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Innovations in Satiety Based on Foods and Ingredients of the Mediterranean Diet to Regulate Overweight and Obesity

Innovaciones en Saciedad Basadas en Alimentos e Ingredientes de la Dieta Mediterranea para el Control del Sobrepeso y la Obesidad

D. David Planes Muñoz

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INNOVATIONS IN SATIETY BASED ON FOODS AND INGREDIENTS OF THE MEDITERRANEAN DIET TO REGULATE OVERWEIGHT AND OBESITY

INNOVACIONES EN SACIEDAD BASADAS EN ALIMENTOS E INGREDIENTES DE LA DIETA MEDITERRÁNEA PARA EL CONTROL DEL SOBREPESO Y LA OBESIDAD

Ph.D. Thesis

David Planes Muñoz

Thesis Tutor & Director:
Dr. Gaspar Ros Berruezo

Thesis Director:
Dr. Carmen Frontela Saseta

Food Science and Human Nutrition Department

Faculty of Veterinary Sciences - International Doctoral School

University of Murcia

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ABBREVIATIONS

α-MSH	α-melanocyte stimulating hormone
AgRP	Agouti Related Peptide
AN	Arcuate nucleus
AUC	Area under curve
BMI	Body max index
CCK	Cholecystokinin
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
EVA	Escala visual análoga
GABA	Gamma amino benzoic acid
GLP-1	Glucagon like peptide 1
IVGD	*In vitro* gastro-intestinal digestion
LPS	Lipopolysaccharide
NPY	Neuropeptide Y
MBM	Minimun basal medium
MD	Mediterranean Diet
MUFA	Mono-saturated fatty acid
NW	Normal weight
OB	Obese
PCA	Principal components analysis
PI2	Protease inhibitor II

POMC	Pro-opiomelanocortin
PUFA	Polyunsaturated fatty acid
PYY	Peptide tyrosine tyrosine
qPCR	Quantitative real time PCR (polymerase chain reaction)
SFA	Saturated fatty acid
TRH	Thyrotropin releasing hormone
VAS	Visual analogue scale
ΔAUC	Incremental area under curve
ΔVAS_{180}	Incremental VAS score at 180 minutes

RESUMEN

El sobrepeso y la obesidad, enfermedades definidas como una acumulación anormal o excesiva de grasa que puede ser perjudicial para la salud, han incrementado su incidencia a lo largo del mundo hasta afectar al 39% y al 13% de la población adulta respectivamente. La causa de estas enfermedades es un desequilibrio entre las calorías ingeridas y las gastadas por el organismo, acumulándose el exceso de estas en forma de grasa. Por esto, el estudio de los mecanismos que regulan el apetito y la saciedad, y por lo tanto la ingesta de alimentos y calorías, puede aportar información valiosa que ayude en la reducción de la ingesta alimentaria, siendo así una novedosa e importante estrategia en la lucha contra el sobrepeso y la obesidad. La regulación de la ingesta alimentaria tiene lugar a nivel del sistema nervioso central, donde el hipotálamo recibe e integra señales de diferentes partes del organismo y en función de estas determina las sensaciones de hambre y saciedad, siendo por lo tanto el encargado de desencadenar la ingesta de alimentos, de determinar la cantidad ingerida y de regular los periodos en los que no existe ingesta alimentaria. Esta estructura nerviosa también se encarga de regular el gasto energético del organismo, factor que también tiene una influencia directa sobre la acumulación de grasa, ya que cuanto mayor sea el gasto calórico, menor cantidad de grasa se almacenara como reserva, y viceversa. Las señales que recibe el hipotálamo le aportan información de la cantidad de alimento, nutrientes y energía que hay en diferentes órganos o tejidos como son el aparato digestivo, la sangre o el tejido adiposo, por lo que los diferentes alimentos, según sus características y composición, no producen igual respuesta saciante. Por todo esto, la búsqueda de alimentos que estimulen en mayor medida la sensación de saciedad puede resultar de gran ayuda para el control de la ingesta y por lo tanto del sobrepeso y la obesidad.

A la hora de elegir alimentos que ayuden a controlar el apetito, también es importante tener en cuenta que estos sean alimentos saludables. La Dieta Mediterránea (DM) ha sido ampliamente estudiada y ha demostrado tener un efecto protector frente a diversas enfermedades, siendo considerada un patrón alimentario saludable, que además, debido a los alimentos y nutrientes que la componen, puede tener un efecto positivo sobre la regulación de la ingesta alimentaria. Por esta razón, el objetivo de esta tesis fue el estudio de las propiedades saciantes de alimentos típicos de la DM para el desarrollo de nuevos alimentos saludables con capacidad de incrementar la saciedad.

La metodología aplicada en esta tesis doctoral se puede dividir en dos partes, una inicial compuesta de ensayos *in vitro* y una parte más extensa desarrollada con estudios en humanos. Por un lado, se estudio *in vitro* el efecto saciante del té verde y la curcumina, y por otro lado, un total de cuatro estudios *in vivo* fueron llevados a cabo para validar encuestas EVA como herramientas para la medición de la saciedad en humanos y para determinar las propiedades saciantes de varios alimentos típicos de la Dieta Mediterránea, o que por sus características puedan ser incluidos en las recomendaciones de este patrón alimentario. Los alimentos estudiados fueron seleccionados debido al potencial efecto sobre la reducción del apetito que la bibliografía actual les atribuye.

El estudio *in vitro* del té verde y de la curcumina fue llevado a cabo exponiendo extractos de estos dos alimentos a las células enteroendocrinas STC-1, las cuales producen y liberan las hormonas saciantes CCK y GLP-1, hormonas que pueden ser usadas como biomarcadores de la saciedad. Los extractos fueron usados de dos formas diferentes, tanto habiendo sido sometidos previamente a un proceso de digestión gastrointestinal *in vitro* (DGIV), como sin haber sido sometidos a dicho proceso. Como controles positivos se usaron proteína de guisante y ácidos grasos de cadena corta (AGCC), los cuales han demostrado estimular la liberación de las hormonas citadas previamente por parte de la línea celular STC-1. La microbiota intestinal ha demostrado ser clave en el estado de salud del huésped, y también se le atribuye una posible relación con la regulación del apetito, por lo que el efecto del extracto de té verde, previamente sometido a un proceso de DGIV, sobre la evolución de la población bacteriana intestinal de donantes humanos fue estudiado en una fermentación colónica *in vitro* durante cuarenta y ocho horas, tiempo durante el que se analizó la evolución de bacterias totales, *Bacteroides, Bifidubacterium, Enterobacteriaceae,* Firmicutes y *Lactobacillus*, así como los cambios de pH y de presión producidos durante el proceso. Como controles positivos se usaron inulina y

D-glucosa.

Las propiedades saciantes de alimentos de la DM fue evaluada en ensayos en humanos usando principalmente escalas visuales análogas (EVA). Las EVA son ampliamente usadas en estudios de saciedad en humanos y sirven para medir las sensaciones subjetivas de apetito y saciedad de una manera rápida y sencilla. La validez de esta herramienta fue demostrada en encuesta en lengua inglesa, habiendo sido validadas también en otros idiomas, pero hasta la fecha, la validez de su uso en castellano no ha sido comprobada, por lo que el primer paso de esta parte de la presente tesis fue desarrollar EVA en castellano para medir las sensaciones de apetito y saciedad y realizar un estudio para su validación en humanos. Además, aprovechando las ventajas que ofrecen las nuevas tecnologías, el grupo de investigación NUTBRO de la Universidad de Murcia ha desarrollado una aplicación para smartphones y tablets, en la que se pueden programar y configurar diversos tipos de encuestas para ser administradas a los participantes en estudios llevados a cabo en humanos. Entre los tipos de encuestas que se pueden configurar en esta App se encuentra las de tipo test, lógico (si/no, verdadero/falso) o EVA. El desarrollo y administración de encuestas usando una app de este tipo ofrece varias ventajas, tanto para los investigadores como para los participantes, pero antes de su aplicación en investigación, realizar una validación del uso de la App para medir saciedad en humanos es necesaria, debido a que en el formato clásico validado en encuestas en papel, las líneas sobre las que se contestan las diferentes preguntas EVA son de 10 cm, pero esta longitud se ve reducida al administrar las EVA en smartphones y tablets, e incluso en función de la posición en que se use el dispositivo (vertical u horizontal). Por lo tanto, un estudio en humanos fue llevado a cabo con el objetivo de validar el uso de la APP para la medida de las sensaciones subjetivas de apetito y saciedad en población humana.

Usando las herramientas previamente validadas, dos estudios en humanos fueron llevados a cabo para analizar las propiedades saciantes de diferentes alimentos típicos de la DM. El primero de estos estudios se centró en tres alimentos complejos típicamente consumidos en la región mediterránea y que aportaban perfiles nutricionales bien diferenciados; gazpacho, ajoblanco y hummus, usando un cuarto alimento, el pan blanco, como alimento de control y de referencia. El segundo estudio se llevó a cabo para encontrar los alimentos más saciantes entre la almendra, la avena, el huevo, la patata, la chufa, la nuez y el pan blanco, este último, nuevamente fue usado como alimento control y de referencia. Los alimentos fueron elegidos por su teórico potencial saciante en base a la bibliografía actual, con un doble objetivo; a) brindar información

sobre cuáles de ellos producen un mayor control del apetito, ya que actualmente no existe demasiada información al respecto entre diferentes tipos de alimentos, y b) seleccionar los mejores candidatos para el desarrollo de nuevos alimentos con gran potencial saciante, los cuales serían probados en nuevos estudios en humanos para verificar su efecto a medio plazo sobre el control de la ingesta alimentaria. La metodología aplicada en ambos estudios fue similar, siendo los alimentos analizados en pruebas de un día, donde los voluntarios llegaban en ayunas a las instalaciones del grupo de investigación NUTBRO y se les servía un desayuno. Un hora después, ingerían el alimentos en estudio, cumplimentando justo antes y durante tres horas después encuestas EVA sobre saciedad y apetito. Una encuesta de palatabilidad fue también cumplimentada justo después de ingerir el alimento en estudio, para controlar el efecto de la aceptabilidad de los alimentos sobre el apetito. En el caso del primero de estos dos estudios, también se realizó una prueba de ingesta *ad libitum* tras las tres horas de estudio, con el fin de medir una posible compensación en la ingesta calórica.

Los estudios *in vitro* mostraron una mayor liberación de ambas hormonas saciantes (CCK y GLP-1) por las células enteroendocrinas STC-1 tras la exposición a ambos extractos de plantas en estudio (té verde y cúrcuma) que para los dos controles positivos utilizados, que en este caso fueron proteínas de guisante y ácidos graso de cadena corta. También se demostró un importante efecto de la digestión gastrointestinal *in vitro* de los extractos vegetales sobre la liberación de hormonas por parte de las células STC-1, ya que durante la exposición a la línea celular de las muestras digeridas y sin digerir, el té verde provocó una mayor liberación de hormonas después de haber sido sometido al proceso de digestión. En el caso de la cúrcuma, el efecto fue el contrario, provocando una liberación de hormonas notablemente inferior el extracto digerido que el que no había sido sometido a dicho proceso. El extracto de té verde mostró buenos resultados de estabilidad al proceso de DGIV, por lo que fue seleccionado para estudiar su efecto sobre la microbiota intestinal de donantes humanos. Los resultados obtenidos tras la fermentación colónica *in vitro* del extracto de té verde no mostraron un efecto importante en la modulación de las poblaciones microbianas analizadas.

En el primero de los estudios con alimentos de la DM realizado en humanos, el gazpacho mostró ser el alimento que mayor supresión del apetito provocó, seguido del hummus, pero en este último caso las diferencias observadas fueron menos evidentes. El último de los estudios incluidos en la presente tesis, también llevado a cabo en humanos, reveló a la avena, seguida de la patata, como los alimentos que mayor respuesta saciante produjeron, sin mostrar el resto

de alimentos efectos significativamente diferentes entre ellos sobre el hambre experimentada por los participantes en el estudio. Un dato importante que merece ser destacado, es que en estos dos últimos estudios, donde las propiedades saciantes de diferentes alimentos fueron comparadas directamente en humanos, los alimentos más saciantes no fueron los que tenían el mayor contenido de proteína o fibra, nutrientes que son considerados según las evidencias científicas, como los que mayor respuesta saciante provocan. Existen más estudios en humanos que no han podido encontrar que la inclusión de alimentos con mayor contenido de proteínas y/o fibra haya resultado en una mayor sensación de saciedad de los participantes, lo que junto a lo observado en esta tesis sugiere que aún queda mucho por estudiar sobre la regulación de la ingesta alimentaria, y que quizás sería interesante estudiar el efecto sobre la regulación de la ingesta, de otros factores menos explorados hasta la fecha, como pueden ser la influencia de la ingesta de micronutrientes o incluso del propio estado nutricional de los individuos.

Con los resultados obtenidos del último estudio desarrollado, donde la avena se monstruo como el alimento más saciante de todos los ensayados, se desarrolló una bebida con alto contenido en fibra y un postre, ambos a base de avena, con el fin de estudiar su efecto sobre la saciedad en un ensayo en humanos a medio plazo, donde estos alimentos se incluirían de forma habitual en la dieta de los participantes durante dos meses, analizando la evolución del apetito, parámetros antropométricos y la microbiota intestinal. Este estudio comenzó en febrero de 2020, pero fue suspendido dos semanas después debido al confinamiento decretado en todo el territorio nacional por motivo de la pandemia COVID-19.

SUMMARY

Overweight and obesity, defined as abnormal or excessive fat accumulation that may impair health, have increased worldwide reaching 39% and 13% of adult population. Enhanced satiety foods could be of great help in regulating energy intake and weight control because they promote reduced food intake. For this reason, the aim of this thesis was to study the satiating properties of several foods, both *in vivo* and *in vitro*, with the goal to develop new foods with increased satiety properties as part of a healthy diet.

This doctoral thesis is divided into two sections. On the one hand, the study of *in vitro* satiating effect of green tea and turmeric extracts, and on the other hand, *in vivo* studies in humans were developed for the evaluation of several types of typical foods of the Mediterranean Diet (MD), a food pattern known for its healthy effects. The *in vitro* study of green tea and turmeric was carried out by exposing this plant extracts to the enteroendocrine cell line STC-1, measuring the satiating effect of these extracts by the quantification of the hormones CCK and GLP-1, released by cells. In addition, to determine the effect of the extract on the gut microbiota, the green tea extract was submitted to an *in vitro* colonic fermentation by using gut microbiota from human donors.

The satiating properties of several MD foods were tested by human volunteers using visual analogue scales (VAS) to measure subjective feelings of appetite and satiety induced by foods. The first step in this part of the thesis was the development and validation of VAS in Spanish, as well as the validation of its use in an application for smartphones and tablets. Two different studies were performed with human participants using the validated VAS surveys, the first

study tested four complex foods typical of the DM; gazpacho, ajoblanco, hummus and white bread. The second study was carried out to find the most satiating foods among almond, oat, egg, potato, tigernut, walnut and white bread. Foods were chosen for their theoretical satiating potential, with the double objective of providing information on which of them offer greater appetite control, and to select the best candidates for the development of new foods with great satiating effect which would be tested in new human studies to verify its real effect in this regard.

In vitro studies showed a higher release of satiating hormones by enteroendocrine cells for both plant extracts, green tea and turmeric than for the used positive controls, showing catechins of green tea a greater stability to digestion than curcumin. Green tea was selected to evaluate its effect on human microbiota, where the obtained results were more modest. Among the four tested complex foods included in MD, gazpacho showed the greatest appetite suppression, followed by hummus, but in this last case the observed differences were less evident. The last study, revealed oat, followed by potato, as the foods with the highest satiating response. Surprisingly, in none of the studies were the most satiating foods those with the highest protein or fibre content, nutrients known to elicit a greater satiating response than other nutrients.

With the obtained results from oat, a high fibre drink and a dessert were developed, in order to include them in the diet of a human study for two months, analysing the evolution of appetite, anthropometric parameters and intestinal microbiota as the output of the experiment. This study was launched in February 2020, but was suspended two weeks later due to the lockdown decreed for the COVID-19 pandemic.

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CHAPTER 1

MODULATION OF SATIETY AS A TOOL IN WEIGHT MANAGEMENT. GENERAL INTRODUCTION

1.1 Obesity and overweight

Overweight and obesity, defined as abnormal or excessive fat accumulation that may impair health, have grown progressively worldwide reaching 39% and 13% of adult population respectively according to data from WHO (WHO, 2017). Both, overweight and obesity, can lead to grave consequences for health, such as a greater risk of suffering cardiovascular diseases, diabetes, muscle-skeletal disorder and even some types of cancer, such as endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon cancer (WHO, 2017). In addition to the previously mention health problems, overweight and obesity also result in a greater economic cost for society (Lehnert, Sonntag, Konnopka, Riedel-Heller, & König, 2013). Obese people have a medical cost approximately 30% higher than people with a normal weight (Withrow & Alter, 2011), and assuming the expense on the gross domestic product in Europe (thirteen western European countries analysed) ranged from 0.09% to 0.61%, with a healthcare burdens of up to 10.4 billion euros (Müller-Riemenschneider, Reinhold, Berghöfer, & Willich, 2008).

Owing to their high prevalence and their high growth prevision, more research to control these illnesses is necessary. It is well known that the principal cause of overweight and obesity is an energetic imbalance between ingested and expended calories, when the amount of calories ingested is greater than the amount of calories expended, accumulating this excess of energy as fat reserves in adipose tissue (Miller, 2019). Because of the high prevalence of this illnesses, and the serious health and economic problems that it produces, strategies to control this situation are of great importance. As the cause of obesity and overweight is a greater calories intake than expenditure, an important factor to influence is the food intake, reason why the study of the appetite and satiety regulation could be of great help to achieve this goal. In this regard, consumption of foods in the daily diet that increase satiety could help to controlling calories ingestion (Blundell, 2010; Yuliana et al., 2011) without causing an important increase of the hunger sensation.

1.2 Appetite and food intake regulation

There are two related, but different precesses involved in the appetite suppression, satiation and satiety, and both of them implicate different mechanisms that are important to know at

the study of appetite regulation. In one hand, satiation is the process that brings a meal to an end, thought a set of processes that inhibit an eating episode. In the other hand, satiety is the precess that maintains the inhibition of the motivation to eat, thus is related with the time that the hunger is suppressed before a new eating event (Blundell et al., 2010). The hunger and satiety mechanisms are regulated at the central nervous system (CNS) through the hypothalamus, where the satiety and hunger centres are located. The hypothalamus integrate autonomic, endocrine and behavioural signals through afferent and efferent pathways between brainstem and peripheral tissues (Miller, 2019). There are several nuclei of the hypothalamus involved in the regulation of appetite, the most important of which is the arcuate nucleus (AN), also involving the paraventricular, dorsomedial and lateral nuclei. As already mentioned, these hypothalamic structures receive signals of different types and places.

In AN, pro-opiomelanocortin (POMC) neurons produce α-melanocyte stimulating hormone (α-MSH) that stimulates MCR4 receptors, also located in this nerve structure. When this receptor is stimulated, there is an increase in satiety and thermogenesis, being related to body weight loss (Mountjoy, 2010; Pritchard, Turnbull, & White, 2002). α-MSH also acts on the paraventricular nucleus, producing the release of nesfatin, oxytocin, thyrotropin releasing hormone (TRH) and corticotropin releasing hormone (CRH), which also increase satiety (Miller, 2019). Another group of neurons also located in the arcuate nucleus, produce Neuropeptide Y (NPY) and Agouti Related Peptide (AgRP). The Y receptors of AN are stimulated by NPY, producing an increase in food intake (Beck, 2006), while AgRP has its effect on the α-MSH receptor, but in this case in an antagonistic way, thus inhibiting it (Nijenhuis, Oosterom, & Adan, 2001). Therefore, these neuropeptides are responsible for increasing appetite. Other neurotransmitters also have an effect on the regulation of appetite or energy expenditure in the hypothalamus, such as Gamma amino benzoic acid (GABA), which reduces energy expenditure, and serotonin and oxytocin, which decrease food intake and, in the case of oxytocin, also increases energy expenditure (Hume & Leng, 2019; Turenius et al., 2009; Voigt & Fink, 2015). This mechanism for regulating appetite and energy expenditure receives information from other nerve structures, such as the amygdala of the limbic system, with emotional information, and the brainstem, which collects information from peripheral signals, hormones and nutrients (Miller, 2019).

These nervous structures, in charge of regulating appetite, collect information from different peripheral tissues through hormones that act on the vagus nerve or directly on specific receptors of the nerve structures themselves. Leptin is a hormone produced by adipose tissue whose

concentration in the blood is directly proportional to the amount of fat, especially subcutaneous. In this way, leptin provides information on the amount of energy reserves in the form of fat in the body (Schaab et al., 2012). It acts directly on the hypothalamus through specific transporters that make it reach the AN and other nuclei, where it stimulates the activity of POMC neurons and decreases Y neurons, thus producing a decrease in intake (Kwon, Kim, & Kim, 2016). Another peripheral hormone highly studied for its effect on satiety and which also acts directly on AN, is insulin. This is produced by the β cells of the pancreas and its release is stimulated by the concentrations of glucose in the blood (Kalivarathan, Saravanan, Levy, & Kanak, 2020). The effect on the hypothalamus is similar to leptin, increasing the activity of POMC neurons and decreasing the Y neurons activity, thus reducing energy intake (Williams, Scott, & Elmquist, 2011).

Several hormones are produced in the digestive system and are released in relation of the presence of food. Enteroendocrine I cells release the hormone cholecystiquinin (CCK) in the duodenum and jejunum, stimulated by satured fatty acids, long chain fatty acids, amino acids, and small peptides (Geraedts, Troost, Fischer, Edens, & Saris, 2011; Miller, 2019). CCK acts on the vagus nerve, carrying information to the brainstem and from there to the hypothalamus, causing a slowdown in gastric emptying and food intake (Berthoud, 2007). The peptide tyrosine-tyrosine (PYY), also released in the intestine, increases satiety, energy expenditure and reduces gastric emptying, acting on receptors of the vagus nerve (Baldock et al., 2007). Glucagon like peptide 1 (GLP-1) is produced by L cells in the intestine by the glucose levels and by the presence of some non digested nutrients in the distal gut, also producing a satiating effects and reducing gastric emptying. In this case, GLP-1 communicates directly with the CNS by specific receptors in brainstem and some hypothalamic nuclei, such as AN and paraventricular nucleus (Secher et al., 2014). Other hormones from gut that increase satiety are oxytomodulin, obestatin or nesfatin (Miller, 2019). The opposite effect is produced by the hormone ghrelin, which is released primarily by the stomach in interdigestive periods. Ghrelin provides information to the hypothalamus through specific receptors or via the afferent vagal pathway, producing an increase in the release of NPY and AgRP, and the inhibition of POMC neurons, with the consequent hunger increase (Delzenne et al., 2010).

To illustrate all the precesses implicated in the food intake regulation, Blundell et al., (2010) proposed the satiety cascade, whose diagram can be seen in Figure 1.1. In this figure it can be seen in summary what type of signals modulate appetite regulation at CNS level, where do

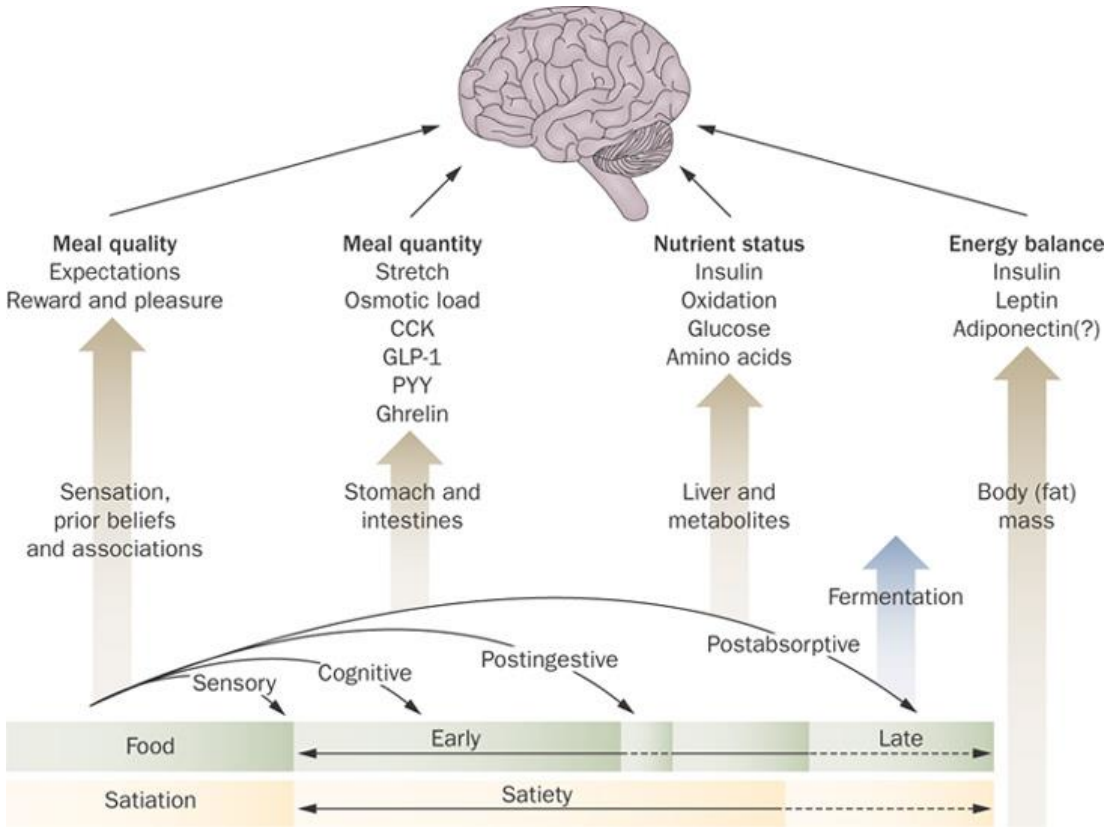

Figure 1.1: Satiety cascade showing the relationship between satiation and satiety, and and the factors implicated in this processes (Blundell, 2010).

these signs come from and at what moment each one of them takes place, before, during or after the ingestion of food.

As it can see, most of these signals provide information to the hypothalamus about the foods and nutrients that are both in the digestive system and in other tissues such as blood and adipose tissue, and the hypothalamus integrates these and other signals, to regulate in this way the hunger and satiety sensations and therefore food intake. As different signals are stimulated by different food component, different types of food and nutrients have a different effect on the regulation of appetite and food intake.

1.3 Satiating effect of foods and their nutrients and components

Several studies on the satiating effect of nutrients have focused on macronutrients, many of them describing proteins as the nutrient that induces a greater appetite suppression, followed by carbohydrates and fats (Veldhorst et al., 2008; Westerterp-Plantenga, Lemmens, & Westerterp, 2012). As mentioned above, the presence of these nutrients and their digestion‘s products

in the digestive tract, stimulate the secretion of hormones that send information to the hypothalamus, thus producing a modulation in the regulation of appetite. The fibre, indigestible carbohydrates, provided by some foods, also has been well studied regarding to its effect on appetite, showing a satiety increase due to several mechanism. Fibre ingestion delaying the gastric emptying, thus producing a higher satiety (Clegg & Shafat, 2010). Fibre also affects the appetite through delaying nutrients digestion and absorption, through physical obstruction of the nutrients in the intestine caused by the presence of insoluble fibre and by increasing the viscosity into the small intestine, effect produced by soluble fibre (Warrilow, Mellor, McKune, & Pumpa, 2019). Another factor that may enhance satiety by fibre, is the production of sort chain fatty acids after the colonic microbiota fermentation, which can interact with GPR43 receptor in the intestinal enteroendocrine cells and produces the release of hormones with satiating effect, such as PYY and GLP-1 (Kimura, Inoue, Hirano, & Tsujimoto, 2014).

Although the satiating effects of the macronutrients have been well studied in both, *in vitro* and *in vivo* studies, when the proportions of proteins, carbohydrates and fats have been manipulated in diets, differences in appetite regulation have been not found (Carreiro et al., 2016), therefore the fact that some macronutrients can further modulate the expression of hunger than others is not entirely clear.

The purpose of the food intake regulation is to supply the organisms with the nutrients it needs to maintain homeostasis and therefore a proper function in the body. For this, carbohydrates, proteins and fats are equally necessary as vitamins and minerals, essential nutrients that have vital functions and that must be ingested regularly. Therefore, it would not be ruled out that could be also internal mechanisms that monitor the amount of micronutrients and that modulate appetite depending on possible deficits. Although less studied in terms of satiety, recently some studies have explored this route offering promising results.

In this regard, studies in humans have shown greater weight loss, lower *ad libitum* intake, and increased PYY plasma levels when the diet has been supplemented with calcium. Similar studies with vitamin supplements in the participants' diet resulted in a greater feeling of satiety (Major et al., 2008). Another interesting finding in this sense is that in obese people, a lower consumption of micronutrients has been observed than in people with normal weight (Poli et al., 2017), the latter group also having greater success maintaining their weight. Regarding this relationship between micronutrient consumption and BMI, it has also been shown that people who regularly consume vitamin and mineral supplements have a lower BMI. Specifically for

vitamin C, a lower plasma concentration has been found in obese patients, a vitamin that is negatively correlated with BMI and has been shown to decrease triglycerides in the blood, which inhibit the transport of leptin through the blood-brain barrier (Aytekin, Godfri, & Cunliffe, 2019; Tremblay & Bellisle, 2015). In a study with 768 participants (Fuhrman, Sarter, Glaser, & Acocella, 2010), they changed from a low micronutrient to a high micronutrient density diet and the experience and perception of hunger were measured before and after the new diet. Results showed decrease in hunger pains, uncomfortable hunger between meals, discomfort it meal was skipped and frequency of hunger, among other findings, concluding that high micronutrient density diet mitigates hunger even with a lower calories diet.

1.4 Studies design to measured satiety

Satiety can be measured by both, *in vivo* and *in vitro* studies, through free access to food and noting when or how much the study subjects eat. In the first case, the participants are allowed to eat whenever they want, taking the time that has elapsed until then as a measure of satiety. In the second case, access to a meal is allowed *ad libitum* at a fixed time, taking the measure of satiety as the amount of food ingested by the subjects (Tremblay & Bellisle, 2015). As mentioned above, there are several hormones involved in the modulation of appetite, so they can be used in the measurement of satiety as biomarkers, both *in vivo*, taking samples of blood or other fluids for measurement, and *in vitro*, through the use of cell lines, such as STC-1, that can produce and release CCK, PYY or GLP-1 (Chang, Chey, Sun, Leiter, & Chang, 1994; Geraedts, Troost, & Saris, 2009; Geraedts et al., 2011). A widely used tool in the study of hunger in humans, are visual analog scales (VAS), used to measure subjective sensations of hunger and satiety through surveys and which have proven their validity and reproducibility for this purpose (Flint, Raben, Blundell, & Astrup, 2000). Among others tools to assess parameters related with hunger in humans, there are also surveys that have been designed to measure eating behaviour, such as the three factor eating questionnaire (TFEQ) (López-Aguilar et al., 2011) or the control of eating questionnaire (CoEQ) (Dalton, Finlayson, Hill, & Blundell, 2015).

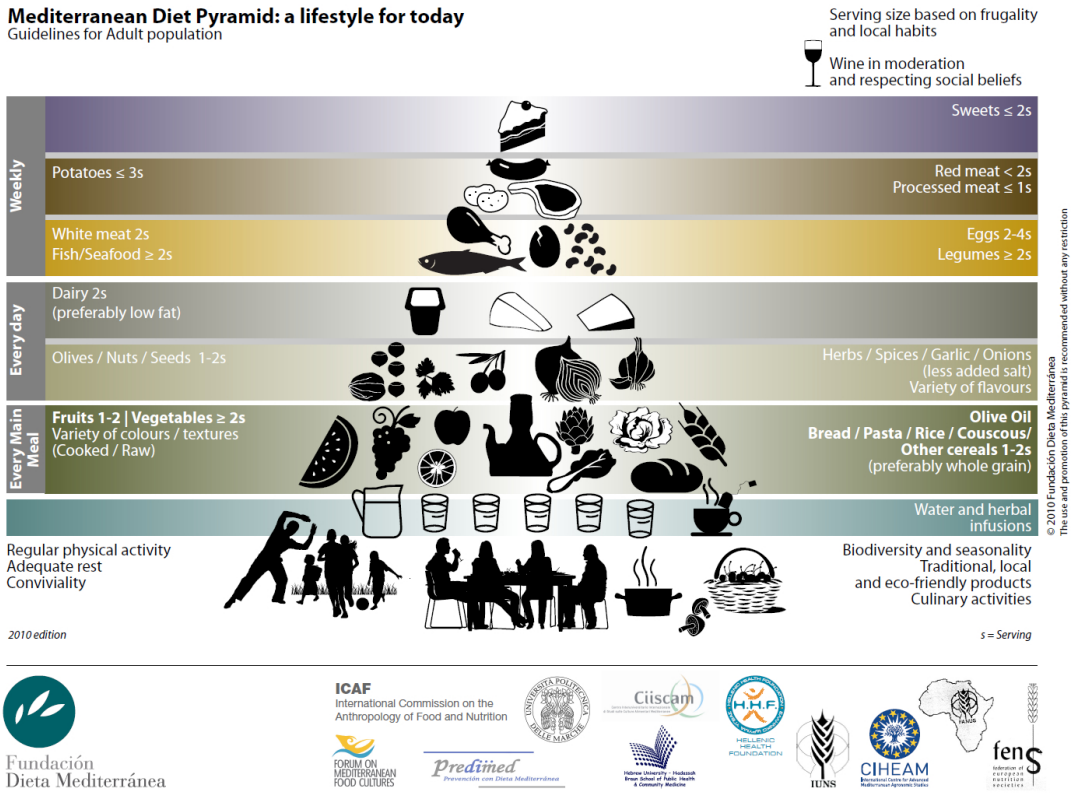

Figure 1.2: Pyramid of the Mediterranean Diet from the Mediterranean Diet Foundation (2010).

1.5 Mediterranean Diet, a healthy food pattern and its relation with satiety

Mediterranean Diet (MD) is considered a healthy food pattern and it has been widely studied in this regard showing a protective effect over several chronic diseases (Serra-Majem et al., 2019). MD is a typical dietary pattern of the countries of the Mediterranean basin, more specifically of those that grow olives and therefore have olive oil as a very present food in their diet. The MD was declared Intangible Heritage of Humanity by UNESCO in 2013 and as for the dietary pattern that follows it is characterized by a high consumption of legumes, nuts, unrefined cereals, fruits, vegetables and fish. Also, as already mentioned, the daily consumption of extra virgin olive oil is a characteristic of the MD. The consumption of dairy products, meats and their derivatives and wine are carried out moderately (Serra-Majem et al., 2019). A graphic way to see the characteristics of this MD eating pattern are the dietary guidelines, such as the Pyramid of the Mediterranean Diet (Figure 1.2) that has been developed by the Mediterranean Diet Foundation.

For many years, MD has been widely studied for its healthy effects and, these studies establish a solid relationship between a greater adherence to the Mediterranean diet and a reduction in the risk of overall mortality, cardiovascular diseases, coronary heart disease, myocardial infarction, overall cancer incidence, neurodegenerative diseases and diabetes (Dinu, Pagliai, Casini, & Sofi, 2018), also specific nutrients and food included in the MD are inversely related with obesity, mental health, respiratory diseases and bone diseases (Serra-Majem et al., 2019). Regarding the relationship between MD and the control of food intake, there are not too many studies on it at present, but focusing on the high amount of dietary fibre that this eating pattern provides and the low caloric density of the foods that compose it, it is hoped that high adherence to MD may help increase satiety and therefore reduce overall calories intake (Schröder, 2007), therefore MD could be a good source of foods that are both healthy while helping to control appetite.

1.6 Objectives

Although there are many works that have studied the satiating properties of both different foods, nutrients, and also other food compounds, there are not many studies that have compared different types of meals or foods among them, in order to also have information on what types of foods cause a better satiating response, information that could be used to include these foods in the diet on a regular basis, in order to achieve diets with greater satiating potential that can be helpful in controlling intake and consequently weight. In addition to this effect, it is important that the foods that are going to be recommended in a diet, are also healthy in all possible aspects, such as typical foods or included in the recommendations for the Mediterranean Diet (Martínez-González et al., 2015; Serra-Majem et al., 2019).

- General objective
  - To identify foods with high satiating capacity of the Mediterranean Diet for the development of new satiating foods from them to achieve greater control of foods intake through the diet.
- Specific objectives
  - To analyse the *in vitro* effect of green tea and turmeric on microbiota population

and satiating hormones release by STC-1 cell line.

- To develop and validate viaual analogue scales (VAS) in Spanish and its use in SatinApp, an app for administering surveys on tablets and smartphones.
- To analyse the satiating capacity in humans of three complex foods typical of the Mediterranean diet, gazpacho, ajoblanco and hummus.
- To identify through a screening in a human study, the most satiating foods among almonds, walnuts, tiger nuts, eggs (omelette), oats and potatoes, for the subsequent development of new satiating foods.

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CHAPTER 2

IN VITRO EFFECT OF GREEN TEA AND TURMERIC ON SATIETY AND ON GUT MICROBIOTA OF NORMAL-WEIGHT AND OBESE DONORS

In vitro effect of green tea and turmeric on satiety and on gut microbiota of normal-weight and obese donors

Abstract: Among different foods, green tea and turmeric have showed some positive effect on weight control and could play a role in food intake regulation where the gastrointestinal tract has a key role through several mechanism such as the effects of enteroendocrine cells or the gut microbiiota, which is related with the host's metabolism and homeostasis, and can be shaped by dietary changes. Plant extract activity can be modified by the digestion process. In order to assess the effect on satiety and gut mircrobiota of green tea and turmeric extracts, an *in vitro* gastrointestinal digestion process was performed. Then, the digested green tea was submitted to the colonic phase by batch fermentation using samples of faeces for gut microbiota from six donors (three with normal weight and three with obesity). Finally, aliquots at 0, 12, 24 and 48 hours were taken for the analysis of gut microbiota of donors. In addition, the effect of digested and non-digested green tea and turmeric extracts on satiating hormones release was assessed by exposing these samples to a STC-1 cell line along 120 minutes. The release of CCK and GLP-1 by the STC-1 cells was similar or higher for the analysed samples compared to the positive controls. Non-digested turmeric showed the largest release of both hormones, but when it was digested, its effect on hormones release was significantly lower. Nevertheless, for green tea hormone release remained high, compared to controls, and stable after digestion, therefore the *in vitro* gastrointestinal digestion process affects the satiety of the samples and digested green tea showed the most stable satiating capacity. In the case of the batch fermentation, green tea produced important variation in pH and gas production, but the analysed microbiota during the colonic digestion process, did not show an improvement in its composition compared to the positive controls.

Keywords: Green tea, Obesity, Satiety, STC-1, Gut Microbiota, qPCR.

2.1 Introduction

2.1.1 Gut Microbiota

Research on the human gut resident microbiota is becoming increasingly important due to its important effect in the host health. The gastrointestinal tract harbours the largest number of bacteria present in the human body (more than 150-fold their eukaryotic nuclear genome) and includes trillions of microorganisms mainly divided into Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobia and Fusobacteria phyla and 90% of bacteria that live in our gut belong to the Firmicutes (60%), Bacteroidetes (15%) and Actinobacteria phyla (Alonso & Guarner, 2013; Tremaroli & Bäckhed, 2012; Wang, Yu, & Adeli, 2020). Other important phyla present in the human intestine are Proteobacteria and Verrucomicrobia, being less frequent the presence of bacterial groups such as Cyanobacteria, Fusobacteria, Lentisphaerae, Spirochaetes (Gangarapu, Yıldız, Ince, & Baysal, 2014). Different studies report that there are individual differences on the intestinal microbiota composition although all people share an important number of basic common microorganisms (Molinaro, Paschetta, Cassader, Gambino, & Musso, 2012; Tremaroli & Bäckhed, 2012). The gut microbiota population affects host carbohydrate, lipid, and amino acid metabolisms that highly contribute to diseases such as obesity, type 2 diabetes, dyslipidemia, non-alcoholic fatty liver disease, gout, vitamin deficiency, and atherosclerosis. Gut microbiota is also highly related with the immune system development in infancy and its adequate maintenance during the life contributing to the host defence against pathogens, cancer prevention and reducing inflammation (Delzenne & Cani, 2011; Lankelma, Nieuwdorp, de Vos, & Wiersinga, 2015; Wang et al., 2020). Moreover, inter-individual differences observed in the gut microbiota can exert important effect on disease processes (Kerimi, Kraut, da Encarnacao, & Williamson, 2020).

2.1.2 Microbiota and weight control

As indicated above, a solid link between gut microbiota and obesity has been found. In this regard, several studies have shown a positive relation between Firmicutes to Bacteroidetes ratio with BMI and obesity (Delzenne & Cani, 2011; Indiani et al., 2018; Koliada et al., 2017); moreover, an improvement on body energy regulation, obesity and metabolic syndrome in presence

of *Bacteroides* and *Bifidobacteria* has been also observed (Fava et al., 2013), suggesting that microbiota is a major contributor to energy homeostasis regulation (Moreno-Indias, Cardona, Tinahones, & Queipo-Ortuño, 2014). An study on obese mice showed a higher Firmicutes population compared to Bacteroidetes population was found, and similar results were observed in human studies where a higher Firmicutes amount was observed in case of obese individuals. Although some studies have shown positive relation between Bacteroidetes and lean individuals, other have not found any relationship in this regard and, then the possible effect of this Phylum on obesity is still not clear (Moreno-Indias et al., 2014). Similar studies in children, reported a bigger amount of *Bifidobacterium* and fewer of *Staphylococcus aureus* when compared normal-weight with overweight children (Kalliomäki, Collado, Salminen, & Isolauri, 2008); meanwhile, in obese woman, larger amounts of *Staphylococcus aureus*, *Escherichia coli* and *Enterobacteriaceae* than in normal-weight were found (Delzenne & Cani, 2011). This relationship found between gut microbiota and body weight could justify the role of microbiota on satiety and food intake control. Regarding to this, short chain fatty acids (SCFAs) produced by gut microbiota, such as acetate, propionate and butyrate have a wide repercussion on host health and have been shown to suppress the food intake activating the vagal afferent pathway (Goswami, Iwasaki, & Yada, 2018).

2.1.3 Gut microbiota metabolites and their relation with satiety

Gut microbiota interacts with the host, mainly, through their metabolites. Some of these metabolites include lactic acid or, as it has commented above, SCFAs such as acetic, butiric or propionic acids. These acids can decrease pH, helping to the development of an adequate microbiota population into the whole intestine (Duncan, Louis, Thomson, & Flint, 2009). Gut bacteria produce and metabolize different types of fatty acids, thus the different SCFAs profiles and quantities can guide us about differences on the gut microbiota population and how to modulate it (Delzenne & Cani, 2011). These SCFAs have some beneficial effects on the host. Colonocytes can use butyrate to produce energy, which is mainly produced by *Clostridium coccoides*, *Lactobacillus spp* and *Bifidobacterium spp*, being important for a correct function of intestinal cells. In case of propionate, it has a key role as a substrate for gluconeogenic and in the lipogenesis reduction, while acetate is a precursor of fatty acids and cholesterol (Moreno-Indias et al., 2014). All these SCFAs can interact with GPR43 receptor, located in intestinal and in adipose tissue and thus producing the release of hormones with satiating effect in the intestine,

such as Peptide Tyrosine-Tyrosine (PYY) and Peptide like glucagon 1 (GLP-1) decreasing the intestinal motility and increasing satiety. In adipose tissue, the interaction with this receptor induces lipolysis, adipogenesis and could also increase leptin release (Kimura, Inoue, Hirano, & Tsujimoto, 2014).

In addition to the metabolites already mentioned, some of these bacteria are also capable of producing some substances with negative effects on health that occurs when there is an altered gut bacterial composition, phenomenon known as dysbiosis and what is related to obesity (Bond & Derbyshire, 2019). In this case, the release of lipopolysaccharide (LPS) by intestinal bacteria, could lead to different inflammatory diseases, infections and also could modify the effect of gut microbiota on energy homeostasis. The LPS is released by Gram negative microorganisms and can affects TLR4 receptor on vagal afferent pathway, inducing the development of obesity, type 2 diabetes and low-grade systemic inflammation. Regarding this, high levels of LPS has been found in obese blood compared with lean individuals (Cani et al., 2007; Saad, Santos, & Prada, 2016; de La Serre, de Lartigue, & Raybould, 2015).

2.1.4 Green tea and turmeric: healthy properties and relation with satiety

Each bacterial species can metabolize certain types of nutrients according with their genetic information and therefore, the diet and its components are main factors in developing and shaping the gut microbiota across the life time (Thursby & Juge, 2017; Moreno-Indias et al., 2014). Indeed, dietary changes could explain approximately 57% of the gut microbiota variations whereas differences in the host genes involved suppose less than 12% in this variation (Zhang et al., 2010). Experiments in mice with a high fat diet showed an increased in Firmicutes, like Mollicutes, and a decreased in Bacteroidetes and in *Bifidobacterium*, whereas calorie-restricted diets showed to reduce the growth of *Clostridium coccoides*, *Lactobacillus spp.*, and *Bifidobacterium spp.* Both in human and mice with a diet rich in fat and lower in fibre during 1 day, induced significant changes in gut microbiota's composition (Moreno-Indias et al., 2014; Delzenne & Cani, 2011).

Green tea consumption has been shown to have a positive effect on human health. Tea contains polyphenols and other components, especially catechins, that have been widely reported to reduce the risk of some diseases such as cancer, cardiovascular diseases, diabetes, obesity or arthritis, being the green tea, among all the tea types, the most interesting in this field (Afzal, Safer,

& Menon, 2015; Khan, Afaq, & Mukhtar, 2008; Khan & Mukhtar, 2008; Suzuki, Pervin, Goto, Isemura, & Nakamura, 2016). Green tea is produced from the *Camellia sinensis* young leaves without fermentation, on the contrary that other teas like black, whose leaves are fermented before sale, causing important modifications on its antioxidant compounds content. Regarding this, the most important active polyphenols of green tea are (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG) and (–)-epicatechin (EC) (Khan & Mukhtar, 2013). Based on this, a typical cup of green tea provides 250–350 mg of tea solids compounds, of which 30–42 % are catechins and 3–6 % caffeine (Mukhtar & Ahmad, 1999). When the tea catechins are consumed, they pass through the small intestine with a minimal absorption and reach the colon where the gut microbiota can metabolize it, producing aglycones and various aromatic acids like phenylvalerolactones and hydroxyphenylpropionic acids (Nakagawa, Okuda, & Miyazawa, 1997). This fact can explain the low bioavailabiity of catechins and the small amount found in human plasma (Khan & Mukhtar, 2013). Then, the effect of these green tea compounds directly on the gut microbiota could benefit gut health throughout shaping the gut microbiota profile, especially when there is a dysbiosis (Bond & Derbyshire, 2019). With this background, green tea consumption could have potentially positive effect on overweight and obesity increasing satiety and reducing food intake. These results have been observed in both, *in vivo* and *in vitro* studies. For example, a study on 14 healthy volunteers, whose that consumed green tea (9 g of loose-leaf in 300 ml water) instead water (control group) in the breakfast, reported an increased satiety and fullness feelings assessed by different surveys (Josic, Olsson, Wickeberg, Lindstedt, & Hlebowicz, 2010).

Turmeric is a spice from the turmeric plant (*Curcuma longa*), that includes the polyphenol curcumin, which has been widely studied for its beneficial effects on health, especially as an antioxidant and anticancer agent (Angelo & Kurzrock, 2009). Regarding its effect on human health there is an study indicating that 400 mg of *Curcuma longa* induced a higher insulin plasma levels than the control, with no differences in the glycaemic index along the 120 minutes of the study time (Wickenberg, Ingemansson, & Hlebowicz, 2010). As previously commented, insulin can increases satiety by interacting at the hypothalamus, therefore it will be interesting to assess the possible effect of this polyphenol in appetite regulation. As is previously commented in Chapter 1, CCK and GLP-1 are hormones produced and released in the intestine as response to food intake and then, can modulate the appetite regulation by increasing satiety. The response of these hormones can be studied *in vitro* by using intestinal cell lines, such as STC-1 enteroendocrine cell line. These cells come from murine mice and are capable to release

CCK and GLP-1 in contact with food and its components (Geraedts, Troost, & Saris, 2009; Geraedts, Troost, Fischer, Edens, & Saris, 2011; Hand, Giblin, & Green, 2012). Also it seems to be of key importance the use of different methods to evaluate differences on the gut bacteria population, as well as on the different metabolites produced by gut microbiota in the presence of different foods and components that reach the intestine. An *in vitro* method used to study the human gut microbiota is batch fermentation, where the assayed bacteria are introduced in sterile bottles with adequate culture medium, together with foods or ingredients to study and then, the modification and evolution of the bacteria population, including changes in pH values, gas production and metabolites production, are measured (Noack, Timm, Hospattankar, & Slavin, 2013).

2.1.5 Objectives

General objective:

- To evaluate the effect of green tea and turmeric after *in vitro* digestion on human satiety.

Specific objectives:

1. To measure the release of CCK and GLP-1 by intestinal cells (STC-1) after *in vitro* gastrointestinal digestion of green tea and turmeric.
2. To evaluate modifications on pH values, gas production and gut microbiota population during 48 hours of fermentation process with green tea by batch experiments.

2.2 Material and method

To evaluate a possible positive effect of green tea and turmeric on STC-1 satiating hormones release and on gut microbiota population by hormone secretion assay and an *in vitro* batch fermentation study (Figure 2.1). For a most realistic way to simulate the intestine environment, samples were previously subjected to a simulated gastrointestinal digestion process (IVGD).

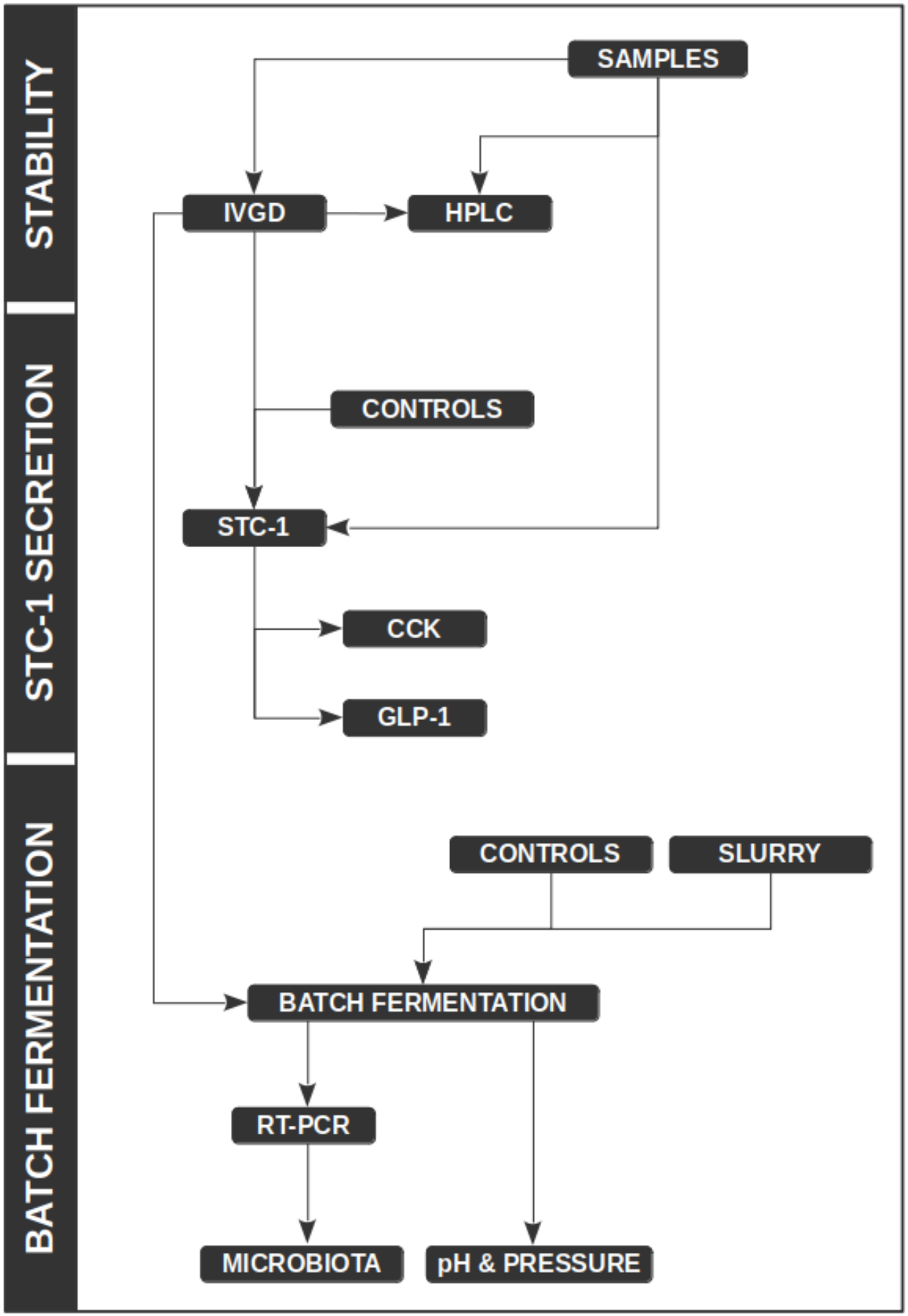

Figure 2.1: Diagram of the study design. IVGD; *in vitro* gastrointestinal digestion, HPLC; high-performance liquid chromatography, CCK; cholecystokinin, GLP-1; clucagon like peptide 1, RT-PCR; real time polymerase chain reaction.

2.2.1 *In vitro* gastrointestinal digestion process

Green tea and turmeric used in this study were provided by Naturex ® (Avignon, France), and in order to determine the stability to digestion of samples and to simulate the environmental conditions in which the food reaches the intestine, an *in vitro* digestion process was run following the Minekus et al., (2014) standardised method. This *in vitro* gastrointestinal digestion was performed including three phases corresponding to oral, gastric and intestinal digestion.

Equipments and reagents

- Lyophilizer Telstar Lyoquest (Terrassa, Spain)
- Precision balance Ohaus Discovery (Parsippany, NJ USA)
- Thermostatic bath Heater Unitronic 320 PR, Barcelona (Spain)
- pH meter Crison micropH 2000, Alella (Spain)
- 6N HCl, Riedel-de Haen (Hanover, Germany)
- NaCl, Probus (Zagreb, Croatia)
- KCl, Panreac (Montcada i Reixac, Spain)
- NaHCO3 1M, Panreac (Montcada i Reixac, Spain)
- CaCl 2 (H 2 O) 2 Fluka (St. Louis, USA)
- KCL Panreac (Montcada i Reixac, Spain)
- KH 2 PO 4 Panreac (Montcada i Reixac, Spain)
- NaHCO 3 Panreac (Montcada i Reixac, Spain)
- NaCL Probus (Zagreb, Croatia)
- MgCL 2 (H 2 O) 6 Panreac (Montcada i Reixac, Spain)
- NH 4 (CO 3) 2 Panreac (Montcada i Reixac, Spain)
- NaOH Probus (Zagreb, Croatia)

- HCL Riedel-de Haën (Hanover, Germany)
- α-amylase, Sigma-Aldrich (St. Louis, USA)
- Pepsin from porcine gastric mucosa, Sigma-Aldrich (St. Louis, USA)
- Bile salts, Sigma-Aldrich (St. Louis, USA)
- Pancreatin from porcine pancreas, Sigma-Aldrich (St. Louis, USA)

Procedure

Seven salt solutions with a concentration of KCL (0.5 M), KH_2PO_4 (0.5M), $NaHCO_3$ (1M), NaCl (2M), $MgCl_2(H_2O)6$ (0.15M), $NH_4(CO_3)_2$ (0.5M) and $CaCl_2(H_2O)_2$ (0.3M) were prepared in milli-Q water and stored refrigerated at 4^oC until assays and then used in the preparation of the three simulated digestive phases, since each juice has different concentration of these salts.

Oral phase A oral juice solution with a final concentration (mmol/L) of 15.09 of KCL, 1.35 of KH_2PO_4, 13.68 of $NaHCO_3$, 0 of NaCl, 0.15 of $MgCl_2(H_2O)6$, 0.06 of $NH_4(CO_3)_2$ and 1.5 of $CaCl_2(H_2O)_2$ was prepared and then salivary α-amylase was added at a concentration of 150 U/mL. The pH solution was adjusted to 7 and 1.6 g of dried sample of each plant extract (50:50 v/v) were mixed well. The mixture of saliva and food solution was maintained for two minutes in the water bath at 37^o C and 60 strokes per minute to simulate the chemical and mechanical processes of oral digestion.

Gastric phase This *in vitro* phase digestion includes a gastric enzyme pepsin mixed at a concentration of 1000 U/mL, with a final concentration (mmol/L) salt solution of 6.9 of KCL, 0.9 of KH_2PO_4, 25 of $NaHCO_3$, 47.2 of NaCl, 0.12 of $MgCl_2(H_2O)6$, 0.5 of $NH_4(CO_3)_2$ and 0.15 of $CaCl_2(H_2O)_2$. Then, this gastric simulated juice was mixed (50:50 v/v) with the total volume from the oral phase digestion. After adjusting the pH to 3, using 1M HCL, the mixture was incubated in the same conditions as for the oral phase, along of two hours.

Intestinal phase For this last simulated digestion phase a salt solution with a final concentration (mmol/L) of 6.8 of KCL, 0.8 of KH_2PO_4, 85 of $NaHCO_3$, 38.4 of NaCl, 0.33 of

Table 2.1: Green tea and turmeric polyphenols stability (%) after their simulated *in vitro* digestion

Compound	Non digested	Gastric phase	Intestinal phase
GA	100±0.7	98.4±2.4	91.4±2.4
C+ED	100±0.8	99.3±2.1	91.4±0.5
GC+EGC	100±1.5	94.8±2.9	90.1±0.5
EGC	100±0.7	85.1±1.7	84.8±0.8
CGC+EGC	100±0.7	93.5±2.6	87.4±2.1
Curcumin	100±0.2	10.2±0.2	3.6±0.1

GA (gallic acid); C (catechin); EC (epicatechin); GC (gallocatechin); EGC (epigallocatechin); ECG (epicatechin galleate); GCG (gallocatechin gallate).

$MgCl_2(H_2O)6$, 0 of $NH_4(CO_3)_2$ and 0.6 of $CaCl_2(H_2O)_2$ was performed. Then, a mixture of pancreatic enzymes (200 U/mL) was added. A bile salt solution with a concentration of 20 mM was added as well and the mixture was mixed (50:50 v/v) with the final product obtained from the gastric phase. Finally, the pH solution was adjusted to 7 by using NaOH 6M, and was incubated for two hours in a water bath at 37^{o}C and 60 strokes/minute. Finished all the process, the final volume obtained from the IVGD was stored at -80^{o}C and then, lyophilised.

2.2.2 HPLC-MS samples analysis

Catechins (green tea) and curcumin (Turmeric), raw and digested, were previously characterized by HPLC-MS using a modified method for detection of different compounds present in green tea (Bedner & Duewer, 2011). This characterization included injection into the chromatograph of digested and non-digested samples . Results of stability to digestion can be seen in Table 2.1.

Equipments and reagents

- HPLC/MS system Agilent 1100 Series HPLC system (Santa Clara, CA, USA)
- Agilent C18 reverse-phase HPLC column (2.1 × 100 mm, 5 μm) (Santa Clara, CA, USA)

- Agilent Chemstation Rev. B.01.03. SR2.
- DataAnalysis software for LC/MSD Trap Version 3.3 (Bruker Daltonik, GmbH, Germany)

Procedure

The analysis was run by HPLC/MS system Agilent 1100 Series HPLC system with a thermostatted μ-well plate autosampler and a quaternary pump, and connected to an Agilent Ion Trap XCT Plus Mass Spectrometer using an electrospray (ESI) interface. Previously to injection, the samples were passed through 0.22 μm HPLC filters. Then, 40 μl of each filtered sample were injected into the column thermostatted at 40 °C, and eluted at a flow rate of 200 μl / min during the whole separation. Mobile phase A (MilliQ water: acetonitrile (95 : 5) with 0.1% formic acid), and mobile phase B (acetonitrile : MilliQ water (90 : 10) with 0.1% formic acid), were used for the chromatographic separation. The initial HPLC running conditions were 5% of B for 5 minutes. The gradient elution programme was from 5% to 100% of B in 25 minutes, followed by 10 minutes at these conditions. Finally, the starting composition of the mobile phases was reached in 5 minutes and the column was equilibrated for 15 minutes before each analytical run. The mass spectrometer was operated in the positive mode for turmeric and the negative mode for green tea, with a capillary spray voltage of 3500 V, and a scan speed of 22 000 (m/z) / s from 150–500 m/z. The nebulizer gas pressure, drying gas flow rate, and drying gas temperature were set at 30 psi, 8 l / min, and 350 °C. Multiple-reaction monitoring, using the MS $\rightarrow$ MS/MS combination of m/z 369.5 $\rightarrow$ 177.0 was used for turmeric detection. In green tea samples, m/z 169.2 $\rightarrow$ 125.0 (gallic acid); m/z 289.2 $\rightarrow$ 205.0 (catechin); m/z 305.2 $\rightarrow$ 261.0 (gallocatechin); m/z 441.2 $\rightarrow$ 331.0 (epicatechin-3-galleate) and m/z 457.2 $\rightarrow$ 331.0 (epigallocatechin-3-galleate) were used to perform a semi-quantification.

2.2.3 CCK and GLP-1 secretion by STC-1 cell line

STC-1 cells were maintained in supplemented Dulbecco's modified Eagle's medium (DMEM) with 4.5% glucose, 10% fetal bovine serum, 2 mM L-glutamine, 100 units / mL penicillin and 100 μL / mL streptomycin. The cells, in passage number 7, were seeded at a concentration of 2700 cells / cm^2 in plastic jars of 75 cm^2 volume, were sub-cultured every 5 days (80–90% confluence) and were maintained at 37 °C in 7.5% CO2 /air humidity. The day of the study,

the cell were in the passage number 15 (McCarthy et al., 2015), and were seeded in two 12-well plates at a density of 12.500 cells / cm^2. Three days after, 1.6 g of each non-digested sample and each control (pea proteins and SCFAs) were mixed with the different digestive fluids used at the IVGD (SSF, SGF and SIF, to obtain the same concentration as that of the digested samples and for their sterilisation, were filtered using 0.2 μm pore filter. Then, the extracts were mixed at 20% with the supplemented DMEM and 2 mL were added in triplicate to each well plate. The plates were incubated at 37° C with 7.5% CO2. Finally, 500 μL aliquots were obtained at 30, 60 and 120 minutes and stored at -80 °C until analysis by ELISA (enzyme-linked immunosorbent assay) using a RayBiotech ELISA kit (Georgia, EEUU) in accordance with the manufacturer's instruction. Data from the blanks (water or water submitted to the IVDG) were subtracted before data analysis. MTT was used to evaluate the cytotoxicity of the samples, which showed that none of the samples was toxic at the concentration used. All experiments were performed in triplicate.

2.2.4 Batch fermentation (large intestine digestion)

To explore changes and modifications on the collected microbiota after exposition to *in vitro* digested green tea, batch experiments technique was used. This technique simulates the colonic digestion environment in hermetically sealed bottles under anaerobic conditions. Inside the bottles, the foods, the gut microbiota from faecal material and the culture medium were deposited and, then, a fermentation process, in similar conditions to the colon was run. In this study, these bottles, with green tea, and inulin and D-glucose as control, were fermented for 48 hours, measuring the pH and taking aliquots at 12, 24 and 48 hours to analyse the microbiota profile.

Equipment and Reagents

- Lyophilizer Telstar Lyoquest (Terrassa, Spain)
- Magnetic shaker
- Autoclave Selecta Presoclave-II
- Stomacher IUL Instrument
- Centrifuge Eppendorf Centrifuge 5804 R

- Biological safety cabinet Telstar Bio-II-A/P (Terrassa, Spain)
- Pressure transmitter CPT6200, WIKA Instruments (Spain)
- pH-meter (Crison, Germany)
- Pepton Conda (Madrid, España)
- Yeast extract Biokar diagnostic (PANTIN FRANCE)
- Tween 80 Sigma-Aldrich (St. Louis, USA)
- L-cystein SAFC (St. Louis, USA)
- NaCL Merk (Darmstadt, Germany)
- NaHCO 3 Panreac (Montcada i Reixac, Spain)
- K 2 HPO 4 Panreac (Montcada i Reixac, Spain)
- KH 2 PO 4 Panreac (Montcada i Reixac, Spain)
- CaCL 2 2(H 2 O) Fluka (St. Louis, USA)
- MgSO 4 7H 2 O Fluka (St. Louis, USA)
- Reaszurin stock solution Sigma-Aldrich (St. Louis, USA)
- PBS Sigma-Aldrich (St. Louis, USA)
- Anaerobiosis bag
- Inulin Aldrich (St. Louis, USA)
- D-glucose Aldrich (St. Louis, USA)

Minimum basal medium (MBM) was used as a culture medium, which allow a low microorganisms growth. The composition of the used MBM (1 L) was 2 g of peptone, 2 g of yeast extract, 0.1 g of NaCl, 0.04 g of K2HPO4 and KH2PO4 , 5 mg of CaCl2, 2 g of L-cystein, 0.5 g of bovine bile, 2 mL of Tween 80 and 1.5 mg of resazurin. Volume prepared was then autoclaved and let at room temperature to finally add 10 μL of vitamin K and 2.5 mL of hemin solution per litre through a 13 mm diameter and 0.22 μm pore size PTFE filter (VWR International, USA).

Procedure

Microbiota was collected from the faeces of six volunteers, three with normal weight (BMI$\geq$18.5 and $<$ 25) and three obese (IMC$\geq$30). The inclusion criteria for the donors were aged between 18 and 45 years, not smokers, had not taken antibiotics, probiotics or prebiotics in last three months and, in the case of female donors, were not pregnant. The faeces were deposited into a sterile and hermetic container by the donor together with an anaerobiosis bag to obtain an environment without oxygen. The container was maintained into a heater at 37ªC until the next step. Three grams of each faeces sample were mixed with 27 mL of phosphate buffered saline (PBS) obtaining a 1:10 (w:v) dilution by using a stomacher.

Then, 15 mL of mixed sample were introduced into a 100 mL volume sterile bottle with 67.5 mL of MBM and the bottle was sealed and incubated at 37ºC in a shaker bath for four hours to obtain a stabilised slurry. After that, 70 mL and the respective ingredients at final volume bottle concentration of 1%, were put into sterilised bottles, by duplicate, for the experiments. These bottles were hermetically sealed and the oxygen inside was replaced by N_2 to ensure the anaerobiosis condition. Two more bottles, with the same content and condition, but without any ingredient, were used as a blank. Finally, to get ready the batch bottles, it was proceeded to inoculate and 7 mL of slurry (microbiota) in every bottle. After that, an aliquot of slurry (7 mL) was taken for every bottle and pressures inside bottles were measured (time 0). Finally, the bottles containing the samples, were incubated into a shaker bath with 37ºC for 48 hours, taking new aliquots and measuring the pressure again at 20 (time 1), 24 (time 2) and 48 (time 3) hours. Steps after sealed the bottles (remove oxygen, inoculate slurry and take aliquots) were done by using a syringe through the septa of the bottle. Aliquots 4 mL were used for measure pH and the remaining sample was centrifuged at 1200 g for 15 minutes. Subsequently, the pellet and the supernatant were separated in two different vials and stored at -80ºC.

2.2.5 DNA extraction and analysis of microbiota

In this study *Bacteroides*, *Bifidobacterium*, Firmicutes, *Enterobacteriaceae*, *Lactobacillus* and total bacteria groups were analysed, by using as a standard *Bacteroides thetaiotaomicron* (DSMZ 2079), *Bifidobacterium longum* (CECT 4503), *Clostridium leptum* (DSMZ 753), *Escherichia coli*, (NUTBRO Collection), *Lactobacillus gasseri* (DSMZ 20077) and *Bifidobacterium*

longum (CECT 4503), respectively.

Equipment and reagent

- NanoDrop-100 spectrophotometer (Thermo Fisher Scientific, Villebon sur Yvette, France)
- 96-well CFX96 Real-Time PCR thermocycler and detection system (Bio-Rad, Madrid, Spain)
- SensiMix T M SYBR No-ROX (Bioline, London, UK)
- Nuclease-free water (AppliChem, Darmstadt, Germany)

Procedure

DNA was extracted from the pellet previously obtained from the batch aliquots by using the protocol described by Boon et al. (2003). To ensure that the laboratory materials and the water used along this process were free of RNase to avoid interferences in the subsequent DNA quantification, all these were autoclaved twice. As a previous step to performance the PCR analysis, the DNA concentration of the samples, was assessed by using the nanodrop at 260/280 nm ratio. To measure the microbiota evolution and changes, quantitative real-time PCR (qPCR) was done. For this process, specific primers, temperatures and targets were used and the summary can be seen in Table 2.2. 1 μL of template DNA, 12.5 μL of SensiMixTM SYBR NoROX (Bioline, London, UK), 0.5 μL of the respective primer (0.2 μM) and 10.5 μL of nuclease-free water were mixed and added to wells (96-well plate). The temperature and time parameters for the thermocycler amplification program were of 95 ^{o}C for 10 minutes for DNA initial denaturation and enzyme activation, and for every of the 40 programmed cycles, 95^{o}C for 15 seconds for denaturation, 60^{o}C for 30 seconds for annealing and 72^{o}C for 45 seconds for extension. The melting curve go from 65 ^{o}C to 95 ^{o}C, increasing 0.5^{o}C every 0.5 seconds, measuring by detecting the fluorescence at the last step of every cycle. Each sample was run in quadruplicate. To quantify the bacteria analysed, standard curves were performed by using serial 10-fold dilution of DNA pure culture, corresponding to 102 to 108 cell (genome) equivalents/mL.

Table 2.2: Specific primers used in this work to target different bacterial groups. AT: Annealing temperature (^{o}C)

Bacteria	**Target**	**Primers sequence 5'-3'**	**AT[a]**	**Bibliography**
Total bacteria	16S	Fwd: GTGSTGCAYGGYYGTCGTCA Rv: ACGTCRTCCMCNCCTTCCTC	60	Fuller et al., 2007
Bacteroides-Prevotella	16S	Fwd: GAGAGGAAGGTCCCCCAC Rv: CGKACTTGGCTGGTTCAG	60	Layton et al., 2006
Bifidobacterium	16S	Fwd: GATTCTGGCTCAGGATGAACGC Rv: CTGATAGGACGCGACCCCAT	60	Gueimonde et al., 2004
Enterobacteriaceae	16S	Fwd: TGCCGTAACTTCGGGAGAAGGCA Rv: TCAAGGACCAGTGTTCAGTGTC	60	Matsuda et al., 2007
Lactobacillus	16S	Fwd: AGCAGTAGGGAATCTTCCA Rv: CATGGAGTTCCACTGTCCTC	60	Rinttila et al., 2004
Firmicutes	16S	Fwd: GGAGYATGTGGTTTAATTCGAAGCA Rv: AGCTGACGACAACCATGCAC	60	Guo et al., 2008

2.2.6 Statistics and data analysis

Normal distribution of the data was checked by using Shapiro-wilk test and the homogeneity of variances was done by F of Snedecor when two groups where tested, Bartlett test for more of two groups and Fligner-Killen test when data had not a normal distribution. For each of the processes in the study design (raw and digested samples) the effect of the ingredient on the release of CCK and GLP-1 into each exposure time was analysed by analysis of variance (ANOVA) and HSD Tukey's multiple comparisons between different ingredients. The evaluation of the effect of *in vitro* gastrointestinal digestion by each ingredient on the release of CCK and GLP-1 was made by Student's t test. The possible association of time of exposure to each sample and treatment, with the CCK and GLP-1 release was carried out through a Pearson correlations ($p < 0.05$). Data obtained from pH evolution was compared for each group (normal weight and obesity) into each time and sample. To find differences among the samples through the time, one way ANOVA (for parametric data) and Kruskal-Wallis test (non-parametric data) were performance, follow by a multiple comparison post-hoc test, HSD Tukey and pairwise comparison with Holm method correction respectively. The total pressure in each bottle was obtained by calculating the pressure increase at each measured time point:

$$\Delta P(KPa) = Pf - Pi \tag{2.1}$$

Being ΔP the pressure increase, Pf the final pressure and Pi the initial pressure.

And adding all the measured increases:

$$TP(KPa) = \sum P(P_1 + P_2 \ldots + P_n) \tag{2.2}$$

Being TP the total pressure and $\sum P$ the addition of each measured time point (12, 24 and 48 hours).

2.3 Results and discussion

2.3.1 Hormone secretion by STC-1 cell line

Table 2.3 shows the results obtained for CCK and GLP-1 release by cells. In the non-digested samples, CCK secretion after 30 minutes of exposure was significantly ($p < 0.05$) higher for the green tea extract (30.2 pg/mL) compared to other samples. After 60 and 120 minutes of exposure, the turmeric extract showed the greatest hormone release. In the case of GLP-1 after exposure to the non-digested samples during 30 minutes green tea extract showed the greatest significant response (54.3 pg/mL). As for CCK, at 60 and 120 minutes of exposure, the highest concentrations of GLP-1 were found for turmeric. There were not differences among the remaining samples. According to the results obtained for hormones released by the STC-1 cell line, non-digested green tea and turmeric showed higher values than the positive controls. Results were in accordance with recent studies that showed a similar trend for green tea (Correa-Betanzo et al., 2014; Josic et al., 2010; Reinbach, Smeets, Martinussen, Møller, & Westerterp-Plantenga, 2009; Song, Aihara, Hashimoto, Kanazawa, & Mizuno, 2015).

Regarding curcumin, turmeric seems to enhance GLP-1 release by enteroendocrine cells despite its low bioaccessibility and absorption (Tsuda, 2015). Gastrointestinal conditions can affect the chemical structure as well as the bioactive compounds function and their solubility and availability (Frontela-Saseta, 2011). With this background, and to assess possible differences between raw and digested samples, digested green tea and turmeric were also studied for gut hormone secretion by STC-1 cells and the results are presented in Table 2.3. In both cases, the amount of hormones released by cells were similar and only statistically significant differences were found for CCK at 30 minutes, where, after turmeric exposition, this hormone showed high release, and for GLP-1 at 120 minutes, being green tea in this case the plant extract that induced the biggest hormone release. In the Table 2.3, the differences in the release of hormones between the digested or non-digested plant extract, are also showed. For green tea exposure, at 30 and 60 minutes, it can be observed a greater CCK concentration when exposed to the non-digested extract. After 120 minutes, result observed was the opposite, obtaining significantly higher release of CCK after the digested extract exposition.

Table 2.3: CCK and GLP-1 secretion (pg/mL) by STC-1 cells exposed to the digested and non-digested samples at different times

	30 minutes		**60 minutes**		**120 minutes**	
Sample	**CCK**	**GLP-1**	**CCK**	**GLP-1**	**CCK**	**GLP-1**
	Non-digested samples					
SCFAs	3.2 ± 0.4^{b}	7.6 ± 0.1^{b}	3.7 ± 2^{b}	7.8 ± 0.7^{b}	2.9 ± 0.5^{a}	8.6 ± 0.6^{b}
Protein	1.1 ± 0.4^{b}	11.5 ± 3.0^{b}	3.0 ± 0.7^{b}	18.7 ± 4.9^{b}	2.6 ± 0.2^{a}	24.6 ± 2.3^{b}
Turmeric	9.2 ± 3.1^{b}	6.2 ± 2.2^{b}	$379 \pm 46^{a}*$	$123 \pm 14^{a}*$	Uq	$347 \pm 125^{a}*$
Green tea	$30.2 \pm 8.3^{a}*$	54.3 ± 20^{a}	$29.5 \pm 0.7^{b}*$	27.0 ± 8.6^{b}	15.1 ± 4.9^{a}	71.1 ± 28.1^{b}
	Digested samples					
Turmeric	$43.3 \pm 11^{a}*$	$62.4 \pm 13^{a}*$	13.3 ± 4.2^{a}	50.5 ± 8.2^{a}	26.6 ± 6.8^{a}	28.9 ± 6.8^{b}
Green tea	9.8 ± 1.6^{b}	48.9 ± 12^{a}	19.3 ± 1.0^{a}	60.6 ± 20^{a}	$29.5 \pm 7.2^{a}*$	$165.7 \pm 52^{a}*$

The results are shown as $\mu \pm SEM$ (n = 3). Within the same exposure time, different superscripts (a–c) denote statistically significant differences ($p<0.05$) in the release of each hormone for different samples. * denotes significant differences ($p<0.05$) for the digested and non-digested samples within the exposure time. Uq: unquantifiable.

For the turmeric extract, the response was contrary to that observed for green tea, being higher ($p < 0.05$) for CCK and non-digested extracts at the last two measured time points. Regarding the amount of GLP-1 after exposure to green tea, it was significantly higher at 120 minutes for the digested extract. Undigested turmeric produced the greatest release of both hormones; however, after exposure to digested turmeric, the amounts of hormones released by STC-1 cells decreased dramatically. As shown by the stability data in Table 2.1, the turmeric was very unstable to the IVGD process, which may explain the lower effect on the cells caused by the digested extract. For its part, digested green tea showed better results in terms of the release of both hormones, as the exposure time progressed. As previously mentioned, green tea catechins showed high stability to the IVGD process. On the other hand, this digestion process could have caused the fibre remains in the green tea extract to release contained catechins and other compounds along the exposure time (Wu, Teng, Huang, Xia, & Wei, 2015).

2.3.2 pH and gas production evolution along the batch fermentation

Metabolites originate from gut microbiota during food digestion includes vitamins, aminoacids and SCFAs among others (Li et al., 2018). These acid metabolites are capable to decrease the environmental pH and thus, a low pH can be used as an indicator of the total gut microbiota activity (Long et al., 2015). The obtained data from the pH evolution through the experimental time grouped by sample and BMI condition are shown in Figure 2.2. As can be seen, pH remained without variations for the fermentation of MBM, being the greatest values observed for 12, 24 and 48 hours ($p < 0.05$) in both group (normal weight and obese) decreasing for the remaining samples, particularly for both positive controls, D-glucose and inulin. Between controls, not statistically significant differences in pH were found in any case. In the case of green tea, pH value observed was higher than that observed for controls at times 12, 24 and 48 h in obesity group, and at 24 and 48h in NW group ($p < 0.05$). The biggest pH decrease, for each group and sample, was observed at 12 hours of fermentation. The pH data indicate a microbial activity in the cases of the green tea and the controls batch fermentation, and a lack of activity for the bottles with only MBM (blank), as expected.

The volatile substances produced into the hermetic bottles through the fermentation process, increase the pressure inside these, so another signal of the microbiota activity (Gonzalez-Bermudez, Lopez-Nicolas, Peso-Echarri, Fronteta-Saseta, & Martinez-Gracia, 2018), through

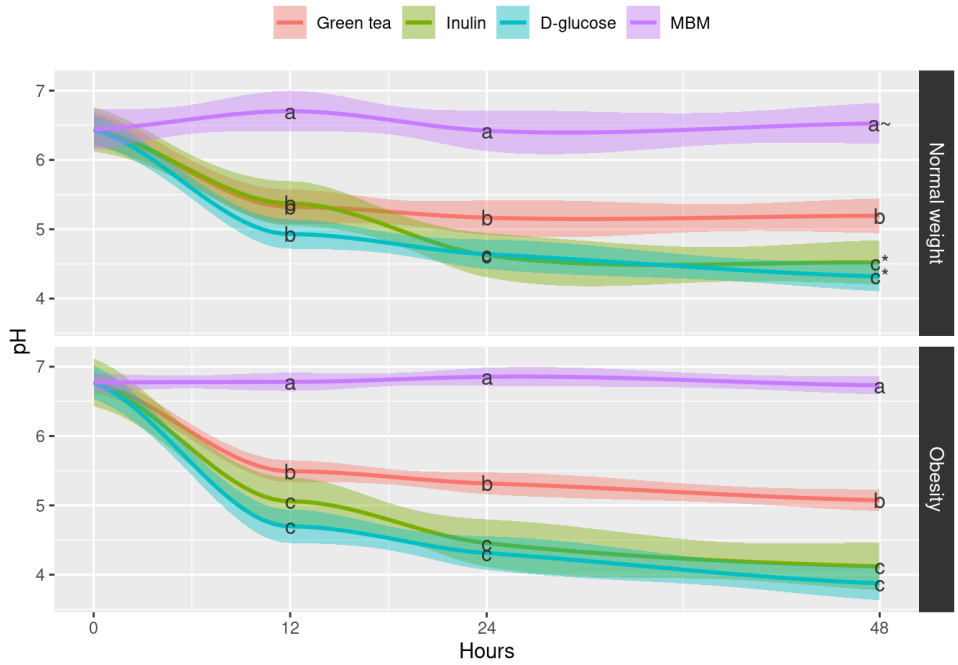

Figure 2.2: pH evolution through time (0, 12, 24 and 48 hours) grouped by donors with normal weight and with obesity. Into each graph and time, letters (a-c) denote statistical significant differences among the samples. Into each time and sample, * and ˜ denote statistical significant differences ($p < 0.05$) and marginal trend ($p < 0.1$), respectively, between the groups (normal weight and obesity). Data are presented as μ (line) and confidence interval (shadow).

the SCFAs production, was the total pressure increment into the bottles in which the batch fermentations were conducted. The total pressure increment is shown in Figure 2.3, where a higher gas production in case of green tea fermented in both, NW and OB donors, compared with the remaining substrates (Inulin, D-glucose and MBM) was observed ($p < 0.05$). Among the controls and blank, statistically significant differences were not found. Neither were significant differences between NW and OB donors for each sample used for its fermentation.

2.3.3 Gut microbiota composition of normal weight and obese donors after fermentation of green tea

Quantification for the gut microbiota bacteria (*Bacteroides*, *Bifidobacterium*, *Enterobacteriaceae*, Firmicutes, *Lactobacillus* and total bacteria) in normal weight donors' group, sample and point time are shown in Table 2.4. For the NW donors at 12 hours of fermentation, statistically significant differences ($p < 0.05$) were found for total bacteria, being the greater amount of these in the case of green tea fermentation, without differences with inulin. With regard to

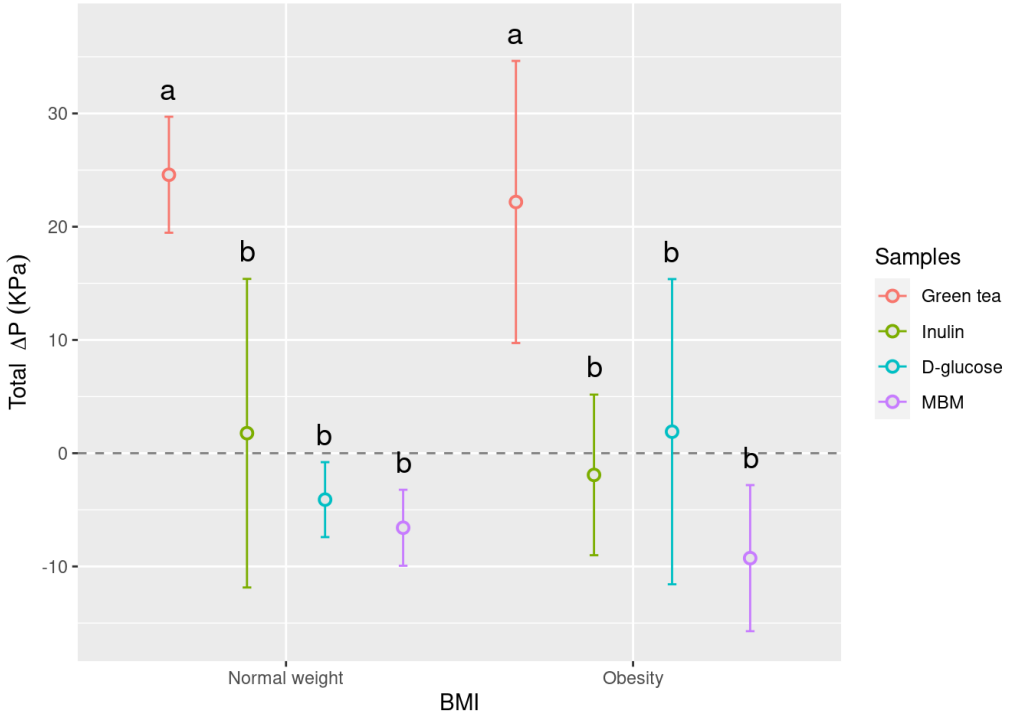

Figure 2.3: Total gas production measured through the pressure for each fermented sample and grouped by donor with normal weight and obesity. Data are presented as $\mu \pm$ S.D. Different letters (a-b) denote statistical significant differences ($p < 0.05$) among the samples into each BMI group.

Bifidobacterium, in the three last times analysed, green tea produced the opposite effect, being the amount of this bacteria gender significantly ($p < 0.05$) low compared to inulin and without statistical differences when compared to D-glucose and blank. For the remaining quantified bacteria of NW donors, significant differences were not found among the three fermented compounds. Similar information for obese donors can be observed in Table 2.5. As can be seen, in the case of OB donors, the trend for *Bifidobacterium* was similar and a smaller quantity of this bacteria was found after green tea fermentation along the 48 hours study, with statistically significant differences ($p < 0.05$) compared with both controls. The amount of *Lactobacillus* also was significantly low for green tea at 24 and 48 hours of fermentation. In the case of total bacteria, the amount observed after green tea exposition was significantly ($p < 0.05$) higher than the blank at 24 hours, without differences when compared to control. However, no significant differences for total bacteria growth among the other analysed times were observed, as well as for the remaining bacteria.

Table 2.4: Evolution of normal weight donor bacterial populations after exposure to digested green tea extract and controls

Time	Sample	Total bacteria	*Bacteroides*	*Bifidobacterium*	*Enterobacteriaceae*	Firmicutes	*Lactobacillus*
0 hours	Green tea	7.68 ± 0.14^{a}	6.72 ± 0.39^{a}	5.63 ± 0.13^{a}	5.51 ± 0.51^{a}	6.16 ± 0.19^{a}	3.65 ± 0.81^{a}
	Inulin	7.68 ± 0.14^{a}	6.72 ± 0.39^{a}	5.63 ± 0.13^{a}	5.51 ± 0.51^{a}	6.16 ± 0.19^{a}	3.65 ± 0.81^{a}
	D-glucose	7.68 ± 0.14^{a}	6.72 ± 0.39^{a}	5.63 ± 0.13^{a}	5.51 ± 0.51^{a}	6.16 ± 0.19^{a}	3.65 ± 0.81^{a}
	Blank	7.68 ± 0.14^{a}	6.72 ± 0.39^{a}	5.63 ± 0.13^{a}	5.51 ± 0.51^{a}	6.16 ± 0.19^{a}	3.65 ± 0.81^{a}
12 hours	Green tea	7.80 ± 0.39^{a}	6.19 ± 0.70^{a}	4.22 ± 0.56^{b}	5.20 ± 0.88^{a}	5.42 ± 0.75^{a}	2.58 ± 0.57^{a}
	Inulin	7.40 ± 0.23^{ab}	6.27 ± 0.37^{a}	5.11 ± 0.54^{a}	4.92 ± 0.57^{a}	6.10 ± 0.55^{a}	3.30 ± 0.85^{a}
	D-glucose	7.18 ± 0.32^{bc}	5.99 ± 0.48^{a}	4.29 ± 0.45^{ab}	5.27 ± 0.39^{a}	5.22 ± 0.45^{a}	3.42 ± 0.98^{a}
	Blank	6.74 ± 0.25^{c}	6.23 ± 0.79^{a}	4.55 ± 0.54^{ab}	5.02 ± 0.36^{a}	4.87 ± 1.24^{a}	3.01 ± 0.15^{a}
24 hours	Green tea	6.55 ± 0.56^{a}	5.58 ± 0.53^{a}	4.35 ± 0.64^{b}	5.03 ± 1.22^{a}	4.71 ± 0.49^{a}	3.05 ± 0.86^{a}
	Inulin	6.88 ± 0.48^{a}	5.94 ± 0.67^{a}	5.49 ± 0.81^{a}	4.88 ± 0.63^{a}	5.44 ± 0.53^{a}	3.15 ± 0.35^{a}
	D-glucose	7.05 ± 0.69^{a}	5.75 ± 0.39^{a}	5.01 ± 0.87^{ab}	5.30 ± 1.00^{a}	5.15 ± 0.60^{a}	3.74 ± 1.14^{a}
	Blank	6.50 ± 0.51^{a}	5.75 ± 0.44^{a}	4.20 ± 0.41^{ab}	5.04 ± 0.74^{a}	4.94 ± 0.37^{a}	3.14 ± 0.34^{a}
48 hours	Green tea	6.98 ± 0.33^{a}	5.71 ± 0.19^{a}	4.35 ± 0.76^{b}	5.07 ± 0.83^{a}	5.27 ± 0.47^{a}	4.56 ± 1.71^{ab}
	Inulin	7.23 ± 0.45^{a}	5.61 ± 0.12^{a}	6.15 ± 0.57^{a}	5.15 ± 0.96^{a}	5.58 ± 0.44^{a}	3.65 ± 0.27^{b}
	D-glucose	7.37 ± 0.47^{a}	5.84 ± 0.41^{a}	5.27 ± 1.09^{ab}	5.56 ± 0.68^{a}	5.34 ± 0.64^{a}	5.55 ± 0.73^{a}
	Blank	6.33 ± 0.44^{b}	5.63 ± 0.46^{a}	4.09 ± 0.34^{b}	5.10 ± 0.21^{a}	5.50 ± 0.34^{a}	3.08 ± 0.64^{b}

Data are presented as Log genome Eq/mL mean values ± SD. Within the same bacteria group and exposure time, letters (a-b) denote statistically significant differences ($p < 0.05$) among the different fermented samples.

Table 2.5: Evolution of obese donor bacterial populations after exposure to digested green tea extract and controls

Time	**Sample**	**Total bacteria**	***Bacteroides***	***Bifidobacterium***	***Enterobacteriaceae***	**Firmicutes**	***Lactobacillus***
0 hours	Green tea	7.85 ± 0.24^{a}	7.10 ± 0.11^{a}	5.65 ± 0.19^{a}	5.82 ± 0.41^{a}	6.63 ± 0.44^{a}	5.02 ± 1.47^{a}
	Inulin	7.85 ± 0.24^{a}	7.10 ± 0.11^{a}	5.65 ± 0.19^{a}	5.82 ± 0.41^{a}	6.63 ± 0.44^{a}	5.02 ± 1.47^{a}
	D-glucose	7.85 ± 0.24^{a}	7.10 ± 0.11^{a}	5.65 ± 0.19^{a}	5.82 ± 0.41^{a}	6.63 ± 0.44^{a}	5.02 ± 1.47^{a}
	Blank	7.85 ± 0.24^{a}	7.10 ± 0.11^{a}	5.65 ± 0.19^{a}	5.82 ± 0.41^{a}	6.63 ± 0.44^{a}	4.38 ± 1.29^{a}
12 hours	Green tea	7.10 ± 0.71^{a}	6.11 ± 0.55^{a}	4.27 ± 0.07^{b}	5.56 ± 0.48^{a}	5.65 ± 1.03^{a}	3.21 ± 1.23^{a}
	Inulin	7.78 ± 0.58^{a}	6.42 ± 0.84^{a}	5.38 ± 1.19^{a}	5.95 ± 0.85^{a}	6.54 ± 0.68^{a}	4.73 ± 1.84^{a}
	D-glucose	7.78 ± 0.52^{a}	6.64 ± 0.72^{a}	5.39 ± 0.88^{a}	6.26 ± 0.95^{a}	6.06 ± 0.96^{a}	5.08 ± 0.93^{a}
	Blank	6.92 ± 0.70^{a}	6.76 ± 0.20^{a}	4.72 ± 0.09^{a}	6.12 ± 1.27^{a}	5.24 ± 0.55^{a}	4.18 ± 1.41^{a}
24 hours	Green tea	7.17 ± 0.45^{a}	6.21 ± 0.28^{ab}	4.20 ± 0.50^{b}	5.85 ± 0.21^{a}	5.71 ± 0.69^{a}	3.80 ± 0.94^{b}
	Inulin	7.67 ± 0.29^{a}	6.62 ± 0.19^{a}	5.90 ± 0.58^{a}	6.29 ± 0.51^{a}	6.39 ± 0.63^{a}	4.87 ± 1.68^{ab}
	D-glucose	7.57 ± 0.43^{a}	6.76 ± 0.46^{a}	5.30 ± 0.98^{a}	6.08 ± 0.76^{a}	6.01 ± 0.72^{a}	5.82 ± 0.76^{a}
	Blank	6.35 ± 0.60^{b}	5.90 ± 0.56^{b}	4.33 ± 0.23^{b}	5.89 ± 1.25^{a}	5.47 ± 0.33^{a}	4.35 ± 1.12^{ab}
48 hours	Green tea	6.86 ± 1.14^{ab}	6.06 ± 0.76^{ab}	4.10 ± 0.61^{b}	5.64 ± 0.82^{a}	5.33 ± 1.58^{a}	4.53 ± 0.34^{b}
	Inulin	7.36 ± 0.21^{ab}	6.66 ± 0.46^{a}	5.63 ± 0.71^{a}	5.75 ± 0.70^{a}	5.96 ± 0.80^{a}	5.91 ± 0.84^{a}
	D-glucose	7.71 ± 0.13^{a}	6.89 ± 0.28^{a}	5.40 ± 0.76^{a}	5.95 ± 0.68^{a}	6.14 ± 0.28^{a}	6.80 ± 0.26^{a}
	Blank	6.21 ± 0.61^{b}	5.40 ± 0.46^{b}	4.26 ± 0.45^{b}	5.35 ± 1.21^{a}	5.01 ± 0.48^{a}	4.38 ± 1.29^{b}

Data are presented as Log genome Eq/mL mean values ± SD. Within the same bacteria group and exposure time, letters (a-b) denote statistically significant differences ($p < 0.05$) among the different fermented samples.

Both, pH evolution and gas pressure production into the batch bottles are indicatives of bacteria activity (Sánchez-Moya et al., 2017). In this regard, results for pH and pressure values seem to indicate a higher bacteria activity in bottles containing green tea compared to those that only contained MBM, and even larger than both controls in the case of the generated pressure. These data agree with the number of total bacteria detected by qPCR indicating that there were a higher quantity of bacteria after green tea fermentation than for MBM, and it was similar to the positive controls along the 48 hours of fermentation.

Focusing in the gas production, which was significantly bigger for green tea fermentation compared to the remaining substrates, it not seems to be in accordance with pH and the presence of total bacteria, since both positive control fermentation resulted in a larger (without significant differences) total bacteria population. This could be explained by the green tea effect on *Bifidobacterium* along this batch fermentation experiment, where this bacteria gender showed the lowest growth in the MBM fermentation, since it has been found a negative relation between *Bifidobacterium* and gas production (Rycroft, Jones, Gibson, & Rastall, 2001). However, several studies shown an increase in the *Bifidobacterium* population after green tea exposition (Ishihara, Chu, Akachi, & Juneja, 2001; Jin, Touyama, Hisada, & Benno, 2012; Liao et al., 2016). In case of *Lactobacillus* similar effect was found after green tea extract fermentation; the amounts of this bacteria gender were similar to those found for the batch bottles with only MBM. Regarding to this, other studies reports show benefit on *Lactobacillus* growth after green tea exposition (Muniandy, Shori, & Baba, 2017), as well as for *Bifidobacterium*. This behaviour in the *Bifidobacterium* and *Lactobacillus* evolution, could be explained by the selenium content of green tea, since it has been found that teas supplemented with selenium, compared to unsupplemented, had a positive effect on the growth of these bacteria (Molan, Flanagan, Wei, & Moughan, 2009), green tea used in the present study did not contain selenium. The phylum Firmicutes has been related with different health problem, including an increase on body weight (Moreno-Indias et al., 2014). In both cases, normal weight and obese groups, the Firmicutes population decreased more after green tea fermentation than for both positive controls, and in a similar way to the experiments run with MBM. Nevertheless, statistical differences were not found in this regard and the analysis of the effect size in Firmicutes changes among the substrates showed a large η^2 (> 0.14) in all the cases. This could indicate a possible non detected effect that maybe due to a small sample, that imply a decrease the statistical power and therefore the possibility to find significant p values. Population changes observed for the remaining bacteria groups were similar among the fermented substrates and the different times

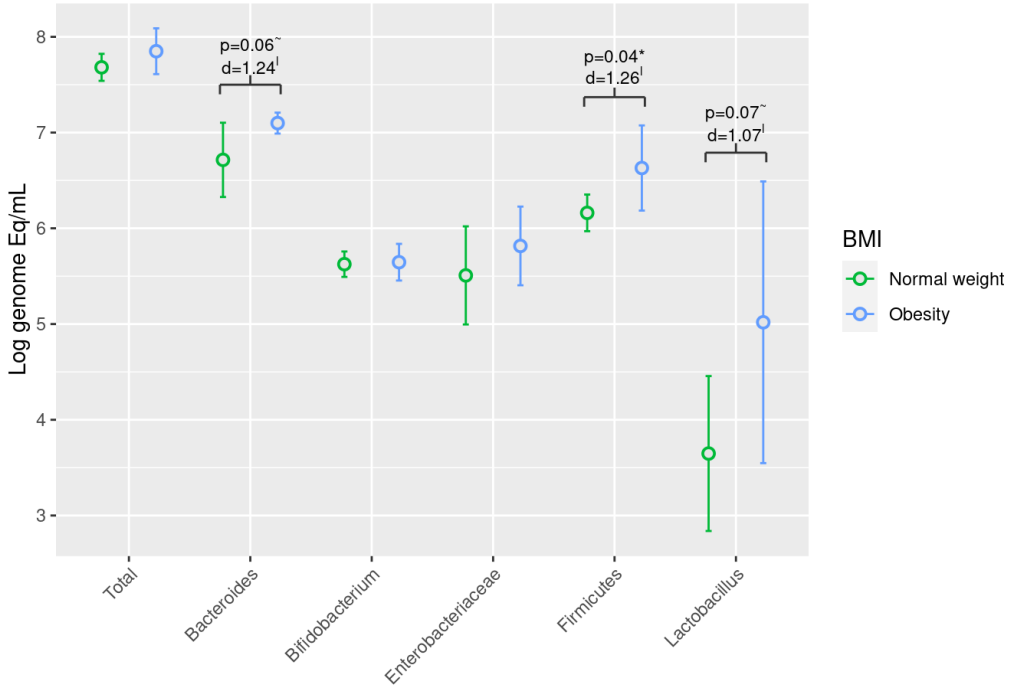

Figure 2.4: Differences in the amount of studied bacteria between the normal weight and obese donors. Data are presented as $\mu \pm$ S.D. * and ˜ denote statistically significant differences ($p < 0.05$) and marginal trend ($p < 0.1$), respectively. l denote a large effect size.

analysed, showing no significant differences ($p < 0.05$).

Several works have found a different microbiota composition between lean and obese individuals (Moreno-Indias et al., 2014; Koliada et al., 2017; Indiani et al., 2018), theorizing about an effect of gut microbiota in the weight control. In this study, the gut microbiota from the NW and OB donors, obtained from faecal samples, was compared before the batch fermentation process to find differences in the microbiota composition of both groups, whose results can be seen in Figure 2.4. The most important difference was found in the amount of Firmicutes and regarding to this, obese donors showed a highest population of this phylum that those volunteers with a lower weight. Bacteroidetes and *Lactobacillus* were found in a larger quantity in OB group, with a marginal trend ($p < 0.01$). Differences on the number of these three group of bacteria were found with a large effect size ($d > 0.8$). There were no significant differences ($p < 0.05$) for the remaining analysed bacteria. In regard with Firmicutes phylum, results were in accordance with the current literature, since this bacteria group is found in higher quantity in individuals with obesity (Koliada et al., 2017; Delzenne & Cani, 2011).

2.4 Conclusions

Green tea, turmeric and their compounds increased CCK and GLP-1 release by the STC-1 enteroendocrine cell line, compared with pea proteins and SCFA (positive control) at the same concentration. This effect on satiating hormones release is affected by the simulated *in vitro* gastrointestinal digestion depending on the ingredient and time of exposure, being the satiating capacity of the green tea extract more stable after the *in vitro* digestion process.

Colonic fermentation of the digested green tea extract induced an increase in the pressure values and a decrease in pH after the fermentation time, especially after 12 hours of fermentation, but in general terms, the exposure to green tea reduced the populations of all the bacteria analysed in a greater extent than the controls, with the exception of fermentation in case of microbiota from donors with normal weight at 12 hours, where an increase in total bacteria was observed. This could indicate that the changes produced in pH and especially in pressure, after fermentation to digested green tea, could be due to the activity of other intestinal bacteria not included in this analysis. The comparison between the microbiota from donors with obesity and donors in normal weight, showed a significantly higher amount of the phylum Firmicutes in obese patients.

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CHAPTER 3

VALIDATION OF VAS SURVEYS TO MEASURE HUMAN SATIETY. DEVELOPING A METHOD FOR FACILITATING USE OF THE SURVEY

Validation of VAS surveys to measure human satiety. Developing a method for facilitating use of the survey

Abstract: To measure hunger and satiety sensations in humans' studies, visual analogue scales (VAS) surveys are one of the most widely used methods, as they have demonstrated their validity and reproducibility in previous studies. But as of today, VAS surveys to measure satiety in humans have not been validated in the Spanish language. In addition, taking advantage of new technologies, our research group (NUTBRO) has developed a computer application (App) for Android OS, to design and apply several types of surveys, in a simpler and more comfortable way, for the volunteers and researchers. Two studies with human participants were conducted to validate the use of the VAS surveys in Spanish language (n=21) and the use of the App to administrate the VAS surveys (n=25). Volunteers ate a standard breakfast, the same for everyone within each study, and later answered the surveys that were provided during a total period of 180 minutes. In the case of the VAS validation study, after filled in the last survey (180 min), the participants had free access to an *ad libitum*, and the entire process was repeated under the same conditions on a different day. IN the case of the App validation, the surveys were completed in three different formats: 1) on a smartphone, 2) on a tablet (both using the app) and 3) with paper and pencil (P&P). The obtained results did not show statistically significant differences ($p < 0.05$) and a high correlation between the different days of study. Although weaker, statistically significant differences between VAS results and *ad libitum* meal intake were found as well. In the case of the App validation, significant differences were not found among any of the three formats (smartphone, tabled and P&P) tested, and a high agreement among the three methods was corroborated by intraclass correlation coefficient. Therefore, based on these results, it can be concluded that the developed VAS in Spanish language and App are adequate and valid to evaluate appetite and satiety.

Keywords: Appetite, Satiety, VAS, Validation, Obesity.

3.1 Introduction

Studying processes involved in food intake, appetite and satiety regulation can provide valuable information for the control of overweight and obesity (Amin & Mercer, 2016; Blundell et al., 2010; Yuliana et al., 2011). Nowadays, there are several forms to measure satiety, both *in vivo* and *in vitro*. In this way, it can be measured in animals and in human's experiments, both with textitad libitum food, as time passes from ingestion to the following meal. Moreover, we can measure satiety through the decrease in food intake of the individuals studied, in the following meal and within a fixed period of time, which tends to be approximately two or three hours (Tremblay & Bellisle, 2015). Concentrations of hormones or other biomarkers related with satiety can also be measured in the plasma of the studied subjects. Regarding *in vitro* tests, it can be carried out using cell lines such as STC-1, which are capable of synthesising and releasing different hormones such as cholecystokinin (CCK), glucagon like peptide 1 (GLP-1) or tyrosine-tyrosine peptide (PYY) (Chang, Chey, Sun, Leiter, & Chang, 1994; Geraedts, Troost, Fischer, Edens, & Saris, 2011; Geraedts, Troost, & Saris, 2009). The visual analogue scales (VAS) are a widely used tool to measure satiety in human, these allow us to measure subjective sensations of satiety in a rapid and comfortable way, and its reproducibility and validity have been previously proved (Flint, Raben, Blundell, & Astrup, 2000). VAS are composed of lines of variable longitude with anchored words in both extremes of the line, at the beginning and at the end, with sentences such as "not at all" or "extremely" (Figure 3.1), which are used as options to answer the subjective questions. VAS to measure satiety tend by questions relating to appetite, fullness, desire to eat and prospective consumption (Neacsu, Fyfe, Horgan, & Johnstone, 2014).

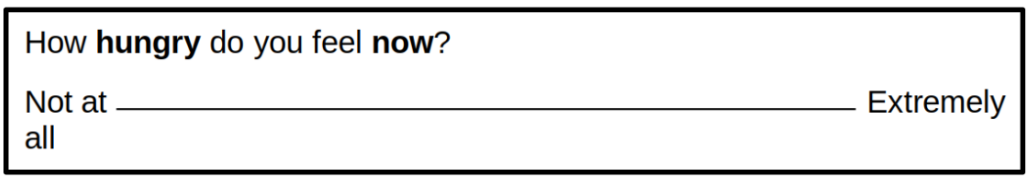

Figure 3.1: Example of P&P visual analogue scale used in the validation of this novel App.

Classically, these kinds of surveys were printed on paper and were filled in by hand, we can call it pen and paper (P&P) format. But this way presents some problems, as the researchers have to measure the distance of the line mark from the beginning of the line, which takes a long time. Then, the obtained data have to be introduced in a data base by the researchers, increasing the human mistake probability. We cannot forget either that this method involves

a waste of paper and other materials, like ink to print. To improve the capture of these data, taking advantage of the new technologies, some software have been developed and validated to help in human satiety studies, like the hand-held electronic data capture (Gibbons, Caudwell, Finlayson, King, & Blundell, 2011), or the iPad visual analogue rating system (Brunger et al., 2015). Nowadays, many people use the operating system Android in their device, for this reason our research group (NUTBRO), from the Human Nutrition and Bromatology area of the University of Murcia, with the support of the IT team, have developed an Android informatics application (App) for smartphones and tablets to measure the sensation of satiety together with other types of surveys, in a more comfortable and simple way, both for the participating volunteers and for the researchers.

Although VAS surveys to measured satiety and hunger sensations were developed and validated in English language (Flint, Raben, Blundell, & Astrup, 2000), to date, these scales have not been validated for use in Spanish. There are 31 countries around the world in which Spanish is spoken, making this language the second most widely spoken in the world by number of natives and the fourth for its use (Ardila, 2020), therefore, validated VAS to the study of appetite and satiety will be an important tool to help researchers from different parts of the world to advance their knowledge within this subject. For this reason, a survey to measure appetite and satiety sensations, based on VAS in Spanish language, was developed with the aim to validate its use and offer a reliable tool to be used in research.

In addition, to be able to use the App to apply appetite VAS in a reliable way, a study was carried out with the aim of checking the App's validity to measure satiety in humans, compared with the pen and paper (P&P) classic format.

3.2 Materials and Methods

3.2.1 Participants

The present study was carried out at the department of Food Technology, Nutrition and Bromatology of the Veterinary Faculty (University of Murcia) on a total of 21, 10 male and 11 female (VAS validation), and 25, 8 male and 17 female (SatinApp validation), healthy volunteers. The inclusion criteria were non-smokers, BMI between 18.5 and 24.9 Kg/m^2, age between 18 and 55

years. None of the volunteers was a professional sports person, was pregnant, had any medical condition or was on medication. All the volunteers gave their written and signed informed consent and the study was approved, according to the Declaration of Helsinki, by the Bioethics Committee of the University of Murcia with the code 2051/2018. The informed consent document approved and used for the participation of volunteers in all the studies that are part of this thesis, can be seen in Appendix A. The recruitment was done by word-of-mouth, e-mail and acquaintances and through posters around the campus and city centre. 21 volunteers in the case of the VAS validation, and 25 volunteers in the case of SatinApp validation, completed follow-up and were included in the results analysis.

3.2.2 VAS surveys characteristics

A survey was designed with the following questions "¿Cómo de hambriento te sientes en este momento?" (How hungry do you feel at this moment?), "¿Cómo de lleno te sientes en este momento?" (How full are you feeling at this moment?), "¿Cómo de fuerte es tu deseo de comer en este momento?", (How strong is your desire to eat at this moment?) and "¿Cuánta comida sientes que podrías comer en este momento?" (How much food do you feel you could eat at this moment?) Under each question, there is a 10 cm line to answer with the expressions "not at all" or "extremely" ("a large amount" for the fourth question) anchored to each end, which respectively refer to the most negative and the most positive rating that evaluator feels regarding the question, an example can be seen in the Figure 3.1.

3.2.3 SatinApp

SatinApp is an application for Android operating system, so it can be used both in smartphones and tablets, developed by the research group NUTBRO of the University of Murcia. This application is used to conduct surveys, with the aim of being a support tool in human research. Researchers have total freedom to design the surveys and questions that compose it, with a total of four different types of questions. One of these types of questions is based on the analogue visual scales used to measure satiety, in which both, the text of the question and the texts anchored on the line are editable by the researcher. In addition, SatinApp also allows the configuration of multiple choice questions, being able to choose the number of answers needed,

and of logical type, with both “true” and “false” options, as well as “yes” or “no” options.

The application is also able to use the alarms of the devices where it is installed, something very interesting for satiety studies where the VAS must be filled in at a specific time. Once all the questions of a survey have been completed, it is sent to the web server of the application allowing the researcher access to the data online and download them in a spreadsheet. The main difference when filling in the VAS is the length of the line, in principle this line is 100 mm, as is the case of the classic format in P&P, but in both smartphones and tablets, the application adapts the size of the line to the screen of the device used, whether it is in vertical or horizontal position. Then, in tablets we can have lines of up to 180 mm and in a smartphone of between 60 (vertical) and 100 mm (horizontal), although it varies according to the model and the inches of the screen.

3.2.4 Procedure

In both validation studies, women participating did not carry out any study day during menstruation and none of the participants performed vigorous sports or drank alcohol the day before each test. For the VAS validation study, the participants arrived in fasting mode, at least since last midnight, to our facilities around 9 am and they filled in the first VAS appetite survey. Immediately after, the participants had a fixed breakfast, consisting in a coffee (or tea) with milk and a toast with olive oil and salt, having fifteen minutes to eat all breakfast. Seven more VAS surveys were filled in by each participant after 15, 30, 60, 90, 120, 150 and 180 minutes from the start of the study. After three hours of the beginning, participants had an textitad libitum meal consisting of beans with vegetables stew, bought from a local supermarket. In each case, the stew amount served and the rest left on the plate by the participants was weighed to calculate the amount of food eaten. The entire process was repeated at least one week apart, to have a test-retest design.

For the SatinApp validation the study began around 9 a.m., in the same conditions previously described, with a fixed breakfast which consisted of a coffee with milk and toast with butter and jam. Previous to the breakfast, each volunteer filled in the same survey (VAS) about appetite three times, one on P&P (classic format), one on smartphone and the last one on a tablet in a random order (using sample function of the R software). This process was repeated immediately after breakfast and twice more with a gap of an hour (0, 15, 75 and 135 minutes). In both

studies, VAS and SatinApp validation, during the time that the study lasted, the participants remained at rest in our facilities. To ensure a good external validity of the results, adequate inclusion criteria were taken into account and the volunteers received the same indications at the beginning of the study, completing the questionnaires themselves, without the presence of the researchers, to avoid some type of influence on the answers.

3.2.5 Statistic analysis

Previously to the statistical analysis, normality and homocedasticity were confirmed through Shapiro-Wilk test and Bartlett-test respectively, sphericity was checked by Mauchly's test. With scores obtained from VAS scores, areas under curve (AUC) were calculated by using the trapezoidal method (equation 3.1).

$$T_n = \frac{b-a}{2n}\left[f(x_0) + 2f(x_1) + 2f(x_2) + 2f(x_3) + \ldots + 2f(x_{n-1}) + f(x_n)\right] \tag{3.1}$$

Where n is the number of trapezoids, x_0 equals a, and x_1 through x_n are the equally-spaced x-coordinates of the right edges of trapezoids 1 through n.

For the VAS survey validation study, AUC and VAS scores at time 0, 15, and 180 obtained from test-retest data were analysed for each question included in the VAS survey. To measure the reliability, intraclass correlation coefficient (ICC) (Huang et al., 2019) were conducted between the data from test-retest. In addition, to verify that there were no differences between the data of the two tests, hypothesis contrasts by using paired Student's t-test, or Wilcoxon signed-rank test when data were not normally distributed, were performed. The relation between the VAS results and the subsequent Kcal intake in the textitad libitum mean, was measured by Pearson's correlations.

In case of SatinApp validation, two way ANOVA was performed to compare the obtained rating, with format and time as a factors. Then, AUC were compared by repeated measures analysis of variance (ANOVA). The reliability among the three different ways of measurement (P&P, smartphone and tablet), were evaluated using ICC. In addition, the correlations between the different formats were compared by Pearson correlation coefficient. All tests were performed with a statistical signification level of p value < 0.05 using the statistical software R version

4.0.3 (R Core Team, 2020). The same software was used to build the graphs shown in the present work.

3.3 Results

3.3.1 VAS surveys validation results

The mean values through the time of the test-retest study for hunger, fullness, desire to eat and prospective consumption can be seen in Figure 3.2. As can be observed, the obtained results from the two different tests were similar.

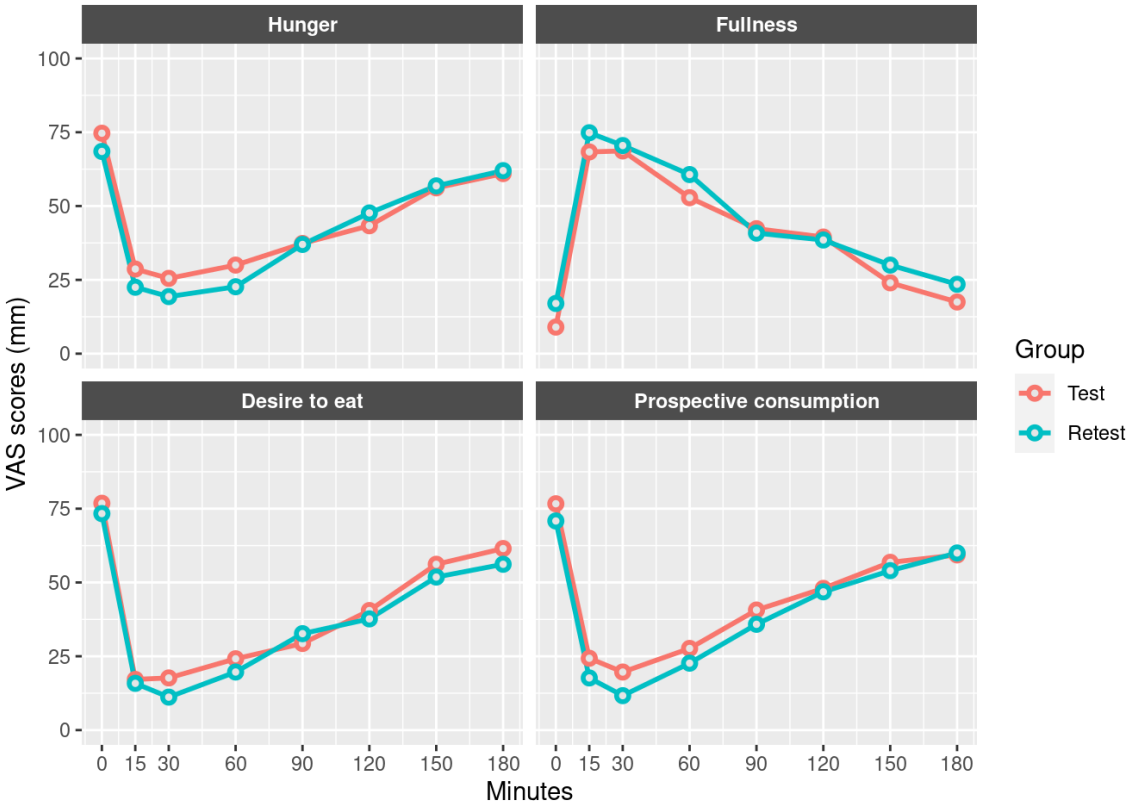

Figure 3.2: VAS appetite and satiety scores along the test-retest assays. Data are presented as mean values ± SEM (n=21).

Appetite and satiety answers through VAS surveys are usually analysed through the AUC values obtained from the VAS scores, that offers a value of the magnitude of sensations measured throughout the study. To obtain a general visual comparison of the obtained curves in the different test days, Figure 3.3 shows the AUC mean values ± SEM and as can be observed, no statistically significant differences ($p < 0.05$) for the contrast hypothesis was observed between

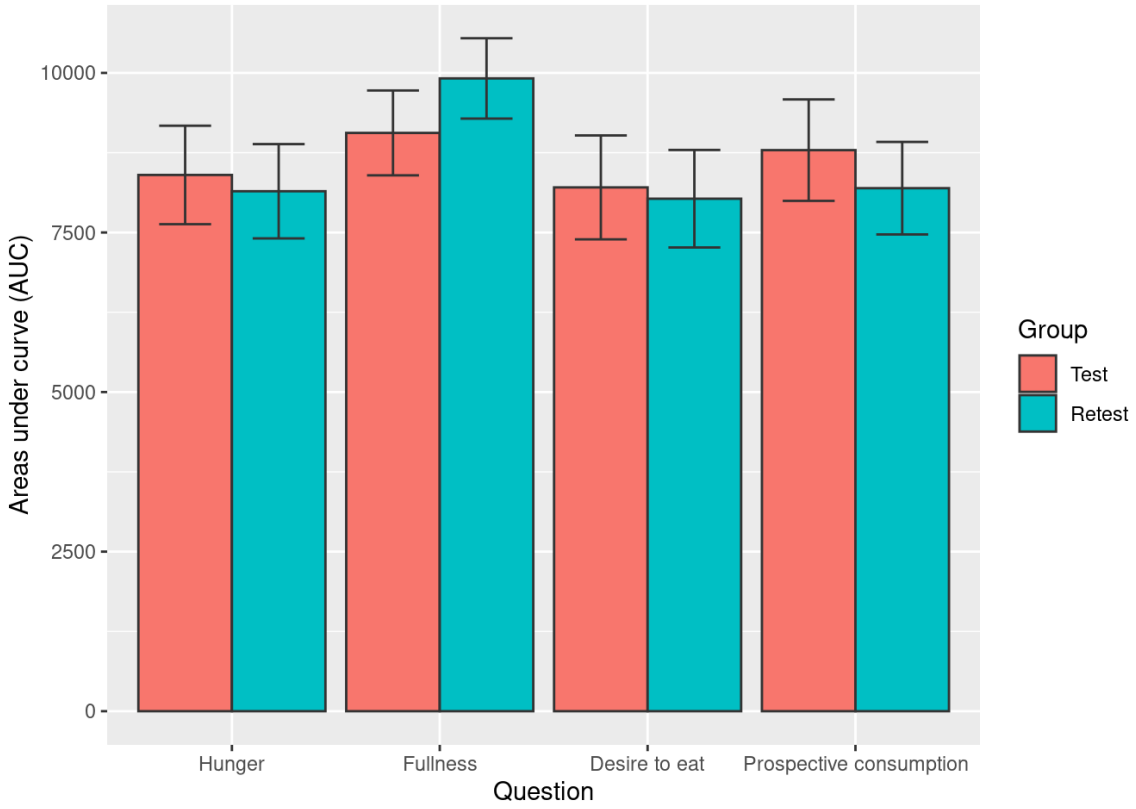

Figure 3.3: AUC appetite and satiety mean values for hunger, fullness, desire to eat and prospective consumption from test and retest assay. Data are presented as mean values ± SEM (n=21). For each question, * denotes significant differences ($p < 0.05$).

test-rest for none of the satiety dimensions analysed.

In addition to AUC, three values from the VAS surveys were included in the analysis of the reliability; the time 0, which is taken in fasting mode, and the maximum (peak) and the minimum (nadir) values between 15 and 180 minutes. Results of the these three VAS points analysis plus the AUC results, for each VAS question between the two performed test can be seen in the Table 3.1. This table is separated in two parts: results for reliability carried out through ICC and results for the hypothesis contrast to verify possible differences between the measures of the two performed tests.

The ICC results shown the reliability between the two test in the same condition. As can be observed in the Table 3.1, where results for AUC and VAS at time 0, peak and nadir are represented, the correlations were different depending on the analysed cases. AUC and peak values demonstrated the highest and significant ($p < 0.05$) intraclass correlations, particularly for hunger and desire to eat, with values above 0.75. In case of fullness and prospective consumption, the observed correlations were smaller. The lowest VAS values (nadir) also showed a good significant ($p < 0.05$) correlation between test-retest results, particularly for fullness. By

Table 3.1: Reproducibility results from test-retest data for the four item included in the VAS survey

	AUC	**Time 0**	**Nadir**	**Peak**
	Intraclass correlation coefficient			
Hunger	0.76*	0.48	0.71*	0.88**
Fullness	0.87**	0.29	0.83**	0.64*
Desire to eat	0.81*	0.49	0.80*	0.86**
Prospective consumption	0.64*	0.58*	0.40	0.51
	Hypothesis contrast for paired samples			
Hunger	0.26	0.52	0.20	0.46
Fullness	0.17	0.30	0.19	0.23
Desire to eat	0.39	0.84	0.68	0.28
Prospective consumption	0.32	0.22	0.36	0.90

For intraclass correlation coefficient, the strength of the correlation is represented. For the hypothesis contrast, the p values are represented.* and ** denote statistically significant differences ($p < 0.05$ and $p < 0.01$), respectively

last, the found intraclass correlations for VAS values at time 0, only showed a significant, but weak result, in case of prospective consumption. For the same satiety questions and variables tested, no statistically significant differences ($p < 0.05$) were found thought the hypothesis contrast. It is worth noting that the peaks values for hunger, desire to eat and prospective consumption take place at the last measured point, at 180 minutes, when participants are hungriest, whilst the peak for fullness take place just after breakfast. On the contrary, in case of fullness, the nadir corresponds to VAS at 180 minutes, when participants feel less full after being three hours without food. Thus, together with AUC, values at 180 minutes showed the best agreement through ICC.

Among the different techniques to measure satiety and hunger sensations, textitad libitum test meal is widely used as well (Bayham, Greenway, Johnson, & Dhurandhar, 2014; García-Vázquez et al., 2019; Geliebter et al., 2015). Thus, to check the validity of the VAS surveys, an textitad libitum meal was served to each participant, just after filled in the last VAS at 180 minutes, the amount of food ingested in each case was noted. Correlations between energy intake and values from VAS surveys are showed in Table 3.2.

In this case, only some weak correlations were found. Between AUC and Kcal ingested, signif-

Table 3.2: Correlation between results obtained from VAS surveys and Kcal consumed in the subsequent *ad libitum* test meal

	AUC	**Time 0**	**Peak**	**Nadir**
Hunger	0.37*	0.01	0.19	0.28~
Fullness	-0.14	-0.10	-0.31*	-0.10
Desire to eat	0.34*	0.02	0.26	0.30~
Prospective consumption	0.38*	0.42**	0.28~	0.44**

*, **, and ~ denote statistically significant differences ($p < 0.05$ and $p < 0.01$) and significant trends ($p < 0.1$), respectively

icant ($p < 0.05$) correlations were observed for hunger, desire to eat and prospective consumption, being AUC the parameter that showed the best relation with the amount of food that the participants ate in the test meal. Similar results existed for nadir, but only this value for prospective consumption was statistically significant. In the case of fullness, a weak significant ($p < 0.05$) correlation was found at 15 minutes.

3.3.2 SatinApp validation results

Figure 3.4 shows hunger, fullness, desire to eat and prospective consumption curves, for the three different formats, P&P, smartphone and tablet, which were used by the volunteers to respond to the VAS survey in the present study. Two-way ANOVA showed that not statistically significant differences ($p < 0.05$) were observed between the three studied formats, neither were there interactions between time and format.

The existence of statistical differences between the three distinct AUC was also checked. Results are showed in Figure 3.5, showing that no differences were found between results obtained for the VAS completed on paper, smartphone or tablet. The agreement between the AUC from three different formats was assessed through the intraclass correlation coefficient and, as can be seen in Table 3.3 results showed correlation above + 0.9 for hunger, desire to eat and prospective consumption, and a correlation of + 0.89 for fullness, which indicates a good agreement between the compared methods.

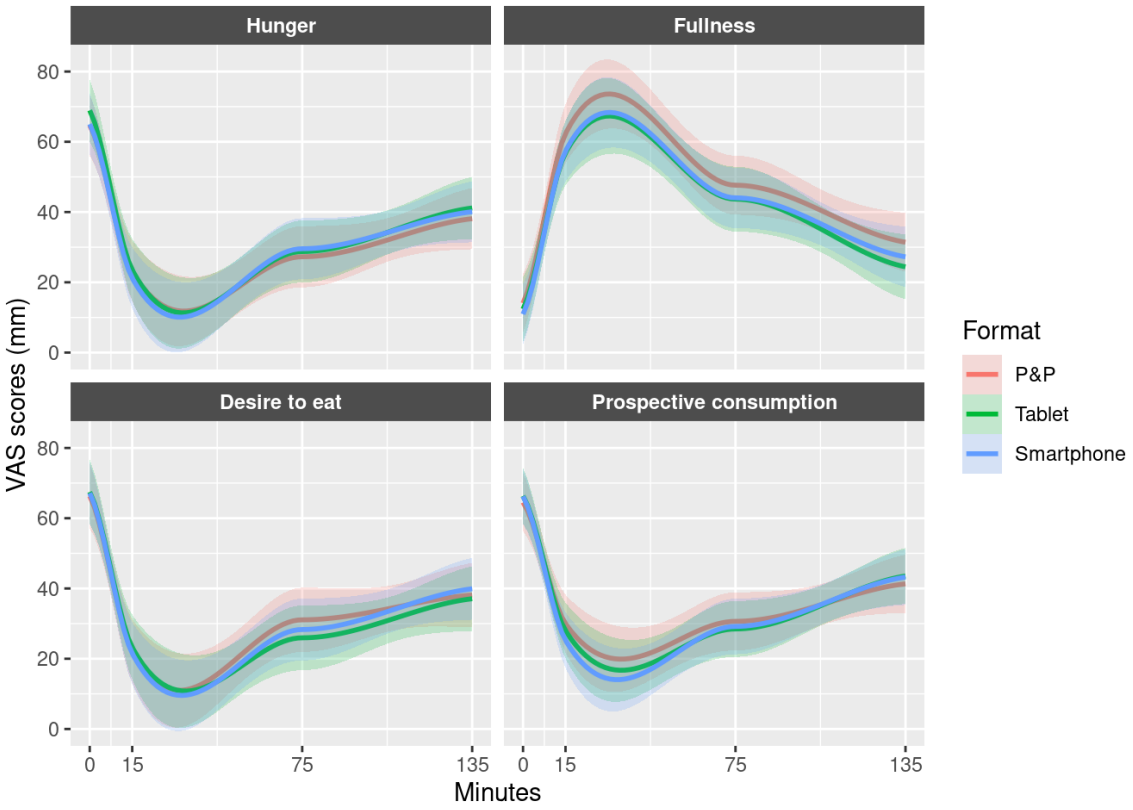

Figure 3.4: VAS rating values over time, for each format and for each question in the present study. Data are presented as mean ± SD.

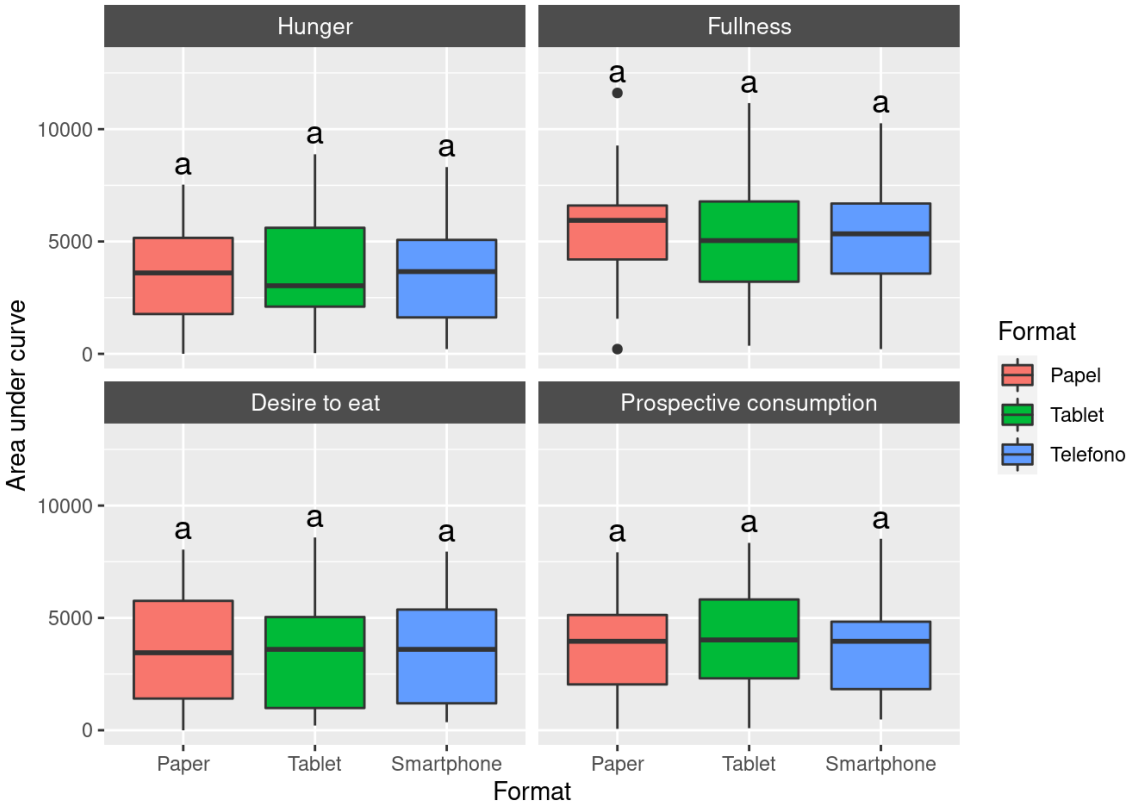

Figure 3.5: Boxplot of the areas under curve obtained for each format and question used to validate SatinApp in the present study.

Table 3.3: Correlations and intraclass correlation coefficients between the AUC of the three VAS formats used for each question.

	Correlations		Intraclass Correlation Coefficients	
Question (AUC)	**P&P vs. Phone**	**P&P vs. Tablet**	**ICC**	**95% CI**
Hunger	0.90***	0.87***	0.91	0.83-0.95
Fullness	0.88***	0.89***	0.89	0.80-0.95
Desire to eat	0.91***	0.90***	0.91	0.84-0.96
Prospective consumption	0.90***	0.95***	0.93	0.87-0.97

3.4 Discussion

Nowadays, visual analogue scales (VAS) are widely used in the study of human satiety (Hobden et al., 2017) and their validations (Flint et al., 2000), where they showed their reproducibility and a good sensitivity are key to can use them adequately. This tool, despite having limitations, has been shown as a good method for these types of studies, especially in intra-subject designs. However, as of today, these surveys have not yet been validated for their use in the Spanish language. In the case of VAS surveys validation, both results, ICC and the curves obtained from the VAS, showed good reproducibility of the VAS surveys in Spanish, and it can be considered normal that the data do not show a greater degree of agreement due to the biological variability itself and between the two different trial days of the study (Flint et al., 2000). None of the analysed parameters showed statistically differences between the two test days. With regard to agreement, ICC demonstrated a good reliability (0.75-0.90) (Koo & Li, 2016) for AUC and for the last analysed point, at 180 minutes in this case, with the exception of prospective consumption, in which case the intraclass correlation was moderate (0.5-0.75) for these two parameters. This could be due to the specific VAS question for prospective consumption, which was "how much food do you feel you could eat at this moment?", which could have been interpreted as both, what is the maximum amount of food that the participants could eat at that moment, and how much food they would like to eat at that moment. Perhaps rethinking the question such as, "How much more would you like to eat at this time?", would have better captured those to be measured, so that in future studies both questions could be included to analyse the behaviour of both variables among themselves and with regarding the rest of the questions. For hunger, fullness and desire to eat, at 15 minutes results also demonstrated good agreement, but in the time 0 results, that had been taken in fasting mode, the correlations were poor and not significant. The same effect could be observed for this time in the appetite VAS

validation in the English language carried out by Flint et al (2000).

Another method to measure satiety is the textitad libitum test meal, where the participants have access to a large amount of food and eat until they do not want more, taking the amount ingested as a measurement of satiety or appetite at that time. This type of test was chosen to check the validity of the VAS surveys; however, it is important to note that is difficult to measure satiety and there is no test that can be used as gold standard. This, could explain the reason although some significant correlations between VAS surveys and Kcal intake were found, these were not as good as for the reliability assessment. In addition, as the time goes on, the capacity of the test meal to measure the satiating effect of the preloads is weaker (Livingstone et al., 2000), therefore, a subsequent meal intake, with a shorter lapse of time after the preload ingestion, could have found better correlations.

In addition, the usual way of administering these surveys has some drawbacks, such as the time needed to evaluate each survey, the waste of materials such as paper or the probability of human error when measuring the results or passing them to a database for analysis. Because of this, some software have been developed and validated to manage VAS in a more comfortable and secure way (Gibbons et al., 2011; Stubbs et al., 2001; Stratton et al., 1998). The new SatinApp computer application, in addition to the advantages that these types of tools suppose, offers some improvements. The application was designed for devices with Android operating system, widespread used in both smartphones and tablets, so that many of the participants can use it directly on their devices, without having to obtain from the researchers a device for this purpose. The design of the surveys is done by the researcher from the web of the application, and is downloaded to the device through a QR code, so the participants do not have the possibility of modifying or altering the configuration of the study design. A problem that some of these applications have had is that when a VAS appears on the screen, the cursor appears with the response marked on the line, which sometimes causes the participant pass to the next question without moving the cursor, which is an error in our data. This problem has been solved in the design of SatinApp, since the cursor does not appear until the touch screen of the device is touched. In addition, the "next" button also appears only when the screen has been touched, making it impossible to move on to the next question without having selected the previous answer.

The present study was designed to verify the validity of SatinApp as a method to perform VAS type surveys textitversus the classical format (P&P), to check if the different screen sizes of

the different types of devices used and, therefore of the line on which to respond can affect the obtained results. The statistical analysis of results did not found significant differences between the P&P format and SatinApp, both in smartphone and tablet, showing a similar sensitivity in all the analysed formats. Regarding the degree of agreement between administering the VAS in the different modalities used, the direct comparison through the ICC showed a high correlation among the three formats tested.

It is important to highlight another advantage of this software, since studies in laboratory conditions and in the "real life" show different characteristics. This App allows studies where very valuable data can be obtained in an ambulatory real context as well as monitoring people under the study conditions (Fahrenberg, Myrtek, Pawlik, & Perrez, 2007), thanks to its portability and the notices when completing the survey, greatly facilitates this type of task.

3.5 Conclusions

Based on results, measure appetite and satiety sensations by VAS surveys in Spanish language, have demonstrated a good reliability and validity and, therefore it can be used as a tool in human satiety studies. In addition, it can be concluded that the informatics application developed by the research group NUTBRO is valid to measure subjective satiety in humans, both on smartphones and tablets, which makes it a valuable tool to carry out studies on satiety in humans. Finally, it is worthy to note that due to the fact that the App allows the design of several surveys, and not only VAS for measured satiety, it could be used for different types of studies and also in different disciplines, such as to measure pain or to perform psychological studies or any other type of research that requires the use of surveys.

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CHAPTER 4

EFFECT OF GAZPACHO, HUMMUS AND AJOBLANCO ON SATIETY AND APPETITE IN ADULT HUMANS: A RANDOMISED CROSSOVER STUDY

Effect of gazpacho, hummus and ajoblanco on satiety and appetite in adult humans: A randomised crossover study

Abstract: Nowadays, overweight and obesity has reached an epidemic level around the world. An interesting strategy to tackle them is the study of the satiety effect of foods and ingredients, which could reduce food and/or calorie intake. Moreover, it is also important that the food used for this purpose can be included as part of a healthy diet. With regard to this, it is well known that the Mediterranean Diet (MD) is a feeding pattern that helps us to maintain good health providing an important and balanced amount of nutrients and active compounds. The aim of this study was to identify MD foods with a high satiating capacity in human volunteers (n = 24). For this purpose, three typical foods of the Mediterranean region, mainly based on vegetables, were selected: hummus, ajoblanco and gazpacho. As a control, white bread was used. After a standard breakfast, the subjective sensation of hunger and satiety for each food was assessed by visual analogue scales (VASs) during three hours. Subsequently, volunteers had *ad libitum* access to a meal. Results indicate that gazpacho showed the highest satiating scores, despite the fact that it was not the food that provided the most macronutrients and fibre amount. More studies of this type are needed in order to determine proportion and/or combination of ingredients of these classical Mediterranean recipes that could enhance human satiety.

Keywords: Appetite; Satiety; VAS; Gazpacho; Hummus; Ajoblanco.

4.1 Introduction

Nowadays, the satiety induced by the consumption of different foods, ingredients or nutrients is being widely studied (García-Flores, Martínez Moreno, Beltrán Miranda, Zepeda-Salvador, & Solano Santos, 2017; Hansen, Andersen, Astrup, Blundell, & Sjödin, 2019; Warrilow, Mellor, McKune, & Pumpa, 2019). Studies indicate that the acquisition of knowledge in this area can facilitate the design of diets or foods to help us reduce appetite, including a lower intake of food and calories and thus being a good strategy in the fight against overweight and obesity. The last being a disease currently in the spotlight for its worldwide relevance. This strategy on human satiety could help to reduce its prevalence and associated health complications, such as cardiovascular disease, diabetes, musculoskeletal disorders and some type of cancers (Cercato & Fonseca, 2019; WHO, 2017). With regard to this, different nutrients and components of foods included in a Mediterranean Diet have been studied to analyse their satiety value and thus help in the control of food intake.

Previous studies have been focused on the study of fibre and macronutrients such as proteins, carbohydrates and fats concluding that proteins and fibre are the most satiating nutrients (Clark & Slavin, 2013; Veldhorst et al., 2008). Moreover, there are also some studies focused on the possible effect of micronutrients on appetite, obtaining very promising results (Tremblay & Bellisle, 2015). However, to date, in this case our knowledge is limited. With regard to this, foods included in a Mediterranean Diet are usually vegetables that, when are frequently consumed, provide beneficial active compounds. Typical foods from this type of diet with a potential to control calorie intake and simultaneously provide healthy properties, could be a good choice in weight management. For this reason, considering their possible satiating potential according to the recent bibliography and their nutritional composition, four typical foods widely consumed in the Mediterranean region were chosen: hummus, ajoblanco, gazpacho and white bread (Hull, Re, Chambers, Echaniz, & Wickham, 2015; Méndez, Rodríguez, Romero, González, & Isasa, 2008; Wallace, Murray, & Zelman, 2016). Moreover, the sensory properties and palatability of foods were also measured since they also play a key role in determining food control intake, whether a food is full of flavour and/or has palatable texture can strongly determine the amount of ingested food (Johnson & Wardle, 2014). Taking this background into account, the aim of this study was to identify the satiety feelings and appetite control capacity of different foods included in the Mediterranean Diet.

4.2 Materials and Methods

4.2.1 Sample description

Ajoblanco, gazpacho, hummus and white bread were chosen to study their effect on human satiety. Hummus is a soft paste made from cooked, mashed chickpeas, blended with tahini, olive oil and lemon juice providing high protein and fibre content (Wallace et al., 2016), both nutrients related with human satiety. Ajoblanco is a cold soup typically from the south of Spain, containing bread, almonds, garlic and olive oil. It was chosen for its almond content, as this type of nut has demonstrated promising results in other satiety studies (Hull et al., 2015). Gazpacho is also a typical cold soup originally from the south of Spain. It is traditionally elaborated with a mix of several vegetables such as tomato, cucumber, pepper, onion, garlic and olive oil. Gazpacho was included in the study due to its fibre and micronutrients (vitamins and mineral) reported high content (Campra et al., 2019; Méndez et al., 2008; Vasilopoulou, Dilis, & Trichopoulou, 2013). All these foods consisting in blended vegetables. White bread based on refined wheat, that basically provides carbohydrates (which have not been described as the most satiating components), and a low amount of proteins and fibre (described as high satiating components), was used as negative control (Tremblay & Bellisle, 2015). Ajoblanco, gazpacho and hummus were elaborated in our facilities following the traditional recipe. White bread was supplied by a local bakery. To calculate the nutrients content of the preload ingested by participants and to prepare the relevant figures relating to nutrient content, food composition tables (Mataix, García, Mañas, Martínez, & Llopis, 2003) and the Easy Diet software (Xyris software, Brisbane, Australia), supported by the Spanish Association of Dietetics and Nutrition, were used.

4.2.2 Study participants

The present study was carried out in the department of Food Science and Nutrition in the Faculty of Veterinary Sciences (University of Murcia) on a total of twenty-four healthy volunteers (9 males and 15 females, aged 30.6 ± 5.58, body mass 62.13 ± 15.32 Kg, height 167.39 ± 9.04 cm, BMI 22.09 ± 2.57 Kg/m^2, fat 22.63 ± 6.86% and muscle 21.20 ± 4.75%. Sample size was calculated using a 95% of confidence level, a variance of 7818770 and a maximum

error of 1195.94, obtained from previous satiety VAS results of our research group (Equation 4.1). The inclusion criteria were the same as those described in the methodology in Chapter 3. The Bioethics Committee of the University of Murcia approved the study in accordance with the ethical principles of the current Declaration of Helsinki with the code 2051/2018. All the volunteers gave their written and signed informed consent before participation in the study. The recruitment of the volunteers was done through posters around the University and other zones of the city of Murcia, word-of-mouth, acquaintances and e-mail contacts. Subjects did not receive any payment for their participation in the study.

$$n = \frac{Z^2_{1-\frac{\alpha}{2}} \times \hat{\alpha}}{\epsilon^2} \tag{4.1}$$

4.2.3 Procedure

This work was a randomised crossover trial, registered in Open Science Framework (osf.io/7bxgz), with three different preloads (gazpacho, hummus and ajoblanco) using white bread as control (Tremblay & Bellisle, 2015). Study design is presented as a flow diagram in Figure 4.1. Each participant arrived to our facilities on four different days (with at least one week of wash-out period), one for each analysed food. Women participating in the study did not carry out any test during menstruation and none of the participants performed vigorous sports or drank alcohol the day before each test. The volunteers came in fasting mode, at least since last midnight, each day to eat breakfast around 9:00 a.m. consisting of 100 ml of black coffee (or 100 ml of green tea) with non-caloric sweetener. One hour later the volunteers filled in the first visual analogue scales (VAS) survey (Flint, Raben, Blundell, & Astrup, 2000) about appetite and satiety feelings and just after that, volunteers consumed the appropriate food. Description of the preload food, the water amount to match the foods volume and the preload foods composition can be seen in Table 4.1.

The order in which every food was tested for every participant was calculated using the function sample of the software R. Then, the participants had the foods in a different order across the study. The amount of ingested food was 10% of the total energy expenditure for each subject in the study, which was calculated using the Harris and Benedict formula (Harris & Benedict, 1918) that is one of the most commonly used equations in clinical practice, being the oldest, and having undergone the most extensive validation (Poli et al., 2016). To approximate the

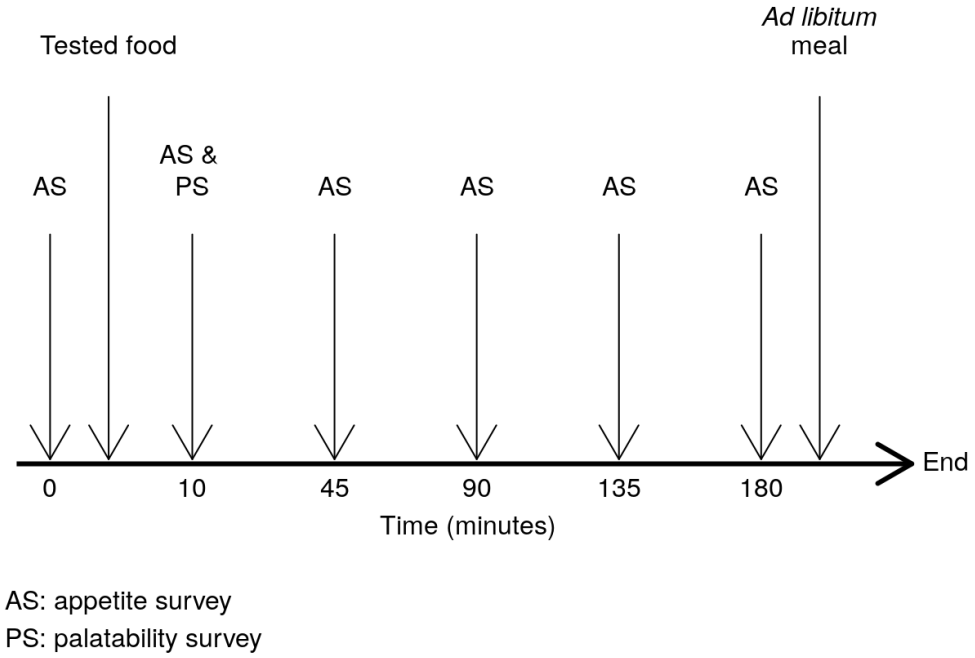

Figure 4.1: Flow diagram representing the different stages of the study design

effect on satiety of the gastric filling, the differences in the volume among the foods assessed were matched using the appropriate amount of water for each case. Just after the volunteers consumed all the food and water, they filled in another two VAS surveys (one about appetite and another about palatability). The amount of water that every participant had during the first day of the study, was annotated to make sure that the participants had the same amount of water on the remaining study days. After that, a new appetite survey was filled in every forty-five minutes, during three hours. Immediately after the last survey, an *ad libitum* test meal was carried out, which consisted of traditional pizza Margherita (241 kcal/100g) whose ingredients were dough, tomato, cheese and olive oil. Three pizzas for each test meal ($\approx$ 1080 g) were baked and cut in small square pieces to avoid the participants knowing the amount of pizza served. Then, the total amount of pizza was weighed and served with the instruction that volunteers could eat as much pizza as they wanted until they were satisfied. When the participants finished eating, the remaining pizza on the plate, was weighed again to calculate the ingested amount. Every participant repeated the entire process three times, one for each remaining tested food in the study.

Table 4.1: Description of the preloads tested in the present study.

	Ajoblanco	**Hummus**	**Gazpacho**	**White bread**
Grams	261.6	93.4	500.4	88.3
Water (g)	238.7	407.0	0.0	412.1
Kcal	208.1	208.1	208.1	208.1
Fats (g)	16.5	13.0	13.3	1.4
Carbohydrates (g)	10.2	15.1	17.9	41.5
Simple sugar (g)	1.3	0.0	15.0	1.8
Fibre (g)	2.2	8.3	5.3	3.7
Proteins (g)	4.8	7.6	4.1	7.3

4.2.4 Assessment of appetite and palatability

The surveys used to measure both, satiety and palatability, were formed by visual analogue scales (VAS), which consisted of the corresponding questions, followed by a 10 cm horizontal line with the expressions "nothing at all" and "extremely" anchored at each end of the line. Participants responded by marking on the line according to their feelings at the time of answering the survey. The VAS survey scale used to evaluate satiety and appetite included four main questions about hunger, fullness, desire to eat and prospective consumption, being a tool validated to measure appetite and satiety sensations (Flint et al., 2000) and widely used nowadays for this procedure (Hägele et al., 2019; Lee, Brett, Chang, de Zepetnek, & Bellissimo, 2019; Sun, Goh, Govindharajulu, Khee-Shing Leow, & Henry, 2019; Duerlund, Vad Andersen, & Victor Byrne, 2019). From these surveys' scores, areas under the curve (AUC), incremental areas under curve (ΔAUC) and incremental VAS score at 180 minutes (ΔVAS_{180}) were calculated as described in the statistical analysis section. All these data give us important information regarding the appetite and satiety sensations during the study. Food palatability (as far as it is another important factor on satiety regulation) was measured through a survey that the participants filled in immediately after eating the four tested foods. Surveys used were formed by VAS as well, consisting of six questions about pleasure of eating the food, desire to eat more, sweetness, savouriness, tastiness and creaminess. To evaluate the possible caloric compensation after the tested foods, the calorie intake at each *ad libitum* meal was also measured.

4.2.5 Data and statistical analysis

With the data obtained from the VAS surveys scores, areas under curve (AUC) and incremental area under curve (ΔAUC), by subtracting the base line (time 0) from the remaining scores, were calculated by using the trapezoidal rule 4.2. Also from the VAS scores, the incremental score at 180 minutes (ΔVAS_{180}) were calculated. Outliers from VAS scores was removed and imputed by using multivariate imputation by chained equations with random forest machine learning method (Shah et al., 2014). Data normality were confirmed through Saphiro-Wilk test. Then, data were compared using multilevel lineal model with preloads as predictor variable and participant ID as random effects variable, setting maximum likelihood as method. Tukey post hoc were used to determine the differences between preloads. Robust bootstrap one-way repeated measures ANOVA for the trimmed means, was used when data did not have normality or sphericity (Checked by using Maunchly's test). The same statistical procedure was followed to analyse the obtained data from palatability. All test were setted with a p value < 0.05. The data and statistical analysis, as well as the figures included in this chapter, were made by using the language and environment for statistical computing R version 4.0.3 (R Core Team, 2020). Two participants did not finish the study due to a change in their work schedules which did not permit them to continue, so all their data were removed from the study to avoid missing values.

$$T_n = \frac{b-a}{2n}\left[f(x_0) + 2f(x_1) + 2f(x_2) + 2f(x_3) + \ldots + 2f(x_{n-1}) + f(x_n)\right] \tag{4.2}$$

Where n is the number of trapezoids, x_0 equals a, and x_1 through x_n are the equally-spaced x-coordinates of the right edges of trapezoids 1 through n.

4.3 Results

Results of this study are divided, on one hand, in the subsequent calorie intake in *ad libitum* meals and, on the other hand, in the data obtained from the VAS ratings (Figure 4.2) to obtain AUC, ΔAUC and ΔVAS_{180}. Moreover, palatability analysis was also performed for each studied food.

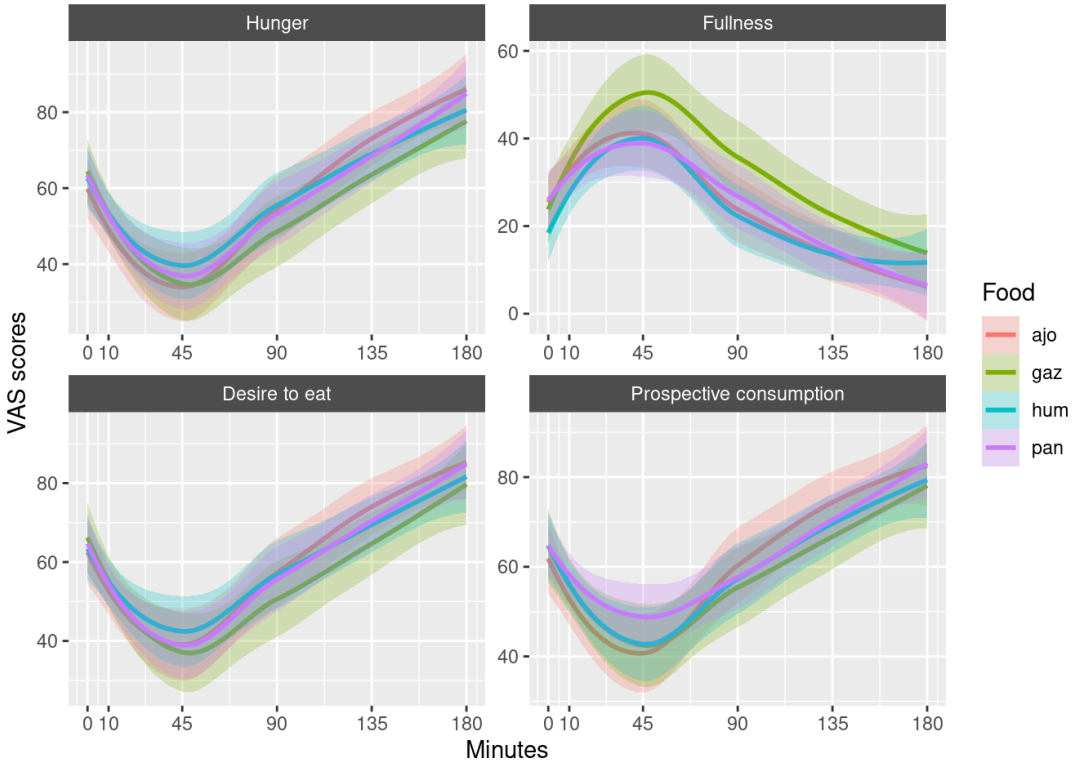

Figure 4.2: Subjective appetite scores after the four preloads test. Data are presented as mean values (n=24)

4.3.1 Palatability assays

An important factor to consider relating to the control intake is the palatability of the food, since more palatable foods have shown to be related with overeating, possibly by a stimulation of the brain circuits related to the reward responses (Johnson & Wardle, 2014). This is the reason why in the present study a palatability survey was carried out immediately after the intake of the foods under study: gazpacho, hummus, ajoblanco and white bread. Participants were asked about the pleasure they felt when eating the respective food, the desire to eat more, the sweetness, the tastiness, the savouriness and the creaminess. As can be seen in Figure 4.3, for volunteers the most pleasant foods were, in this order, gazpacho, hummus, white bread and ajoblanco, with statistically significant differences ($p < 0.05$) between gazpacho and hummus with as opposed to ajoblanco.

Regarding desire to eat more of the corresponding food, ajoblanco was the worst rated, obtaining the remaining food a similar score amongst them and without significant differences. The food scored as sweeter was gazpacho, without significant differences with hummus and white bread; ajoblanco was the lowest scored although no differences were found with hummus

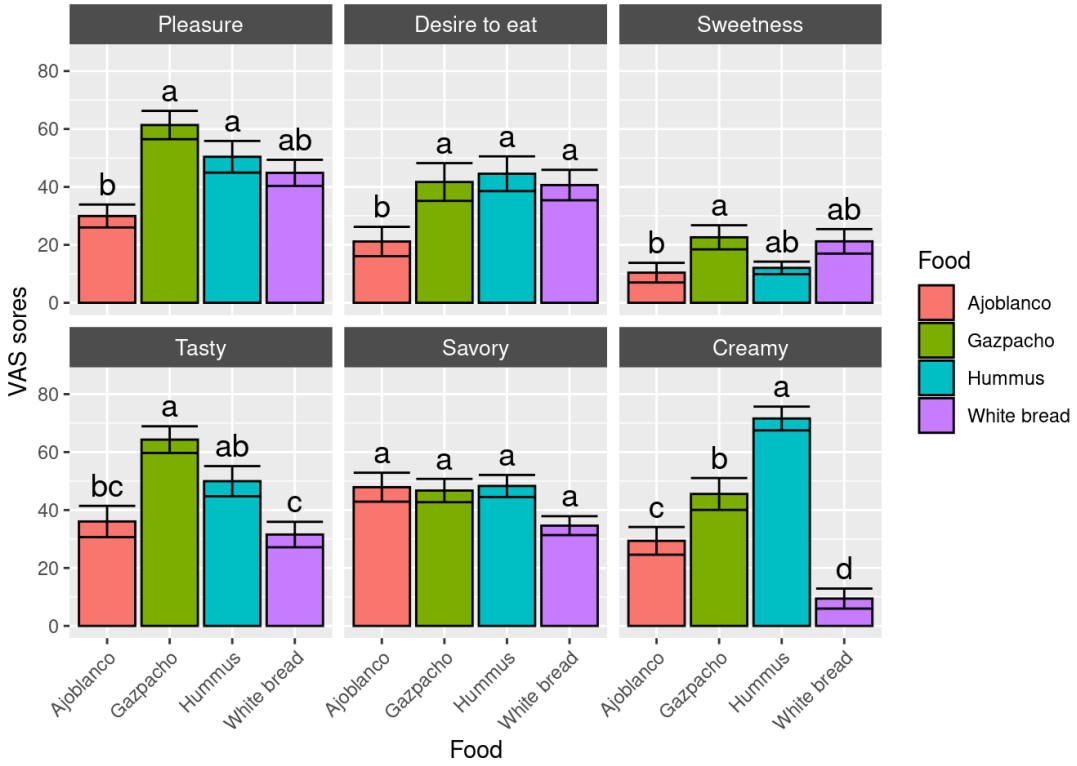

Figure 4.3: Palatability scores measured by VAS for each tested food. Data are presented as mean values ± SEM

and white bread. In terms of flavour, gazpacho was the best rated with statistically significant differences ($p < 0.05$) between it and ajoblanco and white bread. Relating to creaminess, significant differences between all the foods studied were observed, being hummus, the creamiest food followed by gazpacho, ajoblanco and white bread. There were no differences relating to savouriness.

4.3.2 Appetite and satiety sensations analysis from the VAS scores

Data from appetite and satiety VAS surveys related to hunger, fullness, desire to eat and prospective consumption offer an estimation of the appetite and satiety in volunteers during the study time and results can be observed in Table 4.2. According to other studies (Hall, Thomas, & Johnson, 2005; Holt, Brand-miller, & Stitt, 2001; Loria Kohen et al., 2011), white bread was used as reference food compared to the other foods assayed because of its low content in fibre and proteins, that are reported as key components on food intake control. As can be observed in Table 4.2, AUC, ΔAUC and ΔVAS_{180} for hunger, fullness, desire to eat and prospective consumption showed the best satiating mean values when gazpacho was eaten, followed, in most cases, by hummus. Statistical analysis showed no significant differences

Table 4.2: Visual analogue scales (VAS) results expressed as AUC, ΔAUC and ΔVAS score at 180 minutes for each analysed food.

Foods	**Hunger**	**Fullness**	**Desire to eat**	**Prospective consumption**
Area under curve (AUC)				
Ajoblanco	10512 ± 552 $^{a\|\alpha}$	4320 ± 493 $^{b\|\beta}$	11073 ± 569 $^{a\|\beta}$	11122 ± 588 $^{a\|\alpha}$
Gazpacho	9656 ± 791 $^{a\|\alpha}$	5878 ± 660 $^{a\|\beta}$	9973 ± 836 $^{a\|\beta}$	10483 ± 809 $^{a\|\alpha}$
Hummus	10525 ± 657 $^{a\|\alpha}$	4216 ± 476 $^{b\|\beta}$	10722 ± 708 $^{a\|\beta}$	10786 ± 670 $^{a\|\alpha}$
White bread	10277 ± 696 $^{a\|\alpha}$	4415 ± 508 $^{b\|\beta}$	10616 ± 653 $^{a\|\beta}$	11188 ± 548 $^{a\|\alpha}$
Incremental area under curve (ΔAUC)				
Ajoblanco	-2081 ± 851 $^{a\|\alpha}$	1557 ± 421 $^{b\|\beta}$	-2347 ± 773 $^{a\|\beta}$	-1577 ± 901 $^{a\|\beta}$
Gazpacho	-4082 ± 666 $^{b\|\alpha}$	3474 ± 635 $^{a\|\beta}$	-3966 ± 806 $^{a\|\beta}$	-2526 ± 456 $^{a\|\beta}$
Hummus	-2766 ± 790$^{ab\|\alpha}$	2578 ± 549$^{ab\|\beta}$	-2334 ± 736 $^{a\|\beta}$	-2261 ± 608 $^{a\|\beta}$
White bread	-3157 ± 723$^{ab\|\alpha}$	1426 ± 635 $^{b\|\beta}$	-2837 ± 741 $^{a\|\beta}$	-2218 ± 652 $^{a\|\beta}$
Incremental VAS_{180} score (ΔVAS_{180}) (mm)				
Ajoblanco	16 ± 6 $^{a\|\alpha}$	-7 ± 3 $^{b\|\beta}$	8 ± 4 $^{a\|\beta}$	11 ± 5 $^{a\|\alpha}$
Gazpacho	1 ± 3 $^{b\|\alpha}$	2 ± 2 $^{a\|\beta}$	2 ± 3 $^{a\|\beta}$	4 ± 3 $^{a\|\alpha}$
Hummus	8 ± 4 $^{a\|\alpha}$	-1 ± 1$^{ab\|\beta}$	11 ± 4 $^{a\|\beta}$	8 ± 3 $^{a\|\alpha}$
White bread	11 ± 3 $^{a\|\alpha}$	-7 ± 2 $^{b\|\beta}$	9 ± 3 $^{a\|\beta}$	6 ± 3 $^{a\|\alpha}$

Data are represented as mean values SEM. Whitin each measure (AUC, ΔAUC and ΔVAS_{180}) in the same column, different letters (a, b) denote statistically significant differences ($p < 0.05$) among the studied foods. Greek letters ($\alpha - \gamma$) denote large, medium and small general effect size, respectively.

($p < 0.05$) among hummus, ajoblanco and white bread in any analysed cases. For AUC data, gazpacho gave the greatest fullness sensation ($p < 0.05$) compared whit the remaining foods. In the case of ΔAUC, participants reported less hunger sensations and more fullness sensations for gazpacho, compared whit ajoblanco and white bread ($p < 0.05$), without significant differences between hummus and the remaining preloads. Hunger for ΔVAS_{180}, showed gazpacho again as the food that induced the greatest significant hunger suppression among the four preloads. Fullness sensations in this case, showed gazpacho as the most satiating food ($p < 0.05$), but without differences whit hummus.

4.3.3 *Ad libitum* test meal

Three hours after the corresponding food intake, volunteers had an *ad libitum* meal to assess the possible caloric intake compensation. A large amount of pizza Margherita (241 kcal/100g) was baked, weighed and served for each participant on each test day. When the participant finished eating, the remaining amount of pizza was weighed again to calculate the grams and the caloric intake. There were no statistically significant differences in the amount of pizza eaten after ingestion of foods under study. Nevertheless, the same trend obtained from VAS results was observed in the data from test meal, showing that volunteers ate less pizza in the same day that they had gazpacho as food to assay. The amount ingested by participants, represented as a mean ± SEM, were 509.24 ± 178.9 g (1227.27 ± 431.0 kcal) after eating white bread, 502 ± 177 g (1210 ± 427 kcal) after eating ajoblanco, 492 ± 169 g (1187 ± 408 kcal) after eating hummus and 480 ± 172 g (1156 ± 414 kcal) after eating gazpacho.

4.4 Discussion

As can be observed in the results obtained from palatability VAS surveys, gazpacho seems to be the most palatable food for volunteers followed by hummus, which as commented above, could increase the appetite. However, these two foods (gazpacho and hummus) were the ones that showed the best satiety results through the VAS survey, compared to ajoblanco and white bread, therefore it seems that the effects on appetite regulation found in this study could be due to different factors and not only palatability.

The results referring to appetite and satiety sensations, obtained form VAS surveys, seem to indicate that, among the four foods tested, gazpacho was the most satiating food in this study. It is important to consider that these kinds of studies, usually tend to focus on the satiating effect of the macronutrients (protein, fats and carbohydrates) to control hunger. In this regard, it is accepted that, among macronutrients, proteins produce the highest hunger suppression, followed by carbohydrates and fats, respectively (Tremblay & Bellisle, 2015). Also, dietary fibre (non-digestible carbohydrates) present in ingested foods plays an important role in hunger control (Warrilow et al., 2019). However, relating to nutrient content according to recipes of tested foods, gazpacho does not contain the highest amount of either proteins or fibre. Gazpacho

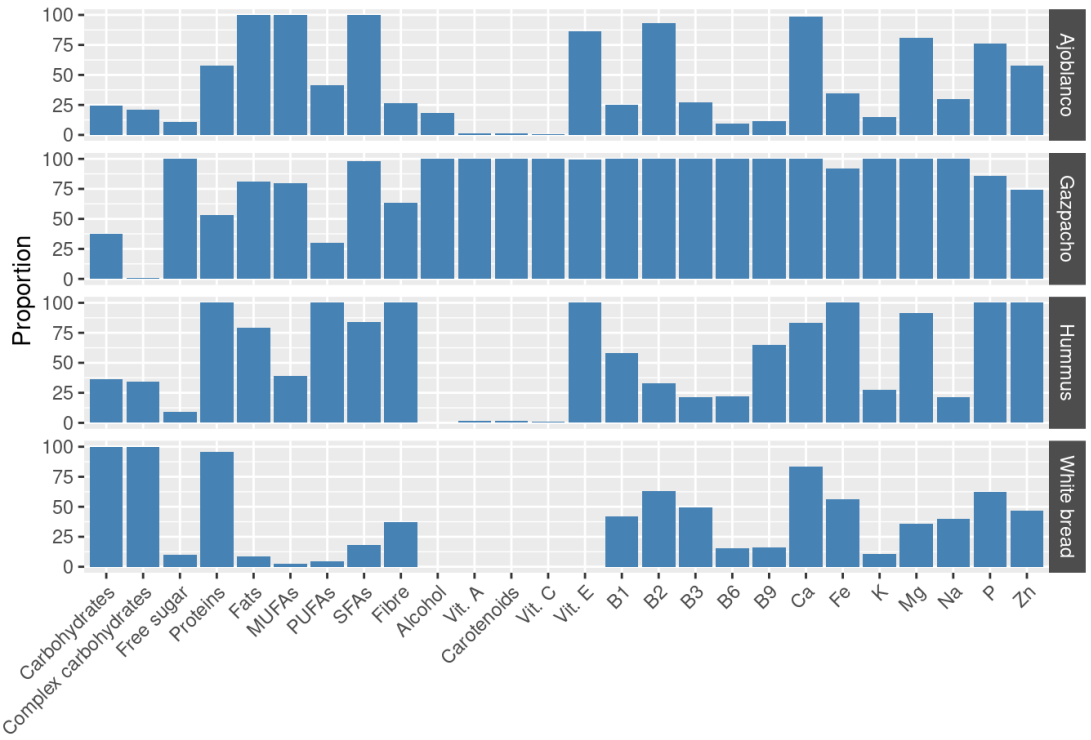

Figure 4.4: Percentage of nutrients provided by the different tested foods in relation to the maximum quantity of each nutrient

induced the greatest appetite sensations suppression, measured by VAS, and it does not seem to be due to caloric content or ingested volume, because the volunteer's intake consisted of the same amount of calories and volume (matched with water). Regarding this, different weights for the different assessed foods were used with the aim of providing the same energy density in all of them. Different satiety studies add water to food to decrease its energy density because satiety is highly affected by the amount of calories. Moreover, it has been reported that drinking water as a beverage together with a meal had a similar effect on satiety as incorporating an equivalent amount of water into the food (Rolls, 2009). In addition, isocaloric foods with different volumes affect satiety (higher volume causes a lower food intake) suggesting a role of energy density in the overconsumption of foods (Rolls, 2000). In Figure 4.4, the total average quantity that volunteers ingested was represented through their nutrient content, being shown as a percentage regarding the highest amount among the four foods.

Therefore, it can be easily observed which food contributed with the greatest amount of each nutrient. As can be seen in Figure 4.4, gazpacho shows the highest amount of free sugar, alcohol and most micronutrients. This cold tomato soup is the second tested food in iron, phosphorus and zinc contents. Therefore, it can be suggested that gazpacho provides the highest amount of free sugar and most micronutrients by calories, compared to the other three studied foods. In

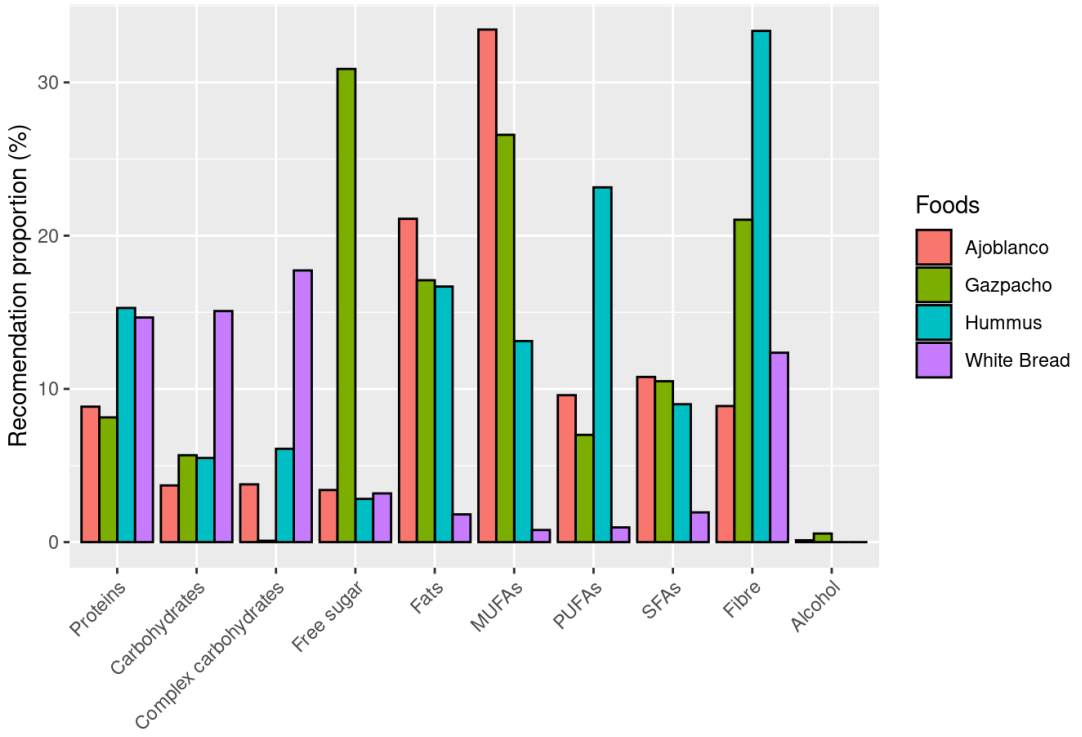

Figure 4.5: Percentage of macronutrients, fibre and alcohol provided by the ingested amount of each tested food, represented as a percentage of the general intake recommendations

Figure 4.5 and Figure 4.6, the amount of nutrients provided by the assayed foods and macro- and micronutrients contents, respectively, can be seen. Results are expressed as a percentage of the Daily Reference Intake (DRI) (García Gabarra, 2006) or general recommendation about each nutrient or substance. As Figure 4.5 shows, gazpacho presents the greater amount of alcohol.

However, as can be seen in this case, alcohol comprises a very small percentage of maximum daily recommendation (less than 1%) and, in any case, alcohol was reported to increase appetite and reduce satiety (Kwok, Dordevic, Paton, Page, & Truby, 2019). Therefore, alcohol should not be considered to clarify the satiating effect observed with gazpacho. Focusing again on gazpacho, a low quantity of proteins, total and complex carbohydrates and PUFAs can be observed, whilst the largest amount of free sugar according to ingredients content, compared with the other three tested foods. Nevertheless, as Figure 4.6 shows, in accordance with Figure 4.4, gazpacho contained the highest DRIs proportion in almost all represented micronutrients, being also the second assessed food that contained the largest amount of the rest of them.

The contribution of carotenoids regarding a general intake recommendation (Biesalski HK et

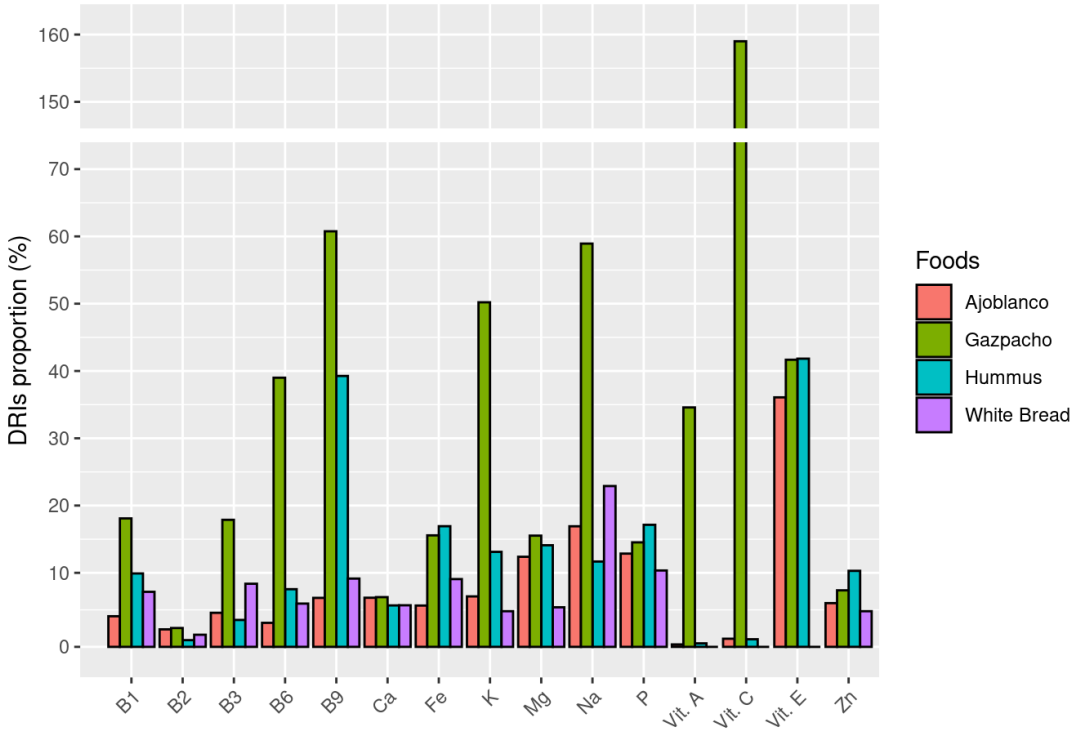

Figure 4.6: Percentage of vitamins and minerals provided by the ingested amount of each tested food, represented as a percentage of the dietary reference intakes (DRI) for each micronutrient

al., 1997), was also calculated, gazpacho being the food that contributed whit the largest amount of that compound followed by humus and ajoblanco, all providing more than the recommend intake. Therefore, regarding the food composition, the most satiating food in this study (gazpacho) stands out for its high contribution of free sugar, vitamins, minerals and other non-nutrient substances like carotenoids. It is important to consider that these compounds can be modified, dissolved and released during digestion and then can modify satiety signalling in different ways when compared with non-digested foods and this fact could explain differences in satiety response (Chambers et al., 2015). This deserves more research to elucidate the effect of digestion on the satiety capacity of foods. Despite current knowledge seems to indicate that regarding food composition, proteins and fibre show the highest satiating capacity (Clark & Slavin, 2013; Westerterp-Plantenga, Lemmens, & Westerterp, 2012), in the present study this effect cannot be confirmed. Among the tested foods, hummus contained the largest amount of protein and fibre meanwhile, gazpacho showed the lowest protein content and the second one with respect to fibre, but in our study, it has demonstrated the most satiating potential. At this point, it is also important to note that the satiating capacity of the different compounds present in foods is not quite clear (Carreiro et al., 2016) and, in the case of proteins, after digestion satiety feelings can be modified in different ways because it is dependent upon peptide

transporters or different technological processes among other factors. Knowledge of the effect of released protein digestion products on satiety is still pending of more research (Santos-Hernández, Miralles, Amigo, & Recio, 2018). In case of high fibre foods, digestion seems to increase satiety (Chambers et al., 2015); however, it is probably that other components of foods generated by breakdown during digestion can also modify feelings of satiety.

A possible explanation, to the satiety effects observed in this study, is the glucostatic theory (Mayer, 1955), which was proposed by Jean Mayer in 1955, and it still stands (Chaput & Tremblay, 2009). According to this theory, glucose receptors in the hypothalamus could detect changes in blood glucose that can regulate appetite and feelings of satiety. In this way, the higher glycaemic levels could induce higher satiety sensations, and vice versa. It is also worthy of note that a high fibre content of foods can slow down the absorption of these sugars, being a factor that enhances medium-term satiety (Mathern, Raatz, Thomas, & Slavin, 2009). However, according with our results, hummus was the food with the highest amount of fibre as can be seen in Figure 4.5. Although gazpacho provided the greatest amount of free sugars, but not the greatest amount of total carbohydrates, which eventually reach the blood as glucose as well. When comparing the glycaemic index (GI) of these foods, it can be observed that both, gazpacho and hummus, have a similarly low GI (Augustin et al., 2016)(Fajkusova et al., 2007). In addition, white bread has been classified as a high GI food (Atkinson, Foster-Powell, & Brand-Miller, 2008) it does not seem that the differences in satiation, in this case, can be attributed only to the amount of sugar. The satiety and hunger sensations ratings measured by VAS through the study time can be observed in Figure 4.2. With regard to hunger, desire to eat and prospective consumption, the results up to 45 minutes, were similar among the different tested foods, after that the scores from gazpacho showed a higher satiety effect. In the case of fullness, the scores for gazpacho were the greatest during the three hours of study. Hummus showed the second greatest VAS scores on satiety, but this effect could be seen at 180 minutes, later than in the case of gazpacho. Therefore, the presence of higher amount of free sugar in gazpacho seems not to be of high importance since, as it has been mentioned above, the GI of hummus and gazpacho were similar. However, the observed effect indicates that gazpacho is more satiating, compared to other foods, as time goes on. Therefore, it is not clear that the highest satiety observed in gazpacho effect could be attributed to the carbohydrate's contribution. As can be observed in Figure 4.6, according references gazpacho provides with the greatest contribution of vitamins and minerals. Although less studied, some researches have observed that micronutrients can play an important role in appetite regulation and weight

management (Aytekin, Godfri, & Cunliffe, 2019; Tremblay & Bellisle, 2015). Probably, the most studied micronutrient in this way, is calcium. *in vivo* studies in rats showed that following a low calcium diet, the animals chose a high calcium beverage (Tremblay & Bellisle, 2015), suggesting a mechanism to mitigate a calcium deficit. Moreover, a calcium and multivitamin supplemented diet has shown a best appetite control (Major, Alarie, Doré, & Tremblay, 2009) and less hunger sensations (Major et al., 2008) in human studies. However, there are few studies on the effect of micronutrients content on satiety of foods. Although a possible explanation for these findings, could point to a compensatory mechanism regarding nutritional status of some micronutrients (Aytekin et al., 2019), the real cause is still unknown. Among the four studied foods in this work, gazpacho contributed, in general terms, the largest quantity of most micronutrients (Figures 4.4 and 4.6), but since the study design did not include a positive control for vitamins and mineral, the satiety effect observed for this food, cannot be attributed to this factor.

With regard to the results obtained from the *ad libitum* test meal, although a similar trend in the test meal than from VAS results can be observed, there were no significant differences that could be due to an excessive time between the preload and *ad libitum* test meal, since after 180 minutes, the possible effects on appetite and intake may have disappeared. Another possible flaws of the study design, regarding the *ad libitum* test meal, could be the choice of food offered for the test. Analysing carefully the data, the mean calories pizza intake by the participants in this study, was almost half (47%) of the total calorie intake that these volunteers need in a day (2501 Kcal), when the regular intake at lunch is around 30 % of the daily calories. It possible that a popular and palatable food, such as pizza, offered *ad libitum*, could lead participants to overeating, therefore, for future studies the *ad libitum* meal will be choose more carefully.

4.5 Conclusions

This study was carried out to determine the satiating effect on humans of different typical foods in the Mediterranean Diet. Gazpacho, hummus and ajoblanco were selected for their satiety potential effect according to their nutritional composition. As a control, white bread was used. The satiating effect was evaluated through VAS and *ad libitum* test meal. VAS surveys results showed gazpacho as the most satiating food, despite the fact that it did not contribute whit the highest amount of proteins, fibre or carbohydrates, compounds that have

been shown to have a satiating effect. Therefore, more studies in this regard are necessary to clarify these effects. Regarding the *ad libitum* test meal, the same trend was observed, but without statistical differences among the tested food, maybe due to an excessive time between the preloads and the *ad libitum* test meal, or to the selected food for the test.

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CHAPTER 5

SATIETY EFFECT OF COMMON MEDITERRANEAN FOODS: A RANDOMISED CROSSOVER STUDY IN ADULT HUMANS

Satiety effect of common Mediterranean foods: a randomised crossover study in adult humans

Abstract: The study of the satiety effect of foods and ingredients is an interesting strategy to help in the weight management. Moreover, it is also important that the food used for this purpose can be included as part of a healthy diet. With regard to this, it is well known that the Mediterranean Diet (MD) is a feeding pattern that helps us to maintain good health providing an important and balanced amount of nutrients and active compounds. The aim of this study was to identify MD foods with a high satiating capacity in human volunteers, with the aim to provide more information in this field and of identifying the most satiating ones for the subsequent development of new foods that included in the diet can help in the hunger control. For this purpose, seven foods that fit the Mediterranean pattern were selected: Almond, egg, oat, potato, tigernut, walnut and as reference food white bread. After a standard breakfast, the subjective sensation of hunger and satiety for each food was assessed by visual analogue scales (VASs) during three hours. Results indicate that oat had the highest satiating scores, following by potato. In addition, the volunteers filled in surveys about food intake, eating behaviour and their anthropometric parameters were measured. Principal analysis component showed an interesting relation among poor quality food intake, loss of control of intake and a higher proportion of body fat.

Keywords: Appetite, Oat, Potato, VAS, Obesity.

5.1 Introduction

An adequate management of overweight and obesity, should include the development of foods that enhancing satiety could help to regulate the energy intake, thus improving the weight control (Lim & Poppitt, 2019). Mediterranean Diet (MD) is considered a healthy food pattern and it has been widely studied in this regard (Serra-Majem et al., 2019). In addition, due to its composition, nutrients and components of this diet pattern could be a good tool to control appetite and manage weight, mainly due to its high fibre content and low calorific density (Schröder, 2007). Based on this, in this chapter typical MD foods have been studied for their possible satiating effects, with promising results.

Nuts are included in MD guidelines indicating that a consumption of 1-2 servings per day is recommended, including in this group almonds, pistachios, walnuts or hazelnuts. This food group has been shown to improve appetite control, probably by its high protein and fibre content (Sabaté, 2003). Among nuts, almonds has been widely studied demonstrating a good hunger control in human studies (Hollingworth, Dalton, Blundell, & Finlayson, 2019; Hull, Re, Chambers, Echaniz, & Wickham, 2015; St-Onge, Campbell, & RoyChoudhury, 2020). Other interesting food including in this group is walnut, which provides a large amount of polyunsaturated fatty acids (PUFAs) and omega-3 (Eteshola & Oraedu, 1996; Maguire, O'Sullivan, Galvin, O'Connor, & O'Brien, 2009) even compared to other nuts, since this type of fat has been described as more satiating than other types of fatty acids (Stevenson, Clevenger, & Cooper, 2015). Moreover, tigernut, is a weed plant of tropical and Mediterranean regions, and although it has not been much studied from the point of view of satiety, it is usually included in the Mediterranean diet and provides with a high amount of fibre and mono-saturated fatty acids (MUFAs) as well (Sánchez-Zapata, Fernández-López, & Pérez-Alvarez, 2012), so it could be an interesting food in this regard.

Regarding eggs, its consumption has been stigmatized for many years due to a not clear link with an increase on cardiovascular diseases, but recent studies show that epidemiological evidence does not support these restrictions, since no relationship has been found between the consumption of eggs and the development of these diseases, resulting in a recent increase in the consumption recommendations for this food (Kritchevsky, 2004). In addition, several studies about satiety have been carried out to determine the effects of eggs consumption on appetite with a promising results, being its strength its protein quality and nutrients contribution

(Bayham, Greenway, Johnson, & Dhurandhar, 2014; Rebello, O'Neil, & Greenway, 2016; Wal, Marth, Khosla, Jen, & Dhurandhar, 2005).

Cereals, along with fruits and vegetables, are the basis of the MD, recommending a consumption of several portions per day. In addition to its fibre content, the endosperm of some cereal such as oat provides with high content of protein and lipid compared to others (Mataix, García, Mañas, Martínez, & Llopis, 2003; Rebello et al., 2016) and these compounds can enhance satiety (Veldhorst et al., 2008; Warrilow, Mellor, McKune, & Pumpa, 2019). Furthermore, the β-glucan, linear polysaccharides present in this grain, have been widely studied for their healthy properties, such as decrease LDL cholesterol levels in plasma (Whitehead, Beck, Tosh, & Wolever, 2014), or its effect on appetite control, showing promising results in this regard (Beck, Tosh, Batterham, Tapsell, & Huang, 2009; Butt, Tahir-Nadeem, Khan, Shabir, & Butt, 2008; Kim, Behall, Vinyard, & Conway, 2006; Rebello et al., 2016).

Another food typically included in MD and widely recommended in this diet are potatoes, which have been demonstrated satiating effect in both, animals and humans experimentations. In rats, diets supplementation with potato powders improve the gut microbiota population, SCFA concentration in distal colon and stimulated the satiating hormone PYY (Wu, Hu, Dai, Che, & Zhang, 2019). In humans' assays, potato have higher satiating values than rice and pasta maybe due to its lower energy density (Zhang, Venn, Monro, & Mishra, 2018), and showed the best results among 38 studied foods (Holt, Miller, Petocz, & Farmakalidis, 1995). Furthermore, the protease inhibitor II (PI2) from potato could reduce the enzymatic activity in digestive system, reducing the satiating hormones degradation, and the potato PI2 have been reported to increased circulating CCK plasma levels, an hormone with satiating effect and modulation of appetite sensation measured by VAS (Flechtner-Mors et al., 2020). With this background, potato could be considered as good candidate to be included in an appetite control diet.

With the aim of selecting a good candidate to develop new foods that can help in the management of appetite control, almond, egg, oat, potato, tiger nut and walnut were chosen to measured their satiating effect in humans. In addition, with the objective to explore a possible relationship between appetite and nutritional status, the diet quality, eating behaviour and body composition were measured to the study participants.

Table 5.1: Description of the preloads eaten for the participants

Grams	Almond	Oat	Omelette	Potato	Tigernut	Walnut	White bread
Eaten food	40.8	236.5	149.3	333.2	50.6	32.8	98.3
Water	359.2	163.5	250.7	66.8	349.4	367.2	301.7
Fats	21.6	4.4	18.1	0.5	12.0	22.5	1.6
Carbohydrates	1.9	42.4	0.0	53.6	21.5	1.3	50.6
Free sugars	1.6	0.6	0.0	2.3	21.5	0.5	3.7
Fibre	5.8	6.8	0.0	6.0	8.8	1.7	3.4
Proteins	8.2	10.8	18.1	8.3	3.1	4.6	8.8

Data are presented as mean values of ingested food. The average amount of dry oats ingested (before boiling) was 64 g.

5.2 Methodology

5.2.1 Sample description

Almond (Pronus dulcis), oat (Avena sativa), egg, potato (Solanum tuberosum), tigernut (Cyperus esculentus), walnut (Juglans regia) and white bread were chosen to study their effect on human satiety. The mean amount of the seven preloads ingested by the participants in this study, and their nutritional contribution is shown in the Table 5.1. To calculate the nutrients content of the preload ingested, food composition tables (Mataix et al., 2003) were used. As mentioned above, almond was chosen due to its good studied result on hunger control, along two more nuts, tigernut and walnut, three types of nuts that have a different nutritional composition. Almond is the richest in proteins content, tigernut in fibre and free sugars and walnut in fats, as can be seen in Table 5.1. Egg provided the highest amount of proteins among the tested food, and potato contributed with the biggest amount of carbohydrates. Oat also provided a high amount of carbohydrates, but with fibre and proteins as well. White bread based on refined wheat, that basically provides carbohydrates (which have not been described as the most satiating components), and a low amount of proteins and fibre (described as high satiating components), was used as negative control (Tremblay & Bellisle, 2015). Tigernuts were kindly supplied by the Regulatory Council "Denominación Origen Valencia", white bread was bought at a local bakery and the remaining studied foods were bought at a local supermarket.

5.2.2 Study participants

The present study was carried out at the department of Food Science and Human Nutrition in the multidisciplinary research centre Pleiades-Vitalis (University of Murcia) facilities, on a total of nineteen volunteers (seven males and twelve females, aged 27.5 ± 5.54, body mass 65.20 ± 12.006 Kg, height 170.71 ± 11.63 cm, BMI 22.171 ± 1.88 Kg/m2, fat 23.93 ± 5.66 %, muscle 23.06 ± 8.91 Kg). Sample size was calculated using a Z = 1.96, 95% of confidence level, a variance of 6554090 and a maximum error of 1234 (Equation 5.1), obtained from previous results of satiety VAS studies. The sample size obtained was n=17 and the recruitment was thirty-three for the possible loss of follow-up of the study by the participants. Nineteen volunteers finished all the study (seven preloads) and twenty-nine of them completed all the surveys and anthropometry.

$$n = \frac{Z^2_{1-\frac{\alpha}{2}} \times \hat{\alpha}}{\epsilon^2} \tag{5.1}$$

Where n is the sample size, Z a constant depending on the confidence interval, α is the confidence interval, $\hat{\alpha}$ is the variance and ϵ is the maximum error.

The Bioethics Committee of the University of Murcia approved the study in accordance with the ethical principles of the current Declaration of Helsinki, with the code 2051/2018. All the volunteers gave their written and signed informed consent before participation in the study. The recruitment of the volunteers was done through posters around the University and other zones of the city of Murcia, word of-mouth, acquaintances and e-mail contacts, with the same inclusion criteria as those described at the Chapter 3 methodology. Subjects did not receive any payment for their participation in the study.

5.2.3 Procedure

This work was a repeated crossover trial, with seven different preloads, consisting of almond, oat, egg (omelette), potato, tigernut, walnut and white bread. White bread was used as a reference food for its expected low satiety potential (Tremblay & Bellisle, 2015). Women participating in the study did not carry out any test during menstruation and none of the par-

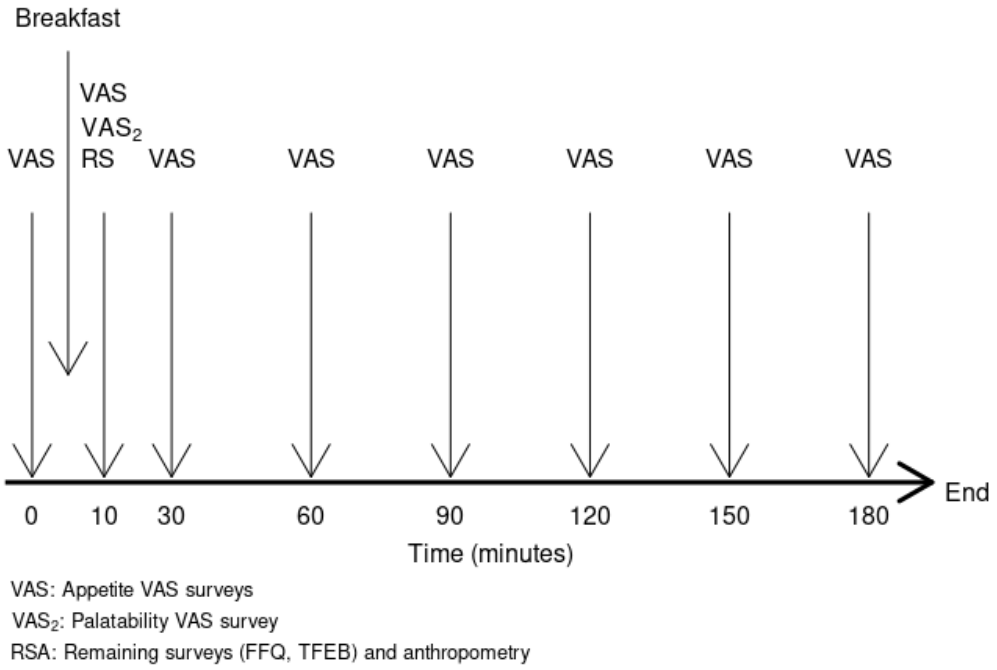

Figure 5.1: Flow diagram representing the different stages of the study design.

ticipants performed vigorous sports or drank alcohol the day before each test. Each participant arrived to our facilities on seven different days (with at least one week of wash-out period), one for each analysed food. The volunteers came in fasting mode each day, at least since last midnight, to eat breakfast around 9:00 a.m. consisting of 100 ml of semi-skimmed without lactose milk, with 50 ml of black coffee (or 50 ml of green tea) and non-caloric sweetener. The coffee (or tea) was served with a white bread toast (100 g) with 5 g of virgin extra olive oil and a pinch of salt ($\approx$ 2 g). The first study day, the participants chose which drink (coffee or tea) they wanted to have with the breakfast in the seven-study day, and they always had the same drink in every study day. One hour later the volunteers filled in the first visual analogue scales (VAS) survey (Flint, Raben, Blundell, & Astrup, 2000) about appetite and satiety feelings and just after that, volunteers consumed the corresponding studied food. The amount of ingested preloads was 10% of the total energy expenditure for each subject in the study, which was calculated using the Harris and Benedict formula (Harris & Benedict, 1918; Poli et al., 2016).

To approximate the effect on satiety of the gastric filling, the differences in the volume among the foods assessed were matched using the appropriate amount of water for each case. Just after the volunteers consumed all the food and water, they filled in another two VAS surveys (one

about appetite and another about palatability). After that, a new appetite survey was filled in every forty-five minutes, during three hours. Participants were allow to drink water during this time, annotating the ingested amount to make sure that they had the same amount of water on the remaining study days. This process was completely repeated by each participant up to seven times, once for each food analysed. Study design is presented as a flow diagram in Figure 5.1. The order in which every food was tested for every participant was calculated using the function sample of the software R. The anthropometric measured (in fasting mode) and the food intake and behaviour questionnaires were completed the first day of the study for each participant.

5.2.4 Assessment of appetite and palatability

The same VAS surveys for assessment of appetite and palatability described in the previous chapter, were used for this study.

5.2.5 Diet quality and eating behaivour assessment

With the aim to analyse the diet quality intake of the study sample, a semi-quantitative frequency food questionnaire (FFQ) was filled in by each participant at the beginning of the procedure. The FFQ is one the most used tool to obtain information about food quality intake (Fuente-Arrillaga, Ruiz, Bes-Rastrollo, Sampson, & Martinez-González, 2010). The used questionnaire consists on 53 items about different food divided in two part, in one hand the amount of rations that are consumed in a day, week or a month and, in the other hand, the size of the corresponding rations ($\frac{1}{4}$, $\frac{1}{2}$, =, $\times 2$, $\times 3$). The amount of the ration (g or ml) for each food, were specified in each item of the questionnaire as a reference for the participants. The three-factor eating questionnaire (TFEQ) (López-Aguilar et al., 2011) was used to determine the eating behaviour of the study participants. This tool includes 51 items that give information about three dimensions; a) food restriction (R), b) disinhibition and the lost of control when eating (D) and c) subjective sensations of hunger and desire to eat (H).

5.2.6 Anthropometric assesment

Height (217 tallimeter, Seca deutschland, Germany) and weight (BF 511 weighing machine, OMRON, Japan), waist, hip, wrist and arm circumferences (201 anthropometric tape, Seca deutschland, Germany), and the bicipital, tricipital, subscapular, suprailiac, abdominal, medial leg and calf skinfolds (Tanner Skinfold Caliper, Holtain ltd., UK) were measured following the protocol of ISAK (Stewart, Marfell-Jones, Olds, & De Ridder, 2011) to obtain BMI, fat proportion by using Brozek equation (Guerra, Amaral, Marques, Mota, & Restivo, 2010) and muscle kg (Alva, Divyashree, Kamath, Prakash, & K, 2020) for each volunteers.

5.2.7 Data and statistical analysis

With the data obtained from the VAS surveys scores, areas under curve (AUC) and incremental area under curve (ΔAUC), by subtracting the base line (time 0) from the remaining scores, were calculated by using the trapezoidal rule (Equation 5.2. Also from the VAS scores peak-nadir, subtracting the minimum score from the maximum score, peak-0, subtracting the score at time zero from the maximum score, and the incremental score at 180 minutes (Δscore180) were calculated. Outliers from VAS scores was removed and imputed by using multivariate imputation by chained equations with random forest machine learning method (Shah et al., 2014). Data normality were confirmed through Saphiro-Wilk test. Then, data were compared using multilevel lineal model with preloads as predictor variable and participant ID as random effects variable, setting maximum likelihood as method. Tukey post hoc were used to determine the differences between preloads. Robust bootstrap one-way repeated measures ANOVA for the trimmed means, or Friedman test were used when data did not have normality or sphericity (Checked by using Maunchly's test). The same procedure was followed the analyse the obtained data from palatability. All test were setted with a p value < 0.05.

$$\int_a^b f(x)dx \approx \sum_{k=1}^{N} \frac{f(x_k - 1) + f(x_k)}{2} \Delta x_k \tag{5.2}$$

Where N is the number of trapezoids, xk is a partition of [a,b] and Δxk is the length of the subinterval.

To explore a relationship among body parameters, appetite, eating behaviour and diet quality, a principal component analysis (PCA) with varimax rotation were conducted. The data and statistical analysis, as well as the figures included in this chapter, were made by using the language and environment for statistical computing R version 4.0.3 (R Core Team, 2020).

5.3 Results and discussion

5.3.1 Palatability assays

Food palatability is shown to be an important factor that influence in the appetite regulation, observing hunger parameters increasing by a high palatable meal (Johnson & Wardle, 2014). To take this factor into account, each participant filled in a palatability survey just after ate each food and results are represented in Figure 5.2. The two first parameters measured about palatability were the pleasure that the participants felt when ate the corresponding food, and the desire to eat more of the corresponding food. As can be seen in Figure 5.2 the best rating in this regard were obtained for almond, omelette and walnut. In case of pleasure, these three foods showed the highest statistically significant differences ($p < 0.05$) values, compared with tigernut and white bread, without differences among the remaining foods.

Regarding "desire to eat" more of the corresponding food, although the differences seem to be higher, robust repeated measured ANOVA did not found differences. As in the last cases, no statistical differences were observed for sweetness and creaminess. Walnut was more savourer than potato ($p < 0.05$), not finding more statistical differences among the remaining food for savouriness. The tastier preload was potato with differences ($p < 0.05$) when compared with tigernut, not being statistical differences for the remaining foods. The total effect sizes were large for pleasure, desire to eat and creaminess, and medium for the remaining parameters of palatability. Regarding palatability, oat and potato were neither the favourite nor the least palatable, meanwhile the results for tigernut, although this food was the sweetest, the other parameters measured showed it as the least palatable food analysed.

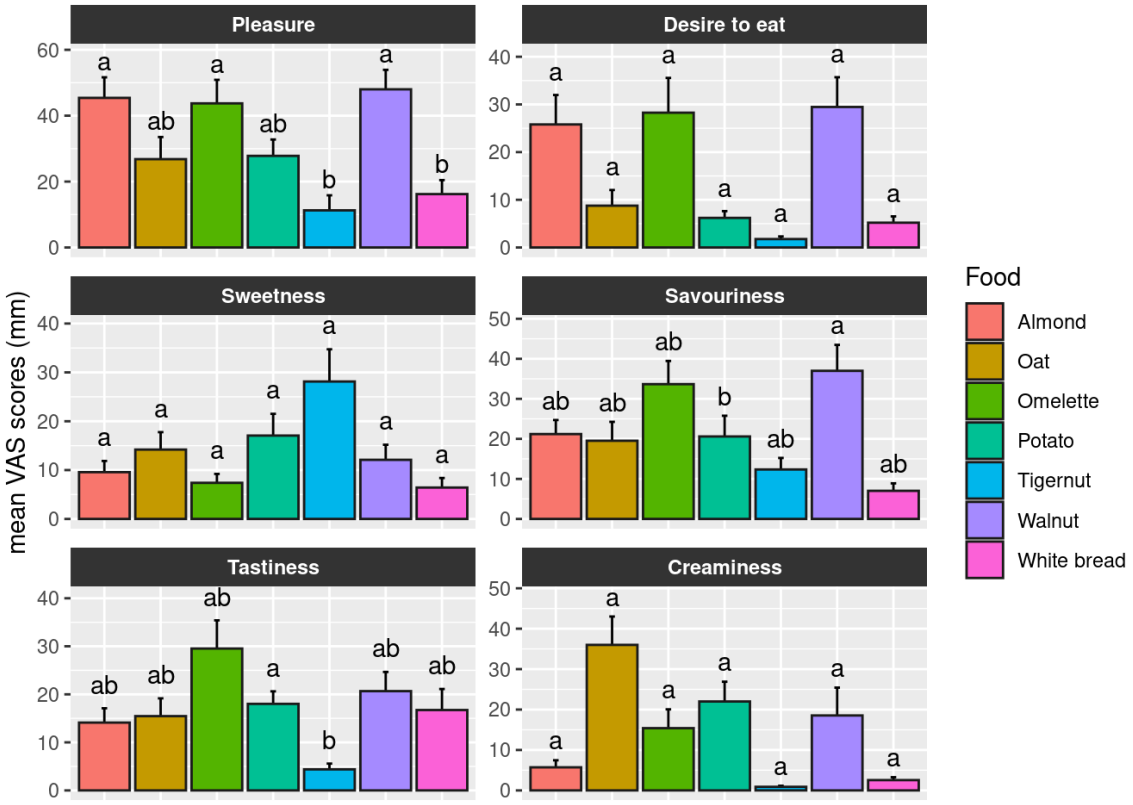

Figure 5.2: Palatability mean results obtained from VAS surveys for each tested food. For every parameter measured, different letters (a-b) denote statistically significant differences ($p < 0.05$) between the seven groups.

5.3.2 Appetite and satiety sensations analysis from the VAS scores

Figure 5.3 shows the mean Δ values (without baseline score) obtained from appetite VAS surveys regarding hunger, fullness, desire to eat and prospective consumption, felt by the participants during the study time.

To summarize the results about appetite and satiety sensation obtained from VAS surveys, a combination of the self-reported sensation was made by subtracting fullness scores from 100, and calculating the average values among this new variable and the scores from hunger, desire to eat and prospective consumption. Same procedure and statistical analysis described in the section 5.2.7 were then carried out, whose results are shown in Figure 5.4.

As can be seen is this figure, oat showed the highest satiety sensation for the analysed parameters, follow by potato and having the remaining foods a lower satiety effect, similar among them. In the case of Peak nadir, there were no statistically significant differences. As can be observed in the Figure 5.4, when the baseline was considered to calculate the results, the differences

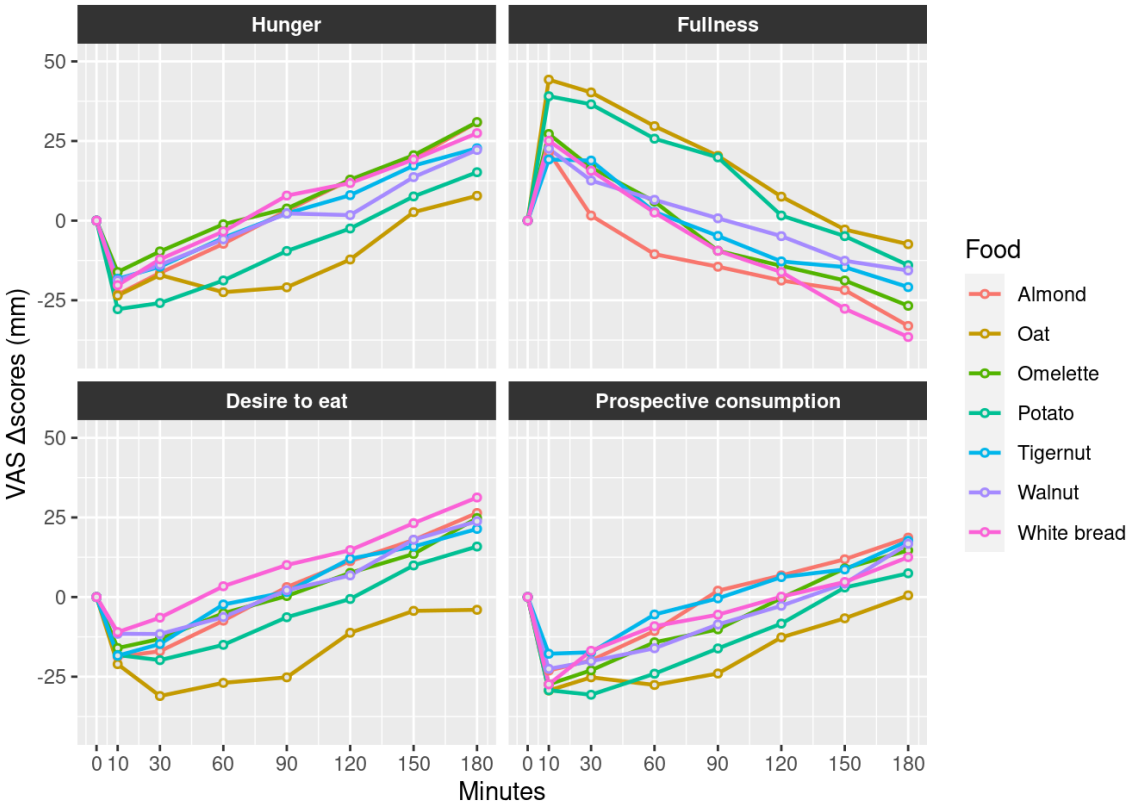

Figure 5.3: Δ values from VAS appetite surveys through the study time (180 minutes) for each studied preload. Data are presented as μ.

were clearer and the effects size were bigger, showing ΔAUC that oat was the more satiating analysed food ($p < 0.05$) over the other foods, except in the case of potato, when statistically significant differences were not found. With regard to potato, this food showed a similar appetite rating than walnut and statistically significant higher values than almond, omelette, tigernut and white bread, being these last four foods the ones that produced the smaller satiety response measured by VAS. The total effects size for ΔAUC was large ($\eta^2 > 0.25$) and the biggest with respect to the remaining parameters analysed.

The detailed VAS results for hunger, fullness, desire to eat and prospective consumption sensations, divided by AUC, ΔAUC, peak-nadir, peak 0 and Δscore180, for the seven analysed foods, are showed in Table 5.2, where results similar to those described for general hunger were found, highlighting clearer differences for the question on the feeling of fullness.

Regarding palatability, studies with human volunteers showed that greater palatability triggers higher short-term intake (Sørensen, Møller, Flint, Martens, & Raben, 2003), therefore it is important take this information into account when satiety effect is analysed (Johnson & Wardle, 2014). The highest satiety response was shown by oat and potato, but neither two foods, in

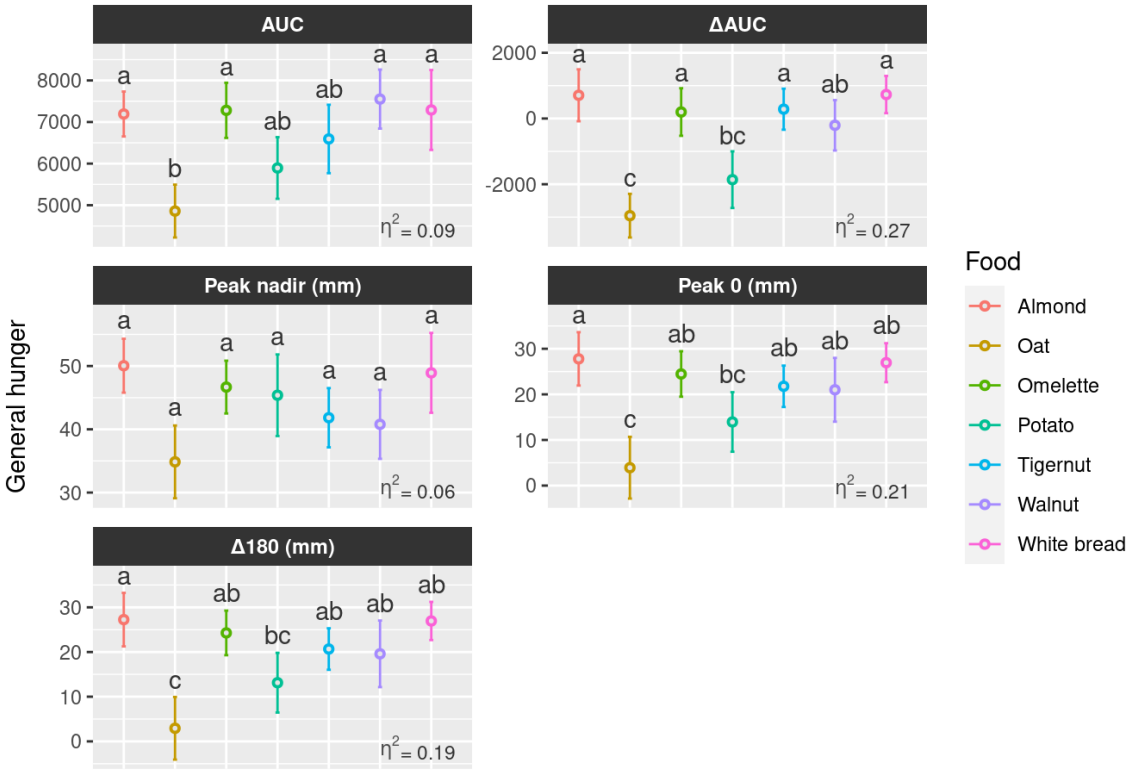

Figure 5.4: Combined results (general hunger) of the questions about appetite and satiety obtained from the VAS surveys. For each analysis showed, letters (a-c) denote statistically significant differences ($p < 0.05$) between the studied food, and η^2 denotes the total effect size.

general, were the least scored in terms of palatability, what could explain this effect. Nevertheless, tigernut was scored as a low palatable food, except for sweetness, but this nut has not had an important satiety response compared to the remaining studied foods. Therefore, lower palatability does not appear to be the cause of the better satiety results for oat and potato.

Human studies have been shown an increase on appetite control improving after eating oat (Geliebter, Astbury, Aviram-Friedman, Yahav, & Hashim, 2014), maybe due to its β-glucan content, fibre that has proven to increase satiety effects in several studies (Beck et al., 2009; Clark & Slavin, 2013). This effect of β-glucan is theorised to be due to viscosity increase when it is eaten, enhancing delaying gastric emptying (Geliebter et al., 2015; Martínez-Villaluenga & Peñas, 2017; Rebello et al., 2016; Wolever et al., 2020). However, although, several studies conclude that oat improve satiety and some of them point to the role of β-glucan in this effect, also there are studies that did not find any differences in appetite ratings when β-glucan was supplemented (Hlebowicz, Darwiche, Björgell, & Almér, 2008; Kim et al., 2006; Wolever et al., 2020), thus the satiating capacity of oats could also be due to other factors. In addition, and compared with the remaining studied food, oat provided with a large amount of proteins

and carbohydrates. A large number of studies about proteins and appetite suggest that this macronutrient has a strong effect on appetite and, based on this, it is considered one of the most satiating component in foods (Geraedts, Troost, Fischer, Edens, & Saris, 2011; Santos-Hernández, Miralles, Amigo, & Recio, 2018; Westerterp-Plantenga, Lemmens, & Westerterp, 2012). Among macronutrients, it seems that after proteins, carbohydrates have the better satiety response (Tremblay & Bellisle, 2015), being oat preload in this study the food that provided with the second larger amount of proteins, after omelette, and with the third larger amount of carbohydrates. So, attending to its composition, and in addition to β-glucan content, the greatest satiety response of oat in this study, could be explained for its good balance in components with effects on appetite suppression.

Potato showed the second-best results in satiety sensations through VAS surveys in this study. In a same manner than oat, potato provided with a good balance of satiating components, being the preload with the biggest amount of carbohydrates, the second of fibre and the third of protein, that as mentioned above, are components that enhance the satiating response. Furthermore, as mentioned previously, potato contains protease inhibitor II that can increase circulating CCK plasma levels and modulate appetite sensations (Flechtner-Mors et al., 2020), by inhibiting proteases activity, which produces a greater stimulation of CCK release, as proteins are degraded with delay. Although, among the remaining preloads there were not significant differences, it should be noted that among the three tested nuts, walnut had best ratings in term of satiety, not being the nut with the greatest amount of protein, fibre or carbohydrates. Regarding the nutrients that walnut provided, the amount of fatty acids should be highlighted. Although fats are not considered a satiating component compared to others (Tremblay & Bellisle, 2015), among different kind of fatty acids some human studies have found different effect on satiety, being MUFA and PUFA more satiating through VAS and increasing CCK secretion more than saturated fatty acid (SFA) (Maljaars, Romeyn, Haddeman, Peters, & Masclee, 2009), and increasing PYY secretion in a greater way after a PUFAs supplementation compared to the other two types of fat (Stevenson et al., 2015), although there are studies as well that find a greater satiating effect after intake MUFAs (Sun, Goh, Govindharajulu, Khee-Shing Leow, & Henry, 2019). Walnut provided with the greatest amount of fat, having the greatest proportion of PUFAs and omega-3 as well (Eteshola & Oraedu, 1996; Maguire et al., 2009). In addition, omega-3 has been reported as a fatty acids than can modulate satiety and then, could be useful in weigh lost in humans studies (Buckley & Howe, 2010; Parra et al., 2008), what could be a explanation of why walnut, providing less amount of proteins, fibre al carbohydrates than the

remaining nuts, showed a best satiety ratings from VAS surveys compared with almond and tigernut.

As commented previously, proteins are considered the most satiating nutrient of foods, however, the preload that provided with the biggest amount of proteins was omelette, but in this study VAS results did not show a good appetite control response, compared to other foods tested. That could be explained by it less amount of other important components, such as fibre or carbohydrates, compared with oat or potato.

Table 5.2: Appetite and satiety results obtained from VAS surveys for the seven studied foods

Question	Almond	Oat	Omelette	Potato	Tigernut	Walnut	White bread
	Areas under curve						
Hunger	$5455 \pm 409^{a\|\beta}$	$3781 \pm 675^{a\|\beta}$	$5658 \pm 768^{a\|\beta}$	$5224 \pm 836^{a\|\beta}$	$4869 \pm 823^{a\|\beta}$	$5638 \pm 833^{a\|\beta}$	$6173 \pm 1072^{a\|\beta}$
Fullness	$6011 \pm 943^{b\|\beta}$	$10534 \pm 758^{a\|\beta}$	$6554 \pm 925^{b\|\beta}$	$10095 \pm 884^{a\|\beta}$	$7183 \pm 1186^{ab\|\beta}$	$5633 \pm 1036^{b\|\beta}$	$7662 \pm 873^{ab\|\beta}$
Desire to eat	$5021 \pm 641^{a\|\beta}$	$3523 \pm 645^{a\|\beta}$	$5744 \pm 787^{a\|\beta}$	$5110 \pm 855^{a\|\beta}$	$5293 \pm 880^{a\|\beta}$	$5590 \pm 804^{a\|\beta}$	$6136 \pm 1162^{a\|\beta}$
Prospective consumption	$6409 \pm 747^{a\|\beta}$	$4294 \pm 713^{a\|\beta}$	$6276 \pm 772^{a\|\beta}$	$5340 \pm 813^{a\|\beta}$	$5393 \pm 919^{a\|\beta}$	$6612 \pm 848^{a\|\beta}$	$6513 \pm 1037^{a\|\beta}$
	Incremental areas under curve						
Hunger	$1193 \pm 611^{a\|\beta}$	$-2251 \pm 845^{c\|\beta}$	$687 \pm 239^{ab\|\beta}$	$-1814 \pm 1033^{bc\|\beta}$	$342 \pm 655^{abc\|\beta}$	$58 \pm 910^{abc\|\beta}$	$869 \pm 812^{ab\|\beta}$
Fullness	$-2101 \pm 1071^{c\|\beta}$	$3202 \pm 884^{a\|\beta}$	$-670 \pm 1129^{bc\|\beta}$	$2559 \pm 1084^{ab\|\beta}$	$-437 \pm 637^{bc\|\beta}$	$-711 \pm 701^{bc\|\beta}$	$-1302 \pm 989^{c\|\beta}$
Desire to eat	$437 \pm 902^{a\|\beta}$	$-3183 \pm 837^{b\|\beta}$	$296 \pm 768^{a\|\beta}$	$-1381 \pm 948^{ab\|\beta}$	$493 \pm 740^{a\|\beta}$	$514 \pm 910^{a\|\beta}$	$1112 \pm 810^{a\|\beta}$
Prospective consumption	$-67 \pm 712^{a\|\beta}$	$-3518 \pm 378^{b\|\beta}$	$-1236 \pm 824^{ab\|\beta}$	$-2460 \pm 784^{ab\|\beta}$	$-163 \pm 780^{a\|\beta}$	$-1094 \pm 766^{ab\|\beta}$	$-939 \pm 637^{ab\|\beta}$
	Peaks nadir (mm)						
Hunger	$55 \pm 4^{a\|\beta}$	$39 \pm 7^{a\|\beta}$	$48 \pm 5^{a\|\beta}$	$49 \pm 7^{a\|\beta}$	$38 \pm 6^{a\|\beta}$	$47 \pm 6^{a\|\beta}$	$50 \pm 8^{a\|\beta}$
Fullness	$58 \pm 6^{a\|\beta}$	$56 \pm 7^{a\|\beta}$	$56 \pm 5^{a\|\beta}$	$58 \pm 6^{a\|\beta}$	$50 \pm 7^{a\|\beta}$	$45 \pm 6^{a\|\beta}$	$65 \pm 5^{a\|\beta}$
Desire to eat	$49 \pm 6^{a\|\beta}$	$37 \pm 7^{a\|\beta}$	$47 \pm 5^{a\|\beta}$	$43 \pm 7^{a\|\beta}$	$45 \pm 6^{a\|\beta}$	$42 \pm 6^{a\|\beta}$	$46 \pm 7^{a\|\beta}$
Prospective consumption	$48 \pm 4^{a\|\beta}$	$33 \pm 7^{a\|\beta}$	$47 \pm 4^{a\|\beta}$	$42 \pm 7^{a\|\beta}$	$40 \pm 5^{a\|\beta}$	$44 \pm 6^{a\|\beta}$	$42 \pm 7^{a\|\beta}$
	Peaks 0 (mm)						
Hunger	$32 \pm 7^{a\|\beta}$	$8 \pm 7^{c\|\beta}$	$29 \pm 5^{ab\|\beta}$	$11 \pm 5^{bc\|\beta}$	$25 \pm 7^{abc\|\beta}$	$25 \pm 8^{abc\|\beta}$	$28 \pm 7^{abc\|\beta}$
Fullness	$24 \pm 8^{a\|\beta}$	$46 \pm 6^{a\|\beta}$	$28 \pm 7^{a\|\beta}$	$43 \pm 6^{a\|\beta}$	$23 \pm 6^{a\|\beta}$	$25 \pm 4^{a\|\beta}$	$28 \pm 7^{a\|\beta}$
Desire to eat	$28 \pm 7^{a\|\beta}$	$3 \pm 8^{a\|\beta}$	$27 \pm 5^{a\|\beta}$	$15 \pm 7^{a\|\beta}$	$25 \pm 6^{a\|\beta}$	$26 \pm 7^{a\|\beta}$	$31 \pm 7^{a\|\beta}$
Prospective consumption	$21 \pm 6^{a\|\beta}$	$-1 \pm 4^{a\|\beta}$	$17 \pm 6^{a\|\beta}$	$7 \pm 5^{a\|\beta}$	$18 \pm 4^{a\|\beta}$	$18 \pm 7^{a\|\beta}$	$10 \pm 2^{a\|\beta}$
	Incremental VAS scores$_{180}$ (mm)						
Hunger	$31 \pm 7^{a\|\beta}$	$7 \pm 8^{c\|\beta}$	$29 \pm 5^{ab\|\beta}$	$13 \pm 7^{bc\|\beta}$	$23 \pm 7^{abc\|\beta}$	$22 \pm 9^{abc\|\beta}$	$27 \pm 7^{abc\|\beta}$
Fullness	$-35 \pm 5^{bc\|\beta}$	$-7 \pm 8^{a\|\beta}$	$-27 \pm 7^{abc\|\beta}$	$-12 \pm 6^{ab\|\beta}$	$-21 \pm 5^{abc\|\beta}$	$-16 \pm 8^{abc\|\beta}$	$-37 \pm 5^{c\|\beta}$
Desire to eat	$26 \pm 7^{a\|\beta}$	$-4 \pm 8^{b\|\beta}$	$25 \pm 6^{a\|\beta}$	$12 \pm 7^{ab\|\beta}$	$21 \pm 6^{ab\|\beta}$	$24 \pm 8^{a\|\beta}$	$30 \pm 7^{a\|\beta}$
Prospective consumption	$19 \pm 7^{a\|\beta}$	$-2 \pm 4^{a\|\beta}$	$15 \pm 6^{a\|\beta}$	$5 \pm 5^{a\|\beta}$	$17 \pm 5^{a\|\beta}$	$17 \pm 8^{a\|\beta}$	$10 \pm 2^{a\|\beta}$

Data are presented as $\mu \pm$ SEM. Within each dimension (hunger, fullness, desire to eat and prospective consumption) letters (a-c) denote statistically significant differences ($p > 0.05$) among the studied foods, and Greek letters (α-γ) denote a respectively small, medium or large total effect size.

5.3.3 Diet quality analysis by using data obtained from frequency food questionnaire (FFQ)

Data from the FFQ were transformed in weekly ration recommendations following the who food pyramid (Antony, 2020), and then the percentage of each food group intake reported by the participants, with respect to weekly recommendations, were calculated, as can be seen in Table 5.3.

Among the thirteen-food group analysed, the studied sample includes approximately the recommended rations of six groups, however, for pastries, sausages, fish and white meats the participants reported eating far more than adequate servings. For the remaining food groups, a deficit in their consumption were detected. This last point is very important, since fruits, vegetables, grains and nuts provide extremely important nutrients for an adequate and healthy nutrition.

5.3.4 Principal component analysis (PCA) among eating behaviour, appetite, diet quality and anthropometric parameters

To explore the possible relationship among eating behaviour, food intake quality and corporal composition, a principal components analysis (PCA) with varimax rotation was made, whose results are represented in Figure 5.5 and Table 5.4. Ten variables from four different measures were included in the PCA. From TFEQ the three variables were included, food intake restriction (restriction), lost of food intake control and overeating (disinhibition) and susceptibility to hunger (hunger), whilst from anthropometric assessment the variables height, weight, BMI, and fat and muscle proportion were included. The diet quality score calculated from the FFQ surveys and the appetite ratings from VAS were included as well for this analysis. The appetite variable was calculated as a mean value, at 0 time, of hunger, fullness, desire to eat and prospective consumption for each participant.

The three first new components (or dimension) obtained from the PCA explained 60% of the variance and were considered for their description. As can be see in Table 5.4, the component 1 was positively related with the three dimension about eating behaviour (TFEQ), specially with disinhibition, with weight and BMI, at expense of fat proportion, and was negatively related

Table 5.3: Mean values of the food groups rations ingested, obtained from the food frequency questionnaire, and their equivalent in proportion (%) of the weekly recommendations for each food group.

Groups	**Mean**	**SD**	**Recomendation**	**Proportion**
Fruits	13.55	14.91	21	65
Vegetables	10.38	8	14	74
Potatos	3.83	11.1	3	128
Olive oil	16.94	12.67	14	121
Grains	22.02	19.38	35	63
Nuts	4.09	5.04	10.5	39
Diarys	18.7	22.87	14	134
Fish and white meats	8.21	6.82	2	411
Eggs	3.94	5.48	3	131
Legumes	1.96	1.58	2	98
Red meats	1.95	3.31	2	98
Sausages	5.6	7.06	1	560
Pastries	32.42	28.61	2	1621

with diet quality. Therefore, the component 1 could be considered a variable that relates a poor diet quality with a bad eating behaviour and their inadequate consequences on corporal composition. Component 2 relates a positive diet quality with a higher appetite (from VAS), height and weight, but not related with fat proportion, so component 2 could be considered a variable about good food intake habits and correct corporal composition. Finally, Component 3 is positively related with restriction, muscle and appetite, being height and fat proportion inversely related with this component.

As can be seen in Table 5.3, the analysed sample had a lack of intake of four food groups, fruits, vegetables, grain and nuts, foods what are the principal source of fibre and some micronutrients such as vitamin C or folates. Fibre, as mentioned above, has shown to be an important satiating component of foods (Chambers et al., 2015); however, the possible effect of micronutrients has not been studied so far. Nevertheless, recent studies in this regard have found some promising results. Tremblay & Bellisle (2015) summarize in their satiety review paper some studies in this way, showing a less caloric intake in an ad libitum meal, and a plasma PYY levels increase when calcium was supplemented in human studies. Moreover, studies in humans have shown

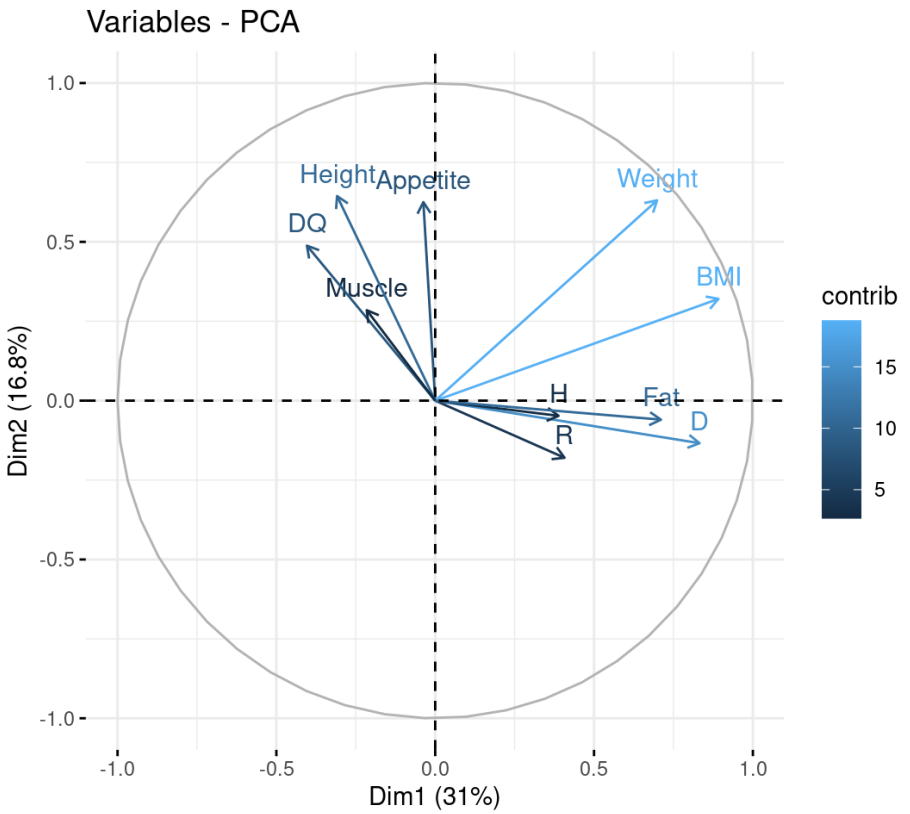

Figure 5.5: Two dimension representation of the principal components analysis. R, D and H represent respectively the intake restriction, desinhibition and hunger dimension obtained from TFEQ. DQ represents the diet qualite calculated from FFQ.

that the group with multivitamins supplementation in a loss weigh program presented a higher satiety sensations compared with the placebo group.

Other study with 768 participants (Fuhrman, Sarter, Glaser, & Acocella, 2010), a low micronutrient diet was compared to a high micronutrient density diet and, the experience and perception of hunger were measured before and after the new diet. Results showed a decrease in hunger pains, uncomfortable hunger between meals, discomfort if meal was skipped and frequency of hunger, among other findings, concluding that high micronutrient density diet mitigates hunger even with a lower calories diet.

Therefore, these two factors could be a possible explanation of the results found in the PCA analysis of the present study, where dimension 1 showed a strong disinhibition when diet is deficient in the mentioned foods group, as well as a weaker relation between this deficiency and the restriction and hunger dimensions obtained from TFEQ surveys. Nevertheless, when diet met dietary needs, a relation with restriction, disinhibition and hunger reported by the participants were not found (dimension 2). In any case, with the present study design, conclusion about the lack of some nutrients and hunger or loss of intake control cannot be drawn and therefore,

Table 5.4: Correlation of each original variable with the first three new dimensions (components). Explained variance of 61%.

Variable	Dimension 1	Dimension 2	Dimension 3
R	0.41	-0.18	0.67
D	0.83	-0.13	0.04
H	0.39	-0.05	-0.22
Diet quality	-0.40	0.49	-0.05
Appetite	-0.04	0.63	0.43
Height	-0.31	0.64	-0.41
Weight	0.70	0.63	-0.14
BMI	0.89	0.32	0.14
Fat	0.71	-0.06	-0.37
Muscle	-0.22	0.28	0.53

more studies with an adequate design for this purpose, are necessary to clarify the effect of an adequate diet on appetite control.

5.4 Conclusions

Six potentially satiating foods, almond, oat, egg, potato, tigernut and walnut, were analysed in a sort-term study in humans to determine which of them produce a higher appetite suppression and satiating effects using white bread as reference food. VAS survey results showed that oat induced the highest satiety response among the studied foods, following by potato. The other assayed foods showed a lower response with similar results among them. Therefore, we can conclude that oat and potato should be highly considered to develop a new food to be included in a diet that could help with appetite control. In addition, the observed relation among diet quality, expressed as a lack in food groups intake, and eaten behaviour, through TFEQ, by using PCA, shows an interesting relation between a poor diet quality and overeating episodes. However, more studies with an accurate design are needed to clarify this fact and to clearly define the possible trend to overeating when exist a lack in the intake of some type of foods.

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CHAPTER 6

GENERAL CONCLUSIONS

- Green tea and turmeric induced high *in vitro* release of CCK and GLP-1 by the STC-1 cell line, being green tea catechins more stable to IVGD. However, *in vitro* fermentation of green tea did not show a significant effect in shaping the analysed gut microbiota.

- Human appetite and satiety sensations in Spanish speaker population was measured by a developed and validated VAS survey, whose results showed good reliability and validity and therefore, it can be used as a tool in human satiety studies. In addition, with the aim of making VAS surveys in human studies easier and safer, an App for smartphone and tablets was developed by the research group NUTBRO. The App validity to measure human satiety sensations was also demonstrated in a human study.

- The satiating response of three different complex foods (gazpacho, hummus and ajoblanco) included in a MD, with different nutritional composition, were compared in a human study. Results showed that gazpacho was the most satiating food measured by VAS surveys, despite the fact that it was not the food that contributed the most protein or fibre, components to which part of the current bibliography confers a great capacity to increase satiety. However, other studies have not been able to corroborate this effect, so it seems clear that more research is needed in the field of appetite regulation to identify other factors that could influence this mechanism.

- Six potentially satiating foods: almond, oat, egg, potato, tigernut and walnut, were analysed in a sort-term study in humans as a screening to choose satiating foods for the development of new foods. VAS survey results showed that oat induced the highest satiety response among the studied foods, following by potato. In addition, a relation among a lack in food groups intake and eaten behaviour was found, showing an interesting relationship between a poor diet quality and overeating episodes.

- Although it was not the main objective of the thesis, it was very interesting to observe that foods showing the greatest effect on satiety in human studies were not the expected ones under a theoretical point of view (considering their composition and the current bibliography). Therefore, we believe that it is worth increasing the research on the effect of other components of food, such as micronutrients (vitamins and minerals), as well as clarifying the possible effect of the nutritional status of individuals on the modulation of food intake.

APPENDIX A

INFORMED CONSENT DOCUMENT

DECLARACIÓN DE CONSENTIMIENTO INFORMADO

D./Dña ……,

de ….. años de edad y con DNI nº ……., manifiesta que ha sido informado/a sobre los beneficios que podría suponer mi participación para cubrir los objetivos de la Tesis Doctoral titulada "TITULO ALTERNATIVO", dirigido por D. David Planes **(Vitalis (Peiades), con el objetivo de conocer el efecto de la alimentación sobre el estado de ánimo, con certificado del Comité Ético de la UM)**, teléfono de contacto: 868889628 y correo electrónico: david.planes@um.es, y financiado por el Ministerio de Economía y Empresa (Gobierno de España).

He sido informado/a de los posibles perjuicios que la participación en dicho proyecto puede tener sobre mi bienestar y salud al haber leído la hoja de información al participante sobre el estudio citado. **(el resto de información se daría en el documento hoja informativa)**

He sido también informado/a de que mis datos personales serán sometidos a tratamiento en virtud de su consentimiento con fines de investigación científica por la Universidad de Murcia. El plazo de conservación de los datos será el mínimo indispensable para asegurar la realización del estudio o proyecto. No obstante, mis datos identificativos, para garantizar condiciones óptimas de privacidad, y cuando el procedimiento del estudio lo permita, podrían ser sometidos a anonimización o seudoanonimización. En todo caso, la información identificativa que se pudiese recabar será eliminada cuando no sea necesaria.

He sido informado/a de que para cualquier consulta relativa al tratamiento de sus datos personales en este estudio o para solicitar el acceso, rectificación, supresión, limitación u oposición al tratamiento podré dirigirme a la dirección protecciondedatos@um.es.Asimismo he sido informado/a de mi derecho a presentar una reclamación ante la Agencia Española de Protección de Datos.

He sido también informado que puedo abandonar en cualquier momento mi participación en el estudio sin dar explicaciones y sin que ello me suponga perjuicio alguno.

Se me ha entregado una hoja de información al participante y una copia de este consentimiento informado, fechado y firmado.

Tomando ello en consideración, **otorgo** mi **consentimiento** a que esta recogida de datos tenga lugar y sea utilizada para cubrir los objetivos especificados en el proyecto.

XXX, a XX de XXXXX de 20XX.

Fdo. D/Dña

Fdo. Investigador: David Planes Muñoz

david.planes@um.es

tlf: 868889628

APPENDIX B

REPORT OF THE BIOETHICS COMMITTEE

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. David Planes Muñoz ha presentado la Tesis Doctoral titulada *"Innovaciones en saciedad basada en alimentos e ingredientes de la Dieta Mediterránea para la regulación del sobrepeso y la obesidad"*, dirigida por D. Gaspar Ros Berruezo y D.ª Mª del Carmen Frontela Saseta, 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 veintinueve de octubre 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: 2051/2018

APPENDIX C

REPORT OF THE BIOSECURITY COMMITTEE IN EXPERIMENTATION

INFORME DEL COMITÉ DE BIOSEGURIDAD EN EXPERIMENTACIÓN DE LA UNIVERSIDAD DE MURCIA

Lucía Periago García, Jefa de Sección de Recursos Humanos de la Investigación y del Plan Propio y en funciones de Secretaria del Comité de Bioseguridad en Experimentación de la Universidad de Murcia

CERTIFICA:

Que D. David Planes Muñoz presentó la memoria de trabajo su Tesis Doctoral titulada *"Innovaciones en saciedad basada en alimentos e ingredientes de la Dieta Mediterránea para la regulación del sobrepeso y la obesidad"*, dirigida por D. Gaspar Ros Berruezo, al Comité de Bioseguridad en Experimentación.

Que dicho Comité analizó toda la documentación presentada, y de conformidad con lo acordado el día treinta de mayo de dos mil diecinueve[1], por unanimidad, se emite INFORME FAVORABLE, desde el punto de vista ético de la bioseguridad en 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 DEL COMITÉ
DE BIOSEGURIDAD EN EXPERIMENTACIÓN
DE LA UNIVERSIDAD DE MURCIA

Fdo.: Francisco Esquembre Martínez

ID: CBE 171/2018

[1] A los efectos de lo establecido en el art. 19.5 de la Ley 40/2015 de 1 de octubre de Régimen Jurídico del Sector Público (B.O.E. 02-10), se advierte que el acta de la sesión citada está pendiente de aprobación

APPENDIX D

IN VIVO EFFECT ON SATIETY AND GUT MICROBIOTA OF OAT-BASED FOODS

As commented in summary and in chapter 5 of this thesis, with the information obtained from the food screening, it was decided to develop foods from oats, which was the food that showed the best results on appetite regulation. Therefore, in collaboration with the Centro Tecnológico Nacional de la Conserva (Molina de Segura, Murcia), an oat-based dessert and a oat drink high in fibre ($> 1.5g/100Kcal$) were developed, whose nutritional composition is shown in the Table D.1.

Table D.1: Nutritional analysis of both oat-based foods. Values per 100 grams

Parameter	**Oat dessert**	**Oat drink**
Saturated fatty acids (g)	0.2	<0.1
Total sugars (g)	5.8	<0.1
Salt (mg)	<0.013	0.02
Fibre (g)	1.2	0.3
Total Fats (g)	1.6	0.1
Carbohydrates (g)	19.9	3.0
Proteins (g)	1.8	0.6
Energy (Kcal/Kj)	103/436	15/65

The aim of this work was to analyse the effect on satiety of both developed oat-based foods, in the medium term, when introduced into the daily diet. The study with volunteers began in mid-February 2020, but was suspended before its total completion due to the lockdown decreed in Spain since March 14, 2020, due to the COVID-19 pandemic. In this appendix, the design of this study is exposed.

With the aim to evaluate the effects on satiety of these two foods, a sample of 34 volunteers was recruited, with the same inclusion criteria described in the Chapter 3 methodology. The study design was a randomised crossover trial, with two different treatments for each participant. A twenty-one days isocaloric diet with the inclusion of the both oat-based foods in the daily intake, and a twenty-one days isocaloric diet without the tested oat foods, with a washout period of three weeks between both diets. To quantify the satiating effects on the participating sample, the follow information was to be collected before and after each diet period:

- Anthropometric measured, such as weight, heigh or subcutaneous fat.
- Stool samples to analyse the gut microbiota evolution.
- Eating behaviour questionnaires.
- Saliva samples to measured the hormone ghrelin.
- Blood glucose and cholesterol.

The study was suspended before neither of the volunteers finished the two treatments, with the intention of doing it again when the epidemiological situation allows it.

APPENDIX E

MERITS AND SCIENTIFIC PRODUCTION DERIVED FROM THIS THESIS

Scientific articles

David Planes Muñoz; Rubén López Nicolás; Carlos Alberto Gonzáles Bermúdez; Gaspar Ros Berruezo; Carmen Frontela Saseta. *In vitro* effect of green tea and turmeric extracts on GLP-1 and CCK secretion: the effect of gastrointestinal digestion. 9, pp. 5245 - 5250. Food & Function, 2018.

David Planes-Muñoz; Carmen Frontela-Saseta; Gaspar Ros-Berruezo; Rubén López-Nicolás. Effect of gazpacho, hummus and ajoblanco on satiety and appetite in adult humans: A randomised crossover study. Foods, 2021.

Communication to international congresses

Effect of gazpacho, humus and ajoblanco on satiety and appetite control on humans. 6th International Conference on Food Digestion. Granada, España. 02-04/04/2019. Infogest. David Planes Muñoz; Rubén López Nicolás; Gaspar Ros Berruezo; Carmen Frontela Saseta.

New Technologies to Evaluate Satiety: Validation Of a Novel App. IV International Student Congress of Food Science&Technology. Valencia, España. 22-23/03/2018. AVECTA. David Planes Muñoz; Rubén López Nicolás; Carmen Frontela Saseta; Gaspar Ros Berruezo.

Satin: SATietyINnivation - Develop of a satiety app to be used in different electronic hardware (smartphone or tablet) along human studies. 8th Food Technology International Symposium. Molina de Segura, España. 09/05/2017. Centro Tecnológico de la Conserva y Alimentación. Rubén López Nicolás; David Planes Muñoz; Graham Finlayson; Catherine Gibbosn; Jason Halford; Joame Harrold; Cesar Leal; Gaspar Ros Berruezo.

Nuevas tecnologías para la evaluación de la saciedad: validación de una App para smartphones y tabletas. Murcia, España. 30/05-01/06/2017. International School of Doctoral Studies (University of Murcia).David Planes Muñoz; Rubén López Nicolás; Carmen Frontela Saseta; Gaspar Ros Berruezo.

Analysis of gut microbiota of normal-weight and obese donors after fermentation of green tea. 5th European Nutrition and Dietetics Conference. Roma, Italia.16-18/06/2016. OMICS. David Planes-Muñoz; Teresa Sánchez-Moya; Rubén López-Nicolás; Carlos Alberto González-Bermúdez; Gaspar Ros-Berruezo; Carmen Frontela-Saseta.

Análisis de la capacidad saciante de legumbres. II Jornadas Doctorales de la Universidad de Murcia. Murcia, España. 31/05-02/06/2016. International School of Doctoral Studies of University of Murcia. Murcia, España. David Planes Muñoz; Teresa Sánchez Moya; Rubén López Nicolás; Gaspar Ros Berruezo; Carmen Frontela Saseta.

Doctoral training

Machine Learning y Big Data en Ciencias Biomédicas (40 horas). Universidad de Granada (2021).

Diseño de experimentos y fundamentos de análisis de datos: introducción a R y RStudio (25 horas).Universidad de Murcia EIDUM (2016).

Métodos de análisis multivariante de datos con R (25 horas). Universidad de Murcia. EIDUM (2016).

R: Contraste de hipótesis y diseño de experimentos (20 horas). Universidad de Murcia, EIDUM (2016).

Representación y tabulación de datos en R (25 horas). Universidad de Murcia, EIDUM (2016).

Metodología de la investigación: Ciencias de la salud (30 horas). Universidad de Murcia (2019).

Procedimiento para la elaboración de informes y documentos científico-técnicos (20 horas). Universidad de Murcia (2019).

Instrumentación científica. Cromatografía y espectometría de masas (30 horas). Universidad de Murcia (2019).

Elaboración de informes científicos y artículos en ciencias sociales y de la salud (25 horas). Universidad de Murcia (2018).

Animal Cell Culture: Basic concepts and applied techniques (20th edition) (100 hours). University of Murcia, SAI (2015).

Escribe tu tesis con latex (25 horas). Universidad de Murcia. EIDUM (2018).

Recursos electrónicos. Gestores bibliográficos (8 horas). Universidad de Murcia. EIDUM (2017).

Gestión avanzada de correo electrónico: Webmail y Thunderbird (2a edición) (10 horas). Universidad de Murcia (2019).

IV Curso escribir ciencia en inglés: Curso práctico sobre redacción científica. La redacción de propuestas académicas y de financiación (25 horas). Universidad Internacional del Mar (2019).

Fundamentos de programación (3.a edición) (40 horas). Universitas Telefónica (2019).

Nutrición y dietética aplicada al ejercicio físico (2a edición) (20 horas). Universidad Central de Cataluña(2019)

Inglés nivel B2. SIDI, Universidad de Murcia (2018).

Procesamiento y edición de imagenes (GIMP) (18 horas). Universidad de Murcia (2017).

Lecciones Prácticas en el diseño y exposición de presentaciones visuales (18 horas). Universidad de Murcia (2017).

Trabajando con la herramienta encuestas (18 horas). Universidad de Murcia (2017).

Software libre: Ofimática con OpenOffice (50 horas). Universidad de Zaragoza (2016).

Stays in other research centres

Queens Medical Centre (Medicine School) - University of Nottingham. Nottingham, United Kingdom. 01/03-31/05/2018.

University teaching

240 hours of class taught at the University of Murcia in the degrees of:

- Human Nutrition and Dietetics
- Food Science and Technology
- Nursing

Direction of the final degree project "Efecto de las semillas de sésamo y chía sobre la saciedad". University of Murcia (2019).

25 hours taught in the course "Estilos de vida saludables: Abordaje desde un enfoque multidisciplinar". Universidad Internacional del Mar (2020).

Member of the evaluation board of final degree projects in the Human Nutrition and Dietetics Degree. University of Murcia (2019).