations (mmol/L) of salts added into simulated salivary fluid (SSF), simulated gastric fluid (SGF), simulated intestinal fluid (SIF).

Salt solution added	SSF pH 7 mmol/L	SGF pH 3 mmol/L	SIF pH 7 mmol/L
KCl	15.09	6.9	6.8
KH ₂ PO ₄	1.35	0.9	0.8
NaHCO ₃	13.68	25	85
NaCl	-	47.2	38.4
MgCl ₂ (H ₂ O) ₆	0.15	0.12	0.33
NH ₄ (CO ₃) ₂	0.06	0.5	-
CaCl ₂ (H ₂ O) ₂	1.5	0.15	0.6
HCl	1.1	15.6	8.4

2.2.2. Gastric phase

The gastric phase of *in vitro* digestion started with the preparation of 30 mL of simulated gastric fluid (SGF). Pepsin was added to gastric solution at a concentration of 1000 U/mL to achieve a final concentration in the mixture of 500 U/mL. CaCl₂(H₂O)₂ was also added to reach 0.075 mM in the final solution. 4 mL of SGF was added to the oral phase volume (50:50 v/v) and the pH was adjusted to 3 by the addition of HCl (1 M). Samples were then incubated in the same conditions as before for 2 hours.

2.2.3. Intestinal phase

60 mL of simulated intestinal fluid (SIF) were prepared with milli-Q water and pancreatin and bile salts were added at a final concentration of 200 U/mL (100 U/mL in

the final mixture) and 20 mM, (10 mM in the final mixture) respectively. Subsequently, $\text{CaCl}_2(\text{H}_2\text{O})$ was added to achieve a final concentration of 0.3 mM. Then, 8 mL of SIF was added to gastric digestion (50:50 v/v), adjusting the pH to 7 using NaOH (1 M). After that, it was incubated for 2 hours as previously. Finally, samples were stored on ice, to slow down enzymatic activity and stored at $-80\text{ }^\circ\text{C}$ until performing the batch culture fermentation.

2.3. In vitro fermentation of milk whey: performing a simulated colonic environment

The batch culture protocol to simulate the colonic fermentation could be read with more detail in chapter 5 (“*In vitro modulation of gut microbiota by whey protein as a strategy to prevent obesity*”). The present study was performed by using as inoculum human faeces from one normal-weight donor (body mass index (BMI) of 20.44 kg m^{-2}) of 32 years old and without any clinic history of intestinal or metabolic diseases. Additionally, donor had not taken any antibiotics, probiotics or prebiotics in the last 3 months, and was not pregnant. All experiments were performed in compliance with the relevant laws and institutional guidelines. The Ethical Research Committee of The University of Murcia approved the study protocol (annex I), and a written informed consent was obtained from each volunteer before donating the faeces.

2.4. Mineral analysis of milk whey

Total ash content was stimulated by total incineration of lyophilised milk whey using the method described in the Oficial Method for Analysis 18th Edition proposed by the Association of Officiating Analytical Chemist (Lynch and Barbano, 1999, AOAC, 2005). This method was based on total destruction of organic matter by calcining the samples of whey followed by a gravimetric determination of the residual content. Two grams of each sample were weighted in duplicate and allocated into crucibles. After that, samples were calcined using a a muffle furnace (Nabertherm D-2804 Lilienthal, Bremen, Germany) at $550\text{ }^\circ\text{C}$ for 8 hours. Next, samples were allowed to cool down in a

dryer and weighted again. Total ashes (% w/w) were calculated by the following equation:

$$\text{Total ashes (\%)} = ((m_2 - m_0)/(m_1 - m_0)) \times 100 \quad \text{Equation 2.1.}$$

where, m_2 corresponded to weight in grams (g) of the crucible containing ashes, m_1 to weight (g) of the crucible containing sample and m_0 to weight (g) of the empty crucible. Results were expressed as an average (%) \pm S.D.

Additionally, mineral content was determined by inductively coupled plasma mass spectrometry (ICP-MS) according to the normalised method proposed to determine thirty-three elements (UNE, 2010). The following elements were measured after an acid digestion: sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), phosphorus (P), iron (Fe), zinc (Zn), selenium (Se), copper (Cu), boron (Bo), aluminium (Al), antimony (Sb), arsenic (As), beryllium (Be), bismuth (Bi), cadmium (Cd), chromium (Cr), cobalt (Co), lead (Pb), nickel (Ni), lithium (Li), sulphur (S), molybdenum (Mo), strontium (Sr), thallium (Ta), titanium (Ti), rubidium (Rb), vanadium (V), silicon (Si) and lanthanum (La).

One gram of lyophilised sample dissolved into 10 mL of distilled water was used for mineral determination. Samples were digested using nitric acid prior analysis. For this purpose, samples were weighted in a Teflon digestion vessel of 25 mL with 4 mL concentrated nitric acid (HNO_3) and 1 mL of 32% hydrogen peroxide (H_2O_2). In the Teflon reactor was added 300 mL of ultrapure water, 30 mL of H_2O_2 and 2 mL of concentrate sulphuric acid. After that, samples were subjected to a microwave program starting with a pressure of 40 bar and increasing 10 bar/min for 30 min. A temperature of 220 °C was achieved and maintained for 20 min. Then, the reactor was cooled, and 25 mL of ultrapure water were added into the tubes. Additionally, standards for calibration curve were prepared using a multi-pattern for Na, K, Ca, Mg, B, Cr, Fe, Ni, Cu, Zn, Cd, Pb, Al, Co, Ti, S, P, As, Mo, Se, Sb, Ti, Be, Bi, V, Sr, Li, Rb, Mn, La and Si at the following concentrations: 0.01, 0.1, 1.0, 5.0, 10.0, 30.0, 60.0 and 100.0 mg/L and a blank (0 mg/L) The ICP model used was the Thermo ICAP 6500 DUO (Thermo

scientific. USA). The ICP-MS operating conditions were the following: nebulizer gas flow 0.91 L/min, radio frequency (RF) 1200W, lens voltage 1.6 V, cool gas 13.0 L/min, auxiliary gas 0.70 L/min. The concentration of each element was directly read in the computer screen based in calibrated curves and expressed as g/100 g in the case of major minerals and mg/Kg in the case of minor mineral components.

2.5. Lactose analysis of milk whey

Lactose content was measured by high-performance liquid chromatography (HPLC) with a refractive index detector (RID) (VWR-Hitachi Elite LaChrom® HPLC system, USA). Chromatographic separation was achieved with a CARBOsep CHO-682 column, with 7 µm particle size; 200 mm x 7.8 mm i.d. (Teknokroma, Barcelona, Spain) at 70°C. The mobile phase was milli Q water with an isocratic elution with a flow-rate of 0.4 mL/min. The injected volume of the sample (previously filtered through a 0.22 µm-pore nylon filter) was 20 µL in duplicate. Peaks were identified comparing retention times with lactose standards. Area of each peak corresponding to lactose was used for the quantitative analysis. Calibration curve for lactose were prepared as followed: 0.1, 0.5, 1.0, 1.5, 2.5, 5.0, 10 % (w/v) (Figure 2.4.). Data analysis was performed using Agilent EZChrom Elite software. Lactose determination was performed in duplicate and the results were expressed as a percentage (% ± S.D.).

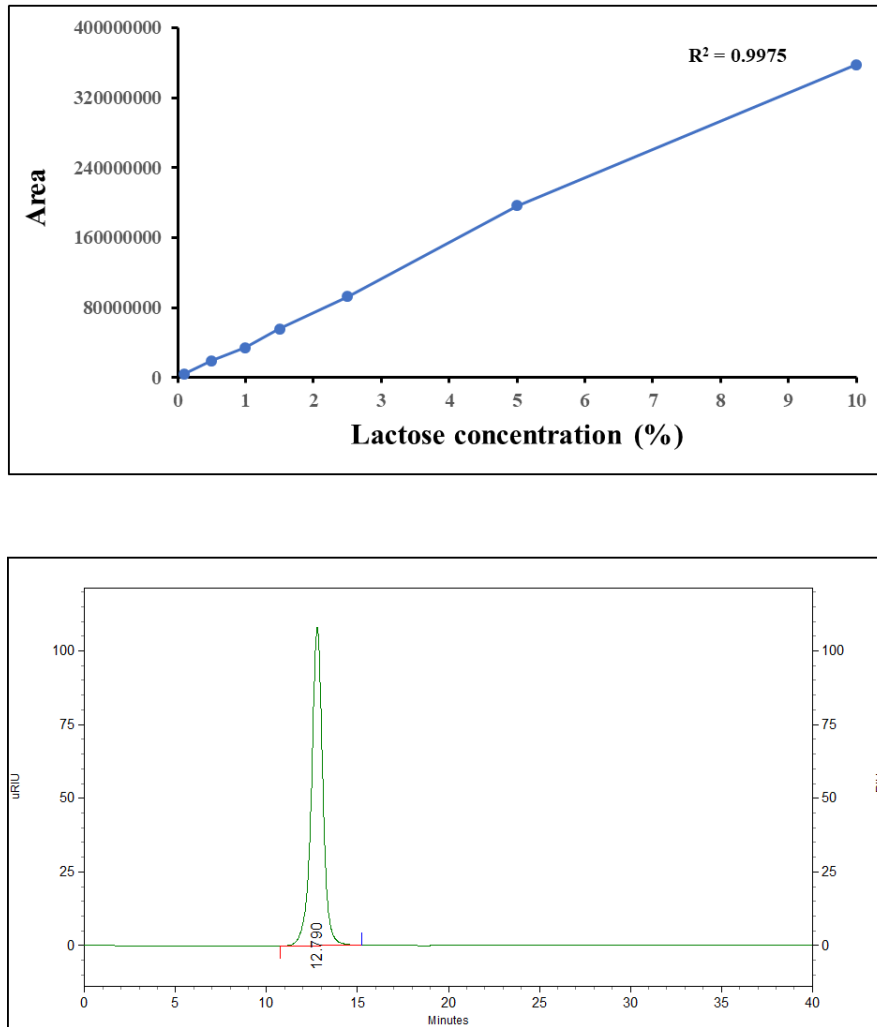


Figure 2.4. A) Calibration curve for determining lactose concentration (% w/v) of whey samples. B) Chromatogram by HPLC/RID of lactose.

2.6. Protein analysis of milk whey

The quantification of protein was evaluated using several methods depending on the phase of the *in vitro* digestion or/and fermentation. Protein of crude whey was determined by using Kjeldahl method because it is a useful method for quantifying high amounts of proteins which were present in lyophilised raw whey. However, after the *in vitro* digestion and fermentation Coomassie-Bradford assay was applied, since it was

more sensitive to determine lesser amount of proteins. Additionally, all samples of whey (crude, digested and fermented) were analysed by HPLC-MS to determine their concentrations of proteins and peptides.

2.6.1. Protein analysis of milk whey by using Kjeldahl method

Nitrogen content of crude whey samples was determined by using Kjeldahl method, and then calculating the initial protein content of samples using the nitrogen conversion factor of 6.38 used in case of dairy products (Lynch and Barbano, 1999). Kjeldahl method included three phases: digestion, distillation and titration. To start experiment, 0.2 g of lyophilised sample of each type of whey were weighted. After that, a digestion was conducted using 15 mL of sulphuric acid (96%), anhydrous sodium sulfate and selenium and heating, firstly at 400 °C and finally at 430 °C (1.5 hours). Digestion allowed to break the nitrogen in the sample to convert into ammonium ions (NH_4^+) which joined to ion sulphate of sulphuric acid giving ammonium sulphate. When digestion finished, the distillation was driven transferring the product of digestion into distillation flasks. Four mL of indicator of proteins and 1 L of boric acid (40%) were added. The solution was neutralised by adding NaOH (32%), dispensed manually, to convert ammonium sulphate into ammonia gas which was liberated from the solution and captured by boric acid. At that point, the low pH facilitated the conversion of ammonia gas into ammonium ion and borate ion (from boric acid). The colour of the solution at this stage was green (Figure 2.5) and the process of distillation was finalised when 150 mL of distilled were recovered. The titration was the final step of the process. The nitrogen content was determined by titration using HCl (0.1 N) into ammonium borate formed in the previous stage (violet colour). Moles of HCl needed to finish the reaction were equivalent to the original nitrogen of whey samples (Equation 2.2). Brute protein (% w/w) was determined by duplicate.

$$\% \text{ Protein} = \frac{(V_2 - V_1) \times 0.1 \times 1.4 \times 6.38}{W} \quad \text{Equation 2.2.}$$

where, V2 was the volume (mL) of HCl achieve the end-point of the reaction in the samples, V1 corresponded to the volume (mL) of HCl used to the blank test, 0.1 corresponded to normality of HCl, 1.4 to the molecular weight of HCl and W corresponded to the weight of each sample (g). A conversion factor of nitrogen into milk proteins was applied (6.38).

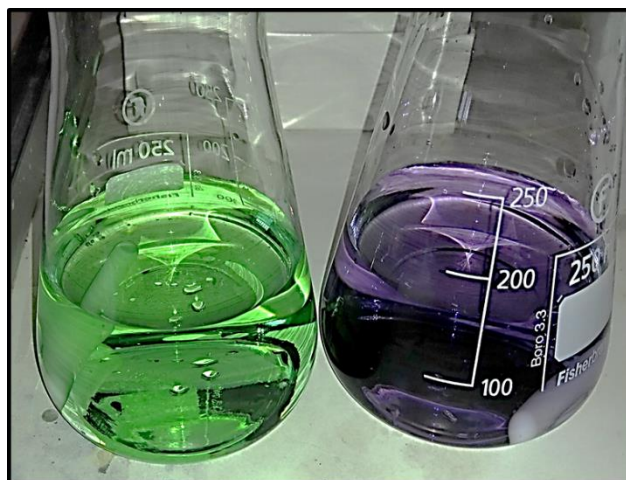


Figure 2.5. Colour variation after titration using HCl (0.1 N). The process finalised when the solution turned violet completely indicating the amount of ammonium absorbed by boric acid.

2.6.2. Protein analysis of milk whey samples by using Coomassie-Bradford

Total protein quantification of digested and fermented samples was measured using the colorimetric method Coomassie (Bradford) protein assay kit (Pierce Biotechnology, USA) based in the principle of protein-dye binding through the microplate protocol for total protein quantification (Bradford, 1976). The acidic Coomassie-dye reagent varies from brown to blue depending on the amount of protein of

the sample. The dye was mainly joined to protein at arginine, tryptophan, tyrosine, histidine and phenylalanine residues (Olson and Markwell, 2007). Five μL of each sample, standards and 250 μL of Coomassie reagent were pipetted in 96-microplate wells. The microplate was shaken and incubated for 10 minutes at room temperature. Absorbance of samples was measured at 595 nm using a UV/Vis spectrophotometer FLUOstar Omega (BMG Labtech, Germany) and the software Fluostar to analyse data. The average of blank absorbance was subtracted from standard and samples. Protein determination was performed in duplicate.

2.6.3. Protein analysis of whey using high-performance liquid chromatography (HPLC).

Liquid chromatography was used to determine the protein content of whey samples as a complement of the previous determinations. HPLC consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermostatted μ -wellplate autosampler. Samples (40 μL) were injected into a C5 reverse-phase (RP)-HPLC column (Discovery Biowide Pore C5, 2.1×100 mm, 5 μm , Supelco), thermostatted at 40 °C, and eluted at a flow rate of 200 $\mu\text{L}/\text{min}$ during the whole separation.

Before injection, samples were centrifuged at 13000 g during 10 min at 20 °C and the supernatant was passed through 0.2 μm HPLC filters. Mobile phase A, consisting of 0.1% formic acid (v/v) in Milli Q water, and mobile phase B, consisting of 0.1% formic acid (v/v) in acetonitrile (ACN), were used for the chromatographic separation. The initial HPLC running conditions were solvent A:B 90:10 (v/v). The gradient elution program was 10% solvent B for 10 min; a linear gradient from 10 to 100% solvent B in 30 min; 5 min at constant 100% solvent B (Table 2.2.). The column was equilibrated with the starting composition of the mobile phase for 15 min before each analytical run. The UV-visible chromatogram recorded at 280 nm was integrated for quantifying the total protein content. The average of blank absorbance was subtracted from standard and samples.

2.6.4. Peptide analysis of whey using high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS).

The separation and analysis of the peptides from whey samples were performed with a HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a μ -wellplate autosampler and a capillary pump. The equip was connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an electrospray interface (ESI).

Whey samples were centrifuged at 13000 g during 10 min at 20 °C. The supernatant was filtered through 0.2 μ m HPLC filters before injection. Samples were injected onto a Waters XBridge BEH300 C18 HPLC column (5 μ m, 150 \times 1.0 mm), thermostated at 40 °C, at a flow rate of 10 μ L/min.

After the injection, the column was washed with buffer A (buffer A: water/ACN (95:5) and 0.1% of formic acid) for 10 minutes and then the peptides were eluted using a linear gradient 0-80% buffer B (buffer B: water/ACN/ (90:10) and 0.1% of formic acid, for 140 minutes. The column was equilibrated with the starting composition of the mobile phase for 15 min before each analytical run (Table 2.2.). The UV-Vis chromatogram recorded at 210 nm was integrated, based in the absorbance of peptide bonds.

The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3500 V, and a scan speed of 8100 (m/z)/sec from 50-2200 m/z, with a target mass of 1000 m/z, and 3 spectra averaging. The nebulizer gas pressure was set to 15 psi, whereas the drying gas was set to a flow of 5 L/min at a temperature of 350 °C. MS/MS data were collected in an automated data-dependent mode (Auto MS mode). The five most intense ions were sequentially fragmented using helium collision-induced dissociation (CID) with an isolation width of 2 m/z and a relative collision energy of 35%.

Table 2.2. Gradient elution program corresponding to quantification of proteins and peptides by using HPLC and HPLC-MS.

Protein		Peptides	
Time (min)	%B	Time (min)	%B
0	10	0	0
5	10	10	0
35	100	150	80
40	100	155	80
45	10	165	0
60	10	180	0

Data processing was performed with a Data Analysis program for LC/MSD Trap Version 3.3 (Bruker Daltonik, GmbH, Germany). To identify peptides, a similar method (Boutrou *et al.*, 2013) was used. All results were submitted to Spectrum Mill MS Proteomics Workbench (Rev A.03.02.060B, Agilent Technologies, Santa Clara, CA, USA), which allowed to identify peptides using a fast database for searching through algorithms to selected validate sequence (NCBI-PubMed). The searching of peptides identification was performed against the major proteins of milk whey (β -LG, α -LA, BSA and Ig).

Finally, after automatic and manual validation of the results, a summary of the identified proteins with the sequence of the digested peptides was reported. Peptides were considered valid after following a selection requirement which ensured the highest level of confidence. These criteria were based on scores threshold and percentages-scored peak intensity (SPI), being higher than 8 and higher than 70%, respectively.

The Spectrum Mill score is based on a points system. This scoring scheme is composed of three main components; the protein score, the peptide score and the Scored Peak Intensity (SPI). Additional measures are provided by unmatched ions (the number of unassigned ions /the number ions from peak detection) and the backbone cleavage score (the number of amino acids cleaved between either *b* or *y* ions). Following peak detection, the MS/MS search algorithm attempts to match and score the most intense

ions in the MS/MS spectrum to an ion type consistent with fragmentation of peptide candidates.

2.7. Statistical analysis

The initial nutritional composition of whey samples from cow, sheep, goat and a mixture of them (60:20:20) was evaluated by one-way analysis of variance (ANOVA) (SPSS v.21.0) and subsequent Tukey multiple comparisons among different samples ($p < 0.05$).

3. RESULTS

Milk whey from several ruminant species (cow, sheep, goat and a mixture of them (60:20:20, respectively)) was analysed over a simulated *in vitro* gastro intestinal digestion and colonic fermentation for 48 hours. The content of ashes and minerals, lactose and proteins (Kjeldahl) was quantified in raw whey as the starting point to know the different nutritional profile through the whole digestion. In addition, protein and peptides were measured in a very sensitive manner in each stage of the static *in vitro* digestion (oral, gastric and intestine phases). Furthermore, the hydrolysis of native proteins were evaluated in a mimicked colonic environment at 12, 24 and 48 hours of fermentation of whey proteins using human faeces as inoculum.

3.1. Initial characterisation of crude milk whey

The nutritional composition analysis of lyophilised whey samples is shown in table 2.3. pH values of all kind of whey were within the normal range that sweet whey should have (5.2-6.7).

Table 2.3. pH measurement and percentage of ashes, protein (Kjeldahl) and lactose of lyophilised whey samples from cow, sheep, goat and a mixture of them (60:20:20)¹.

Sample	pH		Ashes%		Protein%		Lactose%	
Cow	6.70	± 0.00 ^c	10.32	± 0.45	14.20	± 0.26 ^a	73.45	± 0.02 ^b
Sheep	6.11	± 0.02 ^a	8.28	± 0.54	17.60	± 0.53 ^c	71.41	± 0.14 ^a
Goat	6.41	± 0.04 ^b	8.45	± 0.51	15.34	± 0.49 ^b	78.43	± 0.07 ^c
Mixture	6.51	± 0.02 ^b	10.20	± 0.56	14.67	± 0.14 ^a	72.9	± 0.24 ^{ab}

¹Results are expressed as mean of percentage (%) (w/w) ± S.D. Letters a, b and c denote statistically significant differences for a given variable ($p < 0.05$).

It was notable that sweet sheep whey was significant richer in protein compared to cow, goat and mixture whey ($p < 0.05$). Furthermore, lactose content was the highest in the case of caprine whey (78.43±0.07%). Cow whey presented the lowest concentration in proteins (14.20±0.26%), and mixture whey yielded intermediate values of protein and lactose. Ashes content were significant similar among all whey samples. Total elements of milk whey were also determined by using ICP-MS (Table 2.4). As, Bi, Se and Tl were detected in concentrations lower than 0.01 mg/Kg (data not shown). Be, Cd, Co, Cu, Li, Mo, Ni, Pb, Sb, Sr, Ti and V were detected in sheep whey in higher levels compared to other milk whey samples. Besides it was notable the highest concentration of iron in the case of sheep whey compared to other samples (1.55 mg/Kg). In general, the highest concentrations of elements (mg/Kg) corresponded to Fe and Al in case of sheep whey (1.55 and 1.44 mg/Kg, respectively) and Rb in case of goat and mixture whey (1.43 and 1.40 mg/Kg, respectively). Additionally, we found increases in the concentration of Zn in case of mixture whey (0.54 mg/Kg). Several minerals were higher in case of cow, sheep and mixture as in the case of Ca and P compared to goat whey. Conversely, K and Na (g/100 g) were higher in case of goat, cow and mixture whey compared to sheep whey (0.06 and 0.023 g/100 g, for K and Na, respectively).

Table 2.4. Total element of whey from cow, sheep, goat and a mixture of them using ICP-MS.

ELEMENT	COW WHEY	SHEEP WHEY	GOAT WHEY	MIXTURE WHEY
Al (mg/Kg)	0,05	1,44	<0,01	0,67
Be (mg/Kg)	<0,01	0,17	<0,01	<0,01
B (mg/Kg)	0,15	0,10	0,18	0,18
Ca (g/100g)	0,0233	0,0232	0,0097	0,0296
Cd (mg/Kg)	<0,01	0,05	<0,01	<0,01
Co (mg/Kg)	<0,01	0,08	<0,01	<0,01
Cr (mg/Kg)	0,01	0,06	<0,01	<0,01
Cu (mg/Kg)	0,01	0,14	0,01	0,02
Fe (mg/Kg)	0,08	1,55	0,08	0,16
K (g/100g)	0,12	0,06	0,11	0,12
Li (mg/Kg)	<0,01	0,09	0,01	0,05
Mg (g/100g)	0,006	0,005	0,004	0,006
Mn (mg/Kg)	0,49	0,59	0,35	0,48
Mo (mg/Kg)	<0,01	0,31	<0,01	<0,01
Na (g/100g)	0,032	0,023	0,030	0,029
Ni (mg/Kg)	<0,01	0,10	<0,01	0,01
Pb (mg/Kg)	<0,01	0,05	<0,01	0,01
P (g/100g)	0,030	0,026	0,014	0,027
Rb (mg/Kg)	0,63	0,85	1,43	1,40
Sb (mg/Kg)	<0,01	0,19	<0,01	<0,01
S (g/100g)	0,010	0,010	0,006	0,008
Sr (mg/Kg)	0,15	0,31	0,21	0,34
Ti (mg/Kg)	0,01	0,17	0,01	0,01
V (mg/Kg)	<0,01	0,07	<0,01	<0,01
Zn (mg/Kg)	0,16	0,23	0,11	0,54

3.2. Characterisation of digested and fermented milk whey samples

Different raw whey samples were subjected to a harmonised *in vitro* digestion and a simulated colonic fermentation for 48 hours. The objective was to mimic all phases of gastro intestinal digestion, starting from the mouth, reaching the stomach and the small intestine, concluding in the colon.

3.2.1. Protein determination of digested and fermented whey samples

Milk whey protein and its hydrolysis degree were determined in digested and in 48-h fermented samples by using Coomassie Bradford (CB) and RP-HPLC. In table 2.5 it can be observed proteins breaking down along *in vitro* digestion and subsequent 48-h fermentation. In addition, the percentage (%) of protein hydrolysis along the digestion was calculated.

A different pattern of hydrolysis of intact protein can be observed depending on the whey species and the phase of the *in vitro* digestion. When digestion was progressed, lesser concentrations of proteins were obtained due to its hydrolysis by proteolytic enzymes during stomach, small intestine, as well as colon stage. Under the oral digestion, the amount of proteins was similar to initial time, and hydrolysis was not found as expected (data not showed). Protein values during 12 and 24 hours of fermentation were similar to results for concluded fermentation (48 h). After 2 hours of gastric digestion all types of whey samples experimented a remarkable drop in the protein concentration due to the hydrolysis by the acidity and pepsin activity. However, differences can be observed within types of milk whey. Cow whey experimented the lowest percentage of protein hydrolysis ($51.70 \pm 2.16\%$) after gastric digestion. Sheep and mixture whey showed a similar pattern of protein degradation after gastric digestion, although the one corresponding to sheep proteins was slightly lower than mixture whey ($63.02 \pm 0.23\%$ vs $67.54 \pm 1.28\%$). Nevertheless, the percentage of hydrolysis in the case of goat whey after gastric digestion was more severe corresponding to $84.06 \pm 0.83\%$ of protein degradation. Practically, all proteins from goat whey were almost hydrolysed at this stage.

Table 2.5. Hydrolysis of protein fraction from several milk wheys over an *in vitro* digestion and subsequent 48-h of fermentation. Results were expressed as mean±S.D. of percentages (%) of protein hydrolysis related to crude whey and protein concentration (µg/mL).

SAMPLE	PHASE OF DIGESTION	PROTEIN HYDROLYSIS (%)			PROTEIN CONCENTRATION (µg/mL)		
		MEAN	S.D.		MEAN	S.D.	
COW WHEY	Stomach	51.70	± 2.16		4.44	± 0.19	
	Small intestine	75.31	± 0.3		2.27	± 0.02	
	Colon (48 h)	97.95	± 0.38		0.18	± 0.03	
SHEEP WHEY	Stomach	63.02	± 0.23		4.77	± 0.03	
	Small intestine	81.58	± 0.10		2.38	± 0.01	
	Colon (48 h)	98.43	± 0.20		0.2	± 0.02	
GOAT WHEY	Stomach	84.06	± 0.83		1.76	± 0.09	
	Small intestine	91.78	± 0.12		0.91	± 0.01	
	Colon (48 h)	98.23	± 0.36		0.18	± 0.04	
MIXTURE WHEY	Stomach	67.54	± 1.28		3.59	± 0.14	
	Small intestine	84.31	± 0.12		1.74	± 0.01	
	Colon (48 h)	98.19	± 0.26		0.2	± 0.02	

Focusing on terms of protein concentration ($\mu\text{g/mL}$), the initial protein content affected its concentrations after digestion. Sheep whey showed an appreciable protein content ($4.77\pm 0.03 \mu\text{g/mL}$) after 2 h of gastric digestion despite its high hydrolysis. Cow whey presented also higher levels of proteins compared to goat and mixture. As it can be seen, goat whey yielded a dramatic fall in protein concentration after the action of pepsin, being the lowest ($1.76\pm 0.09 \mu\text{g/mL}$).

Furthermore, after 2 h of intestine digestion a similar trend was observed compared to gastric digestion. The degree of hydrolysis found in the case of cow whey regarding the initial protein concentration ($75.31\pm 0.3 \%$) was lesser than in other species. Furthermore, similar percentages of protein degradation were found for sheep and mixture whey. However, goat milk whey yielded the highest percentage of protein degradation after intestinal digestion ($91.78\pm 0.12\%$), in relation to its initial stages.

The concentration of protein measured after intestinal digestion was around half of the concentration showed under gastric conditions in all cases of whey samples. In this sense, goat whey presented the lowest concentration of proteins after 2 h of intestinal digestion ($0.91\pm 0.01 \mu\text{g/mL}$), being noticeably higher specially in the case of sheep, followed by cow and mixture whey (2.38 ± 0.01 , 2.27 ± 0.02 and $1.74\pm 0.01 \mu\text{g/mL}$, respectively).

Finally, after 48 h of fermentation under simulated colonic conditions, percentages of protein degradation were more standardised among all samples compared to gastric and/or intestinal digestion, being near to 97% and 98%. In addition, the concentration of protein under fermentation was extremely lower than other phases of digestion.

The hydrolysis of protein in several phases of digestion was also visualised by reversed-phase HPLC (Figure 2.6) to know the degradation of native protein through several phases of digestion and as a complement for CB protein quantification.

At this point, it is important to consider that a comparison among chromatogram peaks obtained from different species was not resulted adequate since β -LG or α -LA from *bos*, *ovis* or *capra*, showed different absorbances coefficients at the present wavelength (280 nm). Nevertheless, a comparative determination of degree of hydrolysis within each type of whey sample was possible. The milli absorbance units (mAU) of chromatograms within each sample was used to calculate the percentage (%) of protein degradation through the *in vitro* digestion related to mAU obtained from the initial crude whey (Table 2.6). This last estimation together with Coomassie data allowed to shed light on the degradation of proteins within digestion for each whey species.

As we expected, the results derived from RP-HPLC analysis were quite similar to those obtained using CB, being both understood as complementary. Protein chromatograms were descending through the whole digestion progress being different the kinetic of hydrolysis among whey samples.

Table 2.6. Degradation of protein fraction along *in vitro* digestion and fermentation. Results were expressed as percentages (%) of protein hydrolysis related to crude whey.

Sample	% PROTEIN HYDROLYSIS		
	Gastric phase	Intestinal phase	Fermentation 48 h
Cow whey	39.10	74.05	94.92
Sheep whey	70.32	87.63	98.33
Goat whey	82.76	89.90	98.29
Mixture whey	73.77	83.63	96.78

Chromatograms showed that after gastric digestion peaks corresponding to intact protein, were almost disappeared. Nevertheless, after digesting samples with pancreatin and bile salts, little peaks still could be surmised. However, following 48 h of whole fermentation, native protein was completely degraded.

Although the hydrolysis percentages varied from RP-HPLC and CB, the same tendency could be observed, obtaining the highest protein degradation after stomach digestion in goat whey (82.76%), as well as after intestinal phase (90% approximately).

Finally, after 48 h of fermentation, the percentages of degradation obtained from mAU were higher in general, being more than 90%.

All the above results revealed that protein degradation occurred mainly after 2 h of gastric digestion being severe in the case of goat whey, moderate in the case of cow whey and with intermediate values for sheep and mixture.

It was curious that proteins from cow suffered the hydrolysis as in two steps; firstly, a strong hydrolysis after gastric conditions and secondary a moderate hydrolysis after intestinal digestion. After intestinal phase, the hydrolysis of proteins from all whey species was more balanced with regard to previous phases of digestion. After fermentation, percentages of degradation from HPLC revealed an exhaustion of intact proteins at this stage, ranging from 94 to 98%.

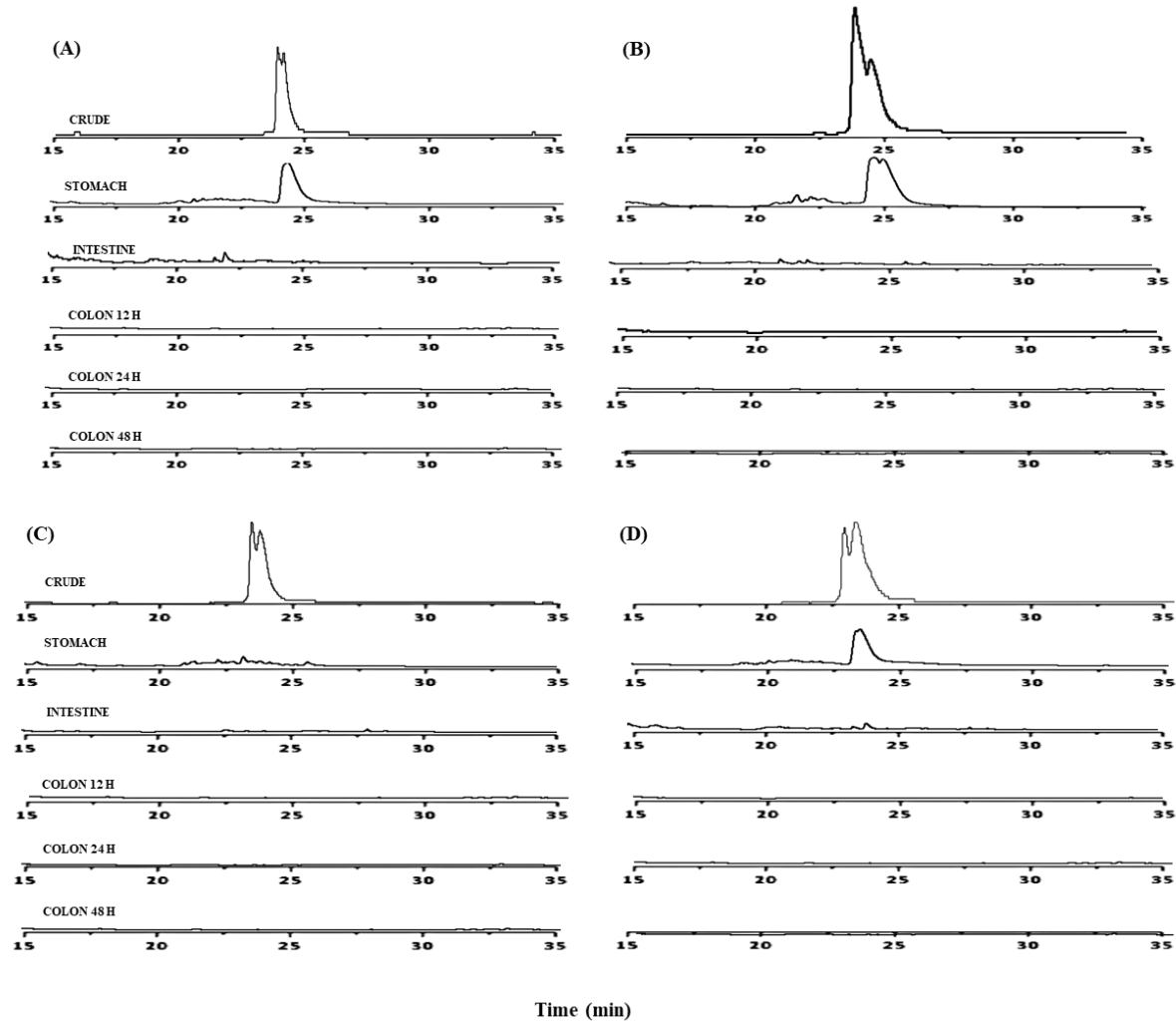


Figure 2.6. Protein chromatograms of digested and fermented milk whey from cow (A), sheep (B), goat (C) and a mixture of them (D).

3.2.2. Peptides determination and biopeptides identification in digested and fermented milk whey samples

Peptide releasing after hydrolysis of protein within several types of milk whey can be observed in figure 2.7. Besides, the chromatogram profile of peptides derived from several types of whey after a simulated gastro intestinal digestion and fermentation can be observed in figure 2.8.

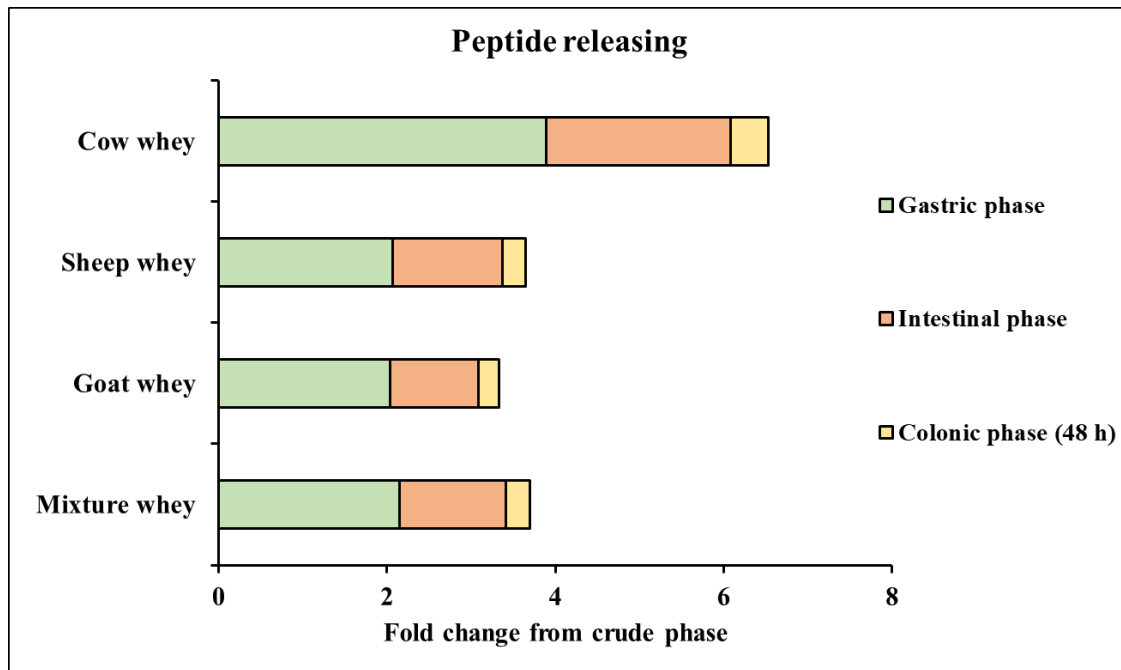
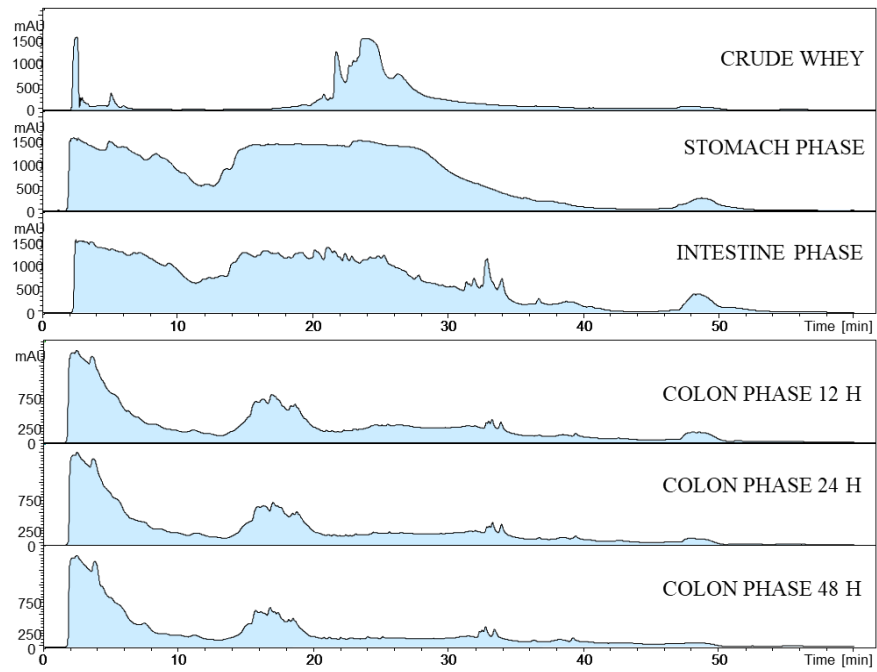
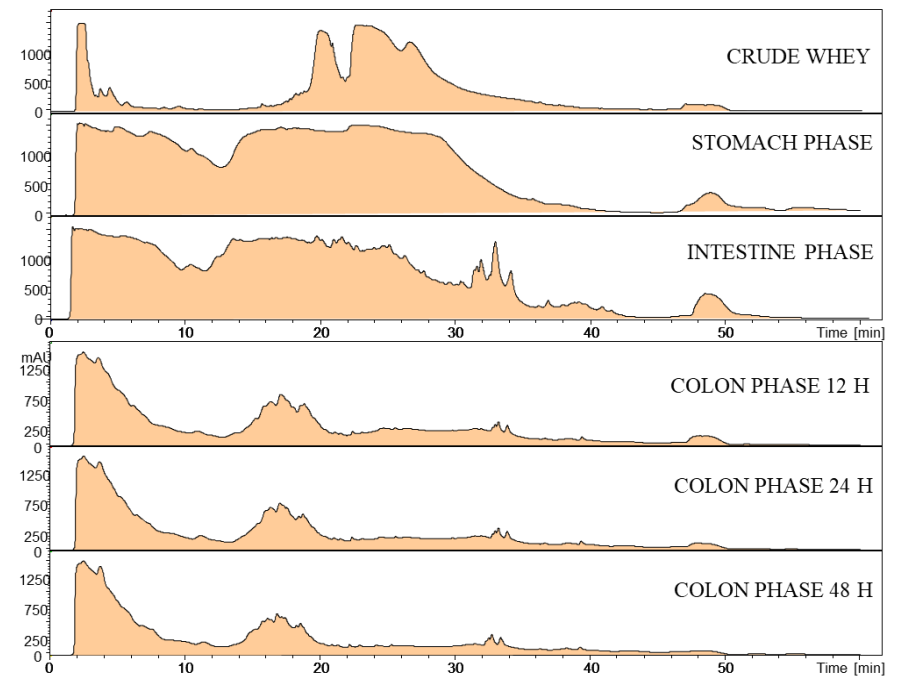


Figure 2.7. Peptide releasing (fold change) derived from protein hydrolysis from cow, sheep, goat and mixture milk whey. Results were expressed as a fold change related to crude whey within each type.

Peptide profile of cow whey



Peptide profile of sheep whey



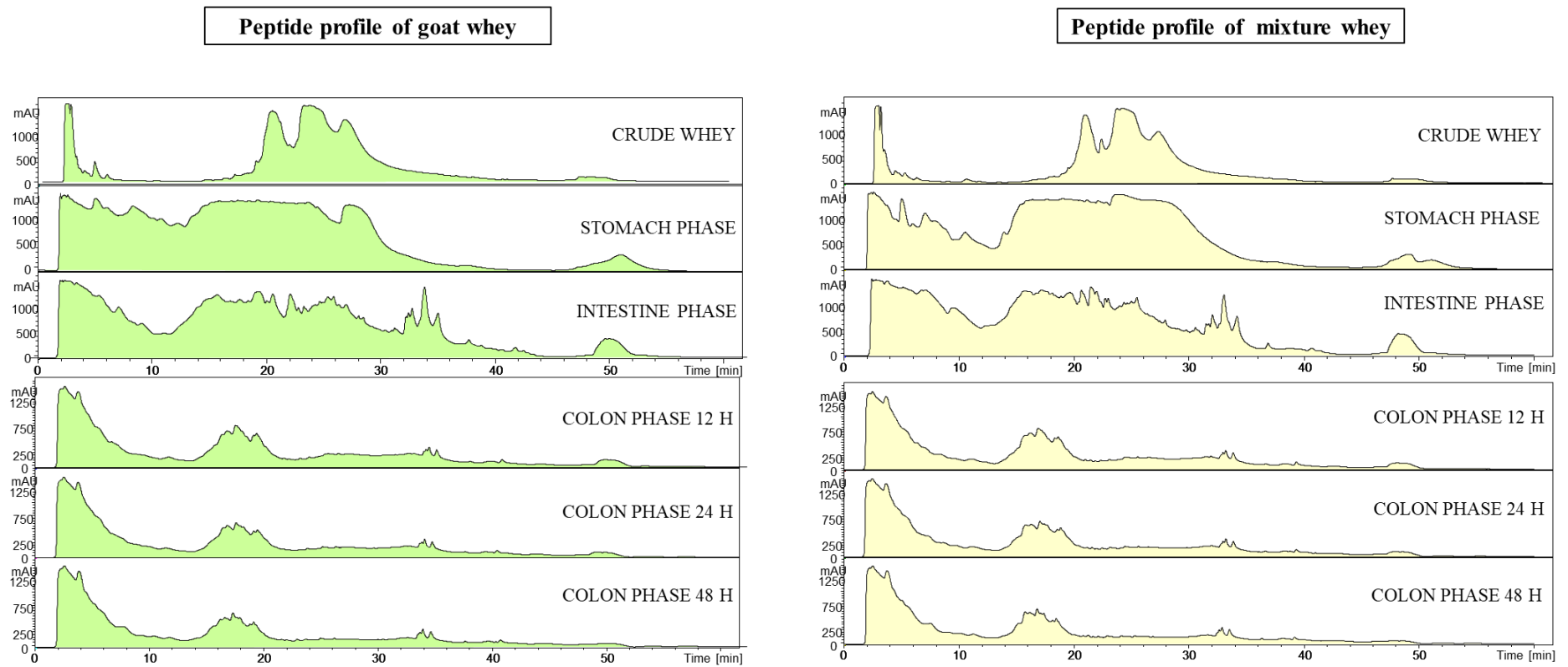


Figure 2.8. Peptides chromatograms of digested and fermented milk whey from cow, sheep, goat and a mixture of them.

After a whole simulated digestion, proteins were degraded into peptides. Fold change values of peptides have been calculated from mAU obtained from HPLC chromatograms in respect to crude phase. The initial point to express the data was considered as zero the crude whey since it was the phase where peptides were not still produced. Comparison among types of whey samples was not considered since peptides derived from different native proteins (β -LG and α -LA from *bos*, *ovium* and *capra*). However, an estimation of peptide production within each type of whey it could result more adequate.

The chromatograms of peptides after gastro intestinal digestion suggested that peptides were released in higher amounts after hydrolysis of protein, specially after gastric conditions, matching with the step of the *in vitro* digestion where a great amount of protein was hydrolysed.

Peptide analysis was also driven using mass spectrometry (MS) and its preliminary identification was performed by comparing with the major proteins from milk whey: β -LG, α -LA, BSA and Ig (Table 2.7). Peptide mass derived from MS was compared using the software Spectrum Mill Proteomics Workbench and the database NCBI.

Total peptides MS spectra were compared against the most important proteins whey form. Following, these detected peptides were identified as valid under exclusion criterion (score>8 and SPI>70%). Peptide identification, as well as its main functions, have been analysed elaborating a homemade database to search the sequence of peptides (oligopeptides, tri o dipeptides) with suitable physiological functions (Table 2.8).

Results of peptide detection and identification showed that after gastric digestion a high number of peptides were detected in milk whey samples corresponding to 67, 66, 60 and 58 for goat, mixture, cow and sheep, respectively. Among these detected peptides, approximately 15% have been identified (validated): 10 for mixture whey, 9 for cow whey and 6 and 5 for sheep and goat, respectively.

Table 2.7. Total detected peptides and identified/validated peptides under exclusion criteria.

SAMPLE	PHASE OF DIGESTION	PEPTIDES DETECTED	PEPTIDES IDENTIFIED	BIOPEPTIDES IDENTIFIED
COW WHEY	Stomach	60	12	9
	Small intestine	56	6	4
	Colon (12 h)	33	3	2
	Colon (24 h)	23	6	3
	Colon (48 h)	24	2	1
SHEEP WHEY	Stomach	58	13	6
	Small intestine	88	23	17
	Colon (12 h)	31	5	5
	Colon (24 h)	34	6	3
	Colon (48 h)	21	0	0
GOAT WHEY	Stomach	67	13	5
	Small intestine	71	6	5
	Colon (12 h)	33	6	5
	Colon (24 h)	24	0	0
	Colon (48 h)	28	2	2
MIXTURE WHEY	Stomach	66	13	10
	Small intestine	57	7	2
	Colon (12 h)	29	9	5
	Colon (24 h)	34	7	3
	Colon (48 h)	28	1	1

After intestinal digestion a great total number of peptides were identified in the case of sheep (88), goat (71), mixture (57) and cow (56) being whey higher compared to gastric digestion in the case of sheep and goat whey. In relation to peptides, finally validated at this phase, sheep whey showed 17 validated peptides. Ultimately, after 48 h of fermentation lesser number of peptides was detected and identified, compared to previous digestion, although several peptides with biological functions were still considered.

Table 2.8. Peptide sequence and biological activities identified in several types of milk whey after a simulated gastrointestinal digestion and colonic fermentation. (A). cow whey; (B): sheep whey; (C): goat whey and (D): mixture whey¹.

Table 2.8. (A). Main peptides identified with known activity from cow whey after digestion and fermentation.

Digestion	Accession	N ^o	Protein	Sequence	Activity
	300508791	1	Beta lactoglobulin	(V)YVEELKPTPEGDL(I)	Antioxidant and ACE inhibitory
	803330671	1	Roundabout homolog 3 isoform X2	(S)LAFSGQNFLQFPNHTLPQNWSPGAVWGLGAL	DPP4 inhibitory
	803322687	1	Receptor-like protein 3 isoform X2	(L)YWFYYKNTVLGSREAPFGGASFNLTMAEHSGNFSCGADNG(L)	DPP4 inhibitory
	803281404	1	Keratinocyte-associated protein 3 isoform X2	(L)ILVGHVNLLLGAVLHGTVLRHVANPRGAVTPE(Y)	DPP4 inhibitory
GASTRIC					
	803280752	1	Leucine-rich repeat neuronal protein 3	(F)IGLHNLLRLHLNSNRLQM(I)	DPP4 inhibitory
	7542833	1	Alpha lactalbumin	(F)LDDDLTDD(I)	ACE inhibitory
	509264350	1	Immunoglobulin mu heavy chain	(S)WVRQAPGKALECLGGISSGGNTGYNPA(L)	DPP4 inhibitory
	586908169	1	MCH class I-related gene protein	(E)YGKDALQRTEPPKVRVNHKETFPGITTL(Y)	DPP4 inhibitory
	75069854	1	Neurotrimin	(L)IFFNVSEHDYGNVTCVASNKLGHNTAS(I)	DPP4 inhibitory
	1211	1	Beta casein	(K)EMPPFK(Y)	ACE inhibitory
	548456349	1	Junctional adhesion molecule A	(L)IVLVPPSKPTINVPSSVTIGTRAVLTCSEKDGSPSEY(R)	ACE and DPP4 inhibitory, and anti-inflammatory
INTESTINAL					
	162797	1	Beta casein precursor	(L)GVSKVKEAMAPKHKEMPPFK(Y)	ACE inhibitory and anti-inflammatory
	803169616	1	Embiigin isoform X6	(T)WYSGNGSVQVPIGVPVNDKYVISGKHANETRLKITQ(L)	DPP4 inhibitory and antioxidant
	803335200	1	Muscle skeletal receptor tyrosine-protein kinase	(K)ITRPPINVKIIEGLK(A)	Antioxidant
COLON 12 H					
	350538267	1	Lymphocyte activation gene 3 protein precursor	(D)LLVAGDRNNFTL(R)	DPP4 inhibitory
	548527469	1	Roundabout homolog 3	(R)GGKLMMSHTFKNDAGMYVCVASNMAGERESGAAKL(V)	DPP4 inhibitory
COLON 24 H					
	803339068	1	Leukocyte immunoglobulin-like receptor	(L)GPVGTIHGGRYRCYGGHLSSEW(S)	ACE inhibitory
	2497311	1	Myelin-oligodendrocyte glycoprotein	(Y)RGRTQLLKETIGEGK(V)	DPP4 inhibitory and antioxidant
COLON 48 H	296475510	¹	ADAMTSL3 protein-like	(L)ARPQLSIPVARL(H)	DPP4 inhibitory

¹Accession: number corresponding to native protein assigned by NCBI PubMed. N^o: number of peptides identified with same sequence corresponding to the same native protein. MHC: major histocompatibility complex.

Table 2.8. (B). Main peptides identified with known activity from sheep whey after digestion and fermentation.

Digestion	Accession N ^a	Protein	Sequence	Activity
GASTRIC	1211	Beta casein	(S)WMHQPPQPLPPTVM(F)	DPP4 inhibitory
	388235	Immunoglobulin gamma-1 chain	(F)IFPPKPKDILT(I)	ACE inhibitory
	7542833	Alpha lactalbumin	(F)LDDDLTDD(I)	ACE inhibitory
	529016080	Matrix-remodeling-associated protein	(V)FESRRRVNVAKKQINPER(W)	DPP4 inhibitory
	529016080	Matrix-remodeling-associated protein	(Q)YVCTAKNLHGADRMVVL(L)	DPP4 and ACE inhibitory
	139948384	MHC class II antigen DS beta	(F)YPGHIEVRWFRNGQEKEAGVVSTGL(I)	DPP4 inhibitory
INTESTINAL	193085052	Albumin precursor	(L)DETYVPKPF(D)	Acetylcholinesterase and ACE inhibitory
	223780	Lactoglobulin beta	(R)VYVEELKPTPEGNLE(I)	Antioxidant and ACE inhibitory
	223780	Lactoglobulin beta	(R)VYVEELKPTPEGN(L)	Antioxidant and ACE inhibitory
	1211	Beta casein	(W)MHQPPQPLPPTVMFPPQSV(L)	ACE and DPP4 inhibitory
	213391437	Alpha s1 casein variant	(Y)NVPQLEIVPK(S)	ACE inhibitory
	803130287	Metalloproteinase-23 isoform X1	(L)AATPDRDSYPRGVAVPLPPHPEA(L)	DPP4 inhibitory
	741939748	T-cell receptor alpha chain V region CTL-F3 isoform X1	(Y)SREQGSCSFANMHSATHSVLL(I)	ACE inhibitory
	803207101	Fibroblast growth factor receptor 2 isoform X6	(L)AGNSIGISFHSAWLTVLPAPVREK(E)	DPP4 inhibitory
	19861780	Versican core Protein	(Y)TLNFEMAQKACVDIGAVIATPEQ(L)	DPP4 inhibitory
	803254576	Receptor-type tyrosine-protein phosphatase F	(K)GDGARSKPKIVTTTGAVFAKNF(R)	DPP4 inhibitory
COLON 12 H	350538267	Lymphocyte activation gene 3 protein precursor	(D)LLVAGDRNNFIL(R)	DPP4 inhibitory
	803209587	Protein sidekick-2 isoform X3	(R)DGRKIPSSR(I)	ACE inhibitory and anti-inflammatory
	165926	Immunoglobulin epsilon-chain	(T)YYNPALKSRLSITR(D)	Antioxidant
	803205793	T-cell surface glycoprotein CD1b-2-like isoform X1	(L)VNELQLKYPFVIQV(I)	Antioxidant
COLON 24 H	975852	Immunoglobulin lambda light chain variable region	(L)GQRVSITCSGSSSNVGLGN(Y)	DPP4 inhibitory
	528964259	CD276 antigen	(Y)RGYPKAEVLWQDQGAPLTGNVTTSQMA NEQGLF(D)	ACE and DPP4 inhibitory
	741916099	Titin isoform X2	(F)QVTWLRNNKEIRSSK(K)	DPP4 inhibitory

Table 2.8. (C). Main peptides identified with known activity from goat whey after digestion and fermentation.

Digestion	Accession	N ^o	Protein	Sequence	Activity
GASTRIC	803340689	1	Casein kinase I isoform beta	(E)YVHRQNLIHRDIKPDNFL(I)	ACE inhibitory
	803129146	1	Hemicentin-1 isoform X6	(T)LECKAAGNPPPV(L)	DPP4 inhibitory
	548506128	1	Protein LOC102179803, partial	(I)ILSDLTNFLKRHGKWSEVPYIQAFWD(L)	DPP4 inhibitory, antioxidant and anti-inflammatory
	803213078	1	Papilin isoform X5	(V)FCTVDDEVYPEHLQCPQPRPADRRPCSLQPC PQTKRYS(Y)	ACE inhibitory
	50872133	1	Killer cell lectin-like receptor	(I)LKLLAVNQTW(L)	DPP4 inhibitory and antioxidant
INTESTINAL	803336288	1	CD48 antigen-like isoform X2	(K)KDSEKGNCHSHNPECTSAVQTIYEKIRR(S)	DPP4 inhibitory and antioxidant
	332078461	1	Interleukin-1 receptor type 1	(L)KDENNE(L)	Antioxidant
	548458821	1	Prostaglandin F2 receptor	(R)ANRRNDVVTSSELLAVMNGDWTLK(Y)	DPP4 inhibitory and antioxidant
	296478922	1	Plexin A2	(A)LVPKQTSSYNIPASASISRTSISR(Y)	ACE and DPP4 inhibitory
	296472694	1	Immunoglobulin	(L)WLLNGSMGLSDGQDGYRVGVDG(L)	DPP4 inhibitory
COLON 12 H	350538267	2	Lymphocyte activation gene 3 protein precursor	(D)LLVAGDRNNFTL(R)	DPP4 inhibitory
	509264415	1	Immunoglobulin delta heavy chain	(C)LGGIGSGGSTLYNRALK(S)	DPP4 inhibitory and antioxidant
	426216050	1	Vascular cell adhesion protein 1-like isoform X1	(G)IYVCEGINQAGISRKEVKL(I)	ACE and DPP4 inhibitory
	548470565	1	Casein beta	(W)MHQPPQPL(S)	DPP4 inhibitory
COLON 48 H	350538267	1	Lymphocyte activation gene 3 protein precursor	(D)LLVAGDRNNFTL(R)	DPP4 inhibitory
	803030174	1	Obscurin-like protein 1 isoform X1, partial	(N)LHQLRRCSPLDAGTYSCVVGMARTGPPVH(L)	DPP4 inhibitory

¹Accession: number corresponding to native protein assigned by NCBI PubMed. N^o: number of peptides identified with same sequence corresponding to the same native protein. MHC: major histocompatibility complex.

Table 2.8. (D). Main peptides identified with known activity from mixture whey after digestion and fermentation.

Digestion	Accession N°	Protein	Sequence	Activity	
	300508791	2	Beta lactoglobulin	(V)YVEELKPTPEGDLE(I)	Antioxidant and ACE inhibitory
	528997546	1	CMRF35-like molecule 4	(T)LEQKKKAFFSGNDSGQWRLW(F)	Antioxidant, ACE and DPP4 inhibitory
	741916099	1	Titin isoform X2	(S)INNYIVEKRDTSTTTWQIVSATVARTTIKACRLKTGCE(Y)	Antioxidant and DPP4 inhibitory
	7542833	2	Alpha lactalbumin	(F)LDDDLTDD(I)	ACE inhibitory
GASTRIC	205831548	1	General transcription factor II-I	(M)LRDQSAVVVQGLPEGVAFKHPENYDLATLKWILENKAGIS(F)	Antioxidant and DPP4 inhibitory
	741948106	1	Signal-regulatory protein beta 1 isoform X1	(E)FKSGPGTHLTVSTKPSPPMVSGPAVRATPEQTVS(F)	DPP4 inhibitory
	359063745	1	High affinity nerve growth factor receptor	(T)IVKSGLRVAPNA(F)	ACE and DPP4 inhibitory
	803338639	1	Killer cell immunoglobulin-like receptor like	(N)FTLGPVTRFHAGSYTCSGFSRSLFEFSRHS DPLQIVVTGV(F)	ACE and DPP4 inhibitory
INTESTINAL	1211	1	Beta casein	(K)EMPPFK(Y)	ACE inhibitory
	803207101	1	Fibroblast growth factor receptor 2	(L)FKLLKEGHRMDKPANCTNEL(Y)	Antioxidant
COLON 12H	350538267	2	Lymphocyte activation gene 3 protein precursor	(D)LLVAGDRNNFTL(R)	DPP4 inhibitory
	209573160	1	Protein kinase C and casein kinase	(R)GRLDGSQLGLYPANY(V)	DPP4 inhibitory
	803226734	1	Hemicentin-2 isoform X2	(R)LTCNATGAPSPLLMWLK(D)	Antioxidant and DPP4 inhibitory
	548527313	1	Immunoglobulin superfamily member 22	(K)KAIXLKS(L)	Antioxidant
COLON 24 H	803334060	1	Titin isoform X54	(K)WTKNGEEVPFDRNVLYRIDK(Y)	Antioxidant and DPP4 inhibitory
	350538267	1	Lymphocyte activation gene 3 protein precursor	(D)LLVAGDRNNFTL(R)	DPP4 inhibitory
	741968632	1	Neural cell adhesion molecule L1	(L)ELHCESQCDSHLKY(S)	Antioxidant
COLON 48H	350538267	1	Lymphocyte activation gene 3 protein	(D)LLVAGDRNNFTL(R)	DPP4 inhibitory

In general, the most important physiological functions found for peptides have been related to ACE inhibition (antihypertensives), DPP4 inhibition (satiety and diabetes), anti-inflammatory peptides and biopeptides showed antioxidant or/and anticholinesterase activity. Moreover, some sequences of peptides have shown several activities within the same large sequence. For instance, peptide YVEELKPTPEGD, derived from β -LG had showed many bioactive sequences such as the dipeptide YV and the oligopeptide LKPTPEGD with ACE inhibitory activity. This peptide also showed the sequence YVEEL and LK which could confer antioxidant activity. Peptide derived from albumin found in case of digested sheep whey, DETYVPKPF, showed potential features as ACE inhibitor (YV) and as acetylcholinesterase-inhibitory activity (KFP). Potent DPP4 inhibitor peptide (IPA) was also detected. Peptide sequence derived from β -LG, (W) MHQPPQPLPPTVMFPPQSV(L) showed ACE inhibitory properties (LPP) and DPP4 inhibitory potential (MHQPPQPL and VL). In addition, peptide GVSKVKEAMAPKHKEMPFPK showed anti-inflammatory activity because of the sequence VKEAMAPK and ACE inhibitory potential due to EMPFPK. Other biopeptides found showed tri-peptides with two potential activities, such as IPP that presented anti-inflammatory and ACE inhibitory activity.

Most validated peptides have shown two major potential functions: ACE and DPP4 inhibition. However, several antioxidant and anti-inflammatory sequences have been also identified among all milk whey samples. Focusing in biopeptides and biological function found in each type of milk whey, a large amount of biopeptides with ACE inhibitory potential has been detected, concretely, in 6 sequences of cow whey, in 18 sequences for sheep whey and in 4 and 8 sequences in the case of goat and mixture whey. These data revealed the potential activity as ACE inhibition of peptides derived from digestion and fermentation of sheep milk whey. Regarding the sequences with DPP4 inhibitory potential, 13 sequences showing satiating potential have been identified in the case of cow and mixture whey, 14 for goat whey and 15 for sheep whey, which could be the one with more potential as satiating ingredient. Finally, the major number of peptides which could release anti-inflammatory peptides were found in cow whey (2), followed by sheep and goat milk whey. None anti-inflammatory sequences have been detected in the case of mixture whey.

4. DISCUSSION

4.1. Initial characterisation of crude milk whey

pH values of milk whey samples assayed in the present study were within the normal range corresponded to sweet whey (Lievore *et al.*, 2015, Giroux *et al.*, 2018, Madureira *et al.*, 2007). However, and being all samples sweet whey, slight differences were due different origin of whey. Features of whey can be basically vary depending on the source of milk. Likewise, nutritional composition of milk may change according to animal species, breed, lactation stage, animal feeding, cheese processing, etcetera (Sanz Ceballos *et al.*, 2009, Hejtmankova *et al.*, 2012, Inglingstad *et al.*, 2010, Tagliazucchi *et al.*, 2018).

In general, the results of the nutritional composition of whey were quite similar to those obtained by other researchers, being the proportion of macronutrients considerably similar (Romo, 2003, de Wit, 1998).

Focusing on differences among types of milk whey, sheep whey contained significantly ($p < 0.05$) more concentration of protein than those from other species. There is scarce literature about the comparative nutritional composition of milk whey, even more in case of milk whey powder. Nevertheless, several authors showed a similar trend when they determined the whey protein content in different types of milk, demonstrating that the summarization of α -LA and β -LG were the highest in sheep compared to cow and goat milk, similarly to our results (Ruprichova *et al.*, 2014, Potocnik *et al.*, 2011). Comparing the protein content of cow and goat milk, similar whey protein composition has been found (Sanz Ceballos *et al.*, 2009). Recently, an study has shown the nutritional composition of cow, sheep and goat sweet whey, being their results of protein composition practically equal than those of the present study, being the highest for sheep, followed by goat and cow (Giroux *et al.*, 2018).

Additionally, lactose concentration values were higher in goat whey, followed by cow, mixture and sheep whey. However, Giroux *et al.*, (2018) found the highest values for lactose in case of sweet and acid sheep whey, followed by cow and goat

whey (Giroux *et al.*, 2018). Lactose concentration could vary depending on lactation period, being lower in the colostrum and at the beginning of lactation (Park *et al.*, 2007).

Total ash content in whey samples were similar when we compared all types of whey, being similar to findings by other authors (Giroux *et al.*, 2018). Total elements of whey samples were also determined by using ICP-MS. Whey was rich in major minerals such as K, Na, P, Ca and Mg and trace elements which may play a notable role in metabolism and physiology (Hernandez-Ledesma *et al.*, 2011). In general terms, focusing in the major minerals in sweet whey, potassium was the highest compared to Na, P and Ca, being Mg the lowest major mineral. These results matching with the major mineral composition of sweet whey described by (Wong *et al.*, 1978). Total element concentration of the types of milk whey assayed in the present study has not been found in the literature. More calcium was found in the case of cow, sheep and mixture whey compared to goat whey. However, Giroux *et al.*, (2018) showed higher Ca content in sweet cow whey followed by goat and sheep samples. This high calcium content could be an interesting tool in terms of satiety (Garcia-Lorda *et al.*, 2005), since calcium intake has been associated to a decreasing food intake in obese and overweight women (Al-Mana *et al.*, 2012). Additionally, increasing dietary calcium could lead to modulate energy metabolism (Zemel, 2004). The remarkable content of iron in the case of sheep whey was also reported by other authors when sheep, goat and cow milk were compared (Hernandez-Ledesma *et al.*, 2011, Zamberlin *et al.*, 2012). Phosphorous value was the highest in the case of cow whey, differently Giroux *et al.*, (2018), who reported the highest level of this mineral for sheep whey.

4.2. Characterisation of digested and fermented milk whey samples

Crude milk whey from several animal species was subjected to a harmonised *in vitro* digestion, amply standardised, followed by a simulated fermentation for 48 h. Relatively few studies have been performed the proteomic profile of these types of, hence few data are available in the literature to compare our results. The digestion of proteins from whey can occur through enzymatic hydrolysis being pepsin in the stomach. In addition, trypsin, chymotrypsin and carboxy and amino peptidases, in the

intestinal lumen of the intestinal medium, were the most important enzymes to protein degradation into bioactive peptides (Madureira *et al.*, 2010, Inglingstad *et al.*, 2010). This bioactive peptides have been demonstrated to have many beneficial effects on nutrition and metabolism (Madureira *et al.*, 2010). Moreover, hydrolysed proteins and peptides could continue to degrade into amino acids under colonic fermentation by the peptidase microbial activity (Macfarlane and Allison, 1986).

4.2.1. Protein determination of digested and fermented whey samples

Intact proteins were moving forward the simulated gastro intestinal tract receiving the first hydrolysis in the stomach, due to acidic medium and pepsin activity, which broke the proteins into peptones, mainly, oligopeptides and some amino acids. Secondly, in the intestinal phase, the action of pancreatic enzymes continued breaking down the peptones into di and tri-peptides and amino acids. In addition, not only protein degradation happened during gastrointestinal digestion, but also proteolytic activity in some groups of bacteria, such as *Bacteroides fragilis* and *Propionibacterium*, and other bacteria belonging to *Firmicutes* such as *Streptococcus*, *Clostridium*, *Bacillus* and *Staphylococcus* (Macfarlane *et al.*, 1988, Macfarlane *et al.*, 1986).

The protein fraction of milk whey has been deeply studied by many researches. Whey proteins represented about 20-30% of the total proteins from milk although with variations depending on species, lactation stage, etcetera. It was composed of complex mixture of globular proteins being two proteins (β -LG and α -LA) the major part of its protein fraction (Madureira *et al.*, 2007).

Regarding gastric and intestinal digestion results, the data obtained from RP-HPC were similar to previous results derived from CB. As a general trend, protein degradation occurred through a whole simulated digestion, especially in the stomach. Several authors have demonstrated that the lower pH of gastric digestion together with the action of pepsin degraded the major part of proteins from whey, especially for a minor proteins (LF, BSA and Ig) (Eriksen *et al.*, 2010). However, β -LG, could resist to gastric digestion since part of the intact protein was observed after stomach digestion in

from of little chromatogram peaks derived from HPLC. These results were in agreement with other authors' that showed that β -LG could resist acidic conditions (Egger *et al.*, 2017a, Egger *et al.*, 2017b, Santos-Hernandez *et al.*, 2018, Inglingstad *et al.*, 2010). Other researches have established that the most resistant protein to digestion was β -LG, being α -LA strongly degraded after gastric digestion independly to pH (Eriksen *et al.*, 2010).

Furthermore, after 2 h of incubation with porcine pancreatin and bile salts, the hydrolysis was continuing. It is probably that the protein fraction digested, by the action of pancreatin in the intestinal digestion, was β -LG. In agreement with this data, other authors showed that after a simulated intestinal digestion, β -LG was almost completely hydrolysed (Egger *et al.*, 2017a). Recently, Santos-Hernandez has described, using the protocol proposed by INFOGEST network, that β -LG from milk whey was completely degraded by pancreatic enzymes within 30 minutes (Santos-Hernandez *et al.*, 2018).

Protein degradation was different among several species of milk whey. After a simulated gastro intestinal digestion, the hydrolysis of proteins was severe in the case of goat whey, showing intermediate values in the case of sheep and mixture whey and showing proteins from cow whey more resistance to its degradation. The hydrolysis degree found in the case of goat whey revealed that its protein fraction was more efficiently degraded which can be translated into more digestibility specially after gastric digestion. This degradation could be due to goat whey presenting a high concentration of α -LA compared to β -LG (Kopf-Bolanz *et al.*, 2014). Contrarily, proteins from cow whey showed more resistance to degradation after acidic pepsin conditions, maybe because this type of whey could have more proportions of β -LG than α -LA. Sheep and mixture whey showed a high hydrolysis after gastric digestion showing intermediate values probably because of their protein's proportions. Tagliazucchi *et al.*, (2018) compared the degradation of milk proteins from cow, sheep and goat an they demonstrated that proteins from milk goat were more efficiently degraded after the action of gastric and duodenal enzymes. Additionally, the remaining of proteins from goat whey compared to cow whey after the *in vitro* digestion showed that β -LG and α -LA from goat whey had higher degradation than cow counterpart (Inglingstad *et al.*, 2010).

Furthermore, after intestinal digestion the hydrolysis degree of cow whey was balanced among all samples of whey. The protein fraction of cow whey could be more resistant to stomach digestion than intestinal digestion, where it was almost completely degraded, suggesting that β -LG may be the majority protein of cow.

Focusing in the protein concentration in its initial stage (raw whey), the highest content was observed in sheep. Despite of a high degradation in sheep protein whey compared to cow, the remaining concentration of proteins after intestinal phase continued being higher. Some authors have suggested that more amount of protein at the initial stages (raw phase) could have effect in terms of digestibility because a low enzyme-to-substrate ratio (Tagliacruzchi *et al.*, 2018). Moreover, goat whey presented the lowest amount of proteins after digestion matching with its high percentage of hydrolysis, which was probably related to its destruction of its major protein, α -LA. Mixture whey (60:20:20 from cow, sheep and goat, respectively) showed intermediate values of undigested proteins after digestion. The proportion of proteins derived from goat which formed the mixture whey could be responsible to the moderate concentrations of proteins after digestion.

Finally, after 48 hours of total fermentation the complete hydrolysis of proteins was evident. Degraded proteins from intestinal stage were even more downgraded by the proteolytic activity of gut bacteria (Macfarlane *et al.*, 1988, Macfarlane *et al.*, 1986).

4.2.2. Peptides determination and biopeptides identification in digested and fermented milk whey samples

Peptides included within the sequence of the parent protein were inactive and proteolysis was needed to break peptide bonds, being then actives (Korhonen and Pihlanto, 2006). It is well known that peptides enclosed into parental protein sequence could be released by gastrointestinal digestion, fermentation by bacteria or/and by the action of specific proteolytic enzymes (Korhonen and Pihlanto, 2006).

Peptide releasing was produced after the proteolytic action under a simulated gastric and intestinal fluids, using the harmonised *in vitro* digestion proposed within COST action INFOGEST networking. Peptides generated after 2 h of gastric digestion were higher than peptides release after intestinal digestion. These results suggest that the main site of peptide releasing took place under 2 h of acid pepsin activity. Additionally, after intestinal digestion, the action of pancreatin and bile salts continued breaking proteins and polypeptides, as other authors have observed (Santos-Hernandez *et al.*, 2018). Finally, after 48-h of fermentation the number of produced peptides was insignificant compared to previous phases of digestion.

Data of cow peptide release showed the same trend than protein degradation, since peptides production was continuing after stomach digestion, taking also place under intestinal conditions. These data suggest that after gastric digestion, a part of proteins was not digested, and they served as substrate for a great peptide generation on intestinal phases. The reason for that results could be found, again, in the protein proportions of this kind of whey, being higher β -LG than α -LA percentages. Several authors have revealed that after incubation of β -LG with pancreatin, the major component of the SIF, resulted in a faster degradation compared to pepsin hydrolysis. This degradation resulted in great formation of peptides over the digestion time (Mandalari *et al.*, 2009).

In general, peptides were detected in a higher amount after gastric and after intestinal digestion than in the case of fermented samples. These data could imply that at colonic stage the major part of peptides were converted into amino acids by the proteolytic activity of gut bacteria.

Several peptide sequences described in the literature for their potential effects on health were compared with the sequences obtained in the present study. Many peptides from milk and dairy products have been described as biopeptides because of their biological functions (Egger and Menard, 2017). Hypertension, hyperglycaemia, obesity and a low-grade systemic inflammation have been related with the development of metabolic syndrome. In this sense, many biopeptides have been described as strategy to prevent these comorbidities. It has been deeply studied the effect of biopeptides on

hypertension, whose action mechanism is the inhibition of angiotensin-converting enzyme (ACE).

Specific sequences have been related directly with the proposed biological effects. All the followed sequences have been identified as a complete sequence or as oligo, tri- or di-peptides in whey samples of this study. ACE inhibitory activity has been described for the sequence which contains tryptophan (W), tyrosine (Y) and/or phenylalanine (F) residues, being at least one in a C-terminal position to inhibit ACE (Abubakar *et al.*, 1998).

Furthermore, the digestion of peptides MAIPPKK and MAIPPK by enzymes could lead to generate a potent ACE-inhibitory peptide, IPP (Manso and Lopez-Fandino, 2003) found in sheep whey. In addition, the tripeptide LKP and the oligopeptide LKPTPEGD have been also identified as ACE inhibitory activity (Fujita and Yoshikawa, 1999) in peptides derived from β -LG of digested sheep and mixture whey samples. The large peptide IFPPKPKDTLT with well-known ACE-inhibitory activity (Ashok and Aparna, 2017) has been identified in sheep whey, derivating from immunoglobulin gamma and also the peptide LEIVPK (Gomez-Ruiz *et al.*, 2002).

It has been demonstrated clearly, the association between peptides and satiety, existing cause and effect. Satiating peptides such as glucagon-like-peptide-1 (GLP-1) were released by nutrient-sensing mechanism by intestinal cells. GLP-1 had proved effect on satiety and in diabetes since its incretin effect, stimulating insulin secretion from β -pancreatic cells. However, incretins could be degraded by the action of dipeptidyl peptidase-4 (DPP4), a protease from intestinal brush-border. Many biopeptides derived from milk and whey had effect on the reduction or inhibition of DPP4, leading to a conservation or activation of the incretin activity (Horner *et al.*, 2016, Nongonierma and FitzGerald, 2016).

About 95% of degradation of GLP-1 could be caused by DPP4 (Iwaniak *et al.*, 2018), therefore peptides with the ability to inhibit DPP4 could be a suitable strategy to prevent type 2 diabetes and obesity. Several peptides with DPP4 inhibitory activity have been reported in the literature such as LPQ and EK (Iwaniak *et al.*, 2018), IW, LW, WL, WI, YI, IV, AV, VA, GL, FW, WF and VL and LV (Le Maux *et al.*, 2015, Tulipano *et al.*, 2011), AV, IG, GI LY (Le Maux *et al.*, 2015). All these peptides have been identified in digested and/or fermented samples in the present study. Moreover, the peptide MHQPPQPL, derived from β -casein, has been identified in digested sheep whey, whereas the biopeptide IPA has been identified in intestinal phase of goat whey (Tagliazucchi *et al.*, 2018). This IPA peptide has been defined as a potent inhibitor of DPP4 (Hsieh *et al.*, 2015).

Other beneficial effects of peptides are anti-inflammatory and immunomodulatory effects (Matar *et al.*, 2003, Chatterton *et al.*, 2013, Tavares *et al.*, 2013). Few studies have investigated the potential anti-inflammatory effect of isolated milk peptides, despite of wide literature on their antimicrobial, ACE and DPP4 inhibitory potential (Tavares *et al.*, 2013). In addition, other authors isolated peptides derived from whey protein, DYKKY and DQWL, with a notable anti-inflammatory activity (Ma *et al.*, 2016). Biopeptide derived from β -casein, VKEAMAPK, has proved inhibiting lipooxygenase *in vitro* (Rival *et al.*, 2001). This specific sequence has been found in digested cow milk whey (intestinal phase). In addition, IPP and VPP, previously described as ACE inhibitors (Manso and Lopez-Fandino, 2003), have also demonstrated anti-inflammatory activity (Nakamura *et al.*, 2013). IPP has been identified in 12-h fermented sheep whey (DGRKIPPSSR) and VPP in cow whey after intestinal digestion. Furthermore, tripeptide VPY, detected in gastric phase of goat digested whey, has demonstrated to reduce the severity of colitis in mice through a down-regulation of pro-inflammatory cytokines in the colon (Kovacs-Nolan *et al.*, 2012).

Other peptides derived from β -LG, such as YVEEL, have shown a potent antioxidant effect (Hernandez-Ledesma *et al.*, 2005). In this study, this peptide has been identified in several digested samples as cow, sheep and mixture whey. The antioxidant

dipeptide LW has been also detected as a part of sequences of α -S1 casein (Tagliazucchi *et al.*, 2018).

Furthermore, biopeptides with DPP4 inhibitory activity, MHQPPQPL, have been identified in fermented (12 h) goat whey samples, as well as peptide LVA in fermented goat and mixture milk whey samples. ACE inhibitory peptides, such as GPV, have been also found in fermented cow whey, and IYV in fermented goat whey. Antioxidant dipeptides (LK) have been detected in fermented mixture whey too. According with these results several authors have reported that fermented goat milk using several species of *Lactobacillus sp.* and *Streptococcus sp.* generated a wide variety of biopeptides with antioxidant and ACE inhibitory properties (Moreno-Montoro *et al.*, 2018).

All types of whey have generated a great number of biopeptides during digestion and even more over a whole fermentation, highlighting those with ACE and DPP4 inhibitory activity, which resulted very interesting for next assays. It was notable the high amount of ACE inhibitory biopeptides in sheep whey. Besides, sheep and goat whey yielded a great number of peptides with satiating potential. Finally, mixture whey showed more sequences with described antioxidant activity but not anti-inflammatory biopeptides.

5. CONCLUSIONS

The initial characterisation of milk whey from cow, sheep, goat and mixture have highlighted that sheep whey was the richest in protein content, meanwhile goat milk whey yielded the highest concentration of lactose. After a simulated *in vitro* digestion and 48-h of fermentation, the hydrolysis of protein into peptides was fairly evident since while digestion was progressing lesser amount of proteins and higher levels of peptides were detected, observing a different behaviour of hydrolysis among all whey samples. Whey proteins from goat showed a high digestibility, meanwhile, proteins from cow whey were more resistant to gastro intestinal digestion.

After a whole digestion, validated biopeptides showed several biological effects such as antihypertensive, satiating, antidiabetic and antioxidant/anti-inflammatory. Sheep whey elicited the highest amount of biopeptides with antihypertensive potential. Moreover, sheep followed by goat generated a high variety of satiating biopeptides which demonstrated potential effectiveness for managing food intake and preventing obesity. Finally, proteins from cow, sheep and goat whey have proved to release peptides with interesting anti-inflammatory activities which could result a suitable strategy to reduce the low-grade inflammation associated with obesity.

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***Potential anti-inflammatory effect of whey through
simulated digestion using an in vitro model of induced
inflammation. CHAPTER 3***



1. INTRODUCTION

The global rate of obesity has doubled in the last years (WHO, 2018). Overweight and obesity are closely related to additional disorders or comorbidities such as type 2 diabetes, cardiovascular diseases, hypertension, nonalcoholic fatty liver and certain cancers, among others (Allison *et al.*, 1999). In consequence, the incidence and prevalence of overweight and obesity should be considered as a public health issue. Furthermore, obesity is the result from a complex scenario among several factors: the individual genetic characteristics and the imbalance between physical activity and calorie intake.

However, in the last years, obesity has also been linked to gut health and immune system. In particular, a low-grade of systemic inflammation, with implications in the development of metabolic syndrome and insulin resistance, has been related to obesity (Hotamisligil, 2006). The cause of this inflammation is not clear yet, but several authors have proposed that dysbiosis (changes in the gut microbiota) and lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria, may have a central role in the progress of obesity and diabetes (Cani *et al.*, 2007). Increases of LPS lead to its translocation through intestinal capillaries towards target tissues. LPS could enhance the release of pro-inflammatory cytokines as tumor necrosis factor-alpha (TNF- α), interleukins 1 and 6 (IL-1 and IL-6), among others (Cani *et al.*, 2007, Kern *et al.*, 2001). The secretion of these pro-inflammatory cytokines may lead to both, systematic and local inflammation in the small intestine and colon (Cox *et al.*, 2015, Cani *et al.*, 2007, de La Serre *et al.*, 2010). Certainly, levels of pro-inflammatory markers are indicative of the inflammatory disease (Li *et al.*, 2018b). Moreover, several studies have demonstrated that the low-grade of gut inflammation would precede the low-grade of systemic inflammation related to diet induced obesity, since pro-inflammatory markers have been identified after high-fat diets in the gut. For instance, some authors showed that when mice conventionally raised specific-pathogen free (CONV) and germ-free mice were fed with a high fat diet, the first one showed increases of body weight, ileal TNF- α mRNA and activation of a nuclear factor kappa

beta (NF- κ B) compared to germ-free mice (Ding *et al.*, 2010). Other researches related increases of interferon-gamma (INF- γ) and IL-17 in the intestinal cells of obese mice (Luck *et al.*, 2015).

In addition, it has been demonstrated that in obesity and diabetes the permeability of intestinal cell barrier is decreased due to metabolic endotoxemia and the reduction of the expression of tight junction proteins (ZO-1 and occluding) (Gregor and Hotamisligil, 2011, Cani *et al.*, 2008). Weakness of tight junctions lead to augmentation of gut permeability and the passage of LPS to systemic circulation.

Nutrients with anti-inflammatory properties could mitigate the mentioned low-grade of systemic inflammation associated to obesity. In this sense, proteins from whey have proved to have effects on immune system modulation, as well as antimicrobial, anticarcinogenic and nutritional effects (Madureira *et al.*, 2007). Among these beneficial activities, it took an special attention their anti-inflammatory activity (Chatterton *et al.*, 2013).

Whey contains about 70-80% of lactose, 9% of proteins and 8-20% of minerals (expressed in a dry basis) (Daufin G., 1998). Proteins from whey represents around 20% of milk proteins recovered after cheese making (by precipitation or rennet coagulation). The protein fraction of whey, described in the previous chapter, has proved to have effects on immune system modulation as well anti-inflammatory effect (Chatterton *et al.*, 2013). In this sense, beta-lactoglobulin, the major protein from whey, could abolish the secretion of IL-6 in rats after duodenum perfusion/ischemia (Yamaguchi and Uchida, 2007). Alpha-lactalbumin also decreased the production of IL-6 in rats and in monocyte/macrophage cell line through inhibition of cyclooxygenase-2 and phospholipase A₂ (Yamaguchi *et al.*, 2009, Yamaguchi and Uchida, 2007). Furthermore, it has been also reported the anti-inflammatory effect of lactoferrin (LF) through inhibiting IL-1, IL-6 and TNF- α induced by LPS in mice (Machnicki *et al.*, 1993). Besides, LF decreased the production of TNF- α , IL-1 β , IL-6, and IL-8 mRNA expression using LPS-inflamed monocytic cells (Haversen *et al.*, 2002).

Digestion of protein and peptide degradation into amino acids have been associated with the suppression of certain pro-inflammatory markers such as IL-8 (Kim *et al.*, 2012, Son *et al.*, 2005, Chatterton *et al.*, 2013). Tavares *et al.*, (2013) demonstrated the anti-inflammatory effect of hydrolysates of whey protein (Tavares *et al.*, 2013). Ma *et al.*, (2016) showed that isolated peptides derived of whey protein exerted a remarkable anti-inflammatory activity, specifically DYKKY and DQWL (Ma *et al.*, 2016). In addition, the anti-inflammatory effect of amino acids, such as glutamine, arginine, cysteine, threonine and methionine, has been deeply demonstrated (Ruth and Field, 2013, Li *et al.*, 2007). All these amino acids play a key role in the maintenance of the growth and integrity of intestinal cells, as well as in regulating the secretion of pro-inflammatory markers (Ruth and Field, 2013).

As it has been mentioned previously, lactose is a disaccharide present in whey milk. The role of lactose and immune system has also been established (Paasela *et al.*, 2014, Cederlund *et al.*, 2013). There are limited studies about the anti-inflammatory potential of lactose. However, in the present study, the anti-inflammatory role of this disaccharide was considered from the point of view of lactose-derived metabolites after fermentation by gut microbiota. During fermentation of whey, lactose was able to act as prebiotic, since it served as substrate for probiotic bacteria to produce galacto-oligosaccharide (GOS) through a transgalactosylation reaction (Aehle, 2007). The anti-inflammatory effect of oligosaccharides has been proved in rats with induced-colitis (Morel *et al.*, 2015, Newburg *et al.*, 2016, Daddaoua *et al.*, 2006). Moreover, lactose could be used as substrate of fermentation by fecal bacteria under fermentation conditions generating short-chain fatty acids (SCFA) (Sanchez-Moya *et al.*, 2017), which could have anti-inflammatory implications.

In this respect, previous studies have reported that the production of the main SCFAs (acetate, propionate and butyrate) depends on carbohydrate fermentation. However, protein and amino acid fermentation also play an important role in this pool of SCFAs as well as branched-chain fatty acids (BCFA) (Macfarlane *et al.*, 1992). SCFAs are mainly produced in the proximal colon in high concentrations (70–140 mM), transported through the distal colon by the intestinal flow. SCFAs are absorbed and mainly used by colonocytes for their metabolic maintenance (Scott and Duncan, 2008).

SCFAs have been associated to multiple biological activities, such as regulation of energy homeostasis and anti-inflammatory activity, among others (Jandhyala *et al.*, 2015). Detailed functions of the main SCFA include acting as an energy source of colonocytes (butyrate); the role in gluconeogenesis (propionate) and in lipogenesis (acetate). However, in the last years an extensive literature has focused in the interplay of SCFA as anti-inflammatory because of their effect on regulating cytokines production (Park *et al.*, 2007, Bailon *et al.*, 2010, Tedelind *et al.*, 2007, Liu *et al.*, 2012).

Chemokines and pro-inflammatory cytokines can be secreted by several immune cells, as well as intestinal epithelial cells (Arai *et al.*, 1998). Pro-inflammatory markers, as IL-8 and TNF- α , can be found in gut inflammation disorders. Moreover, a simulated inflammation using LPS-stimulated RAW 264.7 and Caco-2 cell line has been demonstrated to be adequate to mimic the gut inflammation observed in inflammatory bowel disease (IBD) (Tanoue *et al.*, 2008).

In view of the above, the main aim of the present study was to investigate the anti-inflammatory effect of several types of whey (cow, sheep, goat and a mixture of them) by using a co-culture model of gut inflammation. Furthermore, a secondary objective was to evaluate the anti-inflammatory potential of whey in each stage of a whole *in vitro* digestion (from mouth to colon). The secretion of IL-6, IL-8 and TNF- α of stimulated Caco-2 and RAW 264.7 cell lines after an LPS/ TNF- α induced inflammation has been investigated, as biomarkers of low-grade of inflammation related to metabolic disease. Finally, the integrity of tight junction cell barrier was assessed by measuring the transepithelial electrical resistance (TEER).

2. MATERIALS AND METHODS

2.1. Whey samples

Whey samples were the same that previously have been used, that is: whey from Friesian cow, Segureña sheep, Murciano-Granadina goat and a mixture of them (60% cow, 20% sheep and 20% goat). Similarly, whey samples were defatted and lyophilised (Lyophilizer Telstar Lyoquest. Spain). Finally, samples were stored away from light and humidity. Nutritional characterisation of samples can be seen in chapter 2.

2.2. *In vitro* digestion of whey: from mouth to the colon

In the same way than in the preceding chapter (chapter 2) a simulated gastrointestinal digestion was performed using the harmonised protocol described previously (Minekus *et al.*, 2014). After that, a colonic fermentation was carried out using as inoculum faeces from one normal-weight donor (more detail can be found in chapters 2 and 5 for digestion and fermentation processes, respectively).

2.3. Cell lines culture conditions: Caco-2 and RAW 264.7

The *in vitro* method for induced-intestinal inflammation using a co-culture cell model with Caco-2 and murine macrophages (RAW 264.7) were carried out as other authors have proposed previously (Frontela-Saseta *et al.*, 2013, Tanoue *et al.*, 2008). The human colon adenocarcinoma Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC; number 86010202, Salisbury, UK) and the murine macrophage RAW 264.7 cell line from the European Collection of Cell Cultures (ECACC; number TIB-71, Salisbury, UK).

Prior to the experiment, Caco-2 cells were completely thawed by water-bath at 37 °C. The initial concentration of cells corresponded to 3×10^6 cells/mL. Afterwards, the pool of cells was added to 9 mL of culture medium (Dulbecco's Modified Eagle

Medium (DMEM, Gibco BRL Life Technologies, Paisley, Scotland) and centrifuged (200 *g* for 5 min). DMEM was composed by 4.5 g/L of glucose and it was supplemented with 10% (v/v) foetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin, 2 % glutamine and 1% pyruvate at 37 °C in a 7.5% CO₂ humidified atmosphere and 95% relative humidity (Cell culture CO₂ incubator, Thermo Scientific, USA). Experiments were performed between passages 29 to 35. Caco-2 were maintained in a ventilated and sterile flask (75 cm²) (Transwell, Corning Costar. Sigma-Aldrich. Spain). Cells were subcultured when they achieved a confluence of 80-85% (every 5 days approximately) and the medium was changed every 2 days. Cells counting and viability (number of live cells) were assessed by using a TC10 automated cell counter (Bio-Rad. Spain).

To subculture cells, the medium was completely replaced and trypsin-EDTA solution (0.25%) added to detach cells from polypropylene flask. Hereafter, the pool of detached cells was centrifuged 5 min (200 *g* for 5 min). Then, cells were seeded newly in a 75 cm² flask adding 8 mL of fresh medium. The concentration of cells seeded in each subculture was approximately of 1.5 x 10⁶ cells. Parallely and to know cells viability, 10 µL of the pool of cells were mixed with trypan blue (1:2), since this dye has the potential of staining dead cells.

Twenty-one days before the *in vitro* anti-inflammatory assay, Caco-2 were seeded onto 6- Transwell-insert plates at a density of 0.5 x 10⁶ cells/well. The Transwell-insert plates had the following characteristics: 24 mm of diameter and 0.4 µm of pore size (Transwell, Corning Costar. Sigma-Aldrich. Spain). Medium was changed every 2 days.

In parallel and four days before the experiment, RAW 264.7 cells were thawed as described previously and seeded onto ventilated flasks. Then, a night before the experiment, cells were seeded onto 6-well plates (8 x 10⁵ cells/well) and incubated overnight to ensure their adherence. Both cell lines were often visualised using a phase contrast microscope (Nikon Eclipse TE2000-U, USA) to know their growing rate after seeding (Figure 3.1).

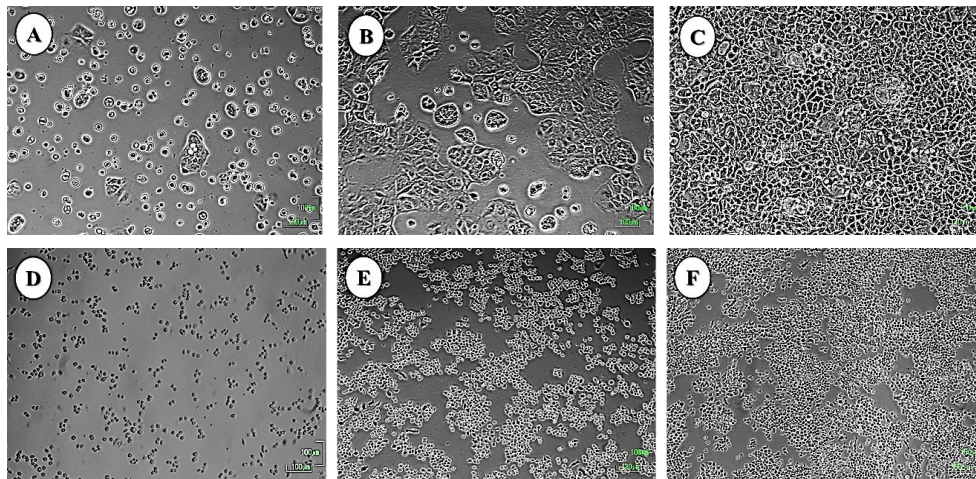


Figure 3.1. Images from inverted phase contrast microscope. Images A, B and C correspond to Caco-2 cells at day 2, 3 and 5 post-seeding. Images D, E and F correspond to macrophages RAW 264.7 at day 0, 1 and 2 post-seeding.

2.4. Assay for *Mycoplasma* detection

An important aspect to consider was to ensure the quality and reproducibility of the present study. Caco-2 cell lines used in this experiment were free of *Mycoplasma*. The contamination of cells with *Mycoplasma* leads to changes in the normal growth of cells such as competition of nutrients, cytogenetic effects, disruption of cellular metabolism, alteration in the immune response, etc. (Rivera-Tapia J.A., 2010). Test of *Mycoplasma* detection was based on the microscopy visualisation of cells after staining with a DNA-fluorescent Hoechst 33258 (Chen, 1977). To perform the analysis, Caco-2 cells, with at least two subcultures which were used and seeded in a four-chamber slide at a density of 5000-7000 cells/well. Later on, cells were incubated for 3 days (37 °C) to ensure the adherence of cells into the wall of the chamber. After incubation, the medium was removed and replaced by Carnoy's fixative, mixed previously with 1:3 of glacial acetic acid and absolute methanol, respectively. The fixative solution remained on the preparation for 5 minutes. After that, the fixative solution was changed, and the same process was repeated for 10 minutes. Finally, the Carnoy's solution was removed and

the preparation dried. 1 mL of the staining solution (1:10, Hoechst 33258 and Hank's balanced salt solution) was added and incubated for 30 minutes at room temperature.

The staining solution was removed, and the preparation was washed 3 times with deionized water and dried. Finally, stained cells with DNA-fluorescent Hoechst 33258 were visualized using a fluorescent microscope (400x). The interpretation of the results consisted in the visualization of bright fluorescent bodies spread onto intracellular or extracellular compartment of cells, having a positive *Mycoplasma* test. Conversely, in the present study, the extra cellular space appeared completely dark, corresponding to absence of *Mycoplasma* (Figure 3.2).

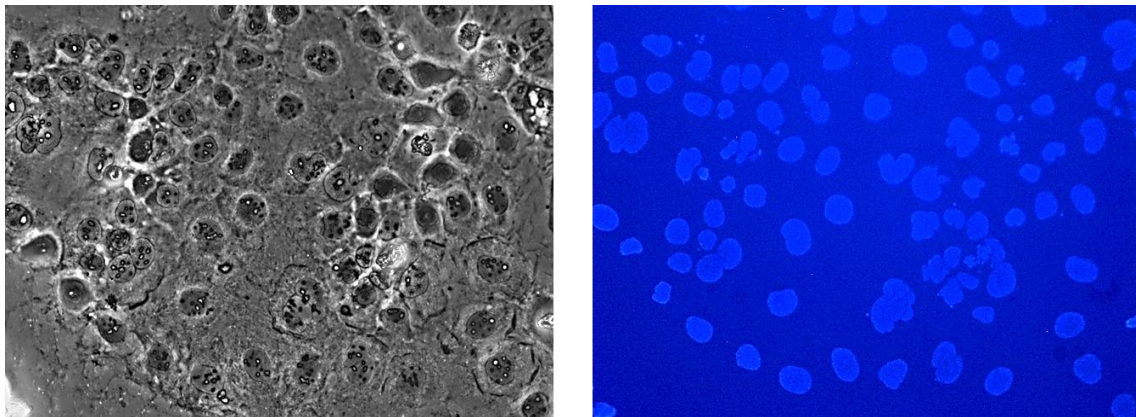


Figure 3.2. Not-stained and stained Caco-2 cells with Hoechst 33258. Absence of fluorescent in the extra cellular compartment and surrounding cellular nucleus. Fluorescence can be observed in the nucleus cell which means negative *Mycoplasma* test.

2.5. Simulating the *in vitro* gut inflammation: Co-culture model Caco-2 and RAW 264.7 cells

To evaluate the anti-inflammatory properties of whey (digested and fermented, D and F, respectively) from different ruminant species, an *in vitro* method to simulate the intestinal inflammation was performed, using a co-culture model of inflammation

with Caco-2 and RAW 264.7 cells (Tanoue *et al.*, 2008, Frontela-Saseta *et al.*, 2013). The general outline of the study can be observed in figure 3.3.

Digested whey (12.5 mg/mL) and 24-h fermented whey (0.125 mg/mL) were prepared and sterilised through a 0.22 μm -pore nylon filter. Then, a treatment solution was elaborated adding 1 mL of filtered ingredient and 9 mL of culture assay medium.

During three days before the experiment, 1 mL of the treatment solution (1:9) was added to Caco-2 in the apical compartment for 3 hours/day to study the anti-inflammatory effect on this cell line.

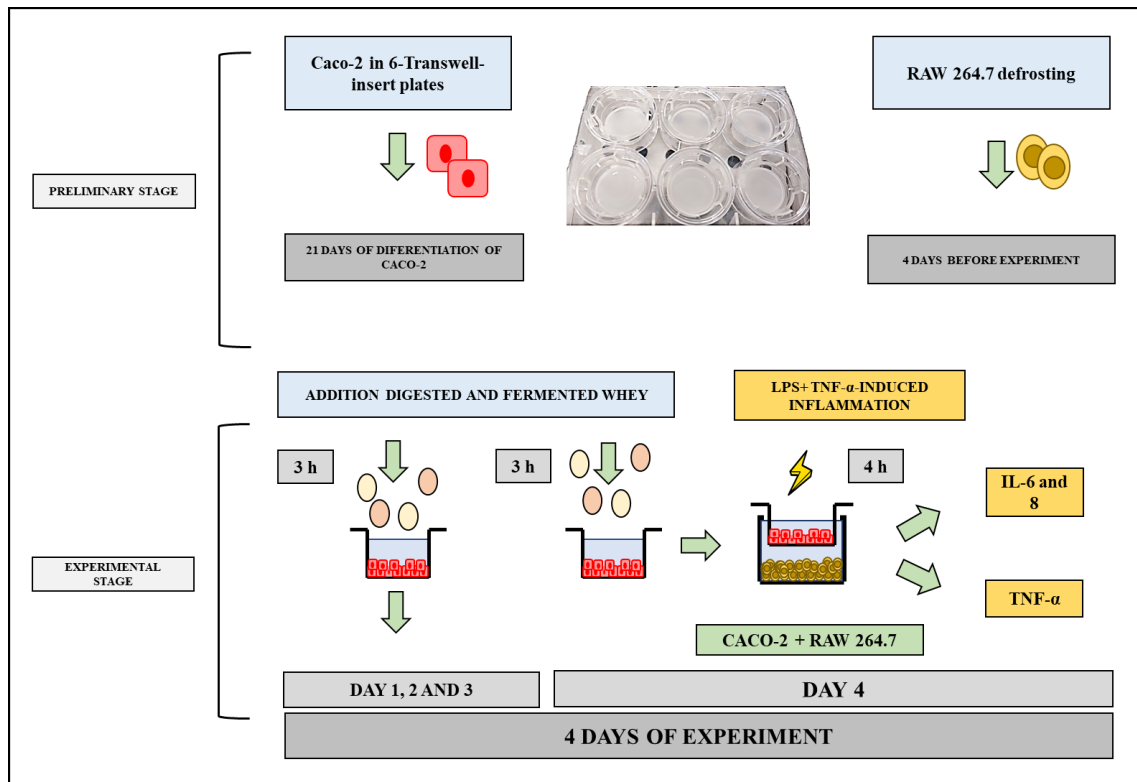


Figure 3.3. General experimental design of anti-inflammatory assay.

On the day of the experiment, Caco-2 were once again incubated with treated ingredient for 3 hours. Then, and upon removed the treatment solution and replaced with new culture medium (1.5 mL), 6-Transwell inset of Caco-2 were co-cultured with RAW 264.7, previously preload into 6-well plates, for 4 hours (Figure 3.4).

In parallel, an inflammatory cocktail was elaborated by the addition of tumor necrosis factor (TNF- α) (10 $\mu\text{g}/\text{mL}$) (TNF- α human recombinant, Sigma-Aldrich, Spain) and lipopolysaccharide (LPS) (1 mg/mL) (LPS from *Escherichia coli* 0127: B8, Sigma-Aldrich, Spain). 1.5 mL of the inflammation mixture were added onto basolateral compartment for stimulating murine RAW 264.7-inflammation for 4 hours. The inflammatory stimulus led to RAW 264.7 cells to produce murine TNF- α and in this way to achieve the secretion of pro-inflammatory cytokines by Caco-2 cells (human IL-6, IL-8 and TNF- α). In addition, TEER resistance was monitored before adding the inflammatory cocktail and during 4 hours of stimuli.

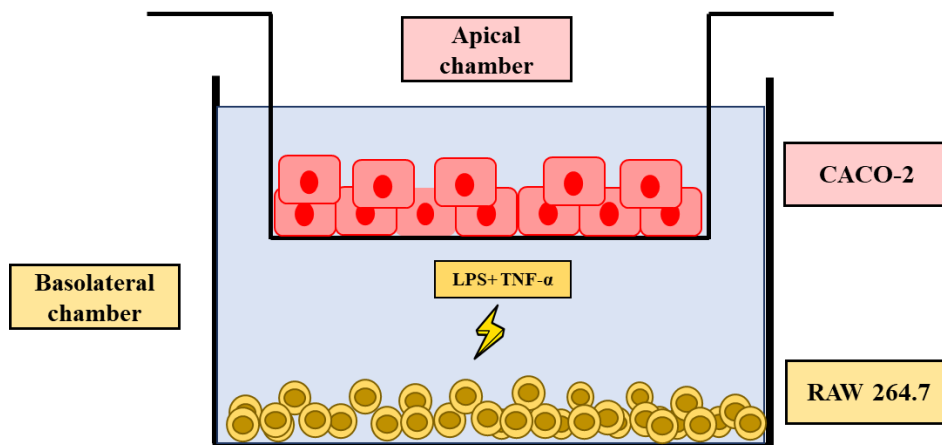


Figure 3.4. The co-culture model using Caco-2 cell line (apical chamber) and murine macrophage (RAW 264.7) (basolateral chamber) to simulated LPS/TNF- α inflammation.

After incubation, aliquots containing 1 mL of supernatant of apical compartment were collected and stored at -80 °C to perform the cytokines analysis (TNF- α , IL-6 and IL-8). Moreover, a positive control (TNF- α and LPS-stimulated Caco-2 and RAW 264.7 cells), a negative control (culture medium, Caco-2 and RAW 264.7 cells) and a blank (culture medium and Caco-2 cells) were assayed. Experiment was run in triplicate.

2.6. Transepithelial electrical resistance (TEER) assay

To determine the integrity of the monolayer formed by Caco-2, a non-invasive method for cells was done: the transepithelial electrical resistance (TEER) assay (Millicell-ERS (Millipore, Bedford, USA)). TEER ($\Omega\cdot\text{cm}^2$) allowed to measure the tight-junctional permeability of cells. In addition, this is a reliable indicator of cell confluence showing a correlation with the paracellular permeability (Hashimoto *et al.*, 1997). TEER measurements were conducted before (time 0 h), in the middle (time 2 h) and after incubation period (time 4 h) with the inflammation cocktail. Values of monolayer resistance lesser than 500 $\Omega\cdot\text{cm}^2$ were excluded. Results were expressed as a percentage (%) of TEER after incubation (time 4 h) with respect to the time 0 h. Determination of TEER was done in triplicate.

2.7. Interleukins and TNF- α analysis

The concentration after *in vitro* inflammation of human interleukin 6 and 8 and TNF- α was determined using the magnetic bead panel MILLIPLEX®MAP method (Millipore Corporation, Billerica, MA 01821 USA). MILLIPLEX®MAP is a technology based on the Luminex xMAP technology. It allowed, using just 25 μL of cell culture supernatant to analyse simultaneously the pro-inflammatory factors produced by Caco-2. The protocol followed was the Human High Sensitivity T Cell Magnetic Bead Panel and the manufacturer recommendations were carried out. This bead-based immunoassay uses magnetic beads microspheres with fluorescent dyes. Then, an analyte of the sample could be captured by the specific bead based in a biotinylated antibody-detection. This mixture (analyte and antibody) was incubated with

a conjugate (Streptavidin-Phycoerythrin) to end the reaction on the external surface of the microsphere. When the reaction was completed, the fluorescent signal emitted by each microsphere was analysed using the software Luminex® xPONENT®. The intra-assay and inter-assay precision of the method correspond to <5% and the inter-assay <15%, respectively. The immunoassay started thawing the cell culture supernatants. Samples and different cell controls were centrifuged to eliminate debris from cells. Several reagents were prepared such as the antibody-immobilised beads, quality controls, wash buffers and the working standards (Table 3.1).

Table 3.1. Concentrations (pg/mL) of seven working standards used to analyse IL-6, IL-8 and TNF α .

Standard	IL-6 (pg/mL)	IL-8 (pg/mL)	TNF α (pg/mL)
Standard 1	0.18	0.31	0.43
Standard 2	0.73	1.22	1.71
Standard 3	2.93	4.88	6.84
Standard 4	11.7	19.5	27.3
Standard 5	46.9	78.1	109.4
Standard 6	187.5	312.5	437.5
Standard 7	750	1250	1750

A volume of 200 μ L of wash buffer was added into each well of 96 well-plate. Then, plate was sealed and mixed on a plate shaker (10 min at room temperature). The wash buffer was removed and 50 μ L of each standard were added, including a background standard (0 pg/mL). Once again, 25 μ L of buffer and 25 μ L of sample were added into appropriate wells. After that, 25 μ L of mixed beads were added to each well. The plate was incubated overnight at 4 °C. Next day, the plate was washed 3 times and 50 μ L of the detection antibodies were added and incubated 1 h. Continuedly, 50 μ L of Streptavidin-Phycoerythrin were introduced into every well. After 30 min of incubation the plate was newly washed. Finally, 150 μ L of Sheath Fluid was added and the plate was run on Luminex® and the software for acquisition data xPONENT®. The median

fluorescent intensity (MFI) results using a five-parameter logistic curve (Figure 3.5) was used to calculate the interleukins and TNF α concentrations. Analysis was conducted in triplicate. The results were expressed as a percentage (%) with respect to the positive control.

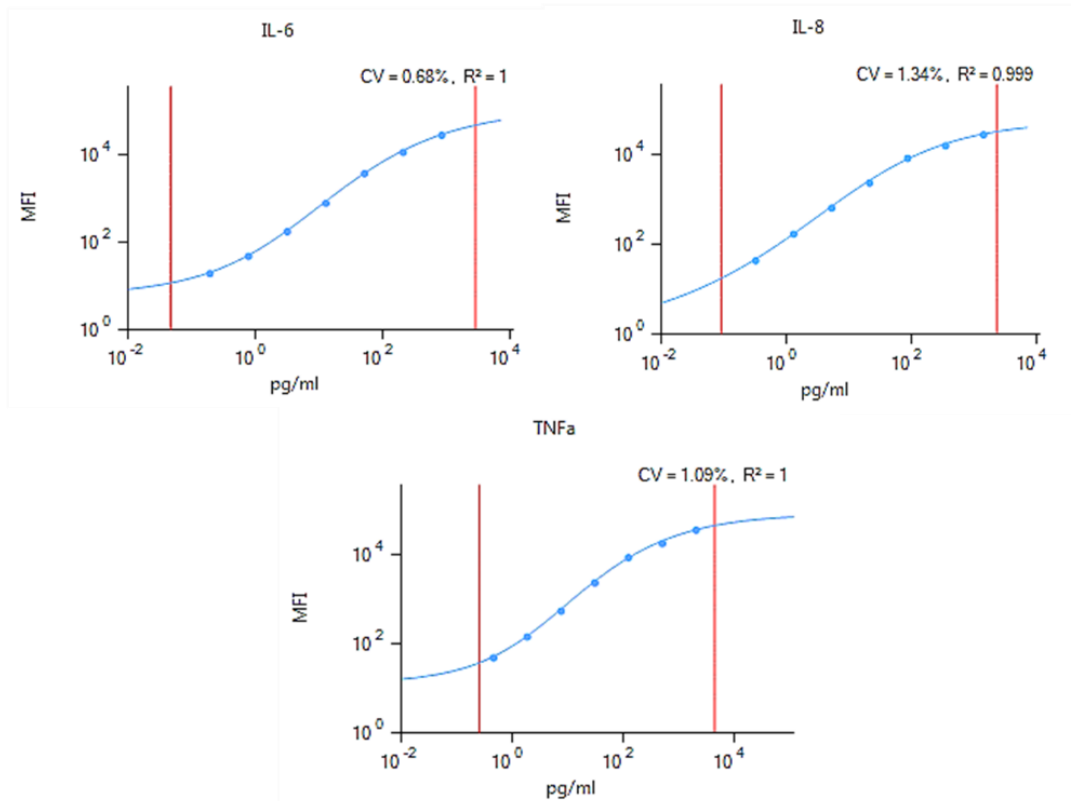


Figure 3.5. Five-parameter logistic calibration curves of interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF- α). CV: Coefficient of variation; MFI: Median Fluorescent Intensity.

2.8. Statistical analysis

Prior to statistical analysis normality and homoscedasticity were confirmed by using Shapiro-Wilk and Levene test, respectively. The anti-inflammatory effect of whey from cow, sheep, goat and a mixture of them (60:20:20) through the *in vitro* digestion, as well as TEER%, were evaluated using one-way analysis of variance (ANOVA) (SPSS v.21.0) and subsequent Tukey multiple comparisons between different samples ($p < 0.05$).

3. RESULTS

3.1. Effect of digested and fermented whey samples on transepithelial electrical resistance of Caco-2 monolayer

The protective effect of different types of digested and fermented whey on Caco-2 cell barrier permeability was evaluated in the present study. The integrity of the Caco-2 monolayer was assayed by transepithelial electrical resistance (TEER) measured before, during and after LPS/TNF- α -induced inflammation (Figure 3.6).

After 4 h of induced inflammation, PC (Caco-2 and macrophages exposed to inflammation) showed a great decrease in % of TEER with respect to initial values (before inflammation) ($p < 0.05$). Conversely, NC (non-inflamed cells) showed an increase in values of resistance after inflammation, which would indicate the preservation of the integrity monolayer. Non-significant differences were found among digested samples and fermented cow and sheep compared to NC and PC. However, FG and FM showed statistically significant differences compared to NC, being similar to PC. Cells treated with all kind of digested samples as well FC and FS, showed a protective effect on cell monolayer integrity with values of % TEER ranging from $99.38 \pm 12.98\%$ to $89.39 \pm 3.91\%$ of preservation regarding the pre-inflammatory values. Treatment of cells with fermented goat and mixture whey induced a reduction of the resistance or increases of permeability, being their percentages of TEER significantly more similar to PC than NC ($p < 0.05$). However, non significant differences were found among all samples, either digested or fermented.

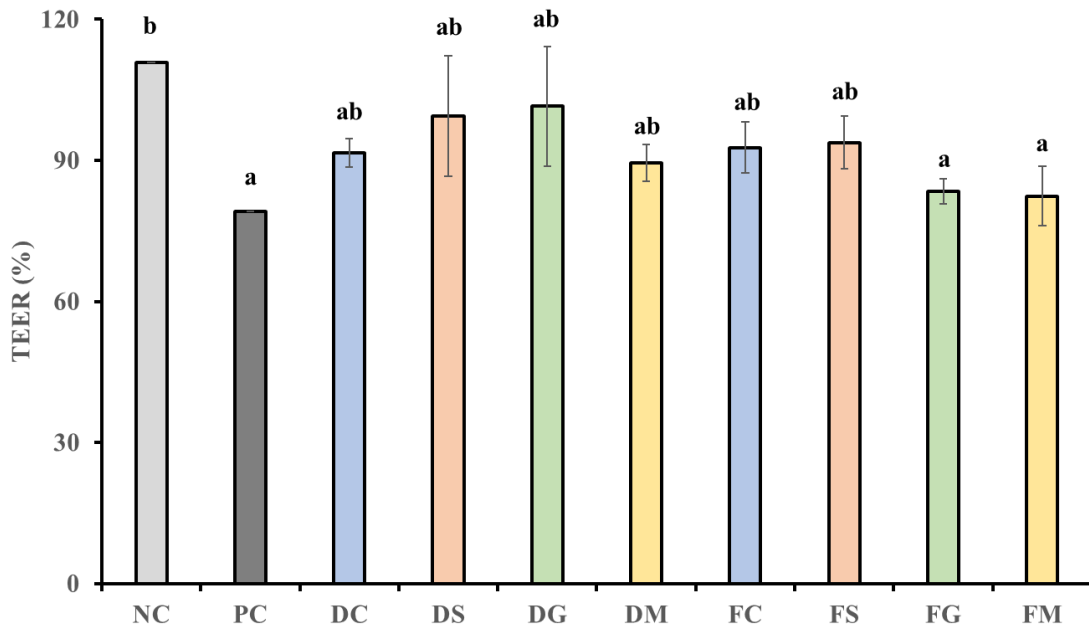


Figure 3.6. Transepithelial electrical resistance (TEER). Results are expressed as mean \pm S.D. of three replicates. TEER value measurements are given as a percentage (%) of preservation relative to the initial TEER value. Letters a and b denote statistically significant differences among different samples ($p < 0.05$). Digested samples from cow, sheep, goat and mixture appear abbreviated as DC, DS, DG and DM, respectively. Fermented samples from cow, sheep, goat and mixture appear abbreviated as FC, FS, FG and FM. Negative control and positive control correspond to NC and PC, respectively.

3.2. Effect of digested and fermented whey samples on interleukin-6 secretion

To determine the *in vitro* anti-inflammatory effect related to obesity of different types of whey on a co-culture intestinal model of inflammation, interleukin-6 secretion was assayed. However, the IL-6 results were under the detection limit in the present study.

3.3. Effect of digested and fermented whey samples on IL-8 secretion

The anti-inflammatory effect of digested and fermented whey samples was also determined by inhibiting IL-8 secretion of Caco-2 cells after LPS/TNF- α -induced inflammation (Figure 3.7).

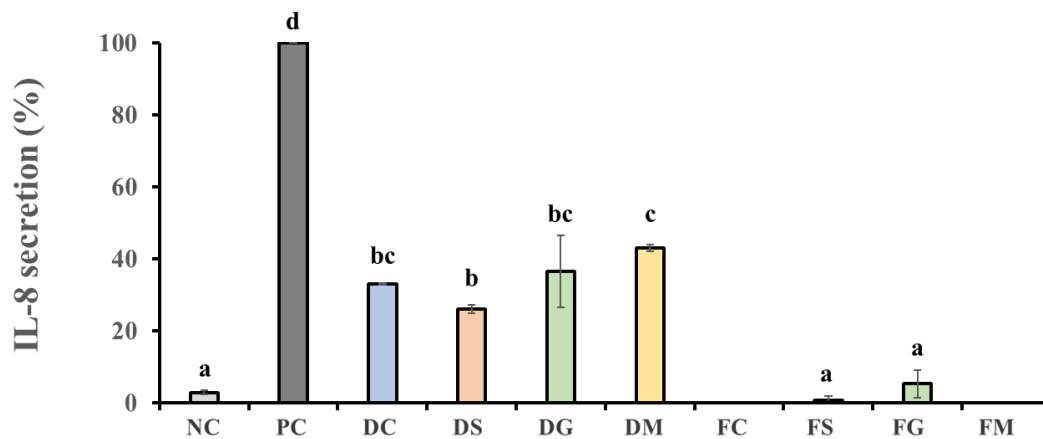


Figure 3.7. Interleukin-8 (IL-8) production by Caco-2 cells. Results are expressed as mean \pm S.D of three replicates. Values are given as a percentage (%) relative to positive control (100%). Letters a, b, c and d denote statistically significant differences among different samples ($p < 0.05$). Digested samples from cow, sheep, goat and mixture appear abbreviated as DC, DS, DG and DM, respectively. Fermented samples from cow, sheep, goat and mixture appear abbreviated as FC, FS, FG and FM. Negative control and positive control correspond to NC and PC, respectively.

As it can be observed, the secretion of IL-8 after 4 h of inflammation was greatly inhibited in both, digested and fermented whey samples in relation to PC ($p < 0.05$). It is also worthy of note that DM whey showed higher percentages of IL-8 than DS whey, fermented samples and NC. However, not statistically significant differences were found when DM was compared to DC and DG whey. It was remarkable that all kind of fermented whey samples had the potential to decrease IL-8 secretion, obtaining similar values to NC (non-inflamed cells) and higher than all types of digested whey samples

($p < 0.05$). In addition, stimulated Caco-2 after LPS/TNF- α inflammation and incubated with FC, FS and FM, secreted minimum levels of IL-8, closer to zero.

3.4. Effect of digested and fermented whey samples on TNF- α secretion

TNF- α was also determined (Figure 3.8) to evaluate the anti-inflammatory potential of whey samples after a simulated whole digestion (from mouth to colon).

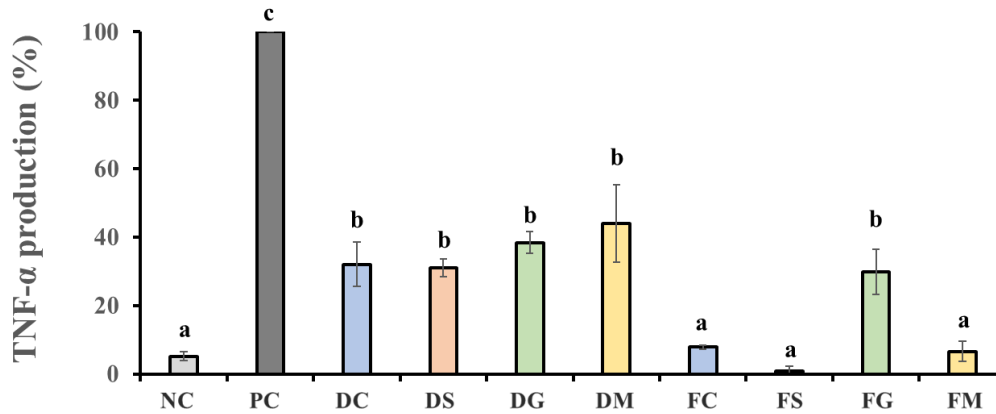


Figure 3.8. Tumor necrosis factor-alpha (TNF- α) secretion by stimulated Caco-2 cells. Results are expressed as mean \pm S.D. of three replicates. Values are given as a percentage (%) relative to positive control (100%). Letters a, b and c denote significant differences among different samples ($p < 0.05$). Digested samples from cow, sheep, goat and mixture appear abbreviated as DC, DS, DG and DM, respectively. Fermented samples from cow, sheep, goat and mixture appear abbreviated as FC, FS, FG and FM. Negative control and positive control correspond to NC and PC, respectively.

Percentages of TNF- α secretion related to PC were quite similar to those obtained for IL-8 since the secretion of these two pro-inflammatory markers are closely related. All types of whey, digested and fermented, strongly inhibited the production of this cell-signalling protein compared to PC ($p < 0.05$), showing a great anti-inflammatory potential. Several differences were found comparing types of whey and phases of

digestion. Percentages of TNF- α released by Caco-2 after incubation with digested whey samples did not show statistical significant differences among themselves. In spite of that, it can be observed the rising trend of DM, similar to the case of IL-8 secretion. However, fermented whey samples from cow, sheep and mixture elicited the highest anti-inflammatory potential according to their lowest percentages of TNF- α , being similar to NC ($7.87\% \pm 0.6$, $0.84\% \pm 1.45$ and $6.55\% \pm 2.96$, respectively). Nevertheless, FG showed higher TNF- α percentages ($29.75\% \pm 6.63$), more similar to digested whey samples, remarking the trend showing in the case of IL-8, in a significant manner.

3.5. Proteins and peptides production through the in vitro digestion

Proteomic data during the *in vitro* digestion can be observed in chapter 2, but it could be adequate to take into account in the present study again (Table 3.2).

The initial concentrations of crude whey proteins were different among all kinds of samples. As we mentioned previously, the nutritional composition of whey milk can vary according to their origin. In fact, crude sheep whey showed the highest percentage of protein ($17.6 \pm 0.53\%$) and crude goat whey, the highest amount of lactose ($78.43 \pm 0.07\%$). However, after the *in vitro* digestion, as well as in the fermentation, different proteins and peptide profiles could be observed due to their degradation. The digestion of protein gave place to a variety of peptides, depending upon the kind of whey. Fifty-six peptides were generated in the case of digested cow whey. In the case of digested sheep whey, 88 peptides were generated after intestinal digestion. Digested goat whey showed 71 peptides and finally, digested mixture whey presented 57 peptides. Lesser proportions of peptides were found after 24 h of fermentation due to the great hydrolysis of protein after the proteolytic bacteria activity.

During 24-h fermentation, peptides from whey may be degraded into amino acids or simply to remain into the fermented sample. It is important to remark that in a simulated digestion several aspects, such as absorption by colonocytes, were impossible to simulate. Thus, peptides presented in the small intestine phase should have three options: to be converted into amino acids by gut bacteria, to be transformed into metabolites by microbiota or to remain in the sample without been degraded. As it can

be observed, digested sheep whey showed a greater number of peptides than other samples. It is worth highlighting that the number of identified biopeptides in fermented goat whey under 24 h of fermentation was zero.

Table 3.2. Determination of proteins, peptides and biopeptides after a whole *in vitro* digestion, simulating the digestion into the small intestine and colon. Protein results are expressed as mean±S.D. (n=2). Letters a, b, c and d denote statistically significant differences among samples ($p<0.05$)¹.

<i>Whey sample</i>	<i>In vitro digestion</i>							
	<i>Small intestine</i>				<i>24-h colon</i>			
	<i>Cow</i>	<i>Sheep</i>	<i>Goat</i>	<i>Mixture</i>	<i>Cow</i>	<i>Sheep</i>	<i>Goat</i>	<i>Mixture</i>
Protein (µg/mL)	2.27±0.02 ^c	2.38±0.01 ^d	0.91±0.01 ^a	1.74±0.01 ^b	0.18±0.03	0.20±0.02	0.18±0.04	0.20±0.02
Peptides detected (n^o)*	56	88	71	57	23	34	24	34
Biopeptides identified (n^o)**	4	17	5	2	3	3	0	3

¹*number of individual peptides detected using Spectrum Mill under exclusion criteria: score>8 and scored peak intensity (SPI) >70%.**number of biopeptides identified with several physiological functions by a manually database.

3.6. Short chain fatty acids in the colonic phase

It is also interesting to know the concentrations of SCFA after 24-h of fermentation using human faeces (Table 3.3), since these bacterial metabolites could exert an important anti-inflammatory effect. As it can be observed in the previous chapter, lactose concentration of whey samples was different. In goat whey, we found the highest lactose concentration, followed by cow, mixture and sheep whey. Lactose *per se* was not relevant as anti-inflammatory, but its role as prebiotic was important.

Regarding this, SCFAs (mM) production was different among whey samples after 24 h of fermentation, being the total concentration of SCFAs statistically significant higher in case of fermented cow samples ($p < 0.05$). Furthermore, individual concentrations of each SCFAs (acetic, propionic and butyric acid) were higher for fermented cow whey, too. Total SCFA, as well as acetic and propionic acid were not different among sheep, goat and mixture whey samples. In addition, the concentration of butyric acid was higher in case of fermented sheep whey compared to fermented goat. Unexpected, the highest concentrations of SCFA have not been found in the case of goat whey which exerted more amount of lactose initially. This finding may be related with the prebiotic effect of other macronutrients and the synergistic effect of the whole components of whey as substrate for bacteria.

Table 3.3. Total SCFAs (mM) and individual SCFAs (acetic, propionic and butyric acid) after 24 h of fermentation¹.

	Total SCFA (mM)	Acetic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
Cow	49.24 ± 3.49 ^b	45.16 ± 3.28 ^b	0.78 ± 0.02 ^b	3.29 ± 0.22 ^c
Sheep	25.27 ± 2.66 ^a	22.66 ± 2.58 ^a	0.45 ± 0.04 ^a	2.14 ± 0.04 ^b
Goat	19.45 ± 1.23 ^a	17.74 ± 1.63 ^a	0.37 ± 0.10 ^a	1.33 ± 0.29 ^a
Mixture	22.82 ± 0.84 ^a	20.53 ± 0.89 ^a	0.39 ± 0.01 ^a	1.89 ± 0.06 ^{ab}

¹Letters a, b and c denote statistically significant differences between substrates for SCFA production ($p < 0.05$). Results are expressed as mean ± S.D. of quadruplicate samples.

4. DISCUSSION

Proteins from whey can be degraded, attending to the whole *in vitro* digestion of the present study, under two circumstances. Firstly, proteins were enzymatically hydrolysed in the stomach and small intestine by the action of pepsine, trypsin and chymotrypsin, generating different peptides from proteins. In subsequent steps of digestion, such as colonic fermentation, the protein degradation into peptides may continue through the peptidase microbial activity (Macfarlane and Allison, 1986).

The effect of degraded proteins and the production of dairy peptides resulted crucial due to their immuno-modulatory and anti-inflammatory potential (Madureira *et al.*, 2007). It has been proved that peptides from milk were able to activate phagocytes and by enhancing of T-cell proliferation or Ig G from lymphocytes. Similarly, peptides derived from native milk proteins had a strong effect on inflammation via modulation of several factors of transcription such as NF- κ B and MAPK (mitogen-activated protein kinase) (Altmann *et al.*, 2016). Moreover, it has been proved the effect of protein hydrolysates on IL-8 production after a LPS stimulation in respiratory epithelial cells (Lands *et al.*, 2010). These authors showed that the interleukin reduction after treatments with whey hydrolysates was higher compared to non-hydrolysed. Piccolomini *et al.*, (2012) showed a similar effect of protein hydrolysates on IL-8 secretion (Piccolomini *et al.*, 2012). More specifically, peptide DQWL had a potent effect on inhibiting interleukin-1 β , cyclooxygenase-2 and TNF- α expression using a LPS- inflammation model murine macrophages RAW 264.7 by the suppression of Nk-B factor (Ma *et al.*, 2016). Tripeptide VPY has demonstrated to reduce the colitis severity in mice through a down-regulation of pro-inflammatory cytokines in the colon (Kovacs-Nolan *et al.*, 2012).

In addition, under fermentation process, the degradation of β -LG into peptides by the peptidase activity of *Lactobacillus paracasei* decreased the stimulation of lymphocytes, IL-4 and INF- γ secretion, while it stimulated the production of anti-inflammatory cytokines as IL-10 (Prioult *et al.*, 2004). The transcription of factor NF- κ B was responsible to modulate the expression of many inflammatory cytokines, such as TNF- α , IL-1, IL-6 and IL-23, among others (Dmitrieva *et al.*, 2016). Other researches proved the anti-inflammatory potential of peptides from whey and fermented milk in mice through decreasing TNF- α and IL-6 (Kume *et al.*, 2012).

Apart from peptides, lactose contained in whey could have an indirect or secondary role as anti-inflammatory. Under fermentation by prebiotic bacteria such as *Lactobacillus sp.*, and *Bifidobacterium sp.*, lactose contained in whey could be enzymatically transformed into galacto-oligosaccharide (GOS) through a transgalactosylation mediated by the enzyme β -galactosidase released by the gut microbiota (Aehle, 2007). Additionally, lactose was used as substrate of fermentation

by fecal bacteria under fermentation conditions generating SCFA, which had anti-inflammatory implications. Moreover, it has been studied the link between lactic acid bacteria (LAB) and inflammation. Metabolites generated by LAB (*Bifidobacterium breve* and *Streptococcus thermophilus*) could suppress the LPS-induced TNF- α secretion by cells, showing an anti-inflammatory effect (Menard *et al.*, 2004).

Previous studies have reported that the production of acetate, propionate and butyrate mainly depends on carbohydrate fermentation. However, protein and amino acid fermentation also plays an important role in this pool of SCFAs and also BCFAs (Macfarlane *et al.*, 1992). SCFAs have been associated to multiple biological activities, such as regulation of energy homeostasis and anti-inflammatory activity (protecting gut barrier function by mucus production and stimulation of tight junctions) (Jandhyala *et al.*, 2015).

However, in the last years an extensive literature has focused on the role of SCFA as anti-inflammatory due to their effect on regulating cell cytokines, for instance IL-6 and IL-8 (Park *et al.*, 2007, Bailon *et al.*, 2010, Tedelind *et al.*, 2007, Liu *et al.*, 2012, Li *et al.*, 2018a). The mechanism proposed to explain the anti-inflammatory effect of SCFA could consist in the activation of the main receptors for fatty acids, FFAR2 and FFAR3, expressed in several types of immune cells (Li *et al.*, 2018b, Hanes *et al.*, 2017, Ang *et al.*, 2016), since some researches have confirmed the pro-inflammatory effect of these receptors (Kim *et al.*, 2013). Other G-protein coupled receptor, GPR109A, has been proposed as inhibitory mechanism of pro-inflammatory cytokines mediated by SCFA (Li *et al.*, 2018b, Chai *et al.*, 2013). The activation of FFAR2 and FFAR3 receptors, could lead to down-regulate the expression of NF- κ B, a mediator in the transcription of pro-inflammatory cytokines (Li *et al.*, 2018b). Thus, the inhibition of NF- κ B by SCFA could produce the suppression of cytokines, being the most potent SCFA, butyrate, followed by propionate and acetate (Tedelind *et al.*, 2007).

It is also worth mentioning that it exists a relationship between the secretion of IL-8 and TNF- α . LPS-stimulated RAW 264.7 located in the basolateral compartment produce TNF- α mainly via TLR4 (Royle *et al.*, 2003, Tanoue *et al.*, 2008), which induces the cytokine release by Caco-2 in the apical chamber (Tanoue *et al.*, 2008). For

instance, fucoidan, a polysaccharide from algae, decreased IL-8 expression through the inhibition of TNF- α in murine macrophages, existing the linkage between two cytokines, demonstrating that TNF- α stimulated IL-8 expression in Caco-2 cells (Tanoue *et al.*, 2008). Results in this study showed this relationship between TNF- α and IL-8, since more inhibition or stimulation of the first one was translated into secretion of the last one.

4.1. Effect of digested and fermented whey samples on transepithelial electrical resistance of Caco-2 monolayer

The potential of digested and fermented whey to maintain the integrity of the Caco-2 monolayer after 4 h of LPS/TNF- α induced inflammation was evaluated. The transepithelial electrical resistance (TEER) was measured to evaluate the permeability and integrity of tight junction of the cellular barrier consisting on a quantitative and non-invasive method widely accepted (Srinivasan *et al.*, 2015). The cellular tight junction or *zonula occludens* (intercellular junction) is a barrier structure presented in endothelial and epithelial cells. This barrier can regulate the permeability to substances in a selective manner. Hence, high values for TEER mean a high preservation of cell monolayer and decreased permeability. A dysregulation of cell permeability gut barrier has been associated to gut inflammation such as IBD, cancer, etcetera (Kanwar and Kanwar, 2009, Soler *et al.*, 1999). It has been proved that intestinal monocellular cells of *lamina propria* could secrete TNF- α in Crohn's disease, which damages the epithelial gut barrier decreasing TEER values (Zareie *et al.*, 2001).

After 4 h of induced inflammation, cells incubated with whey (digested or fermented) were able to maintain TEER values above of 80% of preservation with respect to the pre-inflammatory value. According to the stage of the *in vitro* digestion, no significant differences were found among samples tested. Nevertheless, FG and FM showed a preservation percentage similar to those obtained in LPS/TNF- α inflamed cells (positive control), being opposite to the ones obtained in the negative control. Additionally, the lower gut barrier integrity showed in case the of FG were related with the higher levels of TNF- α found in this sample, maybe due to absence of biopeptides. Furthermore, it has been established that concentrations of butyrate in the intestinal

lumen were related with the regeneration of the gut epithelium (Gilbert *et al.*, 2003, Andoh *et al.*, 1999, Butzner *et al.*, 1996) and in the reparation of intestinal mucosa in induced-colitis in rats (Butzner *et al.*, 1996). Butyrate levels found in case of FG and FM samples after 24 h were lesser than those obtained for FC and FS. All these results together could explain the slightly damage in the paracellular barrier, specially for FG compared to negative control.

As we expected in case of positive control, IL-8 and TNF- α were highly increased while a diminution of TEER values was observed, being established a cause-effect relationship.

Similar studies have demonstrated the protective effect of whey in tight-junction. A dietary supplement based on whey, fibre, fat and micronutrients was assessed to evaluate whether it was effective on preservation of cell gut barrier. Dietary product was digested and tested for 24 h in cells showing a higher TEER values. In addition the gut cell integrity was reconfirmed by increases in the expression of protein ZO-2 (tight junction protein) (Kanwar and Kanwar, 2009).

Results in this study indicated that components of digested and fermented whey from several animal preserved the integrity of the Caco-2 cell barrier into a greater or lesser degree. In this sense, whey protein concentrates (1 mg/mL) showed similar results of TEER to those obtained in the present study to have potential in preserving the Caco-2 cell barrier (Dalziel *et al.*, 2016). Other studies have demonstrated the effect of 0.2% of cheese whey powder digest on Caco-2 cell monolayer integrity (Takano *et al.*, 2007), being in similar concentrations those tested in the present study. Finally, other authors studied two whey proteins (β -LG and bovine serum albumin) into Caco-2-SF cell line to assess their tight junctions. Results revealed that these proteins were more effective than controls and ovalbumin in the maintenance of cell monolayer integrity (Hashimoto *et al.*, 1995).

4.2. Interleukin-8 secretion by the inflamed Caco-2/RAW 264.7 model

The role of IL-8 chemokine in immune system is related to the activation and recruitment of neutrophils, as well as T-lymphocytes and basophils and its activation depends on several stimulus as LPS, IL-1 and/or TNF- α (Harada *et al.*, 1994). Alterations in IL-8 levels could occur in certain pathologies such as colitis and IBD, among others (Mahida *et al.*, 1992). This study has demonstrated that all types of tested whey have the ability to decrease IL-8 secretion after an acute *in vitro* inflammation. However, different degrees of inhibition have been observed depending on the hydrolysis of whey protein and the presence of metabolites such as SCFA. More anti-inflammatory effects were observed when the digestion process went forward. Among all digested samples, sheep whey produced a remarkable decrease in the cytokine production compared, at least, with DM whey. The anti-inflammatory potential of DS whey could be exerted by its protein profile, since this sample contained the highest quantities of proteins ($2.38 \pm 0.01 \mu\text{g/mL}$), peptides detected (88) and biopeptides identified (17) (Table 3.2). Among other whey samples, digested mixture (DM) showed an inhibition of IL-8 similar to that obtained in case of DC and DG, being the protein concentration lesser than DS and a minor number of peptides and biopeptides determined.

These findings suggest the possible role of proteins and peptides in the anti-inflammatory potential of whey protein. There is much research that relates to the anti-inflammatory effect of protein hydrolysates *in vitro* and in human. For instance, a native and a pressurised whey protein isolate hydrolysates was assayed to evaluate the cell viability and IL-8 production, after incubation with H₂O₂. After this exposition, both hydrolysates, native and pressurised, inhibited the production of IL-8 in a dose-dependent manner, showing both, anti-oxidant and anti-inflammatory effects (Piccolomini *et al.*, 2012). In addition, pressurised whey and the novel peptides released after its high-pressure treatments inhibited the production of IL-8 in patients with cystic fibrosis (Lands *et al.*, 2010).

The role of amino acids produced after intestinal digestion could have an important role in the inhibition of IL-8 mRNA expression. It has been proved that

histidine reduced IL-8 in a dose-dependent manner, showing effect in small quantities (Son *et al.*, 2005). The proposed mechanism of inhibition could be explained because histidine could suppress NF-kappaB activation. (Son *et al.*, 2005). Histidine showed slightly higher concentrations in sheep whey compared with cow or goat whey (Rafiq *et al.*, 2016), which could have a role on the whole anti-inflammatory potential of DS, apart from protein and peptide composition. In addition, glutamine, arginine, cysteine, threonine and methionine have been proved on immune gut functions. They play a key role in the maintenance of the growth and integrity of intestinal cells, as well as in regulating the secretion of pro-inflammatory markers (Ruth and Field, 2013). Among these amino acids, previous researches have showed that cysteine and methionine were major in sheep whey than cow and goat whey. However, arginine and threonine were higher in the case of cow, and glutamine in goat compared to other milk wheys. These data suggest that in general, whey is a potent anti-inflammatory ingredient due to several factors which makes it, as a whole, a suitable candidate to ameliorate interleukin secretion.

Regarding fermented samples, Results in this study suggested that the anti-inflammatory effect was exacerbated compared to digested whey, showing fermented samples the highest anti-inflammatory effect, even similar results to NC were obtained. Non-significant differences were found among all fermented whey samples (Figure 3.7). The anti-inflammatory potential of fermented samples could be produced by their peptides, amino acids and SCFAs profile considering as a complex of synergic potential. Few studies have been performed to evaluate the anti-inflammatory effect of fermented whey. Although, an aforementioned study demonstrated non-pressurised and pressurised whey protein inhibited IL-8 secretion, being the pressurised more potent than non-pressurised (Piccolomini *et al.*, 2012). These authors compared the hyperbaric treatment of whey with a fermentation process because of the level of protein degradation, affecting the secondary and tertiary protein structure. In this sense, they confirmed more anti-inflammatory effect attributable to pressurised hydrolysates of whey. Taking into account the potential role of amino acids as anti-inflammatory factors, Katayama *et al.*, (2007) proved the effect on IL-8 secretion of several amino acids such as cystein, alanine, isoleucine, leucine, tryptophan, valine, histidine and

lysine. All tested whey has showed high concentrations of them which could attenuate the secretion of IL-8.

Apart from amino acids, what made fermented samples different to digested ones was its SCFA, peptide profile and more concentration of amino acids. Many researches have focused on the role of specific SCFA as anti-inflammatory. SCFA have a proved effect on regulating cell cytokines IL-6 and IL-8 (Park *et al.*, 2007, Bailon *et al.*, 2010, Tedelind *et al.*, 2007, Liu *et al.*, 2012, Li *et al.*, 2018a). Voltolini *et al.*, (2012) investigated the effect of sodium propionate on the cytokine expression through the activation of FFA receptors. They showed that sodium propionate reduced the expression of IL-6 and IL-8 genes by FFAR2 (Voltolini *et al.*, 2012). Other research demonstrated that LPS-inflamed cells and treated with butyrate showed decreases IL-8 secretion by non-differentiated human colon adenocarcinoma (HT-29 cells) (Bailon *et al.*, 2010). The anti-inflammatory effect of fermented samples has been proved in this study, in part due to these fatty acids. However, we cannot prove if the inhibitory effect was caused by protein breakdown products and/or SCFA. On the one hand, fermented cow whey showed highest levels of SCFA compared to other samples and it was not found lesser IL-8 secretion. On the other hand, the IL-8 secretion, after incubation with FG, was slightly higher than other samples (but non in a significative manner) and the production of SCFA lesser.

With these results, we can confirm that the anti-inflammatory potential of fermented samples was caused by the complex effect of the cocktail composed by peptides, amino acids and SCFA derived from fermentation. Similarly, other authors showed that the anti-inflammatory effect of a nutritional supplement as anti-inflammatory was due to the synergic effect of the nutrients (protein, fat, carbohydrates, etcetera) that composed this foodstuff (Kanwar and Kanwar, 2009).

4.3. Tumor necrosis factor-alpha secretion by the inflamed Caco-2/RAW 264.7 model

The pleiotropic polypeptide tumor necrosis factor-alpha is involved in inflammatory activities and cellular homeostasis. It has a relevant role in several cellular processes such as apoptosis, necrosis and intercellular communication (Blaser *et al.*, 2016).

In this study, results showed that fermented whey samples, except for goat whey, presented anti-inflammatory effect (or TNF- α diminution) higher than in the case of digested samples. As in the case of IL-8 secretion, the inhibition of fermented samples was similar to NC (non-inflamed cells). Both pro-inflammatory markers followed a similar pattern to release according to the type of whey and stage of digestion. The cause may be due to both being related, being murine TNF- α secreted by RAW 264.7 and TNF- α produced by Caco-2 necessary to stimulate IL-8 expression in themselves as in a feedback loop (Tanoue *et al.*, 2008).

As mentioned previously, in general, it has been clearly demonstrated the anti-inflammatory potential of several types of whey through an *in vitro* digestion. However, digested and fermented samples showed different anti-inflammatory potential according to their composition after digestion. Digested samples were richer in proteins and peptides than fermented samples. A formulated supplementary food containing high amount of whey protein, fibre, fat, vitamins and minerals was assessed (digested and undigested) to evaluate its anti-inflammatory potential in THP-1 (LPS stimulated) and Jurkat E6-1 cells. After 4 h of cell incubation (similar to our study), the undigested (85%) and digested (91%) dietary supplement reduced dramatically the level of TNF- α compared to LPS-inflamed cells (Kanwar and Kanwar, 2009). Results are in agreement with this study because of the strong effect of digested protein in abolishing the secretion of TNF- α . It has also been proved the effect of lactoferrin (LF) in descending the secretion of TNF- α in LPS- stimulated kupffer cells (Yamaguchi *et al.*, 2001). Moreover, after injecting bovine LF and a dose of LPS in mice, a decreased of TNF- α was observed in serum (Machnicki *et al.*, 1993).

In the case of fermented samples, the anti-inflammatory potential was caused by two factors: little peptides or amino acids and SCFA. The amino acid composition could be related with a modulation of immune response mediated by reductions of TNF- α , as previously reported in human triathletes after exercise (Bassit *et al.*, 2002). As previously mentioned, it was possible that the absence of biopeptides in the case of FG, together to its specific profile of amino acids, were the reason of a limited inhibition of TNF- α .

Regarding the SCFA profile, it has been demonstrated that LPS-inflamed cells and treated with butyrate showed decreases TNF- α secreted by RAW 264.7 (Bailon *et al.*, 2010). The presence of SCFA may explain the potential of fermented samples in inhibiting inflammation. However, the limited anti-inflammatory effect of FG compared to all fermented samples could be linked to the lesser concentration of butyric acid (mM), at least lesser than in the case of cow and sheep whey. The anti-inflammatory effect of sodium butyrate, sodium phenylbutyrate and sodium phenylacetate was proved by using RAW 264.7 after the stimulation with INF- γ . These inflamed cells produced a raise of IL-10 (an anti-inflammatory cytokine) and the release of nitric oxide synthase (iNOS), IL-6 and TNF- α was diminished (Park *et al.*, 2007). In addition, the anti-inflammatory effect of SCFA seems to be dose and time-dependent, since human umbilical vein endothelial (HUVE) cells treated with different concentrations and several incubation times showed inhibition of IL-6 and IL-8 after LPS or TNF- α activation (Li *et al.*, 2018b, Li *et al.*, 2018a).

5. CONCLUSIONS

The proposed *in vitro* model of induced inflammation has demonstrated, amply, the potential of digested and fermented whey from cow, sheep, goat and mixture to minimize the *in vitro* inflammation. All digested and fermented samples showed a protective effect in cell barrier permeability, being lesser the effect in the case of fermented samples of goat and mixture whey. Fermented whey elicited the highest anti-inflammatory potential by reducing dramatically the secretion of IL-8 and TNF- α , mainly, due to their protein breakdown products as peptides and amino acids, and SCFA. However, fermented samples originated from goat whey did not show the same effect on TNF- α than other fermented presumably because of its SCFA profile and absence of biopeptides. Digested samples from whey and even more, fermented, mostly from cow and sheep whey milk, could be used as a nutritional strategy to preserve the gut barrier integrity and also to mitigate the low-grade of local and systemic inflammation related to obesity and metabolic disorders.

6. REFERENCES

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*Milk whey stimulates the in vitro release of CCK
and GLP-1 through a whole simulated intestinal
digestion. CHAPTER 4*



1. INTRODUCTION

Recent estimations showed that about 2 billion of adults were overweight and obese in 2016, tripling the worldwide prevalence since 1975 (WHO, 2016). Moreover, it is supposed that in Spain the Mediterranean diet is quite usual, but prevalence of overweight and obesity in adults reached 52.7% in 2014 (EUROSTAT, 2014). This 21st century epidemic comprises a significant negative impact in public health due to its association with an increased risk of type 2 diabetes, cardiovascular diseases, certain cancers and a shorter life expectancy (Turnbaugh *et al.*, 2009). Regulation of food intake and a healthy life style are considered as key factors to mitigate this epidemic. With this regard satiety and satiation are two key concepts involved in food intake regulation (Cummings and Overduin, 2007).

Recently, the role that intestine plays in satiety and metabolism has taken growing interest. Enteroendocrine cells (EECs) show different actions such as gut motility control, regulation of insulin secretion and food intake regulation (Gribble and Reimann, 2016). In addition, a wide variety of satiating peptides such as cholecystikinin (CCK) and glucagon-like peptide-1 (GLP-1) are released along gastro intestinal tract from different EECs in response to nutrients.

CCK is primarily produced in I-cells in proximal intestine (duodenum and jejunum), brain and enteric nervous system of the colonic wall (Moran, 2000, Moran-Ramos *et al.*, 2012). CCK secretion is produced in response specially to digested lipids, proteins and mildly to carbohydrates (Cummings and Overduin, 2007, Moran-Ramos *et al.*, 2012). It has several biological activities as the stimulation of gallbladder contraction, stimulation of pancreatic exocrine enzyme secretion and intestinal peristalsis (Lieverse *et al.*, 1995). Furthermore, CCK induces satiety and suppresses hunger in human (Lieverse *et al.*, 1995) and in animal models (Mhalhal *et al.*, 2017, Hosomi *et al.*, 2016) through the inhibition of gastric emptying and then, reducing food intake, via neural or endocrine mechanism (Moran *et al.*, 1997, Moran, 2000).

GLP-1 is an incretin whose major function is the stimulation of insulin secretion after glucose intake. GLP-1 is secreted mainly by L-cells in distal intestine and colon in response to carbohydrates, proteins and fats (Gribble and Reimann, 2016). Its stimulation can be mediated directly by the contact in the distal intestine or indirectly by neuro-humoral mechanism (Brubaker and Anini, 2003, Cummings and Overduin, 2007). Interestingly, GLP-1 is a key mediator in the intestinal negative feedback phenomenon called *ileal brake*, inhibiting proximal motility and delaying gastric emptying. The activation of *ileal brake* results in a reduction of food intake, inhibition of hunger caused by the inhibition of gastric emptying and different intestinal secretions (Maljaars *et al.*, 2008). Furthermore, GLP-1 reduces food intake in humans and animals, increasing satiety and inducing anorexia, possibly through the role of vagal and direct central pathways (Cummings and Overduin, 2007, Turton *et al.*, 1996).

The intestinal secretin tumour cell line, STC-1, originating from murine enteroendocrine tumours, has similar functions as native gut enteroendocrine cells being a reliable and reproducible cell model alternative (McCarthy *et al.*, 2015). STC-1 cell line has been commonly used to test the *in vitro* effect of food or nutrients and satiety hormones release. These cells have the ability to express and secrete gut peptides and hormones involved in food intake and satiety, such as GLP-1 or GLP-2, glucose dependent insulinotropic polypeptide (GIP), CCK, peptide YY (PYY), pancreatic polypeptide, neurotensin and oxyntomodulin, among others (McCarthy *et al.*, 2015).

Considering different satiating efficacies of the macronutrients, protein is the most satiating one (Bendtsen *et al.*, 2013) by reducing food intake and enhancing satiety. However, the effect of proteins in satiation depends on the protein source, even its digestion and absorption (Hall *et al.*, 2003). Whey protein (WP) offers a more satiating effect than other proteins as casein, when both have been studied comparing food intake, postprandial metabolites and gut hormones release (Hall *et al.*, 2003). The reason for that could be found in their features under digestion. As in previous chapters was mentioned, WP could act as a “fast protein” being more satiating in a “short-term”, since it does not coagulate in the acidic condition from the stomach unlike casein (“slow protein”). This resistance to precipitation in the stomach leads to enter quickly in jejunum, raising faster and higher postprandial concentrations of amino acids in plasma

than casein (Boirie *et al.*, 1997). The satiating effect of whey is attributed to its protein fraction, as well as, its peptides and amino acids composition (Madureira *et al.*, 2007, Veldhorst *et al.*, 2009). However, the concentration of proteins depends on several factors such as those that vary the composition of milk (animal species, breed, lactation period and feeding) and/or other factors related with the cheese processing (sweet or acid whey) (Sanz Ceballos *et al.*, 2009, Madureira *et al.*, 2007, Hejtmankova *et al.*, 2012). In addition, galacto-oligosaccharide (GOS) derived of lactose transglycosylation (Keenan *et al.*, 2006, Overduin *et al.*, 2013) and calcium (Garcia-Lorda *et al.*, 2005) have been related with satiety. WP reduces food intake (Pupovac and Anderson, 2002) and stimulates satiating hormones as cholecystinin (CCK) (Schwartz *et al.*, 2000) and GLP-1 greater extent than casein (Aziz and Anderson, 2003, Hall *et al.*, 2003, Veldhorst *et al.*, 2009).

The digestion of whey proteins can occur through enzymatic hydrolysis being pepsin (in the stomach) and trypsin (in the intestinal lumen), the most important ones (Madureira *et al.*, 2010). The bioactive peptides resulting from partial digestion have showed many beneficial effects on nutrition and metabolism (Madureira *et al.*, 2010). In addition, in latter phases of digestion it is possible to obtain protein breakdown products from whey through the peptidase microbial activity (Macfarlane and Allison, 1986). In this sense, in recent years gut microbiota has taken an important role in the regulation of energy homeostasis and food intake. Gut microbiota is a key factor that influences the efficiency of energy harvest from the diet and subsequently the energy storage in the host (Backhed *et al.*, 2004). Moreover, the association between microbiota and host metabolism is so important that bacterial metabolites, such as short chain fatty acids (SCFAs), could regulate the release of gut satiating peptides and subsequently controlling appetite (Tolhurst *et al.*, 2012). The mechanism proposed by which SCFAs act a nutrient-sensing for hormone release is by activation of G-protein-coupled with free fatty acid receptor in EECs, concretely via FFAR2 (GPR43) and possibly FFAR3 (GPR41) (Brown *et al.*, 2003, Tolhurst *et al.*, 2012).

The way in which whey, and its metabolites (derived from digestion and fermentation), triggers the secretion of satiating gut peptides from STC-1 through all the digestion process, mark the starting point for this study. Hence, in this study, the satiety

effect of whey by means of STC-1 cells has been evaluated. We used whey from different species of ruminants (cow, sheep, goat and a mixture of them) through a simulated *in vitro* digestion which mimicked the whole gastro intestinal process.

2. MATERIALS AND METHODS

2.1. Milk whey samples

Four types of mammalian species of milk whey were used in this study: Friesian cow, Segureña sheep, Murciano-Granadina goat and a mixture of the above (60:20:20, respectively). All these whey milks were provided by Palancares Alimentación S.L., a local cheese factory (Murcia, Spain). As in previous chapters, immediately after reception, milk whey samples were defatted by centrifuging at 3.000 g for 15 min and then lyophilised (Lyophilizer Telstar Lyoquest. Spain). Finally, samples were stored away from light and humidity. The nutritional analysis of different raw whey can be observed in table 4.1.

Table 4.1 Nutritional composition of whey samples (on a dry weight basis)¹.

Sample	pH			Ashes%			Protein%			Lactose%		
Cow	6.70	±	0.00 ^c	10.32	±	0.45	14.20	±	0.26 ^a	73.45	±	0.02 ^b
Sheep	6.11	±	0.02 ^a	8.28	±	0.54	17.60	±	0.53 ^c	71.41	±	0.14 ^a
Goat	6.41	±	0.04 ^b	8.45	±	0.51	15.34	±	0.49 ^b	78.43	±	0.07 ^c
Mixture	6.51	±	0.02 ^b	10.20	±	0.56	14.67	±	0.14 ^a	72.9	±	0.24 ^{ab}

¹Results are expressed as mean ± S.D. (n=2). Letters a, b and c denote significant differences for a given variable between the different samples of whey ($p < 0.05$).

2.2. *In vitro* digestion of milk whey: performing digestion from mouth to small intestine

Simulated gastrointestinal digestion was performed in three phases to simulate oral, gastric and intestinal digestions. We used an internationally standardised method proposed by Minekus *et al.*, (2014), described in more detail in chapter 2. One gram of each lyophilised sample of whey was used as starting material, and we also digested 1 g of egg white and 1 g of olive oil as positive controls (Minekus *et al.*, 2014). Afterwards, samples were frozen at -80 °C. Reagents to simulate digestion were supplied by Sigma–Aldrich (St. Louis, MO, USA).

2.3. *Collection of faecal samples*

Faecal sample was collected from one normal-weight donor (body mass index (BMI) = 20.44 kg m⁻²), being the same sample as the one used in chapters 2 and 3. The exclusion criteria for this study was based on not suffering any intestinal and/or metabolic disorders and not taking antibiotics, probiotics and/or prebiotics in the last three months. The present study was conducted in accordance with the relevant laws and current institutional guidelines. As well as, the Ethical Research Committee of The University of Murcia approved the study protocol and a written informed consent was obtained from the volunteer before donating the faeces (Annex I). The fermentation was carried out during 24 h following the protocol described in chapter 5.

2.4. *Measurement of short chain fatty acids (SCFA) by gas chromatography (GC)*

After 24 h of fermentation, SCFA were extracted and analysed, in quadruplicate, from 24-h fermented samples using the method proposed previously (Zhao *et al.*, 2006) following the same protocol described in chapter 5.

2.5. Cell culture conditions

The murine intestinal secretin tumour cell line (STC-1) was kindly provided by Dra Lia Scarabottolo (Axxam, Italy) to University of Murcia. Cells were thawed with a density of 3×10^6 cells/mL and cultured into a ventilated plastic flask of 75 cm² (Transwell, Corning Costar. Sigma-Aldrich. Spain) with supplemented media: Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich, UK), containing 4.5 g/L D-glucose and supplemented with 10% (v/v) of foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were incubated at 37 °C in a 7.5% CO₂ humidified atmosphere and 95% relative humidity (Cell culture CO₂ incubator, Thermo Scientific, USA). The medium was replaced every 3 days and the cells were subcultured when the confluence reached 80-90% (more detailed procedure can be read in chapter 3). Experiment was performed in the passage 25. Cells were visualised by using an inverted phase contrast microscope (Nikon Eclipse TE2000-U. USA) to monitor their growing after seeding (Figure 4.1).

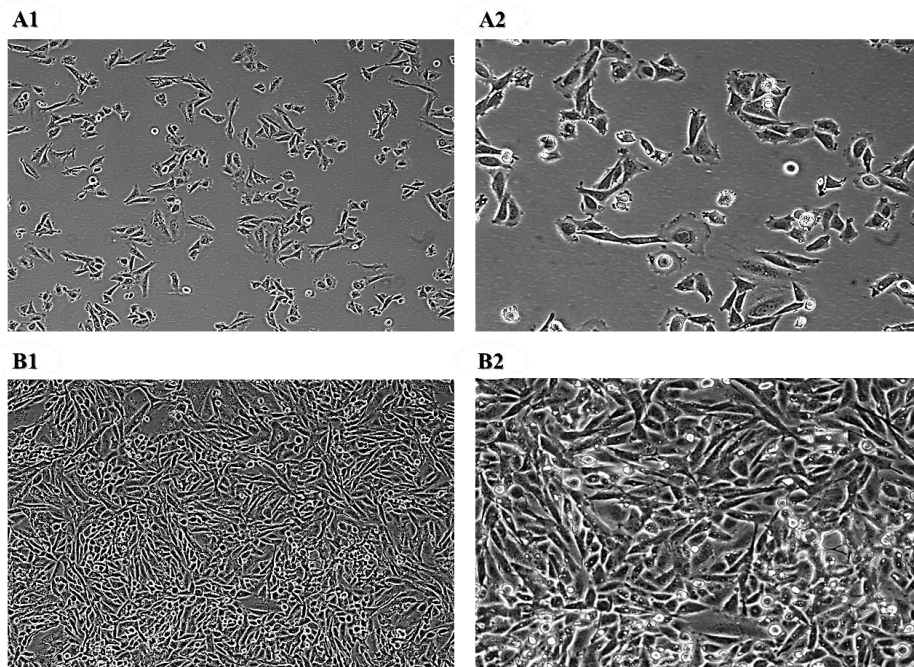


Figure 4.1. STC-1 at 2 days (A), under 10x and 40x objective; and 5 days post-seeding (B) under 10x and 40x objective.

2.6. Secretion assays of CCK and GLP-1

Cells were seeded into 12-well plates (Corning Costar Transwell, USA) at a final density of 8750 cells/cm² and maintained for 3 days. After 72 hours of incubation, the assay was carried out as follow: growing medium was aspirated and 2 mL of a mixture consisting of digested or fermented whey samples and culture medium (1:9) was added.

Samples were filtered-sterilised (0.22 µm) before cell exposition to avoid cell contamination. The final concentration of ingredient exposed to cells were 1% w/v for raw whey (whey just lyophilised), 1% w/v for digested whey and 0.01% w/v for 24-h fermented whey (from cow, sheep, goat and a mixture of them) (Figure 4.2). Experiment was run in three biological replicates. We used egg white powder, olive oil and glucose as positive controls (to test the secretagogue potential of proteins, fats and carbohydrates, respectively), and DMEM as a negative control. Cells were exposed to samples for 2 hours and then, the cellular supernatants were collected and stored at -80 °C until analysis.

Measurements of CCK and GLP-1 secretion by STC-1 were performed by enzyme immunoassay (EIA) kit (RayBiotech, USA). The principle of EIA was based on the competitive binding of biotin-conjugated hormones and samples in the pre-coated anti- CCK or anti-GLP-1 plate. In the assay, a biotinylated hormone is spiked into samples and standards, and 100 µL from each one was added to appropriate well. The optical density (OD) was measured in the spectrophotometer in a 96-wells microplate reader at 450 nm FLUOstar Omega (BMG Labtech, Germany). Thus, a high intensity of colour detected in the spectrophotometer corresponded with lower levels of CCK or GLP-1, and a low intensity of colour to a higher level of CCK or GLP-1 peptide. Five standards were used to perform the calibration curve for each hormone (Figure 4.3). In addition, a positive control and a blank were performed.

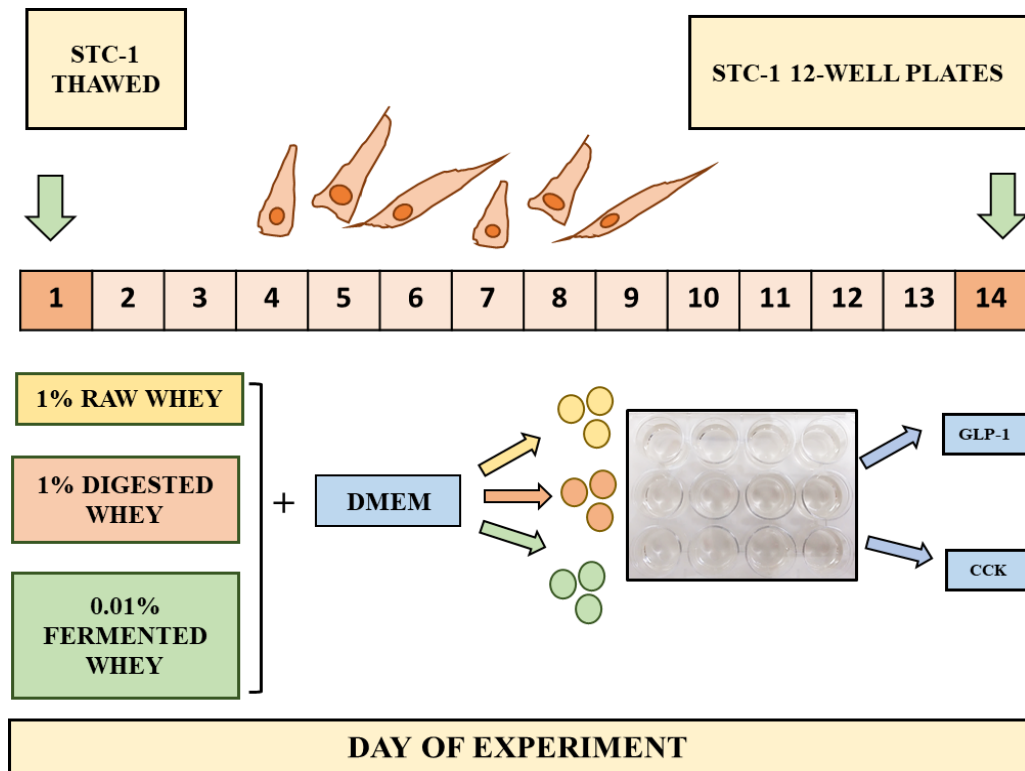


Figure 4.2. Experimental design of satiety hormones secretion.

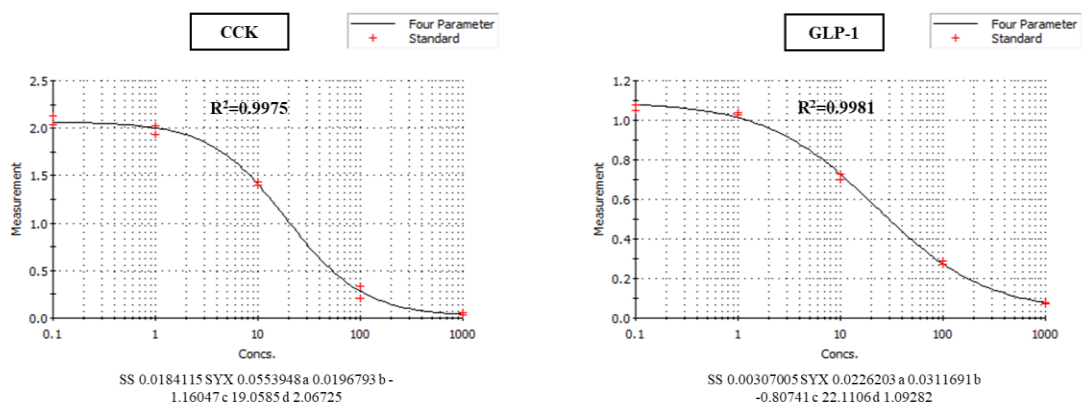


Figure 4.3. Four-parameter logistic calibration curves of CCK and GLP-1.

2.7. Statistical analysis

Prior to statistical analysis normality and homoscedasticity were confirmed by using Shapiro-Wilk and Levene test, respectively. The effect of whey samples from cow, sheep, goat and a mixture of them (60:20:20) through the *in vitro* digestion was evaluated on the release of CCK and GLP-1 by one-way analysis of variance (ANOVA) (SPSS v.21.0) and subsequent Tukey multiple comparisons between different samples ($p < 0.05$).

3. RESULTS

The nutritional composition of whey can vary considering several factors including the origin of the milk, the lactation period and feeding of the animals among others, and also cheese production method (sweet or acid whey cheese) (Giroux *et al.*, 2018, Hejtmankova *et al.*, 2012, Sanz Ceballos *et al.*, 2009).

Attending to nutritional composition of whey, mainly lactose and protein, several studies have found effect of carbohydrates, (ElHindawy *et al.*, 2017, Gribble *et al.*, 2003), proteins (Geraedts *et al.*, 2011) and SCFA (Cani *et al.*, 2004, Tolhurst *et al.*, 2012) in the secretion of satiating hormones using the cell line STC-1. This study was developed with the purpose of identifying to what extent whey could induce the release of CCK and GLP-1, and whether it exists differences according to the type of whey. In addition, the present research performed all phases of a simulated digestion (from mouth to large intestine), to evaluate the ability of undigested, digested and fermented whey to triggers the release of satiating gut peptides.

The use of STC-1 cell line has been reported to be a reliable and reproducible method, predicting what would occurs in an *in vivo* situation. However, it could exist differences in the hormone secretion for the same substance depending on the stage of digestion. The hydrolysis of macronutrients mainly occurs in the stomach and small intestine. Although, in the large intestine, certain gut microbiota's species have

saccharolytic or proteolytic activity (Macfarlane *et al.*, 1986, Macfarlane *et al.*, 1988, Schaafsma, 2008).

3.1. Effect of raw whey samples on GLP-1 and CCK releasing

Comparing the production of both hormones (Figure 4.4), the data showed that CCK secretion by STC-1 was higher than of GLP-1 in all samples of raw whey.

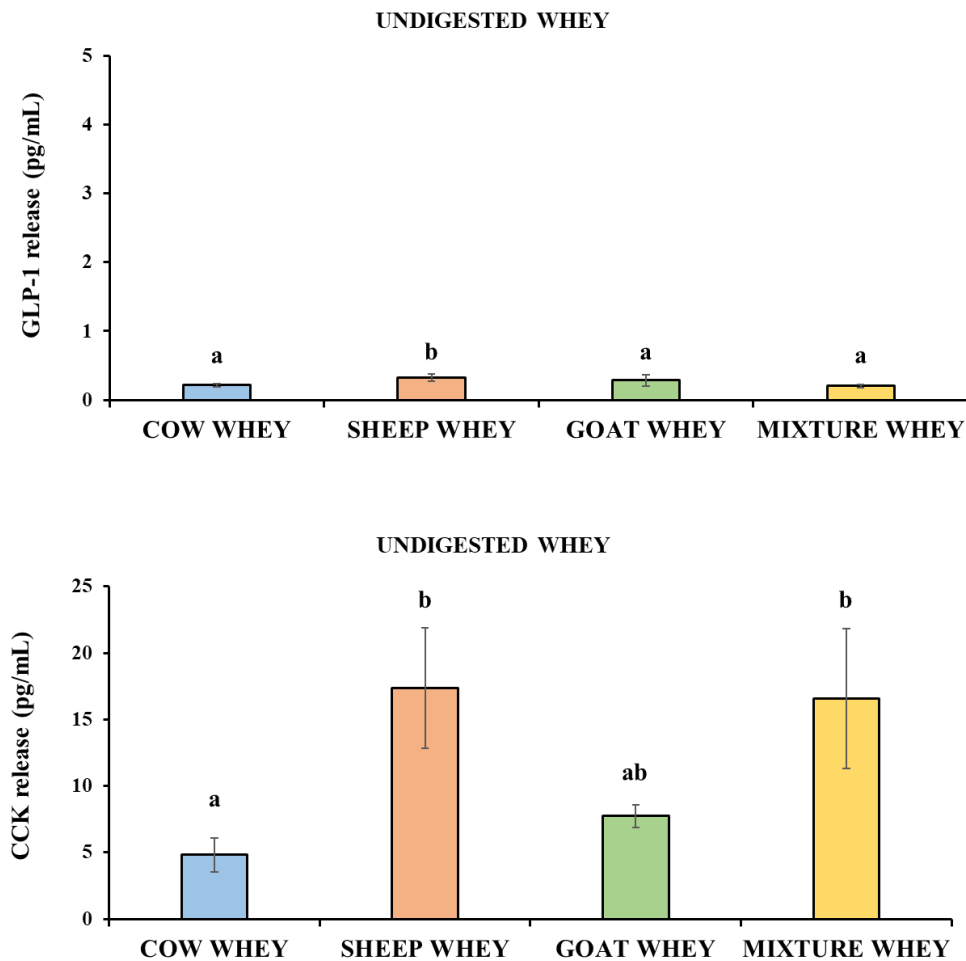


Figure 4.4. Secretion of GLP-1 and CCK from STC-1 cells after 2 h of incubation with raw milk whey. Different superscript letters mean statistically significant differences within types of whey samples ($p < 0.05$). Results are presented as the mean \pm S.D. from triplicate.

The results suggested that the potential as secretagogue of raw milk whey (1%) was trifling in case of GLP-1, except in the case of sheep whey that significantly showed the highest level of GLP-1 (0.32 ± 0.05 pg/mL) even being significantly lower than hormone release after digestion.

Conversely, we obtained a greatest CCK secretory response after exposition with raw whey compared with GLP-1 secretion. Sheep and mixture elicited the highest increases in CCK response (17.37 ± 4.52 and 16.57 ± 5.27 pg/mL, respectively) compared with cow whey, which showed the lowest stimulation (4.82 ± 1.28 pg/mL). Even though, the stimulation of cells under undigested conditions was the lowest compared with other digestion stages (small intestine digestion and fermentation).

To know whether STC-1 cells were appropriately stimulated, intact macronutrients were tested in addition to whey samples (data not shown). We obtained a great production of hormones when cells were stimulated with undigested egg white, and in lesser amount with olive oil and glucose, respectively.

3.2. Effect of digested whey samples on GLP-1 and CCK releasing

After *in vitro* digestion, we tested intestinal aliquots obtaining a quite different hormone productions to those obtained from undigested samples (Figure 4.5).

Similar to previous results, CCK secretion was higher than GLP-1 one, after whey exposition, which demonstrated the satiating potential of whey in stimulating CCK production. It is worthy of note that, digested samples induced higher GLP-1 levels than raw samples. Focusing on GLP-1, goat whey achieved the most stimulation (86.33 ± 4.55 pg/mL) compared to other samples, and the digested cow whey the least one in a statistically significant manner ($p<0.05$).

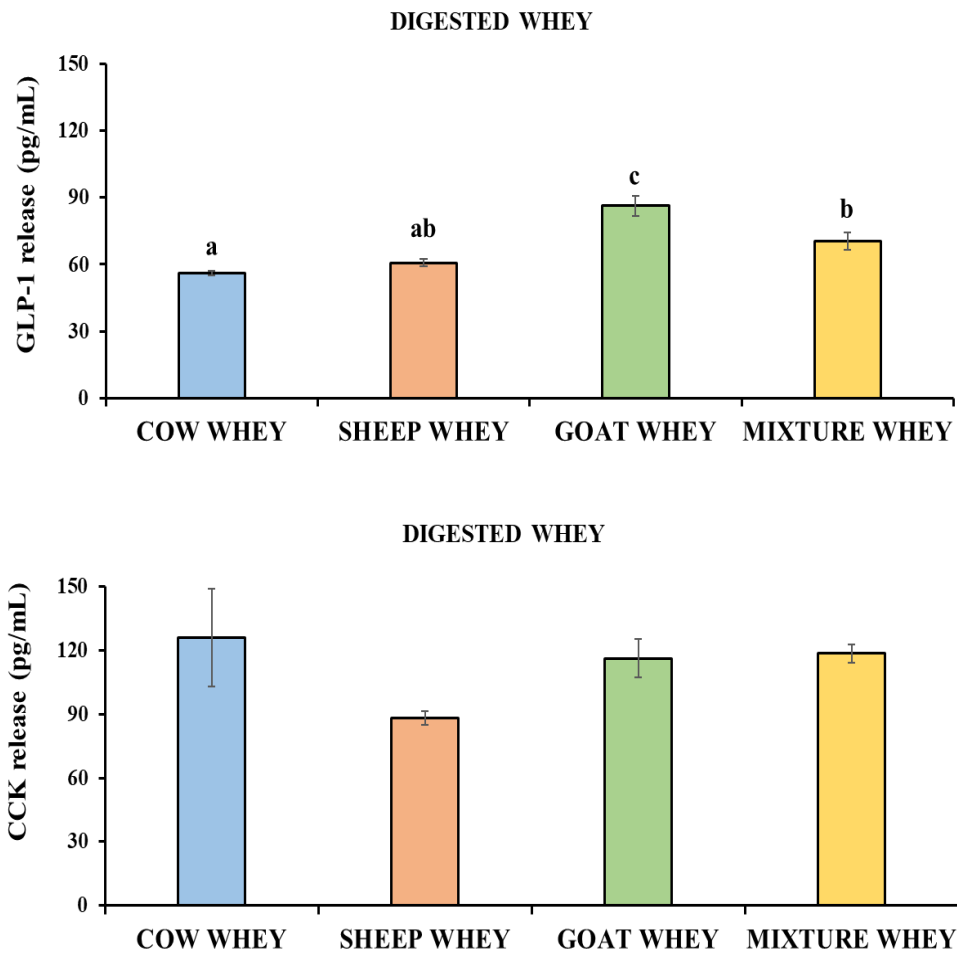


Figure 4.5. Secretion of GLP-1 and CCK from STC-1 cells after 2 hours of incubation with digested milk whey. Different superscript letters mean statistically significant differences within types of whey samples ($p < 0.05$). Results are presented as the mean \pm S.D. from triplicate.

In relation to CCK release, digested samples showed ranges of secretion from 126.13 ± 23.05 to 88.28 ± 3.07 pg/mL in case of cow and sheep whey, respectively. Despite high levels obtained for this hormone, even some interesting differences, no any statistically significant differences comparing types of whey were found in this research. Regarding GLP-1 and CCK secretion induced by positive controls, in both hormones there were found high values when STC-1 cells were stimulated with white egg and olive oil, and lower after glucose stimulation (data not shown).

3.3 Effect of fermented whey samples on GLP-1 and CCK releasing

After 24-h of fermentation the secretion of satiating hormones by STC-1 cells were different comparing both hormones (Figure 4.6)

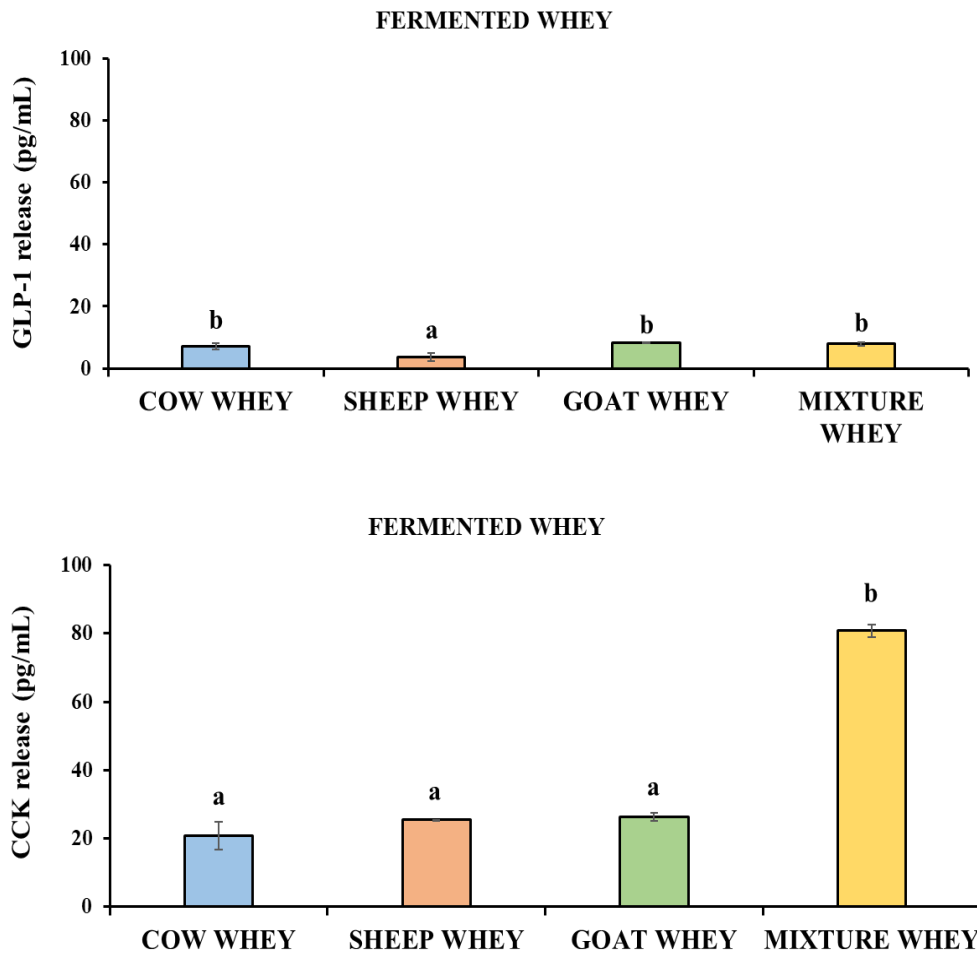


Figure 4.6. Secretion of GLP-1 and CCK from STC-1 cells after 2 hours of incubation with fermented milk whey. Different superscript letters mean statistically significant differences within types of whey samples ($p < 0.05$). Results are presented as the mean \pm S.D. from triplicate.

The secretory response of satiating hormones in this phase showed interesting results, obtaining higher values of CCK than GLP-1. The secretion of GLP-1 was similar comparing cow, goat and mixture whey. Nevertheless, sheep whey induced the lowest satiating response after treatment (3.61 ± 1.26 pg/mL) ($p < 0.05$).

In relation to CCK releasing, mixture whey produced the highest ($p < 0.05$) hormone stimulation (80.78 ± 1.81 pg/mL) being higher than the rest of the samples.

The satiating response under different digestion phases was significantly different. In general, the stimulation of hormones kept the following sequence: the highest secretion was found under small intestine *in vitro* digestion, a moderate stimulation under colonic fermentation phase and a weak response after incubation with non-digested samples.

4. DISCUSSION

4.1. Effect of raw whey samples on GLP-1 and CCK releasing

The starting hypothesis for testing raw ingredients was that several studies have demonstrated that intact proteins could stimulate hormone secretion (Geraedts *et al.*, 2011). In addition, the presence of undigested nutrients in distal portions of intestine (ileum) could lead to activate the named *ileal brake* and reducing food intake by increasing satiating signals. It is a distal to proximal feedback phenomenon in which GLP-1, PYY and oxyntomodulin are mediators, apart from neural signalling (Maljaars *et al.*, 2008). Hence, the stimulation of hormone released by undigested milk whey, due to its high protein content, could be a strategy to regulate satiety. In this sense, microencapsulation could be a suitable option for this regard, promoting a controlled release and targeted delivery of the encapsulated compound, contributing to an increase in its bio accessibility in the gut (Ydjedd *et al.*, 2017).

It was noticeable that the secretion of both satiating hormones was different among all digestion phases, being always higher in case of CCK.

GLP-1 is mainly secreted by L-cells of intestine by nutrient-sensing regulation, being stimulated by hydrolysed carbohydrates, proteins and fats (Gribble and Reimann, 2016). Defatted intact whey is not composed by a single macronutrient, but it also contains lactose and protein. Protein positive control (white egg) induced the highest

production of GLP-1 compared to fat and sugar controls. These findings suggest that GLP-1 stimulation would be mediated by intact protein in a greater extent to sugars or fats. However, results in this study showed that the potential of raw whey (1%) as secretagogue was weak in the case of GLP-1 release, with the exception of sheep whey. These detrimental effects on this hormone production can be due to the cell's exposition to the high levels of lactose and protein of intact whey. Similar results were found by other authors where they study the secretagogue effect of yogurt whey (Gillespie *et al.*, 2015).

Despite low concentrations of GLP-1, it could exist a relationship between amount of hormone and protein content of whey, obtaining the highest peptide secretion with sheep whey, which is the richest one in protein ($17.6 \pm 0.53\%$).

Controversial results have been found in the literature, and additionally, few researches have evaluated the effect of milk whey on secretion of incretin hormones. Some authors have found a positive effect of intact whey proteins on the GLP-1 secretory response using the cell line STC-1 (Power-Grant *et al.*, 2015) and others lesser compared with hydrolysed proteins (Cordier-Bussat *et al.*, 1998). Gillespie *et al.*, (2015) showed a great effect on GLP-1 release after incubating with raw cheese whey (25-50 mg/mL), yogurt whey, intact individual proteins such as β -LG (0.6-10 mg/mL), α -LA (1.25-10 mg/mL) and β -LG-hydrolysates (Gillespie *et al.*, 2015). They found higher effects on GLP-1 releasing after incubation with intact β -LG than chymotrypsin and trypsin β -LG hydrolysates. Milk whey used in the present study was similar to cheese whey used by these authors, but differences were found in tested concentrations: 1% in this research and 2.5-5% in theirs, what could explain different results. However, the effect of intact protein, α -LA was also assayed. They found a secretion effect at concentrations of 0.1-1% of this protein. Similar than before, the concentrations of α -LA used in the present experiment corresponded approximately to 0.003%, being lesser than used by these authors. Additionally, contradictory results were found when they studied dose-dependent GLP-1 release using intact cheese and yogurt whey. More dose of raw yogurt whey led to lesser GLP-1 values, and the contrary to cheese whey, which can give an idea about the complexity of whey as ingredient. It is worthy of note that these researches found higher effect of cheese whey than α -LA and β -LG alone, which

was explained because of the synergistic effect of the different whey components (Gillespie *et al.*, 2015).

The comparison of GLP-1 secretion results of non-hydrolysed protein (undigested whey) and hydrolysed ones (digested whey) suggested that second ones produced higher GLP-1 secretion than intact protein. However, results in this study showed a different tendency than those obtained by Geraedts *et al.*, (2011). These authors indicated that intact protein and, in some cases, partially hydrolysed ones, increases GLP-1 secretion more than completely hydrolysed proteins (Geraedts *et al.*, 2011). They obtained highest levels of GLP-1 after 2 h of incubation with similar intact pure proteins as casein or whey. In this study, the secretion of GLP-1 after the exposition to raw proteins was negligible, except in case of sheep milk whey, maybe because it contains higher protein concentration than the rest of milk whey.

GLP-1 secretion can also be stimulated by carbohydrates, specially glucose. Sugar in crude whey was lactose, a disaccharide composed by glucose and galactose. Nowadays, there is no clear evidence of the potential of lactose on the stimulation of STC-1 to produce GLP-1. Moreover, the effect of crude glucose on GLP-1 releasing was too small to stimulate hormone secretion. Hence, the effect of GLP-1 releasing of intact whey was more attributable to proteins than to sugars.

Conversely, we obtained a greatest CCK secretory response after exposition with raw milk whey compared with the response of GLP-1 secretion. Sheep and mixture whey elicited the highest increases in CCK response comparing with cow milk whey, which showed the lowest stimulation.

Dietary fat and protein are the major physiological stimuli for CCK secretion (Gribble and Reimann, 2016). Specifically, hydrolysed fat (fatty acids) and digested protein have showed stronger effect on CCK secretion than intact nutrients (Moran-Ramos *et al.*, 2012). In the present study, samples were defatted before experiment. As a result, the effect on CCK release was due presumably to protein composition, which was reinforce with results from egg with control (it was the most stimulator of CCK). Thus, the highest protein concentration of sheep whey could explain the CCK response

after exposition with undigested samples, as in the case of GLP-1 secretion. Moreover, cow whey showed the lowest protein content and the lowest CCK stimulation.

Surprisingly, raw mixture whey also showed a high secretagogue effect on CCK, having lesser amounts of protein than in the case of sheep whey. The mixture was composed by 60:20:20 (cow:sheep:goat, respectively), and maybe the synergistic effect of the nutritional composition of samples could explain this response. Again, controversial hypothesis was found. Our results agree to Cordier-Bussat *et al.*, (1997), which suggested that hydrolysed proteins had more effect on CCK release than intact proteins and a mixture of amino acids (Cordier-Bussat *et al.*, 1997). However, *in vivo* studies have demonstrated that intact proteins (lactalbumin and bovine serum albumin) stimulated the CCK release more than hydrolysates of casein, lactalbumin and amino acids (L-phenylalanine and L-tryptophan) and they have not found stimulation with carbohydrates as glucose (Liddle *et al.*, 1986).

4.2. Effect of digested whey samples on GLP-1 and CCK releasing

In the intestinal phase the protein from whey were hydrolysed in polypeptides (peptones), peptides and some amino acids by the action of pepsin in the stomach and pancreatic enzymes in the intestine. Peptones, acid or enzyme hydrolysates of proteins, stimulate the GLP-1 (Cordier-Bussat *et al.*, 1998) and CCK (Cordier-Bussat *et al.*, 1997) secretion. As mentioned before, there is a controversy about which induce higher stimulation of GLP-1/CCK. Our results showed that digested protein (hydrolysates) had more potent effect in GLP-1 and CCK secretion than undigested proteins.

Digested samples provoked higher GLP-1 secretion than undigested whey, supporting the hypothesis that hydrolysed proteins were more stimulators than undigested ones. In relation to protein content, recently, O'Halloran *et al.*, (2018) identified a casein hydrolysate that enhances the GLP-1 production by STC-1 cells in dose-dependent manner and decreases food intake in mice (O'Halloran *et al.*, 2018). In agreement with results in this study, they found increases of GLP-1 after 4 h of exposition the STC-1 cells with the casein hydrolysate (1% w/v) more than with intact

casein (1% w/v). Furthermore, several authors have established that peptones (egg and meat protein hydrolysates) increased GLP-1 secretion in STC-1 cells higher than intact protein (bovine serum albumin) or an amino acids mixture (Cordier-Bussat *et al.*, 1998). The specific effect of peptones on GLP-1 secretion can be related with their load in oligopeptides, which can be an answer to the question of why little peptides and free amino acids did not have a higher effect alone.

Some authors found that the effect of intact β -LG and a shorter hydrolysis with chymotrypsin (30 min) induced similar GLP-1 response, and more time of hydrolysis (90, 120 and 150 min) provoke lesser stimulatory effect (Gillespie *et al.*, 2015). On the contrary, several researches have determined that the effect of digested whey proteins on GLP-1 secretion was lesser than intact whey (Power-Grant *et al.*, 2015). However, these authors used 10 mg mL⁻¹ of a concentrate of whey protein for 4 h of exposition, being a different substrate, concentration and exposition time to the present experiment. Recently, the effect of Irish Cheddar cheese was studied using STC-1 cell line (Kondrashina *et al.*, 2018), obtaining that the optimal dose to achieve GLP-1 secretion is similar to the one we have used. The contradictory results may be due to, in part, the design of experiments: subculture and wash steps, seeding density of cells, number of passages, times of exposition, dose, source of protein and the food matrix, leading to intra and inter experimental variability.

Focusing on the potential as stimulant of GLP-1 on each type of digested samples after 2 h of incubation (1% w/v), goat's milk whey achieved the most stimulation (86.33±4.55 pg/ml) and the digested cow whey the least one in a statistically significant manner ($p < 0.05$). In this study, sheep milk whey yielded the major amount of protein as a raw ingredient (17.6%), but the highest stimulation of GLP-1 was elicited by digested goat whey (15.34% of proteins). Differences could be due to quality *versus* quantity of protein. It has been demonstrated that amino acids as glutamine, alanine and serine stimulated the secretion of GLP-1 (Reimann *et al.*, 2004). The amino acid profile of digested goat whey could increase GLP-1 levels since it has been related to high concentrations (mg/g) of glutamine and serine than cow or sheep whey proteins (Rafiq *et al.*, 2016). This finding could suggest that the secretagogue potential of digested goat whey could be mediated, at least partly, by this amino acid profile.

Furthermore, the aminopeptidase, dipeptidyl-peptidase 4 (DPP4), an enzyme inhibitor of GLP-1, is responsible of GLP-1 degradation *in vivo* (Jakubowicz and Froy, 2013). A water-soluble extract of Irish Cheddar cheese had the ability to inhibit DPP4 showing this inhibition a positive correlation with GLP-1 secretion (Kondrashina *et al.*, 2018). Other research showed that whey protein hydrolysate inhibited DPP4, more potently than intact whey (Power-Grant *et al.*, 2015). Caron *et al.*, (2016) isolated a peptide (VAAA) from *in vitro* gastrointestinal digestion with DPP4 inhibitory properties (Caron *et al.*, 2016). The digestion of whey protein can generate potent bioactive peptides (eg, tripeptide Ile-Pro-Ala) (IPA) responsible to inhibit DPP4 moderately (Tulipano *et al.*, 2011), which could serve to inhibit or ameliorate the GLP-1 degradation.

In another study included in the present manuscript, peptide sequence of digested samples was analysed (chapter 2). Although the number of identified biopeptides with DPP4 inhibitory activity were similar comparing sheep and goat whey, we found the sequence IPA, one of the highest peptides with DPP4 inhibitory activity, only in digested goat proteins. The presence of this tri-peptide could support a higher inhibitory effect on DPP4, which may lead to a preservation of GLP-1 in this sample.

In relation with the release of CCK after incubating cells with digested samples, it was much higher than in case of GLP-1 with ranges that varied from 126.13 to 88.28 pg/mL. Comparing the secretion of digested (1% v/w) with raw samples (1% v/w) the secretion was quite higher after digestion. Despite the highest levels obtained in the supernatant of digested samples for CCK, no statistically significant differences comparing types of milk whey were found in this study. According with these results, several authors did not find differences comparing several protein hydrolysates (from whey, pea, potato, soy and casein) suggesting that it exists a non-specific peptide-sensing mechanism by which STC-1 release CCK (Foltz *et al.*, 2008).

Focusing in the protein content of milk whey, the greatest increases of CCK in the digestion stage, differently to the rest of treatments, can be due to derive of protein degradation. The first hydrolysis of proteins take place in the stomach, due to acidic medium and pepsin activity, which breaks the proteins into peptones, mainly

oligopeptides. Secondly, in the intestinal phase the action of pancreatic enzymes continues breaking the peptones into protein breakdown products, mainly di and tri-peptides and amino acids.

Our data agree to Cordier-Bussat *et al.*, (1997) who determined that peptones (egg, meat, casein and soybean hydrolysates) (1% w/v) directly and in a dose-dependent manner, increased the CCK secretion and *in vitro* CCK gene transcription more than intact proteins or a mixture of amino acids (Cordier-Bussat *et al.*, 1997). Similarly, other researches attributed the stimulation of peptones rather than undigested proteins or amino acids on the CCK release (Nemoz-Gaillard *et al.*, 1998). Moreover, the highest stimulation of CCK by pork peptones using STC-1 cells was associated with the binding of them in the rat small intestinal brush-border membrane and a decreased food intake, suggesting that this peptone could have a bio-peptide effect on CCK release and satiety (Sufian *et al.*, 2006).

4.3. Effect of fermented whey samples and SCFAs production of GLP-1 and CCK release

Using *in vivo* studies, metabolites from digestion (monosaccharides and free amino acid) would be absorbed by intestinal epithelial cells. However, in this *in vitro* gastro intestinal digestion, these products would reach the colon stage, serving as substrate for bacteria fermentation (Macfarlane *et al.*, 1986).

Carbohydrate fermentation leads to produce SCFA, whereas peptides and amino acids derived from the protein degradation, could serve as substrate for bacteria fermentation leading to the production of branched chain fatty acids (BCFAs), *p*-cresol, indole and ammonia. (Rasmussen *et al.*, 1988, Macfarlane *et al.*, 1986). After 24 h of fermentation, cow milk whey yielded the highest amount of total SCFA (49.24 mM), highlighting acetic and butyric acid (these were significantly the highest ones) (Table 4.2). The concentration of propionic acid (mM) was negligible in all samples. The fermentation of lactose and degraded proteins resulted in a high production of acetate, followed by butyric acid and almost negligible propionic acid.

Table 4.2. Short chain fatty acid production after 24h fermentation. Total SCFA concentration, acetic, propionic and butyric acid (mM)¹.

	Total SCFA (mM)	Acetic acid (mM)	Propionic acid (mM)	Butyric acid (mM)
Cow	49.24 ± 3.49 ^b	45.16 ± 3.28 ^b	0.78 ± 0.02 ^b	3.29 ± 0.22 ^c
Sheep	25.27 ± 2.66 ^a	22.66 ± 2.58 ^a	0.45 ± 0.04 ^a	2.14 ± 0.04 ^b
Goat	19.45 ± 1.23 ^a	17.74 ± 1.63 ^a	0.37 ± 0.10 ^a	1.33 ± 0.29 ^a
Mixture	22.82 ± 0.84 ^a	20.53 ± 0.89 ^a	0.39 ± 0.01 ^a	1.89 ± 0.06 ^{ab}

¹Letters a, b and c denote statistically significant differences between substrates for SCFA production ($p < 0.05$). Results are expressed as mean ± S.D. of quadruplicate samples.

There are many biological activities of the SCFA related with metabolism, such as the regulation of energy homeostasis (Puertollano *et al.*, 2014) and satiety (Tolhurst *et al.*, 2012, Cani *et al.*, 2004). In addition, a link between SCFA and GLP-1 secretion have been established through the activation of FFAR2 SCFA receptor, increasing intracellular Ca²⁺, which trigger the GLP-1 secretion (Tolhurst *et al.*, 2012). However, SCFA has not the capacity to stimulate CCK. Thus, CCK may be stimulated by the action of hydrolysed triglycerides into fatty acids, being the length of fatty acids decisive in the stimulation. Longer molecules, higher hormone stimulation, probably through GPR120-coupled Ca²⁺ signalling (Tanaka *et al.*, 2008).

Under colonic digestion, the secretion of GLP-1 was higher than in case of undigested stages, but lesser than in small intestine digestion. These results suggested the higher potential of hydrolysed proteins into peptides to stimulate GLP-1, than the mixture of, amino acids and SCFA produced in the fermentation.

The results of GLP-1 release showed lower level than in the case of CCK, being the results for cow, goat and mixture whey similar. Sheep whey revealed the least GLP-1 stimulation (3.61±1.26). In view of results in this study in relation with GLP-1 secretion under colonic fermentation, it may seem probable that GLP-1 secretion was induced by the additive effect of protein breakdown product (peptides and amino acids) together with SCFA production. It is a fact that fermented samples elicited a high amount of SCFA (acetate), especially in case of cow whey samples ($p < 0.05$). However,

these highest levels of acetate were not enough to explain the secretagogue potential of cow whey, since it was statistically similar to goat and mixture whey. Nevertheless, other metabolites derived from bacterial fermentation such as indole (not determined in the present study) could be responsible of GLP-1 secretion. Indole, a bacterial metabolite of tryptophan, induced an acute stimulation of GLP-1 secretion in GLUTag cell line, but overtime, indole slowed ATP production, leading to a reduction in GLP-1 secretion (Chimerel *et al.*, 2014). In relation with degraded products from proteins, amino acids as glutamine and serine could cause the stimulation of GLP-1, especially in the case of goat whey (Reimann *et al.*, 2004).

The secretory response of CCK in this phase showed interesting results. Mixture whey produced the highest ($p<0.05$) CCK stimulation (80.78 ± 1.81 pg/mL) being higher than the rest of the samples and similar to digested samples. The high levels of CCK in fermented samples, especially in case of mixture whey samples could be due to the oligopeptides and free amino acids derived from protein fermentation. The aromatic amino acids (phenylalanine, tryptophan and tyrosine) have been related as CCK stimulants. Moreover, tryptophan and phenylalanine had a direct effect on the stimulation of CCK secretion (Wang *et al.*, 2011). Whey proteins from cow, sheep and goat have shown to be rich in aromatic amino acids (Rafiq *et al.*, 2016). It was possible that the mixture of them could be the cause of the potent stimulation of CCK by mixture whey.

5. CONCLUSIONS

After considering the results, whey could be a suitable candidate to promote satiety by peptide release. The main findings of the present study have been the differences in the hormone release according to the phase of digestion. Whey, after small intestine digestion showed the highest satiating potential due to its protein degradation, followed by fermented samples, which caused the satiating effect through the synergistic effect of SCFA and protein breakdown products. The least satiating effect was found in undigested whey, suggesting that intact whey protein is not a good satiating candidate, compared to digested proteins. These results shed some light on which phase of digestion whey could have more ability as satiating hormone stimulator, being hydrolysed>fermented>crude, the satiety rank.

Focusing on the potential of each type of whey according to stage of digestion, undigested sheep whey showed the highest GLP-1 release. After digestion, goat whey could be the best stimulator for GLP-1 secretion, maybe due to its peptide profile. Under fermentation stage, mixture whey was the most secretagogue of CCK, although in this case it cannot be attributed the satiating effect to specific component of whey. In general terms, the additive effect of protein, lactose and SCFA could potentiate the global satiating effect of whey through *in vitro* digestion.

It is the first approach to find out how milk whey (considered as an ingredient), and specifically, milk whey from different species, affects the *in vitro* release of satiating hormones. In this study it was shown that milk whey is a suitable ingredient to stimulate satiating hormone release along the whole gastrointestinal digestion, specially under small intestine digestion. Even though, simulated digestion and fermentation are a good candidate to mimic the *in vivo* digestion process, in the human intestinal environment, there exist a variety of complex mechanism between enteroendocrine cells, lumen nutrients-sensing, gut microbiota and their metabolites. Thus, all these actors are involved in satiating hormone regulation and energy homeostasis at some extent. Thus, future studies will be driven to know deeply, how each of them are related with satiety.

6. REFERENCES

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*In vitro modulation of gut microbiota by whey protein
as a strategy to prevent obesity. CHAPTER 5*



1. INTRODUCTION

From 1975 to 2018 the global obesity rate has nearly tripled. In 2016, more than a 1.9 billion adults were overweight, being of these over 650 million were obese (OB). Worldwide estimations indicate that 39% of world adult population were overweight in 2016, corresponding to 39% and 40% for men and women, respectively. However, in the same year, the estimation indicated that 13% of total adult population were OB, (11% of men and 15% of women) (WHO, 2018). Moreover, this pandemic does not solely concern adult population since more than 124 million children and adolescents were obese in 2016 (WHO, 2018). If the observed trends persist, by 2030 the number could rise to a total of 1.12 billion, reaching 20% of the world's adult population (Kelly *et al.*, 2008).

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. Their prevalence is increasing and their global impact for public health is considerable because it is associated with a higher risk of type 2 diabetes, coronary heart disease, certain cancers (Allison *et al.*, 1999) and a shorter life expectancy. Obesity is thought to be a multifactorial disorder. Genetic factors may predispose individuals to overweight and obesity, but a reduction in physical activity, an increase in energy intake (basically fat-rich and energy-dense foods) and an inadequate life style could lead to an imbalance between energy intake and expenditure, resulting in weight gain (Prentice *et al.*, 1989a).

However, the interactions between genetic and environment factors such as diet, style of life and physical activity are the main cause to develop overweight and obesity, but it is not totally explained by these factors. Recently, it has been proposed gut microbiota, the bacteria community that reside in gastrointestinal tract, as a key in this equation (Ley, 2010). An altered gut microbiota composition (also named an aberrant microbiota), a lower diversity and, even, their bacterial genome or microbiome, could

be previous status to develop metabolic disorders such as obesity and diabetes, inflammatory bowel diseases and colon cancer (Blumberg *et al.*, 2012).

Furthermore, microbiota has also been considered as the "forgotten organ" because it affects the absorption and fermentation of nutrients, having a direct association with health and metabolism (O'Hara and Shanahan, 2006). It has been reported an OB and a normal-weight (NW) profile of bacteria. OB microbiota is associated with a low diversity of bacteria population with increases of *Firmicutes* and decreases in *Bacteroidetes*, in obese animals and humans (Turnbaugh *et al.*, 2009, Ley *et al.*, 2005, Ley, 2010, Turnbaugh *et al.*, 2006). Nevertheless, NW is associated with increases in *Bifidobacterium animalis*, *Methanobrevibacter smithii* and phylum *Bacteroidetes* (Million *et al.*, 2012). An important mechanism that could explain the relationship between the gut microbiota and body mass is that bacteria could improve the host's ability to extract energy from the diet and to store it in the adipose tissue (Ley *et al.*, 2005, Ley, 2010, Turnbaugh *et al.*, 2006). In addition, It has been reported a low-grade inflammation in OB people, which is mainly caused by altered microbiota (Hotamisligil, 2006). Thus, treatments to manipulate the gut microbiota could be an interesting approach to prevent and treat overweight and obesity.

Taking the importance of microbiota into account, recent research is focused on the development and study of ingredients able to improve microbiota composition and hence intestinal health (Connolly *et al.*, 2010, Beards *et al.*, 2010, Scott *et al.*, 2013). Moreover, ingredients that could also promote a satiating effect, may contribute to reducing obesity (Kovacs and Mela, 2006, Halford and Harrold, 2012). Thus, ingredients with these two characteristics (improving microbiota and enhancing satiety) have raised interest among the research community. One could be whey milk, as it is a by-product that is easy to obtain from cheese production with high-quality proteins and nutritional value. This by-product, mainly composed by protein and lactose, has interesting effects on satiety and gut protection through increasing satiety and being fermented by colonic bacteria, respectively. The whey protein (WP) fraction has demonstrated to affect satiety by reducing food intake (Pupovac and Anderson, 2002, Froetschel *et al.*, 2001), stimulating satiating gut hormone production, including

cholecystokinin (CCK) (Schwartz *et al.*, 2000) and GLP-1 (Aziz and Anderson, 2003) and slowing stomach emptying (Blundell *et al.*, 2001). Besides, many authors suggest a positive effect of whey milk (specifically protein and bioactive peptides) on gut health, providing non-immune defence against pathogenic bacteria (Yamauchi *et al.*, 1993, Hoek *et al.*, 1997, Shin and Tomita, 2000, Freedman *et al.*, 1998), as well as a probiotic effect (Uchida *et al.*, 2007, Sprong *et al.*, 2010, McAllan *et al.*, 2014). Also, the main fermentable substrates are lactose, peptides and amino acids from whey, which produce short chain fatty acids (SCFAs) and branched chain fatty acids (BCFAs) (Rasmussen *et al.*, 1988).

SCFAs have been associated to multiple biological activities, being the most interesting metabolites produced by gut microbiota such as regulation of energy homeostasis, anti-inflammatory activity (Jandhyala *et al.*, 2015). Main SCFAs show important functions, such as energy source of colonocytes (butyrate); a role in gluconeogenesis (propionate) and in lipogenesis (acetate). In addition, SCFA are related closely with energy intake, promoting satiety, and energy metabolism. The mechanism that could explain the relation between SCFA and satiety is through two G-protein coupled receptors (GPCRs) expressed in the surface of enteroendocrine cells of intestine: free fatty acid receptor 2 and 3 (FFAR2 or GPCR43 and FFAR3 or GPCR41, respectively). These receptors are activated by SCFA, both by propionate, FFAR 2 by acetate and FFAR 3 by butyrate (Brown *et al.*, 2003) and they could have a role increasing gut satiating peptides secretion such as peptide PYY and GLP-1 (Cani *et al.*, 2004, Tolhurst *et al.*, 2012).

Furthermore, whey is also rich in lactose, which is used as a substrate by beneficial bacteria, such as lactobacilli and bifidobacteria (Coppa *et al.*, 2006). They produce lactate that could improve intestinal health, preventing the growth of other pathogens, such as *Salmonella* or *E. coli* (Wells *et al.*, 2005).

Taking everything into account, in this study, we assessed the qualitative and quantitative differences of the gut microbiota in response to different types of whey milk. The composition of the different bacterial genus including SCFA production were

investigated. In the present study stirred and non-pH controlled faecal batch culture fermentation were used. This could have implications for the design of new prebiotic and satiating ingredients based on whey milk and future applications in reducing the overweight and obesity prevalence and incidence.

2. MATERIALS AND METHODS

2.1. Milk whey samples

Four types of mammalian species of whey milk were used in this study: whey from Friesian cow, Segureña sheep, Murciano-Granadina goat and a mixture of the above (60% cow, 20% sheep and 20% goat). All these whey milks were provided by Palancares Alimentación S.L., a local cheese factory (Murcia, Spain). All these samples of whey came from healthy animals. Each kind of whey was previously lyophilised (Lyophilizer Telstar Lyoquest. Spain) and defatted, to finally stored away from light and humidity. Figure 5.1 illustrates the experimental design of the present study, showing the different stages performed.

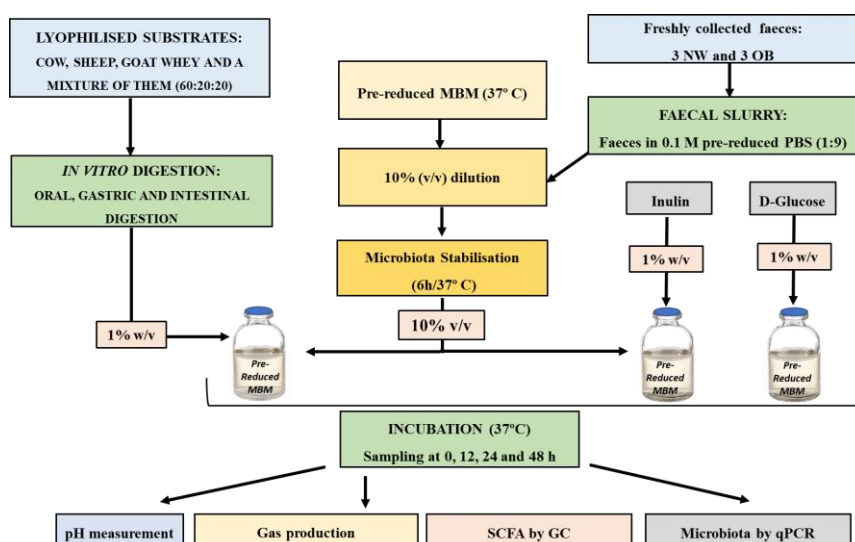


Figure 5.1. Flow diagram of the experimental design showing the different stages carried out in the present study.

2.2. *In vitro digestion of whey (from mouth to the small intestine)*

Simulated gastrointestinal digestion was performed using the harmonised protocol described previously (Minekus *et al.*, 2014). The method followed in the present chapter was the same than used in chapter 2, simulating three phases of digestion: oral, gastric and small intestine digestion.

2.3. *Faecal batch culture fermentation assay*

After performing the previous gastrointestinal digestion, finalised into small intestine phase, a colonic fermentation was included. In this way, the whole digestion process was simulated from mouth to colon stage.

2.3.1. *Faecal sample, medium preparation and faeces stabilisation*

Human microbiota samples were obtained from the faeces of 3 NW and 3 OB volunteers. Donors were between 25 and 45 years, non-smokers, had not taken antibiotics, probiotics or prebiotics in the last 3 months, and were not pregnant. In addition, the volunteers were weight stable (<2 kg change in the past 3 months) and without any clinic history of intestinal disease or metabolic diseases. All experiments were performed in compliance with the relevant laws and institutional guidelines. The Ethical Research Committee of The University of Murcia approved the study protocol (Annex I), and a written informed consent was obtained from each volunteer before donating the faeces. The informed content and information data sheet for participants were in agreement to the Regulation (EU) 2016/679 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data (European Parliament, 2016). Any manipulation of samples containing microbiota throughout the study was conducted in a biological safety cabinet of vertical flow (Biological safety cabinet Telstar Bio-II-A/P. Spain).

Faecal samples were kept at 4 °C in anaerobic conditions and processed within 1 hour after collection to maintain their microbial community composition in optimal conditions (Lauber *et al.*, 2010). Faeces were collected in a sterile sample container and placed with an anaerobic gas generator (AnaeroGen. Oxoid. Thermo Fisher Scientific. Spain) to achieve a rapid environment without oxygen inside the container.

Pre-reduced minimum basal medium (MBM) was prepared containing per litre, peptone water (2 g), yeast extract (2 g), NaCl (0.1 g), KH₂PO₄ (0.04 g), K₂HPO₄ (0.04g), MgSO₄ x 7H₂O (0.01 g), CaCl₂ x 6H₂O (0.01 g), NaHCO₃ (2 g), L-cysteine (0.5 g), bile bovine (0.5 g), Tween 80 (2 mL) and 4 mL of resazurin solution (0.025%; w/v). Resazurin was added as an anaerobiosis/redox potential indicator. Pink colour in the MBM should indicate a high redox potential. Contrarily a colourless a in the MBM are related with a low redox potential, typically that occurs in the intestinal medium (-150 mV) (Rechner *et al.*, 2004). Thereafter, the volume prepared was then autoclaved and allowed to room temperature to finally adding 10 µL of vitamin K and 2.5 mL of hemin solution per liter of culture medium through a 13 mm (diameter), 0.22 µm (pore size) PTFE filter (VWR International, USA) (Olano-Martin *et al.*, 2002, Al-Tamimi *et al.*, 2006). pH was adjusted to 6.5 by adding HCl 37%. Finally, Wheaton serum bottles (Wheaton. DWK Life Sciences Inc. USA) were sealed with silicone septa (20 mm) PTFE (Sigma-Aldrich. Spain) and aluminum seals, using a hand operated crimper W225303 for fastening 20 mm aluminum seals (Wheaton. DWK Life Sciences Inc. USA).

Subsequent step was to perform anaerobiosis into the bottles. Both, PBS and MBM bottles were continuously sparged with oxygen-free N₂ removing O₂ from the Wheaton serum bottle. With this purpose 2 sterile needles were insert into the septum, one of them was used to the N₂ inlet and the other one to displace O₂. Anaerobic condition was noticeable since bottles turned colour due to the reduction of resazurin. Bottles were maintained at 37 °C until use.

The next phase was the stabilisation of faeces. Freshly faeces were quickly processed diluting the faecal material with pre-reduced phosphate buffered saline (PBS)

(1:9 (w/v)). Afterwards the slurry (the mixture of faecal material and PBS) was homogenised in a stomacher (1 min) (Stomacher IUL Instrument. Spain) and inoculated into a Wheaton bottle containing pre-reduced MBM (10% v/v). To achieve the faecal stabilisation, slurry was incubated for 6 hours under anaerobic conditions. Reagents for fermentation were purchased from Sigma-Aldrich (USA) with the exception of peptone water and yeast extract obtained from Conda (Spain) and Biokar diagnostic (France), respectively.

2.3.2. Samples inoculation

Samples corresponded to 1% digested ingredient (cow, sheep, goat and mix whey), using as a positive control inulin (Alfa AESAR, Ward Hill, MA) and D-glucose (Sigma-Aldrich. Spain) as non-prebiotic control (Beards *et al.*, 2010). The stabilised microbiota (faecal slurry described in the previous section) was inoculated at 10% v/v in every sample and maintained in anaerobic conditions for 48 h under fermentation (water shaking bath set at 37 °C). All fermentation samples were assed in duplicate.

2.3.3. Aliquots extraction, gas production and pH determination

At the time of aliquots extraction (0, 12, 24 and 48 h), pH (Crison, Germany) and gas pressure (pressure transmitter CPT6200, WIKA Instruments, S.A.U. Spain) were determined (Manderson *et al.*, 2005, Beards *et al.*, 2010, Arboleya *et al.*, 2013). Gas pressure (kPa) was assayed by inserting a sterile needle connected to a pressure meter (CPT6200. WIKA Instruments, S.A.U. Spain) into the bottle. Gas increment calculation was performed (equation 1) (Sarhini *et al.*, 2011). Total gas production (TP) (kPa) was calculated as the sum of each measuring pressure at time 0, 12, 24 and 48 h per fermented ingredient (equation 2).

$$\Delta P \text{ (kPa)} = P_f - P_i \qquad \text{Equation 1}$$

where ΔP , P_f and P_i corresponded to increase of pressure, final pressure and initial pressure, respectively.

$$TP \text{ (kPa)} = \Sigma P (P_1 + P_2 \dots + P_n) \quad \text{Equation 2}$$

where TP corresponded to total pressure and ΣP to summation of pressures at time 0, 12, 24 and 48 h.

After pH and gas pressure measures, 8 mL of sample were taken. In order to avoid O_2 introduction into the bottle, the extraction was carried out turning the bottle downwards during aliquot extraction.

Then, the supernatant and pellet of samples were separated by centrifugation (1200 g, 15 min) (Eppendorf centrifuge 5804R. Spain) and stored at -80°C until analysis. Finally, supernatant was used to SCFA analysis and the pellet assigned to microbiota quantification.

2.4. Quantification of SCFAs by gas chromatography

The SCFA extraction of digested samples and controls was performed prior to analyses: 100 μL of cell free supernatant was added to 650 μL of a mixture of extra pure formic acid (Scharlau, Spain) (20%), methanol HPLC gradient grade (J.T. Baker, The Netherlands) and 2-ethyl butyric acid (Merk, Germany) (internal standard, 2 mg/ml in methanol) at a ratio of 1:4.5:1. The mixtures were homogenised by vortexing and filtered through a 13 mm (diameter), 0.22 μm (pore size) PTFE filter (VWR International, USA). Then, 1 μL of samples were analysed using gas chromatography (Agilent 7890A) equipped with a flame ionization detector and a NukolTM GC-column (30 m x 0.25 x 0.25 μm) following the method proposed by Zhao *et al.* (2006). The chromatographic conditions are shown in table 5.1. The calibration curve was done using a volatile acid standard mix (Supelco, Bellefonte, PA, USA) of propionic, butyric, *i*-butyric, valeric, *i*-valeric, caproic, *i*-caproic and heptanoic acid, containing a

concentration of 10, 5, 2, 1, 0.5 and 0.25 mM. The calibration curve for acetic acid (Acetic acid glacial. AppliChem Panreac. Spain) was performed at concentrations of 200, 100, 50, 10, 5 and 2 mM. The area of each peak of SCFAs was integrated using Agilent Chemstation Operation software (Santa Clara, USA), and concentrations were calculated by comparing their peak areas with those of the standard and were expressed as mM. Results were expressed by subtracting results at time 0 h, whereby SCFA quantification was nearly negligible. Every sample was run in quadruplicate.

Table 5.1. Chromatographic conditions.

Equipment Parameters	Conditions
Elution Method	Constant pressure injection
Gas flux	He ₂ 25 mL/min (carrier)
	Air 400 mL/min
	H ₂ 30 mL/min
Injection method	Splitless
Volume of injection	2 µL
Oven temperature	80 °C
Temperature ramp	80 °C for 5 min
	5 °C/min until 185 °C
Injector temperature	220 °C
Detector temperature	220 °C
Injector Pressure	58.99 kPa

2.5. DNA extraction and analysis of faecal microbiota from fermented samples

DNA extraction from faecal bacteria was based on the protocol described by Boon *et al.* (2003). All material and water used in this phase was autoclaved twice and were RNase-free. The DNA concentration of each sample was measured at a 260/280

nm ratio, using a NanoDrop-100 spectrophotometer (Thermo Fisher Scientific, Villebon sur Yvette, France). Quantitative real-time PCR (qPCR) was used to characterise the microbiota changes, using specific primers for each bacterial group. The qPCR protocol was performed in a 96-well CFX96 Real-Time PCR thermocycler and detection system (Bio-Rad, Madrid, Spain). Reactions were performed in a volume of 25 μ L, containing 1 μ L of template DNA, 12.5 μ L of SensiMix™ SYBR No-ROX (Bioline, London, UK), 0.5 μ L of each specific primer (0.2 μ M) and 10.5 μ L of nuclease-free water (AppliChem, Darmstadt, Germany). Every sample was run in quadruplicate. The amplification program was run at 95 °C for 10 minutes for DNA initial denaturation and enzyme activation, 40 cycles of denaturation (95 °C for 15 seconds), annealing ((Table 5.2) 60 °C for 30 seconds) and extension (72 °C for 45 seconds). The melting curve was from 65 °C to 95 °C, with an increase of 0.5 °C every 0.5 seconds. The fluorescent products were detected at the last step of each cycle. The bacteria concentration from each sample was calculated by comparing the cycle threshold (Ct) values obtained from standard curves. These standard curves were created using serial 10-fold dilution of pure culture DNA corresponding to 10^2 to 10^8 cell equivalents/ml (genome equivalents/mL). The conversion of the number of bacteria DNA in samples determined by qPCR to theoretical genome equivalents, required the assumption that similarities exists between the amplicon size and the 16S ribosomal RNA gene copy number for each microbial group (Santacruz *et al.*, 2010). The followed bacterial groups were analysed in this study: *Bacteroides* (using *Bacteroides thetaiotaomicron*, DSMZ 2079 as standard), *Bifidobacterium* (*Bifidobacterium longum*, CECT 4503), *Firmicutes* (*Clostridium leptum*, DSMZ 753), *Enterobacteriaceae* (*Escherichia coli*, NUTBRO Collection), *Lactobacillus* (*Lactobacillus gasseri*, DSMZ 20077 and total bacteria (*Bifidobacterium longum*, CECT 4503) (Table 5.2).

Table 5.2. Specific primers used in the study to target different bacterial groups¹

Bacterial group	Target	Primers	AT ^a (°C)*	Reference
Total bacteria	16S	Fwd: GTGSTGCAYGGYYGTCGTCA Rv: ACGTCRTCCMCNCCTTCCTC	60	Fuller <i>et al.</i> , 2007
<i>Bacteroides- Prevotella</i>	16S	Fwd: GAGAGGAAGGTCCCCAC Rv: CGKACTTGGCTGGTTCAG	60	Layton <i>et al.</i> , 2006
<i>Bifidobacterium</i>	16S	Fwd: GATTCTGGCTCAGGATGAACGC Rv: CTGATAGGACGCGACCCCAT	60	Gueimonde <i>et al.</i> , 2004
<i>Enterobacteriaceae</i>	16S	Fwd: TGCCGTAACTTCGGGAGAAGGCA Rv: TCAAGGACCAGTGTTCAGTGTC	60	Matsuda <i>et al.</i> , 2007
<i>Lactobacillus</i>	16S	Fwd: AGCAGTAGGGAATCTTCCA Rv: CATGGAGTCCACTGTCCTC	60	Rinttila <i>et al.</i> , 2004
<i>Firmicutes</i>	16S	Fwd: GGAGYATGTGGTTAATTCTGAAGCA Rv: AGCTGACGACAACCATGCAC	60	Guo <i>et al.</i> , 2008

¹*Annealing temperature.

2.6. Statistical Analysis

Statistical data processing was performed using the statistical program Statistical Package for the Social Sciences (SPSS) v.19.0. Prior to analysis, normality and homoscedasticity were analysed using the Shapiro-Wilk and Levene tests, respectively, setting the level of significance to $p < 0.05$. Results are expressed as means of four measures and SD in the case of bacterial metabolites and SEM in the case of microbiota population. To determine significant differences between the mean values of donors (pH, gas and SCFA production, and bacterial populations) t-tests were used ($p < 0.05$). To establish differences between the substrates and times of fermentation (pH, gas and SCFA production, and bacterial populations) one-way analysis of variance (ANOVA) and a subsequent *post hoc* Tukey test were carried out ($p < 0.05$).

3. RESULTS

The aim of this study was to evaluate pH, gas production, SCFA and microbiota composition in whey samples after *in vitro* digestion followed by batch culture fermentation of the faeces of NW (n=3) and OB (n=3) donors. Results were evaluated comparing both groups.

3.1. pH values and gas production during fermentation

pH values are shown in table 5.3. pH values fell significantly ($p<0.05$) along fermentation respect to time 0 in all fermentation times and samples of whey. There were significant differences between different fermentation times, although there were slightly differences between substrates or groups of donors. The highest reductions were found from 0 to 12 h in all samples. We only found significant differences ($p<0.05$) at the initial time of fermentation between donors, corresponding to an initial value of 6.77 units of pH for OB and 6.44 for NW donors.

Increases of gas are presented in table 5.4. The highest gas production was recorded in all whey samples with respect to controls, especially at 12 hours ($p<0.05$), being less pronounced at subsequent times of fermentation. This increase was marked in the case of cow whey milk, showing the highest increment (72.41 kPa) at 12 h of fermentation was compared with the rest of ingredients in NW donors. There were also significant differences ($p<0.05$) in the increases of gas at 12 and 24 h when 2 groups of donors were compared, corresponding to the largest gas increment to the NW group.

Table 5.3. Fall in pH (units) of batch culture fermentation after 48 h of four types of whey (cow, sheep, goat and mixture). (NW (n=3) and OB (n=3))¹.

Normal-weight				
Time	Cow	Sheep	Goat	Mixture
0 h	6.44 ^d ± 0.02	6.44 ^d ± 0.02	6.44 ^d ± 0.02	6.44 ^c ± 0.02
12 h	4.90 ^c ± 0.03	4.93 ^c ± 0.04	5.06 ^c ± 0.05	5.16 ^b ± 0.02
24 h	4.29 ^b ± 0.03	4.41 ^b ± 0.08	4.54 ^b ± 0.02	4.35 ^a ± 0.04
48 h	3.88 ^a ± 0.07	3.95 ^a ± 0.05	3.99 ^a ± 0.03	4.07 ^{a*} ± 0.03
Obese				
Time	Cow	Sheep	Goat	Mixture
0 h	6.77 ^{d*} ± 0.04	6.77 ^{d*} ± 0.04	6.77 ^{d*} ± 0.04	6.77 ^{d*} ± 0.04
12 h	4.88 ^c ± 0.02	4.94 ^c ± 0.04	5.07 ^c ± 0.01	5.02 ^c ± 0.03
24 h	4.40 ^b ± 0.04	4.99 ^b ± 0.02	4.65 ^b ± 0.01	4.35 ^b ± 0.04
48 h	3.92 ^a ± 0.02	3.98 ^a ± 0.02	4.04 ^a ± 0.01	3.97 ^a ± 0.01

¹Results are expressed as mean ± S.D. Letters a,b,c and d denote significant differences for a given variable between the different times of fermentation per whey milk ($p < 0.05$). * denotes significant differences for a given variable between the different groups of donors ($p < 0.05$).

Table 5.4. Increment of gas (kPa) produced by faecal bacteria from human donors (NW (n=3) and OB (n=3) during fermentation (12, 24 and 48 h) with four types of whey (cow, sheep, goat and mixture)¹.

Δ Gas (kPa) Normal-weight				
Time	Cow	Sheep	Goat	Mixture
12 h	72.41 ^{a*} ± 1.12	65.98 ^{b*} ± 2.34	63.36 ^{b*} ± 3.83	66.38 ^{b*} ± 3.08
24 h	20.66 ^{a*} ± 1.18	23.91 ^{a*} ± 3.73	19.80 ^{a*} ± 1.72	19.93 ^{a*} ± 2.87
48 h	5.06 ^{ab*} ± 0.25	5.63 ^{a*} ± 0.18	4.66 ^b ± 0.47	4.66 ^b ± 0.27
Δ Gas (kPa) Obese				
Time	Cow	Sheep	Goat	Mixture
12 h	45.05 ^a ± 6.75	45.45 ^a ± 5.05	49.21 ^a ± 2.60	54.20 ^a ± 3.69
24 h	7.40 ^b ± 1.10	7.43 ^b ± 1.12	9.90 ^a ± 0.81	7.35 ^b ± 1.24
48 h	3.90 ^a ± 0.42	4.28 ^a ± 0.82	4.31 ^a ± 0.69	4.31 ^a ± 0.76

¹Results are expressed as mean ± S.D. Letters a,b and c denote significant differences for a same time of fermentation and whey ($p < 0.05$). * denotes significant differences for a given variable between the different groups of donors ($p < 0.05$).

Moreover, total gas production (TP) per type of whey and donor was calculated as the sum of gas production during the fermentation (0, 12, 24 and 48 h) (Figure 5.2). Cow and sheep whey fermented with faeces from NW donors showed a TP (kPa) statistically significant ($p < 0.05$) higher than in case of OB donors. However, in the case of sheep and goat whey, TP did not show significant differences between donors.

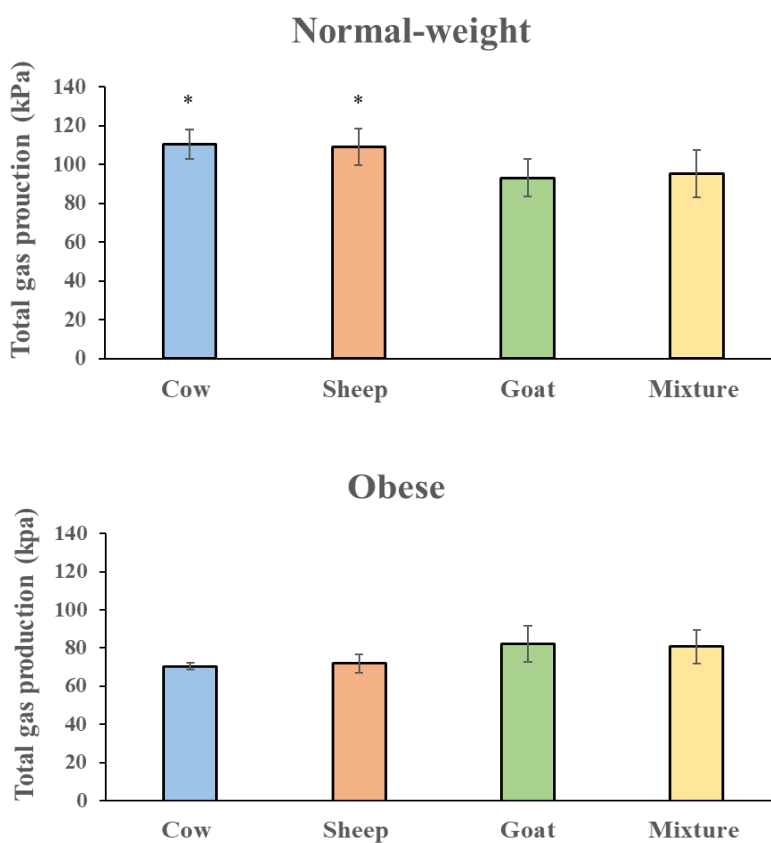


Figure 5.2. Total gas production (kPa) comparison produced by faecal bacteria from human donors (NW (n=3) and OB (n=3) during fermentation (12, 24 and 48 h). Results are expressed as mean \pm S.D. * denotes statistically significant differences for a given variable between the different groups of donors ($p < 0.05$).

3.2. SCFA and BCFA production during fermentation

The total concentration (mM) of fatty acids (acetate, propionate, *i*-butyric, butyrate, *i*-valeric, valeric, *i*-caproic, caproic and heptanoic acid) during the fermentation of several types of whey milk is represented in figure 5.3. The concentration of total fatty acids increased significantly upon fermentation, reaching values near 50 mM in both groups of donors after 48 h of cow, sheep and mixed whey fermentations. Some statistically significant differences ($p<0.05$) were found when fatty acid concentrations from both types of donors were compared. Specifically, NW showed a higher concentration than OB after 48 h of cow and sheep whey fermentations; meanwhile, OB produced more SCFA after 24 h of mixed whey fermentation (43.69 and 25.39 mM, respectively). The molar proportions of the main SCFA (acetate, propionate, butyrate) and minor SCFA (*i*-butyric, *i*-valeric, valeric, *i*-caproic, caproic and heptanoic acid) after fermentation are represented in figure 5.4.

Acetate and propionate showed a tendency to increase with fermentation time, mainly acetate in the case of the OB group. In contrast, butyrate showed a reduction in the relative proportion throughout the fermentation process. Acetate was the most relevant contributor to SCFAs in the NW group, especially at the end of the fermentation. Nevertheless, propionate, butyrate and minor fatty acids were the most important ones in the OB group, showing statistically significance differences ($p<0.05$) between both groups for any whey and time. Comparing the NW and OB SCFA productions, we found statistically significant differences ($p<0.05$) in the case of all SCFAs. The acetate level was significantly higher in NW for the cow and mixed whey at 12 h, while for sheep the high increase (other than for OB) was at 48 h, presenting a relative proportion of 95.99% *versus* 76.66%, respectively. In relation with butyrate, the NW and OB donors showed a marked decreased ($p<0.05$) over the course of fermentations in all samples of whey. We found statistically significant differences ($p<0.05$) after comparing both types of donors, obtaining higher amounts of butyrate in the OB volunteers.

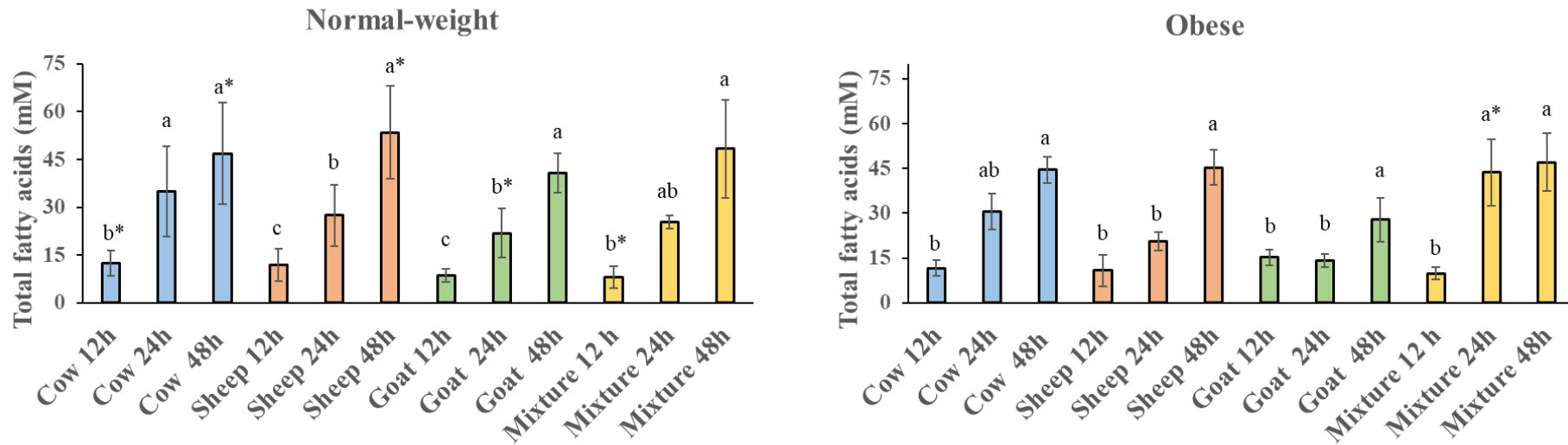


Figure 5.3. Total concentration (mM) of fatty acids (acetate, propionate, *i*-butyric, butyrate, *i*-valeric, valeric, *i*-caproic, caproic and heptanoic acid) for 48 h of fermentation with substrates (cow, sheep, goat and mixture whey milk) in NW (n=3) and OB (n=3) group. Letters a,b,c and d denote significant differences for a given variable between the different times of fermentation per whey milk ($p<0.05$). * denotes significant differences for a given variable between the different groups of donors ($p<0.05$) for 48 h of fermentation with substrates (cow, sheep, goat and mixture whey) in NW (n=3) and OB (n=3) group.

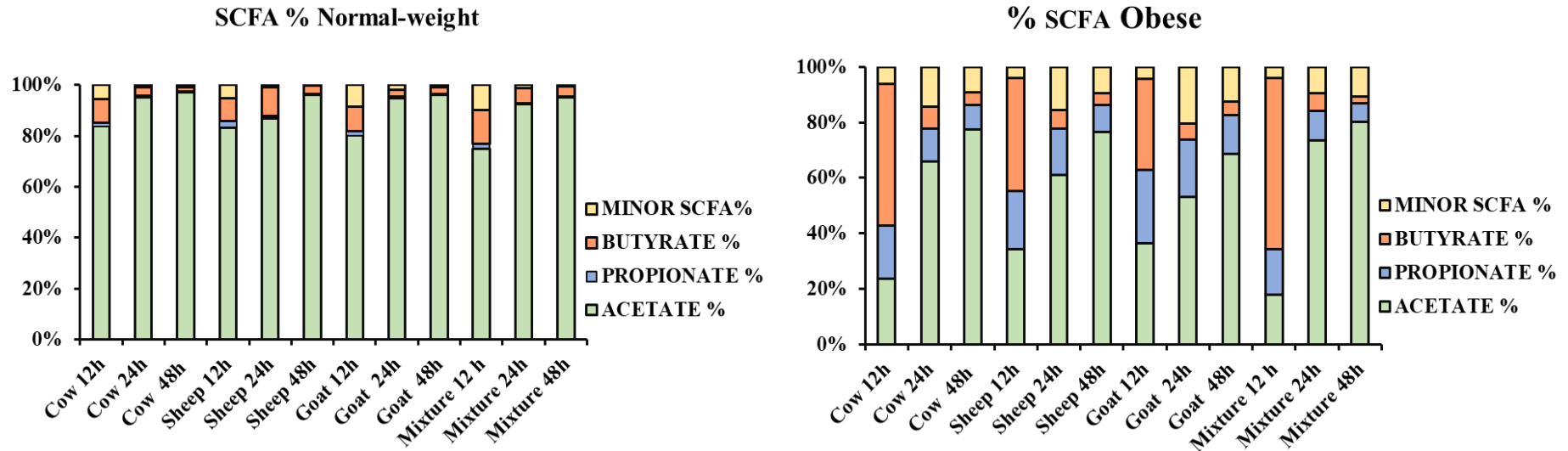


Figure 5.4. Molar proportions (%) of the three major fatty acids (acetate, propionate and butyrate) and minor fatty acids (*i*-valerate, *i*-butyrate, *i*-caproate, valerate, caproate and heptanoic acid) for 48 h of fermentation with substrates (cow, sheep, goat and mixture whey) in NW (n=3) and OB (n=3) group.

It is worth noting the differences after 12 hours of mixed whey fermentation, obtaining 61.2% and 13.4% of butyrate in OB and NW, respectively. In all samples of whey, butyrate showed a substantial reduction near 90%, from 12 to 48 hours. Propionate levels were also significantly higher in the OB than NW donors in all samples of whey. In every whey and donor, propionate increased slightly over fermentations, but its relative proportion decreased due to the high increase in acetate. In relation to the molar proportions of minor fatty acids, *i*-valerate was the main contributor. We found statistically significant higher proportions ($p < 0.05$) in OB donors than NW donors, mainly at 24 h of fermentations. The greatest differences at this time were found for goat whey (20.35% in OB versus 1.92%, in NW donors). Time 48 h also showed significant increases in OB donors compared to NW donors, especially for mixture whey (10.85% vs. 0.64%).

3.3. Analysis of faecal microbiota of NW and OB donors after fermentation

The original microbial population (time 0 h) of both groups of donors is shown in figure 5.5. As mentioned before, OB and NW microbiota differ. This study reinforced this theory, reporting differences in the initial microbiota composition of both groups of donors. Significant differences ($p < 0.05$) were found in *Firmicutes*, *Bacteroides* and *Lactobacillus* being higher in OB than in NW. In the case of lactobacilli, the initial gut difference was marked: 3.64 and 5.01 log genome equivalent/mL in NW and OB, respectively.

Initial microbiota and changes in the bacteria population after 12, 24 and 48 h of fermentation with the different whey milks are shown in table 5.5. The total bacteria population showed statistically significant differences ($p < 0.05$) at 48 h of cow and sheep whey fermentation when both donors were compared. Focusing on the total bacteria evolution over time in each type of donor, we found a significant decrease ($p < 0.05$) from 12 to 48 h for the sheep whey fermentation in NW; significant differences with respect to controls at time 12 h for cow, sheep and mixed whey occurred (data not shown). In the case of OB, we found a significant increase in the total

bacteria after 12 h of cow whey fermentation. However, at 12 h, all whey types showed higher values than the controls (data not shown).

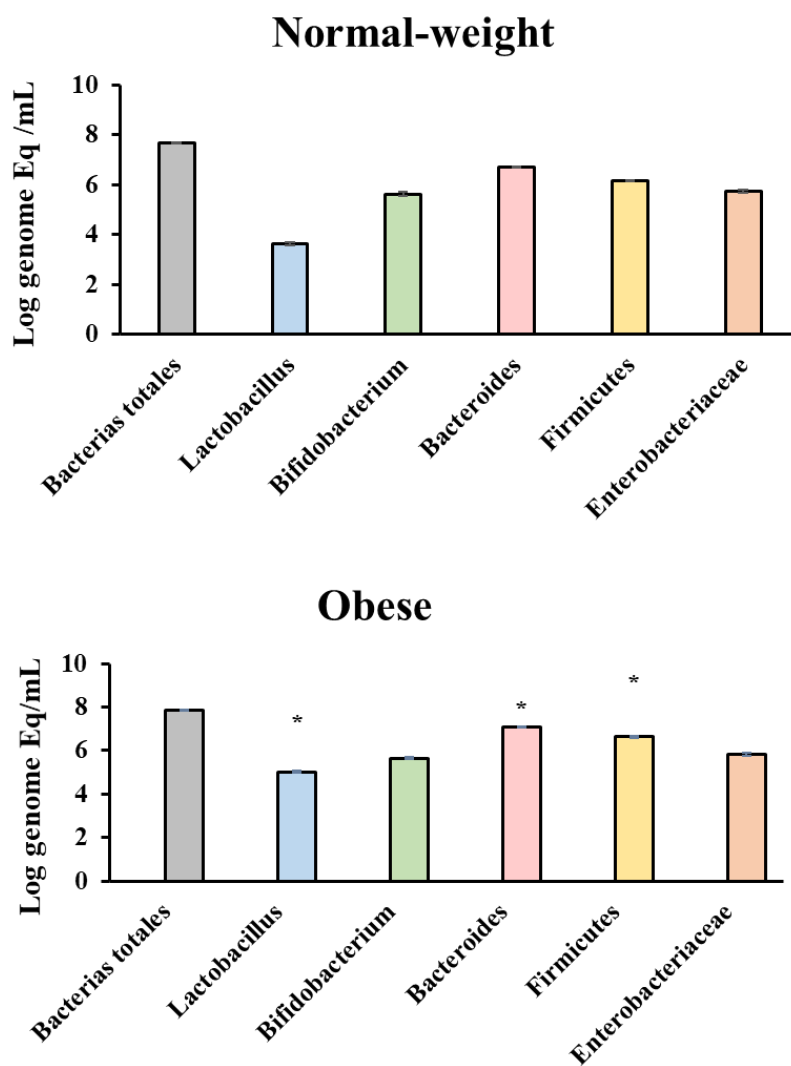


Figure 5.5. Initial microbial population (logarithm genome equivalents/mL) of normal-weight (n=3) and obese (n=3) groups of donors. Bars represent means \pm S.E.M. of quadruplicate cultures. * denotes significant differences for different groups of donors ($p < 0.05$).

Lactobacillus group showed a decreasing tendency in all samples from NW donors, although without any significant differences. Nevertheless, in OB group an opposite effect was found, with increases near 2 log genomic equivalent/mL at time 48 h in the case of cow and sheep (7.1 ± 0.34 and 7.06 ± 0.34 , respectively). All samples of whey during fermentation were significantly different in the OB than in the NW group (except in the case of sheep whey at 12 h), as there was a higher number of lactobacilli in the OB.

Bifidobacterium group showed an approximate increase of 1 log genomic equivalent/mL in all whey and donors, obtaining statistically significant differences in some cases. In NW donors, we found a significant increase in sheep and mixed whey from 0 h to 24 or 48 h, respectively. Furthermore, significantly higher values of bifidobacteria were observed in every whey and at every time compared to the controls (data not shown). In OB donors, we found significant increases with respect to the initial time of fermentation in cases of cow and mixed (12, 24 and 48 h) with differences compared to controls at 24 and 48 h. Differences between both types of donors were not found.

Bacteroides group in NW showed a significant decrease throughout the fermentation (cow, sheep and goat). In OB, the bacteria population descended over time, although not significantly. However, we found significantly higher values of *Bacteroides* in OB than NW in most times and whey samples. *Firmicutes* group data did not show significant increases throughout fermentation, remaining stable in both, OB and NW donors, although the initial value was significantly higher in OB. *Enterobacteriaceae* in NW shown a slight increment in cow and sheep whey at 12 h and a reduction at 24 h. In OB increases occurred at 48 h but not were statistically significant between times of fermentation. However, significant differences occurred between groups of donors, indicating increases in cow and goat whey at 24 and 48 h, and mixed and sheep whey at the end of the fermentation.

Table 5.5. Bacterial populations (log genomic equivalent/mL) from NW (n=3) and OB (n=3) donors after 48 h of fermentation using several types of whey (cow, sheep, goat and mixture). Results are expressed as mean±S.E.M. of quadruplicate cultures. Letters a, b, c and d denote significant differences for a given substrate between the different times of fermentation ($p<0.05$). * denotes significant differences for different donors ($p<0.05$).

		Normal-weight					
	Time	Total bacteria	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Bacteroides</i>	<i>Firmicutes</i>	<i>Enterobacteriaceae</i>
Cow	0	7.68 ± 0.05	3.64 ± 0.33	5.62 ± 0.13	6.71 ^a ± 0.15	6.16 ± 0.19	5.73 ^b ± 0.13
	12	8.01 ± 0.16	3.66 ± 0.59	6.12 ± 0.55	6.45 ^{ab} ± 0.17	6.30 ± 0.26	6.40 ^a ± 0.07
	24	7.63 ± 0.19	3.69 ± 0.33	6.79 ± 0.13	6.07 ^{bc} ± 0.14	6.25 ± 0.26	5.78 ^b ± 0.20
	48	7.49 ± 0.04	3.53 ± 0.37	6.33 ± 0.17	5.65 ^c ± 0.11	5.95 ± 0.19	5.44 ^b ± 0.17
Sheep	0	7.68 ^{ab} ± 0.05	3.47 ± 0.26	5.60 ^b ± 0.05	6.81 ^a ± 0.13	6.10 ± 0.06	5.71 ^b ± 0.10
	12	7.98 ^a ± 0.16	4.28 ± 0.41	5.95 ^{ab} ± 0.41	6.14 ^b ± 0.11	6.26 ± 0.19	6.37 ^a ± 0.10
	24	7.66 ^{ab} ± 0.18	3.89 ± 0.30	6.79 ^a ± 0.17	5.95 ^{bc} ± 0.22	6.33 ± 0.35	5.77 ^{ab} ± 0.29
	48	7.39 ^b ± 0.07	3.16 ± 0.48	6.54 ^a ± 0.16	5.53 ^c ± 0.11	5.87 ± 0.21	5.58 ^b ± 0.08
Goat	0	7.68 ± 0.05	3.64 ± 0.33	5.62 ± 0.13	6.71 ^a ± 0.15	6.16 ± 0.78	5.73 ± 0.13
	12	7.76 ± 0.23	3.31 ± 0.43	6.05 ± 0.51	6.27 ^{ab} ± 0.23	5.84 ± 0.35	6.00 ± 0.21
	24	7.65 ± 0.13	3.14 ± 0.30	6.51 ± 0.13	6.01 ^{ab} ± 0.10	5.78 ± 0.24	5.92 ± 0.21
	48	7.77 ± 0.12	3.18 ± 0.37	6.76 ± 0.19	5.71 ^b ± 0.11	5.87 ± 0.09	5.76 ± 0.09
Mixture	0	7.68 ± 0.05	3.64 ± 0.21	5.62 ^b ± 0.13	6.71 ± 0.15	6.16 ± 0.78	5.73 ± 0.13
	12	8.02 ± 0.17	3.59 ± 0.30	6.26 ^a ± 0.18	6.21 ± 0.14	6.45 ± 0.27	6.36 ± 0.23
	24	7.80 ± 0.19	3.73 ± 0.22	6.65 ^a ± 0.16	5.94 ± 0.29	6.03 ± 0.26	5.85 ± 0.40
	48	7.70 ± 0.18	3.15 ± 0.20	6.44 ^a ± 0.11	5.90 ± 0.22	6.20 ± 0.36	5.61 ± 0.20

		Obese					
	Time	Total bacteria	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Bacteroides</i>	<i>Firmicutes</i>	<i>Enterobacteriaceae</i>
Cow	0	7.85 ^b ± 0.09	5.01 ^{b*} ± 0.60	5.64 ^b ± 0.19	7.09* ± 0.04	6.63* ± 0.18	5.81 ± 0.19
	12	8.44 ^a ± 0.12	5.77 ^{ab*} ± 0.66	6.47 ^a ± 0.34	7.11 ± 0.27	6.83 ± 0.32	6.73 ± 0.35
	24	8.07 ^{ab} ± 0.20	6.05 ^{ab*} ± 0.37	6.70 ^a ± 0.16	6.89* ± 0.24	6.31 ± 0.34	6.73* ± 0.33
	48	8.1 ^{ab*} ± 0.10	7.10 ^{a*} ± 0.34	6.65 ^a ± 0.11	6.68 ± 0.32	6.23 ± 0.34	6.39* ± 0.30
Sheep	0	7.85 ± 0.97	5.01 ^{b*} ± 0.60	5.64 ^b ± 0.19	7.09* ± 0.44	6.63* ± 0.18	5.81 ± 0.16
	12	8.29 ± 0.28	5.46 ^{ab} ± 0.24	6.31 ^{ab} ± 0.24	7.02 ± 0.44	6.74 ± 0.31	6.70 ± 0.46
	24	8.07 ± 0.20	6.07 ^{ab*} ± 0.25	6.57 ^a ± 0.19	6.84* ± 0.29	6.13 ± 0.33	6.75 ± 0.36
	48	8.36* ± 0.14	7.06 ^{a*} ± 0.34	6.62 ^a ± 0.12	6.68* ± 0.31	6.25 ± 0.36	6.52* ± 0.26
Goat	0	7.85 ± 0.09	5.01* ± 0.60	5.64 ^b ± 0.19	7.09* ± 0.04	6.63* ± 0.18	5.81 ± 0.16
	12	8.30 ± 0.14	5.46* ± 0.60	6.22 ^{ab} ± 0.25	7.02 ± 0.30	6.87 ± 0.31	6.72 ± 0.37
	24	8.10 ± 0.15	5.59* ± 0.37	6.43 ^a ± 0.23	6.86* ± 0.29	6.26 ± 0.27	6.8* ± 0.28
	48	7.81 ± 0.20	6.63* ± 0.51	6.29 ^{ab} ± 0.14	6.71* ± 0.26	5.88 ± 0.44	6.51* ± 0.26
Mixture	0	7.85 ± 0.09	5.01* ± 0.60	5.64 ^b ± 0.19	7.09* ± 0.04	6.63* ± 0.18	5.81 ± 0.16
	12	8.18 ± 0.15	5.43* ± 0.55	6.56 ^a ± 0.23	6.72 ± 0.25	6.59 ± 0.36	6.51 ± 0.30
	24	8.08 ± 0.12	5.92* ± 0.39	6.87 ^a ± 0.04	6.88* ± 0.25	6.30 ± 0.29	6.75 ± 0.30
	48	8.06 ± 0.08	6.68* ± 0.55	6.69 ^a ± 0.13	6.76* ± 0.26	6.27 ± 0.33	6.54* ± 0.25

4. DISCUSSION

Whey milk is mainly composed of protein and lactose, and both can act as useful substrates for bacteria and approximately 10% of protein intake reaches the human colon, although it depends on the amount and kind of protein ingested (Cummings, 1997). In the present study, we have evaluated the prebiotic effect of several types of whey milk on human microbiota from NW and OB donors. Large amounts of milk proteins and peptides enter the large intestine where they are degraded in several peptides with well-known physiological effects, such as immunomodulatory and gastrointestinal activity (mineral-binding, anti-appetizing and antimicrobial activity) (Korhonen and Pihlanto, 2006). Based on this, the use of milk whey as ingredient could produce bioactive peptides that can be released by the hydrolysis of digestive enzymes, the action of microorganism proteolytic enzymes (Korhonen and Pihlanto, 2006) or through the hydrolysis of acidolactic proteolytic bacteria such as *Lactococcus sp.* and *Lactobacillus sp.* In gut and other proteolytic bacteria able to degrade digested protein into peptides, such as *Bacteroides fragilis*, *Streptococcus*, *Propionibacterium*, *Clostridium*, *Bacillus* and *Staphylococcus* (Macfarlane *et al.*, 1988, Macfarlane *et al.*, 1986). However, lactose contained in whey has a prebiotic effect for beneficial bacteria and it can be hydrolysed into galactooligosaccharides (GOS) by the enzyme β -galactosidase release by the gut microbiota (*Lactobacillus sp.*, *Bifidobacterium sp.* and *E. coli*) (Aehle, 2007).

A reduction in the pH may reflect SCFA and lactic acid production through the fermentation process, inhibiting the growth of pathogen bacteria and promoting a probiotic effect on gut induced by whey milk. (Cummings, 1981, Coppa *et al.*, 2006) (Belenguer *et al.*, 2007). Regarding to this, *Bifidobacterium* and *Lactobacillus*, that not only decrease the intestinal pH, but also enhance the lysozyme activity and facilitate the disruption of some pathogenic bacteria (Alakomi *et al.*, 2000). Gas production is an indicator of the activities of the total gut microflora. Rycroft *et al.*, (2001) demonstrated that the fermentation of GOS produced lactate and reduced gas production, probably because GOS stimulated the bifidobacteria population, demonstrating that there is an inverse relationship between the presence of *Bifidobacterium* and gas production (Rycroft *et al.*, 2001). According to this, high gas production and low bifidobacteria levels at the

beginning of the fermentation (from time 0 to 12 h) were found, whereas inverse results were found after 48 hours, in our study. Whey is composed of both, lactose and protein, and more proteolytic bacteria would contribute to the total gas production (*Clostridium* and *Streptococcus*). Moreover, *Bifidobacterium* can ferment lactate reducing its availability for gas-producing bacteria, such as *Clostridium* and sulphate-reducing bacteria (Bernalier *et al.*, 1999). The measure of gas production could not be a precise predictor of what occurs in the gut environment since it cannot mimic the interactions of microorganisms and their metabolites and high variability can be observed.

Whey is a complex product that favours cross-feeding because metabolites produced by one type of bacteria serve as substrate for another. For example, lactate and acetate produced by *Bifidobacterium* can be converted to butyrate by another lactate-utilizing and butyrate-producing bacterium, such as *Firmicutes* (Belenguer *et al.*, 2006), highlighting the complexity of the gut ecosystem. These authors suggested that lactate was rapidly metabolised into acetate, butyrate and propionate by the intestinal microbiota at pH values of around 5.9. Nevertheless, at the pH of 5.2 the use of lactate is reduced, while its production is maintained, resulting in lactate accumulation (Belenguer *et al.*, 2007). These data were similar to those found in our research, as values of acetate and butyrate are directly and inversely related with time to the fermentation time, respectively.

The most important metabolites produced by bacteria during fermentation are, directly or indirectly, organic acids such as acetate (produced by *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Enterobacteria*, etcetera), butyrate (*Firmicutes*), propionate (*Bacteroides*, *Prevotella* and *Clostridium*), lactate (*Lactobacillus*, *Bifidobacterium*...), succinate (*Lactobacillus*), together with hydrogen and CO₂ (Cummings, 1981, Duncan *et al.*, 2004, Puertollano *et al.*, 2014). Previous studies have reported that the production of acetate, propionate and butyrate mainly depends on carbohydrate fermentation. However, protein and amino acid fermentation also play an important role in this pool of SCFAs and also BCFAs (Macfarlane *et al.*, 1992). SCFAs are mainly produced in the proximal colon in high concentrations (70–140 mM), transported through the distal colon by the intestinal flow. SCFAs are absorbed and

mainly used by colonocytes for their metabolic maintenance (Scott and Duncan, 2008). These bacteria metabolites have been associated with multiple biological activities, such as the regulation of energy homeostasis, anti-inflammatory activity (Jandhyala *et al.*, 2015, Puertollano *et al.*, 2014) and satiety (Cani *et al.*, 2004, Tolhurst *et al.*, 2012). In addition, the supplementation of butyrate and acetate in diet has shown to suppress weight gain in rodent models (Gao *et al.*, 2009, Yamashita *et al.*, 2007), respectively. Propionate has also been associated with an inhibition of food intake in humans. Moreover, SCFA receptors: FFAR2 and FFAR3, located in the intestine are related to the expression of peptide YY and GLP-1, two of most important hormone mediators in satiety (Cani *et al.*, 2004, Tolhurst *et al.*, 2012). Another action point of SCFAs in satiety is the stimulation of the release of serotonin (5-hydroxytryptamine) through the activation of SCFA receptors, affecting colonic motility and the digestion time, leading to satiety regulation (Sleeth *et al.*, 2010). An antimicrobial function has also been associated with SCFAs producing a decrease on the colonic pH inhibiting the growth of some potential pathogens. Furthermore, it has been described that acetate promotes the release of reactive oxygen species (ROS), which are efficient bactericidal factors (Nicholson *et al.*, 2012). In relation to our results, Shen *et al.*, (2010) used meat as a fermentable substrate in a batch culture fermentation, obtaining similar concentrations of butyric and *i*-butyric acid to our study (Shen *et al.*, 2010). However, our data on acetate, caproate and valerate levels were higher, maybe caused by another whey substrate, such as lactose.

These fatty acids result from protein and amino acid degradation and they could indicate a major degradation ability of microbiota, especially in OB, exposed to several whey proteins. The level of these BCFA often comprises less than 10% of the total SCFA, potentially due to the low level of polypeptides and amino acids reaching the colon compared with non-digestible carbohydrates (Rasmussen *et al.*, 1988). Their effects on the colonic epithelium have been poorly described, they could be probably involved in regulating ionic movements through the colonic epithelial layer (Musch *et al.*, 2001).

In relation to microbiota evolution, it is important to note that obesity is associated with an increase of *Firmicutes/Bacteroidetes* ratio and a decrease in the diversity of microbiota due to both weight and diet (Ley *et al.*, 2005). The initial levels of *Firmicutes*

in our study were consistent with this theory. This group of bacteria is associated with a high ability of harvest energy from diet (Ley *et al.*, 2005) and also related to low-grade intestinal inflammation, leading to the development of obesity and metabolic syndrome (Turnbaugh *et al.*, 2009). Our initial levels of *Bacteroides* counter this assertion. Classically, *Bacteroides* has been associated with NW, overall in the case of a fat- or carbohydrate-restricted diet (it is a bacteria responsive of calorie intake) (Ley *et al.*, 2006), but Patil *et al.*, (2012) found an increase of this bacteria in OB Indian individuals. A recent meta-analysis did not find significant differences in many studies related with this and suggested that the PCR primer used, sample handling or extraction could be the cause of contradictory results (Walters *et al.*, 2014).

Lactobacilli (and also bifidobacteria) have a well-known their probiotic effect on the gut microbiota (Fujimoto *et al.*, 2014). However, several studies have associated *Lactobacillus* ingestion with obesity (Raoult, 2008b, Raoult, 2008a, Raoult, 2009), consistent with our results (initial lactobacilli were higher in OB than NW). Million *et al.*, (2012) confirmed this association when they studied the gut microbiota in OB and NW donors, finding that *Lactobacillus reuteri* was related to microbiota in OB, possibly because this bacterium improves the intestinal capacity to absorb nutrients and increase food conversion (Casas and Dobrogosz, 2000). Nevertheless, other lactobacilli such as *L. paracasei* or *L. plantarum*; *Bifidobacterium spp.* (*B. animalis*) and *Methanobrevibacter smithii* are related to a normal body mass (Million *et al.*, 2012).

Our study has demonstrated amply that liquid whey could be considered a high quality protein source, as well as prebiotic because it contains approximately 5% approximately of lactose, which can be beneficial to increasing probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* (Coppa *et al.*, 2006). Moreover, this by-product from cheese production stabilizes or slightly decreases certain bacteria groups typical in OB people, such as *Firmicutes* and *Enterobacteriaceae*.

The high-quality proteins in whey composition should be taken into account in terms of their health benefits. Not only protein degradation happen during gastrointestinal digestion, but also proteolytic activity in some groups of bacteria, such as *Bacteroides*

fragilis and *Propionibacterium*, and other bacteria belonging to *Firmicutes* such as *Streptococcus*, *Clostridium*, *Bacillus* and *Staphylococcus* (Macfarlane *et al.*, 1988, Macfarlane *et al.*, 1986). Some studies have shown that *Bacteroides* have strong peptidase activity mainly at pH around 6.5 (Wallace and McKain, 1997, Walker *et al.*, 2005). Our data support these results because after 12 h of fermentation the pH lowered to less than 6.5. The reduction in *Bacteroides*, observed in the present study, could be due to the fact that from time 0 to 12 h of fermentation, the pH values fell from 6.5 to approximately 4.9, reducing protease activity and, consequently, the stabilization of *Bacteroides*. Subsequently, the pH values continued falling until they reached 3.9, causing a reduction in these bacteria.

Results showed important changes in beneficial bacteria population such as bifidobacteria and lactobacilli for 48 h of fermentation of whey milk samples. Regarding to this, it should be noted that cow, sheep and the mixture showed the highest prebiotic effect at different times; however, goat whey seems not to exert an important positive effect on the growth of probiotics and the metabolites production in gut. The high concentration of whey from cow (60%) could probably explain the similar trend observed in the mixture of the different whey milks. Increases in *Bifidobacterium* in both donors, OB and NW, correspond to a lower gas production and pH levels. Particularly high levels of bifidobacteria and low pH levels were found at 24 h of fermentation and it can be explained because *Bifidobacterium* could ferment lactose into acetate, lactate and pyruvic acid, which could reduce the intestinal pH. This fact could improve the intestinal health, preventing the growth of other pathogenic bacteria, such as *Salmonella* or *E. coli* (Wells *et al.*, 2005). Consequently, at 24 h the total gas production was not as pronounced as at 12 h because bifidobacteria are non-gas producing bacteria.

The level of *Lactobacillus* observed was very pronounced in OB donors. As we mentioned at the beginning of the discussion, although some authors suggest that *Lactobacillus* (*L. reuteri*) may promote obesity (Raoult, 2008b, Raoult, 2008a, Raoult, 2009, Million *et al.*, 2012), this must be considered with caution because the mechanism by which this probiotic is associated with obesity remains still unclear. Further analysis to

detect specific species of *Lactobacillus* must be carried out to determine better this association.

The increases in probiotic bacteria found in this study are consistent with several previous studies in which it was reported that milk protein and hydrolysed peptides increase *Bifidobacterium* growth (Loennerdal, 2013, Nagpal *et al.*, 2011). Moreover, recent studies have demonstrated that whey protein intake increased the levels of *Lactobacillus* and *Bifidobacterium* in a rat model of colitis, possibly by an amino acid composition-mediated mechanism (Sprong *et al.*, 2010) or specifically increased the *Lactobacillus* population and decreased the *Clostridium* group in mouse model (McAllan *et al.*, 2014). Kobayashi *et al.*, (2011) demonstrated that liquid whey feeding for pigs enhanced probiotic bacteria, while opportunistic pathogen species disappeared (Kobayashi *et al.*, 2011). In addition, the ingestion of milk whey culture produces an active substance (1,4-dihydroxy-2-naphtoic acid (DHNA)), isolated from the whey culture with a bifidogenic effect dose-dependent, improving inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease (Uchida *et al.*, 2007, Suzuki *et al.*, 2006). And is well known that GOS can also selectively promote *Bifidobacterium* (Macfarlane *et al.*, 2008, Davis *et al.*, 2010, Davis *et al.*, 2011, Roberfroid, 2005, Roberfroid *et al.*, 2010) population. Different studies report that specific whey proteins have anti-microbial activity (Freedman *et al.*, 1998, Shin and Tomita, 2000, Yamauchi *et al.*, 1993) through whey-derived peptides' formation by pepsin catalysed lactoferrin to lactoferricin (Hoek *et al.*, 1997). In this regard, polypeptide fractions of α -lactalbumin (LTD1, LTD2 and LCD) have proven to have an effect against *E. coli*, *K. pneumoniae*, *S. aureus*, etc. (Pellegrini *et al.*, 1999). Moreover, milk proteins and peptides such as lactoferrin, lactoperoxidase and lysozyme, have been to provide a non-immune defence against microbial infections (Schanbacher *et al.*, 1997). Regarding obesity, the prebiotic effect of whey is related to obesity prevention (Cani *et al.*, 2009) by increasing *Bifidobacterium* population that could modulate low-grade intestinal inflammation in OB mice by stimulating GLP-2, which reduces cell permeability and therefore the entrance of LPS

5. CONCLUSIONS

The present findings demonstrated that whey from milk of cow and sheep and also mixed, had a prebiotic effect on the gut microbiota of NW and OB donors during batch culture fermentation. These types of whey stimulated the growth of probiotic bacteria, demonstrating a marked bifidogenic effect in both groups of donors. Moreover, whey fermentation led to the production of a wide variety of SCFAs and BCFAs, especially in OB donors, which could be beneficial for colonic health. Supplementing diet with these types of whey can be a promising strategy to modulate the gut microbiota and to stimulate selectively the growth of probiotic bacteria. This could improve intestinal disorders and be an interesting and encouraging approach to the prevention of gut disorders, obesity and related diseases.

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*Separation of protein and lactose from sheep whey using
ultrafiltration membranes. Application of SKK model.*

CHAPTER 6



1. INTRODUCTION

Obesity and overweight have reached alarming levels, potentially achieving 20% of the world's adult population by 2030 (Kelly *et al.*, 2008). Due to the “obesogenic” environment of the actual societies, it is becoming increasingly necessary to develop new strategies that focus on reducing overweight and obesity, as well as a wide range of co-morbidities related, such as type 2 diabetes, cardiovascular diseases, etc (Guh *et al.*, 2009). That approach towards healthy and functional food make necessary the developing of new food products which enhance satiety by reducing food intake without hungry feelings.

Regarding satiety ingredients, protein has been described as the most satiating macronutrient (Bendtsen *et al.*, 2013), having milk whey (MW) an excellent functional and nutritional value. Furthermore, this protein source is a by-product of cheese manufacture or casein production from dairy industry, which makes it even more interesting and profitable as functional food. MW contains 70-80% of lactose, 9% of proteins and 8-20% of minerals, considering in a dry basis (Daufin, 1998). The proteinaceous fraction of whey (WP) has shown effect on satiety by reducing food intake (Pupovac and Anderson, 2002, Froetschel *et al.*, 2001). In addition, whey protein and its biopeptides and amino acids released through digestion have many biological functions such as antimicrobial activities, insulinotropic effect, and prevention of cancer and cardiovascular diseases, among others (Madureira *et al.*, 2007, Jakubowicz and Froy, 2013).

However, the total protein concentration in whey depend on the type (acid or sweet), mammal species (cow, sheep, goat, etcetera), season, lactation period, animal feed and quality of processing (Sanz Ceballos *et al.*, 2009, Giroux *et al.*, 2018, Hejtmankova *et al.*, 2012, Park *et al.*, 2007, Wit, 2001). Furthermore, one of the most important limitations on the use of whey is the high ratio lactose/protein, so many methods have been used to concentrate WP in selective manner. In this context, membrane technology is a suitable/useful tool <for the revalorisation of whey.

In 1960, membrane separation was introduced and it has been successfully used for dairy process design and development through concentration and fractionation of milk components (D'Souza and Mawson, 2005). This methodology constitutes a more ecological, economic and preservative (from a nutritional point of view) alternative compared with traditional and conventional methods of whey concentration, such as thermal evaporation. Depending on membrane cut-off, several methods of whey protein separation have been described: ultrafiltration, diafiltration and nanofiltration (Baldasso *et al.*, 2011). In this work, we have chosen ultrafiltration (UF) since it is capable to separate, in a selective manner, molecules with 1 kDa to 200 kDa (Mistry, 1993) of molecular weights. Moreover, it does not use heat, being more environmentally sustainable than other methods, as well as retention of protein and selective permeation of lactose, minerals, water and other compounds with lower molar mass (Brans, 2006), offering whey proteins with different degrees of purity (Prazeres *et al.*, 2012). Apart from protein, lactose removal could result very suitable for food and biotechnological industry, as well as pharmaceutical applications (as a food preservative, used in infant formulas (Wit, 2001), and as raw material in organic synthesis and biopolymer, among others).

Nevertheless, the process of ultrafiltration can be less capable of achieving product separation after a long-time of operation due to membrane fouling and polarization concentration, reducing the efficiency of the process. Membrane fouling is mainly due to whey components, such as different proteins, fats, minerals, etcetera; therefore, cleaning is an important aspect to preserve selectivity and permeability of membranes. However, several aspects could minimise this fouling such as a whey pretreatment, suitable filtration system and membrane selection, among others (D'Souza and Mawson, 2005). These factors make necessary the improvement of membrane separation processes to minimise these problems in order to obtain a high-quality WP with optimal industry conditions.

Mediterranean countries offer the best possible conditions for the rearing of small ruminants such as ewes and goats mainly due to their meteorological conditions. Indeed, Spain as well as Southern European countries are major producers of high-quality cheeses from sheep and goat (with designation of origin labels). The total cheese

production in Spain in 2016 was of 461.000 tons, being 67.000 and 47.000 tons of sheep's and goat's cheese, respectively (MAGRAMA, 2016) and these vast amounts of whey should be used as a high nutritional meal since it would have been destined as feed for farm animals. Furthermore, protein content of sheep whey has been demonstrated to be higher compared to cow and goat milk whey in the present manuscript (chapter 2) and by many researches (Ruprichova *et al.*, 2014, Giroux *et al.*, 2018, Potocnik *et al.*, 2011, Tagliazucchi *et al.*, 2018). In addition, there are scant researches about membrane process with sheep whey, opposite to vast literature found on bovine whey processing. But the ovine whey has a ratio of total nitrogen/dry matter higher than bovine, being the protein content doubled, making it an interesting by-product to functional food industry (Assenat, 1985).

Taking everything into account, the main aim of this study was to test different UF membranes and studying the influence of operation conditions to produce sheep whey protein (lactose-free) to provide a protein-rich functional food able to enhance satiety, as well as sugar-free product of interest for diabetic patients. The influence of different ultrafiltration membranes, GR60PP (polysulphone and 25 kDa), GR80PP and GR90PP (polyethersulphone, 10 and 5 kDa, respectively) and different operational conditions (pressure and lactose concentration) on lactose separation and protein rejection was studied. We also investigated the viability of using the Spiegler-Kedem-Kachalsky model to predict the rejection of lactose with the different membranes studied.

2. MATERIALS AND METHODS

2.1. Whey

Sweet sheep whey samples were obtained (on three different occasions) from Palancares Alimentación S.L., a local cheese factory from Murcia (Spain). Immediately after reception, ovine whey was defatted by centrifuging at $3.000 \times g$ (gravity) for 15 min and stored at $-80 \text{ }^{\circ}\text{C}$ for further analysis (Eppendorf centrifuge 5804R. Spain). pH value of sheep whey was 5.96 ± 0.03 and its nutritional composition once skimmed corresponded to 44.1 ± 0.73 and $1.8 \pm 0.21 \text{ g L}^{-1}$ for lactose and protein, respectively.

2.2. Membranes

Three ultrafiltration membranes (GR60PP, GR80PP and GR90PP) were supplied by Alfa Laval (Spain) and the specifications are shown in table 6.1. Figure 6.1 shows the appearance of GR80PP membrane before and after use (A and B, respectively).

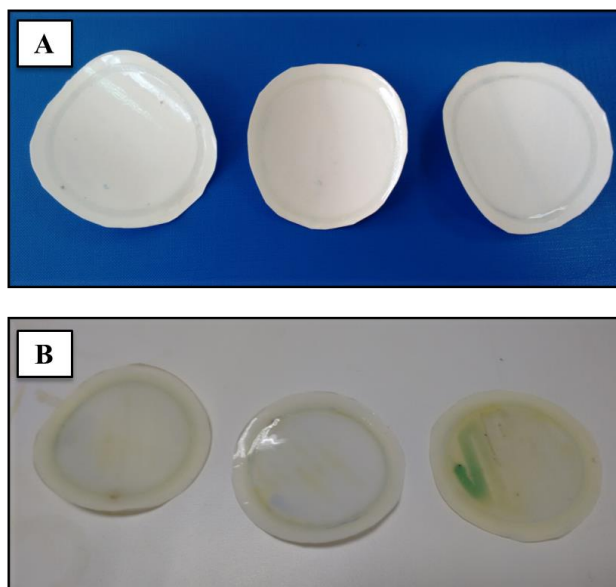


Figure 6.1. Appearance of GR80PP membrane before and after ultrafiltration experiment (A and B, respectively)

Table 6.1. Main characteristics of the membranes used in the experimental flat membrane test module¹.

Provider	Alfa Laval	Alfa Laval	Alfa Laval
Manufacturer	Dow Chemical	Dow Chemical	Dow Chemical
Product denomination	GR60PP	GR80PP	GR90PP
Type	Thin-film composite (UF)	Thin-film composite (UF)	Thin-film composite (UF)
Chemical Composition	Polysulphone	Polyethersulphone	Polyethersulphone
MWCO (kDa)	25	10	5
Pressure (bar)	Production 1-10 Cleaning 1-5	Production 1-10 Cleaning 1-5	Production 1-10 Cleaning 1-5
pH range (reference a T^a 25°C)	Production 3-10 Cleaning 1-13	Production 3-10 Cleaning 1-13	Production 3-10 Cleaning 1-13
T^a range (°C)	5-75	5-75	5-75
T^a Max (°C)	80	80	80

¹*Molecular weight cut-off (MWCO). Temperature (T^a)

2.3. Ultrafiltration equipment

Tests were performed in an MMS Triple System Model F1 flat membrane test module (MMS AG Membrane Systems, Switzerland). The experimental unit consisted of a thermostated stainless-steel feed tank with a capacity of 0.8 L designed for a maximum operating pressure of 40 and a flat sheet membrane module (specific area 8.48 cm²) supporting the membranes. Figure 6.2 shows a diagram of the experimental unit (Hidalgo *et al.*, 2016).

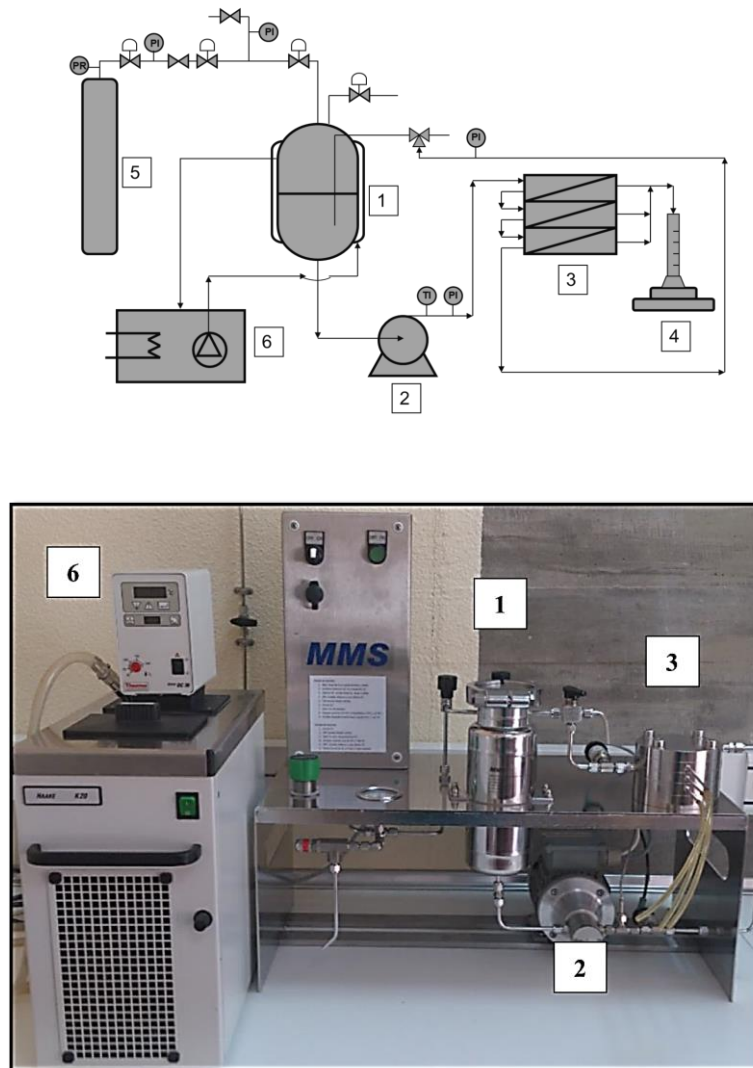


Figure 6.2. Flow diagram of ultrafiltration test unit. (1) feed tank, (2) pump, (3) membrane module, (4) scale, (5) nitrogen cylinder, (6) cryostat. (Hidalgo *et al.*, 2016).

Experimental units were provided with a pressure pump to drive the sheep whey solution through the membrane. The pressure of the system was obtained by a nitrogen cylinder. Monitorization of pressure and temperature was performed by the Triple System Software and PC Lenovo (SL 500. Windows XP).

2.4. Experimental procedure

Water and aqueous solutions of/from sweet ovine whey were treated in the test module in which the feed stream was separated into two streams: one purified “permeate” and the other concentrated “concentrate”. Concentrate was recycled into the feed tank. Permeate was collected at intervals of 3 to 5 minutes. Experiments could reach the steady-state, which was considered to be reached when the difference between concentration values in the permeate stream in three consecutive measurements was lower than 3%. Rejection percentages and permeate fluxes were calculated as an average value of the last three measurements. All the experiments were run in duplicate and standard deviation values of about 3% were obtained for the whole set of data.

The following experimental conditions were maintained unchanged throughout the experimental series: feed solution 0.8 L, temperature 22 ± 0.5 °C and assay time of 30 minutes. Figure 6.4 illustrates the experimental design of the present study, showing the different stages performed. Three different UF membranes, GR60PP (polysulphone and 25 kDa), GR80PP and GR90PP (polyethersulphone, 10 and 5 kDa, respectively) were tested at different operating pressures (4, 6, 8 and 10 bar) and at different lactose concentrations (normal, concentrated and diluted whey). Water permeability was assayed before and after experiment. Samples for analysis were removed at intervals of 3 to 5 minutes per membrane experiment.

Aqueous solutions of sweet ovine whey (protein concentrations ranging between 1.27 kg/m^3 and 2.34 kg/m^3) were treated in the test module at operating pressures varying from 4 to 10 bar for the ultrafiltration, varying the feed lactose concentration from 1.5 to 6.15 % (figure 6.3).

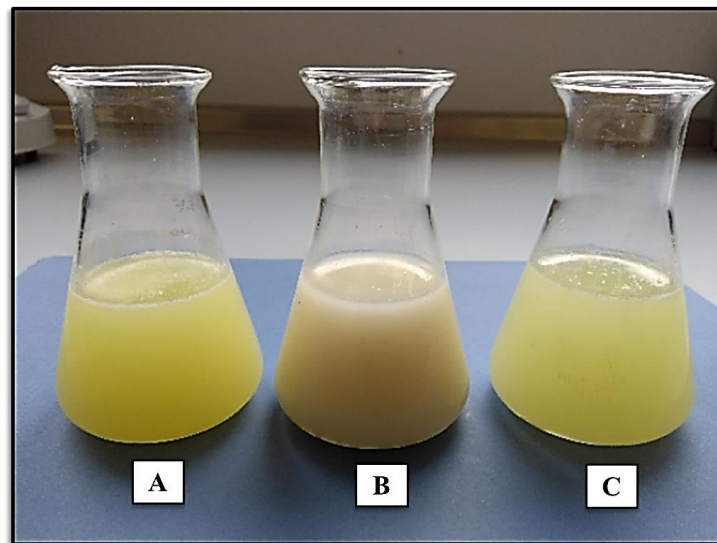


Figure 6.3. Sweet sheep whey tested using three UF membranes: A) normal whey, B) concentrated whey and C) diluted whey (4.4%, 6.15% and 1.5% of lactose, respectively).

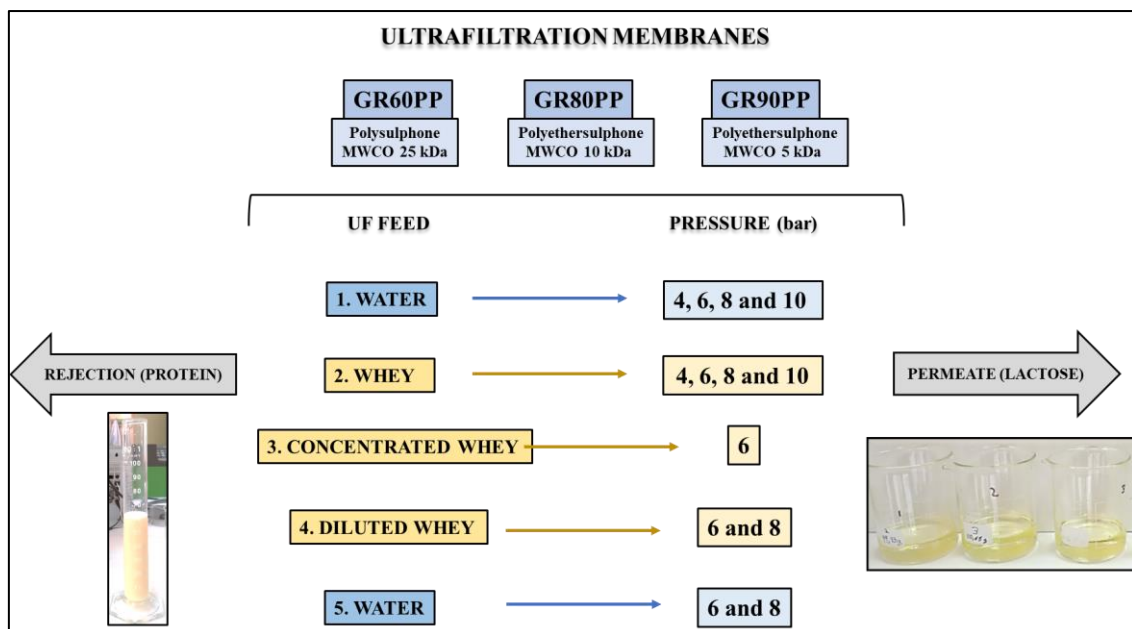


Figure 6.4. Flow diagram of the experimental design.

2.5. Membrane characterisation

For an initial characterisation of the membranes used in the study, the water permeability coefficients (A_w) were obtained. To determine of A_w , distilled water was used as feed and fluxes were measured at pressures from 4 to 10 bar for all ultrafiltration membranes tested.

The water flux (J_w) depends on the hydraulic pressure applied across the membrane, ΔP , according Eq. (1) (Bhattacharya and Ghosh, 2004).

$$J_w = A_w \cdot (\Delta P) \quad (1)$$

The water permeability coefficient was measured in the native membrane (previous to each experiment), as well as, at the end of all assays for each condition and membranes tested.

2.6. Analytical methods

Lactose content was measured by high-performance liquid chromatography (HPLC) with refractive index detector (RID) (VWR-Hitachi Elite LaChrom® HPLC system, USA) similarly than methodology proposed in chapter 2.

Protein quantification was measured using the colorimetric method Coomassie (Bradford) protein assay kit (Pierce Biotechnology, USA) based in the principle of protein-dye binding through the microplate protocol (Bradford, 1976) equally than in chapter 2. pH was measured using a digital pH-meter Crison MicropH 2001 (Crison, Germany).

3. THEORETICAL BACKGROUND

Membrane performance was measured in terms of membrane rejection coefficient, R (%), and permeate flux, J_p .

Lactose and protein rejection coefficients, R , reflect the membrane ability to separate protein and lactose in the feed solution, and it is given as a percentage:

$$R = \left(1 - \frac{c_p}{c_f}\right) \times 100 \quad (2)$$

where C_p and C_f are the lactose concentration in the permeate and feed streams, respectively (Chandrapala *et al.*, 2016, Galanakis *et al.*, 2014, Koros *et al.*, 1996). The permeate flux was calculated by measuring the quantity of permeate collected over a certain time period and dividing it by the membrane area used for filtration. Due to lactose passed through membrane and recovered into permeate feed, the lactose separation was defined as:

$$\text{Lactose separation (\%)} = 100 - R \quad (3)$$

The transport phenomena of ultrafiltration, nanofiltration and reverse osmosis membranes in the pressure-driven process can be described by irreversible thermodynamics. In general, the transport equations for the components through a membrane are two: the diffusion and the convection components. Although, modifications to solution-diffusion theory through the addition of a convection term to describe mass transfer through membrane defects, have been used to model solute flux through porous RO and NF membranes with some success (Wendler *et al.*, 2002). Another approach is based on the irreversible thermodynamic approach proposed by Onsager, Kedem–Katchalsky, and Spiegler, where the membrane is considered to be a “black box” and mass transfer is expressed in terms of driving forces and membrane characteristics are mostly left out (Nghiem *et al.*, 2004).

3.1. Spiegler-Kedem-Kachalsky model

Initially, the Spiegler-Kedem-Katchalsky (SKK) model was developed to be applied in reverse osmosis. However, several authors have used it successfully in nanofiltration processes, and it has been suggested, but not tested, that it could also be used in ultrafiltration technology (Bhattacharya and Ghosh, 2004). According to the SKK model, for a system involving a single solute in aqueous solution, solute retention can be described by three transport coefficients:

Specific hydraulic permeability, A_w

Local solute permeability, P_s

Reflection coefficient, σ

Permeability is the flux of a component (solvent or solute) through the membrane per unit of driving force (the operating pressure). The reflection coefficient is a measure of the degree of semi permeability of the membrane (Bhattacharya and Ghosh, 2004).

The Spiegler–Kedem–Kachalsky (SKK) model states that the fluxes of solute and solvent are directly related to the chemical potential differences between the two sides of the membrane. The chemical potential gradient is caused by a concentration or pressure gradient. Solvent transport is due to the pressure gradient across the membrane and solute transport is due to the concentration gradient and/or convective coupling of the volume flow (Bhattacharya and Ghosh, 2004).

The transport equation expressed by Spiegler–Kedem–Kachalsky model is as follows (Spiegler and Kedem, 1983, Katchalsky and Kedem, 1962, Pontie *et al.*, 2003):

For solvent:

$$J_w = A_w \cdot \left(\frac{dP}{dx} - \frac{d\Pi}{dx} \right) \quad (4)$$

For solute:

$$J_s = B_s \cdot \frac{dC_s}{dx} + (1 - \sigma) \cdot J_w \cdot C_s \quad (5)$$

Diffusion is represented by the first term in Eq. (5) and the second term of the same equation represents the contribution of convection to the transport.

The following assumptions are made:

- The Spiegler–Kedem–Katchalsky model adequately predicts the transport of solute and solvent, regardless the type of solute and its charge, solvent and membrane.
- The pressure and concentration gradients are the driving forces.
- The solute present in the system is semipermeable to the membrane.
- In the layer thickness polarization concentration, the solute has a value that is independent of the diffusion and mass transfer coefficients.
- A_w , P_s and σ are constants across the membranes, hence the equation for the integration of Eqs. (4) and (5) of the membrane can be simplified.

The simplified version of model transport equations can be written as (Murthy and Gupta, 1997):

$$J_w = A_w \cdot (\Delta P - \sigma \cdot \Delta \Pi) \quad (6)$$

$$J_s = B_s \cdot (C_m - C_p) + (1 - \sigma) \cdot J_w \cdot C_s \quad (7)$$

J_w and J_s are, respectively, the solvent flux and the solute flux; ΔP and $\Delta \Pi$ define, respectively, the pressure drop and osmotic pressure differences across the membrane; C_m and C_p are, respectively, the solute concentrations at the membrane surface and in the permeate; C_s is the logarithmic mean concentration of the solute between the feed and permeate; A_w is the permeability of pure water.

The observed rejections can be explained by SKK theory as follows:

$$R_{obs} = \frac{\sigma \cdot (1-F)}{1-(\sigma \cdot F)} \quad (8)$$

where R_{obs} is the observed rejection and F is a parameter that depends on the solvent flux, the rejection coefficient and solute permeability coefficient according to the expression:

$$F = e^{-\left(\frac{1-\sigma}{P_s} \cdot J_w\right)} \quad (9)$$

The Spiegler–Kedem–Katchalsky model proposes a relationship between the flux of solvent (J_w) and the logarithm of solute membrane parameters, taking into consideration the observed rejection (R_{obs}) and the reflection coefficient (σ) values in the following equation:

$$\ln \left[\left(\frac{1}{1-\sigma} - \frac{1}{1-R_{obs}} \right) \cdot \frac{(1-\sigma)}{\sigma} \right] = -\frac{(1-\sigma)}{P_s} \cdot J_w \quad (10)$$

From Eq. (7), and using the condition that $C_f = C_m$, the following expression can be obtained:

$$P_s = \frac{J_s - (1-\sigma) \cdot J_w \cdot C_s}{(C_m - C_p)} \quad (11)$$

Substituting the value of B_s given by Eq. (11) into Eq. (10) gives expression (12):

$$\ln \left[\left(\frac{1}{1-\sigma} - \frac{1}{1-R_{obs}} \right) \cdot \frac{(1-\sigma)}{\sigma} \right] = -\frac{(1-\sigma)}{a-b \cdot (1-\sigma)} \cdot J_v \quad (12)$$

where

$$a = \frac{J_s}{(C_m - C_p)}$$
$$b = \frac{J_w \cdot C_s}{(C_m - C_p)}$$

Using a numeric method, the values of the reflection coefficient (σ) that make Eq. (12) equal to zero were obtained. From this coefficient (σ), the solute permeability (P_s) coefficient can be calculated.

4. RESULTS

4.1. Membrane characterisation

Membrane characterisations were performed with distilled water. Figure 6.5 shows the water permeate fluxes for the three membrane assays as functions of operating pressure. It can be observed that, as expected, the water flux increased linearly with the operating pressure ($r^2=0.998$). The values of permeability coefficients depend primarily on chemical composition of membrane and secondary its molecular weight cut-off (MWCO). Table 6.2. shows the initial A_w coefficients for the different ultrafiltration membranes assays. As expected, the greatest differences in permeability coefficients were observed between the membranes with different compositions: GR60PP (polysulphone) *versus* GR80PP and GR90PP (polyethersulphone). For those with the same material (GR80PP and GR90PP) the differences were marked by MWCO, due the GR90PP is 5 kDa and GR80PP is 10 kDa.

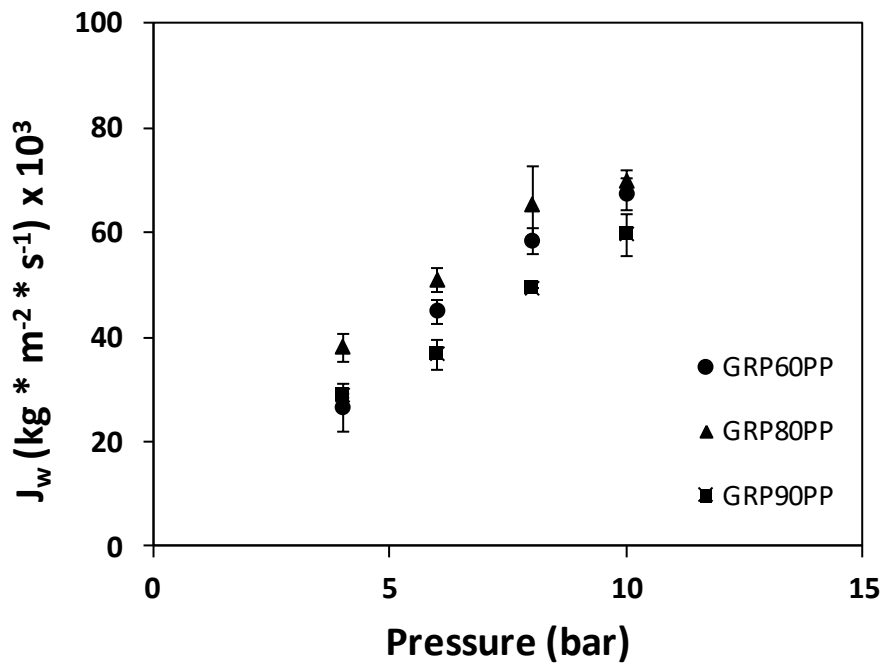


Figure 6.5. Water flux vs operating pressure. (●) GR60PP, (▲) GR80PP and (■) GR90PP.

Table 6.2. Water permeability coefficients (A_w) obtained at the beginning and end of experiments for the different membranes.

	Membranes		
	GR60PP	GR80PP	GR90PP
A_{wo} ($m s^{-1}$)	$6.69 \cdot 10^{-8}$	$5.38 \cdot 10^{-8}$	$5.06 \cdot 10^{-8}$
A_{wf} ($m s^{-1}$)	$5.71 \cdot 10^{-8}$	$4.29 \cdot 10^{-8}$	$4.83 \cdot 10^{-8}$
F_w (%)	14.75	20.18	4.52

A comparison was made between the initial and final experiments, and a fouling parameter, F_w , based on water permeability coefficients, was obtained as:

$$F_w = \frac{(A_w)_0 - (A_w)_f}{(A_w)_0} \times 100 \quad (13)$$

where subscript 0 is the initial value and subscript f is the final value (Hidalgo *et al.*, 2016).

Solvent permeability decreased for the three ultrafiltration membranes, presumably due to fouling phenomena on the membrane surface (Macedo *et al.*, 2011). The GR80PP membrane showed the highest fouling parameter, which could be due to interactions between membrane and whey solution, and concentration of polarization phenomena.

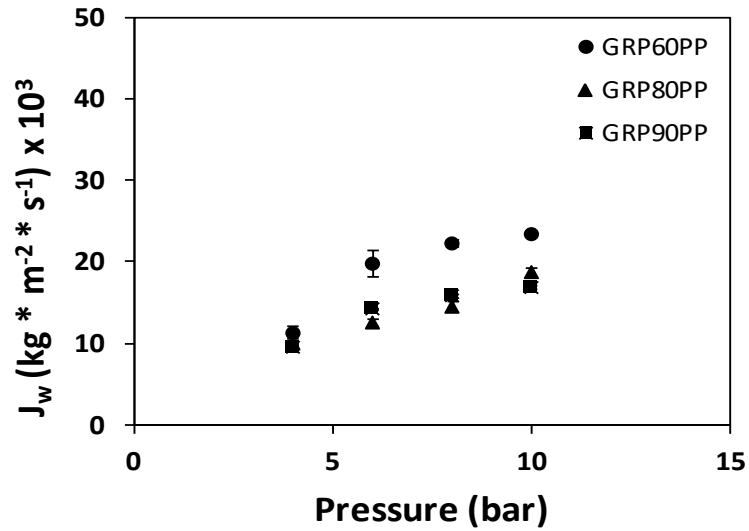
4.2. Influence of operating pressure in the ovine whey treatment and lactose separation

The effect of pressure on the lactose separation in ovine whey was also performed in the three membranes, whose results can be observed in figure 6.6 (A).

As expected, a higher pressure induced a higher permeate flux, with values obtained for GR60PP being higher than for GR80PP and GR90PP. Two membranes made with polyethersulphone showed similar trend between them. The higher permeate flux of the GR60PP membrane may also be due to the chemical composition and the molecular weight cut-off. When the effect of operating pressure on lactose separation was studied, no statistically significant differences were found in the case of GR60PP and GR90PP, which gave 85 and 80% lactose separation from the whole whey, respectively, at all the pressures tested. However, GR80PP decreased the lactose separation capacity when higher pressures were assessed (figure 6.6 (B)). It is important to mention that neither of the membranes nor different pressures tested affected protein

separation, since this macronutrient was absent in all whey permeate. As a result, protein recovery was 100%.

(A)



(B)

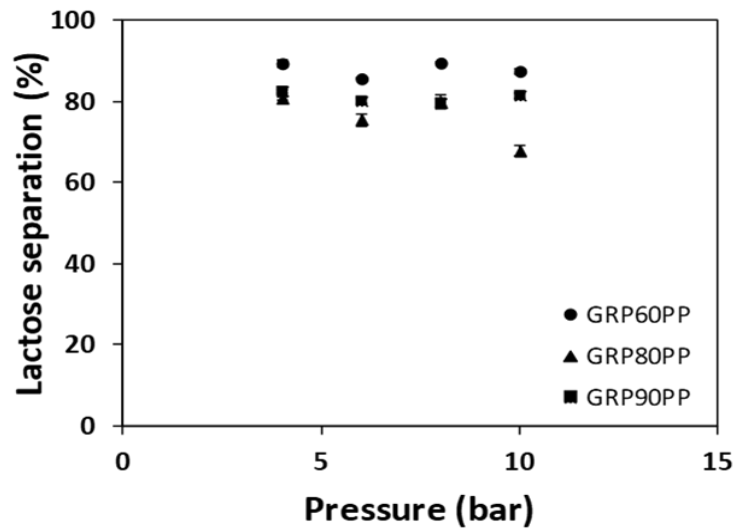
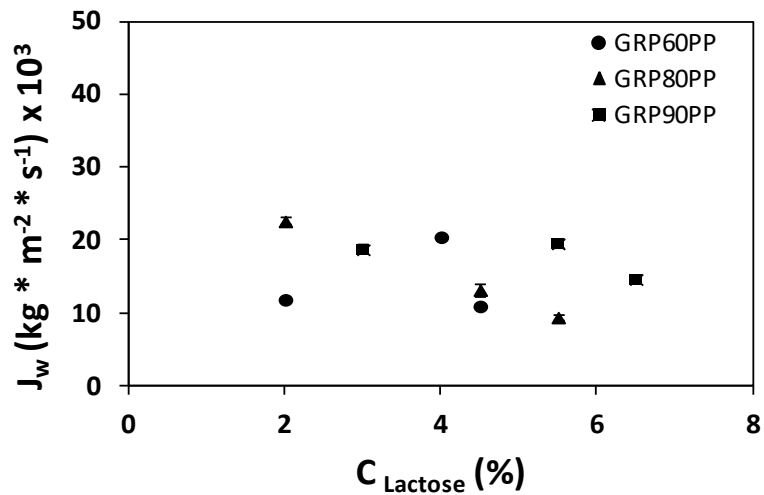


Figure 6.6. Variation in permeate flux (A) and lactose separation coefficient (B) with pressure for: Lactose concentration (3.5 ± 0.05 %) and pressure (4 to 10 bar). GR60PP membrane (●), GR80PP membrane (▲) and GR90PP membrane (■).

4.3. Influence of lactose concentration in the whey protein separation treatment

During the period of ultrafiltration, the concentration of lactose and proteins increased, changing the environment of liquid to be filtrated. The effect of whey concentration on the permeate flux for the different UF membranes is shown in figure 6.7 (A).

(A)



(B)

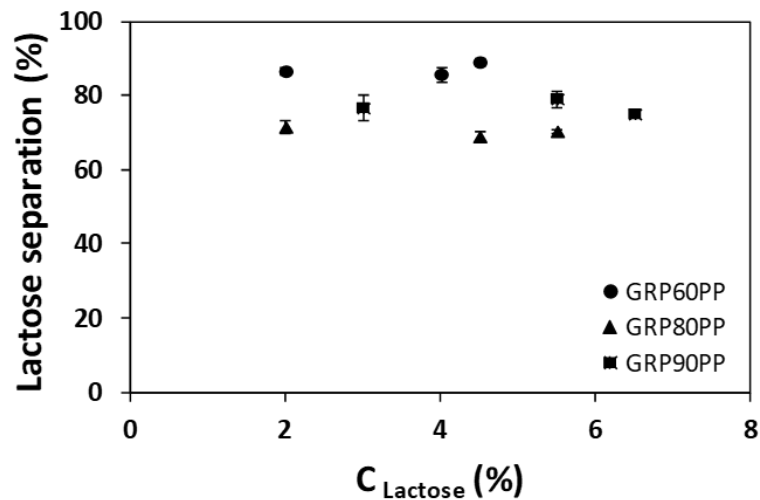


Figure 6.7. Variation in permeate flux (A) and lactose separation coefficient (B) with lactose concentration for pressure = 6 ± 0.5 bar. Range of whey concentration between 1.5 to 6.15 % of lactose. GR60PP membrane (●), GR80PP membrane (▲) and GR90PP membrane (■).

As it can be observed, in general, the permeate flux decreased when the lactose concentration increased, although different membranes showed significant variations.

No statistically significant effect of the lactose concentration in lactose separation into the permeate feed was observed. Due to lactose separation remaining constant independently of the concentration in the feed solution, around 80% of lactose was removed (figure 6.7 (B)).

4.4. Fitting the Spiegler–Kedem–Katchalsky model

For each experiment using different experimental conditions and different compounds, the reflection coefficient (σ) and the solute permeability coefficient (P_s) were obtained. Average values were calculated as the mathematical mean using (SPSSv.19) statistical program. The average values of the reflection coefficient (σ) and the solute permeability coefficient (P_s) obtained for the different membranes are shown in table 6.3.

Table 6.3. Model constants for the three membranes studied.

	Membranes		
	GR60PP	GR80PP	GR90PP
σ	0.271	0.811	0.762
P_s	$2.72 \cdot 10^{-2}$	$2.98 \cdot 10^{-2}$	$3.20 \cdot 10^{-2}$
r^2	0.9418	0.9743	0.9954

Figure 6.8 shows the theoretical and experimental rejection coefficients from different membranes. As it can be observed, real and hypothetical values are quite similar and fit perfectly to the diagonal.

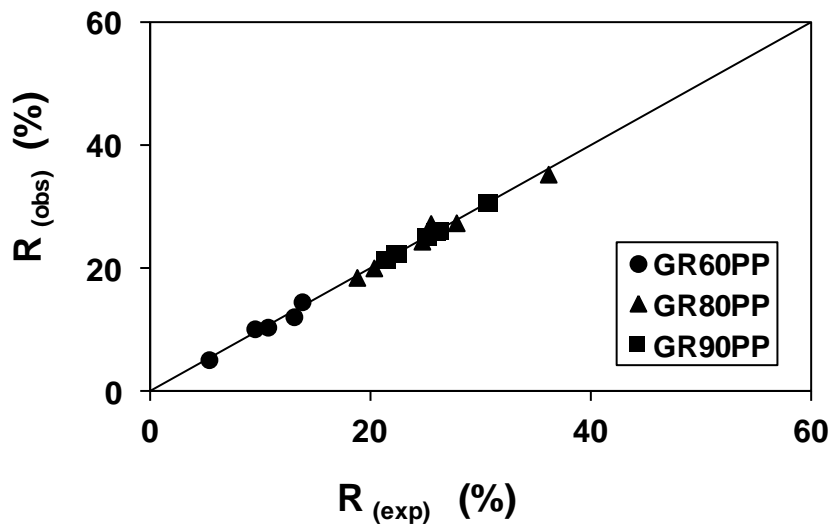


Figure 6.8. Experimental and model retention coefficient for GR60PP membrane (●), GR80PP membrane (▲) and GR90PP membrane (■).

5. DISCUSSION

5.1. Membrane characterisation

It could be observed, as it was expected, how the water flux increased linearly with the operating pressure ($r^2=0.998$). Solvent permeability decreased for the three ultrafiltration membranes, presumably due to fouling phenomena on the membrane surface (Macedo *et al.*, 2011). The GR80PP membrane suffered most fouling-despite its intermediate MWCO value.

5.2. Influence of operating pressure in the ovine whey treatment and lactose separation

The permeate flux increases with pressure for three membranes, which means that the fouling or polarization effects were less intense than it could be expected (Van der Bruggen *et al.*, 1999, Galanakis *et al.*, 2014). It is noteworthy that the whey-permeate flux was smaller than the water flux, suggesting that the concentration-polarization effect was very significant (especially in the case of GR80PP and GR90PP) for whey at the initial concentration and that this effect increased with operating pressure.

When the whey and water-permeate fluxes were compared for the same operating conditions, a statistically significant difference ($p < 0.05$) was observed, being lower in the case of whey-permeate flux. Similar results were found in the literature, where the whey permeate flux was lower than the water flux at all pressures (Rektor and Vatai, 2004, Atra *et al.*, 2005, Butylina *et al.*, 2006, Baldasso *et al.*, 2011, Macedo *et al.*, 2011, Galanakis *et al.*, 2014). Possible causes for these different fluxes could be due to the presence of concentration of polarization induced by polar molecules like sugars (Galanakis *et al.*, 2014).

All these results were better than those described in the literature by Baldasso *et al.*, (2011) and Sluková *et al.*, (2016). The first authors used a UF-6001 membrane made of polyethersulphone with an MWCO of 10kDa, obtaining, with the best strategy used, a protein concentrate of around 70%. Meanwhile, Sluková *et al.*, (2016) used a tubular ceramic ultrafiltration membrane of 50 kDa, obtaining a high recovery of protein (81%) and good reduction of lactose during the UF step. The same authors obtained a 37% level of lactose remaining in the rejected fraction in a single-step, which was reduced to 14% by diafiltration. Similar results were obtained by Galanakis *et al.*, (2014). when using the scenery combined process (20 kDa-polysulphone, GR70PP, and 2 kDa-polyethersulphone, GR95PP, membranes) (Galanakis *et al.*, 2014). Their recovery of proteins (87-90%) and the rejection of non-reducing sugars (39-32%) were quite lower than the obtained in the present study. In this research, when the

GR60PP membrane was used at a similar pressure, only 13% lactose remained in the protein after a single-step.

5.3. Influence of lactose concentration in the whey protein separation treatment

The decrease with GR90PP was directly proportional, while the GR80PP flux was stable and only decreased at higher concentration of lactose. In the case of polysulphone membrane, a different trend was observed, the lowest flux values being obtained with the highest lactose percentage. Those variations could be explained by the sum of two effects: the increase in solute flux due to the increase in feed concentration and the decrease in water flux as a consequence of the increase of $\Delta\Pi$.

Similar results have been obtained by researchers working with other types of membranes (Baldasso *et al.*, 2011, Atra *et al.*, 2005, Smit, 2003, Galanakis *et al.*, 2014). In addition, other authors concluded that the higher protein concentration, the lower permeate flux, mainly due to the higher osmotic pressure and the greater accumulation of solute molecules in the polarized layer, increasing its thickness and, consequently its resistance to permeation (Rektor and Vatai, 2004, Bacchin *et al.*, 2006, Cheryan, 1986).

Several authors have investigated the separation of whey components using ultrafiltration in association with discontinuous diafiltration to concentrate and to purify the whey proteins (Baldasso *et al.*, 2011, Slukova *et al.*, 2016). However, none of them took into account the effect of sugar concentration on the separation of proteins and sugars (mainly lactose). In this study, it was noticed an increase in the lactose concentration in the feed liquid, therefore, the effect of this increase was assayed on protein rejection.

When the lactose feed concentration increased, the permeate concentration also increased in the case of GR60PP membrane hence, there was no significant change in lactose rejection. With the GR90PP membrane, no changes were found, while GR80PP showed a slightly decrease in permeate concentration. These results agreed with

Galanakis *et al.*, (2014) when using GR70PP (polysulphone) and GR95PP (polyethersulphone).

5.4. Fitting the Spiegler–Kedem–Katchalsky model

According to the bibliography, Cuartas-Ubire *et al.*, (2010) applied the Kedem-Spiegler model to predict lactose rejection using a nanofiltration membrane (Desal 5 DL) and obtained a high reflection coefficient, and low solute permeability coefficient (Cuartas-Urube *et al.*, 2010). The different results obtained are explained by the different chemical composition of membrane and different MWCO.

Taking into account all the results for the different membranes tested, the final correlation coefficient between observed and predicted values (with SKK model) of $r^2=0.9892$ was obtained. Although this model is usually used in nanofiltration, it also fitted perfectly in the present study, and it can be considered a very interesting approximation for predicting different coefficients in the separation of protein and lactose from sweet sheep whey using ultrafiltration membranes at least in the studied range of pressure and lactose feed concentrations.

Regarding the solute permeability coefficient (P_s), an inversely proportional relation was found with the molecular weight cut-off of the membrane tested - the higher the MWCO, the lower the P_s .

6. CONCLUSIONS

The results using three specific membranes (GR60PP, GR80PP and GR90PP), different pressure conditions and concentrations of feed solution suggest that the ultrafiltration of sheep whey could be a suitable alternative for its problematic separation, contributing to the little knowledge that exists on the sheep whey.

Taking the results into account, the main conclusions of this work were that the range of pressure used had no significant effect on lactose separation in the case of GR60PP and GR90PP. However, GR80PP membrane showed a decreased permeation pattern when higher pressures were assessed. Furthermore, increasing the volume and concentration of the feed phase did not affect lactose permeation, which remained constant during the UF process. The separation of protein was not affected by the type of membrane tested or range of operating pressures assessed, with 100% recovery being obtained in all cases.

The UF process itself led to a reduction of permeate flux when lactose concentration increased, the GR80PP membrane being the most affected in this respect. Cleaning the membranes with water after the UF assays restored initial conditions, and the same membrane could be used several times.

Finally, the Spiegler–Kedem–Katchalsky model (applied to different membranes) can be used to predict, accurately ($r^2=0.9892$), the effect of different experimental conditions on the separation of lactose and protein from sheep whey using UF. This model represents a suitable tool for predicting the permeation pattern for a given membrane and sheep whey and it illustrates the effect of different process conditions. In this way, the by-product can be optimized for use as functional food.

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*In vivo satiating potential and anti-obesogenic effects
of Mediterranean foods: goat whey, egg white, tiger
nut and nopal. CHAPTER 7*



1. INTRODUCTION

The prevalence of obesity and overweight is increasing dramatically in recent years. If the trend persists, the absolute number of obese could rise to a total of 1.12 billion, reaching 20% of the world's adult population in 2030 year (Kelly *et al.*, 2008). Furthermore, obesity is related with a wide range of comorbidities due to its association with an increased risk of type 2 diabetes, cardiovascular diseases, certain cancers and a shorter life expectancy (Turnbaugh *et al.*, 2009). Although obesity is due to a multifactorial factor, an imbalance between calories ingested and expended for an extended period, together with a sedentary lifestyle, are the major causes (Agnoli *et al.*, 2018). Regulation of food intake and the promotion of a healthy life style shape the key to mitigate this epidemic. Moreover, the adherence to a healthy diet, together with the inclusion of ingredients capable to enhance satiety into the diet is the two key strategies to prevent and treat overweight and obesity.

Regarding with the first point of the equation, an example quite representative of healthy diet is the Mediterranean diet. Several studies have proved the effect of this diet on body weight reduction, especially when it is energy-restricted and associated with physical exercise (Esposito *et al.*, 2011). Recently, Agnoli *et al.*, (2018) have showed that the adherence to a Mediterranean diet was related to a reduced risk to develop overweight and obesity, being associated to reduced body weight and waist circumference (Agnoli *et al.*, 2018). Furthermore, a systematic review of intervention trials highlighted the potential of this diet to reduce obesity and related chronic diseases risk (Bendall *et al.*, 2018).

A traditional Mediterranean diet should include the use of olive oil, a high consumption of fruits and vegetables, bread and cereals, pulses and nuts being preferable little processed. Furthermore, yogurt and cheese consumption are abundant in this type-diet, especially from Mediterranean species such as goat and sheep. In addition, whole egg has been included in this type of diet few days per week (Serra-

Majem *et al.*, 2004). Some of these Mediterranean ingredients have an especial interest in terms of satiety and body weight regulation because its macronutrient composition.

Satiety and satiation are two key concepts involved in food intake regulation (Cummings and Overduin, 2007). Satiety and satiation intensity and duration may vary according the potential of the physiological signals generated by the action of food and the nature of food *per se* (Blundell *et al.*, 1987). Nevertheless, very complex mechanisms are involved in the control and regulation of food intake, energy homeostasis and metabolism.

Many researches have established the potential of proteins reducing food intake and promoting the body weight loss and body weight maintenance. Certainly, comparing the satiating effect of macronutrients, proteins have shown the most effectiveness (Lejeune *et al.*, 2006, Veldhorst *et al.*, 2008, Smeets *et al.*, 2008, Westerterp-Plantenga *et al.*, 2009). Satiating effects and body-weight regulation of proteins can be explained through a multifactorial approach such as by stimulating gut hormone, digestive effects, postprandial amino acids levels, by enhancing thermogenesis and energy expenditure (EE), etcetera (Drummen *et al.*, 2018).

Particularly, most of researches have been focused in the role of whey proteins as a satiety ingredient. The effect of whey on satiety has been attributed to its protein fraction, beta-lactoglobulin (β -LG), alpha-lactalbumin (α -LA) and glycomacropeptide (GMP), among other proteins. Furthermore, its biopeptides, amino acids derived from gastrointestinal digestion, as well as calcium, could be involved in satiety (Veldhorst *et al.*, 2009, Garcia-Lorda *et al.*, 2005, Madureira *et al.*, 2007).

Other source of proteins of excellent quality are the derived from egg white, specifically, ovalbumin, ovotransferrin, ovomucoid, lysozyme and ovomucin (Chang *et al.*, 2018). Many biological effects have been attributed to egg protein because the physiological effect of its protein fraction and biopeptides (antihypertensive, antimicrobial, immunomodulatory, antilipidemic, etcetera) (Jahandideh *et al.*, 2016, Mine, 2007, Matsuoka *et al.*, 2014). The effect of egg protein in increasing satiety and promoting weight loss has been previously demonstrated (Vander *et al.*, 2005, Vander

Wal *et al.*, 2008, Semon *et al.*, 1987). However, contradictory results have been found when several sources of proteins have been compared to egg. For instance, Lang *et al.*, (1998) showed that none difference on satiety was found comparing egg albumin, casein, gelatin or soy protein (Lang *et al.*, 1998). Nevertheless, several researches have indicated opposite results (Anderson *et al.*, 2004, Semon *et al.*, 1987, Pal and Ellis, 2010).

Another nutrient with satiety properties could be fibres, which are a wide group of carbohydrates formed by soluble or insoluble and fermentable or non-fermentable fibre (Burton-Freeman, 2000). In general terms, dietary fibre consists of dietary plant-based non-degraded under the digestion process and being fermented into large intestine. Dietary fibre includes soluble fibre as inulin, mucilage, gum, resistant starch, pectin, etcetera; and insoluble fibre as cellulose, hemicellulose and lignin. Dietary fibre has been responsible to produce enhances of volume, bulking, water-holding capacity, viscosity and fermentability, obtaining relevance its role as prebiotics (Burton-Freeman, 2000, Lockyer and Stanner, 2019). Fibre could promote satiety and satiation since its specific features, for instance, its viscosity confers satiety and its effect on gastric bulking produces satiation. Furthermore, the chewing of foods rich in fibres results in an increased production of saliva that enhances the stomach distension inducing satiety. In addition, fibres may reduce or delay the absorption of other nutrient through the intestinal digestion which led to these macronutrients trigger their satiety signals in a longer time (Slavin, 2010). Furthermore, in the intestinal lumen, dietary fibre has the capacity to bind dietary fat in a complex avoiding fat absorption, so it facilitate fat excretion in faecal material (Uebelhack *et al.*, 2014). In large intestine, gut bacteria utilize these components producing SCFAs, and other metabolites that could stimulate the production of satiety-inducing hormones such as GLP-1 and PYY.

These findings have been considered as the starting point of the present chapter. Most researches have focused on the role of cow whey proteins and limited publications have published the particular effect of different whey sources by using *in vivo* experiments. Then, it was imperative to evaluate the satiating effectiveness and the role on obesity of types of whey different to cow. With this purpose, goat whey has been selected in the present experiment to determine its effect on satiety using an obese mice

model. Furthermore, other ingredients usually consumed in the Mediterranean diet have been selected, too. Egg white protein has been selected, apart from the wide literature that endorses its satiating effect, because previous experiments (chapter 4) demonstrated its effect of CCK secretion. Regarding to the well-known effect of fibre on satiety, two Mediterranean ingredients based on fibre have been included in the present manuscript, tiger nut and nopal. The main effects on obesity and satiety of these derived-plant ingredients have been widely described in the general introduction (chapter 1).

This study has the main objective of evaluating the satiating and anti-obesogenic effect of several ingredients from Mediterranean regions: goat whey protein, egg white protein, tiger nut and nopal. The experiment has been conducted by using obese C57BL/6J mice, fed along 11 weeks with different diets based on those ingredients. Particularly, we designed two high-protein diets, based on goat whey or egg protein, and two high-fibre diets formulated with tiger nut and nopal. Several satiety markers as feeding pattern, cumulative food and energy intake and circulating satiating peptides have been determined to know deeply the effectiveness suppressing hunger of each ingredient. In addition, we investigated whether the consumption of selected ingredients could revert the “obese phenotype” previously induced to animals. With this purpose, several parameters related to energy homeostasis and obesity have been analysed too, such as energy expenditure, blood lipids, organs morphology and body-weight composition.

With the purpose of providing sufficient and complete relevant information used in this study, the “Animals in Research: Reporting *In Vivo* Experiment” (ARRIVE) guidelines was followed, as well as provided a concise description about the present animal research (Kilkenny *et al.*, 2010). The 20 items of the ARRIVE’s checklist have been considered to elaborate the present manuscript, from the title (item 1) until funding (item 20).

2. MATERIAL AND METHODS

2.1 Ethical considerations

The experiment design was carried out in accordance with the directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and approved by the Animal Experimentation Ethics Committee of University of Murcia, Spain (CEEA code number: 238/2016 and 458/2018) (Annex II).

2.2 Animals

Forty-four male C57BL/6J mice, at 7 weeks of age and average weight of 25.85 ± 1.48 g, were used. They were provided by the animal facility of University of Murcia (purchased in Envigo, Spain). C57BL/6J mice are a strain commonly used as a model of diet-induced obesity (DIO) in research of non-leptin deficient obesity. This strain, when is fed with *ad libitum* high-fat diet, shows similar comorbidities to human metabolic syndrome (obesity, hyperinsulinemia and insulin resistance, hyperglycaemia, hypertension and hyperphagia) (Collins *et al.*, 2004).

2.3 Sample size

The number of animals used in this study was 44. After a randomized allocation of mice in collective cages, 6 experimental units were formed, corresponding to 5 groups (n=8) and 1 control group (n=4). The estimation of sample size was based on the outcomes derived from pilot experiment as well as based on several publications consisting in a similar experimental designs and topics (Pilvi *et al.*, 2008, Schellekens *et al.*, 2014).

2.4 Experimental design

Mice were randomly housed in 6 groups (5 of them with 8 mice per collective cage) and 1 group of 4 animals. This randomized distribution of the animals was kept until the end of the study. The experiment had a total duration of 11 weeks (Figure 7.1).

The whole study was divided into three steps: a phase of adaptation (10 days≈1.4 weeks) was necessary to acclimate the animals to the new living conditions and avoiding the slightest stress. Afterwards, and for 4 weeks, five groups (n=8) were fattened receiving a high-calorie diet, leading to obesity by induced-diet (DIO). The optimum weight used to consider DIO was achieved when animals were 13-week-old with a mean weight of 40 mice of 36.65 ± 0.84 g.

Following the DIO stage, the experimental phase was performed. It consisted in four groups (n=8) of obese mice were fed with the experimental diets for 11 weeks (Figure 7.1). The experimental diets were design with Mediterranean foodstuff, typically consumed in the South and Southeast Spain. These diets were based on proteins such as whey (W) (n=8) and egg (E) (n=8) diet, and diet based on high fibre content such as diets formulated using tiger nut (TN) (n=8) and nopal (N) (n=8). As well as, a control (submitted to DIO) fed with an isoenergetic diet were performed, named internal control (IC) (n=8). In parallel, a non-obese group, (external control (EC) (n=4)) were maintained during all time with standard diet in order to know the normal physiology and metabolic parameters of an adult mouse.

During the experimental period, as well as adaptation and DIO stage, several measurements were carried out, such as food and water intake determinations, body weight measurement, behaviour recording, blood extraction for biochemical and hormone quantification, metabolic cages stay, indirect calorimetry assay and computerised tomography visualisation (CT) (Figure 7.2). At the final of the study, mice were slaughtered by over anesthetization of gaseous isoflurane, and tissues and organs of interest were extracted. (Isoflurine 1000 mg/Kg. Fatro. Spain). Blood was collected in LH/Li heparinized tubes of 1 mL (Aquisel. Spain) and plasma was separated by centrifugation 4.000 g for 15 min at 4 °C (Eppendorf centrifuge 5804R. Spain) and frozen at -80 °C.

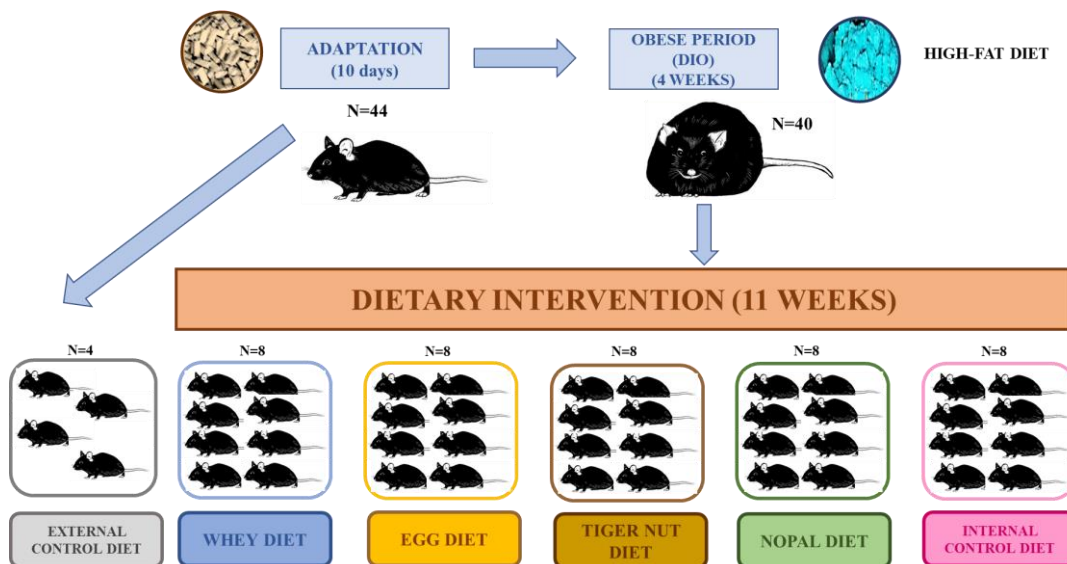


Figure 7.1. General experimental design abstract of the experimental design used in the present study.

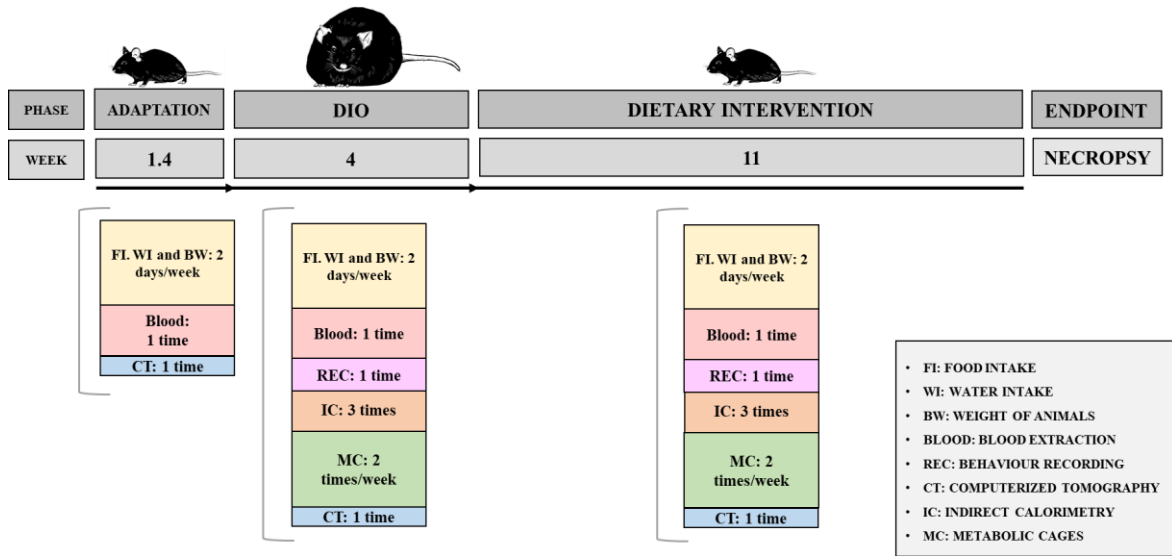


Figure 7.2. Time line and interventions performed during the several phases of experiment.

2.5 Housing and husbandry

Mice were maintained in top-filtered plastic cages allocated in ventilated racks (Tecniplast Touch SLIM Plus, Italy). Cages were continuously ventilated (75-times/min) by an entry-exit air system. The air was filtered by a high efficiency particulate air (HEPA). The temperature and relative humidity of the room were controlled being 23 ± 1 °C and 65-67%, respectively. Light-dark cycle was controlled, corresponding 12 hours of light and 12 hours of darkness. Water and food were available *ad libitum* throughout the study. Cages were changed twice a week and the fibre of the bedding material (Lignocel, Germany) was always saved in a small amount. With this regard, mice had environmental enrichment, consisting in thick plastic tubes, to avoid boredom and fights between animals.

2.6 Diets

Mice were fed with a standard pelletized mice diet *ad libitum* (Teklad Global 14% Protein Rodent Maintenance Diet. Envigo. Spain) during the adaptation period to all mice, as well as in the experimental phase to external control (EC). The nutritional composition is presented in table 7.1. During the DIO phase, mice were fed with a pelletized high-fat diet providing 60% of energy from fat *ad libitum* (Teklad Custom Diet TD.06414 60/fat. Envigo. USA).

The formulation of experimental diets was performed as isocaloric ones and their composition could be observed in table 7.2. The general appearance of each type of experimental diet can be seen in figure 7.3.

Table 7.1. Composition (% w/w) of mice standard diet for adaptation period and external control diet, as well as high-fat diet¹.

Diet	14% Standard diet	High-fat
Protein (%)	14.3	23.5
Carbohydrate (%)	48.0	27.3
Fat (%)	4.0	34.3
Energy density (Kcal/g)	2.9	5.1
Protein (En% Kcal)	20	18.3
Carbohydrate (En% Kcal)	67	21.4
Fat (En% kcal)	13	60.3

¹En%: percentage of energy estimated as metabolizable energy. It was calculated based on Atwater factors corresponding to 4 Kcal/g for proteins and carbohydrates and 9 Kcal/g for fat content.

Table 7.2. Composition (% w/w) of experimental diets used in the experiment¹.

Diet	W	E	TN	N	IC
Protein (%)	37.5	36.1	17.9	19.2	14.8
Carbohydrate (%)	46.9	47.4	43.6	47.6	49.9
Fat (%)	4.4	3.1	13.3	9.1	11.9
Energy density (Kcal/g)	3.7	3.6	3.6	3.5	3.6
Protein (<i>En%</i> Kcal)	39.6	39.8	19.6	21.9	16.1
Carbohydrate (<i>En%</i> Kcal)	49.6	52.3	47.6	54.4	54.5
Fat (<i>En%</i> Kcal)	10.6	7.7	32.7	23.6	29.2

¹ W: whey diet; E: egg diet; TN: tiger nut diet; N: nopal diet; IC: internal control diet. *En%*: percentage of energy estimated as metabolizable energy. It was calculated based on Atwater factors corresponding to 4 Kcal/g for proteins and carbohydrates and 9 Kcal/g for fat content.

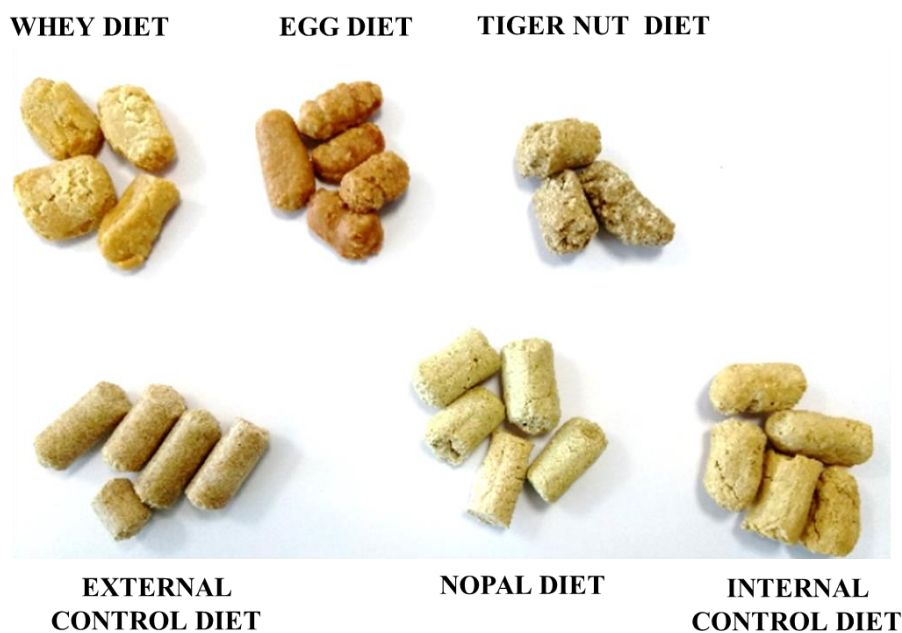


Figure 7.3. Appearance of the mice experimental diets (W, E, TN and N), EC and IC.

The percentages of total energy of each macronutrient (protein, carbohydrates and fat) in experimental diets are shown in figure 7.4.

All experimental diets were formulated using commercial diets as a basis (Envigo, Spain). As well as, the different diets were made manually in the laboratory.

The elaboration of the experimental diets followed the next steps:

- a) Crushing of the pelletized basis diet using a laboratory mill (Robot coupe R. France).
- b) Mixing and kneading the studied ingredients and the powdered basis diet using distilled water.
- c) Pelletizing manually the mixture and drying in a forced convection oven (50 °C) (Binder, Germany).

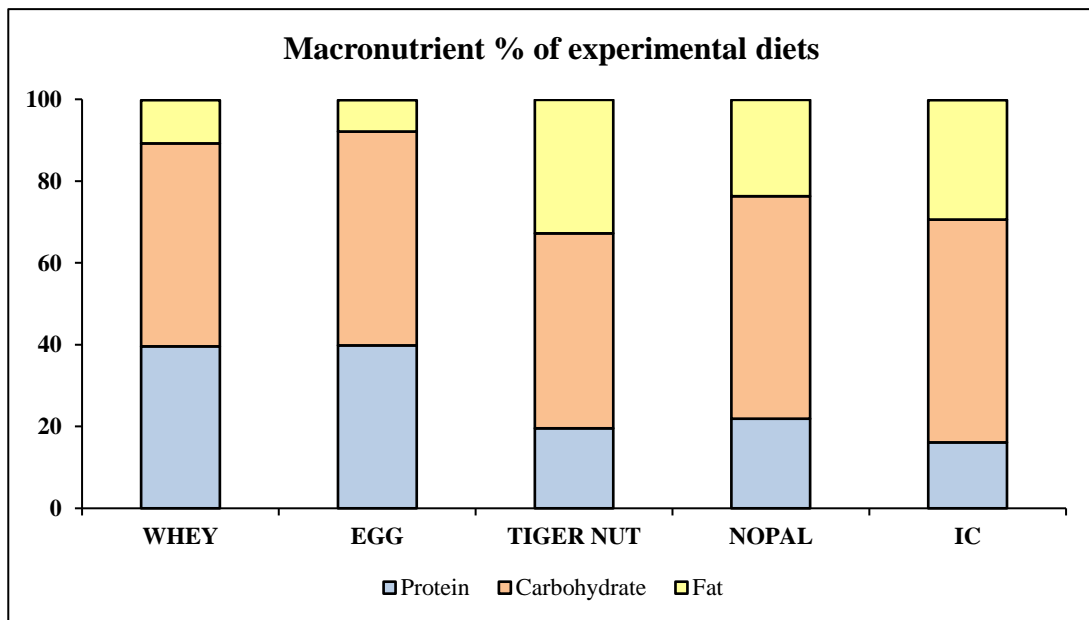


Figure 7.4. Percentage (%) of macronutrient composition (protein, carbohydrates and fats) in total calories of experimental diets (W: whey, E: egg, TN: tiger nut, N: nopal) and internal control (IC).

All diets were manufactured as pellet with similar size and hardness. The formulation and elaboration of the diets were indicated below:

- a)** High-protein diets: Whey (W) and egg (E) diets. These diets were elaborated using as basis a commercial diet completely free of protein (Teklad Custom Diet TD.93328 protein free diet. Envigo, Barcelona, Spain). Afterward, goat whey protein concentrate (Swanson. USA) containing 75% of protein or powdered egg white protein (Advanced egg protein. Ovofull. Huevos Inmaculada Fitness. Spain) (84% protein) were incorporated until obtaining diets with approximately 40% of metabolizable energy from protein. Accordingly, macronutrient of the diet was adjusted to provide isocaloric energy respect to other diets.

- b)** Adequate protein/high-fibre and high-fat diet: Tiger nut diet (TN). Tiger nut (Regulating Council of the Denomination of Origin Valencia. Spain) was mixed (40% w/w) with a standard mice pellet (Envigo.Spain). Macronutrients of the diet were adjusted to provide the same energy density that the other experimental diets (3.6 Kcal/g). Final dietary fibre content of the diet corresponded to 12% w/w.

- c)** Adequate protein/high-fibre diet: Nopal cactus powder (SaludViva Superalimentos. Spain) was mixed (25% w/w) with a standard mice chow diet (Envigo.Spain). As in the case of TN diets, macronutrients of the diet were adequately adjusted to provide the same energy density that the other experimental diets. Final dietary fibre content of the diet corresponded to 16% w/w.

- d)** Internal control diet (IC). The control diet was elaborated using as a basis standard mice chow diet (Teklad Global 18% Protein Rodent Diet. Envigo. Spain).

2.7 Food intake, water intake and body weight measurements

Through all stages of experiment (adaptation, DIO and dietary intervention) water and food intake and body weight (BW) were monitored each 2 days. Food intake (g/d) were calculated subtracting the weight of the previous measured to the new measured weight and expressed per animal. In addition, cumulative water and food intake for 11-weeks (mL/d and g/d, respectively) and daily caloric intake (Kcal/d) were calculated. Furthermore, animals were weighed every 2 days, between 10:00 and 11:00 a.m., using a precision weight scale (0.1 g) (Highland Adam 600 x 0.01 g, UK).

2.8 Food pattern evaluation

Meal pattern of four animals per group was monitored by video recording in the dark phase for 12 hours, from 08:00 p.m. until 08:00 a.m, similarly to other authors have previously described (Bensaid *et al.*, 2002). The methodology for the recording included as follows (Figure 7.5):

- i.** Two night-vision lamps with infrared led (Clover electronics IR045. Korea).
- ii.** Three methacrylate diffuser screens. The diffusion of the light was needed to optimise the night-vision recording.
- iii.** Electronic equipment: a webcam for laptop with a sensor resolution of 640 x 480 pixels (Trust. China); a webcam motion detector software v 2.0. to record only when the mice is on the trough area. Recording was monitored in a non-stop way for 60 seconds. Additionally, a laptop (HP ENVY laptop-17t. USA) and a hard-disk to store videos (2.5" USB HDD25BL13 3GO. Spain) were used. The lens of the webcam was adapted to allow the night-vision under infrared light.

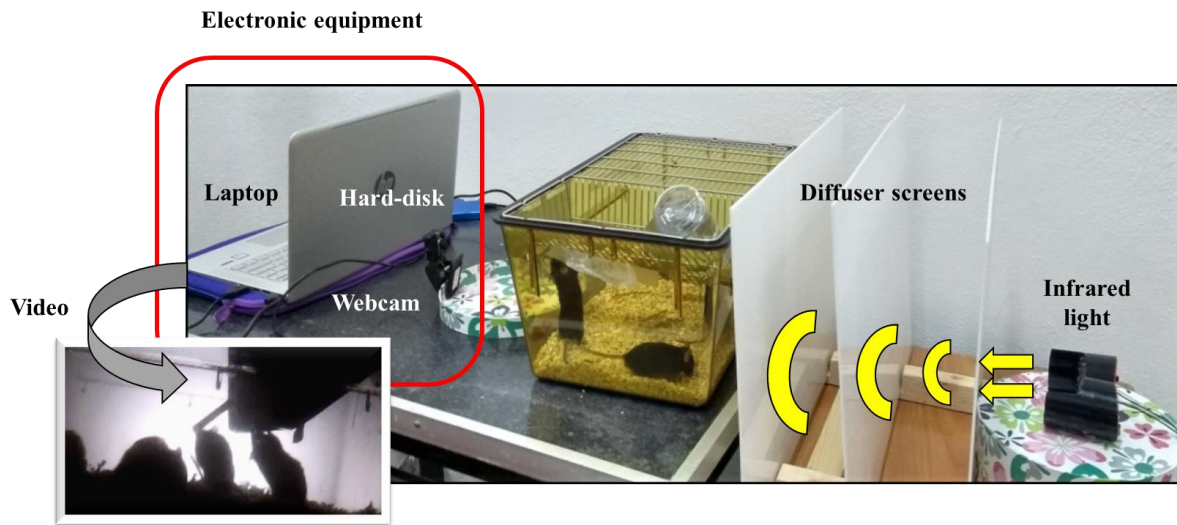


Figure 7.5. Food pattern evaluation by nocturnal recording.

Under behaviour recordings, mice had free access to food and water, environment enrichment, etc. Feeding behaviour was monitored such as number of times they ate (number of meals); meal duration (min) calculated as the time from mice started to eat until the end of each meal and inter-meal interval duration (min) (IMI). Meal quantification has been carried out by considering that a meal ought to be characterised by at least 13 seconds of ingestion (Ayaso *et al.*, 2014). To consider a new meal a minimum time of 5 minutes should be lapsed from the prior meal. These specific time has been previously considered as adequate to quantify the nighttime feeding pattern (Castonguay *et al.*, 1986).

2.9 Metabolic measurements: Indirect calorimetry

Respiratory quotient (RQ) was calculated by indirect calorimetry. It was obtained based on the amount of oxygen consumed and carbon dioxide produced. This quotient was monitored individually under non-sleepiness arousal period. Measurement of RQ was assayed in triplicate for each group in every phase of experiment (DIO and dietary intervention period). Animals were allocated in specially designed plastic cages at room temperature (23° C) where oxygen consumption and carbon dioxide expiration

were analysed continuously, every 3 min during 1 h. Before starting the measurements, the gas analyser was calibrated, and mice were weighted and acclimated for 30 min. The O₂ and CO₂ analyser used was CheckPoint II (PBI Dansensor Fresh Thinking. Spain) having a measurement range of 0-100%, a resolution of 0.1% for O₂ and CO₂ and a measurement time of 15 seconds.

RQ was calculated as the quotient VCO₂/VO₂. Caloric value (Kcal/LO₂) (CV) and EE (Kcal/h) was calculated following the next equation (Weir, 1990, Lusk, 1928):

$$CV \text{ (Kcal/LO}_2\text{)} = 3.815 + (1.232 \times RQ), \text{ and}$$

$$EE \text{ (Kcal/h)} = CV \times VO_2 \text{ (L/h)}$$

EE and VO₂ measures were normalised to Kg of body weight (BW), being EE expressed as Kcal/Kg BW/h and VO₂ as mL/Kg BW/h.

2.10 Collection of faeces and urine in metabolic cages

Mice were housed in individual metabolic cages, (Tecniplast Group. Italy) equipped with a separation system for feces and urine, twice a week for 10 weeks (Figure 7.6). Mice had free access to water and food for all experiment. Faecal and urine collection had 2 different purposes in this study. The first one was to collect them to know the total amount of feces and urine excreted/24 h. The second purpose was only to collect feces and urine to perform future analysis. Faeces and urine samples were weighted at the time of collection and stored in plastic tubes at -80 °C. Renal function was monitored weekly using urine strips (KRUUSE VET-10. Korea); 100 µL of urine were used to detect absence or presence of nitrites, the amount of urobilinogen, glucose, bilirubin, ketones, blood, protein and leukocytes. In addition, specific gravity (SG) and pH were determined.

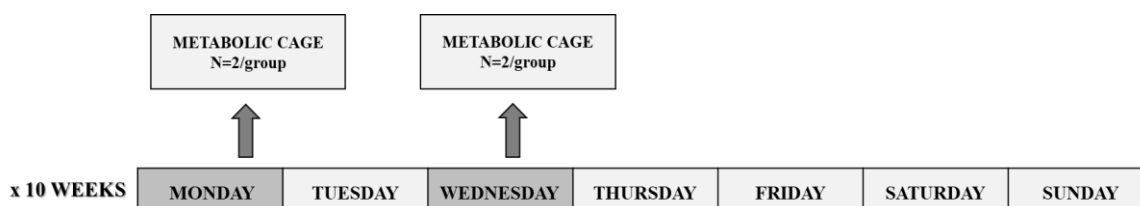


Figure 7.6. Weekly distribution scheme in the metabolic cages.

2.11 Plasma analysis

Blood was withdrawn from facial vein through several stages of the study: at the beginning (adaptation period), at the end of DIO phase and at the final of dietary intervention. Plasma was separated by centrifugation 4.000 g for 15 min at 4 °C and frozen at -80 °C to further analysis.

Total cholesterol (TC), HDL and LDL cholesterol were analysed by spectrophotometry (500 nm) using an analyzer A25 Biosystem (Biosystem. Barcelona. Spain). The detection limit was 4.2 mg/dL (0.109 mmol/L), the repeatability was 0.7 % and 0.6 % for mean concentrations of 153 mg/dL and 220 mg/dL, respectively. The repeatability within laboratory was 1.4 % and 1 % for previous mean concentrations.

The detection limit for LDL cholesterol was 0.44 mg/dL (0.01 mmol/L). The intra-assay repeatability for mean concentration of 59 mg/dL and 97 mg/dL was 0.6 % and 0.7 %, respectively. The repeatability within laboratory was 2.5 % and 2.2 % for these mean concentrations. The detection limit of HDL cholesterol was 1.83 mg/dL (0.048 mmol/L). For a mean concentration of 53 mg/dL and 73 mg/dL the repeatability was 0.6 % and 0.7 %, respectively, whereas the repeatability within laboratory was 2.7 % and 2.6 % for their previous values, respectively. Results were expressed as mg/dL.

A mouse metabolic magnetic bead panel (Milliplex Map kit. EMD Millipore Corporation. Germany) was designed to analysed GIP and amylin. The Luminex analyser MAGPIX (The Netherlands) was used to capture and detect the magnetic

beads, and the Luminex Xponent (The Netherlands) software was used to data acquisition and analysis.

The principle of this immunoassay consisted in using magnetic microspheres coated with a specific antibody (anti-GIP beads and anti-amylin beads). The target molecule from the test sample added (10 µL) was captured by the specific bead after 20 h of incubation at 4 °C under shaking. Afterward, a biotinylated detector antibody was added to recognize their specific epitopes and bind to the appropriate analyte. After 30 min of incubation, streptavidin conjugated and the fluorescence protein, phycoerythrin, were introduced in each well completing the reaction on the surface of each bead. Finally, and after washing the plate, samples were analysed with Luminex analyser. Based on the spectral properties of each magnetic microsphere and the amount of fluorescence, the concentration of each hormone (pg/mL) was determined. Two controls, a blank (0 pg/mL) and seven standards were used to elaborate the calibration curve of each hormone. Calibration curves for hormones can be observed in figure 7.7. For data interpretation a five parameters logistic curve was used (Logistic 5P Weighted), being the general formula as follows:

$$Y = a + \left(\frac{(b - a)}{\left(1 + \left(\frac{(x - c)^d}{d} \right) \right)} \right) f$$

Analyses were run in duplicate. The assay allowed the quantification of hormones without cross-reactivity between specific antibodies for each analyte. Regarding the sensitivity of the metabolic magnetic bead panel, the minimum detectable concentration was 1 pg/mL for GIP and 19 pg/mL for amylin.

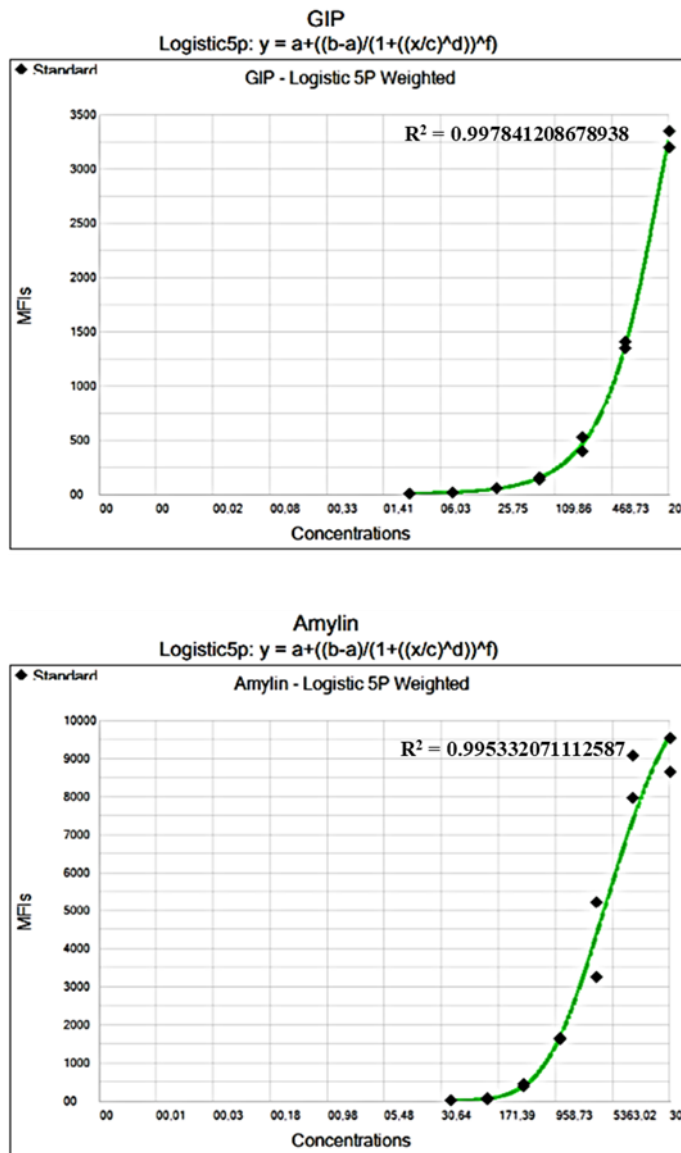


Figure 7.7. Five parameters logistic calibration curve for GIP and amylin

Enzyme immunoassay (EIA) kit was used to quantify CCK levels (RayBiotech. USA). The principle of EIA was based on the competitive binding of biotin-conjugated CCK and samples in the pre-coated anti-CCK plate (immobilised). In the assay, a biotinylated CCK peptide is spiked into the samples and standard and 100 μ L of each mixture was added to appropriate well. After incubation, plate was washed to remove the non-bonded complexes.

Then the chromogenic substrate, horseradish peroxidase (HRP)-streptavidin was added to catalyze the interaction, given place to a color product. More intensity of color was proportional to the amount of bonded biotin-labeled CCK, and inversely proportional to the amount of CCK in samples. The optical density (O.D.) was measured in the spectrophotometer in a 96-wells microplate reader at 450 nm (BioTek Synergy HT. UK). Thus, a high intensity of color detected in the spectrophotometer corresponded with lower levels of CCK, and *vice versa*. A four parameters logistic regression calibration curve was performed to obtain the result (pg/mL). The formula used is described as:

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

A positive control, a blank (0 pg/mL), a total binding (biotinylated-CCK) and five standards were used to perform the calibration curve. Standards concentrations corresponded to 1000, 100, 10, 1 and 0.1 pg/mL of CCK. Figure 7.8 shows the calibration curve used for CCK quantification. The minimum detectable concentrations of CCK were 0.2 pg/mL, and the intra-assay coefficient was <10% reproducibility and inter-assay of <15%. The assay was run in duplicate.

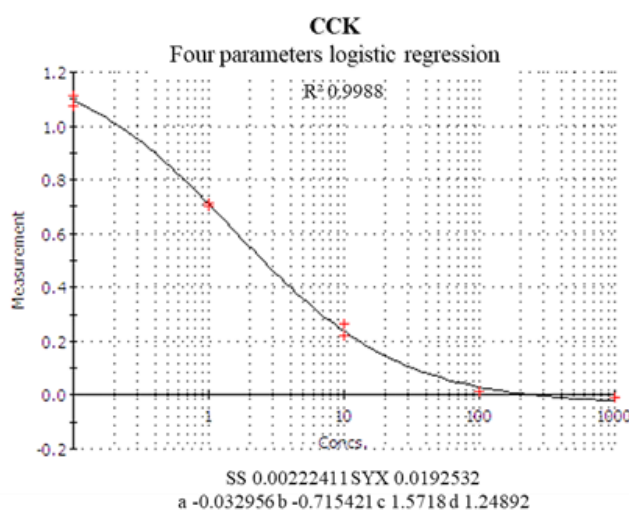


Figure 7.8. Four parameters logistic calibration curve for CCK.

2.12 In vivo body composition analysis

Total volume and fat volume (cm³) were determined in a non-invasive way using computed tomography scan (CT) (Albira Bruker BioSpin. Corporation of Spain). The CT image acquisition was performed using an X-ray energy of 0.4 mA and a voltage of 45 kVp. CT was performed before the beginning of study (n=6 of adaptation or initial period), after DIO (n=5) and at the end of the dietary intervention (n=4 per each group; W, E, TN, N, EC and IC). Prior the CT, mice were anaesthetised with inhalation anaesthesia using isoflurane (Isoflurin 1000 mg/kg. Fatro. Spain) at a dose of 2.5%. The whole CT scan was performed from nose to the end of the tail, including the bed and the inhaler of anaesthesia. Figure 7.9 summarises the entire process of acquiring and processing images. The software used to image processing from CT were PMOD Technologies LLC (Switzerland) and the software Volview v3.2 (Kitware, USA) to create three-dimensional (3D) images (figure 7.10).

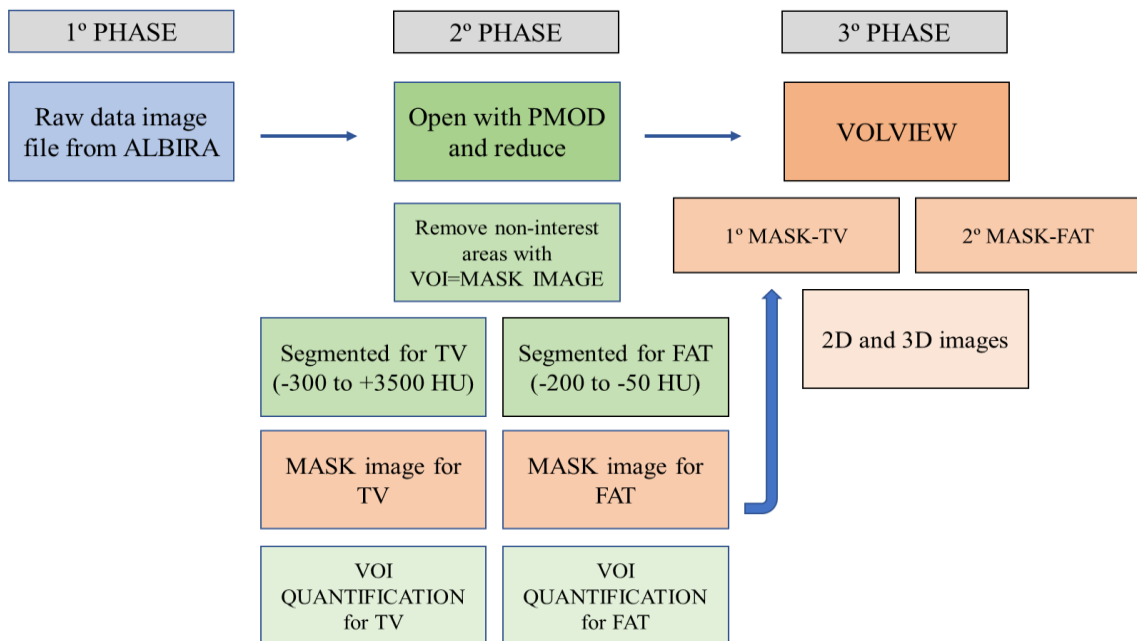


Figure 7.9. Summary of the entire proceedings to acquire and analysis of images.

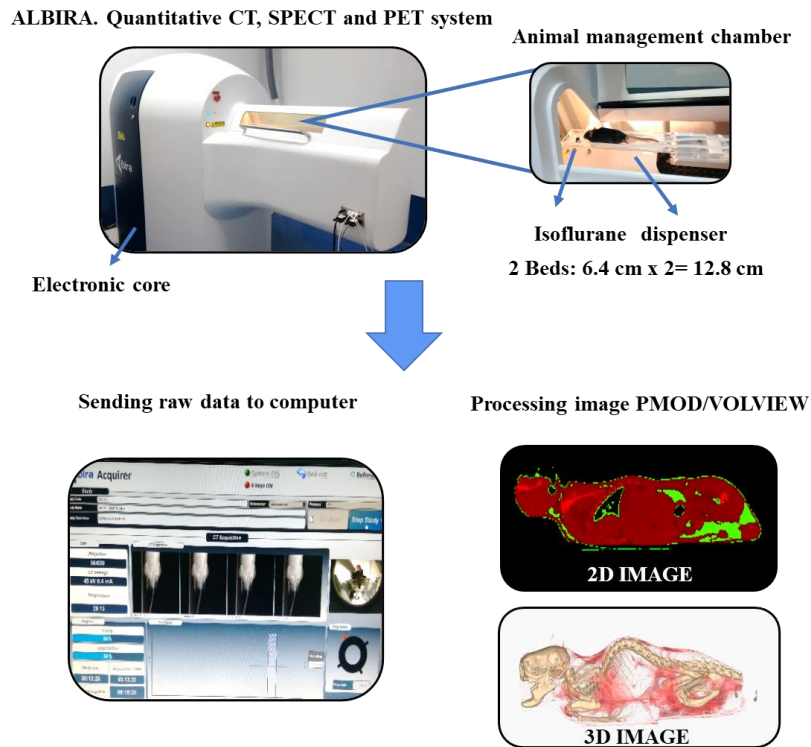


Figure 7.10. First phase: Measurement of body composition using CT Albira Bruker and software to image processing (PMOD and Volview).

Next phases to reconstruction and analysis of images consisted of the following steps:

A) *Image reconstruction and analysis*

The generated images from Albira were processed using PMOD from the measure of total volume (TV) and fat volume (Sasser *et al.*, 2012). Raw images from Albira were firstly reduced to facilitate the PC work. Afterwards, reduced images were masked to eliminate non-interest areas such as the bed, the inhaler of anesthesia and the tail. For this purpose, volume of interest (VOI) were generated from nose to the top of the tail (near of the first vertebrae of the tail). Then, mask images were segmented to check TV of mice, which means the selection of a range of Hounsfield units (HU) from -300 to +3500. The same procedure to analyze total fat was assayed, but changing the range of HU, since in case of fat the HU ranged from -200 to -50 HU (Sasser *et al.*,

2012). Then, we obtained the TV (cm³), fat volume (cm³) and fat/TV ratio for each animal. CT x-ray is able to recognise different tissue densities expressed as HU. For example, -1000, 0 and +1000 HU correspond to densities of air, water and bone, respectively. Segmented images were not eroded or/and dilated since the peripheral fat was interest area for this study.

B) Visualization of 2D and 3D images

When mask images of total volume and fat were acquired by PMOD, 3D images were performed using Volview. Firstly, the mask images of TV were analysed as a first component and secondarily, a second component (mask images of fat) were added. Modifying scalar colour and opacity mapping, 3D images were generated applying for the fat (second input) red colour to distinguish clearly from the body. Moreover, two-dimensional (2D) images were also obtained, in a similar way to as described previously, but assigning red colour to whole body and green for fat (Figure 7.11).

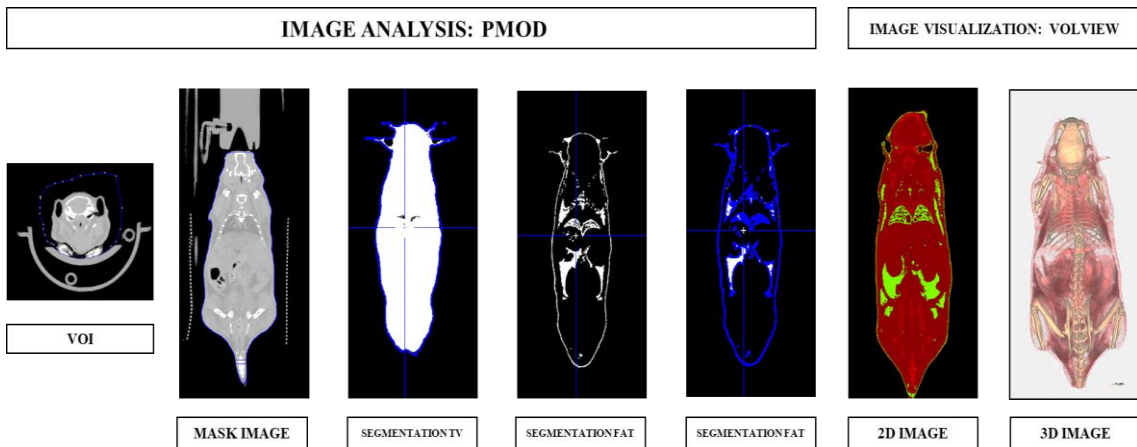


Figure 7.11. Image analysis and visualization using PMOD and VolView, respectively. VOI image corresponds to the first stage to image analysis being drawn the volume of interest and eliminating non-interest areas. Mask image corresponds to the image after selected all VOI. Segmentation of TV and FAT corresponds to the segmentation in a range of -300 to +3500 HU and -200 to -50 HU, respectively. 2D and 3D images are acquired after using Volview software including two components: TV and FAT.

2.13 Necropsy: organs and tissue collection

Organs were carefully dissected and rinsed continuously in saline solution. Afterward, organs were snap-frozen in liquid nitrogen and stored at -80 °C or collecting in 10% of formaldehyde depending on the further analysis. Specifically, the organs stored at -80 °C were heart, stomach, small and large intestine, part of the liver, portions of the total abdominal fat, also named white abdominal fat (WAT) including retroperitoneal, epididymal and perirenal fat, brown adipose tissue (BAT), and right soleus muscle. However, to the histopathological analysis the target organs were: part of the liver, portions of white and brown adipose tissue (WAT and BAT, respectively) and the left soleus muscle and they were collected in 10% of formaldehyde. The weight (g), length (cm) and size (cm²) of each organ was recorder and expressed as a function of body weight (figure 7.12). Samples of intestine for future tissue gene expression analysis were excised conscientiously, separating duodenum, jejunum and ileum, as well as colon, caecum and rectum. Luminal contents of each portion of gastrointestinal tract were collected, weighted and stored at -80 °C to next analysis.

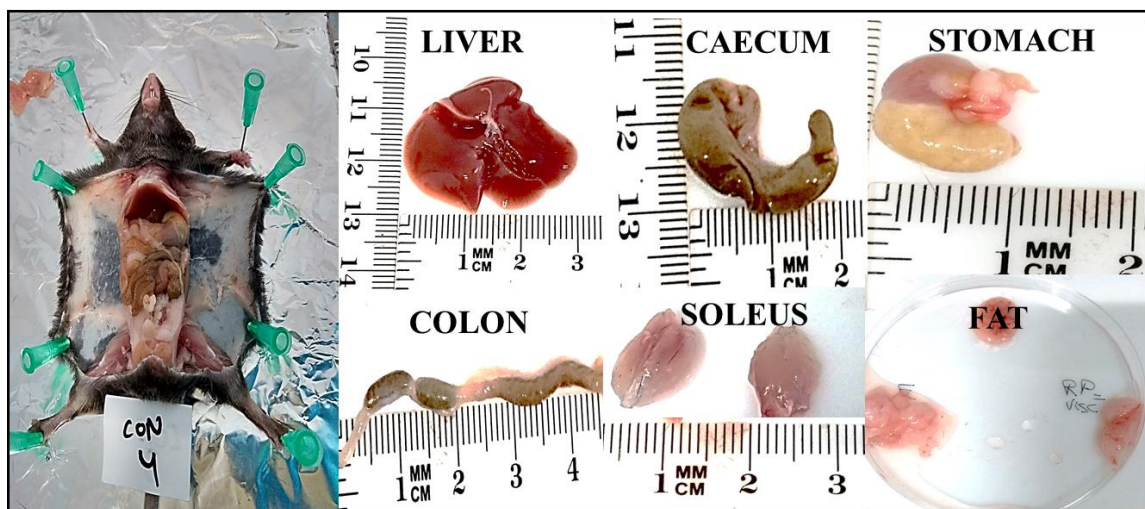


Figure 7.12. Organ and tissue collection and measurement of tissue of interest.

2.14 Statistical analyses

Statistical data processing was performed using the program Statistical Package for the Social Sciences (SPSS) v.19.0. (California, USA). Prior to statistical analysis normality and homoscedasticity were confirmed by using Shapiro-Wilk and Levene test, respectively. Statistical significance was assessed by One-way ANOVA followed by multiple comparison using a *post hoc* test (Tukey HSD test). Differences with $p < 0.05$ were considered statistically significant. Results are expressed as means \pm S.D. or means \pm S.E.M. Pearson's correlation coefficient r was used to quantify a relationship between two sets of data.

3. RESULTS

3.1 Body weight gain, food and fluid intake under DIO phase

Mice were fattened prior the dietary intervention for 31 days using a high-fat diet. This type of diet provided an energy density of 5.1 Kcal/g, corresponding 60% of total energy from fat. This diet was given to achieve an excessive weight just before the dietary intervention. The body weight (BW) before and after DIO stage were 26.64 ± 0.91 and 36.65 ± 0.84 g, respectively. The cumulative BW gain during DIO phase was of 10 g with respect to the initial weight, fattening approximately 0.32 g per day. Figure 7.13 summarises the weight gain of mice during DIO phase. Values are expressed as an average of BW estimated each 2 days. Daily food and fluid intake along DIO phase are showed in figure 7.14, indicating that the average of food and water intake per animal ($n=40$) corresponds to 3.04 ± 0.56 g and 3.99 ± 0.36 mL, respectively. The cumulative average of energy intake of mice receiving high-fat diet was 15.51 ± 2.87 Kcal/d.

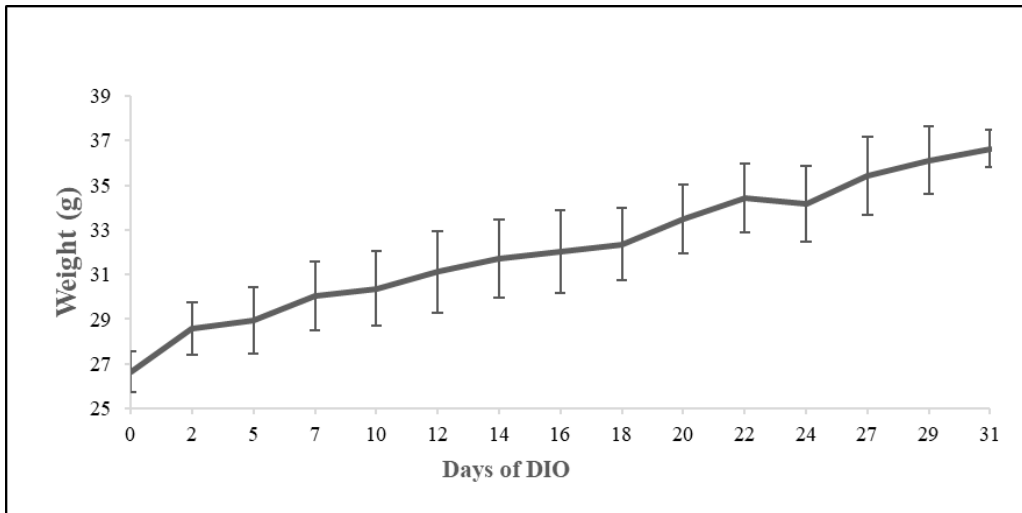


Figure 7.13. Body weight (g) under DIO stage for 31 days. Values are means \pm S.D. of two-weekly measurements, n=40.

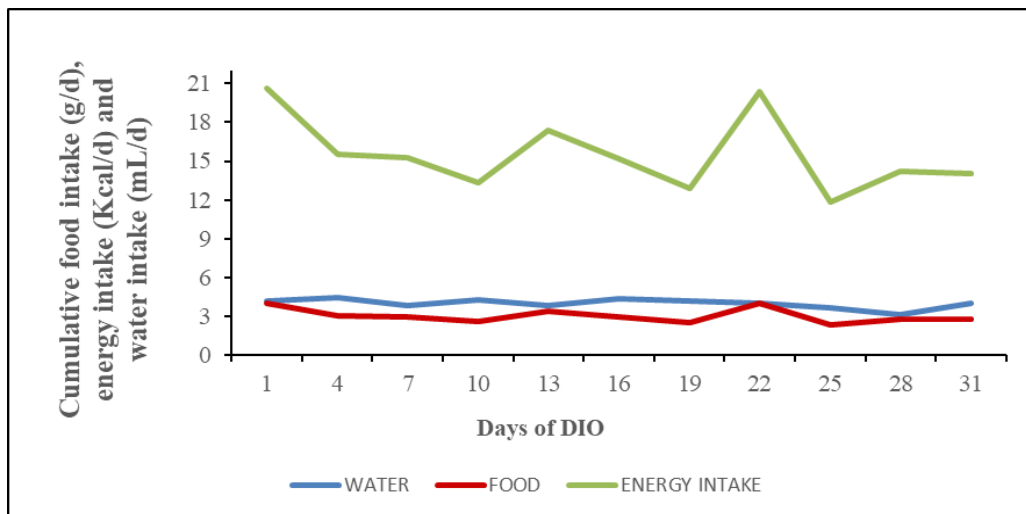


Figure 7.14. Cumulative food (g) intake, energy intake (Kcal) and water (mL) intake per animal of n=40 mice after 31 days receiving a high-fat diet.

3.2 Body weight, food and fluid intake of external control (EC) mice group during experiment

Animals belonging to the EC were never submitted to DIO phase. This group was maintained under “normal” conditions, without dietary interventions and fed with a standard chow diet. The objective was to have a group of animals (n=4) which played the role of “real” control, allowing to know how mice strain C57BL/6J would be in the adult age. The evolution of mice belonging EC during 16 weeks of total duration of the experiment is shown in figure 7.15.

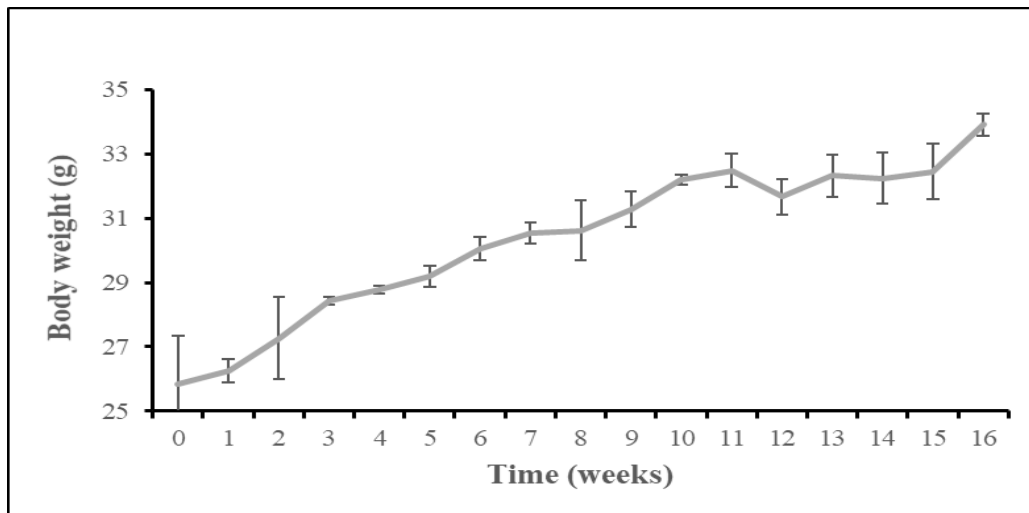


Figure 7.15. Body weight (g) of EC group during 16 weeks of experiment. Values are means±S.D. of two-weekly measurements, n=4.

It is worth considering how was the “normal” weight of mice, understanding “normal” as animals not subjected to dietary interventions. Animals started the experiment at the age of 7 weeks weighing 25.85 ± 1.48 g and they ended the study at the age of 23 weeks weighing 33.9 ± 0.33 g. During 16 weeks of the whole experiment EC group gained 8.05 g, receiving a standard chow diet (2.9 Kcal/g of food). Cumulative food and water intake, as well as energy intake, are shown in figure 7.16. The average of daily food intake (g) and water (mL) per animal for 16 weeks corresponded to 3.49 ± 0.36 and 5.21 ± 0.58 , respectively. The average daily energy intake per animal (n=4) corresponded to 10.12 ± 1.01 Kcal/d.

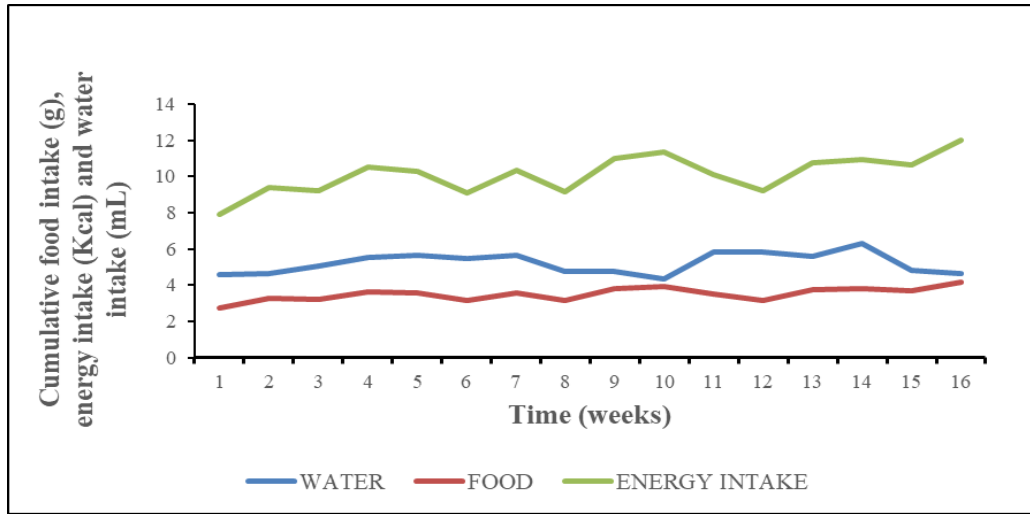


Figure 7.16. Cumulative food (g) intake, energy intake (Kcal) and water (mL) intake of external control (n=4) during 16 weeks of experiment.

3.3. Body weight, food and fluid intake of experimental groups under dietary intervention

After 11 weeks of dietary intervention using several types of diet based on high-protein composition (W and E) or high-fibre (TN and N) different cumulative water of food intake results can be observed in Table 7.3.

Cumulative water intake (mL/day/mouse) over 11 weeks of treatment, showed that diet based on nopal produced the highest water ingestion (6.59 ± 1.72 mL/d) compared to all experimental diets, obtaining important differences among them. Mice fed with goat whey protein were the second one, followed by animals with egg diet. Finally, TN and IC groups drank similar volume of water, but statistically significantly lower than other groups. It was notable the descended water intake of IC respect to N group, being about half of total millilitres.

Both, cumulative food intake (g/d) and cumulative energy intake (Kcal/d) were significantly reduced in case of W, E and N respect to TN and IC. Moreover, no significant differences were found comparing two high-protein diets and nopal diet; nor between TN and IC.

Differences in BW evolution (gain or loss of BW) were significantly ($p < 0.05$) marked after 11 weeks of treatment under different isoenergetic diets (Figure 7.17). The weight reduction was highly noticeable in the case of N, E and W group, especially between the first and second week. Around the fourth week the fall of weight was considerably high in N group, becoming more stable about the fifth and sixth week. The weight decreased was quite similar among these three experimental diets, stabilizing weight after 3-4 weeks.

Interestingly, W, E and N groups did not gain BW through the experimental intervention remaining under their specific initial BW. However, in the case of TN, the weight reduction was notable in short-term achieving the maximum weight loss around the middle of the experiment (fifth week) to continue increasing after that. As we can observe, IC experimented a slight reduction in BW around the firsts three weeks and they began to stabilize and gain BW over eight weeks.

Table 7.3. Total body weight (BW), food and water intake, energy intake and feed efficiency rate of C57BL/6J mice fed with whey (W), egg (E), tiger nut (TN), nopal (N) and internal control (IC) diet, after 11 weeks of dietary intervention ¹.

Parameter	Dietary group									
	W		E		TN		N		IC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Initial BW (g)	36.24 ± 0.33 ^a		35.70 ± 0.41 ^a		36.25 ± 0.16 ^a		37.35 ± 0.49 ^b		37.71 ± 0.48 ^b	
Final BW (g)	32.79 ± 0.42 ^{c*}		31.72 ± 0.19 ^{b*}		37.03 ± 0.48 ^{d*}		28.64 ± 0.16 ^{a*}		40.64 ± 0.32 ^{e*}	
BW gain (g)	-3.45 ± 0.38 ^b		-3.97 ± 0.17 ^b		0.78 ± 0.44 ^c		-8.70 ± 0.14 ^a		2.93 ± 0.32 ^d	
Water intake (mL/day)	5.88 ± 1.08 ^c		5.11 ± 1.11 ^b		4.36 ± 0.56 ^a		6.59 ± 1.72 ^d		3.70 ± 0.46 ^a	
Food intake (g/day)	2.85 ± 0.54 ^a		2.53 ± 0.55 ^a		3.34 ± 0.52 ^b		2.78 ± 0.74 ^a		3.42 ± 0.57 ^b	
EI (Kcal/day)	10.55 ± 2.01 ^a		9.13 ± 2.00 ^a		12.05 ± 1.87 ^b		9.75 ± 2.59 ^a		12.33 ± 2.07 ^b	

¹ Values are expressed per animal and dietary group. Data are shown as a mean ± S.D. for twice determinations per week (n=8 mice). Different letters were used to indicate significant differences within each row at $p < 0.05$. * indicates differences between final BW respect to initial BW.

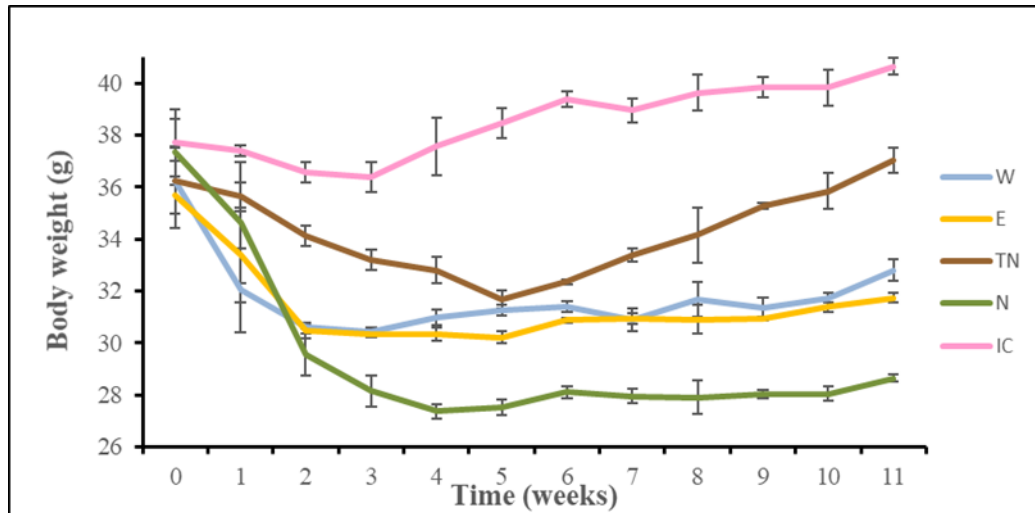


Figure 7.17. Body weight (BW) evolution along 11 weeks of dietary intervention: whey (W), egg (E), tiger nut (TN), nopal (N) diets and internal control (IC). Results are means \pm S.D. of BW measurements each 2 days for 11 weeks. BW (g) data as expressed per mouse (n=8).

At the final of the dietary intervention, all experimental diets showed significant differences of BW gain respect to DIO phase and even respect to IC (Figure 7.18). The highest decrease of body weight compared to initial stages was obtained after the supplementation of N diet (-8.70 ± 0.14 g) corresponding approximately to 23% of BW reduction respect to its obese condition. In the case of egg (-3.97 ± 0.17 g) and W diets (-3.45 ± 0.38 g) body-weight loss were significantly similar between them, corresponding the reduction of BW to 10% approximately. TN diet did not elicit weight loss but also it was slightly increased after 11 weeks of treatment (0.78 ± 0.44 g). Despite this data, increase of BW showed by TN was significantly lower than showed by IC group, which increased body weight (2.93 ± 0.32 g). We must consider that mice were 23 weeks old (the age of mice after experiment), which showed increases of BW induced, maybe by an age effect, being 4.27 ± 0.33 g (external control, data not shown). Similarly, the BW gain of IC can be explained, mainly, by the age. Then, the decreases of BW induced by W, E, N diets and the stabilization elicited by TN are even more substantial.

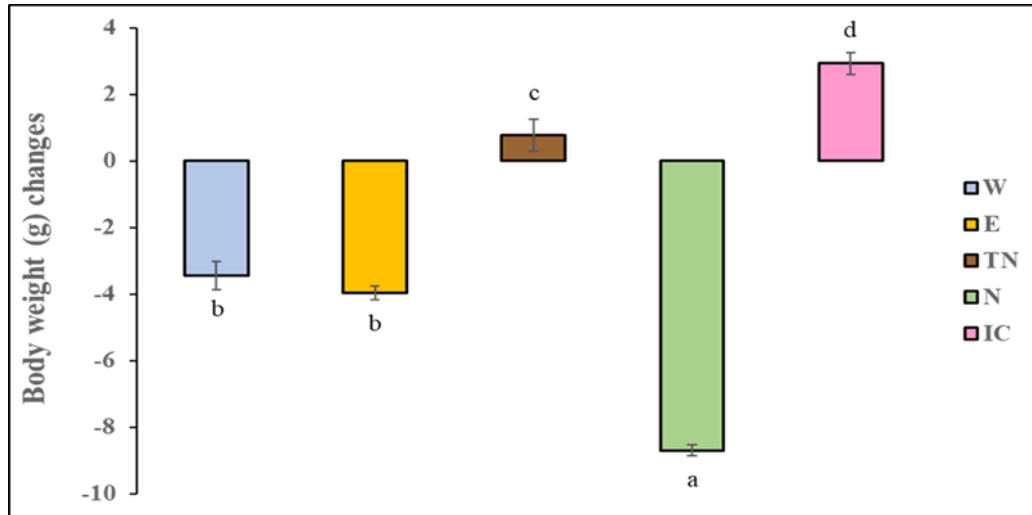


Figure 7.18. Body weight changes after 11 weeks of dietary intervention: whey (W), egg (E), tiger nut (TN), nopal (N) diets and internal control (IC). Results are means \pm S.D. of BW measures every 2 days (n=8).

3.4. Evaluation of urine and faecal content

Urine and faecal were collected twice per week, when animals remained individually in the metabolic cage. Urinary nitrogen gave us an idea of the oxidation of substrates during dietary intervention; meanwhile, faecal collection has been carried out to determine the gut microbiota profile and their metabolites after specific diets. In addition, total volume of urine and mass of faecal material for 24 h were monitored (table 7.4).

Urine test strips was used to ensure that mice presented an adequate renal and hepatic function along the experiment, showing normal values during the whole study. However, differences in urine pH and specific gravity (SG) were detected due to the different types of ingredients tested. Concretely, urine pH of animals fed with W and E diets were slightly more acid compared to other groups. These differences was due to the different protein or vegetable concentration on the diet. Groups with the highest urine excretion (mL/24 h) were W and E coinciding the low SG with these diuresis (1914.66 \pm 500 and 1984.50 \pm 621.53 mL/24 h of urine, respectively) (Table 7.4). Nopal group showed a high diuresis although significantly lower compared to W and E diets.

However, the total volume for 24-h was three-fold of IC. Tiger nut group presented a descended urine excretion compared than other experimental diets and similar to IC.

Regarding to faecal depositions, differences in the consistency and volume were detected at first sight (Figure 7.19). As it can be observed in Table 7.4, the difference of weight of faeces was notable. N group exhibited a high faecal excretion (0.58 ± 0.16 g/24h of faeces) during the stay into the metabolic cage. It is worthy of mentioning that fecal material produced by N diet showed a greenish and bright appearance and it was highly voluminous compared to other groups.

Table 7.4. 24-h Urine and faecal material deposition of individual animal during the stay in the metabolic cages ¹.

Group	Urine (mL/day)	Faeces (g/day)
Whey	1914.66±500 ^c	0.23±0.08 ^a
Egg	1984.50±621.53 ^c	0.23±0.07 ^a
Tiger nut	391.87±216.19 ^a	0.40±0.18 ^b
Nopal	1351.25±256.79 ^b	0.58±0.16 ^c
Internal control	431.87±150.35 ^a	0.23±0.15 ^a

¹ Results were calculated as a mean±S.D. two-weekly measurement during 10 weeks. Different letters indicate significant differences within each column at $p<0.05$.



Figure 7.19. General appearance of faecal deposition from mice fed with several types of diet.

3.5 Feeding pattern assessment

Mice recorded during 12 hours of dark phase showed differences of social behaviour and eating pattern under *ad libitum* conditions (Figure 7.20). All groups of animals were recorded along the experiment (DIO, experimental groups and EC).

Despite social behaviour was not monitored *ad hoc*, we could observe great differences between different dietary groups. Animals fed with a high-fat diet (DIO) showed an aggressive behaviour and high levels of anxiety, during 12-h of recording. They seemed jealous and fighting for the water or/and food. Similar pattern was recorder in case of IC and to a minor extent in TN group. Several researches have suggested that high-fat diets could induce aggressivity in male rats and mice possibly mediated by increases in serum estradiol (HilakiviClarke *et al.*, 1996). For these groups, food and water could have a social value more than a nutritional value. Surprisingly, W, E, N and EC groups showed a more sociable behaviour. In addition, mice from W, E and N groups were more active during the recording; and these animals were climbing up and running from one side to another into the cage. Furthermore, it was notable time to water-access, specially in the case of N group. The distribution and duration of eating events during the recording period (from 08:00 p.m. until 08:00 a.m) of several dietary groups can be observed in the figure 7.21.

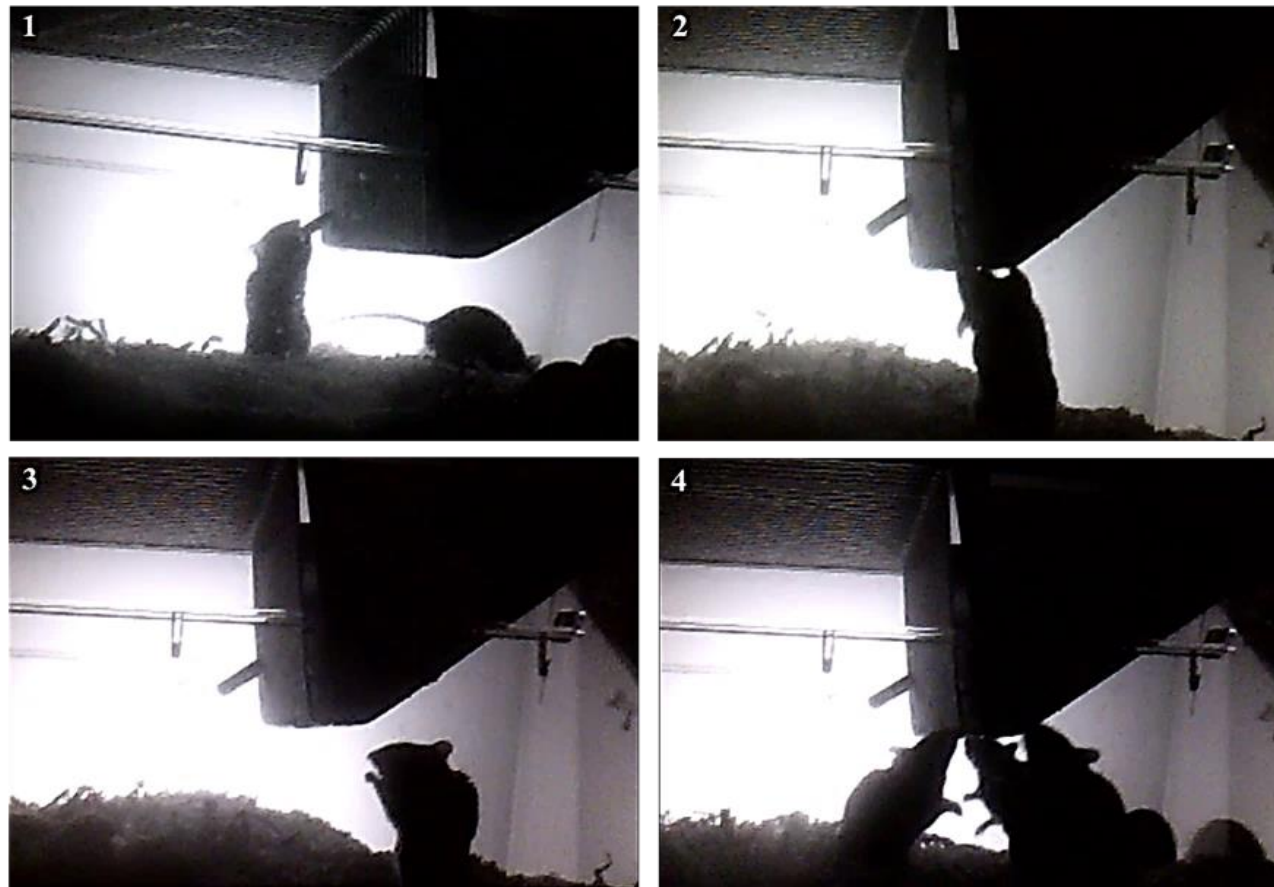


Figure 7.20. Images of feeding behaviour of mice reordered under dark phase using infrared light (n=4). 1) Mouse drinking water; 2 and 3) Mouse having a meal; 4) Three mice eating at the same time.

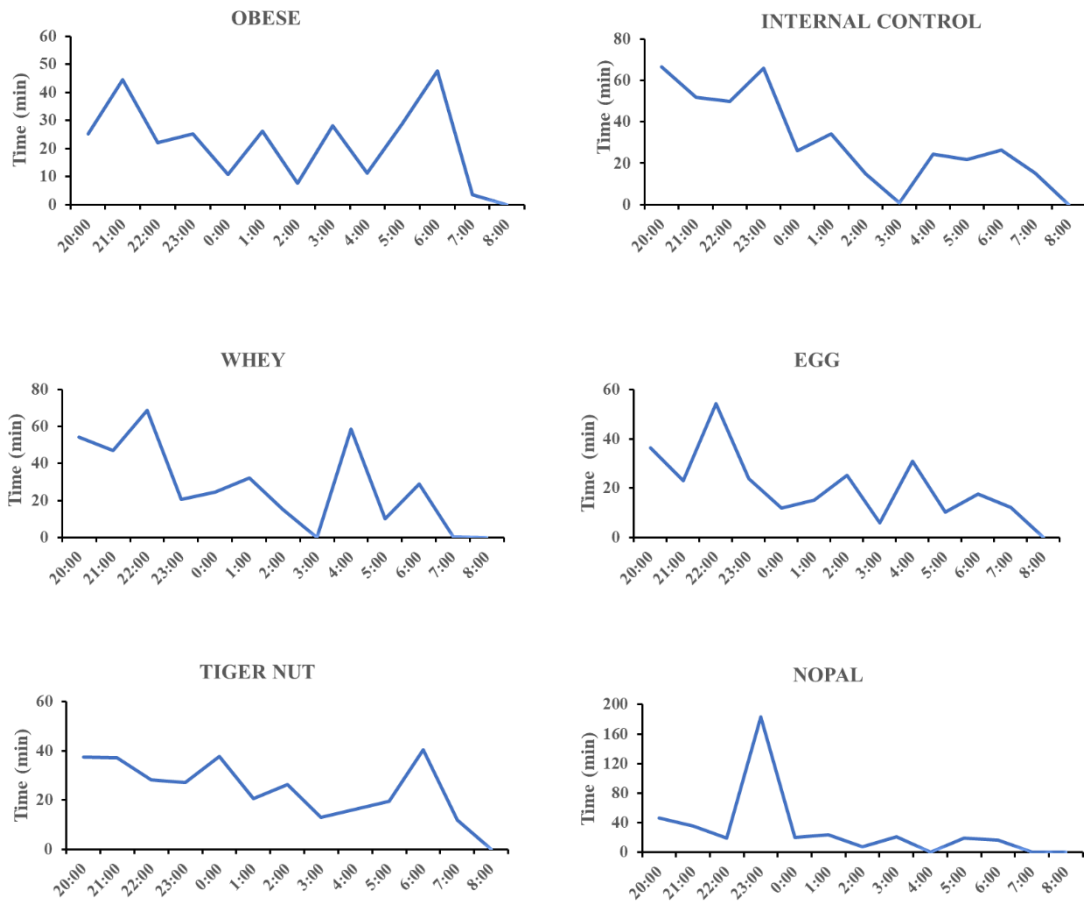


Figure 7.21. Duration (min) and distribution of eating episodes for 12 hours of video recording of mice belonging to DIO, internal control, whey, egg, tiger nut and nopal experimental diets.

Feeding behaviour showed differences among dietary groups. In general terms, all groups with the exception of nopal, showed a steady rhythm along the recording. Approximately, great number of events was concentrated in two times, from 21:00-23:00 p.m. and 03:00-07:00 a.m. Interestingly, N group had unique great food binge from 22:00 until 00:00 p.m. Contrarily, TN showed large feeding events, specially concentrated from 05:00 to 07:00 a.m. These differences highlighted the different feeding pattern among all kind of diet.

It is worth mentioning that during DIO period, mice gnawed in a non-stop way but spending few minutes into each meal. The cause was due that the commercial high-fat diet was presented softer than the other diets having a buttery consistency. That consistency led to animals eating food easier and faster than other dietary groups. General feeding pattern of mice fed with different types of isocaloric diet can be observed in figure 7.22.

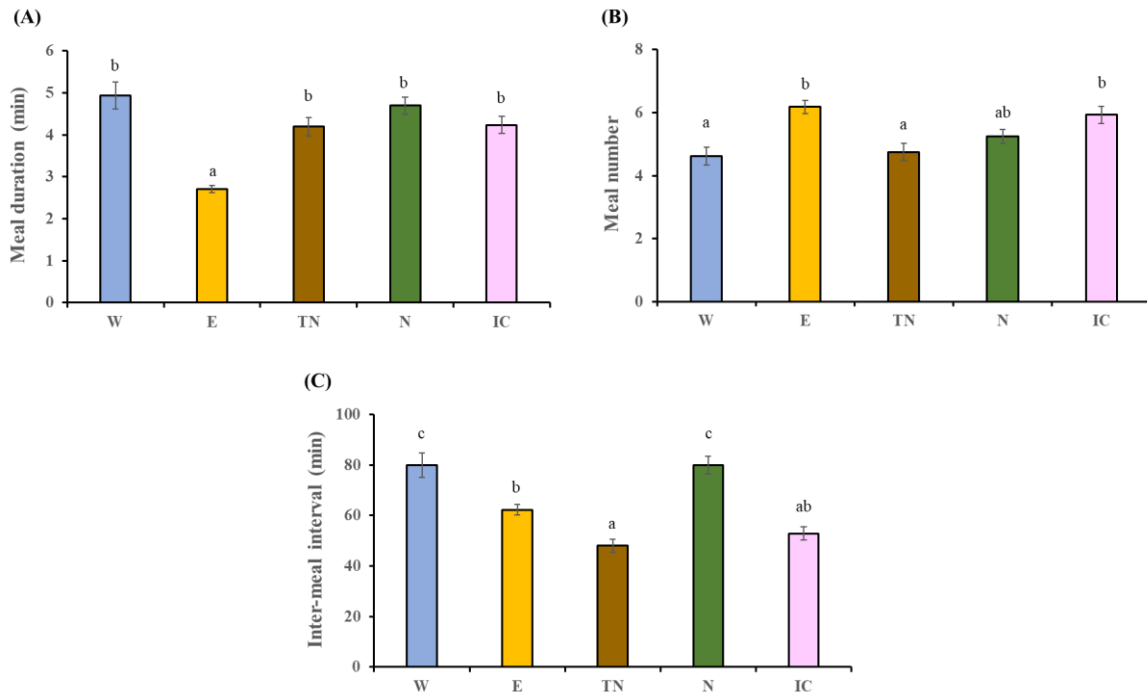


Figure.7.22. (A) Meal duration, (B) meal number and (C) inter-meal interval at nocturnal phase of mice during W, E, TN, N and IC diets. Results are expressed as a mean \pm S.E.M., n=4. Different letters indicate statistically significant differences ($p < 0.05$).

Feeding behaviour of all experimental diets was compared among themselves and in relation to IC. Regarding to meal duration (min), W, TN and N showed similar values than IC, but higher than in the case of E diet, being this one the group that least time spent eating. Furthermore, IMI of W and N was surprisingly exactly equal (79.84 ± 4.86 and 79.89 ± 3.48 min, respectively), and significant longer than other diets.

Egg group presented values of IMI significantly higher than TN group (62.27 ± 2.1 min). However, no significant differences were found by comparing E, TN and IC among themselves. The number of meals under 12 h of recording also revealed differences among dietary groups. W and TN group showed fewer meals compared to E and IC, having N group an intermediate result.

3.6 Necropsy: organs collection analysis.

Immediately after the processing of slaughtering animals, tissues and organs of interest were quickly measured, frozen in liquid nitrogen and stored. The relative weight, area and length of tissues and organs are shown in tables 7.5 and 7.6.

The relative heart weight (g/100 g BW) was significant higher in case of W group compared with IC, but not comparing with the rest of the dietary groups (E, TN, N and EC). The relative weight of empty stomach was higher in case of TN compared with IC and N groups ($p < 0.05$), and similar to W and E groups. The relative weight of liver was increased in W, E and N diets compared to IC. However, IC and TN showed similar weights of the liver. It was noticeable that the relative weight of the liver in the W group was approximately 2 grams greater than IC (5.65 ± 0.44 vs 3.68 ± 0.77 g/100 g BW), and the highest comparing all diets.

Table 7.5. *Post-mortem* relative tissue weights (g/100 g B.W.) of mice fed under experimental diets (W: whey, E: egg, TN: tiger nut, N: nopal) and IC (internal control) after 11 weeks of dietary intervention ¹.

Diet group	W	E	TN	N	IC
	Tissue weight (g/100 g B.W.)				
Heart	0.66±0.05 ^b	0.59±0.07 ^{ab}	0.56±0.09 ^{ab}	0.56±0.06 ^{ab}	0.50±0.13 ^a
Stomach**	0.70±0.09 ^{ab}	0.70±0.13 ^{ab}	0.84±0.11 ^b	0.66±0.14 ^a	0.55±0.11 ^a
Liver	5.65±0.44 ^c	4.63±0.46 ^b	4.06±0.25 ^{ab}	4.51±0.21 ^b	3.68±0.77 ^a
Total visceral fat deposition	2.02±0.32 ^a	3.29±0.84 ^a	7.45±1.93 ^b	2.57±0.38 ^a	8.68±1.84 ^b
Retroperitoneal fat	0.40±0.09 ^a	0.73±0.27 ^a	2.25±0.56 ^b	0.64±0.18 ^a	3.32±1.01 ^c
Perirenal fat	0.16±0.07 ^a	0.32±0.11 ^a	0.62±0.16 ^b	0.16±0.07 ^a	0.71±0.18 ^b
Epididymal fat	1.46±0.20 ^a	2.25±0.64 ^a	4.58±1.18 ^b	1.77±0.16 ^a	4.65±0.92 ^b
Brown adipose tissue	0.49±0.11 ^a	0.62±0.12 ^a	0.83±0.23 ^b	0.56±0.09 ^a	1.01±0.11 ^b
Soleus muscle	0.60±0.09 ^c	0.54±0.04 ^{abc}	0.50±0.03 ^{ab}	0.56±0.05 ^{bc}	0.45±0.07 ^a
SI*	3.63±0.33 ^{ab}	4.06±0.78 ^b	3.62±0.28 ^{ab}	4.82±0.21 ^c	3.17±0.35 ^a
Large intestine**	1.30±0.13 ^a	1.94±0.2 ^b	1.24±0.13 ^a	2.06±0.21 ^b	1.17±0.16 ^a
Colon**	0.68±0.11 ^{ab}	0.64±0.10 ^a	0.60±0.13 ^a	0.88±0.14 ^b	0.70±0.17 ^{ab}
Caecum**	0.49±0.09 ^a	1.08±0.20 ^b	0.50±0.07 ^a	0.99±0.21 ^b	0.35±0.07 ^a
Rectum**	0.12±0.01 ^a	0.24±0.05 ^b	0.14±0.04 ^a	0.21±0.06 ^b	0.12±0.03 ^a

¹*Small intestine (SI) corresponds to the sum of duodenum, jejunum and ileum. ** The hollow organs were weighted after being emptied. Data are presented as the mean±S.D. for n=8. Different letters indicate statistically significant differences ($p<0.05$) within each row.

Table 7.6. *Post-mortem* relative tissue area (cm²/100 g BW) and length (cm/100 g BW) of mice fed under experimental diets (W: whey, E: egg, TN: tiger nut, N: nopal) and IC (internal control) after 11 weeks of dietary intervention ¹.

Diet group	W	E	TN	N	IC
Tissue area (cm²/100 g BW)					
Stomach	4.38±0.61 ^c	3.85±0.62 ^{bc}	4.34±0.70 ^c	2.62±0.59 ^a	2.88±0.64 ^{ab}
Liver	19.22±1.23 ^c	10.63±1.95 ^a	15.51±3.02 ^{bc}	15.38±3.73 ^{bc}	15.11±2.91 ^b
Tissue length (cm/100 g BW)					
SI*	109.74±6.46 ^{bc}	112.78±11.14 ^{bc}	99.86±9.69 ^{ab}	120.76±9.72 ^c	93.78±13.53 ^a
LI*	25.25±1.22 ^{ab}	28.91±3.26 ^{bc}	22.13±3.31 ^a	33.60±3.16 ^c	24.40±4.09 ^{ab}

¹*Small intestine (SI) corresponds to the sum of duodenum, jejunum and ileum. *Large intestine (LI) includes colon, caecum and rectum. Data are presented as the mean±S.D. for n=8. Different letters indicate statistically significant differences ($p<0.05$) within each row.

Total visceral fat or white adipose tissue (WAT) deposition was calculated as the sum of retroperitoneal, perirenal and epididymal fat, corresponding approximately to 20%, 10% and 70% for the total visceral fat, respectively. WAT was considerably higher in TN and IC mice compared to W, E and N group. It was worth mentioning that total visceral fat of IC was >75%, >60% and >70% higher than W, E and N, respectively. However, non-significant differences were showed comparing internal control to TN (>15%). Separating all components of WAT, it can be observed that TN and IC followed a similar pattern. However, in the case of retroperitoneal fat it can be observed statistically significant differences between these groups, showing IC the highest values (3.32±1.01 g/100 g BW), having one g more of fat than TN. These data shed an idea of the fat distribution in case of TN group, corresponding to more amount into epididymal pad than other locations.

The relative total fat deposition (g/100 g BW) (white adipose tissue, WAT) and interscapular brown adipose tissue (BAT) (g/100 g BW) shared the same tendency. Figure 7.23 shows a graphical comparison of WAT and BAT differences from dietary groups. In both cases, W, E and N were similar among themselves and lower than IC and TN. When increased WAT, augmentations of BAT also has been observed. The highest BAT relative weight ($p<0.05$) was found again in the case of TN and IC.

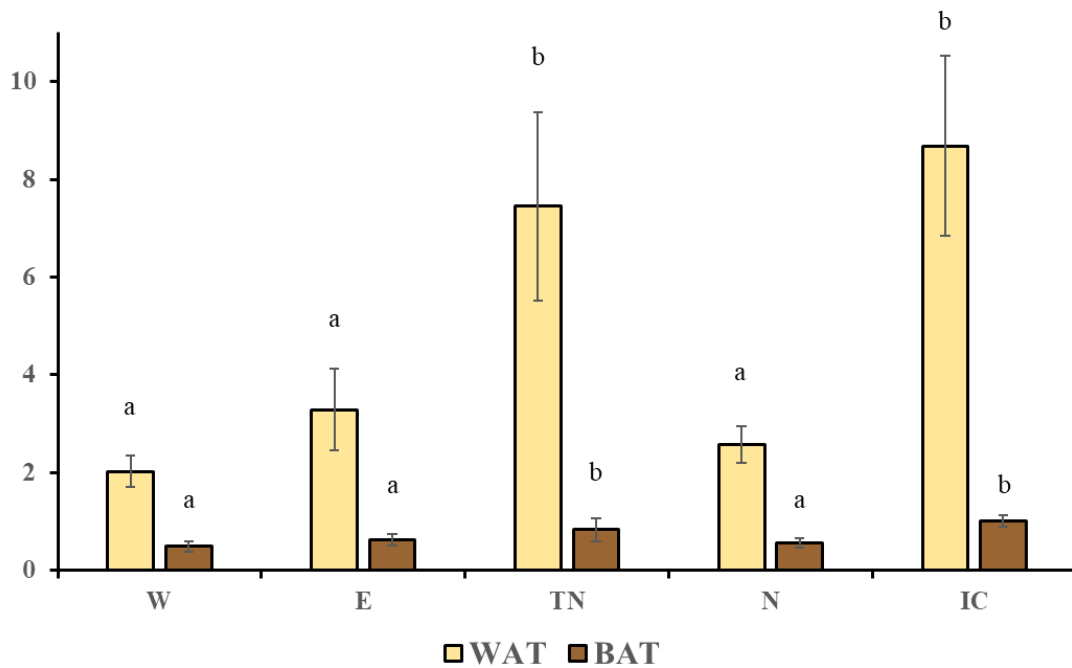


Figure 7.23. Total fat deposition (white adiposse tissue and brown adipose tissue, WAT and BAT) after 11 weeks of dietary intervention. Whey (W), egg (E), tiger nut (TN), nopal (N) diets and internal control (IC). Data are presented as the mean \pm S.D. for n=8. Different letters indicate statistically significant differences ($p<0.05$).

The relative soleus muscle weight was higher in the case of W group and similar compared to E and N group. Soleus from N group was not significant different to E and TN groups but it was higher compared to mice of IC.

The relative weight of small intestine (g/100 g BW) was calculated by summing the dissected portions of duodenum, jejunum and ileum after emptying them. Nopal group presented the highest weight being approximately 34% higher compared to IC. In addition, the weight of the emptied small intestine of E group was significantly higher than in the case of IC but not different to W and TN groups. Regarding to relative weight of large intestine (sum of dissected portions of colon, caecum and rectum) were significantly different in the case of N (2.06 ± 0.21 g) compared with all dietary groups and EC (data not shown), except with E group. According with these results, the weight of colon, caecum and rectum were equivalent to 50%, 40% and 10%, respectively of total large intestine weight (g/100 g BW). Detailing more deeply, the weight of caecum and rectum were the highest in the case of E and N and statistically significant different to all group ($p < 0.05$). However, colon weight of N was significantly higher than E and TN group, although it was not statistically significant higher than W and IC. These findings give an idea of the proportions of the weight of large intestine. Nopal and E groups showed the highest weight in caecum and rectum, but the weight of the colon not contributed the total weight of large intestine, being the total sum higher in E and N compared to other groups.

Relative areas of organs, expressed as a function of mice BW ($\text{cm}^2/100$ g BW) showed significant differences among experimental groups. Stomach area was lower in the case of N comparing to all experimental diets except for IC. Stomach area showed in the case of W and TN was significantly higher to N and also to IC group. This finding matched with the relative weight of the empty stomach that also was higher in case of TN compared with IC and N groups, and more similar to W and E diet.

The relative area of liver were the lowest in the case of E group, and quite different to all groups. The area of liver from W group was higher compared to IC, being almost 4 cm^2 the difference. These data resulted interesting since the weight and area of W groups was superior to IC. However, the relative high weight of the liver in the case of E diet compared to IC was not reflected in terms of area. These findings supported the idea of a compacted liver, but not increased in volume was shown in the case of E group.

The lengths of the gastrointestinal tract (cm/100 g BW) was measured by dissecting meticulously the small intestine and large intestine (colon, caecum and rectum). Whey, egg and nopal groups showed higher length of SI than in the case of IC group. However, the longest small intestine was found in the case of N group compared to IC (120.76 ± 9.72 and 93.78 ± 13.53 cm/100 g BW, respectively). Length results were in line with weight results of SI, showing E and, especially N, higher values compared to IC. Furthermore, the total length of large intestine was higher in case of N compared with experimental groups and control, except for E diet. The difference of length was quite notable comparing N and IC (33.60 ± 3.16 vs 24.40 ± 4.09 cm/100 g BW). Length of large intestine showed in the case of E group was higher compared to TN but not different than W diet and control.

3.7 In vivo body composition analysis

CT analysis was assayed at the beginning of the experiment (adaptation period), after DIO phase (obese), as well as in mice after 11 weeks of dietary intervention. Figure 7.24 provides representative CT two-dimension (2D) images of total volume (in red) and fat composition (in green) of adaptation, DIO phase and different groups after dietary intervention (including the internal control) using Albira CT system. Image integration was performed by using segmented fat and total volume acquired previously by PMOD and Volview software's. Figure 7.25 (A) and (B) shows three-dimension (3D) images constructed by using segmented fat and total volume data integrated, similarly to 2D, by PMOD and Volview. Figure 7.26 shows the general physical constitution of animals just after slaughtering.

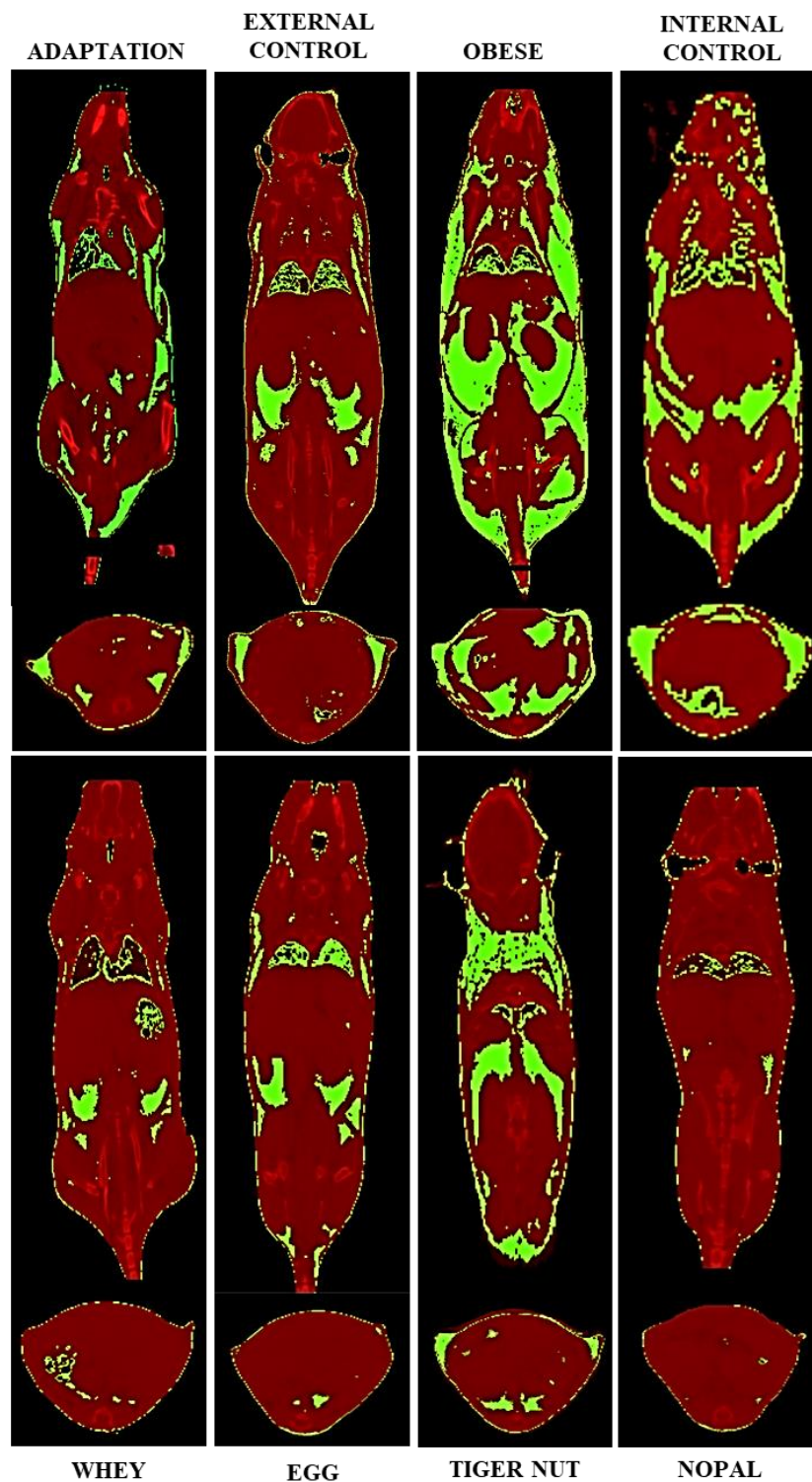


Figure 7.24. CT two-dimension images of total volume (in red) and fat composition (in green) of adaptation, DIO and dietary intervention groups. Images are displayed in frontal plane (Y axis in coronal direction) and transversal plane (X axis in axial).

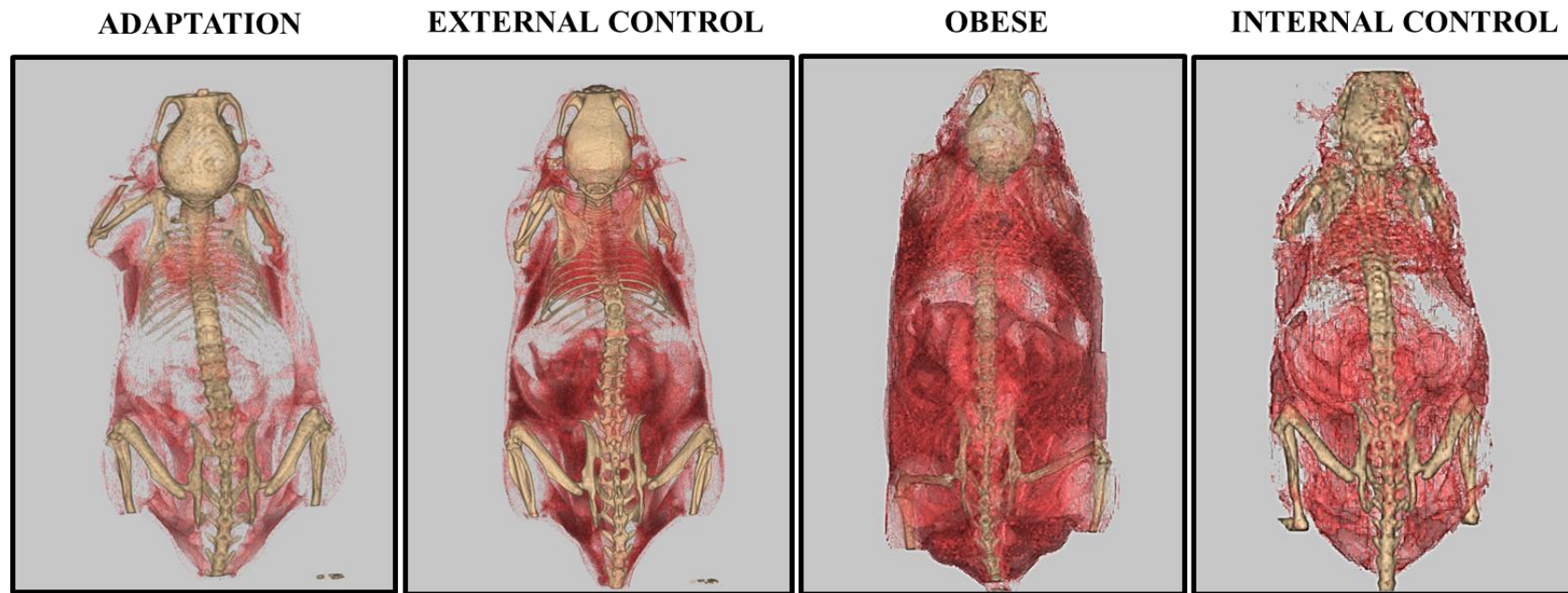


Figure 7.25. (A). CT images were acquired by using PMOD and analysed and displayed as three-dimensions (3D) using PMOD and Volview software's. Images are displayed in frontal plane (Y axis in coronal direction).

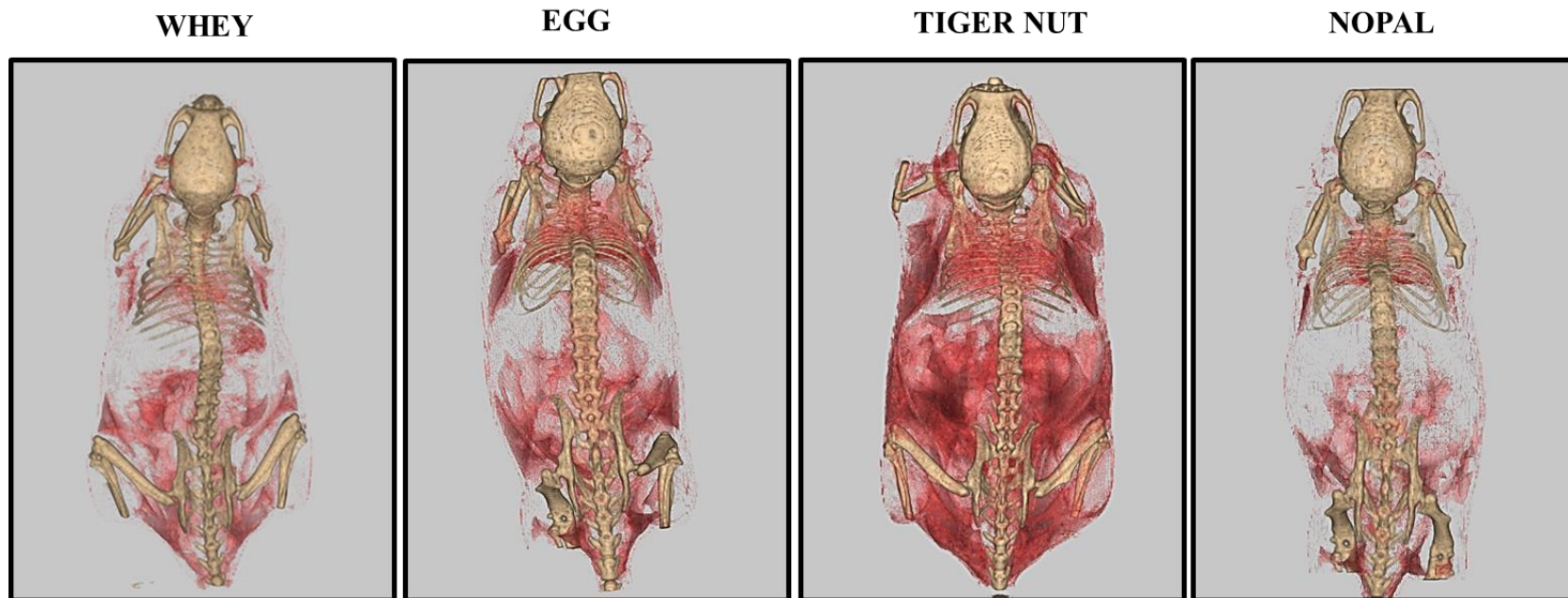


Figure 7.25. (B). CT images were acquired by using PMOD and analysed and displayed as three-dimensions (3D) using PMOD and Volview software's. Images are displayed in frontal plane (Y axis in coronal direction).

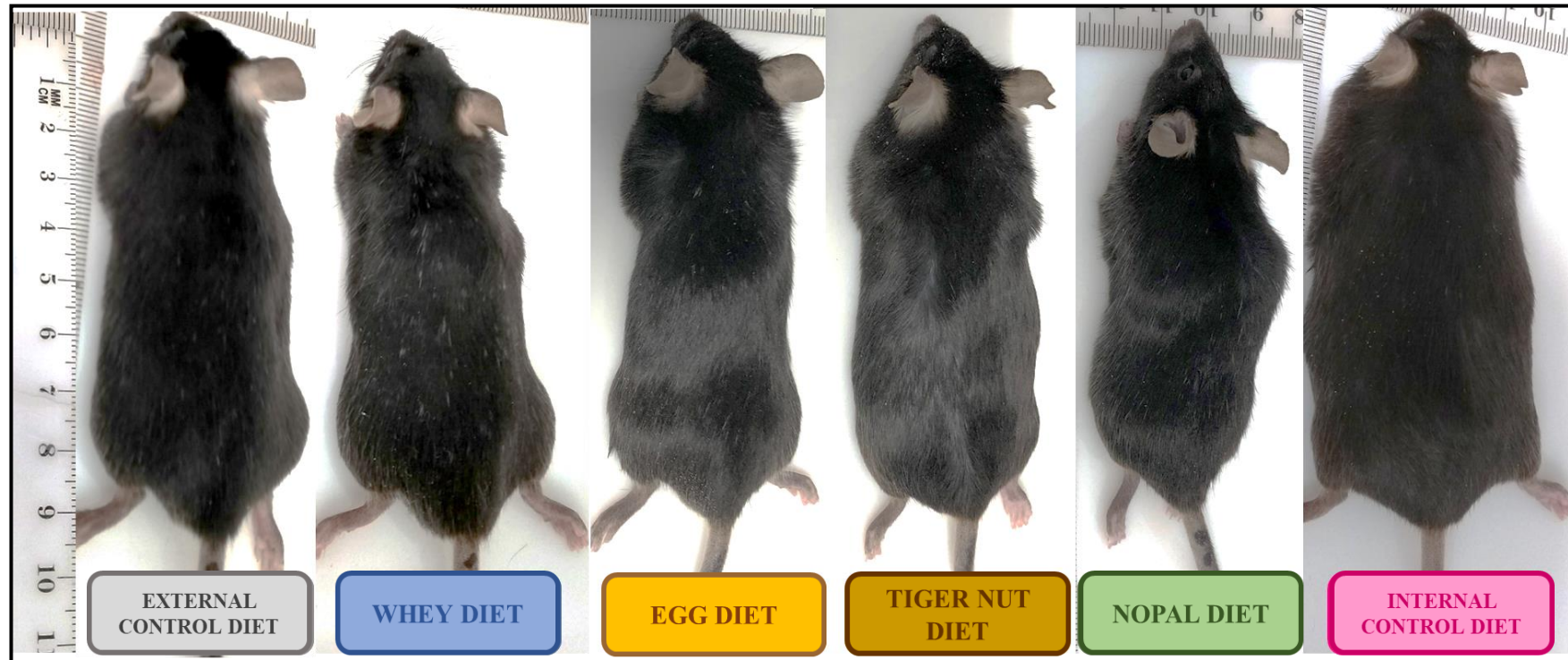


Figure 7.26. General physical constitution of animals fed with whey, egg, tiger nut, nopal and both controls just after slaughtering.

CT analysis allowed determining in an acute and non-invasive way the TV and fat deposition of animals. The fat deposition among all animal group, provided by 2D and 3D images, were evident. When fat was segmented, subcutaneous, peripheral and visceral fat was included to know more detailed the total fat composition. In 2D images from obese, TN and IC mice showed a great proportion of fat (green) related to TV (red). It can be noted the presence of adipose tissue, for instance, into the lungs because the air/tissue interface, being an artefact due to the x-ray attenuation coefficient of tissue and air. Sometimes, the organ adjacent to air bags as lungs or intestines can be recognised as adipose tissue because the large difference between HU of air (-1000 HU) and these organs (-50 and +200 HU in the case of lungs) (Lubura *et al.*, 2012, Assini *et al.*, 2015). Together with 2D images, 3D images showed a sharp vision of the localization of body fat. It can be observed the exacerbation of adipose tissue in the case of obese animals and how the fat mass was distributed in all tissues (subcutaneous, BAT, abdominal, visceral, etc). Moreover, 3D images of W, E and N groups exhibited a restricted fat deposition compared to EC, TN and DIO animals, corresponding the main fat focus to BAT (interscapular region), perirenal and epididymal. It can be observed the lesser amount of subcutaneous adipose tissue showed in the case of W, E and N groups. Additionally, total volume (TV), fat adiposse tissue and fat/TV ratio (cm³) were calculated derived of segmentation of fat and TV using PMOD VOI analysis (Table 7.7).

CT was performed in three times. At the begining of the experiment (when mice was 9 weeks), at the end of DIO phase (when mice got obese) and in the moment previous to slaughter at the end of the experiment. Furthermore, total volume, total fat and fat/TV ratio of mice in these phases were calculated. It can be observed the statistically significant difference ($p<0.05$) between animals in the adaptation period, obese stage and after dietary intervention. TV of DIO mice was significant higher than the initial stages and in the case of experimental groups, except for IC, which showed more similarties with obese animals. TV of TN was significant similar to EC and IC animals, and higher than W, E and N groups. It was quite notable the small TV of N and E groups (23.30 ± 0.96 cm³ and 26.12 ± 1.86 cm³) significantly similar to animals at the

begining of the experiment ($23.97 \pm 2.29 \text{ cm}^3$). Images of animals just after slaughtering also showed notable reductions in the size of mice belonging to W, E and N group.

Table 7.7. Total volume, total fat and total fat (TF)/total volume (TV) ratio (TF/TV) of adaptation, obese animals (DIO) and mice belonging to experimental diets ¹.

Group and phase	Total volume (cm³)	Total fat (cm³)	TF/TV ratio
Initial	23.97±2.29 ^a	2.14±0.81 ^a	0.09±0.03 ^a
Obese	37.11±0.71 ^e	13.21±1.09 ^c	0.36±0.03 ^c
Whey	28.15±1.28 ^{bc}	1.99±0.55 ^a	0.07±0.02 ^a
Egg	26.12±1.86 ^{ab}	2.96±1.28 ^a	0.11±0.05 ^a
Tiger nut	32.47±2.41 ^d	7.61±2.33 ^b	0.23±0.07 ^b
Nopal	23.30±0.96 ^a	2.50±0.64 ^a	0.11±0.03 ^a
Internal control	33.97±1.32 ^{de}	8.00±1.77 ^b	0.23±0.04 ^b
External control	30.93±2.64 ^{cd}	3.54±1.29 ^a	0.11±0.03 ^a

¹ Results were calculated as mean±S.D. (n=6 for adaptation, n=5 for DIO and n=4 for experimental diets and controls). Different letters were used to indicate significant differences within each column at $p < 0.05$.

Total fat composition was highly different among all groups. As it can be expected, obese mice showed the highest proportions of fat compared to experimental groups, which confirmed that mice achieved an adequate obese condition. Mice fed with IC and TN showed an enhanced fat mass corresponding to the highest fat deposition compared to all experimental diets, being quite remarked the differences. W, E and N animals showed the lowest proportion of fat (cm^3) and significantly comparables to EC and to initial phases of the experiment. These findings shed an idea of the great degree of fat weight lost of these experimental groups. Comparing to IC, total fat deposition of W and N was <75%; <63% in the case of E group and <5% for TN. Furthermore, a marked reduction comparing with the initial obese stage was observed, corresponding the fat reduction to <84%, <77%, <81%, <42% for W, E, N and TN groups, respectively. In relation to total fat/TV ratio, the trend was equal. The ratio followed this descended sequence: DIO animals>IC and TN>E, N, EC and W, becoming these last

ones similar to initial stages (adaptation period). Surprisingly N, E and EC shared the same ratio among themselves and also in the case of TN and IC.

An statistical analysis was performed to study the relationship between fat deposition parameters studied *in vivo* and post-mortem (*ex vivo*). Pearson’s correlations were conducted to correlate the fat composition determined *ex vivo* (post-necropsy) and *in vivo* (CT analysis) (Table 7.8.). The correlation among *in vivo* fat (cm³) measured by CT, TF/TV ratio derived from CT, weight of the animals (g) after slaughting, *ex vivo* total fat (g), including BAT and WAT, and *ex vivo* BAT and WAT were performed. A level of significance of $p < 0.01$ was found for all correlations.

Table 7.8. Pearson’s correlation test to determine the relationship between several fat deposition parameters determined *in vivo* and *ex vivo* ¹.

Correlation (r)	TF/TV ratio	Animal weight	Ex vivo total fat	WAT	BAT
<i>In vivo</i> fat	0.99	0.78	0.98	0.97	0.89
TF/TV ratio		0.72	0.97	0.96	0.89
Animal weight			0.81	0.81	0.77
<i>Ex vivo</i> total fat				1.00	0.90
WAT					0.88

p value	TF/TV ratio	Animal weight	Ex vivo total fat	WAT	BAT
<i>In vivo</i> fat (cm ³)	5.98E-19	8.05E-06	2.82E-16	8.70E-16	4.73E-09
TF/TV ratio		8.54E-05	2.06E-14	5.92E-14	4.75E-09
Animal weight			1.28E-06	1.66E-06	1.03E-05
<i>Ex vivo</i> total fat				2.84E-31	2.78E-09
WAT					1.86E-08

¹*A level of significance $p < 0.01$ was established to Pearson’s correlation test.

A significant positive correlation was determined between the fat deposition parameters studied both *in vivo* and *ex vivo*. Total fat deposition (BAT and WAT) obtained in the necropsy was strongly correlated with the measurement of fat deposition by using CT (*in vivo*) ($r = 0.98$, $p < 0.01$). *In vivo* fat measurement, determined by a non-invasive method (CT), was modestly correlated ($r = 0.78$, $p < 0.01$) with the weight of

the animal just after slaughtering. TF/TV ratio obtained from *in vivo* data showed a positive relationship when it was compared with total fat determined in the moment of the necropsy ($r= 0.97$, $p<0.01$). Additionally, WAT and BAT showed a positive relationship between fat proportion obtained *in vivo* ($r= 0.97$ and $r= 0.89$, $p<0.01$, respectively). These positive correlations revealed the effectiveness of CT technology to determine the *in vivo* total fat deposition.

3.8 Metabolic parameters by indirect calorimetry

During DIO phase and under dietary intervention respiratory quotient (RQ) was determined by indirect calorimetry in steady-state conditions. RQ is defined as the quotient between CO₂ produced and O₂ consumed by mice for an specific time (RQ = VCO₂/ VO₂). Volume of oxygen was prior measured and thereafter the following metabolic parameters were calculated: RQ, calorific value (CV) and energy expenditure (EE). Mice from DIO phase were also included (data not shown) because it was interesting to know the metabolic parameters modification after a weight gain period and subsequent dietary intervention.

Firstly, the volume of oxygen consumed (VO₂ (mL/kg BW/h) (metabolic rate)) was estimated to calculate the heat production due to nutrient oxidation (figure 7.27.). Volume of oxygen mobilised from obese animals was 2098.68±60.62 mL/kg BW/h and it was significant similar to values obtained for TN and IC. However, volume of oxygen consumed by mice fed with W was higher (3713.39±39.33 mL/kg BW/h) compared to control (IC), obese animals (DIO), TN and N group. Furthermore, VO₂ obtained from TN and IC groups was significantly similar and notably lower than other experimental diets.

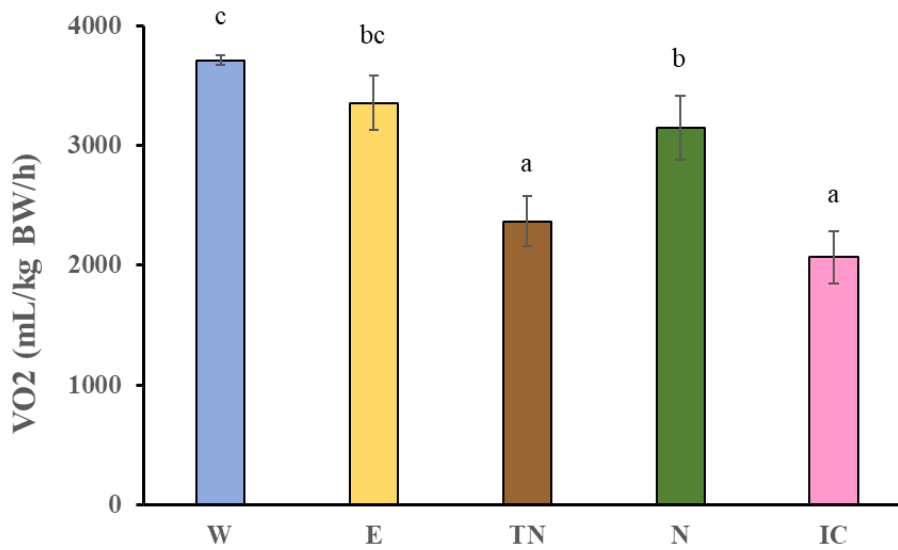


Figure 7.27. Oxygen consumption (mL) per mice expressed as a function of Kg of BW. W (whey), E (egg), TN (tiger nut), N (nopal) and IC (internal control). Data are presented as means \pm S.D. for n=3. Different letters were used to indicate statistically significant differences $p<0.05$.

After measuring VO_2 and CO_2 , their quotient was calculated (Figure 7.28.). RQ may reflex the substrate oxidation of nutrients from diet. It is worth to mentioning that RQ ranging from 0.7 and 1.0 means a fully fat and carbohydrates oxidation, respectively. Intermediate values could indicate protein oxidation (0.835) (Flatt, 1987). However protein oxidation, due to its complexity, is usually estimated by urinary nitrogen excretion (over 24 hours). RQ from obese animals showed a lower values compared to all experimental diets (0.72 ± 0.02) due to the high-fat proportions of the obese diet. When high-protein groups (W and E) were compared, similar RQ (0.87 ± 0.02 and 0.86 ± 0.02 , respectively) was obtained, being lower than the other experimental groups ($p<0.05$). These results could indicate that proteins are being oxidized and being the major fuel-substrate. However, RQ values are merely approximative about substrate oxidation, since certainly to have an accurate substrate oxidation estimation nitrogen from urine is needed. Additionally, RQ results from N, TN and control were no significantly different among themselves, showing close RQ results (from 0.93 to 0.96).

RQ derived from these last diets were increased compared to the experimental diets based on protein, thus is, W and E.

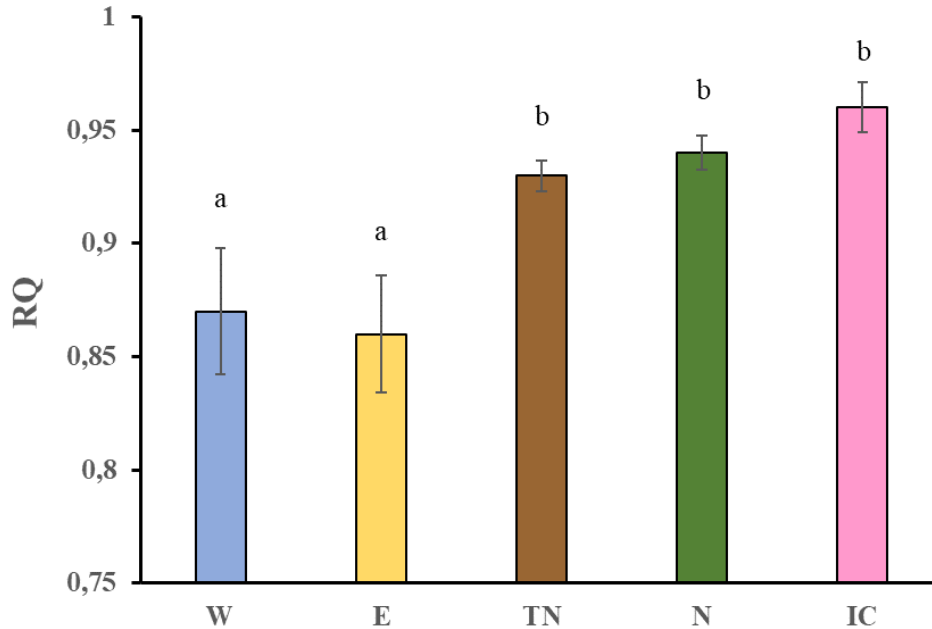


Figure 7.28. Respiratory quotient (RQ) measured individualized. W (whey), E (egg), TN (tiger nut), N (nopal) and IC (internal control). Data are presented as means±S.D. for n=3. Different letters were used to indicate statistically significant differences at $p<0.05$.

Lastly, EE (Kcal/Kg BW/h) could be calculated (Figure 7.29.) based on individualized VO_2 . EE related to BW in the case of obese mice were decreased respect to W, E and N diets. However, EE from DIO was significant similar to values obtained in TN and IC groups. W, E and N diet showed increased values of EE being similar among them. It was remarkable the difference respect to IC, being the EE of W group considerably higher. Similar to previous parameters measured, no significant differences in EE were found comparing TN and IC diets (11.64 ± 1.07 and 10.03 ± 1.37 Kcal/Kg BW/h, respectively).

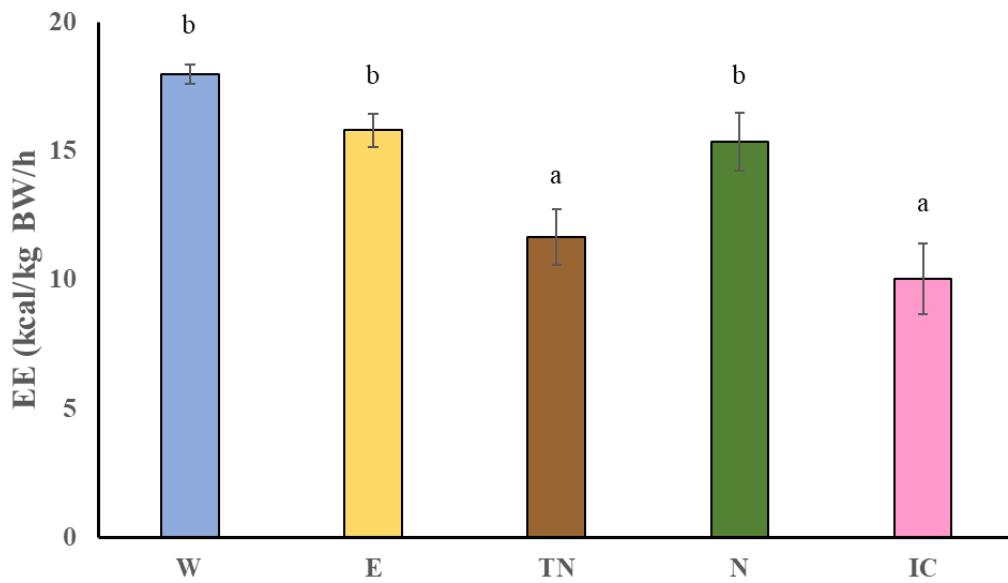


Figure 7.29. Energy expenditure (EE) calculated as a function of Kg of BW (Kcal/Kg BW/h). W (whey), E (egg), TN (tiger nut), N (nopal) and IC (internal control). Data are presented as means±S.D. for n=3. Different letters were used to indicate statistically significant differences at $p<0.05$.

3.9 Blood lipids analysis

Fasting serum total cholesterol (TC) of mice subjected to intervention diets can be observed in figure 7.30. Total cholesterol level was the highest when animals were fattened for 4 weeks with a high-fat diet (obese data not shown), corresponding to 208.5 ± 2.12 mg/dL. This result was taken into account in the core dataset to compare the effect of several intervention diet after obesity induction stage.

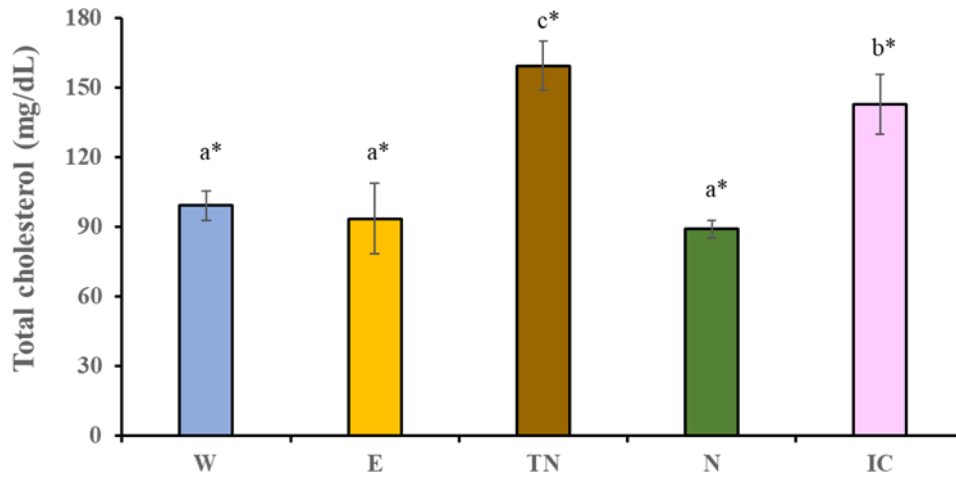


Figure 7.30. Total serum cholesterol (TC) (mg/dL) of n=8. Results are expressed as a mean±S.D. Different letters were used to indicate significant differences among several experimental diets ($p<0.05$). * indicates significant differences respect obese stage ($p<0.05$).

Animals over DIO phase were fed with a high-fat diet and the great cholesterol increase was the reflex of this type of diet, as well as the weight gain and fat accumulation. External control, which was fed with a standard diet during whole experiment, was also analysed obtaining 125.5 ± 8.58 mg/dL of TC. This result was similar to a reference of biochemical parameters using the same strain of mice at 16 weeks old and using the same method for blood extraction (124.57 ± 12.88 mg/dL) (Fernandez *et al.*, 2010).

All experimental diets showed significant TC reductions respect their previous obese condition. Furthermore, total cholesterol concentrations after 11 weeks of dietary intervention showed significant differences among all types of experimental diets. Total cholesterol from TN diet, although it showed a significant reduction compared to DIO phase, presented undesirable level of cholesterol concentrations (159.42 ± 10.59 mg/dL). It was significantly higher than in the case of W, E and N diets and, even than IC diet (143 ± 12.87 mg/dL). Interestingly, serum TC resulted from W (99.14 ± 6.30), E (93.55 ± 15.13) and N (89 ± 3.81) diets were under the levels showed in the case of EC (fed with standard diet with lower caloric content), and much lower than DIO stage.

The TC reductions of W, E and N groups related to IC were <30%, <34% and <37%, respectively. Comparing TC levels of experimental diets to obese stage the decreases of TC were <52% for W, <55% for E, <23% for TN and <57% for N.

Additionally, low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were analysed in serum of mice fed with experimental diets (Figure 7.31.).

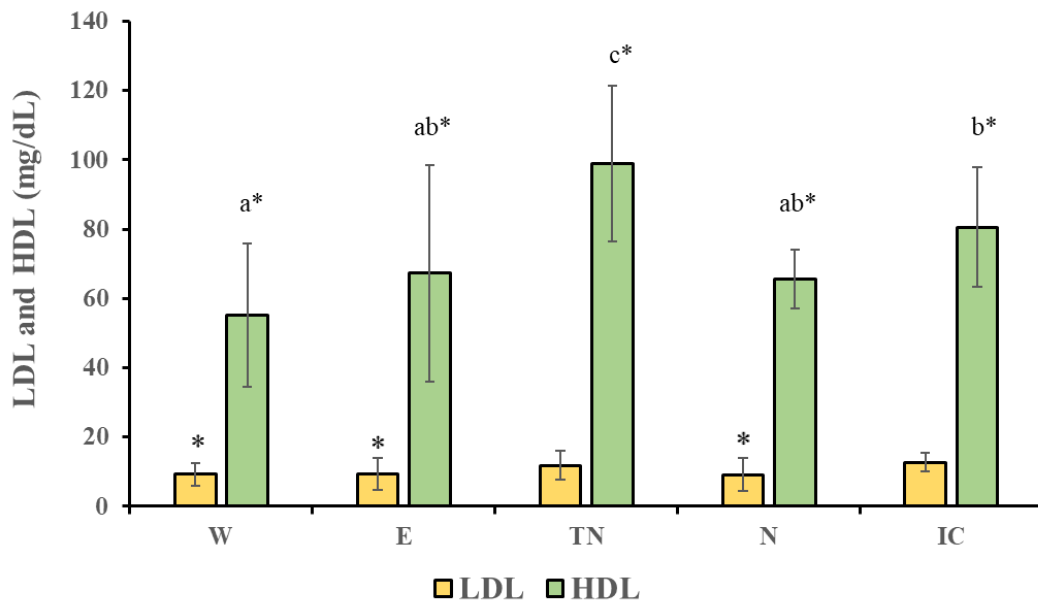


Figure 7.31. LDL and HDL cholesterol (mg/dL) of n=8. Results are expressed as a mean±S.D. Different letters were used to indicate significant differences among several experimental diets ($p<0.05$). * indicates significant differences respect obese stage ($p<0.05$).

Newly, data of HDL-C showed by obese animals were significantly higher (130.41 ± 2.12 mg/dL) compared to experimental diets. Furthermore, comparing the effect of different diets on HDL-C, great differences were found. TN showed the highest level of HDL-C, meanwhile W diet produced the lowest level. IC showed statistically similar results to E and N groups. No significant differences were found in LDL-C concentrations among assessed diets. However, the comparison between

intervention diets and DIO animals (18.4 ± 2.12 mg/dL) revealed that W, E and N showed significant decreases respect obese stages, approximately minor than 50%.

3.10 Hormone analysis

Plasma hormone concentrations after DIO stage and through 11 weeks of dietary intervention were determined (Figure 7.32.). Concretely, GIP and amylin were analysed by using metabolic magnetic bead panel (Luminex analyser MAGPIX), and enzyme immune-assay in the case of CCK.

GIP was significant higher in DIO phase (160.02 ± 12.65 pg/mL) compared to experimental diets, but not different to IC group (149.30 ± 38.01 pg/mL). No significant differences were found comparing GIP levels among W, E, TN and N diets. However, it was notable that IC showed GIP values high increased compared to N and E groups.

W, TN and N showed significant lower levels of amylin compared to IC, meanwhile egg white diet presented increased values (134.79 ± 19.68 pg/mL) respect to TN, and significant similar to IC (172.25 ± 27.61 pg/mL).

Values of CCK (pg/mL) were the highest in the case of W and N groups (49.97 ± 3.9 and 52.24 ± 1.68 pg/mL, respectively). CCK level in W and N groups was quite higher than IC group (15.94 ± 1.22 pg/mL). E, TN and both controls presented a notable decreased concentrations compared to W and N groups.

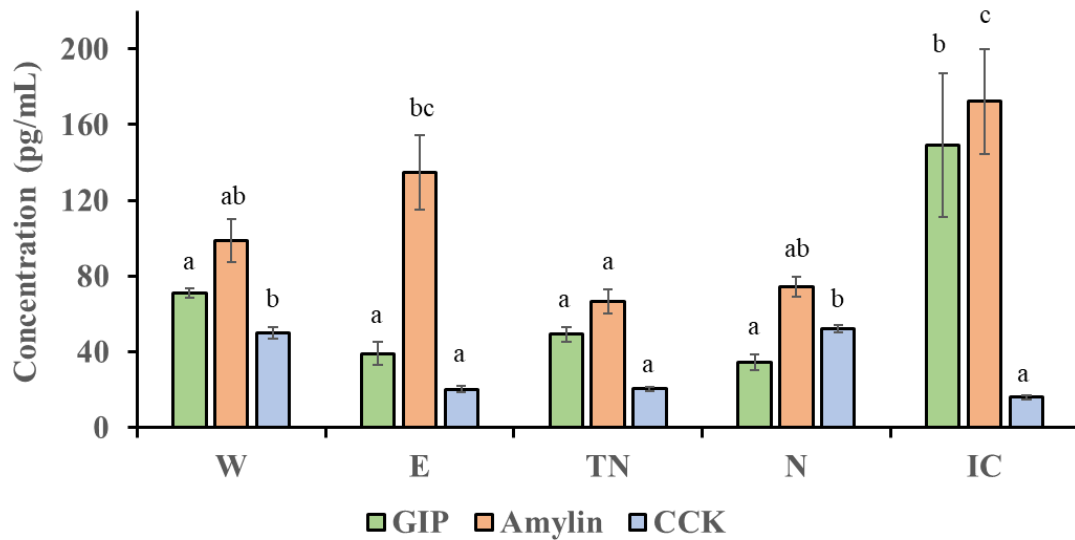


Figure 7.32. GIP, amylin and CCK plasma concentration (pg/mL). Results are expressed as a mean±S.E.M. (n=8). Different letters were used to indicate significant differences in hormone concentration among several experimental diets ($p < 0.05$).

4. DISCUSSION

4.1 Body weight gain, food and fluid intake under DIO phase

Mice fed with a high fat diet (60% of energy provided from fat) gained approximately 0.32 gram per day. The average food intake was slightly lower than in the case of animals fed with standard chow diet (EC), but the energy intake was greatly higher.

The use this type of strain obesity related to high-fat diet has been proposed previously. C57BL/6J mice have showed suitable features to develop metabolic syndrome, which includes obesity, hyperinsulinemia, hyperglycaemia and hypertension. However, an interesting singularity of this strain is that when a restricted diet was returned, animals become lean. In addition, the progression of the metabolic syndrome in this strain of mice has been similar to human (Collins *et al.*, 2004).

The main mechanism that explain the acute BW gain after 31 days can be explained apart from the hyperphagia, because the low physical activity and by the high feed efficiency showed (ratio between BW gained and calories intakes) (Collins *et al.*, 2004, Brownlow *et al.*, 1996, Parekh *et al.*, 1998). Similar daily average of BW gain (0.32 g/d) was related by other authors who considered DIO with a BW gain of 7 grams or more over 21 days (Stanton *et al.*, 2011).

4.2 Body weight, food and fluid intake of external control (EC) mice group during experiment

External control showed a BW gain during the whole duration of the experiment of 0.5 grams per week, corresponding the weight gain to 8.05 g for the total experiment (17 weeks). It is worth considering how was the “normal” weight of C57BL/6J mice, understanding “normal” as animals not subjected to dietary interventions. Furthermore, if just the time of dietary intervention carried in parallel for experimental groups is taking into account, mice of EC gained 4.27 ± 0.33 g for 11 weeks. These data results quite interesting since it revealed that the standard BW gain of animals belonging C57BL/6J and 23 weeks of age should weight 33.9 ± 0.33 g. The general tendency of this mice strain was to fatten over the time, so mice got overweight as they age. In addition, the physiological summary data provided by The Jackson Laboratory for C57BL/6J mouse at the age of 24 weeks was 34.0 ± 2.7 g, being similar than in our case (The Jackson Laboratory, 2019).

4.3. Body weight, food and energy intake of experimental groups under dietary intervention

Several researches have been conducted sharing a similar experimental design to our study. Prior, mice were fattened for four weeks. When animals were gained 10 grams (approximately 37% of BW), different types of isoenergetic and energy-restricted diets related to DIO were supplied. The percentage of energy provided by the experimental diets supposed a restriction about 30% respect obese diet.

The first week, mice fed with W, E, N and TN diets showed a reduction of food intake regarding to previous obese diet. In addition, it is worth mentioning that all experimented diet, including IC, showed a reduction on food intake specially the first week of the experiment and subsequent BW reductions. This effect was mainly due to the differences in palatable and energy densities comparing to high-fat diet received previously. This effect has been widely described by other researches, especially in the case of diets based on proteins. All studies concluded that a necessary adaptation to the ingestion of new diet due to differences in previous diet composition.

In relation to a possible taste aversion, concretely with high protein diets, it was not observed in the present study since normalised ingestion was achieved apart from visualised the feeding pattern under recording sessions. Moreover, the proportions of proteins used in the present study have been within a non-taste aversion rate (50% and 70%), which have been proved to not induce this effect in rats (Bensaid *et al.*, 2003, L'Heureux-Bouron *et al.*, 2004).

During a long-term dietary intervention (11 weeks), differences in food intake and hence, in energy intake was observed. Concretely, mice fed with high-protein diets (W and E) and N diet showed a reduced food and energy intake compared to TN and IC diets. Overall results of this study have proved that these groups of animals showed a reduction of food intake (monitored by recording) by lower number of meals in case of W and N groups and increased inter-meal interval in case of W, E and N diets. In addition to features of feeding pattern that suggest more satiety and satiation in these groups, it has also been supported with postprandial satiating hormone secretion as CCK or the suppression of orexigenic peptides as GIP. Furthermore, pre-absorptive factors such as an elevated water intake in these three groups could influence further food ingestion as below is explained. These results could be explained by the satiating potential demonstrated by high-protein diets (W and E groups) and because the hunger suppression effect of fibre and phenolic compound showed in the case of N diet.

Focusing in food and energy intake results, W, E and N mice presented lesser values respect to TN and IC. As it has mentioned, protein is the macronutrient with most satiating potential, suppressing food intake and the later meal to major extent than carbohydrates or fats (Lejeune *et al.*, 2006, Veldhorst *et al.*, 2008, Smeets *et al.*, 2008).

This suggestion was correlated with the present findings, since whey and egg protein diets elicited a similar and a great potential suppressing food intake. Moreover, high-diet protein diets affect both short and long-term satiety, as this study has demonstrated. This mechanism could be partly explained because protein enhances thermogenesis, increases of fat-free mass and lowering energy efficiency in overfeeding conditions (Westerterp-Plantenga, 2008, Westerterp-Plantenga and Lejeune, 2005, Halton and Hu, 2004). According to our results, several researches have proved the decrease on food intake of high-protein diets. Freudenberg *et al.*, (2012) showed that C57BL/6J mice fed with 50% of protein for 20 weeks, presented a reduced food intake compared to mice fed with adequate protein (10%). Similar to the present experiment, they assessed isocaloric diets, and the daily energy intake of animals provided with high-protein diets was lower than normal protein group (52.4 ± 0.5 vs 63.2 ± 0.7 kJ/d) (Freudenberg *et al.*, 2012). These authors explained the reduced energy intake showed by high-protein groups because the satiating potential of proteins specifically due to the leucine amino acid. Although differences in satiety according to the type of protein source have been proposed (Anderson *et al.*, 2004, Gilbert *et al.*, 2011), none differences on food or energy intake, comparing whey or egg white proteins, have been described in the present study. However, several researches have reported differences comparing whey and white egg protein, differently than our results. For instance, Pal *et al.*, (2010) showed a lower rating of hunger and a reduced food intake after a whey meal compared to tuna, turkey or egg meals. That experiment was conducted in humans who received four protein meals in separates occasions. In contrast, the present experiment was performed over 11 weeks and using an animal model (Pal and Ellis, 2010). Moreover, Anderson *et al.*, (2004) showed an increased cumulative energy intake after egg albumen preload compared to whey protein, producing the last one a suppression of food intake for 2 hours.

Furthermore, the effect of fibres on suppressing food intake have been widely hypothesised (Blundell and Burley, 1987). Parnell *et al.*, (2012) demonstrated that high-fibre diets (10% and 20%) decreased food intake dose-dependently and consequently reduced body weight (Parnell and Reimer, 2012). The effect has been related mainly with the viscosity, water-binding capacities and fermentability of fibres (Tan *et al.*, 2016). Separating two concepts, satiety and satiation, several researches have suggested that satiety and food intake inhibition may be related to its viscous properties, meanwhile satiation and gastric fullness more associated to its bulk properties (Burley *et al.*, 1987, Gustafsson *et al.*, 1995, Rigaud *et al.*, 1998). Furthermore, the satiating potential of fibres continues beyond postabsorptive status since, the fermentation of them into colon by gut bacteria led to the production of SCFA and satiating peptide releasing (Brown *et al.*, 2003, Tolhurst *et al.*, 2012). The effects of high volume produced into gastro intestinal tract by fibres may reduce gastric emptying and gut transit time, which affects satiating signals such as CCK. It is well known that fibres in the gut affect the absorption of nutrients such as fats and carbohydrates. These disruption of the absorption lead to prolong the time needed to these macronutrient would trigger satiety signals (Burton-Freeman, 2000).

Regarding to the reduced energy intake showed by mice fed with *Opuntia* powder, several researches have found similar results in animals models (Sanchez-Tapia *et al.*, 2017). Tan *et al.*, (2016) showed a decreased food intake in rats fed with high concentrations of fibre, mainly due to the bulk and viscosity capacities and the postprandial releasing of satiating hormones related to SCFA production (Tan *et al.*, 2016). However, Cysneiros *et al.*, (2018) only showed reductions of food intake in rats by using nopal supplementation the first week of the experiment, of the total of 15 weeks. Despite to the modest inhibition of food intake, they demonstrated great BW reductions (Cysneiros *et al.*, 2018). These results was explained by the especific composition of nopal, concretely, alkaloids and flavonoids (Yoon *et al.*, 2011). In addition, several phenolic compound presents in nopal could be contributed together with fibre to reduce food intake and increase water ingestion. It has been proved the effect of phenolic compound by reducing the secretion of orexigenic hormones as amylin and ghrelin and contrarily increasing satiating hormones as CCK, GLP-1 (Panickar, 2013).

However, tiger nut diet did not show a significant reduction in food intake and energy intake over 11 weeks of experiment. The firsts weeks of experiment animals fed with “chufa” showed a reduction in food intake. However, considering cumulative food intake over 11 weeks, these decreases were compensated by a subsequent increased in consumption. In addition, tiger nut showed a great percentage of dietary fibre, but also a high concentration in fats and high GI. All together could depress the satiating effect of fibre as results have shown.

Regarding to BW loss, results have showed interesting findings. Certainly, the highest BW reductions have been showed for animals fed with a high-protein diets as well as nopal group. Moreover, not only BW reductions have been proved, since a maintenance of BW has been showed. Furthermore, all experimental groups showed variations of BW respect to initial stages. None of groups exhibited increases of BW respect to the age-related weight (4 grams). In addition, none group showed increases respect to IC, which suggest the effectiveness of diets on reducing BW. Under dietary intervention, non-treated mice (IC), experimented a BW reduction from first to third week, being then stabilised and some increased until week 11. Differently to IC, all experimental diets showed decreases of BW from first to third or fifth week, to a minor or greater extent. From this point W, E and N groups maintained BW. However, mice fed with tiger nut experimented an augmentation of BW, which may be reflected in the total BW gain respect to other diets. These findings suggest that TN produced a modest, but not negligible effect on BW reduction. Following this argument, animal fed with “chufa” should gain at least 4 grams over 11 weeks (similar to EC) or about 3 grams (similarly to IC). Nevertheless, this group showed a BW gain just of 0.78 grams, remaining almost equal that initial stages. These data were due the reduction of BW experimented until the half of the experiment, since from first week to fifth week mice loss about 5 grams of BW. Overall findings indicate that “chufa” can be an interesting ingredient to promote BW reductions in short-term, since in a long-term, tiger nut was not able to maintain the loss of weight until the end of the experiment. With this respect, only one study has revealed the potential role of tiger nut on BW management, concretely, Moon *et al.*, (2012) showed that a supplementation (10%) of defatted tiger nut in mice fed with a high-fat diet, produced a significant reduction of body-weight gain (Moon *et al.*, 2012). This result can not be compared to our results because they

used deffated chufa differently to our experiment. The modest effects of tiger nut in BW reductions could be related to their content in fibre. However, its high GI ranging from 83.3 to 95.9% (Ijarotimi *et al.*, 2018), togheter with its increased fat concentration, providing more than 32% of energy, could led to slight reductions of BW compared to other type of diets. However, future analysis will be conducted to know the specific role of fibre from chufa in the production of SCFA and the subsequent satiating hormone releasing.

4.4. Evaluation of urine and faecal content joint to the cumulative water inatke

The supplementation of different ingredients such as proteins and fibres into the diets was reflected on the subsequent urine and faecal excretion. 24-h water intake per mice was higher in the case of N, followed by W and E, being lesser in TN and IC. The urine excretion was the highest in the case of W and E, but surprisingly, N was slightly lower, but higher than in the case of TN and IC.

It is well-known that water requirements can vary depending on nutrient composition of the diet. Specifically, high-protein diets enhance thirsty sensation compared to other macronutrients such as carbohydrates and fats (Kaunitz *et al.*, 1956). Le Magnen (1992) showed that the ratio of millilitres of water intake to grams of food ingested was highly increased in the case of consumption of high-proteins diets rather than high-carbohydrates or high-fat diets (Magnen, 1992). These findings were in line with the results of cumulative water ingestion in the case of high-protein diets compared to TN and IC. The cause may be due the physicochemical features of proteins since them require high amount of water to be mixed into the mouth. However, our results showed that N diet produced an increased intake of water compared to W and E. A balance between liquid input and output may explain why N group showed the highest water intake and not the highest urine excretion. These increased water ingestions were further excreted by urine due to the diuretic effect of nopal and eliminated in form of retained water into faeces due to the high fibre content of *Opuntia*. Certainly, these excessive water intakes observed in three groups may be related with a lower desire to eat since the great volume of water into the stomach.

High urinations and acidic urine were observed in the case of high-protein diets by measured with a urine analysis strip compared to other diets. Together with the high excretion of urine, other researches have showed similar results. A lower pH and a great urine excretion have been described after the supplementation with casein in rats (Amanzadeh *et al.*, 2003). The cause of the exacerbated urine excretion matched with the high osmolarity load of high-protein diets which may led to a high excretion of urine together with a higher thirsty sensation.

Followed by two high-protein diets, N group showed a great volume of urine. The explanation can be due to the diuresis effect of nopal. Rats treated with infusions made from cladodes, fruits or flowers of *Opuntia* showed an acute diuresis and uric acid excretion after intervention. The diuretic effect was mediated though the effect of K⁺ ions and other compounds as flavonoids and ascorbic acid, which may stimulate the diuretic effect (Galati *et al.*, 2002). Furthermore, high-fibre diets could enhance water ingestion due to an excess of water retained into faeces, existing a relationship between redistribution of water into faeces and water intake. For instance, it has been proved that dogs fed with high percentages of sugar-beet fibre increased water consumption 50%, using guar gum 18% and with inulin 39% (Diez *et al.*, 1998).

Certainly, an enhanced faecal excretion measured as daily increasement of faecal mass into the metabolic cage was found in the case of high-fibre diets (N and TN) compared to control or high-proteins diets assayed in the present experiment. It is worth mentioning that faeces were measured directly without being dried, then, the real mass faecal volume was weighted. Both high-fibre diets, N and TN, provided high concentrations of soluble and insoluble fibres, being the total amount major in the case of N (12% and 16%, respectively). A higher increments of faecal material have been marked in the case of N than TN diet. This results was due the difference in the fibre concentration of each diet. Fibres increases the faecal bulk since their water holding capacity being more voluminous and containing more amount of water (Shimotoyodome *et al.*, 2005). Interestingly, a crossover clinical investigation in human volunteers showed an exacerbated fat excretion into faeces due the high fibre content and calcium of nopal (Uebelhack *et al.*, 2014). This increase in volume and frequency could be related to an increased fat excretion, in line with the body weight reductions

showed by this group. Other experimental diets showed a decreased faecal excretion compared with these groups because a lower percentage of fibre composed these diets (5%).

4.5 Feeding pattern assessment

With the purpose of knowing in a deeply manner how different dietary intervention could affect satiation and satiety, the feeding behaviour of animals was monitored. In human studies, the use of visual analogue scales (VAS) to determine appetite profile, desire to eat, fullness sensation and satiety is often useful (Veldhorst *et al.*, 2009). However, in animal models, appetite profile could be less readily noticed. A suitable strategy to assay the feeding pattern in animals can be defined by several parameters as meal number, meal size, inter-meal interval (IMI) and duration of each meal. Several studies have proved its usefulness using animal models (Burton-Freeman *et al.*, 1997, Castonguay *et al.*, 1986). In line with this argument, satiation can be defined as the process that brings eating to an end, in fact it is so-called as intra-meal satiety (Tremblay and Bellisle, 2015). Differently, satiety is the inhibitory mechanism that leads to cessation of further eating. Satiety prevents the return of hunger and increases fullness sensation after a meal has finished (inter-meal satiety) (Blundell *et al.*, 2001). Whereas satiation refers to meal duration, satiety is more related to the inter-meal interval (Ayaso *et al.*, 2014). In a practical manner, an enhanced satiety is translated in terms of increased IMI as well as reductions in meal number; whereas satiation is indicated more by the reduced meal size and meal time (Ayaso *et al.*, 2014).

Focusing on the present results, in general, feeding pattern parameters have been within the similar ranges showed by other researches (Girardet *et al.*, 2011, Ayaso *et al.*, 2014, Yu *et al.*, 2009). When animals were obese, a shorter meal duration related to experimental groups was due mainly to the buttery consistency of the high-fat diet. In addition, IMI value was considerably lowest in obese animals. Bake *et al.*, (2014) showed that mice fed with a high-fat palatable diet, a reduction in the control of food intake was quite marked caused by a strong hyperphagia (Bake *et al.*, 2014).

Feeding pattern of IC group was considered as a reliable start point due to this group was supplemented with a chow diet in isocaloric condition with respect experimental diets. In general, a reduced IMI and superior meal number have be found for IC compared to W and N diet nd TN diet, respectively. These findings suggested that W, N and TN showed more satiating potential, in diferent degrees, compared to IC. Furthermore, E diet showed a number of meals lower than IC which confirm the satiation potential of this ingredient respect to control.

Diet based on goat whey protein gave place to a desirable satiating profile due to two key points: increased IMI and reduced meal number. These results matched with the higher secretagogue potential whey on CCK showed in the present study. Quite similars results have been showed by other authors who used the same strain of mice than our study and percentages of energy provided by whey protein of 35% compared to soy and gluten protein. These researches manifested that animals fed with whey also showed an increased average duration of each meal (Yu *et al.*, 2009). This was coincident with our experiment where mice belonging W spent more time in each meal compared to E and EC. Furtermore, other researches proved that diets based on proteins had a strongest satiating potential (Bensaid *et al.*, 2002).

Under 12-h of nocturnal recording and taking into account IMI values, whey diets elicited more satiating potential than other groups including egg, except in the case of nopal. In this sense, differences in IMI has been proved when two proteins have been tested (soy and whey) being the hypothesis that the source of protein may vary satiating potential of the diet correlated with our study (Yu *et al.*, 2009). Additionally, the reduced number of meals can be translated in terms of enhanced satiety. Increases of meal duration also was observed, that could suggest lesser satiation. However, the cause may be expalined to causes more related to the functional features of whey protein. Several authors showed similar increases in time duration of meals due to the high viscosity of pure protein that could lead to spend more consuming-time in each meal (Yu *et al.*, 2009, Ye *et al.*, 2004). Other researches have showed the same pattern using a high-protein preload which led to an increased duration of meal, reduced number of meals and then, decreases of the ingestion rate (Bensaid *et al.*, 2002).

Diet based on egg white powder was similar in terms of protein concentration than W diet, but changing the protein source. Different results of feeding pattern has been found in the present study. Several researches have noted differences on satiety according the type of protein (Anderson *et al.*, 2004, Hall *et al.*, 2003, Pal and Ellis, 2010). Particularly, Anderson *et al.*, (2004) proved a more sustained supression of food intake (60 min and 2 h later) after whey preload compared to egg albumen in young men. Despite the differences, egg diet showed an interesting satiating profile. These group of animals demonstrated to consume lesser time eating than all diets, which suggest an increased satiation. Nevertheless, decreased in IMI revealed lower satiating potential compared to nopal or whey diets. Moreover, its increased number of meals (quite similar to obese animals) could suggest lesser satiety effect compared to W and TN diets. As main differences, we found more satiation potential and lesser satiety features in E than W protein. These particular behaviour led to mice to supress the ongoing intake within the meal ingestion. Despite the increased meal frequency of E diet, the IMI, taking as a marker of satiety, was prolonged compared to TN diet, which suggested that E diet exhibited more satiating potential than these group.

Differences between two protein groups could be explained because the specific origin of protein can lead to trigger of satiety signals leading to more or less satiety features. Several authors have demonstrated, in humans, similar differences after diet supplementation with egg or whey proteins, showing the last one a reduced rating of hunger compared to egg albumin diet, as previously was mentioned. One explanation to answer this question could be found in the large variation in amino acids composition of proteins such as phenylalanine, lysine and tryptophan. For instance, the amino acid lysine has been associated with reductions in body weight gain, decreases in food intake and in meal number mediated by CCK and GLP-1 stimulation (Tome *et al.*, 2009, Ayaso *et al.*, 2014). The effect of phenylalanine showing great fullness sensations mediated by CCK stimulation, has been also proved in humans (Ballinger and Clark, 1994). Additionally, tryptophan supplementation has been linked with reductions in food efficiency and food intake mediated by stimulation of serotonin in the brain, which led to decrease the meal number and increased satiety (Leibowitz and Alexander, 1998, Ayaso *et al.*, 2014). Moreover, the role of tryptophan has not related only with serotonin stimulation since it has showed an stimulatory effect on the releasing of gut peptides as

CCK and GLP-1, thus reducing meal number (Tome *et al.*, 2009). With respect to amino acid profile of whey and egg, higher concentrations in these three amino acids have been found in the case of whey proteins compared to egg proteins (Delaney, 1976, Lewis *et al.*, 1950, Pal and Ellis, 2010). Differences in feeding pattern of both high-protein experimental diets could be mainly explained by amino acid composition, rather than on CCK and amylin releasing. However, cumulative energy intake over 11 weeks of experiment has not been significant different between E and W diet, suggesting the complexity of satiety, including other satiating hormones, features of protein source, social factors, etc.

Tiger nut diet showed weak results in terms of satiety. The meal time duration was similar to other diets, except E diet. However, the number of meals was as reduced as W and N diet, but being the IMI duration markedly reduced. These data support the idea that TN diet provided a modest satiation compared to E diet and lesser satiety compared to N and W diets. These finding were in part related to hormone stimulation determined in this study. The lower amylin concentrations compared to E group can be related with a reduced satiation and the lower levels of CCK with a poor satiety compared to N and W diets. It may be the high percentage of fat and high GI on tiger nut which made imposible to trigger a satiating response. In fact, it has been demonstrated that exist an inverse relationship between GI-insulin index and satiety-CCK secretion (Holt *et al.*, 1992).

Diet based on nopal showed a feeding pattern more similar to W diet than other types of diet, even the high-protein diet based on egg white. Meal number was reduced compared to control and IMI prolonged, which togheter, supported the theory that nopal promotes satiety. As in the case of whey, feeding pattern fits perfectly with plasma CCK data, suggesting that the main mechanism of food intake suppression have been mediated by this gut peptide. The main explanation for the food intake inhibiton of nopal was the same that for CCK stimulation, thus, due its composition in polyphenol and fibre (Al Shukor *et al.*, 2016, Bourdon *et al.*, 2001). Furthermore, several researches have demonstrated the suppressive effect of fibres on food intake in rats. According to our results, high concentrations of fibres produced an increased IMI and lower meal numbers than a control diet (Tan *et al.*, 2016).

4.6 Necropsy: organs collection analysis.

The real capacity of the stomach could not be measured directly due to the low size of gastric mice. However, gastric area and weight were determined to estimate its theoretical capacity. These two parameters were just higher in the case of TN compared to control.

Satiety and the regulation of food intake are mediated by complex signals, which included satiating peptides and the gastric capacity, among many others. Stomach acts as a reservoir to limited or extend food intake. More stomach capacity requires more amount of food intake to achieve satiety signals (Geliebter *et al.*, 2004, Geliebter, 1988). Moreover, a higher gastric capacity has been described in obese subjects compared to non-obese (Granstrom and Backman, 1985, Geliebter, 1988). However, animals fed with tiger nut did not show more food intake than IC group despite the stomach differences between two groups.

The relative weight of the liver was higher in all experimental groups compared to IC. Comparing liver weight with a reference (4.21 ± 0.38 % of BW) we can observed that relative weight of the liver were under the normal range, although being slightly higher and lower in the case of W and IC, respectively (The Jackson Laboratory, 2019). There is a relationship between high-protein diets and increases of liver weight. Freudenberg *et al.*, (2012) demonstrated that C57BL/6J mice fed under high-protein conditions (50% w/w) showed increases in the relative liver weight (g/100 g BW) compared to adequate-protein diet (10% w/w). These authors showed relative liver weights similar to our results, being the weight of liver >15% higher in high-protein mice than control mice. However, these animals fed with a high-protein diet showed decreased of triglycerides and glycogen concentrations into the liver; as well as a decreased liver lipogenesis (Freudenberg *et al.*, 2012). Noatsch *et al.*, (2011) also found an association between higher liver weight and high-protein diet in C57BL/6J mice. They did not found triglycerides or glycogen concentrations higher in the case of high-protein diets in accordance with previous authors (Noatsch *et al.*, 2011). Metabolites of nitrogen into the liver could be available to promote hepatocyte hypertrophy using these types of diets (Schoknecht and Pond, 1993). Other researchers suggest the importance

of liver in energy expenditure, being approximately 5-10% of the total energy expenditure (Baldwin *et al.*, 1980). These increases could be related to higher energy expenditure showed in the case of high-protein diets and also in the case of N diet. Our results showed a clear trend to be higher in the case of W and E diet, however, the first group showed a higher relative weight of the liver than second group. Several authors have showed differences in liver weight comparing two sources of proteins, concretely, whey and soy. These researches proved that weights of livers from obese mice fed with 35% of energy from whey hydrolysates was higher to soy hydrolysates (Aoyama *et al.*, 2000).

The relative soleus muscle weight of W group was similar to E and N group, but higher than the rest of mice. Similar to our results, other authors have been shown that after a high-protein supplementation using C57BL/6J, the final body fat was reduced compared with an adequate protein diet for 2 weeks and the total lean body weight was increased compared to control. The explanation to these lean or muscle development was due by leucine amino acid. This amino acid led to stimulate muscle protein synthesis via mammalian target rapamycin (mTOR) pathways (Freudenberg *et al.*, 2012). Other research demonstrated increases of 7.4% of body lean and 32% of body fat reduction after 11 weeks of high-protein diet. The results were explained by the action of leucine too (Shertzer *et al.*, 2011). The supplementation of leucine alone, using a low-protein diet, has proved to stimulate the protein synthesis in skeletal muscle and most visceral tissues using neonatal pig as an animal model (Torrazza *et al.*, 2010).

Apart from leucine, the role of glutamine in protein synthesis and inhibition of catabolism in muscle has been reported (Jepson *et al.*, 1988). Glutamine has shown increase the muscle glycogen leading to increasing the muscular strength and potential (Williams, 2005). High content in leucine has been obtained in the composition of goat whey protein and egg white powder corresponding to 8.62 mg/100 g and 6.8 mg/100 g, respectively. Nopal powder showed decreased levels of leucine compared to protein ingredients (1.3 mg/100 g). However, increased levels of glutamine have been described in nopal (17.3 mg/100 g) (Stintzing and Carle, 2005) and whey (14.23 mg/100 g) compared to egg white (10.5 mg/100 g). These concentrations may explain the increased weights of soleus in case of W and N respect to E group.

The relative total fat deposition (g/100 g BW), named WAT (white adipose tissue) was calculated by weighing retroperitoneal, perirenal and epididymal fat after dissection. Moreover, BAT was subtracted from interscapular area and hence weighted. These two fatty deposits are involved in the regulation of energy homeostasis: energy intake and energy expenditure. However, the functions of each of them are different. WAT is an energy storage and it is involved in hormone (insulin, leptin, etcetera) and cytokine secretion to modulate finally BW. Excess of WAT, unequivocally, lead to obesity. Conversely, BAT is considered as a thermogenic tissue and it is responsible to adaptative thermogenesis in response to DIT or cold (Gao *et al.*, 2009). Moreover, BAT is inversely related with body-weight gain (Cypess *et al.*, 2009). A decrease in the total fat deposition including, WAT and BAT, was found in W, E and N groups respect to TN and IC. The highest increase of BAT was found in the case of TN and IC being consequence of the general increments of fat, affecting both fat pad, WAT and BAT. It may be explained because more caloric ingestion and lesser EE in these groups. However, not all fat excess was due to energy intake since previous studies have proved that the fat deposition into the body were strongly dependent to intrinsic factors of the diets such as proportions of energy from each macronutrient, rather than the total energy intake (Monsanto *et al.*, 2016, Ferro Cavalcante *et al.*, 2014). In line with this argument TN and IC diets showed a higher percentage of energy provided by fat compared to W, E and N diets.

In agreement with our results several authors have demonstrated that high-protein diets are related to a reduced fat deposition (Petzke *et al.*, 2007, Petzke *et al.*, 2005, Huang *et al.*, 2008, Zhou *et al.*, 2011). Furthermore, the exposition to diets based on a very high-protein proportions (50%) lead to decrease feed efficiency and hence, diminishing fat pad deposition without detrimental effects on kidney function (Lacroix *et al.*, 2004). Mice exposed high-protein diets also showed a decrease in the relative WAT mass compared to adequate-protein diet (Freudenberg *et al.*, 2012). Our results are aligned with the results obtained by Zhou *et al.*, (2011). They studied the body composition of rats nourished with high-protein diets (using similar proportions of macronutrients to our) showed lower amounts of fat than control. Finally, Batterham *et al.*, (2006) demonstrated that low-fat-high-protein diets are more effective to reduce inguinal fat pad than low-fat-normal-protein, high-fat-high-protein and high-fat-low-

protein using *Pyy* null mice (Batterham *et al.*, 2006). The explanation to fat mass loss under high-protein diets is mainly mediated by several factors: *i*) the widely proved satiating effect of protein than carbohydrates or fats. Proteins suppress hunger and help to maintain a lower energy intake as it can be showed previously; *ii*) thermogenic effect of dietary protein than enhance EE after high-protein preload. The high EE, together with the lower energy intake, are responsible for the weight loss, and particularly, for the reduction of fat mass.

With regard to the lesser proportions in WAT and BAT found in N group, it has been demonstrated, using obese mice, that dietary fibre reduced white adipose tissue and the size of adipocytes after 10 weeks of consumption (Wang *et al.*, 2018). Apart of the proved effect of fibre reducing energy intake, these authors suggested that dietary fibre may diminish the overexpression of metabolic genes involved in obesity through modulation of microbiota (Wang *et al.*, 2018). Retroperitoneal fat pad showed in N group was similar to W and E diet but lesser than in the case of TN and IC. These results are aligned that found by Moran-Ramos *et al.*, (2017) who found a significant decrease in retroperitoneal fat after long-term consumption of nopal cladodes in rats fed with high-fat diets. In addition, the size of adipocyte was lesser in animals fed with nopal respect to non-treated. The decreases in total fat deposition exhibited by N, were additionally related to the highest EE showed in this group of animals joint to the lower energy intake, which lead to a negative energy balance (Moran-Ramos *et al.*, 2017). Moreover, a meta-analysis has proved the effect of *Opuntia* in reducing the percentage of body fat (Onakpoya *et al.*, 2015). Furthermore, nopal showed intrinsic features involved in the promotion of fat excretion by its fibre content. As it has been mentioned previously, dietary fibres have the capacity to bind dietary fat in a complex not being the fat available for absorption. This fat binding may contribute to suppress fat absorption to be excreted in faecal material. This proposal mechanism has been proved previously by other researches who performed a crossover clinical investigation using 20 volunteers (Uebelhack *et al.*, 2014). The showed that after supplementation with 2 tables containing 500 mg each one of cactus fibre (Litramine IQP G-002AS) (thrice daily), the faecal fat excretion was marked compared to controls.

In addition, at this stage it is imperative taking in consideration the role of calcium in fat metabolism. The amount of fat excretion showed by volunteers was exacerbated, corresponding to 15.11 g of fat excreted daily. These data supposes about 136 Kcal/day lost after nopal consumption (Uebelhack *et al.*, 2014). Cladode of nopal showed large quantities of calcium and magnesium ranging amount from 235 to 5520 mg/100g (El-Mostafa *et al.*, 2014). It has been demonstrated that augmentations of adipocyte intracellular calcium promotes lipogenesis; triglycerides storage and it could inhibit lipolysis. However, increments in dietary calcium induce a suppression in calciotropic hormones and, thereby a reduction in intracellular calcium into adipocyte which lead to a reduction in the lipid storage (Al-Mana *et al.*, 2012, Kovacs and Mela, 2006, Zemel, 2004). Additionally, and together the fat binding of fibres, the role of calcium in fat storage is promoted the formation of complex with non-absorbed fat, producing the fat excretion (Papakonstantinou *et al.*, 2003). Hence, the major mechanism of nopal to promote fat loss could be mediated by the role of fibre and calcium.

Regarding to BAT, it has been associated with non-shivering thermogenesis in hibernating animals, cold-adaptating animals, rodents (throughout their lives), neonates and man (although it could experiment a regression after birth) (Skala *et al.*, 1970, Rothwell and Stock, 1997). The development of the thermogenic BAT could contribute to dissipate heat from the body (Seale and Lazar, 2009) improving energy balance and promoting weight loss, mainly because of its implication in the uncoupled mitochondria respiration through uncoupled protein 1 (UCP1) (Lowell *et al.*, 1993). Furthermore, activation of BAT after cold exposition lead to uptaking lipids into BAT and hence promoting a clearance of plasma tryglicerides (Yuan *et al.*, 2017, Bartelt *et al.*, 2011). As we mentioned, the relative weight (g/100 g BW) of the interscapular brown adipose tissue (BAT) was different among dietary groups. W, E and N were similar among themselves and lower than IC and TN. Contrarily, TN and IC showed a great increase of BAT compared with the other experimental diets. Moreover, W diet group presented a relative WAT and BAT even lower than in case of EC (data not shown). Brito *et al.*, (1992) found similar results to ours when compared a high-protein diet vs a control diet in rats. They demonstrated that rats fed with high-protein diets exhibited a lower BAT weight compared to those fed with a control diet (335±21 vs 543±27 mg, expressed per

100 g BW, respectively) (Brito *et al.*, 1992). They also found that the amount of mitochondrial protein and mitochondrial cytochrome oxidase activity in case of high-protein diet were lesser than in control group. It has been proposed that the amount of protein in the diet could lead to a sympathetic suppression (lowering the turnover of norepinephrine) and finally, leading to a decrease in BAT (Brito *et al.*, 1992). Besides, Petzke *et al.*, (2007) found that rats fed with high-protein-normal-fat showed a lower BAT (g) and a higher EE (Kj/(h x Kg) similarly to our results. Furthermore, they found a positive correlation between UCP1 expression in the BAT (Petzke *et al.*, 2007). The gain of the BAT found in TN and EC compared to other diet groups was not reflect of an increased EE, but was due to the massive fat deposition. Possibly, BAT showed in the case of DIO, IC and TN were not activated, since results of EE of these groups showed a lower thermogenic effect.

The relative mass and length of the whole large intestine were the highest in N group although significant similar to E group. Colon weight was the highest in N group, significant different to all experimental diets but not different to IC. In addition, caecum weight was the highest in case of N diet and similar to E. Caecum changes can be reflecting of the gut fermentation (SCFA) and microbiota diversity. The high fibre contains in N group diet (16% w/w) may explain the increased weight and length of the large intestine. Poor fermentable fibre-high-fat diets are associated with a reduction in the weight and length of the colon (Chassaing *et al.*, 2015). High-fibre diets (10%) and very high-fibre diets (20%), produced increases of weight of caecum and colon in rats dose-dependently (Parnell and Reimer, 2012). Moreover, it has been demonstrated that rats nourished in a long-term with diets containing nopal showed a higher caecum weight and caecum content (Moran-Ramos *et al.*, 2017). These finding could be due to the higher fermentation and SCFA production (acetate, propionate, *i*-valerate, *i*-butyrate and valerate) and gut microbiota diversity after nopal consumption. SCFAs have been associated to multiple biological activities, such as regulation of energy homeostasis, anti-inflammatory activity (Jandhyala *et al.*, 2015), prevention of obesity and satiety stimulation (Cani *et al.*, 2004, Tolhurst *et al.*, 2012). In addition, a link between SCFA and GLP-1 secretion has been established through the activation of FFAR2 SCFA receptor (Tolhurst *et al.*, 2012). The proliferative effect of fibre on the intestinal growth (weight and length) was coupled to increases of satiety hormones (proglucagon

expression and plasma GLP-1). Chassaing *et al.*, (2015) demonstrated that the absence of dietary fibre was correlated to an atrophy of colon and caecum leading to the development of an obese phenotype microbiota-dependent. Then, we could suggest that fibre of N group could be responsible in part of preventing the obese phenotype by a protective effect of gut environment related with the possible production of SCFA and gut satiating hormones.

However, TN group diet contained around 12% of fibre. We expected to have found increases of weights and lengths of colon and caecum than in N group as Parnell *et al.*, (2012) showed with a similar dose of fibre in rats. Unexpected results from E group were also surprising, but not due the fibre content. Recently, it has been found a relationship between high protein diets (based on pea protein) and the caecum size maybe consisting in a great mass of fermentable microbiota. They also found a positive correlation between the production of SCFAs (acetate and propionate) and the caecum size (Adam *et al.*, 2016).

4.7 *In vivo* body composition analysis

Two major locations of white fat depots have been described, subcutaneous fat and visceral fat. Fat deposits have been quantified *ex vivo* through a direct measurement of the fat after animal slaughtering, and *in vivo* by using CT technology. Both measurements of fat have showed a strong correlation. Several authors have demonstrated, as in our experiment, the accuracy of this innovative methodology to quantify fat deposition (Assini *et al.*, 2015, Sasser *et al.*, 2012, Lubura *et al.*, 2012). In addition, this technique has allowed measure the total fat deposition when animals were obese without the needed to slaughter them. Results derived from CT revealed great reductions of fat mass of all dietary groups related to when they were obese. Differences among IC and mice fed with W, E and N have also proved, matching with previous *ex vivo* results.

Whey, E and N groups showed a similar volume of fat and TF/TV ratio. However, in relation to total volume it can be noted comparing mice belonging different groups. It was clear the reduction of volume in case of nopal group respect to all experimental diets, EC and DIO phase. They showed total volumes significant comparable to adaptation phase. These data suggest that these three groups (W, E and N) experimented a similar fat loss, although the total volume of N group was significant lower respect to all groups. Furthermore, these decreases of volume could not be produced by a lesser muscle development of nopal group, since similar soleus weight has been described compared to W and E group. The explanation of differences in volume were correlated rather to the water-holding effect of fibre and the diuretic activity of nopal, which consequently led to a lesser size of animals. Several researches have shown its diuretic and hypotensive activity (Galati *et al.*, 2002, Bakour *et al.*, 2017). Bisson *et al.*, (2010) demonstrated that the consumption in rats of 240 mg/Kg/d of an extract of nopal for one week provoked a high increase in urine volume which marked a trend in BW reduction. These authors have even demonstrated that the diuretic effect of nopal was comparable to standard diuretic drugs as hydrochlorothiazide (Bisson *et al.*, 2010). These finding may indicate and comparing with W and E diets, that part of the acute BW loss exhibited by mice fed with nopal was mediated because the diuretic effect of this plant. Total BW decreases could be due to the putative effect of nopal on fat reduction plus its diuretic effect.

4.8 Metabolic measurements using indirect calorimetry

Metabolic parameters such as RQ, VO₂ and EE can be determined by direct or indirect calorimetry and non-calorimetric methods. Direct calorimetry estimates the amount of heat released from the body, whereas the indirect calorimetry indicates the amount of heat produced by oxidation caused by biochemical process. However, determination of nitrogen form urine (24 hours) apart from CO₂ and O₂ measurements by indirect calorimetry, may be more accurate to calculate energy expenditure and net substrate oxidation (Brouwer, 1957, Weir, 1990).

During breathing, each liter of oxygen consumed corresponds to an specific amount of heat produced depending on what nutrient from diet is oxidizing (Jequier *et al.*, 1987). RQ equal to 1 indicates a pure carb oxidation (glucose) whereas RQ values near to 0.7 are reflects of fat oxidation. Intermediate values could indicate protein oxidation (0.835) (Flatt, 1987) or mixed fuel use. However, RQ not always is affected by substrate oxidation or ventilation since physiological conditions may affect it such as famine situations, DIO, alcoholism and diabetes sates could give place to RQ lesser than 0.7. Overfeeding can lead to a limited fat oxidation, elevating RQ level. In addition, gluconeogenesis (synthesis of glucose from amino acids) usually showed a RQ of 0.8, as well as, under ketosis conditions RQ may range close to 0.7 (Jequier *et al.*, 1987).

Independently of substrate metabolism, under standard conditions, the catabolism of 1 L of oxygen leads to conversion to 4.7 Kcal of heat (Kleiber, 1975). RQ could give a relative idea about substrate oxidation, indicating how much energy is being expended using as a fuel-substrate proteins, carbohydrates or/and fats.

RQ data can be difficult to interpret when mixed meals have been assayed. In addition, RQ from this experiment was not assayed under 24 hours since it was performed during 1 hour of steady-state conditions. In consequence, the RQ data should be interpreted cautelosly because they give us just and approximation of the relative fuel-substrate oxidation (fat or carbs) for a given moment.

RQ results after 11 weeks of dietary intervention and RQ data under DIO stage were different, suggesting that different substrate oxidation was taking place. Similar RQ values than obtained under DIO phase have been showed by other researches when animals were fed with high-fat diets (Sanchez-Tapia *et al.*, 2017). Animals fed with a high-fat diet (DIO) showed a lesser RQ compared with mice from experimental stage. Maybe, a higher fatty acid oxidation could taken place, reducing RQ.

High-protein-low-fat diets (W and E) were based on a high proportions of goat's whey and egg white powder, while fats were diminished and maintained the proportions of carbohydrates in a similar quantities than experimental diets. Hence, RQ from W and

E diet could be lesser than non high-protein diets due to protein proportions, but more precise way to calculate substrate oxidation were needed. These RQ would indicate that proteins are the major oxidised macronutrient at specific moment, suggesting that a combination of oxidations from a high moite of proteins and carbohydrates respect to fats were taking place. RQ determinations of several mixed diets were performed showing values of 0.82 for a high-protein-high fat diet, 0.88 for a high-protein-normal-fat diet or 0.97 for a adequate-protein-normal fat diet (Petzke *et al.*, 2007). Accordingly, Zhou *et al.*, (2011) found RQ values in rats, after consumption of high-protein diets (whey or soy), almost equal to our results (around 0.85) and lesser than control (between 0.91 and 1.0) (Zhou *et al.*, 2011). Moreover, Noatsch *et al.*, (2011) assayed using the same mice strain as we used and, in agreement with our results, they reported a diminished RQ of mice fed under high-protein diets (0.876 ± 0.008) than in adequate protein controls (0.917 ± 0.009) (Noatsch *et al.*, 2011).

RQ results from TN, N, and IC were similar, ranging from 0.93 to 0.96. These values of RQ could suggest that endogenous carbohydrates were catabolised to a large extent as the major substrate at this moment. Energy (%) provided from fats in case of TN, N and IC was 32.7%, 23.6% and 29.2%, respectively and maybe may led to a slight decrease in the RQ of TN group. Despite these marked differences in diet, non significant differences were showed. Similar RQ data was also found by other authors when compared different mixed diets in mice. RQ value from the control diet used by this author was quite similar to RQ obtained in the present study (0.95 and 0.99) (Klaus, 2005).

Increases in the oxygen consumption suggest that heat production or thermogenesis were taken place. Volume of oxygen consumed related to BW was higher in the case of W group and E (high-protein-low-fat diet) and N compared to TN, IC and obese stage. In agreement with our results, higher VO_2 related to BW was found when rats were fed with high-protein diets (Petzke *et al.*, 2007). Hence, diets based on high-protein could lead to produce a high thermogenic effect due to increases in oxygen consumption which was translated into high EE requirements. Moreover, N showed an oxygen consumption increased respect to TN and EC. The explanation can be related to its high fibre concentration and phenolic compounds. It has been demonstrated that diet

based on high amount of fibre may lead to increase oxygen consumption (Wang et al., 2018). Recently, rats fed with a high-fat diet supplemented with sucrose and 5% of nopal showed increases in oxygen consumption compared to control (Sanchez-Tapia *et al.*, 2017).

In connection with weight-specific EE (Kcal/Kg BW/h) results, significantly differences were found, corresponding the highest EE to W, E and N groups. These mice showed clearly a negative energy balance, being the Kcal ingested minor than Kcal expended at that specific time. Although EE measurements were in a punctual time, it was probable that increases on EE accompany these groups over all experiment due to the BW loss and decreased fat deposition. Several authors have demonstrated that the high-protein diets were then main responsible of EE increasing, being the main macronutrient that rise EE under *ad libitum* conditions (Petzke *et al.*, 2007). Moreover, high-protein diets have a clear effect on DIT related with satiety. The ingestion of proteins induces a thermogenic effect higher than carbohydrates or fats, increasing the body temperature, which leads to feel fullness or satiety. This affirmation has been repeatedly demonstrated both in human in animal models (Due *et al.*, 2004, Westerterp-Plantenga, 2003, Halton and Hu, 2004, Mikkelsen *et al.*, 2000, Petzke *et al.*, 2007). A possible explanation could be that the organism is no able to store a great amount of proteins, resulting in a rapid metabolisation. In addition, the synthesis of proteins is energetically costly since urea production, peptide bound synthesis and gluconeogenesis require high amount of ATP (Robinson *et al.*, 1990, Halton and Hu, 2004). Augmentations in EE caused by protein intake may lead to induce uncoupling respiration by increasing of proton-pump in the liver. Uncoupling may lead to a lesser production of ATP and hence increased the amount of heat per volume of oxygen consumed (Forslund *et al.*, 1999).

In agreement with that hypothesis, W and E group exhibited the highest EE comparing to tiger nut and control, suggesting that high-protein-low-fat diet could be increases EE related to BW, maybe due to increases of DIT. The assumption proposed that high-protein diets increasing EE (Kcal/Kg BW/h) under *ad libitum* condition and testing isocaloric diets can be accepted based on our results. Mikkelsen *et al.*, (2000) also verified that high-protein based on pork or soy increased EE subjects, being animal

protein most elevated EE-24h showed compared to vegetal protein, suggesting that the source of protein affected EE (Mikkelsen *et al.*, 2000).

Interestingly, N diet also exhibited a higher EE (Kcal/Kg BW/h) compared to TN and IC. Finally, EE related to BW in case of TN was lower compared with high-protein diets and N, and similar to IC and DIO energy expenditure. It is remarkable this similitude, since these diets were formulated to be isoenergetic diets. The low proportion of proteins and the elevated BW of these animals could be responsible to provoke lowers EE. Energy intake was lesser in groups where the EE was higher. Additionally, diets based on high amount of fibre, as in case of nopal diet, could lead to increase EE in long-term animal studies (Wang *et al.*, 2018), suggesting that fibre can be used as therapy to modulate energy homeostasis. Other authors have demonstrated the increased metabolic rates and energy expenditure on obese CB57BL/6J fed with a high-fat diet enriched with 0.3% and 0.6% of *Opuntia* extract mediated by the enhance amount of extract polyphenols (isorhamnetin glycosides) (Rodriguez-Rodriguez *et al.*, 2015). Interestingly, quercetin, a phenolic compound of nopal (El-Mostafa *et al.*, 2014), produced increased in EE at 3 week of treatment in mice when a high-fat diet was supplemented with 0.8% of this flavonoid (Stewart *et al.*, 2008). Rutin, other majoritary flavoniod of nopal, showed an increased EE without produce any change in energy or water intake in DIO mice and in genetically obese mice (*Db/Db*). This augmentations were due mainly a reduction of general adiposity after treatment and by activation of BAT markers (UCP1) and lipolysis (Yuan *et al.*, 2017, Hu *et al.*, 2017).

4.9 Blood lipids analysis

Cholesterol is present in tissues of organism as well as in lipoproteins. It can be present as free cholesterol or in long chain fatty acids. Two carrier can transport cholesterol in the blood, and the relative concentration of each carrier could predispose to develop risk of cardiovascular diseases. HDL-C mainly derived from endogenous synthetised cholesterol into the liver and the cholesterol obtained from intestine which is derived from diet. These sites are the major productors of HDL-C, corresponding the production to 70-80% for liver (Sontag *et al.*, 2013). Its function is mainly to transport

cholesterol discarded or expelled by cells to the liver to be converted into bile acids (Harini and Astirin, 2009). The role of this lipoprotein is essential in the protection against atheroma plaque since removes the the excess of cholesterol towards the live reducing the development of atherogenesis (Harini and Astirin, 2009). Contrarily, LDL-C has the function to transport cholesterol from liver to tissues to be finally incorporated to cell membranes. In addition, LDL-C is the major source for atherosclerotic lipids storage (Ivanova *et al.*, 2017).

Metabolic syndrome could be explained as a combination of factors that enhance the risk to suffer adverse events such as type 2 diabetes and cardiovascular diseases. Some symptoms of metabolic syndrome are related to the decreased levels of HDL-C (<40 mg/dL for men and <50 mg/dL for women), increased levels of triglycerides (>150 mg/dL) and enhanced levels of LDL-C (Kocak *et al.*, 2019, Fan *et al.*, 2019), among others. The combination of high levels of LDL-C and triglycerides and low levels of HDL cholesterol constitutes the so-called “proatherogenic lipoprotein phenotype”, features of diabetes and metabolic syndrome status (Eckel *et al.*, 2005, Ivanova *et al.*, 2017, Fan *et al.*, 2019).

Differences on lipid profiles between mice and human have been related. Whereas mice exhibited the major part of lipoproteins as HDL (atheroprotective), human showed more LDL levels (atherogenic) (Camus *et al.*, 1983). For this reason, data from mice have not been compared with human results available in the literature.

Experiments using C57BL/6J strains have demonstrated that the TC profile corresponds in a high part to HDL-C than LDL-C, being the first one approximately 70% of the TC fraction (Yin *et al.*, 2012). Reference values for TC has been described previously by other authors corresponding to 124.57 mg/dL (Fernandez *et al.*, 2010). This value was coincident with the results showed by mice of EC and consequently being under normal parameters. DIO animals received a diet providing 60% of energy from fat, being the source predominantly saturated fat (lard fat). The purpose of feeding animals with a high-fat diet had indeed converted them in obese animals showing the respective hyperlipidemia profile of this status. Then, the goal was how the experimental diet may improve this obese phenotype.

Certainly, TC reductions have been showed after 11 weeks of supplementation with different types of diet. Experimental diets were formulated in energy-restriction respect to high-fat diets, being the caloric density different between diets and DIO. The reduction in TC was expected due the lower caloric supplementation. However, the interesting point was how the different diets would improve the lipid parameters. Although all experimental diets were formulated in isocaloric manner, the proportion of metabolizable energy provided for each macronutrient was different. For instance, energy provided by fat in TN diet was replaced in W and E diet by protein.

The present results may reveal that TN diet was not capable to reduce significantly the TC levels under normal conditions, despite to show lower levels compared to DIO phase. Diet based on tiger nut was composed on 40% of powder from whole tiger nut. This diet contained more than 13 g/100 g of fat, which meant to metabolizable energy more than 32% of energy from fat. The fatty acid profile of TN is quite similar to olive oil (17% SFA, 73% MUFA and 9% PUFA), but higher in SFA. With this regard it has been amply studied that SFA could provoke increases of TC and specially LDL-C, whereas unsaturated fatty acids gave place to the opposite effect and being both fatty acids responsible to diet-induced hyper or hypocholesterolemia, respectively (Wilson *et al.*, 2000). Tiger nut also provided a high percentage of oleic acid within the MUFA fraction. The lowering-cholesterol effect of oleic acid was demonstrated previously (Mattson and Grundy, 1985) but maybe the highest concentration of SFA could enhance lipids profile. However, not only dietary fats could affect serum lipids. Several longitudinal analyses have suggested the relationship between increases of TC and LDL-C levels with GI of carbohydrates from diet. The connection may be explained because carbohydrates with high GI could lead to increases blood insulin concentrations which may drive to increase cholesterol and LDL-C (Ma *et al.*, 2006). Additionally, a meta-analysis has corroborated that diets high in sugar may promote increases in triglycerides, TC, LDL-C and HDL-C and blood pressure independently of the effect of sugars on body weight (Te Morenga *et al.*, 2014, Fattore *et al.*, 2017). Certainly, there is a consensus about the effect of fibre on body weight management and the lower effect on lipids profile. However, about half of tiger nut corresponded to sugar despite its high content in fibre. The amount of fibre of tiger nut maybe was insufficient to lower blood lipids compared with the supposed increases

provoked by its fat and sugar content. Thus, the additive effect of fat and sugar profile could resonate on the lipid profile of animals fed with this type of diet. In addition, few studies have demonstrated the protective effect of tiger nut in blood lipids. Some of them have proved effect on blood lipids in animals using defatted tiger nut (Moon *et al.*, 2012) or supplementing the diet with a tiger nut-oil based (Ibitoye *et al.*, 2018). However, these results were non matching with our experiment, since the dietary intervention of that study was performed under caloric restriction diet (Moaty *et al.*, 2012).

High-proteins diet (W and E), as well as diet based on nopal, have been proved to have an excellent protector effect on fasting lipids profile, being TC levels notably lower than the previous obese stages and even IC. An explanation for reduced fasting TC in these three groups can be the lesser proportions in fat content, as well as energy provided by fat compared to TN and IC. However, hereunder a deep explanation about the role of each type of diet-based ingredient has been taken into account. Internal control diet showed TC levels upper normal range and significant similar to TN diet. IC diet provided 29.2% of energy from fat, which could produce these increments of cholesterol together with the increased food intake that this group showed.

Additionally, normalised levels of HDL-C found in the literature for the same strain of mice at similar age was of 61.86 mg/dL (Albers *et al.*, 1999). All diets exceeded this level except W group. Certainly, in the case of TN the highest levels of HLD-C were compatibles with the general increase of TC since the major lipoprotein of cholesterol corresponded to HDL lipoprotein.

Finally, LDL-C level has been previously described in the literature, considering within the normality values approximately of 10.05 mg/dL (Albers *et al.*, 1999). W, E and N levels were under this reference. Although no significant differences have been found comparing experimental diets, LDL-C of W, E and N were significant reduced from their obese condition. Several studies have demonstrated the potential of each ingredient used in this experiment as lowering-cholesterol. To understand adequately the potential of whey protein, egg white protein and nopal decreasing blood lipid, a detailed explanation has been proposed hereunder.

Diet supplemented with goat whey protein powder showed desirable levels of TC and LDL-C. The beneficial effect of whey protein has been demonstrated in animal models and human interventions (Tranberg *et al.*, 2013, Pal *et al.*, 2010, Zhang *et al.*, 2018, Freudenberg *et al.*, 2012). The mechanism proposed whereby whey has showed a positive effect in lipids could be related to *i*) the effect of whey proteins on depressing *de novo* genesis of cholesterol in the liver (Zhang and Beynen, 1993), *ii*) augmentation of faecal excretion of neutral steroids and bile acids mainly because the join with the peptide fraction (Lovati *et al.*, 1990), *iii*) role of beta-lactoglobulin inhibiting the absorption of cholesterol in the intestine (Nagaoka *et al.*, 1992, Pal *et al.*, 2010), *iv*) by the effect of whey proteins in suppressing the gene expression related to cholesterol and fatty acids synthesis and absorption (Chen and Reimer, 2009) and *v*) by the specific profile of protein and peptides and of branched chain amino acids (BCAA) derived from whey proteins (Tranberg *et al.*, 2013, Hlais *et al.*, 2012).

In relation with our results, Nagaoka *et al.*, (1992) showed similar results. They fed rats with a diet based on whey protein. Serum TC obtained in that study was 92.8 ± 7.6 mg/100 mL and lesser than controls. Similarly, Noatsch *et al.*, (2011) demonstrated that mice, after 14 weeks of supplementation with a high-protein diet based on whey, presented TC results (92 ± 6 mg/dL) quite comparable to our data and being lowered to respect diets providing adequate protein (Noatsch *et al.*, 2011). Weisse *et al.*, (2010) also found that after administration of other dairy protein (casein) the total levels of cholesterol, HDL-C and LDL-C were decreased and maybe it was due to the specific decrease of HDL-C (Weisse *et al.*, 2010) similarly to our data. None of these authors suggested an explanation about the effect of dairy proteins on decreasing HDL-C.

Animals fed with a diet based on egg white protein also presented an improvement of blood lipid profile respect to previous obese stages. E diet resulted in a high proportion of energy provided by protein (40%) and lower energy given by fats (7.7%). Traditionally the increased consumption of egg has been associated by increasing levels of cholesterol; however, white egg was cholesterol-free. Several researches have highlighted the beneficial effect of egg white protein (EWP) on lipid metabolism in animals and human (Yamamoto *et al.*, 1993, Asato *et al.*, 1996,

Matsuoka *et al.*, 2014, Matsuoka *et al.*, 2008). Several pathways may explain the cholesterol-lowering effects of EWP showed in the present study: *i*) peptides derived from EWP digestion. Undigested peptides produced in the lumen by protein digestion could bind steroids to be excreted in faeces, reducing serum cholesterol levels (Yamamoto *et al.*, 1993, Asato *et al.*, 1996, Sugano *et al.*, 1990), *ii*) the physicochemical features of EWP which suppress the intestine cholesterol absorption by inhibiting the micellar formation. This could lead to avoiding cholesterol absorption in the gut and promoting the excretion of faecal neutral sterols (Matsuoka *et al.*, 2008). Cholesterol absorption depends on its efficient incorporation into bile acid micelles from the solid phase in the intestine. Thus, EWP could inhibit the micellar solubility of cholesterol from the solid phase mainly through the action of ovalbumin, ovotransferrin and ovomucin (Matsuoka *et al.*, 2008, Nagaoka *et al.*, 2002) *iii*) Other explanation has been related to the role of protein *per se* and specific amino acids contained in egg such as cystine (Asato *et al.*, 1996).

Finally, diet based on 25% of powder from *Opuntia cladodes* showed superb results on lipid profile. Additionally, this type of diet provided the most percentage of dietary fibre among all diets (16%). Nopal is also rich in amino acids, polyphenols, vitamins A, B, C and E, minerals and polyunsaturated fatty acids. The protective effect of nopal as lowering-cholesterol has been widely revealed in human and using animal models (El-Mostafa *et al.*, 2014, Sanchez-Tapia *et al.*, 2017, Fratimunari *et al.*, 1983, Guevara-Cruz *et al.*, 2012, Galati *et al.*, 2003). A systematic review and a metanalysis of randomised clinical trials have showed that supplementation with *Opuntia ficus indica* induced reductions in the percentage of body fat and total cholesterol (Onakpoya *et al.*, 2015). Effect of lower dose (12%) that used in the present study (25%) proved effect in animal models, diminishing 34% values of LDL-C compared to controls (Medellin *et al.*, 1998). Surprisingly, this percentage has been almost equal to obtain in this study (37%). A recent study showed similar results (84.6 ± 3.6 mg/dL) to our data (89 ± 3.81 mg/dL) when rats were fed for 10 weeks with 4% of nopal fibre (Moran-Ramos *et al.*, 2017). These data suggest that the effect of nopal by reducing cholesterol was not dose-dependently. Authors suggested that the beneficial effect on blood lipids may be mediated by the low glycaemic index of nopal, its fibre content and polyphenols. This suggestion could led to produce a notable change in gut microbiota

being the effect of altogether rather than separately causes of this effect. The mechanism proposed to explain the beneficial effect of diet-based on nopal could be due by synergistic action of the followed factors: *i*) decreases in oxidative stress mediated by the antioxidant capacity of polyphenols presented in *Opuntia* (isorhamnetin and kaempferol), suggesting an interaction between nopal polyphenols and reactive oxygen species (ROS) in the liver, *ii*) through a mediated effect of adiponectin. This anti-inflammatory adipokine was enhanced after nopal supplementation and it has been related to limit the accumulation of fat into the liver and, in turn, with the prevention the accumulation of triglycerides in this organ, *iii*) by beneficial effects on insulin sensitivity due its low glycaemic index (Moran-Ramos *et al.*, 2012a) and *iv*) by its fibre content. Soluble fibre, specially pectin, and insoluble fibre has been involved in reductions of TC and LDL-C mediated by the bind of fibre to cholesterol or bile acids inhibiting the formation of micelles and reducing the lipids uptake. Consequently, the reduction of TC leads to a hepatic up-regulation of LDL receptors and thus enhancing LDL-C. Other role of fibre on lowering cholesterol could be due the fermentation of fibre producing SCFA that ultimately led to an inhibition of hepatic fatty acid synthesis (Brown *et al.*, 1999, Kristensen *et al.*, 2012, Sanchez-Tapia *et al.*, 2017, Rodriguez-Rodriguez *et al.*, 2015).

4.10 Hormone analysis

Satiety and satiation are produced by sensorial stimulus, pre-absorptive and post-absorptive signals. These complexes of signals could trigger the releasing of satiating peptides. When the nutrient chemoreceptors and stretch receptors in the gastro intestinal tract are stimulated, by the presence of nutrient-specific meal (protein, carbohydrates and fat) (meal quality) or by changes in volume (meal quantity), lead to the releasing of “satiety peptides” and “satiety hormones”. These post-ingestive signals within the gut trigger the releasing of hormones into gastro intestinal tract such as ghrelin, CCK, PYY and GLP-1. They act as potent cues for satiation and medium-term satiety through their impact on stomach emptying, by stimulating afferent signals to the CNS or directly influencing CNS function. Nutrients as proteins and non-digestible carbohydrates that reach the colon could serve as substrate for gut microbiota. These fermentation

produces short-chain fatty acids (SCFA) that could trigger the releasing of satiating gut peptides such as GLP-1 and PYY (Cani *et al.*, 2009). Finally, the brain integrates all satiating signals from the periphery of organism.

These signals reflect energy input (energy intake) and energy output (EE) balance, which drive to suppress hunger or contrarily continue eating. A complex of signals and nutrient-sensing stimulus explains satiety and satiation mechanism. All together confers satiety effectiveness to a specific nutrient. Several satiety and satiation signals have been determined in the present study. Most of them could be stimulated or suppressed according to the type of nutrient and also depending on the body fat storage. Then, most of hormone investigate act as satiating signals or/and adipose signals. Concretely, incretin GIP and satiating peptides or hormones as CCK and amylin have been studied.

Glucose-dependent insulintropic polypeptide (GIP), named originally as gastric inhibitory polypeptide and re-named as glucose-dependent insulintropic polypeptide, is a peptide secreted by K enteroendocrine cells of proximal small intestine. Traditionally, its main action has been related as incretin since it is responsible to have the ability of increase insulin by stimulation of β -cells in pancreas. However, there is evidence of GIP showed other functions apart from pancreatic activities. This peptide has been related to the inhibition of gastric secretion and gastric emptying, but to achieve these effects, supraphysiologic dose are required (Fehmann *et al.*, 1995). GIP has a key role promoting overnutrition and obesity. Additionally, it has been proved that GIP may stimulate fat deposition in adipocytes via enhancement of lipoprotein lipase (Moran-Ramos *et al.*, 2012b). The proposal explanation to answer to the link between GIP and obesity is that high-fat diets promote the hyperplasia of K cells and an inhibitory GIP releasing induced by insulin (Ebert *et al.*, 1979), leading to a gene overexpression of GIP reflecting high levels into intestine and circulating blood (Bailey *et al.*, 1986, Flatt *et al.*, 1983, McClean *et al.*, 2007). However, specific nutrients in the intestinal lumen can also trigger GIP releasing. There is secreted postprandially and the major secretagogues nutrients are digested fats, hydrolysed carbohydrates and, although poorly studied compared with the last one, protein hydrolysates and amino acids (Tseng *et al.*, 1994, Althage *et al.*, 2008, Parker *et al.*, 2009).

The highest levels of GIP in the case of obese animals after being treated with a high-fat diet which matched with the high-caloric intake of this group. A similar trend has been observed in the case of mice belonging to IC, showing GIP levels increased respect to experimental diets. These increments in both groups could be due to the high content of fat showed in case of obese diet (Ebert *et al.*, 1991) and secondarily by the increased fat deposition showed by DIO animals and IC group. Additionally, it was possible that these obese phenotype may led to a enhanced hyperplasia and hypersecretion of GIP on K cells of these two groups (Flatt *et al.*, 1984).

At this point, the question is to know how experimental diets reversed the acute levels of GIP presented after obese period and even compared to control. All experimental diets yielded lower GIP levels, maybe related with the lesser food intake and lower proportions of fat deposition of these animals compared to DIO and IC groups. Attending to a nutrient-sensing GIP mechanism explanation, glucose and fat have been proved to be strongest GIP stimulators (Ebert *et al.*, 1991, Sykes *et al.*, 1980). Nevertheless, it seemed that neither high carbohydrates nor fats presented in the case of TN could elevate GIP. This fact could be related with the high levels of fibre contained in tiger nut. With this respect it has been proved reductions on GIP by soluble fibres as guar (Morgan *et al.*, 1990). Additionally, the highest protein content of W and E diets could not increase GIP as other researches have demonstrated previously (Yoder *et al.*, 2010). These authors evaluated the effect of nutrients on GIP secretion by using a lymph fistula rat model, showing that GIP increased dose-dependent after fat or carbohydrate administration, but not with protein (Yoder *et al.*, 2010). Focusing in the specific role of nopal to ameliorate the secretion of GIP it has been proved the antihyperglycaemic effect of nopal mediated in part by the descended stimulation of GIP (Lopez-Romero *et al.*, 2014). Recently, Sánchez-Tapia *et al.*, (2017) has demonstrated the inhibitory effect of nopal on GIP secretion in obese rats fed with a high-fat diet enriched with 5% of nopal. Concretely, they showed a reduction of 68% of GIP due to the nopal effect. Descended results could be explained because the low glycaemic index of nopal (32%), fibre and antioxidant composition (Lopez-Romero *et al.*, 2014). Moreover, hypersecretion of GIP may produce hyperinsulinemia followed by fat accumulation and obesity. These finding suggest that the assayed experimental diets were able to ameliorate the high GIP levels showed under obese stage mostly due to

their non-obese stage. Results shed light of the promising use of whey, egg, tiger nut or nopal to reduce levels of GIP, which may improve glucose and insulin homeostasis.

Amylin is secreted by β -cells in pancreas and is co-secreted with insulin in response to nutrients and it is absent in type 1 diabetes (Young and Denaro, 1998). In general, amylin showed the same response to nutrients than insulin. However, controversial findings have been shown regarding to the nutrient-sense amylin releasing. Several authors have proposed that it could be secreted in response to glucose load (Thomaseth *et al.*, 1997, Mitsukawa *et al.*, 1990). Other researches have demonstrated that amylin secretion is strongest stimulated by fat and carbohydrates rather to diet derived-protein (Michel *et al.*, 2007). Contrarily, amylin levels were higher increased in obese cats after consuming a high-protein diet compared to high-fat or high-carbohydrate diet. The effect was maybe mediated by the action of amino acids in β -cells (Martin *et al.*, 2010).

Among physiological functions of amylin, it has been described a complementary action to insulin effects over glucose homeostasis. However, it has been proved that amylin could antagonise insulin secretion. In addition, amylin could inhibit glucagon secretion, lipase, amylase and it is related to the inhibition of gastric emptying (Reda *et al.*, 2002, Young *et al.*, 1995). The inhibition of gastric emptying by amylin results interesting in terms of satiety. Augmentations of food into the stomach produced gastric distension and via afferent signal trigger satiety signals in the brain. Apart from these vagal signals, the inhibition of gastric emptying produced by amylin may delay the absorption of other nutrients. Additionally, it has been demonstrated the direct effect of amylin on food intake. It seems amylin has a synergic effect together CCK to suppress appetite by a central mechanism. In contrast, amylin may suppress the effect of the orexigenic hypothalamic neuropeptide Y (NPY). Furthermore, intrahypothalamic increases of amylin has been related with increases in serotonin, suggesting the important role of neurotransmitter in the satiating effect of amylin (Hita *et al.*, 2006) (Gutzwiller *et al.*, 1994).

Several studies have demonstrated the role of amylin of food intake suppression and BW loss. Concretely, amylin decreased food intake in diabetic and non-diabetic

mice after an intraperitoneal or intracerebrovascular injection. This effect could be explained because amylin reduced the feeding-induced by insulin (Morley and Flood, 1991). Moreover, it has been related the specific role of amylin on the ending of meal-duration, or so-called satiation (Lutz, 2010). Other studies have suggested that, additionally, amylin could increase IMI time under certain conditions (Arnelo *et al.*, 1996). Finally, increases of EE have been showed after amylin administration. These effect was accompanied of reductions on food intake and BW loss, specifically for fat mass (Mack *et al.*, 2007).

Amylin showed the highest levels in IC. Furthermore, E group elicited an increased plasma concentration of amylin, similar to IC and higher compared to mice belonging TN group. Few researches have explained the role of specific nutrients on amylin release, but contrarily, there is a wide literature related the secretion of this peptide with body adipose composition. This peptide may have two roles, satiating hormone and also acts as adipose signal. Firstly, it can induce satiation leading immediately to end the duration of a meal. However, its role as adipose signal may increasing EE and promoting BW loss (Lutz, 2010). Our results suggest that IC mice showed great increases of amylin due to its excessive body fat composition. Obese subjects may show higher levels of amylin caused by a down-regulation of amylin receptors; this is an amylin resistance (similarly to an insulin resistance). It has been demonstrated that the hyperglycaemia and high corticosteroid levels showed often by obese subjects may mediate in the exacerbated secretion of amylin (Young and Denaro, 1998, Reda *et al.*, 2002). It has been suggested that the distinction whether amylin acts as a satiety or adipose signal. The difference may be related with the decreased on the meal duration and meal size. Surprisingly we determined high concentrations of amylin in E group, matching with the higher satiation showed. Additionally, the decreased food intake, increased EE effect and BW loss were mediated partially by the action of amylin in E group. Differently, no satiating effect of amylin has been showed in the case of IC, suggesting that increases in that peptide were related with the high fat deposition.

Cholecystokinin is released in the I-cells of the proximal small intestine in response to dietary protein and fat and their degraded products, being lower stimulated by carbohydrates. This peptide promotes digestion through bile and enzyme released

and also slows gastric emptying, the so-called "*duodenal brake*" mechanism; ie, adjustments of stomach and gut motility after food ingestion, contraction of pylorus and gastric distention (Crawley and Corwin, 1994, Moran, 2000). CCK also showed suppression of food intake in animal models and human (Pedersen *et al.*, 2000, Hall *et al.*, 2003). The presence of nutrients in the stomach and intestine generates a complex of physiological responses that lead to meal end. The satiety signal generated by mechanical stimulus in gastro intestinal tract or by chemical stimulation produced by nutrients activating the satiating centre in CNS (*nucleus tractus solitarius*) via vague nerve. CCK suppresses hunger after a meal, reducing food portions, and it can be used as a suitable satiating marker (de Graaf *et al.*, 2004, Hita *et al.*, 2006). Most CCK stimulants of all experimental diets have been whey and nopal. Surprisingly, the other protein diet, egg, did not showed the capacity of triggering the releasing of CCK in this manner. Likewise, tiger nut or IC did not stimulate CCK secretion.

Several studies have described the potent effect of protein on CCK release in general (Geraedts *et al.*, 2011, CordierBussat *et al.*, 1997) and whey in particular as CCK secretagogue (Liddle *et al.*, 1986, Pedersen *et al.*, 2000). Results of chapter 4 demonstrated the potential of several types of milk whey in stimulating CCK by using STC-1 cells. The secretagogue effect of whey protein is mediated by the intrinsic characteristic of protein. Concretely, glycomacropeptide, form whey protein, has a strong effect on CCK releasing in rats (Pedersen *et al.*, 2000). Other researches have proved the effect of specific amino acids (phenylalanine and tryptophan) as CCK stimulators (Wang *et al.*, 2011, Ballinger and Clark, 1994). As we mentioned, the satiating potential of protein could vary depending on the source of protein. In this sense, Hall *et al.*, (2003) showed an increased effect (60%) of whey on CCK release compared to casein. The different satiating potential, being *a priori* both ingredients similar proteins was due to their postprandial amino acid profile. Whey proteins were rapidly digested acting as "fast protein", leading to a high peak of plasma amino acids, which it is closely related with high peaks of CCK. Differently, casein seemed to be a "slow" protein, coagulating in the stomach due to its precipitation by gastric acid (Billeaud *et al.*, 1990). As a result, overall gastric emptying time for casein appeared to be longer and a smaller post-prandial increase in plasma amino acids compared with the non-coagulating whey protein. The present study has proved clear differences on CCK

secretion between whey protein and egg white protein. Previous experiments (chapter 4) described a potent effect of CCK releasing after exposed STC-1 cells to digests from liquid egg white compared to digests from whey milk (data not shown). Certainly, whey milk used in that experiment contained lactose and lesser amounts of protein, since it was not a protein concentrate. However, the present experiment has been conducted by using a whey protein concentrate and an egg white protein concentrate mixed into a meal and assayed *in vivo*, being different circumstances than assayed in chapter 4. Trigazis *et al.*, (1997) suggested that chicken egg albumin suppressed food intake on rats mediated by CCK secretion (Trigazis *et al.*, 1997). Other studies showed that the exposure on rat intestinal mucosa cells of several protein hydrolysates from soybean protein, egg white and wheat gluten stimulated CCK. However, the stimulation was lesser compared to soybean hydrolysates (Nishi *et al.*, 2001). Differences in CCK response could be due because the different digestibility of proteins as Hall *et al.*, (2003) described. Furthermore, the protein quality and amino acid composition of goat whey and egg white is different. For instance, GMP is not present in egg, and aromatic amino acids such as phenylalanine and tryptophan were greater in goat whey than egg protein. Nutritional information of two commercial proteins used in this experiment showed that the concentration of phenylalanine and tryptophan in goat whey were 2390 and 1246 mg/100 g. Contrarily, the concentrations of these amino acids in commercial egg white powder were 4.7 mg/100 g and 1.3 mg/100 g for phenylalanine and tryptophan. Pal *et al.*, (2010) determined the satiating effect of whey and egg proteins (and other proteins) and they reported higher satiating effect of whey compared to egg mainly due its amino acid composition, demonstrating increased concentrations of tryptophan and phenylalanine in whey proteins compared to egg proteins (Pal and Ellis, 2010).

Weak CCK stimulation has been showed in the case of TN group. Many researches have corroborated that proteins exert most stimulatory effect on CCK than carbohydrates or fats in rats (Trigazis *et al.*, 1997, Liddle *et al.*, 1986). Certainly, it has been described the effect of hydrolysed triglycerides into fatty acids and CCK simulation. The length of the fatty acids is a determinant on the CCK secretion, being needed at least of 12 carbons (McLaughlin *et al.*, 1999). Regarding to our results, it may be possible that the whole effect of the TN diet as a complex diet (high-fat, high-fibre

and high-GI) could interfere to achieve an enhanced CCK secretion of each compound of the diet.

As we mentioned, the relationship between fibres and CCK secretion has been established. It has been related that the consumption of bean contained into a meal not only caused a high secretion of CCK but also was maintained a long time compared to low fibre meals (Bourdon *et al.*, 2001). Moreover, food that exhibited a low GI index potentiates more CCK stimulation than foods providing a high GI. Interestingly, the strongest CCK stimulation by nopal may be mediated, in part, by its phenolic constituents. *Opuntia* is rich in phenolic acids and flavonoids (El-Mostafa *et al.*, 2014, Stintzing and Carle, 2005). For instance two flavonoids presented in nopal, quercetin and kaempferol, have proved to be great stimulators of CCK in STC-1 cells (Al Shukor *et al.*, 2016). Moreover, other researches have confirmed the CCK secretagogue effect of flavonoids and isoflavones in rats (Panda and Shinde, 2017, Zhang *et al.*, 2009).

5. CONCLUSIONS

The present study has demonstrated the satiating potential and the ability to revert an obese condition of several Mediterranean ingredients (whey, egg white, tiger nut and nopal). Obese mice fed with high-protein diets (goat whey and egg white) and nopal exhibited decreases in food and energy intake along 11 weeks of treatment. Body weight and total fat storage were notably reduced after whey, egg and nopal consumption and maintained over the time. Food intake also may be related to promote satiety sensations, as well as diuresis could have an additive effect in the exacerbate body weight reduction of nopal group.

However, the satiating and anti-obesogenic effect of tiger nut was only maintained in short-term, showing final BW reductions lower than in the case of other Mediterranean ingredients.

All these findings have been supported by a complex mechanism that included increases in energy expenditure, the analysis of feeding pattern after a meal and the circulating satiating or orexigenic hormones in each group. In general terms, two high-protein diets have showed satiety effectiveness, the feeding pattern and hormone secretion revealed that whey provoked more satiety than satiation and the contrary in case of egg. Moreover, high-fibre diet based on nopal presented a similar satiating and anti-obesogenic features than goat whey proteins.

Finally, all experimental diets induced improvements of blood lipids related to obese stage demonstrating the capacity to alleviating cholesterol levels. Although, all of Mediterranean diets tested in obese mice over 11 weeks have demonstrated beneficial effect on satiety and body weight management, whey, egg white and nopal exhibited promising effect on regulating markers related to obesity and satiety signals.

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*Scientific production derived from this PhD Thesis
and from collaborations*



SCIENTIFIC PRODUCTION DERIVED FROM THIS PhD THESIS

1. Scientific articles

Sánchez-Moya T., Ros-Berruezo G. and López-Nicolás R. (2016). Ingredientes y alimentos mediterráneos con efecto saciante. Revista CTC (Centro tecnológico nacional de la conserva y alimentación); 65: 14-21.

Sánchez-Moya T., López-Nicolás R., Planes D., González-Bermúdez C.A., Ros-Berruezo G. and Frontela-Saseta C. (2017). *In vitro* modulation of gut microbiota by whey protein to preserve intestinal health. Food & Function. Royal Society of Chemistry; 8(9): 3053-3063. DOI: 10.1039/c7fo00197e.

2. Scientific dissemination

Sánchez-Moya T. and López-Nicolás R. (2016). ¿Cómo engañar al estómago? Cosas que debes saber. Eroski Consumer. http://www.consumer.es/web/es/alimentacion/aprender_a_comer_bien/2016/03/28/223508.php.

3. Oral communications

Sánchez-Moya T., Frontela-Saseta C., López-Nicolás R. and Ros-Berruezo G. (2016). Gut microbiota associated with prebiotic effect of whey protein. 5th European Nutrition and Dietetics Conference. Foodomics. Journal Nutrition and Food Science. 6:3 (Supl): 69. DOI: 10.4172/2155-9600. C1.020. Rome (Italy).

Sánchez-Moya T., López-Nicolás R., Frontela-Saseta C. and Ros-Berruezo G. (2016). Whey protein fermentation as a predictor of satiety. II Jornadas Doctorales de la Universidad de Murcia. Escuela Internacional de Doctorado de la Universidad de Murcia (EIDUM). Murcia (Spain).

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Sánchez-Moya T., López-Nicolás R., Frontela-Saseta C. and Ros-Berruezo G. (2018). Milk whey stimulates the *in vitro* production of CCK and GLP-1 through a whole simulated intestinal process. IV Jornadas Doctorales de la Escuela Internacional de Doctorado de la Universidad de Murcia (EIDUM). Murcia (Spain).

3. Poster communications

Sánchez-Moya T., López-Nicolás R., Frontela-Saseta C. and Ros-Berruezo G. (2015). Ingredientes de interés en saciedad y obesidad. I Jornadas Doctorales de la Universidad de Murcia. Escuela Internacional de Doctorado de la Universidad de Murcia (EIDUM). Murcia (Spain).

Sánchez-Moya T., López-Nicolás R., Frontela-Saseta C. and Ros-Berruezo G. (2016). Quantification of whey protein after *in vitro* digestion: from mouth to colon. Improving Allergy Risk Assessment Strategy for New Food Proteins. imPARAS. COST ACTION FA- 1402. Warsaw (Poland).

Sánchez Moya, T., López Nicolás, R., Planes Muñoz, D., González Bermúdez, C.A., Marínez Graciá, C., Ros Berruezo, G. and Frontela Saseta, C. (2017). Efecto prebiótico del suero de leche sobre la microbiota de donantes con normopeso y obesos.

Número de comunicación: 252/31. VIII Workshop. Sociedad Española de probióticos y prebióticos (SEPYP). Santiago de Compostela (Spain).

Sánchez-Moya T., López-Nicolás R., Frontela-Saseta C. and Ros-Berruezo G. (2017). Ingredientes y alimentos mediterráneos con efecto saciante como preventivos del sobrepeso y obesidad / Mediterranean ingredients and foods with a satiating effect as a preventive of overweight and obesity. Comunicación nº10. 8th Food Technology International Symposium. Centro Tecnológico Nacional de la Conserva y Alimentación (CTC). Murcia (Spain).

Sánchez-Moya T., Planes D., López-Nicolás R., González-Bermúdez C.A., Frontela-Saseta C. and Ros-Berruezo G. (2018). Whey protein fermentation as a predictor of satiety. 4th International and 5th national student Congress of Food science and Technology. Asociación Valenciana de Estudiantes y Profesionales en Ciencia y Tecnología de los Alimentos (AVECTA). Valencia (Spain).

Sánchez-Moya T., López-Nicolás R., López-Fernández A., Frontela-Saseta C. and Ros-Berruezo G. (2018). Whey milk stimulates the *in vitro* production of CCK and GLP-1 through a whole simulated intestinal process. 4th International and 5th national student Congress of Food science and Technology. Asociación Valenciana de Estudiantes y Profesionales en Ciencia y Tecnología de los Alimentos (AVECTA). Valencia (Spain).

Sánchez-Moya T., Hidalgo A.M., Frontela-saseta C., Ros-Berruezo G. and López-Nicolás R. (2018). Análisis de la separación de lactosa y proteína en suero de oveja mediante ultrafiltración. Aplicación del modelo Spiegler–Kedem–Katchalsky. 1º Congreso de jóvenes investigadores en ciencias agroalimentarias. CIAIMBITAL. Centro de investigación de agrosistemas intensivos mediterráneos y biotecnología agroalimentaria. Almería (Spain).

SCIENTIFIC PRODUCTION DERIVED FROM COLLABORATIONS

1. Scientific articles

Ydjedd S., Bouriche S., López-Nicolás R., Sánchez-Moya T., Frontela-Saseta C., Ros-Berruezo G., Rezgui F., Louaileche H. and Katy D.E. (2017). Effect of *in vitro* gastrointestinal digestion on encapsulated and non-encapsulated phenolic compounds of carob (*Ceratonia siliqua L.*) pulp extracts and their antioxidant capacity. Journal of Agricultural and Food Chemistry, American Chemical Society Publications. 65(4): 827-835. DOI: 10.1021/acs.jafc.6b05103.

Sánchez-Moya T., Martínez-Hernández V.M., López-Nicolás R., González-Bermúdez C.A., Ros-Berruezo G., and Frontela-Saseta C. (2018). Effect of soaking and inositol phosphate content on *in vitro* mineral availability in edible seaweeds. Journal of Applied Phycology. 1-9. DOI: 10.1007/s10811-018-1677-x.

2. Oral communications

Caballero-Valcárcel A.M., Martínez-Miró S., Madrid-Sánchez J., González-Bermúdez C.A., Sánchez-Moya T., Santaella-Pascual M. and Martínez-Graciá C. (2016). Changes in gut microbiota after a period of repletion with different iron supplements weanling pigs. II Jornadas Doctorales de la Universidad de Murcia. Escuela Internacional de Doctorado de la Universidad de Murcia (EIDUM). Murcia (Spain).

Ydjedd S., López-Nicolás R., Sánchez-Moya T., Chaalal M., Frontela-Saseta C., Ros-Berruezo G., and Katy D.E. (2018). Effet préventif *in vitro* des extraits phénoliques de la pulpe de caroube sur l'inflammation intestinale: Effet de la digestion gastrointestinale. Séminaire National: Durabilité au service de notre Alimentation, Santé et Environnement. Université A. Mira-Bejaia. Bejaia (Argelia).

Ydjedd S., López-Nicolás R., Bouriche S., Sánchez-Moya T., Frontela-Saseta C., Ros-Berruezo G., Rezgui F., Chaalal M., and Kati D.E. (2018). Effect *in vitro* gastrointestinal digestion on the antioxidant potential of encapsulated and non-encapsulated phenolic carob pulp extracts and their bioavailability. Séminaire International des Sciences Alimentaires (SISA). Constantine (Argelia).

3. Poster communications

Sánchez-Moya T., López-Nicolás R., Planes D., Peso-Echarri P. and González-Bermúdez C.A. (2013). Influence of Pine Bark Extract Polyphenols on Intestinal Microflora. IUNS 20th International Congress of Nutrition. International Union of Nutritional Science y Sociedad Española de Nutrición. 1156-1556. Granada (Spain).

Planes-Muñoz D., Sánchez-Moya T., López-Nicolás R., González-Bermúdez C.A., Frontela-Saseta C. and Ros-Berruezo-G. (2015). Analysis of gut microbiota of normal-weight and obese donors after fermentation of green tea. IV International Conference on Foodomics. 66. DOI: 978-88-902152-7-8. Cesena (Italy).

Sánchez-Moya T., Planes-Muñoz D., López-Nicolás R., González-Bermúdez C.A., Ros-Berruezo G. and Frontela-Saseta C. (2016). Analysis of gut microbiota of normal-weight and obese donors after fermentation of green tea. 5th European Nutrition and Dietetics Conference. a: Foodomics. 6:3 (Supl). 101. DOI: 10.4172/2155-9600. C1.021. Rome (Italy).

Planes-Muñoz D., Sánchez-Moya T., López-Nicolás R., Frontela-Saseta C. and Ros-Berruezo G. (2016). Analysis of satiety capacity of dietary pulses. II Jornadas Doctorales de la Universidad de Murcia. Escuela Internacional de Doctorado de la Universidad de Murcia (EIDUM). Murcia (Spain).

Martínez-Gracia C., Santaella-Pascual M., Caballero-Valcárcel A.M, González-Bermúdez C.A. and Sánchez-Moya T. (2017). Can iron fortification affect gut microbiota? The use of weaned piglets as a model. 4th International Conference on Nutrition & Growth. Shift 1: Infancy. 207. Amsterdam (The Netherlands).

Caballero-Valcárcel A.M., Frontela-Saseta C., Guerriero G., Sánchez-Moya T, Hausman J.F., Santaella-Pascual M. and Martínez-Graciá C. (2017). Different iron sources in infant nutrition induce changes in gene expression of iron transporter at the intestinal level. 4th International Conference on Nutrition & Growth Shift 1: Infancy. 204. Amsterdam (The Netherlands).

López-Fernández A., Frontela-Saseta C., Sánchez-Moya T. and Ros-Berruezo G. (2018). Establishment of a sensory detection threshold trial for yeast fermentation of orange juice. 4th International and 5th national student Congress of Food science and Technology. Asociación Valenciana de Estudiantes y Profesionales en Ciencia y Tecnología de los Alimentos (AVECTA). Valencia (Spain).

Ydjedd S., López-Nicolás R., Sánchez-Moya T., Frontela-Saseta C., Ros-Berruezo G., Hayette L. and Katy D.E. (2018). Effet de la digestion gastro-intestinale *in vitro* sur les extraits phénoliques de pulpe de la caroube (*Ceratonia siliqua* L.) et leur activité antioxydante. First International Congress on Biotechnologies for Sustainable Development. CIBSDD. Boumerdes (Argelia).

López-Nicolás R., Sánchez-Moya T., Teixeira-Guedes C., Planes-Muñoz D., Ros-Berruezo G. and Frontela-Saseta C. (2019). Mediterranean foods to enhance human satiety. 6th International conference of food digestion. INFOGEST. Granada (Spain).

Marín-García A., López-Nicolás R., Sánchez Moya T. and Martínez-Graciá C. (2019). Mineral Availability from edible insects. 6th International conference of food digestion. INFOGEST. Granada (Spain).

Summarizing, the overall scientific contribution derived from this PhD has been 1 indexed article, 1 article published in a non-indexed journal, 1 scientific dissemination, 4 oral communications and 7 posters. As well, total research contribution apart from this Thesis has been 2 indexed articles, 3 oral communications and 10 posters.

INTERNSHIP IN FOREIGN RESEARCH CENTRE

Centre: School of Nutrition and Translational Research in Metabolism (NUTRIM). Maastricht University. Maastricht (The Netherlands).

Period of stay: 1 February 2017- 1 May 2017.

Project title: Effects of 2 yr moderate to high protein-diet on energy expenditure, substrate oxidation, sleep-architecture, circadian rhythm, and cardiovascular health - a PREVIEW respiration-chamber-study.

Funding Organisation: ERASMUS PLUS. University of Murcia (Spain).

ANNEXES



ANNEX I



INFORME DE LA COMISIÓN DE ÉTICA DE INVESTIGACIÓN DE LA UNIVERSIDAD DE MURCIA

Jaime Peris Riera, Catedrático de Universidad y Secretario de la Comisión de Ética de Investigación de la Universidad de Murcia,

CERTIFICA:

Que D.^a Teresa Sánchez Moya ha presentado la Tesis Doctoral titulada *"Capacidad saciante y efecto antibesogénico de alimentos mediterráneos. Evaluación in vitro e in vivo"*, dirigida por D. Gaspar Ros Berruezo y D. Rubén López Nicolás, a la Comisión de Ética de Investigación de la Universidad de Murcia.

Que dicha Comisión analizó toda la documentación presentada, y de conformidad con lo acordado el día cuatro de junio de dos mil dieciocho, por unanimidad, se emite INFORME FAVORABLE, desde el punto de vista ético de la investigación.

Y para que conste y tenga los efectos que correspondan firmo esta certificación con el visto bueno del Presidente de la Comisión.

Vº Bº
EL PRESIDENTE DE LA COMISIÓN DE ÉTICA DE INVESTIGACIÓN DE LA UNIVERSIDAD DE MURCIA

Fdo.: Francisco Esquembre Martínez

ID: 1964/2018

ANNEX II



Código CEEA: 458/2018

INFORME DE COMITÉ ÉTICO

INFORME DEL COMITÉ ÉTICO DE EXPERIMENTACIÓN ANIMAL

DATOS DEL CENTRO

Nombre: **CEEA Universidad de Murcia**Número de Registro del Centro: REGA **ES300305440012**Título de la Tesis Doctoral: "*Capacidad saciante y efecto antibesogénico de alimentos mediterráneos. Evaluación in vitro e in vivo*"Alumna: D.^a Teresa Sánchez Moya

Directores de la Tesis Doctoral: D. Gaspar Ros Berruezo y D. Rubén López Nicolás

Título del Proyecto en el que se enmarca la Tesis Doctoral: "*Estudio del efecto saciante del suero de leche en ratones C57BL/GJ*"

Investigador responsable: D. Gaspar Ros Berruezo

Aspectos que han sido considerados para su evaluación:

- Capacitación del personal investigador
- Idoneidad del procedimiento en relación a los objetivos del estudio.
- Metodología empleada
- Posibilidad de conseguir conclusiones válidas con el menor nº posible de animales
- Consideraciones de métodos alternativos
- Idoneidad de las especies seleccionadas
- Supervisión, Criterios de Punto Final y Finalización del Procedimiento

Una vez evaluado el procedimiento antes mencionado, atendiendo a los puntos indicados y de conformidad con lo acordado el día 4 de junio de 2018, el Comité Ético de Experimentación Animal de la Universidad de Murcia, **INFORMA FAVORABLEMENTE** sobre la realización de dicho procedimiento.

D^a. Nuria García Carrillo

D. Francisco Esquembre Martínez

Secretaria en funciones CEEA

Presidente CEEA



