UNIVERSITAT ROVIRA I VIRGILI

FOOD INTAKE MODULATION BY INSECT PROTEIN THROUGH THE INTERACTION WITH THE ENTEROENDOCRINE SYSTEM

Alba Miguéns Gómez

Doctoral Thesis

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Doctoral Thesis

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FAIG CONSTAR que aquest treball, titulat "**Food intake modulation by insect protein through the interaction with the enteroendocrine system**", que presenta **Alba Miguéns-Gómez** per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament Departament de bioquímica i Biotecnologia d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "**Food intake modulation by insect protein through the interaction with the enteroendocrine system**", que presenta **Alba Miguéns-Gómez** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento Departamento de Bioquímica y Biotecnología de esta universidad.

I STATE that the present study, entitled "**Food intake modulation by insect protein through the interaction with the enteroendocrine system**", presented by **Alba Miguéns-Gómez** for the award of the degree of Doctor, has been carried out under my supervision at the Department Department of Biochemistry and Biotechnology of this university.

Tarragona, 02/03/2022

El/s director/s de la tesi doctoral
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Sé feliz

"The greatest glory in living lies not in never falling,
but in rising every time we fall"

Nelson Mandela

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SUMMARY

Nowadays, animal protein and derived products are the most extended ones for human consumption as high-quality protein sources. Due to the overpopulation, its demand is expected to increase globally in the next years. However, meat production carries numerous negative implications for the environment. For this reason, the search for alternative environmentally-friendly produced protein sources is becoming more important. Insects have been characterized as a good source of high-quality protein, containing a significant amount of good quality fats, minerals and vitamins. Although many beneficial effects on health have been described, scarce information is available about their bioactivity at the intestinal level. Hence, in this thesis we aimed to characterize the ability of the insect protein to modulate the enterohormone secretion and subsequent food intake, comparing it with more traditional protein sources, and to determine if it can have long-term effects on this enteroendocrine function.

We showed that insect protein, from *Alphitobius diaperinus*, can modulate the enterohormone secretion *ex vivo* in intestinal samples producing a secretory profile with some differences compared with the secretome obtained from almond and beef proteins. Some of the secretions have been related to the amino acid composition of the respective protein sources. When the different protein sources were acutely administered to rats, we observed that the food intake from the insect group was increased compared with the almond and beef groups, although the protein dose was adjusted for the three treatments. After a chronic administration, this increase in food intake was lost after a week of treatment. The same occurred with *Tenebrio molitor*, which meant that the observed effect was not species-specific. In human subjects, the administration of a protein preload from the insect *A. diaperinus* modulated food intake similarly to an almond preload. We observed an increased protein intake after both preloads with a lower increase in energy intake after the insect protein preload.

In conclusion, insect protein is a promising candidate to be considered as a bioactive ingredient intended for people who need to increase their food intake.

RESUMEN

En la actualidad, la proteína animal y los productos derivados son los más extendidos para el consumo humano como fuentes proteicas de alta calidad. Debido a la sobrepoblación, se espera que su demanda aumente a nivel mundial en los próximos años. Sin embargo, la producción de carne conlleva numerosas implicaciones negativas para el medio ambiente. Por esta razón, la búsqueda de fuentes alternativas de proteínas producidas de forma respetuosa con el medio ambiente es cada vez más importante. Los insectos se han caracterizado como una buena fuente de proteínas de alta calidad, además de contener una cantidad importante de grasas de buena calidad, minerales y vitaminas. Aunque se han descrito muchos efectos beneficiosos para la salud, se dispone de escasa información sobre su bioactividad a nivel intestinal. Por lo tanto, en esta tesis nos propusimos caracterizar la capacidad de la proteína de insecto para modular la secreción de enterohormonas y la posterior ingesta de alimentos, comparándola con fuentes de proteínas más tradicionales, y determinar si puede tener efectos a largo plazo sobre esta función enteroendocrina.

Mostramos que la proteína de insecto, de *Alphitobius diaperinus*, puede modular la secreción de enterohormonas *ex vivo* en muestras intestinales produciendo un perfil secretor con algunas diferencias en comparación con el secretoma obtenido a partir de proteínas de almendra y carne. Algunas de las secreciones se han relacionado con la composición de aminoácidos de las respectivas fuentes de proteínas. Cuando las diferentes fuentes de proteína se administraron de forma aguda a ratas, observamos que la ingesta del grupo de insecto aumentó en comparación con los grupos de almendra y carne, aunque la dosis de proteína se ajustó para los tres tratamientos. Tras una administración crónica, este aumento en la ingesta se perdió después de una semana de tratamiento. Lo mismo ocurrió con *Tenebrio molitor*, por lo que el efecto observado no fue específico de especie. En sujetos humanos, la administración de una precarga de proteína del insecto *A. diaperinus* moduló la ingesta de manera similar a una precarga de almendras. Observamos un aumento en la ingesta de proteínas después de ambas precargas

con un menor aumento en la ingesta de energía después de la precarga de proteínas de insectos.

En conclusión, la proteína de insecto es un candidato prometedor para ser considerado como un ingrediente bioactivo destinado a las personas que necesitan aumentar su ingesta de alimentos.

RESUM

Les proteïna animal i els productes derivats són els més estesos per al consum humà com a font de proteïna d'alta qualitat. A causa de la superpoblació mundial, s'espera que la seva demanda augmenti a nivell mundial en els propers anys. Tanmateix, la producció de carn té nombroses implicacions negatives per al medi ambient. Així, la cerca de noves fonts de proteïnes produïdes de manera respectuosa amb el medi ambient és cada cop més important. Els insectes s'han caracteritzat com una bona font de proteïnes d'alta qualitat, que també contenen una quantitat important de greixos de bona qualitat, minerals i vitamines. Tot i que s'han caracteritzat molts efectes beneficiosos sobre la salut, es disposa d'escassa informació sobre la seva bioactivitat a nivell intestinal. Per tant, en aquesta tesi hem volgut caracteritzar la capacitat de la proteïna d'insecte per modular la secreció d'enterohormones i la posterior ingesta, comparant-la amb fonts de proteïnes més tradicionals, i determinar si pot tenir efectes a llarg termini sobre aquesta funció enteroendocrina.

Vam demostrar que la proteïna d'insecte, d'*Alphitobius diaperinus*, pot modular la secreció d'enterohormonas *ex vivo* a l'intestí produint un perfil secretor diferent de l'obtingut a partir de proteïnes d'ametlla i vedella. Quan les diferents fonts de proteïnes es van administrar de manera aguda a rates, vam observar que la ingesta del grup d'insectes augmentava en comparació amb els grups d'ametlla i vedella. Després d'una administració crònica, aquest augment de la ingesta es va perdre després d'una setmana de tractament. El mateix va passar amb *Tenebrio molitor*, indicant així que l'efecte observat no era específic de l'espècie. En humans, l'administració d'una precàrrega de proteïne de l'insecte *A. diaperinus* va modular la ingesta de manera similar a una precàrrega d'ametlla. Vam observar un augment de la ingesta de proteïnes després de les dues precàrregues amb un menor augment de la ingesta d'energia després de la precàrrega de proteïne d'insecte.

En conclusió, la proteïna d'insecte és un candidat prometedor per ser considerat com un ingredient bioactiu destinat a persones que necessiten augmentar la seva ingesta.

LIST OF ABBREVIATIONS

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AA	Amino acid
ACE	Angiotensin-Converting Enzyme
AgRP	Agouti-Related Protein
ALA	Alanine
AMPs	Antimicrobial peptides
ARC	Arcuate nucleus
ARG	Arginine
ASP	Aspartic acid
BW	Body weight
CART	Cocaine- and Amphetamine-Regulated Transcript
CCK	Cholecystokinin
CNS	Central Nervous System
CYS	Cysteine
DPP-4	Dipeptidyl peptidase-4
EECs	Enteroendocrine cells
GHG	Greenhouse gas
GHSR	Growth Hormone Secretagogue Receptor
GI	Gastrointestinal
GIP	Gastric Inhibitory Polypeptide
GLN	Glutamine
GLP-1	Glucagon-like peptide 1
GLU	Glutamic acid
GLY	Glycine
GOAT	Ghrelin O-acyltransferase
HIS	Histidine
HPRO	Hydroxyproline
ILE	Isoleucine
INSL-5	Insulin-like peptide 5
LEU	Leucine

LYS	Lysine
MET	Methionine
MUFA	Monounsaturated fatty acids
NPY	Neuropeptide Y
PHE	Phenylalanine
POMC	Proopiomelanocortin
PRO	Proline
PUFA	Polyunsaturated fatty acids
PYY	Peptide YY
SER	Serine
THR	Threonine
TRP	Tryptophan
TYR	Tyrosine
VAL	Valine
WHO	World Health Organization

INTRODUCTION

INTRODUCTION

1. Enteroendocrine System Modulation by Proteins

1.1 Introduction to the Enteroendocrine System and its Role on Food Intake Control

The main function of gastrointestinal (GI) tract is the digestion and absorption of nutrients. It is also directly connected to the external environment, acting as a biochemical and physical barrier that prevents the diffusion of toxins, pathogens and allergens from the lumen to the tissues, hence playing a key role in the immune system [1,2]. Moreover, it is also in charge of another relevant function, the enteroendocrine activity, which is coordinated by the enteroendocrine cells (EECs). These are specialized secretory cells scattered throughout the mucosal epithelium of the gut that constitute the largest endocrine organ in the body, even though EECs only represent 1% of the cells in the GI epithelium [3–5]. These secretory cells express more than 30 different hormones along the GI tract.

The secreted peptides can act in a paracrine way, interacting with other neighbouring EECs and other cell types locally, they can be released into the bloodstream reaching distant targets, or they can act on the site of release directly on nerve terminations [5,6]. The intestine is considered our second brain, and it is connected to the central nervous system (CNS) through what is known as the gut-brain axis [7]. The EECs secrete hormones into the bloodstream in response to nutrients, which informs the brain about the energy status of the body. Then, an answer is integrated and transmitted back to the intestine, regulating different physiological functions in the GI tract such as motility, digestion, absorption and food intake [8].

These enterohormones can interact with the CNS via the circumventricular organs in the hindbrain and hypothalamus, with evidence that some intestinal peptides cross the blood-brain barrier [9,10]. This plays a key role in the control of food intake mediated by two main different sets of neurons that reside in the

hypothalamic arcuate nucleus (ARC). On the one hand, the neuropeptide Y (NPY) and agouti-related protein (AgRP) neurons, stimulate food intake and reduce energy expenditure. On the other hand, the proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons inhibit food intake and increase energy expenditure [8,11].

Traditionally, EECs are divided into two groups based on their morphology (**Figure 1**). In the first group there are open type cells, which are those that exhibit a microvilli-covered surface in the apical side in contact with the lumen. Thus, ingested dietary compounds interact with the nutrient-sensing machinery, located predominantly in the apical side of many enteroendocrine cells, leading to the secretion of different enterohormones [6,12]. In the second group we find closed type cells that are not in contact with the lumen. These cells sense the luminal content through neuronal and humoral mechanisms. Some EECs have been shown to have neuropod extensions in their basolateral surface. These extensions contact the enteric nervous system establishing communication between EECs and the nervous system [13–16].

In the classical conception, EECs were classified into different sub-types according to the main hormone they produced (e.g., I- and L-cells if they produced cholecystokinin (CCK) or glucagon-like peptide-1 (GLP-1) respectively), known as the "one cell—one hormone hypothesis". Nevertheless, many studies have shown the ability of individual EECs to co-express a wider range of enterohormones. The co-expression of CCK, GLP-1, gastric inhibitory polypeptide (GIP), peptide YY (PYY), neurotensin and secretin in intestinal cells from mice has been reported [17], as well as the co-expression of GLP-1 and GIP in human duodenum [18], among others [19,20]. However, since the typical nomenclature of EECs (the letter code naming) is well established in the literature, it is still used when specifying cell populations that share the expression of a particular hormone. Despite this overlapped enteroendocrine production, the secretion of each hormone is concentrated in a specific region of the gut. Ghrelin is mainly secreted in the stomach and the proximal duodenum,

GIP and CCK are produced in the upper small intestine and GLP-1 and the PYY are released mainly from the distal gut (**Figure 2**). This distribution was established in human tissues, and it is also strongly correlated with the distribution of murine EECs populations [21–23].
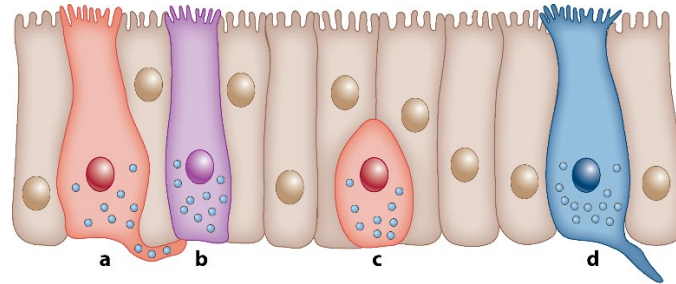
Figure 1. Different enteroendocrine cell (EEC) types in the gut epithelium. (a) Open-type EEC communicating in a paracrine way with a (b) neighbouring EEC through a basolateral extension. (c) Closed-type EEC. (d) Open-type EEC with a neuropod basolateral extension to communicate with neuronal termini. Adapted from Reimann *et al*. 2016 [24].

The more studied enterohormones are mainly GLP-1, CCK and PYY as anorexigenic hormones that reduced food intake, and ghrelin as an orexigenic hormone that stimulates food intake. They modulate the digestive process but also different metabolic processes such as glucose and energy homeostasis and food intake by modulating satiety and hunger. Each EEC responds differently to nutrients, which characterize each nutrient as a strong or weak stimulus for each enterohormone depending on the nutrient receptors present in the EEC families. All together, they constitute the enteroendocrine system.
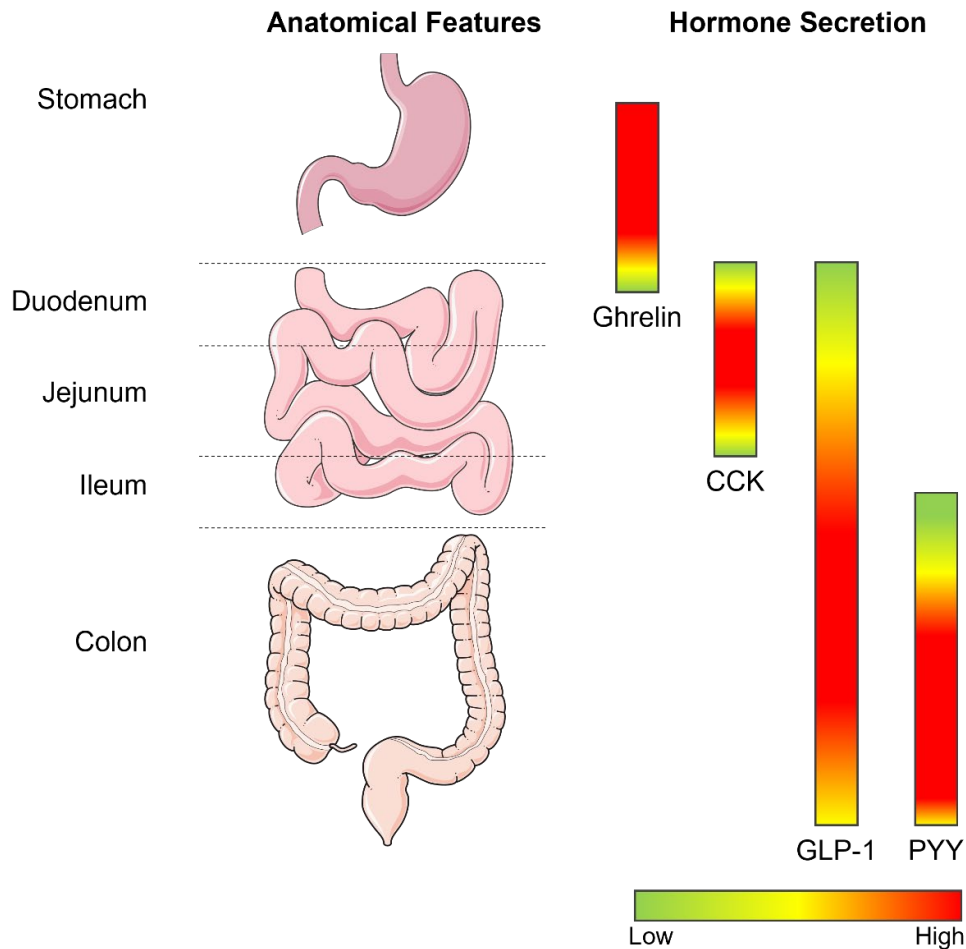
Figure 2. Anatomical features on the gastrointestinal tract (left) and specific secretion profiles of the enterohormones ghrelin, CCK, GLP-1, PYY (right) in humans.

1.2 Enterohormones

1.2.1 GLP-1

GLP-1 is a satiating enterohormone secreted in response to nutrients. In the present review entitled *GLP-1 regulation by food proteins and protein hydrolysates*, we analyse the main functions of this hormone, as well as its implications in health. We also detail the nutrient stimulus that modulates its secretion with special attention to protein.

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Glucagon-like peptide-1 regulation by food proteins and protein hydrolysates

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Abstract

Glucagon-like peptide-1 (GLP-1) is an enterohormone with a key role in several processes controlling body homeostasis, including glucose homeostasis and food intake regulation. It is secreted by the intestinal cells in response to nutrients, such as glucose, fat and amino acids. In the present review, we analyse the effect of protein on GLP-1 secretion and clearance. We review the literature on the GLP-1 secretory effects of protein and protein hydrolysates, and the mechanisms through which they exert these effects. We also review the studies on protein from different sources that has inhibitory effects on dipeptidyl peptidase-4 (DPP4), the enzyme responsible for GLP-1 inactivation, with particular emphasis on specific sources and treatments, and the gaps there still are in knowledge. There is evidence that the protein source and the hydrolytic processing applied to them can influence the effects on GLP-1 signalling. The gastrointestinal digestion of proteins, for example, significantly changes their effectiveness at modulating this enterohormone secretion in both *in vivo* and *in vitro* studies. Nevertheless, little information is available regarding human studies and more research is required to understand their potential as regulators of glucose homeostasis.

Introduction

The gastrointestinal tract is responsible for the digestion and absorption of nutrients, and acts as a barrier against luminal pathogens. Moreover, the gastrointestinal tract cooperates in controlling the metabolism through hormones secreted from enteroendocrine cells, which are the body's largest endocrine organ[1]. Enteroendocrine cells are capable of responding to luminal content because their apical side has chemosensing machinery such as taste receptors (TASR), G protein-coupled receptors (GPCR), specific transporters and channels. Their secretory products are stored in characterised secretory vesicles, before being secreted through the basolateral membrane by exocytosis[2,3]. When luminal content moves through the gastrointestinal tract, specific macronutrients stimulate the chemosensing machinery, which leads to the modulation of gut hormone release. Gut hormones exert their effect via vagal nerve or endocrine/paracrine signalling, through the interaction of specific receptors expressed in different tissues of the body. These hormones, which are mainly glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), peptide YY (PYY), gastric inhibitory polypeptide (GIP) and ghrelin, influence the functioning of the digestive tract, but also modulate insulin secretion from the pancreas, the energy storage of adipose tissue and neuronal signalling in appetite centres in the brain to mediate the regulation of food intake by terminating hunger and inducing satiety.

Since dietary compounds modulate enterohormone secretion, and given the central role of enterohormones in body homeostasis, such an interaction could have beneficial health implications[4]. In this context, protein and protein hydrolysates are currently being studied to determine their effects on GLP-1 modulation, either through secretion or clearance, which may influence the processes regulated by this hormone such as regulation of glycaemia homeostasis and food intake control. The nutrient-sensing machinery of carbohydrates and lipids is better understood than the detection and pathways followed by protein digestion. The main reasons for this gap in knowledge is the redundant signalling in the gut for the different protein digestion products and the complexity of protein digests[5]. Here we review the literature on this subject in order to determine if the evidence supports differential effects of food proteins on GLP-1 profile. We will introduce the relevance of GLP-1 signalling on health. Then we will focus on the effects on GLP-1 secretion of proteins and its hydrolysates, and the suggested mechanisms. Finally, we will briefly review the use of protein hydrolysates as dipeptidyl peptidase-4 (DPP4) inhibitors. We compile a

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significant number of scientific studies to highlight the importance of the different protein sources, the hydrolysis conditions applied to them, and the resulting digestion products.

Relevance of glucagon-like peptide-1 signalling in health

There is evidence to suggest that specific enterohormones administered at physiological concentrations can influence the appetite of rodents and human subjects (for a review, see Murphy & Bloom[6]). Likewise, the effects of gut hormones on food intake and body weight have been observed in bariatric surgery (such as Roux-en-Y gastric bypass), which induces a huge increase in GLP-1 and peptide YY (PYY) secretion and is used to treat obesity[7]. Therefore, the modulation of enterohormone signalling may be an important target in the prevention of obesity and related/associated pathologies. Moreover, endogenous gut hormones regulate appetite physiologically, unlike the drugs that are currently available, which mainly influence the central neurotransmitter systems. Therefore, gut hormone-based therapies might lead to fewer side effects[6].

Furthermore, modulation of endogenous incretin hormones (GLP-1 and GIP) could be an interesting strategy for preventing and/or managing type 2 diabetes mellitus (T2DM)[8]. T2DM is the most common endocrine disorder, characterised by insulin resistance and impaired insulin secretion, and it is one of the fastest growing non-communicable diseases in the world[9]. The main goal in the treatment of T2DM is to keep blood glucose levels within the normal physiological range. In this regard, GLP-1 and GIP are therapeutically interesting peptides because they are important mediators of glycaemic homeostasis, as they are responsible for approximately 50–70 % of the total insulin secreted after glucose intake[10]. GLP-1, together with GIP, is responsible for the incretin effect, since it binds to GLP-1 receptor in β-cells in the pancreas leading to an increase in intracellular Ca and a subsequent insulin secretion in response to glucose[11]. It has also been shown that GLP-1 enhances markers of proliferation and differentiation, and decreases markers of apoptosis in the pancreas of Zucker diabetic rats[12,13]. Furthermore, GLP-1 improves the glycaemic profile by inhibiting glucagon secretion and improves glucose disposal in peripheral tissues[10]. In that way, for patients with T2DM, a non-pharmacological therapeutic approach could be achieved by targeting these incretins (GLP-1 and GIP) through protein- and protein hydrolysate-based strategies. This approach would be mainly focused on increasing GLP-1 levels rather than stimulating GIP because in these patients the responsiveness of their β-cells to GIP action is decreased[14]. Furthermore, only GLP-1 exerts an appetite-suppressing effect, while GIP does not seem to do the same[10]. Accordingly, many incretin-based therapies focus on using GLP-1 analogues, promoting endogenous GLP-1 secretion or using DPP4 inhibitors.

DPP4 is a ubiquitous aminodipeptidase that exists essentially as a membrane-anchored cell-surface enzyme[15]. It is expressed throughout the body tissues, such as kidneys, the gastrointestinal tract, liver, pancreas, and the endothelial and epithelial cells on the vascular bed. Its soluble form is found in plasma and therefore it is in close proximity with hormones circulating in the blood[16,17]. The main activity of DPP4 is to remove N-terminal dipeptides from polypeptides[18], which preferably have a proline or alanine in the second position from the N-terminal. Some of the main DPP4 substrates are GLP-1 and the other incretin hormone GIP, which are peptides with N-terminal Tyr–Ala and His–Ala, respectively[19]. The intact GLP-1 is rapidly hydrolysed by DPP4 into a shorter, inactive form, once it reaches the plasma. GLP-1 has a half-life of 1–2 min[18]. Only 25 % of the active GLP-1 reaches the portal circulation and subsequently the liver, where a further 40–50 % is digested by the DPP4 in hepatocytes. This means that only 15 % of the secreted GLP-1 enters the systemic circulation and may reach other tissues, such as the pancreas or the brain[20]. Therefore, DPP4 is responsible for inactivating more than 80 % of the secreted GLP-1[18]. Studies focus not only in the development of DPP4-inhibitory drugs, but also on peptides derived from food sources with DPP4-inhibitory capacity.

Although pharmacological compounds are being studied[21], natural compounds might be used to prevent the development of overweight- and obesity-related problems from early preclinical stages through interaction with the enteroendocrine system[22].

Dietary regulation of glucagon-like peptide-1 secretion

Nutrient ingestion is the primary physiological stimulus for inducing GLP-1 secretion by L cells, located in the ileum and colon in the human gastrointestinal tract. GLP-1 secretion occurs in a biphasic pattern, which consists of a rapid release in 15–30 min after a meal, followed by a second minor peak that occurs in 60–120 min. Enteroendocrine cells have been shown to respond to carbohydrates, lipids and proteins.

Glucose and fat have been reported to be strong GLP-1-secretagogues after they have been ingested[23], or directly administered into the intestine[24,25] or into perfused ileal segments[26]. In the murine model, glucose-stimulated GLP-1 release is blocked using Na-dependent GLUT-1 (SGLT-1) knock-out mice and SGLT-1 inhibitors[27,28], which suggests that glucose metabolism uses glucose transport via SGLT-1 to induce GLP-1 secretion. It has also been proposed that sweet taste receptors (T1R2, T1R3) are involved in the glucose-sensing mechanism, but there is still some controversy about whether this is so[29,30]. On the other hand, it has been reported that G-protein-coupled receptors (GPCR) are activated by dietary fat to stimulate GLP-1 release, including GPR40 and GPR120 by medium-chain fatty acids, long-chain fatty acids and long-chain unsaturated FA; and GPR41 and GPR43 by SCFA (for reviews, see Hirasawa *et al.*[31] and Reimann[32]).

Other food components could also modulate GLP-1 secretion. Flavonoid structures, present in several vegetables, also stimulate GLP-1 secretion[33]. In both *ex vivo*[34] and rat models[35], these compounds have been shown to improve the metabolic status altered by a cafeteria diet treatment[36].

Effects of proteins on glucagon-like peptide-1 secretion

Dietary proteins undergo digestion by gastric (pepsin) and pancreatic (chymotrypsin and trypsin) proteases and membrane

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digestion by peptidases associated with the brush-border membrane of enterocytes. The different digestive proteases cleave the peptide bonds at preferential positions. The primary endproducts are dipeptides and tripeptides, which will enter the cell through peptide transporters. Free amino acids are also released after luminal protein digestion and after peptide hydrolysis within the intestinal cells, and then exit across the basolateral membrane via specific amino acid transporters.

GLP-1 release is activated by luminal intestinal chemosensors, which could be reached by peptides of different sizes, mixed with free amino acids.

Studies in human, animal and enteroendocrine cells have shown increased GLP-1 secretion by free amino acids such as L-phenylalanine, L-alanine and L-glutamine[37,38] and L-asparagine[39]. The effect of glutamine has been confirmed in healthy, obese and diabetic human subjects[40,41]. Tolhurst et al.[42] demonstrated this effect in isolated mouse L cells and reported that the mechanisms were associated with an increase in cyclic AMP (cAMP) and cytosolic Ca^{2+} levels. They also found evidence to suggest that electrogenic Na-coupled amino acid uptake is responsible for initiating membrane depolarisation and voltage gated Ca^{2+}, while a second pathway increases intracellular cAMP levels. Young et al.[43] also reported similar results with L-proline, L-serine, L-alanine, L-glycine, L-histidine, L-cysteine and L-methionine in the STC-1 cell line.

When analysing the effects of protein on GLP-1 release, many studies focus on the effects of protein hydrolysates, produced by the hydrolysis of food protein with commercial enzymes (summarised in Tables 1–3). Sometimes, especially in in vitro studies, these are digestive enzymes that simulate intestinal digestion. However, many different hydrolysates are obtained through treatment with enzymes other than pepsin, chymotrypsin or trypsin. Protein hydrolysis can have two main benefits: (1) protein will be more quickly digested after intake; and (2) bioactive peptides[44–52] might be released. Thus, the degree of protein digestion may impact the capability of protein to stimulate GLP-1 release, as discussed below.

In vitro studies on the STC-1 cell line showed a clear stimulation by whole dairy proteins (whey, casein, α-lactalbumin, β-lactoglobulin)[53–55]. Moreover, the stimulation of GLP-1 by whey protein β-lactoglobulin in STC-1 cells was partially lost when treated with trypsin (β-lactoglobulin 7·3-fold increase and hydrolysates 2–5·8-fold increase, all v. vehicle control), and totally lost when digested with chymotrypsin for 60 min or more[54]. In the same cell line, the stimulatory effects of whey protein on GLP-1 were lost after extensive hydrolysis with microbial (not described) enzymes, or after a simulated gastrointestinal digestion that included a 90-min treatment with pepsin and a 150-min treatment with Corolase PP[56]. Another study showed that treating whey or casein with trypsin or DPP4 for 30 min did not lead to any loss of GLP-1-stimulatory properties[53].

In humans, dairy protein is one of the most studied protein sources involving GLP-1 secretion. Intraduodenal infusion of whey protein hydrolysate has stimulated plasma GLP-1 in lean and obese subjects[57], reduced glucose concentration and suppressed energy intake[58] compared with saline. In these studies, hydrolysed, rather than intact, whey protein was selected because it more closely resembles partially digested protein.

Also in patients with T2DM, a whey preload increased GLP-1 secretion, lowered plasma glucose levels and increased the insulin response[59,60] compared with water and sucralose, respectively. It has been shown that whey, casein and casein hydrolysates increase GLP-1 secretion[61-63]. However, there is no agreement about whether there are any differences between their effect on GLP-1 secretion. Hall et al.[62] showed that 120 min after being ingested, whey protein induced a 2-fold increase in postprandial GLP-1 levels compared with casein protein. On the other hand, when comparing whey, casein and their hydrolysates, Calbet & Holst[63] showed that the release of GLP-1 was not influenced by the source or hydrolysis process. Also, a commercially available whey protein hydrolysate showed a higher GLP-1 release 30 min after an oral glucose tolerance test (OGTT) than did casein glycomacropeptide (CGMP), but not compared with whey isolate or α-lactalbumin-enriched whey[64] (incremental AUC_{30min} median; 593 (hydrolysate), 270 (CGMP); $P=0·045$). Thus, the studies performed with whey and whey hydrolysates do not show any differences in the effects of the two sources in terms of GLP-1 secretion. Calbet & Holst[63] suggested that this is because the dairy protein hydrolyses rapidly in the intestine and there is a subsequent rise in peripheral amino acids independent of the fractionation.

Other protein sources have also been shown to stimulate GLP-1 release in vivo. A similar rise in rat plasma GLP-1 levels, comparable with that caused by dairy protein, has been observed after pea-protein meals[65]. Furthermore, also in rats, pea protein and pea-protein hydrolysate have been shown to similarly stimulate GLP-1 release, although the hydrolysate showed stronger eating-inhibitory properties[66] (total energy intake: 63 (SEM 6) kJ, 46 (SEM 3) kJ, 67 (SEM 5) kJ after pea protein, the hydrolysate and the control, respectively). In vitro studies with STC-1 cells showed that intact pea protein increases GLP-1 release. On the other hand, various pea-protein hydrolysates obtained by enzymic hydrolysis with subtilisin were tested, and only one of them maintained its GLP-1-secretory capacity[53].

Cereal protein has also been shown to stimulate GLP-1. Maize protein zein (a major maize protein) hydrolysate attenuated glycaemia in rats under the intraperitoneal glucose tolerance test, associated with enhanced secretions of GLP-1 and GIP[67] compared with water. In vitro (GLUTag cells), zein hydrolysate was shown to stimulate GLP-1 release more than egg albumin, country bean and meat hydrolysates[68]. However, the type of hydrolysis was different in the various sources, so the effect of the protein source per se cannot be concluded from this paper. The stimulation of GLP-1 secretion by maize zein hydrolysate in GLUTag cells is not affected by treatment with pepsin/pancreatin for 60 min, although it is reduced after pronase treatment[67] compared with the positive control, KCl 70 mM. The authors suggested that the hydrolysate is not further cleaved by pepsin treatment (the degree of hydrolysis was only 8·6 %).

Oral administration of rice protein hydrolysates also increased total GLP-1 in plasma, and improved glycaemic response in rats[69] (the control used was 2 g/kg of glucose solution). In the same study, rice protein hydrolysates (degree of hydrolysis 5–10 %) stimulated GLP-1 in GLUTag cells, with the potency depending on the enzyme and the time of digestion[69] compared with the blank treatment. The effect of the whole rice

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Table 1. Stimulation of glucagon-like peptide-1 (GLP-1) secretion by protein and protein hydrolysates in humans

Protein	Hydrolysis conditions	Subjects	n*	Protein dose	Secretion	Increment *v.*	Reference
Turkey	Intact protein	Healthy subjects	8	Ingestion of 352 g	↑	Fat isoenergetic meal	[23]
Whey	N.D.	Obese and lean men	12	Intraduodenal infusion of 24 g Intraduodenal infusion of 48 g	↑	Saline	[57]
Whey	N.D.	Healthy men	16	Intraduodenal infusion 8 g Intraduodenal infusion 24 g Intraduodenal infusion 48 g	↑	Saline	[58]
Whey	Intact protein	T2DM subjects of both sexes	21	Ingestion of 17 g	↑	Sucralose	[59]
Whey	Intact protein	T2DM subjects of both sexes	15	Ingestion of 50 g	↑	Water	[60]
Casein Whey	Intact protein	Overweight to obese men and women	24	Ingestion of 30 g	↑	Time 0	[61]
Casein	N.D.						
Whey	Intact protein	Healthy men and women	9	Ingestion of 48 g	↑	Casein	[62]
Whey	Alcalase/53°C/pH 8·0/–† + Neutrase/53°C/pH 7·0/–† Intact protein	Healthy men	6	Stomach infusion of 36 g	↑	Time 0	[63]
Casein	Alcalase/53°C/pH 8·0/–† + Neutrase/53°C/pH 7·0/–† Intact protein						
Whey	N.D.	T2DM subjects of both sexes	11	Ingestion of 45 g	↑	CGMP-enhanced whey	[64]

↑, GLP-1 secretion is incremented *v.* the control, specified in each row; N.D., hydrolysis conditions not described; T2DM, type 2 diabetes mellitus; CGMP, casein glycomacropeptide.
* Number of subjects per group.
† Time not known.

Table 2. Stimulation of glucagon-like peptide-1 (GLP-1) secretion by protein and protein hydrolysates *in vitro**

Protein	Hydrolysis conditions	Cell line	Protein treatment	Secretion	Increment v.	Reference
Egg albumin	N.D.	STC-1	2 h	↑	KRB	[26]
Meat			2·5–20 mg/ml			
Meat	N.D.	Small-intestinal cultures	2 h	↑	Saline with 0·1 % BSA	[37]
			5·0–50 mg/ml			
			2 h	–		
			0·5 mg/ml			
Milk	N.D.	Small-intestinal cultures	2 h	↑		
Vegetables			5·0 mg/ml			
Casein	Intact protein	STC-1	2 h	↑	Hanks' buffered salt solution	[53]
Codfish			1·0 mg/ml			
Egg						
Pea (DPS†)						
Wheat						
Whey						
Ovomucoid	Intact protein	STC-1	2 h	–		
Pea (Pisane†)			1·0 mg/ml			
Pea (SM†)						
Soyabean						
Casein hydrolysate	N.D.	STC-1	2 h	–		
			1·0 mg/ml			
Egg	N.D.	STC-1	2 h	↑		
			1·0 mg/ml			
Pea	Subtilisin/–‡/pH 8·0/–‡ + PSE/–‡/pH 6·0/–‡	STC-1	2 h	–		
			1·0 mg/ml§			
Pea (HP90†)	N.D.	STC-1	2 h	–		
Wheat			1·0 mg/ml			
Yoghurt whey	Intact protein	STC-1 pGIP/neo	3 h	↑	HEPES	[54]
			5·0–25 mg/ml			
	Intact protein	STC-1 pGIP/neo	3 h	↓		
			50–100 mg/ml			
Cheese whey	Intact protein	STC-1 pGIP/neo	3 h	–		
			5·0–10, 100 mg/ml			
			3 h	↑		
			25–50 mg/ml			
β-Lactoglobulin	Intact protein	STC-1 pGIP/neo	3 h	↑		
			0·63–10 mg/ml			
			3 h	–		
			0·31 mg/ml			
	Chymotrypsin/37°C/pH 7·4/30 min Trypsin/37°C/pH 7·4/30–150 min	STC-1 pGIP/neo	3 h	↑		
			10 mg/ml			
	Chymotrypsin/37°C/pH 7·4/60–150 min	STC-1 pGIP/neo	3 h	–		
			10 mg/ml			
α-Lactalbumin	Intact protein	STC-1 pGIP/neo	3 h	–		
			0·31–0·63 mg/ml			
			3 h	↑		
			1·3–10 mg/ml			

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Table 2. (*Continued*)

Protein	Hydrolysis conditions	Cell line	Protein treatment	Secretion	Increment v.	Reference
Casein	Intact protein	STC-1 pGIP/neo	3 h 0·31–10 mg/ml	↑	HEPES	(55)
α-Casein β-Casein	Intact protein	STC-1 pGIP/neo	3 h 0·16–5·0 mg/ml	↑		
κ-Casein	Intact protein	STC-1 pGIP/neo	3 h 0·16–0·31 mg/ml	–		
			3 h 0·63–5·0 mg/ml	↑		
α-Casein	Chymotrypsin/37°C/pH 7·4/30–150 min	STC-1 pGIP/neo	3 h 5·0 mg/ml	–		
	Trypsin/37°C/pH 7·4/30–150 min	STC-1 pGIP/neo	3 h 5·0 mg/ml	↑		
	Pepsin/37°C/pH 2·3/30–150 min					
β-Casein	Chymotrypsin/37°C/pH 7·4/30–150 min Trypsin/37°C/pH 7·4/30–150 min	STC-1 pGIP/neo	3 h 5·0 mg/ml	–		
	Pepsin/37°C/pH 2·3/30–150 min	STC-1 pGIP/neo	3 h 5·0 mg/ml	↑		
Whey	Intact protein	STC-1	4 h 10·0 mg/ml	↑	KRB with 10 mм-glucose	(56)
	Pepsin/37°C/pH 2/90 min + Corolase PP/37°C/pH 7·5/150 min	STC-1	4 h 10 mg/ml	–		
Whey DH32	N.D. Pepsin/37°C/pH 2/90 min + Corolase PP/37°C/pH 7·5/150 min	STC-1	4 h 10 mg/ml	–		
Whey DH45	N.D. Pepsin/37°C/pH 2/90 min + Corolase PP/37°C/pH 7·5/150 min	STC-1	4 h 10 mg/ml	–		
Maize zein	Papain/55°C/pH 7·2/60 min Papain/55°C/pH 7·2/60 min + Pepsin/37°C/pH 1·85/60 min + Pancreatin + trypsin/37°C/pH 8·2/120 min Papain/55°C/pH 7·2 + Pronase/37°C/pH 7·0	GLUTag	1 h 10 mg/ml	↑	HEPES	(67)
Maize zein	Papain/55°C/pH 7·0/60 min	GLUTag	1 h 2·0 mg/ml	–	HEPES	(68)
			1 h 5·0–20 mg/ml	↑		
Egg albumin	N.D.	GLUTag	1 h 5·0 mg/ml	–		
BSA	Intact protein					
Meat	N.D.					
Bean	Pepsin/37C/pH 1·9/10 min					

Table 2. (*Continued*)

Protein	Hydrolysis conditions	Cell line	Protein treatment	Secretion	Increment *v.*	Reference
Rice endosperm	Papain/55°C/pH 7·2/60 min Pepsin/37°C/pH 1·85/30 min	GLUTag	1 h 10 mg/ml	↑	HEPES	[69]
	Pepsin/37°C/pH 1·85/60 min	GLUTag	1 h 10 mg/ml	–		
Rice bran	Papain/55°C/pH 7·2/60 min	GLUTag	1 h 10 mg/ml	–		
	Pepsin/37°C/pH 1·85/30 min Pepsin/37°C/pH 1·85/60 min	GLUTag	1 h 10 mg/ml	↑		
Wheat (770 Da fraction)	N.D.	GLUTag	2 h 0·1–0·25 mg/ml	–	Saline	[70]
			2 h 0·5–1·0 mg/ml	↑		
Wheat (7740 Da fraction)	N.D.	GLUTag	2 h 1·0 mg/ml	–		
Wheat gluten	N.D.	GLUTag	1 h 5 mg/ml	–	HEPES	[71]
			1 h 10 mg/ml	↑		
α-Lactalbumin	N.D.	GLUTag	1 h 5 mg/ml	–		
			1 h 10 mg/ml	↑		
Wheat gluten	N.D. + Pepsin/37°C/pH 1·85/30–60 min + Pancreatin/37°C/pH 8·2/60–120 min	GLUTag	1 h 10 mg/ml	↑		
α-Lactalbumin	N.D. + Pepsin/37°C/pH 1·85/30–60 min + Pancreatin/37°C/pH 8·2/60–120 min	GLUTag	1 h 10 mg/ml	↑		
Cuttlefish viscera	Intact protein + Salivary fluid	STC-1	2 h 13 mg/ml	–	Baseline	[72]
	H‖/50°C/pH 8·0/4 h + Salivary fluid	STC-1	2 h 13 mg/ml	↑	UCVP + salivary fluid	
	H¶/50°C/pH 8·0/4 h + Salivary fluid	STC-1	2 h 13 mg/ml	–	UCVP + salivary fluid	
	H‖/50°C/pH 8·0/4 h + Salivary fluid + Pepsin/37°C/pH 2·5–3/120 min	STC-1	2 h 13 mg/ml	–	UCVP + IVD	

Table 2. (*Continued*)

Protein	Hydrolysis conditions	Cell line	Protein treatment	Secretion	Increment *v.*	Reference
	H¶/50°C/pH 8·0/4 h + Salivary fluid + Pepsin/37°C/pH 2·5–3/120 min	STC-1	2 h 13 mg/ml	↓	UCVP + IVD	
	H‖/50°C/pH 8·0/4 h + Salivary fluid + Pepsin/37°C/pH 2·5–3/120 min + Pancreatin/37°C/pH 7·0/120 min	STC-1	2 h 13 mg/ml	–	UCVP + IVD	
	H¶/50°C/pH 8·0/4 h + Salivary fluid + Pepsin/37°C/pH 2·5–3/120 min + Pancreatin/37°C/pH 7·0/120 min	STC-1	2 h 13 mg/ml	↓	UCVP + IVD	
Bovine Hb	Intact protein + Salivary fluid	STC-1	2 h 13 mg/ml	↑	HEPES	(73)
Bovine Hb	Salivary fluid + Pepsin/37°C/pH 2·5–3·0/60–120 min	STC-1	2 h 13 mg/ml	↑	HEPES	
	Salivary fluid + Pepsin/37°C/pH 2·5–3·0/120 min + Pancreatin/37°C/pH 7·0/30–120 min					
	Salivary fluid + Pepsin/37°C/pH 2·5–3·0/120 min + Pancreatin/37°C/pH 7·0/120 min	STC-1	2 h 5·0 mg/ml 2 h 10 mg/ml			
Meat	N.D.	GLUTag	2 h 1·0–50 mg/ml	↑	Baseline	(74)

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Table 2. (Continued)

Protein	Hydrolysis conditions	Cell line	Protein treatment	Secretion	Increment v.	Reference
Salmon skin gelatin	Alcalase/50°C/pH 7·0/4 h Alcalase + Flavourzyme/50°C/pH 7·0/4 h	GLUTag	2 h 2·5 mg/ml	↑	Glucose 2 mM	(76)
	Promod/50°C/pH 7·0/4 h	GLUTag	2 h 2·5 mg/ml	–		
	Alcalase + Flavourzyme/50°C/pH 7·0/4 h + Pepsin/37°C/pH 2·0/90 min + Corolase PP/37°C/pH 7·0/150 min	GLUTag	2 h 2·5 mg/ml	–		
Salmon trimmings	Alcalase/50°C/pH 7·0/4 h	GLUTag	2 h 2·5 mg/ml	–		
	Alcalase + Flavourzyme/50°C/pH 7·0/4 h	GLUTag	2 h 2·5 mg/ml	↑		
	Promod/50°C/pH 7·0/4 h	GLUTag	2 h 2·5 mg/ml	↓		
	Alcalase + Flavourzyme/50°C/pH 7·0/4 h + Pepsin/37°C/pH 2·0/90 min + Corolase PP/37°C/pH 7·0/150 min	GLUTag	2 h 2·5 mg/ml	↑		
Meat	N.D.	NCI-H716	2 h 20 mg/ml	↑	KRB with 0·2 % BSA	(82)
Chicken feet	Neutrase/25°C/pH 7·0/24 h	STC-1	2 h 5 mg/ml	↑	HEPES	(99)
		Ileum explants	1 h 15 mg/ml	↑	KRB with 10 mM-glucose	

↑ GLP-1 secretion is incremented v. the control, specified in each row; –, GLP-1 secretion is not altered v. the control, specified in each row; ↓ GLP-1 secretion is reduced v. the control, specified in each row; BSA, bovine serum albumin; Corolase PP, a porcine pancreatic enzyme preparation; DH32, 32 % degree of hydrolysis; DH45, 45 % degree of hydrolysis; DPS, Dutch Protein Services; H, hydrolysis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IVD, in vitro digestion with pepsin and pancreatin, always indicates the same hydrolysis conditions as the protein that is compared with; KRB, Krebs–Ringer modified buffer; N.D., hydrolysis conditions not described; PSE, proline-specific endoprotease; UCVP, undigested cuttlefish viscera protein.
* The salivary fluid does not contain enzymes.
† Pea protein origin: DPS, from Dutch Protein Services; Pisane, from Cosucra; SM, from Nutralys; HP90, from Triballat.
‡ Temperature or time not known.
§ This pea hydrolysate did not stimulate GLP-1 secretion; nor did the 10 kDa permeate. Nevertheless, the supernatant fraction obtained after centrifugation increased GLP-1 secretion compared with the control.
‖ Hydrolysis with cuttlefish hepatopancreas digestive proteases.
¶ Hydrolysis with cuttlefish smooth hound intestine digestive proteases.

Regulation of glucagon-like peptide-1

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Table 3. Stimulation of glucagon-like peptide-1 (GLP-1) secretion by protein and protein hydrolysates in animals

Protein	Hydrolysis conditions	Species	n*	Protein dose	Secretion	Increment v.	Reference
Egg albumin	N.D.	Wistar male rats	7–9	Jejuno-ileum administration of 25 mg/ml Jejuno-ileum administration of 50 mg/ml Colon administration of 25 mg/ml Colon administration of 50 mg/ml	↑	Saline	(26)
Salmon skin gelatin	Flavourzyme/50°C/pH 7·0/4 h	Sprague–Dawley male rats†	12	5 weeks Oral administration 300 mg/d	↑	Water	(47)
Porcine skin gelatin	Flavourzyme/50°C/pH 7·0/6 h	Sprague–Dawley male rats	12	6 weeks Oral administration 300 mg/d	–	Water	(48)
	Flavourzyme/50°C/pH 7·0/6 h	Sprague–Dawley male rats†		6 weeks Oral administration 300 mg/d	↑		
Halibut skin gelatin	Flavourzyme/50°C/pH 7·0/4 h	Sprague–Dawley male rats†	11	4 weeks 750 mg/kg/d	↑	Water	(49)
Tilapia skin gelatin	Flavourzyme/50°C/pH 7·0/6 h	Sprague–Dawley male rats		4 weeks 750 mg/kg/d	–		
Whey Pea	Intact protein	SPF Wistar male rats	9	Oral administration of about 3 g/kg BW	–	Sucrose	(65)
Pea	Intact protein N.D.	Sprague–Dawley male rats	10	Intragastric infusion of 136 mg/ml	–	Saline	(66)
Maize zein	Papain/55°C/pH 7·2/60 min	Sprague–Dawley male rats	7–10	Oral administration§ of 2 g/kg BW	↑	Water	(67)
Meat	N.D.	Sprague–Dawley male rats	7–10	Oral administration§ of 2 g/kg BW	–		
Maize zein	Papain/55°C/pH 7·2/60 min	Goto-Kakizaki male rats	6–7	Oral administration‡ of 2 g/kg BW	↑		
Whey	Papain/55°C/pH 7·2/60 min	Goto-Kakizaki male rats	6–7	Oral administration‡ of 2 g/kg BW	–		
Maize zein	Papain/55°C/pH 7·0/60 min	Sprague–Dawley male rats	6–9	Duodenal administration of 100–250 mg/ml Ileal administration of 100 mg/ml Ileal administration of 250 mg/ml Duodenal loop administration of 300 mg/ml Jejunal loop administration of 300 mg/ml Ileal loop administration of 300 mg/ml	↑ – ↑ –	Water Time 0	(68)
Rice endosperm	Pepsin/37°C/pH 1·85/30 min	Sprague–Dawley male rats	4–6	Oral administration of 2 g/kg BW Oral administration‡ of 0·1–1·0 g/kg BW Oral administration‡ of 2 g/kg BW Oral administration§ of 1–2 g/kg BW	↑ – ↑ ↑	Water	(69)
Rice bran	Pepsin/37°C/pH 1·85/30 min	Sprague–Dawley male rats	4–6	Oral administration of 2 g/kg BW Oral administration‡ of 0·1–1·0 g/kg BW Oral administration‡ of 2 g/kg BW	↑ – ↑		
Wheat (770 Da fraction)	N.D.	Sprague–Dawley male rats	8	Oral administration§ of 2 g/kg BW	↑	Saline	(70)
Wheat gluten α-Lactalbumin	N.D.	Wistar/ST male rats	5–7	Oral administration of 1 g/kg BW	–	Water	(71)
Meat	N.D.	Wistar male rats	6	Duodenal infusion of 50 mg/ml	↑	Baseline	(75)
Lysozyme	Alcalase/60°C/pH 8·0/6 h	ZDF male rats	9	Oral administration of 1 g/kg BW	–	Untreated rats	(100)
Maize zein	Papain/55°C/pH 7·0/60 min	Sprague–Dawley male rats	6–8	Ileal administration§ of 250 mg/ml	↑	Water	(101)
Meat	N.D.						

N.D., hydrolysis conditions not described; ↑, GLP-1 secretion is incremented v. the control, specified in each row; –, GLP-1 secretion is not altered v. the control, specified in each row; SPF, specific pathogen-free; BW, body weight; ZDF, Zucker diabetic fatty.
* Number of animals per group.
† Sprague–Dawley streptozotocin-induced diabetic rats.
‡ Changes in plasma GLP-1 after oral administration of the protein under the oral glucose tolerance test.
§ Changes in plasma GLP-1 after oral administration of the protein under the intraperitoneal glucose tolerance test.

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FOOD INTAKE MODULATION BY INSECT PROTEIN THROUGH THE INTERACTION WITH THE ENTEROENDOCRINE SYSTEM
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protein was not assessed. The authors found that GLP-1 secretion was weaker after 60 min digests with pepsin in rice endosperm protein hydrolysates than after 30 min digests, which suggests that oligo- or larger peptides, rather than small peptides or free amino acids, might be responsible for this stimulation. The results for wheat protein were just the opposite. In GLUTag cells, a low-molecular fraction of wheat protein hydrolysate enhanced GLP-1 secretion while a high-molecular fraction did not[70]. The low-molecular fraction of wheat protein hydrolysate had a glucose-lowering effect mediated by GLP-1 in rats[70] after an oral administration compared with 0·9 % NaCl. Also, in another study in a distal enteroendocrine cell model (GLUTag cells), the effect of wheat hydrolysate on the stimulation of GLP-1 secretion was largely enhanced by pepsin/pancreatin digestion relative to the blank[71].

For other protein sources, *in vitro* studies also showed that GLP-1-secreting activity of digested protein was greater than that of the original source. In a study performed with cuttlefish (*Sepia officinalis*) viscera, a hydrolysate (obtained from digestion with cuttlefish hepato-pancreatic enzymes) was found to exert GLP-1-secreting action while the undigested protein did not[72]. These results were found with the samples solubilised in saliva, but they were subjected to further *in vitro* simulated gastrointestinal digestion (including treatment with pepsin and pancreatin). Results showed that gastrointestinal digestion increased the GLP-1-secretory effects of both the hydrolysate and the initially undigested protein, leading to no differences between the hydrolysate and the non-hydrolysate gastrointestinally digested samples. Also, intestinal digested bovine Hb protein had a greater effect on GLP-1 release than partially digested protein (saliva and gastric digest) in STC-1 cells[73].

Taken together, all these studies prove that several protein sources increase GLP-1 secretion, which is associated to benefits such as food intake or glucose homeostasis regulation. *In vivo* studies do not fully clarify whether previous hydrolysis of the protein sources with commercial enzymes leads to stronger GLP-1-secreting effects. *In vitro* data show that many protein sources, including purified proteins, activate GLP-1 release. However, digestion as it might physiologically happen upon protein intake might stimulate or reduce the effect of the undigested protein, depending on the original source. This suggests that some high-molecular-weight peptides might reach enteroendocrine cells and activate GLP-1 secretion, while in other cases the lower-molecular-weight peptides or the amino acids released after digestion are responsible for the secretion.

Mechanisms involved in the effects of protein as glucagon-like peptide-1 secretagogue

The mechanisms through which the proteins and peptides released after protein hydrolysis (either 'synthetic' or simulated digestion) act as secretagogues are still not fully understood, but several pathways have been shown to be involved. Studies on the mechanisms through which protein and protein hydrolysates stimulate GLP-1 secretion are carried out using *in vitro* (i.e. enteroendocrine cell lines such as STC-1 and GLUTag) and *ex vivo* (i.e. perfused intestine and intestinal explants) models, and also primary cultures.

Many of the studies that focus on the mechanisms that stimulate GLP-1 secretion use commercial meat peptones, that is meat hydrolysates produced by the digestion of meat with proteolytic enzymes which lead to a complex mixture of partially metabolised proteins.

With this protein source, it seems that one key player in the oligopeptide stimulation of GLP-1 release is peptide transporter 1 (PepT1) (Fig. 1). Meat peptone was shown to stimulate GLP-1 secretion in mouse colonic primary culture through PepT1-dependent uptake, followed by an increase in intracellular Ca, and activation of Ca-sensing receptor (CaSR)[74]. Very recently Modvig *et al.*[75] used isolated perfused rat small intestine to study GLP-1 secretion stimulated by meat peptone. The sensory mechanisms underlying the response depended on di-/tripeptide uptake through PepT1 and subsequent basolateral activation of the amino acid-sensing receptor (CaSR) (Fig. 2). CaSR might also be activated by free amino acids taken up from the intestinal lumen by different amino acid transporters[75].

It has been pointed out that it is difficult to determine the PepT1-dependent oligopeptide-sensing pathway in GLUTag and STC-1 cell lines, because the expression of endogenous PepT1 is lower than in native L cells[74]. Therefore, the effects of peptones observed in both cell lines may be due to the free amino acids that some of these peptones contain, as has been suggested in an *in vitro* study on the effects of salmon hydrolysate[76] carried out in GLUTag cells. However, other studies on these cell lines do not share this view. As mentioned above, GLP-1 secretion is activated by dairy proteins[53–55], low-molecular-weight wheat (with less than 1 % free amino acids)[70], intact pea-protein[53] or peptin-resistant zein hydrolysate[67]. Furthermore, three synthetic peptide sequences (ANVST, TKAVEH and KAAT) were reported to be able to enhance GLP-1 secretion in STC-1 cells[77]. The authors concluded that the incretin effect of proteins is associated with the amino acid profile, but the specific amino acid motif that triggers GLP-1 secretion stimulation was not determined. Thus, receptor or peptide transporters other than PepT1 expressed in STC-1 and GLUTag cells might be involved in the peptide stimulation of GLP-1. For instance, one of the mediators suggested was the G protein-coupled receptor family C group 6 subtype A (GPRC6A)[70] (Fig. 3).

Protein hydrolysates are also detected by the umami receptor (T1R1–T1R3 heterodimer)[78] (Fig. 4) and G protein-coupled receptor 92/93 (GPR92/93)[79], which leads to the release of the gut-derived satiety factor cholecystokinin. There is no direct evidence of umami stimulation and GLP-1 secretion, but the T1R1 receptors were co-expressed with GLP-1-expressing STC-1 cells[80], which suggests that umami receptors play a role in GLP-1 signalling.

An increase in intracellular Ca has been reported to be a pathway activated by protein hydrolysates to mediate GLP-1 secretion. Pais *et al.*[37] reported that meat peptone-stimulated GLP-1 secretion from primary L cells was also associated with Ca influx through voltage gate Ca channels (Fig. 3). In NCI-H716 human enteroendocrine cells, tetrapeptides, but not single amino acids or any of the dipeptides, tripeptides and pentapeptides tested, were found to induce a robust and selective $[Ca^{2+}]_i$ response associated with increased secretion of GLP-1[81].
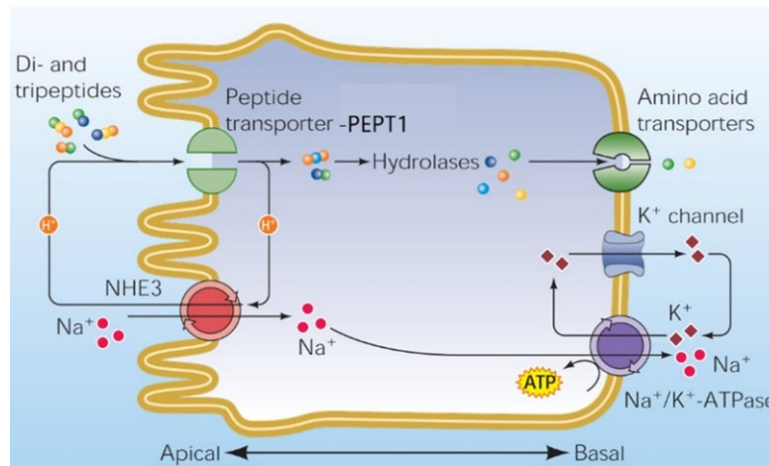
Fig. 1. The intestinal transporter form PEPT1 (SLC15A1) is located in apical membranes with a functional coupling to the apical Na^+/H^+ antiporter (NHE3) for pH recovery from the peptide-transport-induced intracellular acid load. Adapted from Daniel *et al.*[103].
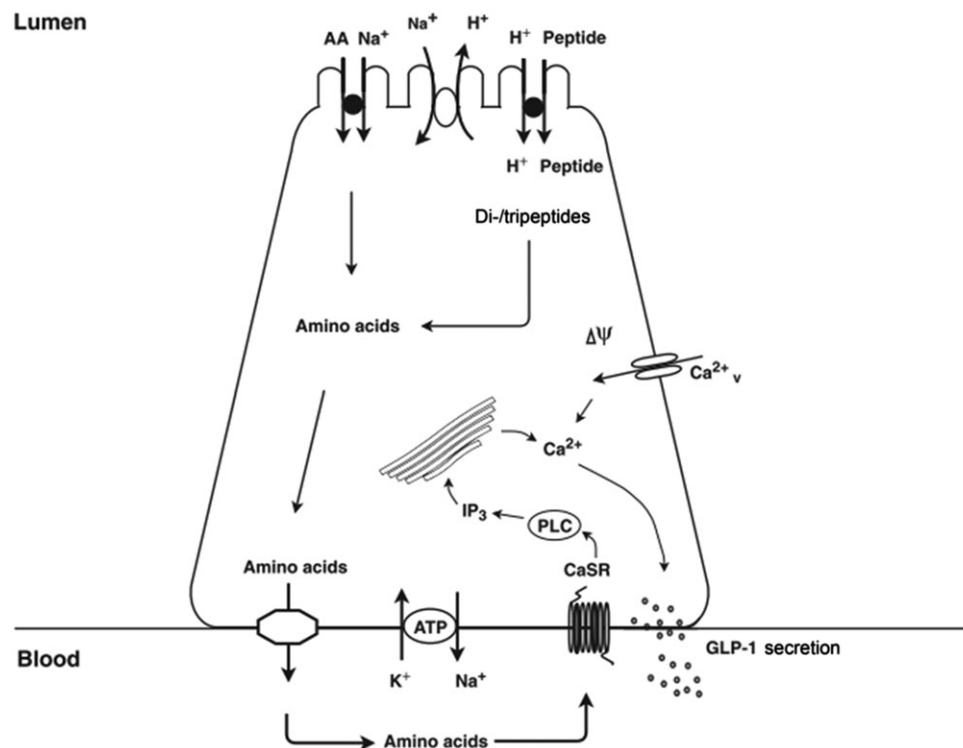
Fig. 2. Illustration of the endocrine L cell and the proposed mechanisms by which peptone stimulates glucagon-like peptide-1 (GLP-1) release. Di-/tripeptides are taken up by PepT1 and are degraded by cytosolic peptidases to their respective amino acids (AA). Intracellular amino acids are then transported to the interstitial side through basolateral amino acid transporters, wherefrom they stimulate the L cells by activating amino acid sensors, like calcium-sensing receptor (CaSR), situated on the basolateral membrane. IP_3, inositol trisphosphate; PLC, phospholipase C. Adapted from Modvig *et al.*[75].

Moreover, these effects were not observed in either STC-1 or in GLUTag rodent cells. Interestingly, in the same paper, the authors showed that casein protein hydrolysate elicited an increase in GLP-1 without modulating intracellular Ca.

It has been suggested that GLP-1 secretion is mediated by other intracellular pathways such as extracellular signal-regulated kinase 1/2 (ERK1/2), mitogen-activated protein kinase (MAPK) and p38 MAPK, activated by peptones and mixtures of essential amino acids in NCI-H716 cells[82].

Altogether, the studies show that which signalling pathways are involved in GLP-1 secretion by different peptide mixtures will depend on the peptide length, the sequences and/or the amino acid composition, and whether there are free amino acids in the mixture. Furthermore, the model studied has to be carefully considered since there are differences in the expression of key genes (such as pepT-1) and some effects might depend on the vectoriality of the system (the capacity to differentiate basolateral and apical processes).

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Fig. 3. Signalling through G protein-coupled receptor family C group 6 subtype A (GPRC6A) in β- or gut cells. GPRC6A can be directly activated by amino acids and use calcium as an allosteric regulator. IP$_3$, inositol triphosphate; PLCβ, phospholipase Cβ; GLP-1, glucagon-like peptide-1; VDCC, voltage-dependent calcium channel. ‡ Described in enterocyte L cells of the small intestine. Adapted from Wauson et al.[104].
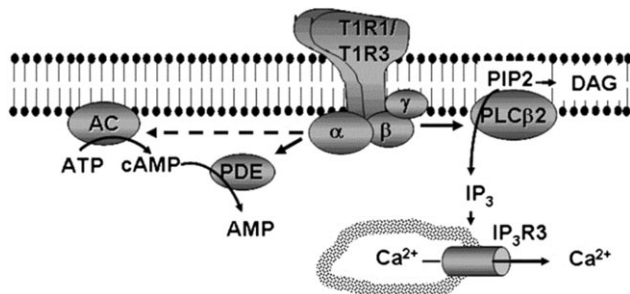
Fig. 4. The T1R1/T1R3 heterodimer is coupled to a heteromeric G protein, where the Gbc subunit appears to mediate the predominant leg of the signalling pathway. Ligand-binding activates Gbg, which results in activation of phospholipase Cβ2 (PLCβ2), which produces inositol trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ activates IP$_3$ receptor type 3 (IP$_3$R3) which results in the release of Ca^{2+} from intracellular stores. AC, adenylyl cyclase; cAMP, cyclic AMP; PDE, phosphodiesterase; PIP2, phosphatidylinositol 4,5-bisphosphate. Adapted from Kinnamon[105].

Protein bioactivity on glucagon-like peptide-1 clearance

Like the studies on the effects of protein on GLP-1 secretion, most of the studies on the effects of protein on DPP4 inhibition are performed with protei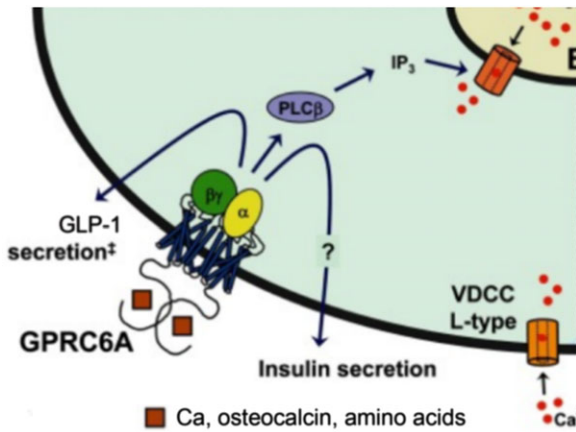n hydrolysates. Over the past few years, bioactive peptides have shown their potential as DPP4 inhibitors, a research area that is currently expanding. In vitro simulated gastrointestinal digestion has been reported to produce DPP4-inhibitory protein hydrolysates[83,84]. Also, hydrolysis with a range of enzymes is used to release DPP4-inhibitory peptides[69,85–90]. Thus, a wide range of protein sources has been used to obtain hydrolysates, for which DPP4-inhibitory activity has been screened mainly in vitro.

Research has shown that the amino acid sequence plays a much greater role in DPP4-inhibitory activity than other physicochemical parameters such as length, isoelectric point, hydrophobicity and net charge[91,92]. DPP4 preferentially cleaves substrates that bear proline or alanine at their P$_1$ position (Xaa-Pro and Xaa-Ala; where Xaa represents any amino acid)

and also acts on substrates that bear other residues, such as glycine, serine, valine and leucine[93]. Hydrophobic and basic residues at the P$_2$ position enhance the affinity for cleavage compared with acidic residues[94]. The presence of tryptophan residue at the N-terminal position increases the susceptibility to cleavage. Although the residues at the N-terminal position may have a major impact by inhibiting DPP4, the authors pointed out that the C-terminal amino acid also affects the potency of DPP4 because it is involved in the interaction with the enzyme[95].

To date, some studies have been carried out on the in vivo DPP4-inhibitory effects of the hydrolysates and peptides from dietary proteins. Peptides derived from milk and bean proteins, which have been shown to inhibit the activity of DPP4 in vitro, were also found to have glycaemic effects in mice[96,97] as plasma glucose levels decreased after an OGTT. A β-casein-derived peptide LPQNIPPL found in Gouda-type cheese with in vitro DPP4-inhibitory effects has also been tested with animal models. Oral administration of this octapeptide resulted in 1·8-fold lower postprandial glucose AUC; however, insulin plasma levels did not differ[98]. In these studies, the authors did not measure plasma DPP4 activity, so it is not known whether the lower blood glucose was caused by inhibition of DPP4 activity. Chicken feet hydrolysates with DPP4-inhibitory activity in vitro improved hyperglycaemia in diet and aged models of glucose homeostasis impairment[99].

As well as hydrolysates from milk and bean protein, in in vivo models hydrolysate from the egg protein lysosyme has also shown a 25 % reduction in blood serum DPP4 activity and a trend towards higher serum GLP-1 levels after 90 min in diabetic rats undergoing chronic treatment[100]. Streptozotocin-induced diabetic rats were used to evaluate the effects of porcine skin gelatin hydrolysates[48], Atlantic salmon skin gelatin[47], and halibut and tilapia skin gelatin[49]. In all these studies, diabetic animals showed reduced blood glucose levels during OGTT, increased plasma insulin and active GLP-1 levels, and reduced plasma DPP4 activity after a chronic treatment with these proteins compared with water. Diabetic rats treated for 42 d with a daily dose of 300 mg/kg of porcine skin gelatin showed their plasma glucose AUC reduced from 30 000 to 28 000 mg × min/dl (1665 to 1554 mmol × min/l), insulin levels increased 2-fold, active GLP-1 levels reduced from 15 to 13·5 pM and DPP4 activity reduced by half[48]. In another study in which the animals were treated for 35 d with a daily dose of 300 mg/kg of Atlantic salmon skin gelatin hydrolysate, blood glucose levels were reduced to less than 200 mg/dl (11·1 mmol/l) during OGTT, insulin levels increased 3-fold, active GLP-1 levels increased 1·6-fold and DPP4 activity was reduced from 115·5 to 82·6 % (lower than in normal rats)[47]. When these animals received a 30 d treatment involving a daily dose of 750 mg/kg of halibut (HSGH) or tilapia skin gelatin hydrolysate (TSGH) the plasma glucose was lower than 200 mg/dl (11·1 mmol/l) in the TSGH-treated group. When TSGH was administered, insulin levels were 1·56 g/l, higher than that of HSGH (1·14 g/l) and the diabetic control group (0·43 g/l). The active GLP-1 plasma levels of the diabetic control rats (5·14 pM) were lower than those for TSGH-treated group (13·32 pM) and for HSGH-treated group (7·37 pM) and the DPP4 activity reduced from 115·5 in the diabetic group to 86·6 and 71·6 % in the HSGH- and TSGH-treated groups, respectively[49].

Moreover, rodents receiving halibut and tilapia skin gelatin hydrolysates also showed increased total GLP-1 levels. Therefore, the findings of this study suggest that these hydrolysates exert their anti-hyperglycaemic effect via dual actions of DPP4 inhibition and GLP-1 secretion enhancement. Similarly, the ileal administration of zein protein hydrolysate to rats was found to potentiate the incretin effect when administered before an intraperitoneal glucose tolerance test, resulting in decreased glucose concentration, increased insulin levels, decreased plasma DPP4 activity, and increased total and active GLP-1 secretion compared with water[101]. Rice-derived peptides were likewise found to act via dual action. Oral administration increased plasma GLP-1 levels compared with water during an intraperitoneal glucose tolerance test, and ileal administration reduced plasma DPP4 activity and increased the ratio of active GLP-1 to total GLP-1[69] in rats. *In vitro* studies also showed dual mechanisms for protein hydrolysates; both enhanced GLP-1 secretion and inhibited DPP4, as has been shown for the cuttlefish (*Sepia officinalis*) viscera protein hydrolysate and bovine Hb hydrolysate[72,77], whey proteins[56] and chicken feet hydrolysate[99]. Therefore, these two mechanisms might also take part *in vivo* for some protein sources, leading to an increase in active GLP-1 and improve glycaemia.

Human studies, although limited, offer some evidence that food-derived peptides, mostly from dairy protein, act as DPP4 inhibitors[102]. It was shown that a whey preload, consumed before the breakfast meal, reduced glucose levels by 28 % and increased insulin and total GLP-1 levels by 105 and 141 %, respectively, compared with water. Nevertheless, no significant differences in plasma DPP4 activity were found. This could be interpreted as whey protein acting as an endogenous inhibitor of DPP4 in the proximal small intestine, but not in the plasma (intestinal DPP4 activity was not assessed)[60]. Further studies are needed to examine the potential of casein- and whey-derived peptides, as well as peptides derived from other sources, to act with DPP4 inhibitors in human subjects.

Conclusions

Food proteins target the enteroendocrine system. They directly enhance GLP-1 release from enteroendocrine cells. Current studies suggest that the source of the protein might lead to differences in GLP-1 secretion, although there is not enough literature to enable the different proteins to be compared. The effect of gastrointestinal digestion can also enhance or decrease GLP-1-secreting capacity depending on the protein type. Thus, it is important to consider this digestion when discussing the effects of protein on GLP-1 secretion *in vitro*. In addition, peptides with DPP4-inhibitory effects can be released during the digestion process, which could modulate the life span of target enterohormones. However, whether this hydrolysis remains important after intestinal digestion *in vivo* remains to be clarified. Thus, the use of protein/protein hydrolysates to ameliorate situations of glucose derangements is promising, but more research, specifically human studies, is required to define the most effective sources/treatments.

Acknowledgements

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1.2.2 CCK

Introduction

CCK expressing cells are open type-cells (traditionally named as I-cells) located predominantly in the duodenum and proximal jejunum, less abundant in the distal jejunum and sparse in the ileum [23,25,26]. This enterohormone is encoded by the *cck* gene and synthesised as a 115 amino acid (AA) preprohormone. Then, CCK undergoes post-translational processing leading to different circulating truncated forms including CCK-8 (the most potent peptide), CCK-33, and CCK-58 (the most abundant molecular form) [27,28]. The different forms of CCK interact with the specific receptors CCK-1, more abundant in peripheral tissues, and CCK-2, found centrally, triggering the biological actions of this peptide. Thus, CCK can act either as a hormone or as a neuropeptide.

CCK is released 10-15 minutes after meal ingestion, with lipids and protein being the most potent stimulus and carbohydrates the least after duodenal infusions of nutrients or oral administration [29–31]. Proper digestion of fats is required for CCK stimulation since it has been reported that fatty acids with a chain length greater than 12 carbons are effective stimulators of CCK, while those with a smaller chain length are not good secretagogues [32].

This hormone is an important regulator of the digestive process. The primary functions of CCK are the stimulation of pancreatic enzyme secretion and gall bladder contraction, and inhibition of gastric emptying [33]. But an increase in CCK plasmatic levels also induce a reduction of food intake in rats and humans, which characterize CCK as a satiating hormone [34–36].

CCK secretion by food proteins

As above mentioned, proteins are potent stimulus for CCK secretion. Duodenal infusions of 24, 48 or 84 g of whey protein hydrolysate significantly increased CCK secretion and reduced food intake when compared with saline, both in lean and obese subjects [30,37]. This CCK rise in plasma was accompanied by an increase in GLP-1 secretion. The same effects were reported by other authors

when intact whey protein was administered orally compared with an isoenergetic load of casein [38]. Geraedts *et al*. also reported an increase in CCK secretion after an oral or intraduodenal administration of intact pea protein. They observed a reduction in food intake 2 hours later but just after the intraduodenal administration [39]. Experiments *ex vivo* with human duodenal samples also reported the stimulatory effect on CCK after a treatment with pea, codfish and wheat protein, while the same amount of egg or ovomucoid did not cause this secretory effect. In rats, any of these protein sources stimulated CCK secretion *ex vivo* [40].

Intact casein, orally administered to rats, significantly increased CCK secretion more efficiently than lactalbumin and bovine serum albumin protein, an effect that was lost when these proteins were previously hydrolysed [41]. Lewis and Williams reported the same loss of the CCK secretory ability for casein when hydrolysed after duodenal infusion [42]. On the contrary, β-conglycinin, one of the major components of soy protein, was observed to increase CCK in plasma after duodenal infusion in rats and to reduce food intake only if hydrolysed. This increase in CCK levels was reproduced *in vitro* with intestinal rat cells [43]. Then, although some authors defend that intact protein is a more potent stimulus for CCK secretion in rats than hydrolysate protein, some discrepant results make it difficult to fully accept this statement.

In vitro with STC-1 cells, an intestinal cell line from mice, intact and hydrolysed proteins have been reported to increase CCK levels in the medium. Intact casein, pea and wheat were effective while their hydrolysed forms were not [44]. On the other hand, soy, potato, casein, whey, pea, egg, and pork hydrolysates significantly increased CCK secretion [44–47]. Under physiological circumstances, intact protein is not likely to reach the small intestine. Nevertheless, there are technological approaches that could protect intact proteins from degradation to allow them to interact with the nutrient sensors in the intestinal mucosa.

Apart from protein and its hydrolysates, AAs have been extensively studied regarding CCK secretion. Several authors have reported that intraduodenal and intragastric infusions of L-TRP or L-LEU increased CCK release and reduced food intake in healthy and non-diabetic obese subjects, albeit the LEU dose required is higher than the TRP dose [48–51]. The same results were reported for L-PHE but not for D-PHE [52], which suggest a stereospecific effect of AAs, also reported for TRP, LEU and GLU *in vitro* in STC-1 cells [53]. However, no increase in CCK plasmatic levels was reported after gastric administration of L-PHE or L-TRP in rats [41].

Among the exposed results we can conclude that proteins and derivates have a strong potential for CCK stimulation and food intake modulation. Nevertheless, the selection of the model to perform the experiment, the species, the study design, and the hydrolysis conditions of the samples are relevant aspects to consider when studying the CCK secretory capacity of proteins.

1.2.3 PYY

Introduction

PYY is often co-expressed with GLP-1 in open-type enteroendocrine L cells. Their abundance increases distally from the ileum to the colon while their presence in the upper small intestine is scarce [22,23,54,55]. PYY in plasma is present mainly in two different forms: PYY (1–36), which represents the 60% of circulating levels and is first secreted, and PYY (3–36), the active endocrine form (40% of the circulating levels) that results from the cleavage by the dipeptidyl peptidase-4 (DPP-4) [56]. Both forms are key mediators of the "ileal brake," an inhibitory feedback mechanism that controls the transit of a meal through the gastrointestinal tract to optimize nutrient digestion and absorption by inhibiting gastric and pancreatic secretions and proximal intestinal motility [57].

PYY is secreted postprandially in proportion to the energy load of the meal and is also secreted at baseline levels during fasting [58,59]. Lipids seem to be the most potent stimulus for PYY secretion, with the chain length of the fatty-acids

being an important factor , which must be greater than or equal to C12 to trigger its release [59–62]. Protein seems to be less effective in stimulating PYY but still significant [62–65]. Carbohydrates also seem to stimulate PYY secretion via the interaction with TAS1R1/TAS1R3 sweet taste receptors [66,67]. Plasmatic PYY levels increase 15-30 min after food intake, reaching maximum levels at 60-90 min [68–70]. Although PYY and GLP-1 are mostly produced in the same EECs, their secretory patterns are different since DPP-4 inactivates GLP-1 and activates PYY, and because the PYY secretion seems to be higher than the one of GLP-1 in the distal intestine, reported in rats [71,72].

The receptors that mediate the physiological effects of PYY belong to a family of NPY receptors, named Y1, Y2, Y4 and Y5 in humans, that are expressed in a wide range of tissues, including the intestine, neurons, and nerve fibres [73]. The main physiological functions of this enterohormone are the modulation of appetite and energy intake and gastric emptying [74]. Food intake modulation is mediated by the interaction of PYY (3-36) with the Y2 receptor, more expressed in the hypothalamic arcuate nucleus [75,76], while the administration of PYY (1-36), acting through the Y1 and Y5 receptors, has been reported to induce an increase in energy intake in rats [56,77]. In any case, PYY secretion is considered a satiety signal since after bariatric surgery, its elevated levels have been correlated with a reduced appetite [78] and improved insulin secretion [79], results also reported in healthy humans after the intravenous administration of this hormone [58,80]. However, these orexigenic effects have been the focus of the debate for this hormone, since several authors failed in reproducing them when administered exogenously [81–83].

PYY modulation by food proteins

The stimulation of the enteroendocrine PYY secretion via membrane nutrient receptors has been less extensively studied than other enterohormones. Moreover, protein is not considered one of the most potent stimuli, although there is evidence describing the PYY stimulatory capacity of proteins. Van Avesaat *et al*. showed that an ileal infusion of casein increased PYY secretion

compared with saline in healthy subjects [65]. Hydrolysed whey protein has been shown to increase PYY levels [84]. Also in obese patients, a high protein preload, composed of whey and soy protein, increased PYY secretion [62], as well as it did a pea protein preload [39]. In this study, it was also observed a food intake reduction but only when the protein was administered intraduodenally. Other examples are present in the literature. A high protein meal acutely increased PYY release in obese and lean subjects [85]. For obese women during energy restriction, a greater PYY response was reported after a high protein compared with a high carbohydrate meal [86]. In mice, a long term protein supplementation in combination with a low or a high-fat diet significantly increased PYY plasma levels and reduced food intake [85]. A chronic high protein diet administered to rats found no differences in plasma PYY levels between whey and soy proteins, although whey protein reduced food intake [87]. Conversely, in rats following a lifetime of a high-whey diet, PYY expression was down-regulated compared with high casein or soy protein diets [88]. Other two studies have reported no differences in the PYY response to different protein sources: whey and casein administration combined with glucomannan [89], or whey compared with pea protein hydrolysate, and casein, in combination with whey protein [90].

Some authors have reported no effect on PYY secretion after a protein administration. Rubio *et al.* showed that an acute oral administration of hydrolysed gelatine did not modulate PYY secretion in both obese and lean subjects [91]. However, most of these results are found working with AAs. L-CYS did not modulate PYY plasmatic levels when administered both oral and intraperitoneally in mice and rats. When administered to humans, no effect was observed either [92]. The same was reported with LEU after an intraduodenal infusion in lean men [48]. But not all the studies with AAs obtained the same results for this enterohormone. Steinert *et al.* showed an increase in PYY secretion following a TRP infusion [49,51].

Given the scarce information regarding PYY modulation by food proteins, it is difficult to characterize the best protein to stimulate its secretion. It is possible

that the type of the protein could also influence the PYY response to this macronutrient. Nevertheless, further studies are needed to fully elucidate the more potent stimuli for this hormone.

1.2.4 Ghrelin

Introduction

Ghrelin, known as the "hunger hormone", is the only well-characterized hormone implicated in the orexigenic signalling. It is produced along the gastrointestinal tract, with a higher abundance in the gastric fundus, where it is produced by closed-type EECs, while in the rest of the intestine it is mainly produced by open-type cells where they act as nutrient sensors [93]. The cells that secrete ghrelin were traditionally named X/A-like cells (P/D_1 cells in humans), where the 'X' referred to the originally unknown hormone produced and 'A-like' because of the similarities in the morphology between its secretory granules and the pancreatic A cell granules [94,95].

Apart from ghrelin, insulin-like peptide 5 (INSL-5) has been recently proposed as an anorexigenic hormone [96]. Their levels have been reported to be elevated during fasting and they return to the regular levels after refeeding. Nevertheless, there are still some gaps in the knowledge that prevents the scientific community from fully considering this peptide as orexigenic.

Ghrelin is a 28-AA acid hormone that requires post-translational processing, consisting of an acylation, to transform into its active form and interact with the growth hormone secretagogue receptor or ghrelin receptor (GHSR) [97]. This processing is carried out by the ghrelin O-acyltransferase (GOAT), mainly produced in the stomach and intestine [98,99]. Thus, GOAT levels are responsible for the circulating levels of active ghrelin also named acyl-ghrelin. The plasmatic levels of this hormone are increased before a meal and food intake trigger its reduction [100–102]. Some authors have shown that carbohydrates suppressed ghrelin secretion stronger than lipids [103,104] while others reported that ghrelin suppression is dependent on the energy content of the meal rather than the

macronutrient composition [105]. Other authors have reported the importance of both the energy content of the meal as well as the nutrient type, protein being the most effective one and lipid the least, in the reduction of ghrelin secretion [106,107]. Then, the discrepancies regarding nutrient stimulus for ghrelin secretion make it difficult to establish the more potent stimulus for this hormone.

The physiological functions of this hormone are triggered by its interaction with the GHSR [94,108]. The main established functions of this hormone are related to energy metabolism. There is evidence of ghrelin and its effect on growth hormone secretion in humans [97,109], food intake modulation also causing an increase in hunger feeling [110–112], body weight (BW) [113–115], energy expenditure [116,117], glucose homeostasis [118], and gastrointestinal motility [119–121]. The fact that the proximal X/A-like cells are close-type cells makes it difficult to decipher the exact mechanisms by which ghrelin release is regulated in response to nutrients. There is evidence *in vivo* that shows the implication of both sympathetic and parasympathetic neurons as a positive stimulus for these cells [122,123], while hyperglycaemia, insulin and somatostatin from gastric cells seem to be inhibitory regulators of ghrelin secretion [124–126]. On the other hand, sensing of all three macronutrients seems to be implicated in its secretion at the intestinal level.

Ghrelin modulation by food proteins

The hierarchy characterizing the most potent stimulus to limit ghrelin secretion remains still under discussion in the literature. Bowen *et al*. reported in a first study a decrease in plasma ghrelin levels, in overweight men, 60 minutes after an acute oral administration of whey or casein protein compared with an isoenergetic preload of carbohydrates [127]. In a subsequent study with healthy men, they described the same effect after the ingestion of a similar dose of protein (50 g) from whey, soy or gluten compared with glucose [128], which was also reported for women by Chungchunlam *et al*. [129]. Chronically, diet supplementation with whey protein, but no soy protein, has been shown to reduce fasting ghrelin levels after 23 weeks of administration compared with an

isocaloric carbohydrate-rich meal [130]. Other authors have also observed the greater effect of a high protein meal in reducing ghrelin secretion compared with high fat or a high carbohydrate meal after the protein preload [107,131]. Unexpectedly, in the same study, the authors observed an increase in plasma ghrelin after the protein preload one hour after the administration [131]. Nevertheless, an increase in ghrelin secretion after protein ingestion has been previously reported in several studies. In two different studies with healthy subjects, Erdmann *et al*. observed an increase in plasma ghrelin after a high protein meal while the high carbohydrate and the high-fat meals reduced its levels [132,133].

Contrary to these results, several authors have reported the absence of the effect of a protein dose in modulating ghrelin levels in different *in vivo* conditions. Geraedts *et al*. reported a reduction in food intake after an intraduodenal administration of intact pea protein in lean and obese men, but no effect on ghrelin levels [39]. Similarly, an acute oral administration of hydrolysed gelatine did not modulate plasma ghrelin in both lean and obese subjects [91]. Even the double protein dose, but from chicken breast, did not affect ghrelin levels compared with lipid and glucose preloads, which reduced its secretion [134]. In another group of study, with obese and normal-weight adolescent girls, a high protein meal did not alter ghrelin secretion [135]. The same absence of effect in the modulation of ghrelin was observed in mice after a long term dietary supplementation with protein [85].

These discrepancies regarding the protein effect on ghrelin secretion do not seem to be related to the protein source. As described previously, different proteins (whey, casein, soy, and gluten) exerted the same result in the modulation of ghrelin when administered in the same dose [136,137]. More sources have been found supporting this fact. Diepvens *et al*. described no differences in ghrelin secretion when comparing whey and pea hydrolysate, a combination of both protein sources and a combination of casein with whey protein [90]. Moreover,

also different protein sources reported the same absence of effect on ghrelin modulation in human subjects [39,91,134].

Studies with AAs are also found in the literature. In healthy men, the intraduodenal infusion of TRP and LEU reduced ghrelin levels more than GLN, which did not exert any effect [51]. Surprisingly, the same author did not observe this effect previously in another study with the same characteristics for both LEU [48] and TRP infusions [49]. CYS has been shown to reduce ghrelin levels in plasma compared with water and GLY after an acute oral administration in both human subjects and rodents (rats and mice) [92]. On the contrary, in sheep, duodenal infusions of GLU, GLN, LYS, THR and VAL have been reported to enhance ghrelin secretion [138]. A different study supports these results for LYS since they reported a reduced ghrelin expression in piglets under LYS restricted diets [139]. In rodents, PHE administration led to a reduced secretion both *in vivo* and *ex vivo* [92,140,141].

Thus, neither the protein source nor the macronutrient type do not seem to be the only ones responsible for the ghrelin secretion. The lack of knowledge regarding the nutrient-sensing mechanisms of the ghrelin producing cells makes the interpretation of the results difficult. However, some protein sources can be considered as candidates for reducing ghrelin secretion, intended for people with overweight problems, as well as candidates for increasing ghrelin secretion, intended for people who need to increase energy intake.

2. Insects as an Alternative Protein Source

2.1 Need to Search for Alternative Protein Sources

The world population is predicted to reach 9.7 billion in 2050 [142]. Feeding this whole population will require food production to be doubled to assess the future demand. The most traditionally consumed protein source is meat and its derived products [143]. They also suppose an important source of other nutrients, including vitamins and iron, among others. These nutrients can be supplied in

sufficient amounts by including the recommended pieces of fruit in our diet. Nevertheless, in countries where access to these foods is limited, eating meat can protect against malnutrition [144]. Western diets are characterized by a high intake of dairy products, meat and eggs. As a consequence, the consumption of saturated fat and red meat exceeds dietary recommendations [145]. To assess this big demand for protein, nowadays large livestock farms are being used. The billions of animals for food production contribute between 12% and 18% of the total greenhouse gas (GHG) emissions, with cattle being responsible for 70% of these emissions [146]. Moreover, livestock production also has a negative impact on water pollution and scarcity, on the use of land and its nitrification [147,148]. For these reasons, livestock is one of the main contributors to climate change. Thus, the big challenge of feeding this growing population needs to be addressed through more environmentally friendly food production approaches.

Reducing the demand for livestock products is a powerful mitigation tool, as supported by several authors [149–153]. The production of 1 kg of beef requires 50 times more land and about 15 times more water than the production of 1 kg of grain or vegetables. These calculations do not include the water necessary for the forage intended to feed these animals, which can increase the water requirement by 100 times [154,155]. Hence, reducing by fifty per cent the consumption of these products would considerably reduce the GHG emissions by 25-40% in the European Union [145]. This can be achieved by incorporating more vegetable proteins into our diet instead of meat proteins, as well as changing ruminant products to lower impact species, like pigs and poultry [156–159]. Another approach to mitigate the current food and feed production system impact is the use of alternative protein sources with more sustainable production. One suggested alternative protein source is *in vitro* cultured meat. Some authors consider that its production could have a lesser impact than the conventionally produced meat [160–162]. Nevertheless, there are still some weak points with regards to the scalation of its culture to an industrial level [163] and controversy about its lesser environmental impact exist [164,165]. Plant-based meat substitutes have gained interest in the last few years. Examples of plant materials

used to develop meat analogues are legumes, cereals and oilseeds [166]. One of the most used ones is soy protein, which has the advantages of a fibre matrix and gelling properties that resemble meat [167]. Soy product derivates include tofu, tempeh and yuba, which use is now more extended [166,168]. Other proposed alternative proteins are seaweed [169] and duckweed [170], mycoprotein [171], microalgae [172] and insects [173,174]. Lately, insects have been gaining more interest as an alternative protein source. They are more suitable for industrial production as well as being more economically viable, which makes them a better alternative than the other available options [175].

2.2 Why Eating Insects?

The consumption of insects, known as entomophagy, has been a common practice in many countries for a long time. Nowadays, there are still many regions where insects form part of the traditional diets, majorly in Africa, Asia and Latin America [176–178]. Nevertheless, the idea of insects as food is still mainly rejected in western countries due to a cultural barrier. People understand entomophagy as a primitive and disgusting practice [179]. Moreover, the lack of knowledge about this novel food makes its acceptance as part of our diet difficult, despite presenting several nutritional and environmental advantages [175].

2.2.1 Environmental Benefits of Edible Insects

Regarding the environment, rearing insects presents many advantages in comparison with traditional livestock. One of the most remarkable is the feed conversion efficiency, i.e., the feed needed to produce a 1 kg increase in weight. The conversion rates vary depending on the animal. Typically, 1 kg of live animal weight requires 2.5 kg, 5 kg and 10 kg of feed for chicken, pork and beef respectively [180]. Contrary, the required feed necessary to rear insects is much less. In the case of 1 kg of crickets, just 1.7 kg of feed are needed [181]. This conversion rate for insects is still higher if we adjust it for edible weight. Normally, the entire animal cannot be eaten, for example, the bones. Thus, insects become even more effective in producing food. It is estimated that the edible part

is up to 80% for crickets, 55% for chicken and pigs and 40% for cattle, which means that crickets are 12 times more efficient than cattle (**Figure 3**) [178]. Another advantage is the lower production of GHG and ammonia. As mentioned before, traditional livestock is responsible for 18% of GHG emissions, which is higher than the contribution of the transport sector [182]. The insect species considered for human consumption are calculated to produce about 100 times lower GHG emission levels compared with pigs and cattle [183]. However, this data needs to be interpreted cautiously, since these results were obtained from small-scale laboratory studies and large-scale comparisons should be performed with attention. Water is a key factor when speaking of food production. Agriculture is estimated to consume about 70% of freshwater worldwide [184]. A major part of the produced grain is intended to feed farm animals. Then, the water requirements to produce 1 kg of meat is more than 100 times higher than the requirements to produce the same amount of insect protein, taking into account the water needed for forage and feed production for animals [184–186].

For all the above-exposed reasons, insects can be considered as a very promising alternative protein source with a high environmentally-friendly production compared with the traditional animal protein production system.
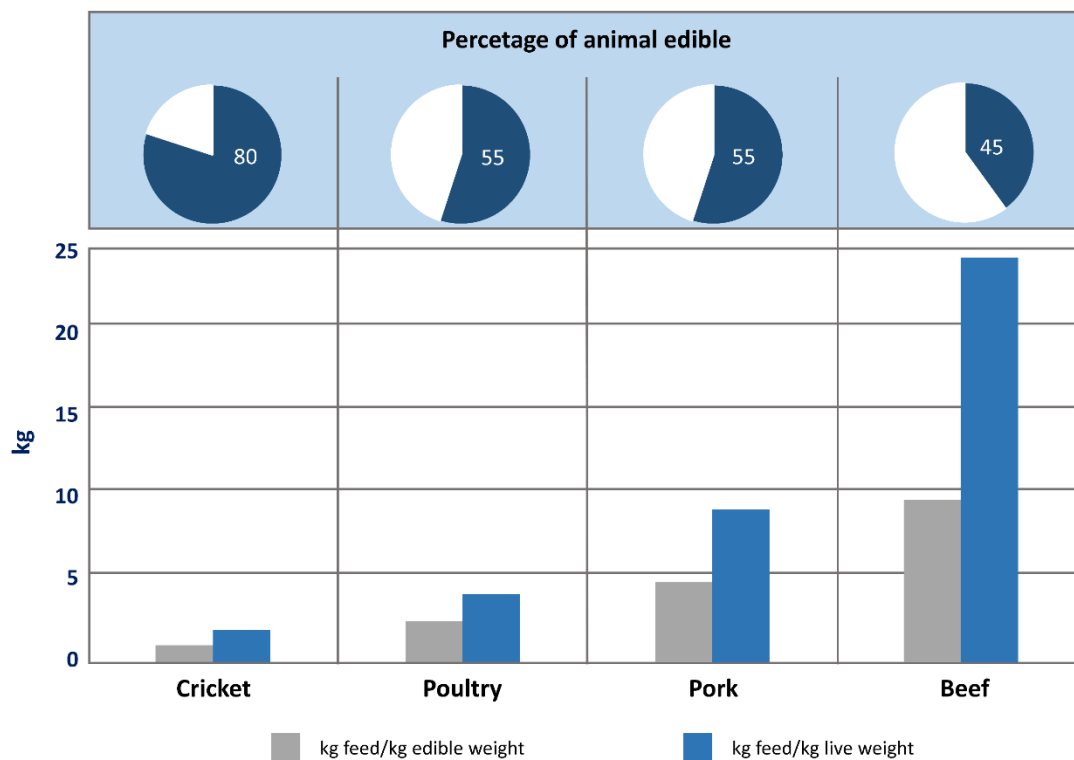
Figure 3. Efficiencies of production of conventional meat and crickets. Adapted from Van Huis *et al.*2013 [178].

2.2.2 Nutritive Value of Insects

In the body of the literature, there are many references characterizing insects as a highly nutritive food [187–189], most of the edible insect species being rich in protein, fat and essential AAs, as well as minerals and vitamins [188]. The protein content of many insect species is about 60% [190]. Its digestibility is highly variable and it is affected by chitin [191], a polysaccharide compound and the main component of the exoskeleton of insects [188]. In a study with the insect species *Tenebrio molitor*, the protein digestibility was assessed in different fractions, showing that the supernatant fraction had higher protein digestibility compared to the whole-insect or the pellet fractions [192]. Yi *et al*. concluded that insect protein content was comparable with conventional meat products [193]. Furthermore, edible insects, in general, meet the AA requirements of the World Health Organization (WHO) providing the necessary amount of essential AAs

[194,195]. Fat is the second most abundant macronutrient found in insects, ranging from 13% to 33% among the characterized insect species [188]. Besides, insects have also been reported to contain high amounts of monounsaturated fatty acids (MUFAs) and/or polyunsaturated fatty acids (PUFA) [196,197]. Finally, some species have been characterized as good sources of minerals and vitamins [197–199].

Although these nutritional values are widely variable between insect species, gender, developmental stage, diet, the environment and the feed [188,190,200–203], insects are still considered as high-quality food and a good alternative protein source.

2.2.3 Bioactivity of Edible Insects

The evidence about the beneficial effects of edible insects on health are substantially growing. The antioxidant capacity of insects and their hydrolysates has been extensively studied [204–207]. As an example, extracts of grasshopper, silkworm, and cricket have an antioxidant capacity that is 5 times greater than the antioxidant capacity of fresh orange juice *in vitro* [208]. Other authors have reported the anti-inflammatory and antioxidant capacity of insects and the therapeutic potential for Alzheimer's [209–211]. Stull *et al.* reported the anti-inflammatory effect of consuming 25 g/day of whole cricket powder during six weeks in healthy adults. This supplementation reduced systemic inflammation by decreasing plasma TNF-α, but also supported the growth of probiotic bacteria [212]. Some insect species have demonstrated their antihypertensive activity. A protein hydrolysate from *Alphitobius diaperinus* (a beetle from the Tenebrionidae family) showed the ability to inhibit the angiotensin-converting enzyme (ACE) [213]. Diet supplementation with *Tenebrio molitor* reduced blood pressure, heart rate and coronary perfusion pressure in spontaneously hypertensive rats along with neuro-protective effects [214]. It has been also reported the antimicrobial activity of some insect species, based on the action of antimicrobial peptides

(AMPs), that are short peptides that can act against bacteria, viruses, fungi or parasites [215,216].

Insects have also been studied as a treatment to ameliorate metabolic syndrome related pathologies. Chronic supplementation with *Protaetia brevitaris* larvae for seven weeks, on mice fed a high-fat diet, improved their health status by reducing BW gain, epididymal and subcutaneous fat weight, lipid droplet accumulation in liver and LDL levels among other effects [217]. Ahn *et al.* showed that an ethanolic extract of *Gryllus bimaculatus*, together with a high-fat diet, did not affect BW, epididymal fat or HDL and LDL levels in obese rats, but it reduced total and abdominal fat weight, glucose levels and protein and lipid oxidative damage in the liver, caused by the diet [218]. Other authors have also reported the effectivity of insect consumption to counteract the effect of diabetes in mice, increasing blood glucose reduction rate and reducing blood glucose during tolerance tests, improving fasting blood glucose and insulin levels [219,220]. Products derived from insects have also been studied because of interestin them as bioactive compounds. Glycosaminoglycan, a polysaccharide found in crickets, when administered to diabetic mice caused a reduction in blood glucose and LDL-cholesterol levels based on the antioxidant action of catalase, superoxide dismutase and glutathione peroxidase [221]. Chitin and its derived products like chitosan have demonstrated antimicrobial, antioxidant, anti-inflammatory, anticancer and immunostimulatory activity [222].

However, the effect that eating insects could have on the enteroendocrine system and food intake modulation has hardly been studied. In subjects under resistance training, supplementation with a bar containing *Alphitobius diaperinus* protein tended to reduce total energy intake compared with supplementation with an isoenergetic carbohydrate-rich bar [223]. One of the most studied insects is *T. molitor*. In male rats, an administration of 300 mg/kg of BW of *T. moli*tor for 28 days did not modify the BW, but showed a tendency to increase with the 1000 and 3000 mg/kg of BW doses, that was accompanied by an increase in food consumption [224]. Other authors reported no effects on food intake or BW after

four weeks of administration in Wistar Kyoto male rats [225], or in female rats after chronic treatment for 90 days [226]. On the contrary, in obese Zucker rats, a diet supplemented with *T. molitor* caused an increase in food intake and BW accompanied by lipid-lowering effects in the liver [227]. Therefore, the lack of knowledge about the bioactivity of insect protein at the intestinal level is noticeable. Generally, no adverse side effects have been reported after consumption of insects, which is supported by its extensive traditional consumption in other areas of the world as Latin America, Asia and Africa. The risks associated with their consumption are very low, and mostly related to their allergenicity [228]. Besides, it has been described as healthy as meat products [229]. Nevertheless, there is still a necessity to better understand the effects that insects, as a novel food, can exert on the organism. Thus, the characterization of the bioactivity of insects could motivate people to include this alternative protein source as part of their daily diet.

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HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

In the last decade, insect protein is emerging as an alternative solution to conventional livestock since its production presents environmental advantages. The nutritional value of insects has been extensively characterized and they are considered as an abundant source of good quality protein. Since dietary compounds modulate enterohormone secretion and food intake, studying the bioactivity of this alternative protein source at the intestinal level is of great importance to elucidate the potential health implications of including insect protein in our diet.

The gastrointestinal tract is responsible for the digestion and absorption of nutrients. But the gastrointestinal tract has also other functions. Among them, dietary compounds interact with the chemosensory machinery located in the apical side of the enteroendocrine cells that are scattered along the gastrointestinal tract, leading to the secretion of different enterohormones. These hormones, which are mainly GLP-1, CCK, PYY and ghrelin, modulate the digestive process but also different metabolic processes such as glucose and energy homeostasis and food intake by modulating satiety and hunger.

Protein is considered the most satiating macronutrient. During its digestion, different small peptides and free amino acids are released, which interact with the nutrient-sensing machinery, modulating the enteroendocrine secretion. Dairy protein is one of the most studied protein sources. Whey, casein and different derived hydrolysates have been reported to increase GLP-1, CCK, and PYY plasma levels in human subjects. Other protein sources have also demonstrated different ability to enhance gut hormone secretion *in vitro* and *in vivo*. However, little is known about the bioactivity of insect protein at the intestinal level.

Altogether, **we hypothesized that bioactive compounds derived from an alternative protein source, insect protein, can modulate the enterohormone secretion, and subsequently food intake, differently from other protein**

sources and that they can have long-term activity on the enteroendocrine function of the gastrointestinal tract.

To demonstrate our hypothesis, we have defined these specific objectives:

1. To study the effects of insect protein on the enteroendocrine system and its effectiveness on food intake modulation acutely in rats.

The nutritional value of insects has been extensively characterized. Some species are contemplated as rich sources of high-quality protein. Since protein is considered the most satiating macronutrient, we aimed to study the bioactivity of this alternative protein source at an intestinal level. Therefore, we aimed to compare the acute effects of insect protein with the other protein sources on the enteroendocrine secretory profile *ex vivo* and the effects on food intake *in vivo* in rats.

2. To test the acute effects of an insect protein preload on food intake in human subjects.

Animal models are a valuable tool to perform a first screening of the effects of the administration of a new compound and to study the implicated mechanisms. Nevertheless, our last objective is to translate these results to the human population to establish the benefits of insect protein administration in the target group. As there is a lack of knowledge regarding the bioactivity of this new food after its ingestion in humans, we aimed to study the effects of an acute administration of insect protein preload on food intake together with the analysis of appetite and other sensations, assessed by established questionnaires.

3. To analyse if a chronic administration of insect protein can exert changes in the enteroendocrine system and modulate food intake.

Insect protein is considered an alternative protein source to be included in our diet. Hence, we aimed to characterize the effects of a chronic administration of insect protein *in vivo*, to see if the potential acute effects on food intake, planned

to be addressed in objective 1, could be maintained over time. We also wanted to analyse if the effects on food intake could be translated to body weight modulation, and to clarify the mechanisms involved in such effects.

RESULTS

PART 1

Study of the effects of insect protein on the enteroendocrine system and its effectiveness on food intake modulation acutely in rats.

Manuscript 1

Gastrointestinally Digested Protein from the Insect *Alphitobius diaperinus* Stimulates a Different Intestinal Secretome than Beef or Almond, Producing a Differential Response in Food Intake in Rats

Published in Nutrients, 7 August 2020

Manuscript 2 *

Molecular Composition of Lipid and Protein Fraction of Almond, Beef and Lesser Mealworm After *in vitro* Simulated Gastrointestinal Digestion and Correlation with the Hormone-Stimulating Properties of the Digesta

Submitted to Food Research International, 3 February 2022

* Most of this work has been accomplished together with my colleague Francesca Accardo (University of Parma). Only the results related to the enterohormone secretion and food intake modulation by protein samples belong to this thesis. The other results belong to the future thesis of my colleague.

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MDPI

Article

Gastrointestinally Digested Protein from the Insect *Alphitobius diaperinus* Stimulates a Different Intestinal Secretome than Beef or Almond, Producing a Differential Response in Food Intake in Rats

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Abstract: In this study we compare the interaction of three protein sources—insect, beef, and almond—with the gastrointestinal tract. We measured the enterohormone secretion ex vivo in human and pig intestine treated with in vitro digestions of these foods. Insect and beef were the most effective in inducing the secretion of CCK, while almond was the most effective in inducing PYY in pig duodenum. In the human colon, almond was also the most effective in inducing PYY, and GLP-1 levels were increased by insect and beef. The three digested proteins reduced ghrelin secretion in pig duodenum, while only insect reduced ghrelin secretion in human colon. We also found that food intake in rats increased in groups fed a raw insect pre-load and decreased when fed raw almond. In conclusion, the insect *Alphitobius diaperinus* modulates duodenal and colonic enterohormone release and increases food intake in rats. These effects differ from beef and almond.

Keywords: dietary protein; in vitro digestion; gut; enterohormones; food intake; insect; almond; beef

1. Introduction

The world population is predicted to reach 9.7 billion inhabitants in 2050 [1]. This presents a major challenge that can be faced through more environment-friendly food and feed production models. In the twenty-first century, insects as food and feed are emerging as an alternative solution to conventional livestock. The mass production of insects is economically viable and presents some environmental advantages, such as the high feed conversion efficiency, emission of fewer greenhouse gases and less ammonia, and they require less land and water than cattle rearing [2,3]. As food or feed, insect protein could complement animal protein to address the challenge of feeding a rising population [4,5].

Consuming the recommended quantity of good-quality protein is essential for optimal human growth, development, and health [6]. Some research has characterized insects as an abundant source of

high-quality protein and essential amino acids [7]. Although entomophagy has been a long-standing part of life in many cultures [2], other societies still reject the practice primarily due to cultural biases towards consuming insects and a lack of knowledge about this novel food [2]. The nutritional value of insects has been extensively documented [8,9]. In addition to protein quality and quantity, they have high-quality fat content with high MUFA and/or PUFA content, and are rich in minerals and vitamins [10–12]. Although this composition varies depending on the insect's species [9], sex [13], and diet [14], edible insects are considered a nutritious alternative to traditional animal protein derived from livestock. However, there is a lack of information about their bioactive properties. Some studies, mostly *in vitro*, have examined the effects of edible insects on human health, including their interaction with microbiota [15], antihypertensive peptides [16], and their antimicrobial function [17], among other health benefits [18,19]. Nevertheless, the possible effects of insect intake at the gastrointestinal level have rarely been the focus of study.

The gut is the largest endocrine organ in the body [20], and secreted enterohormones are involved in a wide range of physiological and metabolic processes, such as appetite regulation, gastric motility, and glucose homeostasis. The enteroendocrine cells (EECs) present in the gastrointestinal tract interact with nutrients and secrete hormones in response to food ingestion, acting as chemosensors of the lumen content [21]. EECs are scattered throughout the gastrointestinal tract, and the hormones they produce are concentrated in specific regions of the gut, according to their roles in regulating these physiological functions [22,23]. Each gut hormone appears in the bloodstream based on its individual temporal profile, which is determined by patterns of food intake, nutrient absorption, and the distribution of EECs along the intestinal tract [24,25]. Dietary compounds can modulate enterohormone secretion. For example, protein hydrolysates, or even intact protein, induce the secretion of satiety hormones. In the body of literature on proteins, dairy protein is one of the most studied: casein and whey proteins have been shown to increase GLP-1, CCK, and PYY plasma levels in human subjects [26,27]. Other plant and animal protein sources have also demonstrated their capacity to enhance gut hormone secretion in in vitro and in vivo experimental conditions [28–30].

In this study, we aimed to compare how animal proteins from insects and beef and plant protein from almonds interact with the gastrointestinal tract. We focused our analysis on how they act on the enteroendocrine system and food intake regulation. We assayed their effects on the secretome in ex vivo models of human and pig intestine treated with in vitro digestions of these protein sources. We next assayed the satiety capacity of raw insect, beef, and almond in vivo in rats.

2. Materials and Methods

2.1. Chemicals and Reagents

Raw insect (Buffalo *Alphitobius diaperinus* powder) and IPC (*Alphitobius diaperinus* insect protein concentrate powder) were provided by Protifarm. Beef (a lean portion, Protifarm NV, Ermelo, the Netherlands) was purchased at a local market (Mercat Central, Tarragona, Spain) and almond (*Prunus dulcis*) flour was provided by Borges Agricultural & Industrial Nuts (BAIN). The nutritional composition of these samples, provided by producers or bibliography [31], is presented in Table S1. All the samples were stored in the dark at −20 °C for optimal conservation.

Chemicals, porcine digestive enzymes (α-amylase, pepsin, and pancreatin, Sigma-Aldrich, Madrid, Spain) together with bile salts were purchased from Sigma-Aldrich (Madrid, Spain). Pancreatin contains enzymatic components including trypsin, amylase, lipase, ribonuclease, and protease.

The ELISA kits for acyl-ghrelin (catalog no. EZRGRA-90K), total ghrelin (catalog no. EZGRT-89K), and total GLP-1 (catalog no. EZGLPT1-36K) were purchased from Millipore (Billerica, MA, USA). ELISA kits for CCK (catalog no. EKE-069-04), PYY (catalog no. FEK-059-03), and human PYY (catalog no. FEK-059-02) were purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). We used them according to the instructions provided by producers that could be easily obtained in the websites

of each enterprise with detailed references (see Table S2). We measured all the samples in the sample plate for each hormone (intra-assay variation detailed in Table S2).

2.2. In Vitro Digestion

Foods were digested according to the INFOGEST harmonized protocol [32], first published in 2014 by Minekus et al. [33], which consists of the simulation of the three main stages of in vivo digestion: the oral, gastric, and intestinal stages. In our study, we used samples digested up to the gastric phase (gastric digestion) and totally digested samples (intestinal digestion). The two types of digestion occurred in parallel. Food quantity was adjusted by protein content in order to achieve the same ratio of protein per volume for all the samples (0.12 g protein/mL simulated saliva).

2.2.1. Gastric Digestion

The different foods were minced with 20 mL of simulated saliva using a mincer (Ultra-Turrax T25; IKA Werke, Staufen, Germany) for 2–5 min. Amylase was added after this step (75 U mL^{-1}) and mixed with the minced food for 2 min. Then, 20 mL of simulated gastric juice containing pepsin (2000 U mL^{-1}) was added and mixed for 120 min. Half of the gastric digestion samples were stopped at this point, and the other half went on to the next step.

2.2.2. Intestinal Digestion

To 20 mL of gastric digestion sample, 20 mL of simulated intestinal juice containing pancreatin (100 U mL^{-1}) and bile salts (10 mM) was added and incubated for 120 min.

We also applied the same procedures to all four food samples but without enzymes as a negative control for digestion, and as an enzyme control we followed the procedure using only the enzymes and simulated fluids without food. Finally, we placed all the digestions and the controls in a 90 °C bath for 20 min to stop the enzymatic reactions. After that we minced and centrifuged all the samples (4000 rpm, 5 min, 4 °C) to discard the undigested fractions: a pellet for all samples and an upper layer of fat for the almond and insect digestions. The samples were stored at −20 °C.

2.3. Characterization of Digestion Products

The glucose and triglyceride contents of the digested samples, negative controls, and enzyme controls were quantified using commercial kits obtained from QCA (Amposta, Spain). A BCA kit was used (Pierce, Thermo Fisher Scientific) to measure protein content.

SDS-PAGE electrophoresis was performed to test the digestion process. Thirty microliters of digested sample (protein concentration of 2 µg/µL) was mixed with 10 microliters of 4 × sample loading buffer (125 mM Tris HCl (pH 6.8), 2.5% (*w/v*) sodium dodecyl sulfate (SDS), 0.1% (*w/v*) bromophenol blue, 25% (*v/v*) glycerol, 25% (*v/v*) β-mercaptoethanol) and heated at 100 °C for 5 min. From this, fifteen microliters were loaded on a 16% polyacrylamide gel. A molecular weight marker (Page Ruler, Thermo Fisher Scientific) and undigested samples were included on each gel. Gels were then stained with colloidal Coomassie Blue (Bio-Rad Laboratories).

2.4. Tissue Collection

2.4.1. Pig Tissue

Intestinal segments of duodenum were obtained from three female pigs (*Sus scrofa domesticus, Landrace X Largewhite)* that were slaughtered for meat production at a local slaughterhouse. The pigs were commercial breeds that weighed approximately 120 kg at slaughter and had been fasted for approximately 24 h prior to slaughter. The procedure for collecting and preparing the tissue for the experiment was previously established by Ginés et al. (2018) [34]. Five minutes after slaughter, the intestines were excised and segments of the anatomical regions required for the experiment (duodenum) were placed in ice-cold oxygenated (95% O$_2$, 5% CO$_2$) Krebs–Ringer bicarbonate (KRB)

buffer (pH 7.4) with D-mannitol 10 mM (Sigma Aldrich, Madrid, Spain). The tissues were promptly transported to the laboratory at 4 °C and immediately used for the experiments. The time elapsed between excision and the beginning of the experiments was about 30 min.

2.4.2. Human Tissue

Human colon segments were collected from 10 donor patients of both sexes (median age 65 years) who had undergone colon surgery due to colorectal carcinoma. Only subjects who satisfied the study criteria were selected. The exclusion criteria were the consumption of anti-inflammatory drugs, alcohol abuse, and intestinal bowel disease or celiac disease, as these would alter intestinal functioning. The characteristics of the patients included in this study are summarized in Table S3 of the supplementary material. All donor patients provided informed consent and the study was approved by the Clinical Research Ethics Committee (CEIC) of the Hospital Universitari Joan XXIII in Tarragona (CEIm 101/2017). Healthy tissues not needed for diagnostic purposes were excised from the proximal and distal colon. After resection, these colon tissues were transferred from the hospital within 30 min in ice-cold oxygenated KRB buffer (pH 7.4) with D-mannitol 10 mM. This tissue collection protocol was previously established by González-Quilen et al. (2020) [35].

2.5. Ex Vivo Experiments with Intestinal Segments

After rinsing the pig duodenum with KRB buffer, it was mounted on a plastic tube to facilitate stripping—the removal of the serosal and outer muscular layers—with a scalpel. The intestinal tube was then sliced longitudinally and circles of tissue with a diameter of 14 mm were taken using a biopsy punch. This process took approximately 15 min. The sample was kept at a low temperature with cold buffer and an ice-cold bath during the entire procedure. We then started the secretion study. We placed each duodenum circle in a well (24-well plate) containing 1 mL of KRB buffer with D-mannitol 10 mM pre-warmed to 37 °C for 15 min. After this pre-incubation period, the buffer was replaced by the same volume of pre-warmed treatments, diluted in KRB buffer containing glucose 10 mM (Sigma Aldrich, Madrid, Spain), protease inhibitors aprotinin 100 KIU (Sigma Aldrich, Madrid, Spain) and amastatin 10 µM (Enzo Life Sciences, Madrid, Spain), and 0.1% BSA (Sigma Aldrich, Madrid, Spain). The control group was treated with the same solution (KRB buffer with glucose) without any additions. The incubation period was 60 min for the PYY and CCK secretion studies and 90 min for the active ghrelin secretion study. In this case, as we were studying the enterohormonal secretion profile in pig duodenum, we used the gastric digestions as treatments. We simultaneously assayed all the treatments in the same animal tissue. These tissue explants were divided into five treatment groups: control (*n* = 3 replicates), enzyme control, insect, beef, and almond (*n* = 2 replicates). For all the digested samples, the protein content was adjusted to a dose of 15 mg/mL, as we have previously reported its effectiveness in modulating enterohormone secretion in intestinal segments [36]. The enzyme control was diluted by half, using the same dilution factor as for the digested insect sample to have a similar enzyme concentration as in treatment groups. After the incubation period, the medium was collected and stored at −80 °C in different aliquots for further analysis.

The tissue processing and the experimental protocol used for the human colon was largely the same as described for the pig tissue. The colon explants, measuring 5 mm in diameter, were placed in a 48-well plate, previously filled with 400 µL KRB buffer with D-mannitol. In this experiment, we used the intestinal digestions diluted to 5 mg/mL protein content. For each human donor sample, we obtained six colon explants that were divided into six groups: control, enzyme control, insect, IPC, beef, and almond. The enzyme control was diluted by one-third as in the digested insect sample. After a 30 min incubation period, the medium was collected and stored at −80 °C in different aliquots.

2.6. Food Intake Study in Rats

Ten female RccHan:WIST rats (8 weeks old, 220–240 g) were obtained from provider ENVIGO (Castellar del Vallés, Spain). Upon arrival, the animals were housed in pairs for a week and then

individually in animal quarters for another week to get them accustomed to oral administration. The rats had free access to food, standard chow (Teklad2014 from ENVIGO), containing (by energy) 20% protein, 13% fat, and 67% carbohydrates, and tap water. Room temperature was maintained at 22 °C with a 12 h light/12 h dark cycle (lights from 7:00 am to 7:00 pm). The animals were used in the experiments after this two-week acclimation period. The Animal Ethics Committee of University Rovira i Virgili (Tarragona, Spain) approved all procedures (CEA-OH/10715/3).

We tested three different protein sources: insect, beef, and almond. The design of the study involved the treatment of the ten animals in six different experimental days (Figure S1). In each experimental day two of the three protein sources were tested (5 animals per group). There was a washout period of a minimum of two days. Then, the rats were redistributed randomly in two other different groups to again test two of the three protein sources. After six experimental days, each rat was treated at least twice with each protein. We administered each protein source 20 times. The experimental design ensured that the animals received the same protein more than once. For this reason, for the calculations we considered the mean of each of the 10 animals (each one with 2–4 administrations of each protein source).

To test the protein sources each experimental day, animals were food deprived starting at 5:00 pm. We administered the protein load at 6:00 pm every treatment day. Rats were administered the different protein sources dissolved in the vehicle by controlled oral intake with a syringe. As vehicle, we used an artificial saliva with the same composition as described by Minekus et al. [33]. All animals received a dose of 300 mg protein/kg of body weight (BW). They had free access to food again when the dark cycle began at 7:00 pm. Food intake was measured 3, 12, and 20 h after the dark cycle began.

Control food intake of all the animals was measured twice during the washout periods.

2.7. Statistical Analysis

The results are expressed as the mean ± standard error of the mean (SEM). The sample size (n) for each variable is indicated in the corresponding figure description. A one-way ANOVA test and Tukey's post-hoc test were used for multiple comparisons. p-values < 0.05 were considered statistically significant. These calculations were performed using XLSTAT 2020.1.2 software (Addinsoft, New York, NY, USA).

3. Results

3.1. Characterization of Digested Samples

To ensure that the digestion protocol worked properly, we analyzed the protein hydrolysis with SDS-PAGE electrophoresis. As Figure 1 shows, the protein pattern between digested (+) and non-digested (−) samples was very different. The number and intensity of bands in the non-digested samples was much more pronounced than in the digested samples. The bands in the samples from the gastric phase (Figure 1a) were more intense for the digested samples than for the intestinal digested samples (Figure 1b). As the digestive process progressed the proteins became smaller, generating more peptides, and the bands become fainter. These small peptides are difficult to visualize in gel electrophoresis, and this is why the digested samples appear with less intensity in Figure 1b.

It is important to know a food's composition in order to explain the effect that it can have at an intestinal level. We therefore examined the composition of both the gastrically and intestinally digested foods. Table 1 shows that after gastric digestion, the protein content was similar across our samples, as we defined. Since the digestion process itself diluted all the components to half at the beginning of each phase, after the intestinal phase, both protein and glucose levels were lower than they were in the gastric phase. As we did a centrifugation step to obtain homogeneous samples with which to treat the intestines, we obtained an upper lipidic phase and a precipitate with the debris and undigested remnants. This separation procedure had a clear effect on the triglyceride levels of the gastric digestions. They were very different from one another, with different proportions of

triglycerides than in the original raw samples (Table S1). The triglyceride levels were higher after the intestinal phase, as expected due to the presence of bile salts.
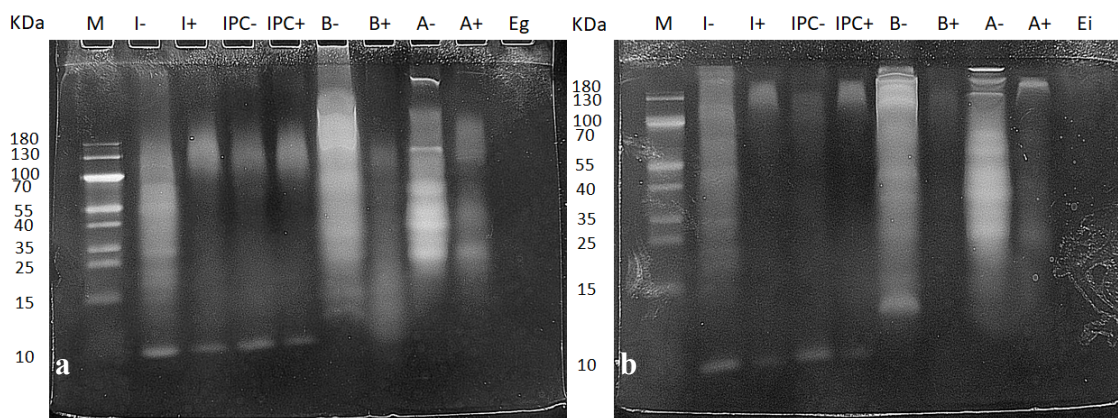

Figure 1. Protein hydrolysis of food after gastric (**a**) and intestinal (**b**) digestion. Samples marked with "+" are the digested ones and the samples marked with "−" are the negative controls for each digestion. The protein load was adjusted to 30 µg of protein in each lane, except for the enzyme controls, which were used without dilution. A molecular weight marker (10–180 kDa) was included. I, insect; IPC, insect powder concentrate; B, beef; A, almond; Eg, gastric enzyme control; Ei, intestinal enzyme control; M, molecular weight marker.

Table 1. Protein, glucose, and triglyceride composition of digested food samples after gastric and intestinal in vitro digestion.

Samples		Protein mg/mL	Triglycerides mg/mL	Glucose mg/mL
	Insect	26.60	5.84	0.20
	IPC	31.49	15.30	0.22
Gastric digestion	Beef	21.60	0.92	0.09
	Almond	26.55	2.24	0.12
	Enzymes	0.65	0.05	n.d.
	Insect	15.02	8.97	0.09
	IPC	14.38	12.91	0.08
Intestinal digestion	Beef	9.28	0.70	0.06
	Almond	10.29	9.44	0.1
	Enzymes	0.62	0.05	n.d.

n.d.: not determined.

3.2. Insect Gastric Digestion Stimulated Pig Duodenal Enteroendocrine Secretions Differently than Beef or Almond

To evaluate the stimulation produced by these digested extracts on enterohormone secretion, we first tested the gastric in vitro digested samples on intestinal duodenum pig segments. Our results (Figure 2) showed that insect and beef digestions stimulated the secretion of CCK more than almond or the control. Additionally, insect and beef showed a similar effect on PYY secretion, but almond was the most effective at increasing PYY secretion. All of the foods tested significantly reduced ghrelin secretion, with beef being the most effective and insect the least. The enzyme control did not affect hormone release, as expected.
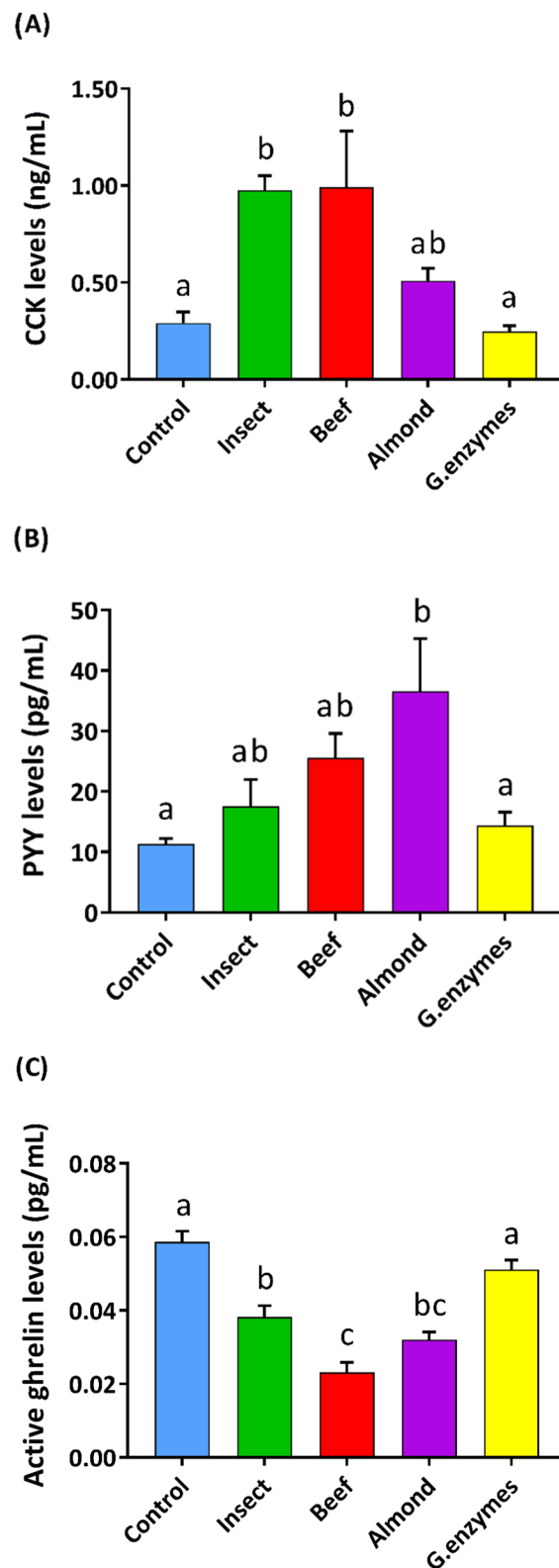
Figure 2. Effect of different gastric digestions (15 mg protein/mL) and the enzyme control on CCK (**A**), PYY (**B**), and active ghrelin (**C**) pig duodenum secretion in explants. The incubation period was 60 min for CCK and PYY and 90 min for active ghrelin secretion, all after 15 min of stabilization. The sample size was control (KRB buffer with D-glucose 10 mM) $n = 9$, treatments $n = 6$. Results are expressed as the mean ± SEM. One-way ANOVA and Tukey's post-hoc test were used for multiple comparisons. Different letters (a, b, c) indicate significant differences (p-values < 0.05).

3.3. Insect Intestinal Digestion Induced GLP1 Secretion and Reduced Ghrelin Secretion in Human Colon Samples

We were able to use human colon samples for the purpose of studying intestinal digestions. We also assayed *in-vitro*-digested IPC, as it is a commercially available product intended for human consumption. Because our focus was on the colon, and the amount of protein that reaches this site in vivo is expected to be lower, we used a smaller quantity of protein (5 mg protein/mL) with these samples. Figure 3 shows that raw insect and beef were the most effective in inducing the secretion of total GLP-1 (tGLP-1), as a measure of all GLP-1 secreted by these samples. IPC also increased tGLP-1 similarly to insect (0.19 ± 0.04 and 0.12 ± 0.02 pg/mL of GLP-1 for insect and IPC, respectively). PYY levels only increased after treatment with almond digestions. In the case of total ghrelin levels, the insect and IPC treatments (49.48 ± 3.41 and 43.35 ± 3.51 pg/mL, respectively) statistically limited ghrelin secretion compared to the control and to the other samples.
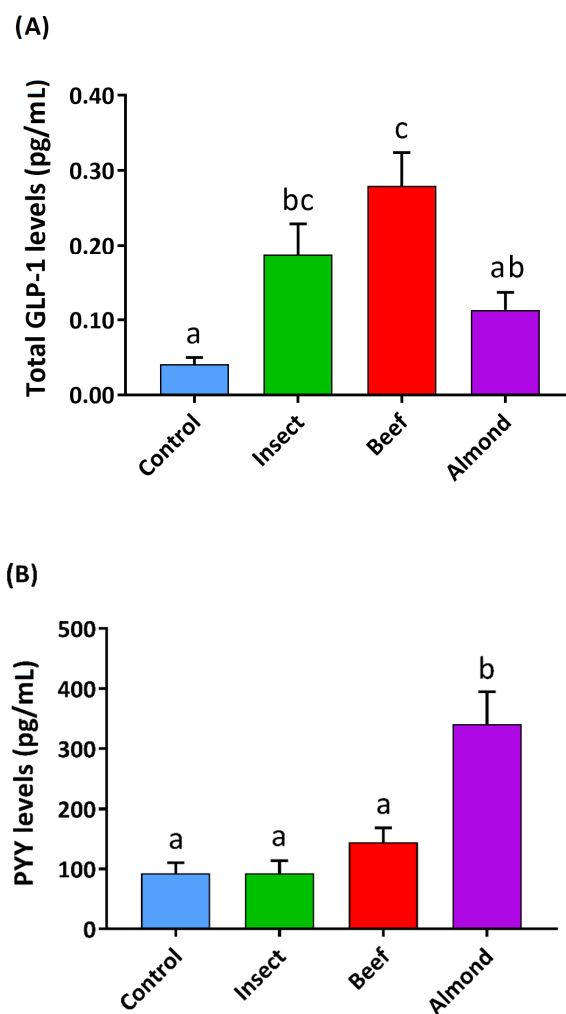
(A)

(B)

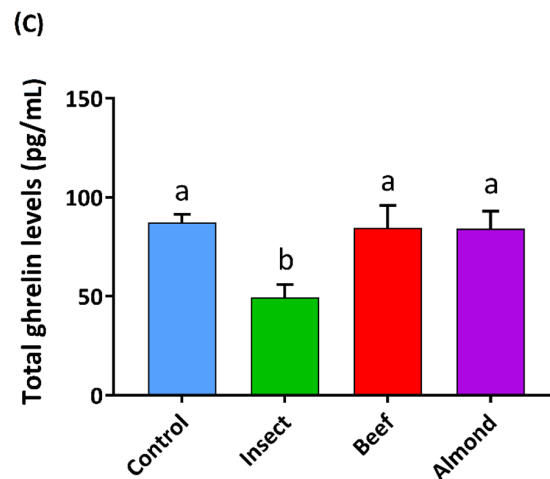**Figure 3.** *Cont.*

(C)

Figure 3. Effect of intestinal digestions of different foods (5 mg protein/mL) on total GLP-1 (**A**), PYY (**B**), and total ghrelin (**C**) human colon secretion in explants. The incubation period was 30 min after 15 min of stabilization. The sample size was $n = 10$ for control and treatment groups. Results are expressed as the mean ± SEM. One-way ANOVA and Tukey's post-hoc test were used for multiple comparisons. Different letters (a,b,c) indicate significant differences (p-values < 0.05).

3.4. Buffalo Alphitobius diaperinus Powder Stimulated Food Intake in Rats

As we found different secretions for the different assayed protein sources, we investigated their in vivo effects on food intake. We measured the energy intake in rats (Figure 4) after the oral administration of an equal protein dose of the three different foods (300 mg protein/kg BW, which corresponded to kJ (kcal) per rat: 2.7 (0.64) for insect, 2.0 (0.48) for beef, and 9.5 (2.27) for almond (composition detailed in Table S4). We observed that 3 h after the animals began eating (four hours after the treatment), the almond-treated rats ate less, while the insect-treated rats ate somewhat more compared to the control and beef groups. This effect was maintained twelve hours after the lights were switched off. In the final intake measure, 20 h after the start of the dark period, we observed that the almond-treatment group continued to consume less food than the other groups. The beef group showed a slight tendency towards higher intake than the control group, and the insect-treated group significantly increased its energy intake compared to the control group. These results suggest that after an administration of an equal dose of protein, insect protein stimulates food intake while almond protein provides the greatest satiating effect. The effect of insect and beef was also evident at 20 h if the energy content of the protein load (i.e., the total energy intake of each animal including the protein dose) is considered (Figure S2).
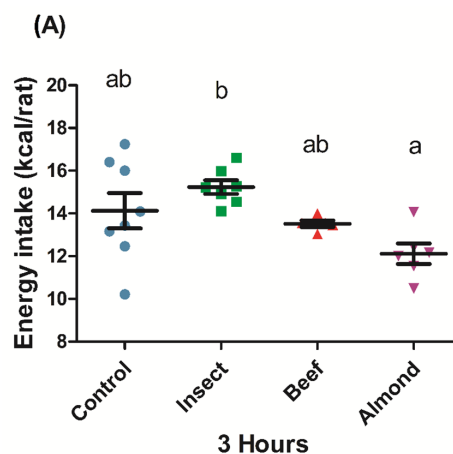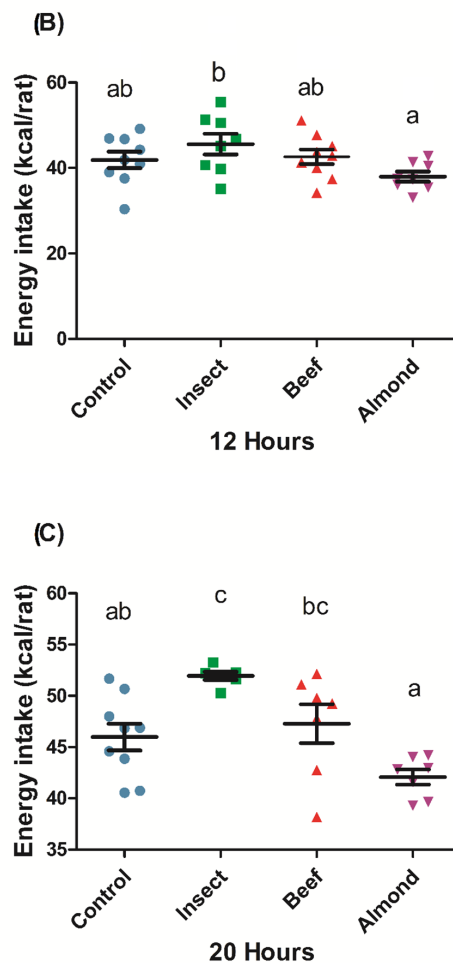
(A)

Figure 4. *Cont.*

Figure 4. Energy intake after oral administration of a protein dose (300 mg/kg BW). Orally digested samples from insect, beef, or almond were administered to rats. Food intake was measured at three different intervals: 3 h (**A**), 12 h (**B**), and 20 h (**C**) after the beginning of the dark cycle. The control was an equivalent volume of tap water. The sample size was $n = 10$ rats per group. The results are expressed as the mean ± SEM. One-way ANOVA and Tukey's post-hoc test were used for multiple comparisons. Different letters (a,b,c) indicate significant differences (p-values < 0.05). The circle, square, and triangle means feeding with different source of proteins.

4. Discussion

In the present study, we compared the enterohormone secretion profile of three different *in-vitro*-digested protein sources, working on ex vivo systems of pig and human intestine. Our results show that the different protein sources produce a different secretome in both types of intestinal segments as well as differing effects on food intake responses in rats.

To test the effects of insect, beef, and almond on different intestinal segments (duodenum and colon), the food was first subjected to an in vitro digestion process, using a standardized methodology [32]. Our results for the SDS-PAGE electrophoresis are consistent with those of other authors [32]. Using beef samples [37], almond samples [38], or the same insect we used [39], they started with a similar pattern of proteins in the original sample and they disappeared similarly to ours after the different digestions. We can therefore assume that the protein in our samples was successfully hydrolyzed.

We assayed the duodenal enterohormone response to gastric digestion products working with pig samples. This species is considered a close and valid model for human studies [40], which facilitated the set-up for the procedure. Pig duodenum has been reported to produce high secretions of PYY, together with CCK secretions and measurable amounts of acylated ghrelin [41]. Our results showed

that digested insect, almond, and beef significantly modulated the duodenal enterohormone profile, increasing the release of PYY and CCK and reducing that of ghrelin. In addition, the enterohormone secretion profile was different for each one, even though the treatments were performed with the same quantity of protein (15 mg/mL). This suggests that the total amount of protein is not the only factor responsible for the secretion of these satiety hormones. CCK secretion has been reported to be stimulated by lipids and protein [23,42], and PYY is secreted in proportion to caloric intake [42,43]. The reduction of ghrelin levels is proportional to the energy load and macronutrient content, and its suppression is greatly promoted by protein and less effectively by lipids [44,45]. Considering the composition of the digestion (Table S5), the largest component is protein, followed by triglycerides and glucose in a much smaller proportion. The differences in triglycerides also implies that the energy load of each treatment it is different: the insect is the most caloric, followed by almond and beef. Our results show that the increased secretion of PYY and CCK or the inhibition of ghrelin secretion did not correlate with the caloric content or triglycerides of the different food sources, suggesting that the quality of the protein is the primary factor responsible for the observed differences.

As observed with the duodenum, neither the energy load nor the macronutrient composition of the intestinally digested food (Table S6) explains the effects on enterohormone secretion in the colon. We also found differences among foods in PYY and ghrelin secretions, as well as in tGLP-1 secreted in this intestinal segment. We worked with complex mixtures of dietary components that underwent a transformation during the digestion process. We simulated the in vivo digestion process with the INFOGEST harmonized protocol [32], which is very close to in vivo pig protein hydrolysis, as demonstrated by Egger et al. [46]. It has been shown that protein hydrolysates have different effects on the enteroendocrine system, mainly due to the presence of specific peptides [47]. The amino acids and di-, tri-, and oligopeptides generated during protein digestion interact with EECs [25]. These resulting peptides differ depending on the source of the protein, the enzymes present, and the physicochemical conditions of the process. Here we include a new protein which has not been previously assayed on enterohormone secretions. Each intestinal segment responds to nutrient intake differently. In addition, the compositions of gastric and intestinal digestion are different, and this is likely true regarding the resulting peptides as well. Further characterization of the hydrolysis products of our different samples will help to elucidate the primary factor responsible for each enterohormone secretion.

Differences between the secretomes could give rise to differing physiological effects, such as changes in food intake patterns. Protein has been reported to have a good satiety capacity compared to other macronutrients [29,48]. In this study we show that beef, almond, and insect produced different effects on food intake after the administration of an equivalent amount of protein. We found that the orally digested almond was the most satiating in rats. This could be due to the fact that the caloric load administered for almond was higher than that for the other foods (Table S4). The total energy intake, including the caloric load of the treatments, did not differ from the control (Figure S2). The satiating effect of almond disappeared in the overall energy intake, which was similar to that observed in other experiments where, although it had the highest satiety quotient, almond total energy intake did not differ from the other treatments [48]. We did not anticipate finding that administering insect protein would increase food intake, even when considering the total energy intake. These differential effects cannot be explained by the secretome found in the ex vivo studies, since, overall, we observed an increase in anorexigenic hormones and a reduction in orexigenic ghrelin in proportions that did not correlate with the effects on the intake of each food. Actually, in vivo hormonal profile is complex and depends not only on the direct effects of food stimulation of receptors, but also on the indirect paracrine effects of secreted hormones, the inactivation of these hormones, and other factors. In this paper, we did not aim to fully decipher the mechanism of action of each protein source, but we show that they acted differently on the secretome and that they had different effects on food intake. Differences in food intake after the administration of an equivalent protein dose have previously been reported in the literature. In a study in rats, a high-protein meal composed of egg white suppressed food intake while a wheat-gluten-based meal containing the same percentage of protein, fat, and carbohydrates

increased food intake [49]. Other examples are found in the literature for human studies. A whey pre-meal was found to similarly reduce food intake to the same protein load of casein [50], soy, turkey, egg, or tuna [51,52]. In the digestion process, as previously discussed, proteins are hydrolyzed in different ways according to their physicochemical properties. A clear example is the difference between whey protein, which enters the jejunum mostly intact, and casein, which appears mainly in the form of degraded peptides, attributed to the precipitation of casein in the acid media of the stomach and the longer exposure to gastric hydrolysis [53]. Therefore, another important factor that may explain these differences in food intake regulation could be the digestibility of each food. It can limit the derived products of hydrolysis (i.e., peptides and amino acids), which can modify the secretome obtained in each case. Of the indices that evaluate protein quality in relation to digestibility, one of the most widely adopted is the protein digestibility corrected amino acid score (PDCAAS) [54]. Raw almond has been classified with a PDCAAS value of around 45 [55] and beef with a value of 92 [56], approaching the maximum of 100. Little is known in this regard for insect species, and each species has very different particularities. The chitin present in insects has been shown to affect protein digestibility [57]. In a study with the insect species *Tenebrio molitor*, the protein digestibility was assessed in different fractions, showing that the supernatant fraction had higher protein digestibility compared to the whole-insect or the pellet fractions [58].

5. Conclusions

In conclusion, in this study we demonstrated that insect, beef, and almond modulate food intake differently when administered in equal amounts of protein: *Alphitobius diaperinus* increased food intake in rats, while almond limited it. We also showed that the digestion of these food sources modulates enterohormone release. The analysis of the amino acid and peptide composition of gastric and intestinal in vitro digestion could lead to a better understanding of the enterohormone secretion profile we obtained with these ex vivo experiments. The quantity, source, bioavailability, and hydrolysis of proteins are key points that affect their interaction with the EECs, in both ex vivo and in vivo conditions.

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Molecular Composition of Lipid and Protein Fraction of Almond, Beef and Lesser Mealworm after *in vitro* Simulated Gastrointestinal Digestion and Correlation with the Hormone-Stimulating Properties of the Digesta

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ABSTRACT

The current production of meat presents many disadvantages for the environment and much research focuses on alternative protein sources. Insects are novel protein sources highly valued for their nutritional and sustainable potential. However, many aspects concerning biological and nutritional proprieties of the insects after digestion, in comparison with other protein sources, are still overlooked. In this work, a comparative study on three different protein sources, namely almond, lean beef and insect *Alphitobius diaperinus* (lesser mealworm), was performed after *in vitro* simulated gastrointestinal digestion. An in-depth characterization of the chemical composition of the solubilized protein and lipid fractions of the digesta was performed by applying different analytical techniques, including chromatographic methods coupled to mass spectrometry and ^{1}H NMR spectroscopy. Then, the biological activity of the digesta was investigated focusing on the enterohormone secretion of GLP-1 by *in vitro* cell tests. Moreover, the possible correlations of PYY, ghrelin, GLP-1 and CCK release and rats' food intake with the molecular composition of the digesta were also examined. Results indicated a comparable level of solubilized protein from lesser mealworm larvae and lean beef digestion, together with a good lipolysis rate. Insects proved to be good meat equivalent, in terms of solubilized proteins and amino acid profile. Almond gave the least solubilized protein, while it presented a high lipolysis rate similar to the one measured in lesser mealworm at the end of the digestion. A positive correlation was observed between the biological activity and the amino acid composition of the solubilized protein fraction. The increase of GLP-1 hormone secretion was mostly related to His, HPro, Lys, Leu, Met, Cys amino acids when present in the digestate mixture. The composition of amino acids in insect digesta seemed to have specific effects on enterohormone release of CCK, total ghrelin in the human colon, and the modulation of food intake in rats.

Keywords: gastrointestinal digestion; *in vitro* digestibility; hormone release; food intake; almond; beef; insect.

1. INTRODUCTION

Nowadays, there is a continuous growth in the global protein demand, driven by the increase of the global population and the socio-economic shifting of dietary preferences [1]. Indeed, many researchers are focusing their attention on the use of new, sustainable and high-value protein sources. Among them, edible insects are considered good candidates to be new protein sources for feed and food applications. Insects are appreciated for their nutritional value, quite similar to the traditional animal product, having a good amount and a good nutritional quality of their main constituents, namely proteins [2–4] and fat [5–7].

The nutritional quality of a given food is influenced by its composition in essential nutrients, including its ability, once ingested, to be properly absorbed by the organism to satisfy the body's requirements.

Nutrient-gut interactions define the effects, other than the nutritive ones, that food can have in our organism. To study the bioactivity of foods is necessary to understand the underlying mechanisms of these nutrient-gut interactions. Ex vivo and in vitro techniques are essential to pursue this purpose before translating the results to the clinic [8].

Current methods of static or dynamic *in vitro* gastrointestinal digestion can simulate the complex digestive process of food. Therefore, *in vitro* digestion from the chemical and biochemical point of view becomes a valuable tool to determine the bioaccessibility [9] of food matrices in terms of enzymatic hydrolysis and solubilization of their components. Indeed, the availability of nutrients after digestion can be evaluated on the soluble fraction of the digestate. The soluble fraction can be studied through various analytical techniques and the value of the soluble fraction is calculated based on the total amount of the nutrient [10]. Working with digested samples is fundamental in secretory studies with intestinal models, as in physiological conditions the food always reaches this tissue already digested.

An example of one of the most employed *in vitro* static methods is represented by the harmonized INFOGEST protocol [11,12], used in this work to obtained digested protein and lipid fractions of selected foods and insects.

From a nutritional point of view, the value of a protein depends mainly on its amino acids composition and digestibility [13]. Dietary proteins can be found in animal (e.g. eggs, dairy products, meat, fish, insects) and vegetable (e.g. cereals, legumes) sources. Animal proteins are considered to be high-quality among other food sources since they have more balanced proportions of essential amino acids than plant-based food. Furthermore, the animal-derived proteins are appreciated for their higher digestibility, about 95% more than the 80-85% exhibited by those from vegetable sources [14].

Most dietary lipids consist of triacylglycerols (TG), which are composed of glycerol esterified to three fatty acid chains of varying length, degree of unsaturation and location within the TG molecule [15].

The nutritional value of dietary lipids, in terms of energy source and their role on human health, is strongly affected by their fatty acid profile [16]. Saturated fatty acids (SFAs), such as palmitic and stearic acids, are mainly supplied by animal fat (i.e., meat, seafood, milk, cheese, eggs, etc.) but also by some plant oils (i.e., coconut and palm oils). Meat content in SFAs depends on animal species, growth, and environmental conditions [17]. On the other hand, lipids derived from plants (e.g., olive oil) or marine algae generally contain higher levels of unsaturated fatty acids (such as oleic, linoleic and linolenic acids), while fish oils are most notable for containing the omega 3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DPA) [18].

The availability for absorption of fatty acids into the human digestive system largely depends on the extent of lipolysis after digestion as well as their stereospecific distribution in the TG, as, for example, long-chain SFAs, located at the outer positions sn-1 and sn-3, have limited absorption [19].

The information on the molecular composition of gastrointestinal digesta both at the protein and lipid level is still very scarce for insects, in comparison with other common protein sources. Therefore, the study of the changes of the chemical and biological properties after gastrointestinal digestion has become a challenge.

In our previous published work [20], on the same protein sources, the interaction between different proteins sources and the gastrointestinal tract was evaluated, concerning the enterohormone secretion in human and pig intestine membrane (i.e., *ex vivo*) and food intake regulation in rats (i.e., *in vivo*). The data obtained in our previous investigation underline the different hormone secretory profiles of all the digested protein sources tested as well as the different modulation of food intake in rats, although no information on the molecular composition of the digesta was obtained.

In this study, lesser mealworm larvae (*Alphitobius diaperinus*), almond (*Prunus dulcis*) and lean beef (*Bos taurus*) were evaluated for their raw composition, and the percentage of released lipids and proteins was measured after having performed *in vitro* simulated gastrointestinal digestion. Degree of hydrolysis of both lipid and protein fractions was also measured and related to the solubilisation after the digestion process. Then, the previously observed biological properties were related to the compounds present in the digesta, to better understand the relation between the food digestate composition and hormones released. In addition, the ability of the digesta to affect the secretion of GLP-1 enterohormone was evaluated and related to its specific amino acid composition.

2. MATERIALS AND METHODS

2.1. Solvent and reagent

20x XT MES running buffer, 20x XT reducing agent, 4x XT sample buffer, Coomassie brilliant blu protein stain powder R-250, Criterion TMXT Bis-Tris Precast Gel (12% Bis-Tris, 13.3 x 8.7 x 0.1 cm), and Precision Plus Protein

Standards from BIO-RAD (Hercules, CA, USA). Quant-iT™ Protein Assay Kit was purchased from Invitrogen (Carlsbad, CA, USA). Kjeldahl defoamer was purchased from Merck (Darmstadt, Germany). Hydrogen peroxide, Kjeldahl tablets catalyst (3.5 g/tablet), sulfuric acid (96%), Ultrapure water obtained with Milli-Q® system, The ELISA kit for total GLP-1 were purchased from Merck Millipore (Burlington, MA, USA). Aspartic acid, bile salts, boric acid, Cysteine, formic acid (>95%), Glutamine, hydrochloric acid (37% HCl), L-Isoleucine, Methionine sulfone, NAC (N-acetyl-cysteine), DL-norleucine, OPA (o-phthaldialdehyde), pancreatin (containing enzymatic components including trypsin, amylase, lipase, ribonuclease, and protease) from porcine pancreas, porcine α-amylase, porcine pepsin from gastric mucosa, SDS (Sodium dodecyl sulfate), Tryptophan and the amino acids standards for biological assay were purchased from Sigma Aldrich (St. Louis, MO, USA). Amino Acid Standard Mixture (2.5 mM) was purchased from Thermo Scientific (Waltham, MA, USA). Kjtabs VS defoamer was purchased from VELP Scientifica (Usmate Velate, Italy). Acetonitrile (ACN) and Copper (II) oxide were purchased from VWR Chemicals (Radnor, PA, USA). AccQ•Fluor Reagent Kit for Amino Acid Analysis was purchased from Waters (Milford, MA, USA).

2.2. Sample selection

Raw insect larvae belonging to lesser mealworm species (*Alphitobius diaperinus* powder) were provided by Protifarm (Protifarm NV, Ermelo, the Netherlands). Beef (*Bos taurus*), a lean portion, was purchased at a local market (Mercat Central, Tarragona, Spain) and almond (*Prunus dulcis*) flour was provided by Borges Agricultural & Industrial Nuts (BAIN). All the samples were stored in the dark at −20ºC for optimal conservation. We chose to compare insects as a new protein source with beef, as one of the traditional protein sources and almond as a vegetal protein source.

2.3. *In vitro* digestion

Foods were digested according to the INFOGEST harmonized protocol [21], first published in 2014 [12], which consists of the simulation of the three main stages

of the *in vivo* digestion: the oral, gastric, and duodenal stages. In our study, we used samples digested up to the gastric phase (Oral-Gastric (OG) digestion) and gastrointestinal digested samples (Oral-Gastric-Duodenal (OGD) digestion). The two types of digestion occurred in parallel. Food quantity was adjusted by protein content to achieve the same ratio of protein per volume for all the samples (0.12 g protein/mL simulated saliva).

Oral-Gastric digestion. The different foods were minced with the simulated saliva using a mincer (Ultra-Turrax T25; IKA Werke, Staufen, Germany) for 2–5 min. Amylase was added after this step (75 U mL−1) and mixed with the minced food for 2 min. Then, the simulated gastric juice containing pepsin (2000 U mL−1) was added to a final ratio of 50:50 (v/v) and mixed for 120 min at 37ºC. The OG digested samples were stopped at this point, and the OGD samples went on to the next step.

Oral-Gastric-Duodenal digestion. The OG digested samples were added with the same volume of simulated intestinal juice containing pancreatin (having a trypsin activity of 100 U mL−1) and bile salts (10 mM) to obtain a final ratio of 50:50 (v/v) and mixed for 120 min at 37ºC.

We also applied the same procedures to all three food samples but without enzymes as a negative control for digestion, and as an enzyme control, we followed the procedure using only the enzymes and simulated fluids without food. Finally, we placed all the digestions and the controls in a 90 °C bath for 20 min to stop the enzymatic reactions. After that, we minced the samples to avoid the protein clotting formed after the heating. Then we centrifuged all the samples (3220*g*, 5 min, 4 °C) to discard the undigested fractions: a pellet for all samples and an upper layer of fat for the almond and insect digestions. The samples were frozen at -80 °C and freeze-dried. After that there were stored at -20 ºC.

2.4. Determination of residual moisture

The determination of the relative moisture was performed on each lyophilized sample. 0.5 g of each sample, in duplicate, was dried at 105 °C until reaching a constant weight.

2.5. Lipid analysis

2.5.1. Determination of lipids content

The determination of fat content on lyophilized raw, gastric and gastrointestinal digested samples using Soxhlet extraction was performed. The fat content was extracted by Soxhlet apparatus (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy) using 1 g of each lyophilized sample and 70 mL of diethyl ether. The following method was used for the beef and almond samples: 30 minutes of extraction, 60 minutes of washing and 15 minutes of recovery. For the insect, the method used included: 60 minutes of immersion, 30 minutes of washing and 15 minutes for recovery.

2.5.2. Determination of fatty acids profile

Soxhlet lipid extracts obtained from samples before simulated gastrointestinal digestion (A RAW and I RAW) were subjected in duplicate to acid-catalyzed transmethylation according to the ISO 12966-2:2017 protocol [22], slightly adapted. Briefly, 100 mg of fat were added to 0.5 mL of H_2SO_4/MeOH (1:15 v/v) and heated at 100 °C for 3 hours. After cooling, fatty acid methyl esters (FAMEs) were recovered with 5 mL of n-hexane containing 0.2 mg of internal standard tetracosane. This solution was diluted to match the linearity range of the GC–MS instrument, by taking 100 µL and diluting with 800 µL of n-hexane. Then, samples were split-injected (1 µL) on a Thermo Scientific Trace 1300 gas-chromatograph (Thermo Scientific, Waltham, Massachusetts, USA) carrying a SUPELCOWAX® 10 capillary column (30 m x 0.25 mm x 0.25 µm, Supelco, Bellafonte, USA) coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). The concentration of each detected fatty acid was calculated in relation to the concentration of the internal

standard, after calculating the response factors using the Supelco 37 Component FAME Mix (Sigma Aldrich, Saint Louis, MO, ®USA). Finally, results were expressed as relative percentage of total FAMEs.

2.5.3. ¹H NMR analysis of lipid classes before and after simulated gastrointestinal digestion

¹H NMR analysis was performed on the same samples analyzed for fatty acid profiles and the Soxhlet lipid extracts of samples before (A RAW and I RAW) and after simulated oral-gastric phase (A OG, I OG), simulated oral-gastric-duodenal phase (A OGD, I OGD) and, as control, the simulated gastrointestinal fluid (blank). For NMR analysis, about 100 mg of fat were dissolved in 0.8 mL of deuterated chloroform (CDCL3) in a 5mm glass tube. ¹H NMR spectra were registered on a Bruker Avance III 400 MHz NMR. Spectrometer (Bruker BioSpin, Rheinstetten, Karlsruhe, Germany) operating at a magnetic eld-strength of 9.4 T. Spectra fi were acquired at 298 K, with 32 K complex points, using a 90° pulse length and 3 s of relaxation delay (d1). 128 scans were acquired with a spectral width of 9595.8 Hz and an acquisition time of 1.707 s. The relaxation delay and acquisition time allow the complete relaxation of the protons, allowing their integrals for quantitative purposes. The whole zone ranging from 0.87 to 2.90 ppm plus signal centered at 5.35 ppm were used as determinant indicative of total fatty acid moles, both free and bound. For the glycerol esters, the specific signals (listed in **Table 5**) were integrated. Integrals were normalized for the number of hydrogens contributing to the specific signal. In the case of fatty acid integral, the mean number of hydrogens in fatty acids was inferred from the mean fatty acid composition obtained by GC analysis, and it was found as 33.27 for almond and 32.83 for insects. The normalized areas obtained were converted as relative molar percentages.

Quantification of the lipolytic products

To determine the molar percentage (mol %) of free fatty acids (FFA), the total fatty acid molar percentage was subtracted from the contribution of fatty acids bound to triacylglycerols (TG), diacylglycerols (DG) and monoacylglycerols

(MG), according to the simple relation previously reported (Nieva-Echevarría *et al.*, 2014):

$$[FFA] = [TFA] - (3[TG] + 2[DG] + 1[MG])$$

The extent of lipid digestion

The extent of lipid in vitro digestion has been estimated as the degree of transformation of TG (TTG%), which considers the hydrolysis that occurred in TG, according to the following equation reported [23]:

$$TTG\% = 100 \left[(mol\% \ TGi - mol\% \ TG)/ \ mol\% \ TGi \right]$$

where mol% TGi is the molar percentage of TG initially present in the sample.

2.6. Protein analysis

2.6.1. Protein content

The protein content was evaluated for raw and digested samples using the official Kjeldahl method (DKL heating digester and UDK 139 semiautomatic distillation unit, VELP SCIENTIFICA) according to European Regulation EC 152/2009, the nitrogen was quantified and subsequently multiplied by the conversion factors respectively: almond 5.18 [24], beef 5.57 [25] and insect 6.25.

2.6.2. Electrophoretic profile

The protein pattern of raw and digested samples was evaluated using SDS-PAGE analysis. 0.01 g of each lyophilized sample in 10 mL of water/acetonitrile (50:50 ratio) solution was dissolved. Each sample was mixed with an overhead shaker for 1 h at room temperature and centrifuged for 15 min at 3220g and 4 °C. A rate of all the samples corresponding to 30 µg of protein (quantified by Quant-iT™ Protein Assay) was dried under nitrogen flux. Each sample was added with 25 µL of a solution consisting of sample buffer xT 4X, reducing agent 20x and ultrapure water, subsequently loaded into the electrophoretic gel. The electrophoretic run was performed at a constant voltage of 150 V using a Mini-Protean II electrophoresis chamber (Bio-Rad, Hercules, CA, USA). The main proteins were visualized on the gel by staining through Coomassie Blue.

2.6.3. Evaluation of the degree of hydrolysis in digested samples

The degree of hydrolysis (DH) is the percentage of severed peptide bonds compared to the number of total peptide bonds. The DH % was calculated using the OPA method as previously described [26,27] to obtain a solution that absorbs at a wavelength of 340 nm, using a UV-VIS spectrophotometer (B530 JASCO, Oklahoma City, OK, USA). The calibration curve was prepared using L-isoleucine from 0 to 2 mg / mL. The OPA assay was carried out using 3 µL of the digested sample (or digestion blank), 17 µL of milli-Q water and 2.4 mL of OPA/NAC reagent. During digestion proteins were hydrolyzed, a high number of free amino groups determines a greater protein digestibility. The percentage of the degree of hydrolysis was calculated as reported below:

$$DH\% = \left(\frac{N_{free}}{N_{total}}\right) x\ 100$$

N_{free} was calculated from OPA and N_{total} corresponded to the total nitrogen moles present in the solution before hydrolysis, calculated by the Kjeldahl method.

2.7. Total amino acid

For evaluation of total amino acid profile, 0.1 g of lyophilized sample (raw and digested) were added with 6 mL of 6 M HCl, mixed and hydrolyzed for 23 hours at 110 °C. Subsequently, the samples at room temperature were added with 7.5 mL of 5 mM norleucine water solution, used as internal standard. In conclusion, in a volumetric flask (250 mL) the samples were filtered up to the mark. 10 µL of the final solution obtained, from total amino acid procedures, were derivatized using AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA, USA) and analyzed by UPLC-MS as previously reported [28].

In addition, another protocol for the determination of the sulfur-containing amino acids (methionine and cysteine) was used in the present study. 0.05 g of each lyophilized sample was added with 0.2 mL of fresh performic acid (previously mixing 95% formic acid and hydrogen peroxide in a 90:10 ratio and incubating 1 hour at room temperature and 1 hour at 4 °C). The samples were

incubated in an ice bath at 0 °C for 16 hours. Subsequently, 0.03 mL of pure hydrobromic acid (48 wt % in water) was added, mixed and dried under nitrogen flux. The dry matter was resuspended by the addition of 0.6 mL of 6 M HCl, mixed for 1 min under nitrogen flux and incubated at 110 °C for 23 hours. Finally, the samples at room temperature were added with internal standard (0.75 mL of 5 mM norleucine), filtered and made up to the mark in a 25 mL volumetric flask with demineralized water. The samples were derivatized using AccQ-Tag Ultra Derivatization Kit (Waters, Milford, MA, USA) and analyzed by UPLC-MS. Tryptophan was determined as previously described [29] reducing the amount of sample to 0.1 g.

2.8. Protein and peptides identification by high-resolution mass spectrometry

Each lyophilized digested sample was analyzed in high-resolution mass spectrometry using tandem mass spectrometry analysis to identify the main protein and peptide release after OG and OGD digestion. µHPLC (Dionex Ultimate 3000, Sunnyvale, CA, USA) coupled to Orbitrap LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used in the present study.

The condition parameters were previously reported [30] except for the loading flow: 30 µL/min, 98% eluent A and 2% eluent B and for protein identification the precursor ion tolerance: 10 ppm.

2.9. Correlation with biological properties of the digested mixtures

A heat map was created to visualize the total amino acid content associated with every source (insect, almond, beef) based on the Wilcoxon rank-sum test, logistic regression, and random forest classification. The different samples as well as the amino acids were reordered by hierarchical clustering. On top of the heatmap a dendrogram of the experiments is given, that is a tree showing the order in which the samples are merged. Samples with similar outcomes are grouped next to each other. The dendrogram on the left of the heatmap gives the similarity between the amino acid percentage in the samples.

Unsupervised principal component analysis (PCA) was performed with XLSTAT 2021.2.1 software (Addinsoft, New York, NY, USA) to assess relationships between the enterohormone secretions obtained from intestinal tissue explants, previously published [20], and the amino acid composition of the digested samples and between the food intake from rats and the amino acid composition of the raw food administered. Principal components (PC) were considered significant if they contributed >50 % to the total variance.

2.10. Cell culture

GLUTag cells were kindly donated by Prof. Staels (Institut Pasteur de Lille, Lille, France) with permission of Prof. Drucker (Lunenfeld-Tanenbaum Research Insitute, Toronto, Canada). The cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 1 g L−1 glucose, supplemented with 10% fetal bovine serum (Sigma-Aldrich, Madrid, Spain), 100 U mL−1 penicillin, and 100 mg L−1 streptomycin (Lonza, O Porriño, Spain) and incubated under a 5% CO_2-humidified atmosphere at 37 °C.

2.11. GLP-1 secretion studies

GLUTag cells were plated onto 24-well plates precoated with matrigel (Lonza, O Porriño, Spain) at a density of 200.000 cells/ml 24 h before the secretion study. Cells were then washed twice with HEPES buffer and treated for 2h with the mix of the amino acids more abundant in the OGD digested beef (His, HPro, Lys, Leu, Met, Cys with a concentration of 0.34, 0.11, 0.42, 0.88, 0.22, 0.34, 0.26 mg/ml respectively) or vehicle (HEPES buffer). After the treatment the medium was collected and stored at -80ºC until the determination of total GLP-1 by ELISA kit. Cells were lysed with RIPA buffer and lysates were analyzed for total protein content (to control for an equal number of cells). At least three replicates using cells with different cell passage numbers were performed for each experiment, including at least three wells for each condition in every replicate. Cells with passage numbers below 30 were used.

2.12. Statistical analysis

Data analysis was performed using SPSS version 26.0 software (SPSS Inc., Chicago, IL., USA). The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's-b test for the homogeneous variance of sample to define significant differences between samples ($p < 0.05$). For GLP-1 secretion analysis Students' unpaired t-test was used and only differences with P-values <0.05 were considered significant.

3. Results and discussion

3.1. Composition of the food matrices: lipids, protein and amino acid profile

3.1.1. Lipids and protein content

Lipids were analyzed in all samples by the Soxhlet method, to evaluate the total amount of lipid fraction before digestion. Furthermore, also the nitrogen content was determined in raw samples by the Kjeldahl method in all samples and converted to protein content by multiplying for specific conversion factors. The reported conversion factors for almond and beef were used [24,25] (5.18 and 5.57 respectively). On the other side, the commonly used 6.25 conversion factor was used for insects, due to its extensive use by insect-producing companies. It is any way to be reminded that previous papers already demonstrated that this conversion factor is not the most accurate to determine the protein content of insects [3,31], due to the specific amino acid composition of insect, and mostly to their content of chitin (nitrogen-containing polysaccharide): both features lead to an overestimation of the actual protein value when the 6.25 factor is used.

The amount of lipids and proteins found in raw samples, on dry weight, is reported in **Table 1**. Lipid and protein content for lesser mealworm (*Alphitobius diaperinus*) samples were in agreement with previous literature data [32].

For all raw samples statistical differences were observed. Lipids in almonds showed the highest value, followed by insects, whereas their amount was found

to be very low in raw beef. On the contrary, the protein content on the dry matter was higher in beef than in insects, with almonds showing the lowest value.

Table 1. Lipid and Protein percentages (on dry weight) in raw samples.

Sample Name	Lipid content (% dw) (Soxhlet)	Protein content (% dw) (Kjeldahl)
Almond	56.7 ± 0.2	22.3 ± 0.4
Beef	6.6 ± 0.1	77.4 ± 0.1
Lesser mealworm	28.5 ± 0.5	58.8 ± 0.2

dw, dry weight.

3.1.2. Fatty acid profile

The almond and insect raw lipids were characterized for their fatty acid profiles by GC-MS. Results are reported in **Table 2**. The analysis was not performed in the case of beef considering the negligible amount of lipids contained in the sample.

In both cases the values found agreed with previous literature data [33]. The fatty acid composition of almond oil consisted primarily of oleic acid (C18:1 cis-9) at about 60%, followed by linoleic acid (C18:2n-6) at about 30% and only trace amounts of α -linolenic acid (C18:3n-3; ALA). The remaining 10% of the fatty acid profile is constituted by saturated fatty acids (SFA), such as lauric (C12:0), myristic (C14:0), C15:0, palmitic (C16:0) (the most abundant at 7%) and stearic (C18:0) acids. Regarding insect samples, the main fatty acids were represented by oleic and linoleic acids at about 30% each and palmitic acid (C16:0). Minor amounts of α-linolenic acid (C18:3n-3; ALA) were also detected. This composition is in line with what has already been reported for *Alphitobius diaperinus* [32,34], performing the same extraction method, except for the above reported minor amount of α-linolenic acid detected.

Table 2. Relative percentages of fatty acids detected in almond and lesser mealworm larvae oils. Fatty acids were determined as methyl esters after methylation in acidic media performed in duplicate. Relative coefficient of variation (CV%) ranged from 2% to 10%.

Fatty Acid	Raw almond	Raw Lesser mealworm
C12:0	0.1	n.d.
C14:0	0.5	1.1
C15:0	0.1	0.2
C16:0	7.1	27.2
C16:1 cis-9	0.9	n.d.
C18:0	2.4	6.8
C18:1 cis-9	59.4	34.3
C18:2n-6 (LA)	29.6	28.0
C18:3n-3 (ALA)	n.d.	2.3
Σ SFA	10.1	35.4
Σ MUFA	60.3	34.3
Σ PUFA	29.6	30.3

LA: Linoleic Acid; ALA: α-Linolenic Acid; SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids.

3.1.3. Total amino acid profile

Total amino acid content is shown in **Table 3** with the results expressed as g of amino acids / 100 g of food matrix (on dry weight). From the total amino acids, it was possible to calculate the true protein content of every matrix, by calculating the total mass of the residual amino acid moieties, i.e., the mass of the amino acid after subtraction of a water molecule (since the amino acids are bound in a condensed form in proteins). This latter method for protein content gives indeed more realistic results than the one based on nitrogen determination through Kjeldahl analysis and subsequent multiplication for a predefined factor to obtain the protein mass. In particular, the use of conversion factors not perfectly tailored to the proteins under analysis, and the presence of non-proteic sources of nitrogen, are definite sources of error in the Kjeldahl analysis. Obviously, this

source of error can be avoided if we deduce the protein content only considering the amounts of total amino acids, which are the protein constituents.

Table 3. Total amino acid profile in raw samples (g/100 g dry weight).

Amino Acid	Almond	Beef	Lesser mealworm
Ala	1.1 ± 0.1	4.43 ± 0.003	4.1 ± 0.1
Arg	2.8 ± 0.2	6.1 ± 0.2	2.41 ± 0.01
Asp	2.8 ± 0.2	7.37 ± 0.02	5.1 ± 0.1
Cys	0.6 ± 0.1	2.6 ± 0.1	1.27 ± 0.01
Glu	6.9 ± 0.3	12.57 ± 0.05	7.0 ± 0.1
Gly	1.53 ± 0.02	4.7 ± 0.2	2.7 ± 0.1
His	0.7 ± 0.1	3.4 ± 0.2	1.5 ± 0.1
HPro	0.0	0.9 ± 0.1	0.0
Ile	0.73 ± 0.03	3.3 ± 0.1	2.08 ± 0.05
Leu	1.7 ± 0.1	7.2 ± 0.1	3.96 ± 0.04
Lys	0.47 ± 0.01	3.4 ± 0.2	1.3 ± 0.1
Met	0.21 ± 0.01	2.56 ± 0.02	0.96 ± 0.02
Phe	1.5 ± 0.1	4.4 ± 0.3	3.1 ± 0.1
Pro	1.1 ± 0.1	3.8 ± 0.2	3.70 ± 0.03
Ser	1.20 ± 0.02	4.18 ± 0.01	3.04 ± 0.07
Thr	0.79 ± 0.02	4.8 ± 0.1	2.77 ± 0.02
Trp	0.11 ± 0.01	0.29 ± 0.02	0.33 ± 0.0003
Tyr	0.86 ± 0.01	3.7 ± 0.4	4.99 ± 0.04
Val	0.97 ± 0.01	4.0 ± 0.1	3.10 ± 0.01
Total amino acids	26.0 ± 0.7	83.52 ± 0.03	53.3 ± 0.2
Total protein content[a]	22.3 ± 0.7	71.81 ± 0.03	45.8 ± 0.2

No significant differences were found between samples after one-way ANOVA followed by Tukey's-b test ($p > 0.05$).

[a] Derived from total amino acid content considering the molecular mass of the residual amino acid moieties (i.e., without a water molecule).

Thus, the data obtained on total protein content evaluated by amino acids composition was compared to the protein amount calculated by the Kjeldahl method. For almonds, no significant differences were observed, the protein content was in perfect agreement between the two methods. For beef and insect significant differences were observed between the two methods. Indeed, for beef, the values obtained by Kjeldahl were slightly overestimated compared with total protein calculated by total amino acid (6% more with Kjeldahl), very likely due to the presence of nitrogen-containing compounds naturally present in meat such as carnosine, anserine and creatine (non-proteinogenic origin) [35–37]. In stark contrast, the lesser mealworm protein content obtained by Kjeldahl was highly overestimated (30% more with Kjeldahl): this can be ascribed, as already anticipated above, to the 6.25 factor used to convert the amount of nitrogen in protein (not the correct one given the amino acid composition), and to the fact that chitin is also contained in the insect biomass, yielding a consistent amount of non-proteic nitrogen wrongly included in the Kjeldahl analysis.

3.2. Characterization of solubilized lipids and proteins after digestion

Samples underwent simulated gastrointestinal digestion according to the procedures detailed in the experimental section (2.3) and already reported in the previous paper [20]. Lipids and proteins were quantified after digestion in the soluble part of the digested samples. It can be assumed that the substance solubilized during the digestion phases is closely related to the substance available for absorption after digestion, thus the percentage of protein and lipids solubilized can be related to protein and lipid digestibility in the various matrices [38].

3.2.1. Solubilized lipids after digestion

The lipid content (% dry weight) after OG and OGD digestion of insect and almond were obtained by the Soxhlet method. Beef samples were not analysed, as giving the very low content of lipids in the original raw samples, would have led to unreliable data. Results are reported in **Table 4** as percentage of solubilized lipids relative to the total amount of lipids originally present in the sample.

Table 4. Solubilized percentage of lipids (as referred to starting raw material) after OG and OGD digested samples.

Sample Name	Solubilized lipids after OG (%)	Solubilized lipids after OGD (%)
Almond	20 ± 2	30 ± 1
Lesser mealworm	16 ± 0.2	23 ± 1

OG, oral-gastric, OGD, oral-gastric-duodenal

Given that the OG digestion contains no lipases, the solubility data obtained after OG is likely to be the same as the original raw sample, possibly increased by the triglyceride hydrolysis induced by the acidic environment. Anyway, even after the duodenal phase, which contained lipases, the increase in solubilized lipids was somehow limited, implying a low activity of the lipase (possibly due to poor accessibility of the enzyme) or to an intrinsic poor solubility of lipids also in form of free fatty acids.

^{1}H NMR analysis was performed to evaluate the lipid class distribution in almond and larvae before and after OG and OGD digestion. To obtain quantitative data on the relative amount of each lipid class, a simple procedure based on ^{1}H NMR spectroscopy was used, adapted for the identification and quantitation of different lipid species in the analysed oils, including triglycerides (TG), diglycerides (DG), monoglycerides (MG) and free fatty acids (FFA), as described above [39]. The quantitative molar proportions of the different acyl groups present in the lipid extracts of the analysed samples are reported in **Table 5**.

From the above data, it clearly emerges that TG after digestion markedly decreased as compared to the raw samples, while FFA (at 76.5 % in digested larvae and 39.4 % in almond oils), followed by total MG (as the sum of 1-MG and 2-MG, at 17.6 % in digested insect and 20.8 % in almond oils) became the predominant lipid classes in both the digested matrices.

Table 5. Relative molar percentages determined by ^{1}H NMR of the different glycerides and free fatty acids present in solubilized lipid fractions of lesser mealworm and almond before (RAW) and after both simulated OG and OGD) digestion. Coefficient of variations (CV%) max 5%.

Lipid class	Lesser mealworm			Almond		
	RAW	OG	OGD	RAW	OG	OGD
1,2-DG	2.8	3.9	1.0	2.6	2.9	6.9
2-MG	0.7	1.4	0.0	0.0	0.2	3.6
1,3-DG	2.1	2.4	1.4	1.4	2.1	2.9
1-MG	3.6	8.8	17.6	1.5	3.7	17.2
TG	67.9	24.5	3.5	94.5	78.4	30.1
FFA	22.8	59.1	76.5	0.0	12.7	39.4

OG, oral-gastric, OGD, oral-gastric-duodenal

[1] Simulated gastro-intestinal fluid.

However, by comparing the distribution of the lipid classes, a difference in the relative abundance of the FFA among the two matrices can be observed, which resulted to be higher in insect lipids than in almond lipids, both before (22.8% and 0.0%, respectively) and after digestion (76.5% and 39.4%, respectively). Indeed, as previously reported, the natural content of FFA in vegetable oils is generally limited (< 10%) and increases with increasing storage time, temperature, and humidity [40]. On the other hand, the high free fatty acid content determined in raw lesser mealworm lipids is probably due to the high concentration of lipase enzyme, consequently its strong activity depending on killing method and storage, as previously reported for other insects [6]. Anyway, this higher amount of FFA in insects did not affect lipid solubility, as seen above, which resulted in quite similar for both matrices.

It is to be noted a consistent increase in free fatty acids, in both matrices (but in a more consistent way in insects) also after gastric digestion, which, as said above, given the absence of any lipase in that phase, can only be due to the hydrolysis induced by the acidic environment.

3.2.2. Protein profile after digestion

The protein pattern of undigested and digested samples, including negative digestion control (same digestion mixtures, but without enzymes), is shown in **Figure 1**. All raw undigested samples showed clearly separated protein bands. The most abundant proteins found in almond seed were Prunin 1 (61 kDa) and Prunin 2 (63 kDa) which under reducing conditions yielded the acidic and the basic subunits at 42 kDa and 20 kDa, as previously described [41]. The most intense bands of the proteins found in raw beef were shown at ~250 kDa and over 37 kDa corresponding to Myosin (220 kDa) and Actin (41-42 kDa). However, other lighter bands were visible from 15 kDa to 150 kDa region. For raw lesser mealworm, two main bands were visible at 25 kDa and over 37 kDa, but the incomplete insect database hampered a clear-cut attribution of proteins.

For almond and beef, after OG digestion only weak protein bands were observed, with intense smearing in the low molecular weight region, indicating the presence of peptides coming from digested proteins. For insect samples, after OG digestion the main bands originally present in the raw sample did not weaken, suggesting pepsin resistance. Anyway, in all samples the protein bands completely disappeared after OGD digestion. Negative digestion control showed intact protein bands, confirming that their disappearance was due to the action of proteolytic enzymes.

3.2.3. Protein solubilization and hydrolysis degree after digestion

To characterize protein digestibility, the solubilized fractions after digestion was determined by the Kjeldahl method, total and free amino acid and degree of protein hydrolysis, the results are reported in **Figure 2**. The nitrogen content obtained by Kjeldahl was converted to protein content for all samples, by using the same conversion factors reported in section 3.1.1 and compared with the protein amount originally present before digestion, by calculating the percentage of solubilized proteins, analogously to lipids. Protein hydrolysis degree was also calculated by using the OPA method (details in the experimental section 2.6.3.) and was associated with the solubilized protein content. The amount of

solubilized proteins via total amino acids measurement was obtained by relating the amount of solubilized total amino acids related to the starting value of total amino acids.
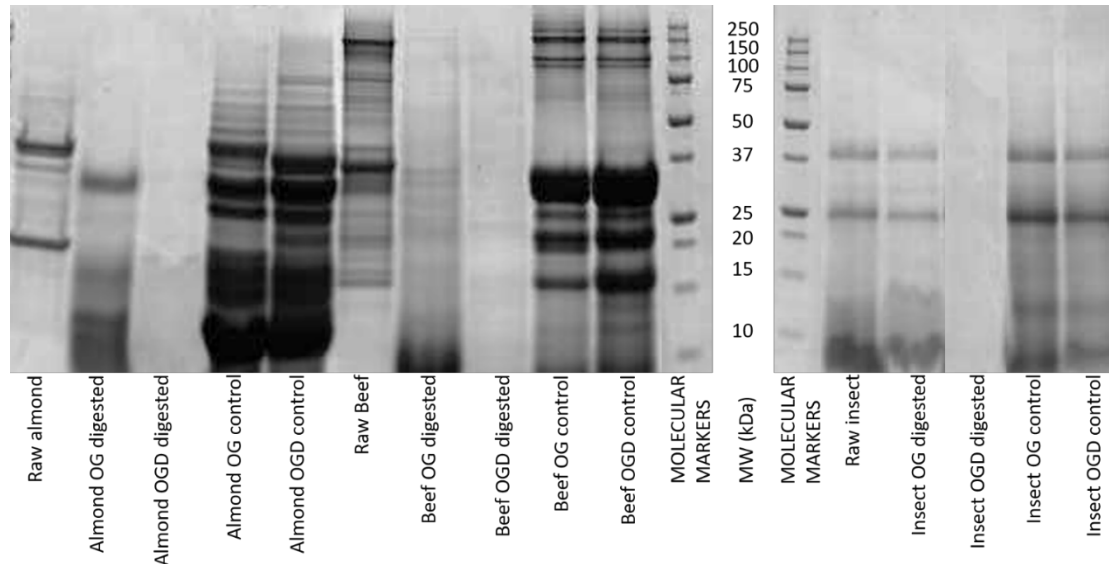

Figure 1. SDS-PAGE electrophoretic pattern of Almond, Beef and Insect in undigested (raw) and digested form (OG, oral-gastric; OGD oral-gastric-duodenal). The figure includes each negative digestion control, i.e. the proteins treated in the same OG and OGD mixtures, but without enzymes. Negative digestion control bands are more intense due to the interference of the digestion mixtures.

For almond and insect samples, protein solubilization increased when comparing the OG phase to the OGD phase, and also the degree of hydrolysis followed the same trend, indicating that such solubilization is strictly linked to their susceptibility to being cleaved during the digestive process. For the beef sample, no statistically significant differences between OG and OGD digesta were detected, possibly due to the action of proteolytic enzymes involved during the *in vitro* simulated gastric phase.

Both the amount of the solubilized proteins and the degree of hydrolysis indicated a higher digestibility of beef, followed by almond and lesser mealworm more or less at the same level. Free amino acids were also determined, but their amount turned out to be very low and scarcely significant in all samples, being

about 1% of the dry mass in beef and almond digesta, and about 10% of the dry mass in insects digesta.
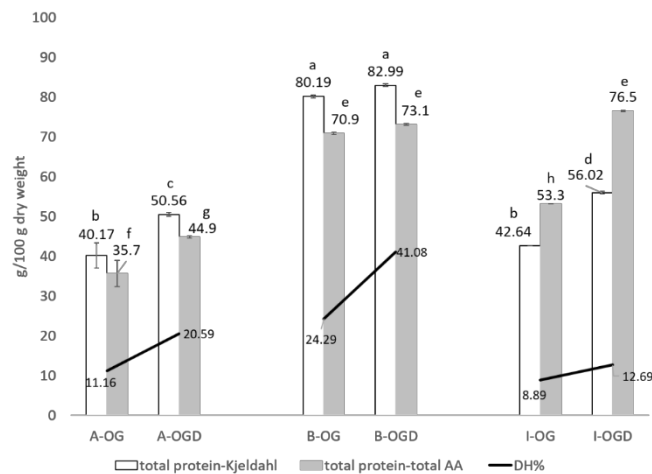

Figure 2 Solubilized protein percentage (as referred to starting raw material) after oral-gastric (OG) and oral-gastric-duodenal (OGD) digested samples calculated by both Kjeldahl and total amino acids method and protein degree of hydrolysis (DH%) in almond (A), beef (B) and insect (I). Different letters mean statistically different samples ($p < 0.05$; one-way ANOVA followed by Tukey's-b test).

The data obtained for almond and beef samples showed that the Kjeldahl method lead to a slight overestimation of the actual protein content, as compared to the protein calculated from the sum of amino acids (11-12% higher values with Kjeldahl), indicating the presence of small amounts of non-proteinogenic nitrogen in the digestive mixtures.

In stark contrast, for the lesser mealworm samples the degree of protein solubilization was found to be much higher when calculated on the total amino acid amount (37% higher than the value obtained by Kjeldahl). Again, this is likely due to the presence of chitin: when calculating the solubilization degree by using Kjeldahl, the total initial protein figures also include chitin, whereas solubilized proteins do not (chitin is not digested and therefore does not solubilize). In this way, based on Kjeldahl data, the protein solubilization after digestion is grossly underestimated. This bias completely disappears when using total amino acid as a way to calculate proteins, which allows the correct calculation of both initial proteins and solubilized proteins. Thus, after this re-

evaluation of protein solubilization, the bioavailability of insect proteins reached a level comparable to the one of beef.

Also, the amino acid analysis allowed to compare the percentage of solubilization for every single amino acid, to outline if any amino acid had some positive or negative preference in solubilization during digestion, as compared to all the others. Anyway, for all samples, there was no selection effect for amino acids in the digestion phases (data not shown), meaning that all amino acids were solubilized in the same way yielding a total amino acid distribution in solution (hence a nutritional score) very similar to that originally present. This also means that there is no particular bias in the protein digestion, and all the proteins present are digested in the same way, confirming what was already seen in the SDS-PAGE analysis which showed the complete disappearance of all proteins after digestion.

3.2.4. Protein and peptides identification by high-resolution mass spectrometry

To complete the molecular characterization of solubilized protein fraction, the identification of the main peptides present in the soluble fraction of the digested samples was performed by high-resolution mass spectrometry on LTQ-Orbitrap, both for OG and OGD digested samples (details in the experimental section). The reason to perform such an analysis was to identify, through the peptides detected, the main proteins undergoing digestion. This serves a twofold purpose: first, identifying the main proteins present in the food matrices, and also, by comparison with literature data, assess if those proteins undergo digestion (hence we observe peptides coming from them) or they are somehow resistant (hence no peptides from those proteins are generated).

All detected peptides are reported in the **Supplementary Material (S1)**. The protein databases for Almond, Bovine and Insect were used for the identification of the peptides and to identify the proteins which originated them. The main identified proteins, according to the number of identified peptides and their intensity, are reported in **Table 6**. The scarce presence of protein sequences in the

insect databases limited the attribution of proteins and peptides for insect samples.

Table 6. Summary of main protein attribution and number of peptides identified for each digested sample.

Sample	Main Identified Proteins (code-name)	N. Identified per protein	Coverage per protein %
Almond OG	•Q43607- Prunin 1 •A0A4Y1S2I9- RmlC-like	133 53 20	75 45 17
Almond OGD	cupins superfamily protein •A0A5E4EZP4- Vicilin	101 28 5	60 36 8
Beef OG	• A0A1K0FUF3- Myoglobin • Q5KR49- Tropomyosin	18 20 56 138	66 64 55 53
Beef OGD	• P68138- Actin • Q9BE40- Myosin	2 8 25 79	18 16 37 21
Insect OG	•D6W8Q9-Pupal cuticle protein •A0A0L7QKC9- Actin •P80681-Larval cuticle protein	6 19 2	42 35 26
Insect OGD	•A0A084VLA6-Actin •A0A482VEP8- Uncharacterized protein •P80681- Larval cuticle protein	14 3 5	31 30 17

N, number of peptides.

The main proteins identified, i.e. the ones generating the most abundant peptides, were clearly the most abundant proteins originally present for each sample, i.e. seed storage proteins for almond, and muscle proteins in beef and insect [42–44]. This means that the solubilized fraction is composed mostly of peptides coming from the most abundant proteins, which, generate most of the

soluble nitrogen fraction after digestion clearly indicating that there is not a particular resistance to digestion in the main proteins from the mixture, somehow confirming the data obtained by gel. This also indicates that the lower solubilization observed for almond proteins is due to a generic resistance of all the protein fractions, possibly induced by the diverse matrix or the different amino acid composition, and it is not due to specific resistance to the digestion of specific proteins in the fraction.

Based on the identified peptides after in vitro digestion, bioactive dipeptides (e.g., RF or GF) can be identified inside the sequences of larger peptides prunin, vicilin, myosin and actin. These dipeptides have been found to control food intake, in particular relating to the stimulation of CCK secretion, as previously reported by Tulipano [45] for dairy proteins. Also, peptides derived from muscle proteins showed bioactivity as antihypertensives or ACE-inhibitors in previous work [46].

3.3 Correlation between amino acid composition and biological properties of the digested mixtures

In a previous paper [20], the ability of digested mixtures from the same biomass matrices to affect hormone secretion at the intestinal level was measured. Here, a further analysis was performed to identify a possible relationship between the amino acid composition and the biological effects previously reported.

A heat map was initially constructed to analyse features and sample clustering simultaneously. The differences in amino acid composition for almond, beef and insect samples, both raw and submitted to *in vitro* digestions, were analysed. **Figure 3** shows that in all the conditions (raw or digested) insect and beef are clustered together, indicating that there are more similarities in the amino acid composition between these protein sources and that they are more different compared with almond amino acid composition.

To understand whether there was a relationship between the amino acid composition and the biological effects previously observed we performed a PCA

analysis. The PCA results for all the variables analysed are shown in **Table S2** in the supplementary material. The projections in the plane of the amino acid composition of the OG digestions and the enterohormone secretion (ghrelin, CCK and PYY) data obtained from the treatment of pig duodenum explants with the different protein digestions are shown in **Figure 4**. The distribution of the protein sources in the space based on the different variables is represented in the corresponding right panel. Axis 1 was responsible for explaining 63.48% of the total variance, while axis 2 explained 35.52%. The first principal component (PC1) clearly discriminated between insect and almond gastric digestions. Overall, PC1 was characterized, on the one hand by Gly, Arg, Glu, Asp together with PYY, which were grouped on the left side of the graphic. On the other hand, we observed that Ser, Val, Trp, Ala, Tyr, Pro, Ile, Thr, Phe and CCK were grouped on the right side of the PC1. These groupings indicate a positive association between CCK and the amino acids located on this side of the panel, which are most abundant in the protein fraction coming from digested lesser mealworm. By contrast, CCK was negatively associated with Arg, Glu and Asp, more abundant in the protein fraction coming from digested almonds. PYY was positively associated with Gly, Arg, Glu and Asp, all abundant in the protein fraction coming from digested almonds, and negatively with Ala, Trp, Ser and Val. The contribution of PC2 explains the differences between insect or almond, and beef. PC2 is mainly described by HPro, Lys, Leu, His, Cys, Met, and Thr, located in the upper part of the graphic, and active ghrelin and Phe, located in the lower part of the graphic. These amino acids are the most abundant in the protein fraction coming from digested beef. The distribution of the variables in PC2, clearly showed that active ghrelin was negatively associated with those amino acids.
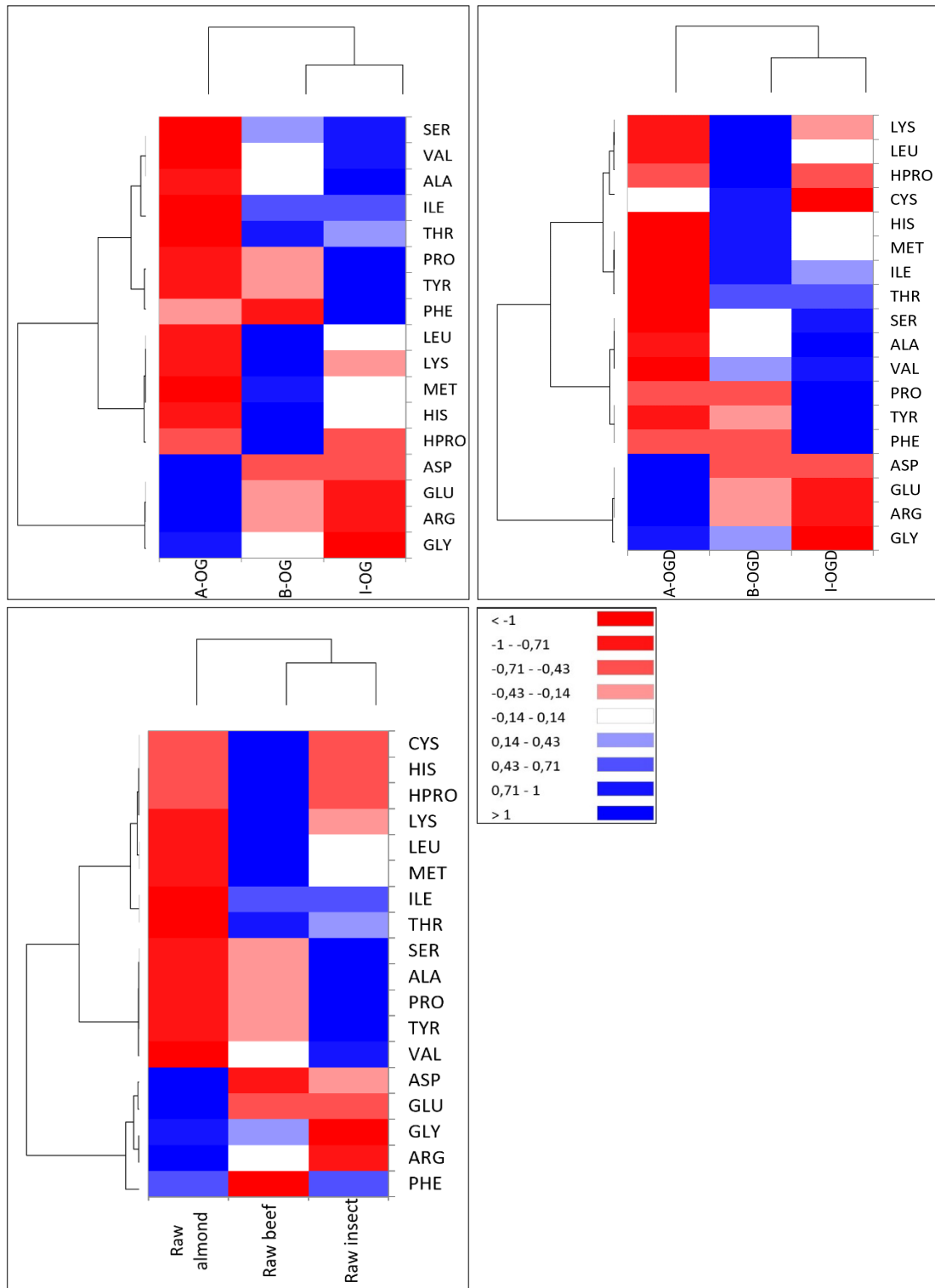
Figure 3. Heat map of the amino acid data in raw, oral-gastric (OG) and oral-gastric-duodenal (OGD) digested samples of almond (A), beef (B) and *Alphitobius diaperinus* insect (I). In the subsequent rows, red hues represent decreased concentrations and blue hues increased concentrations. Colour intensity increases proportionally to the magnitude of the change.
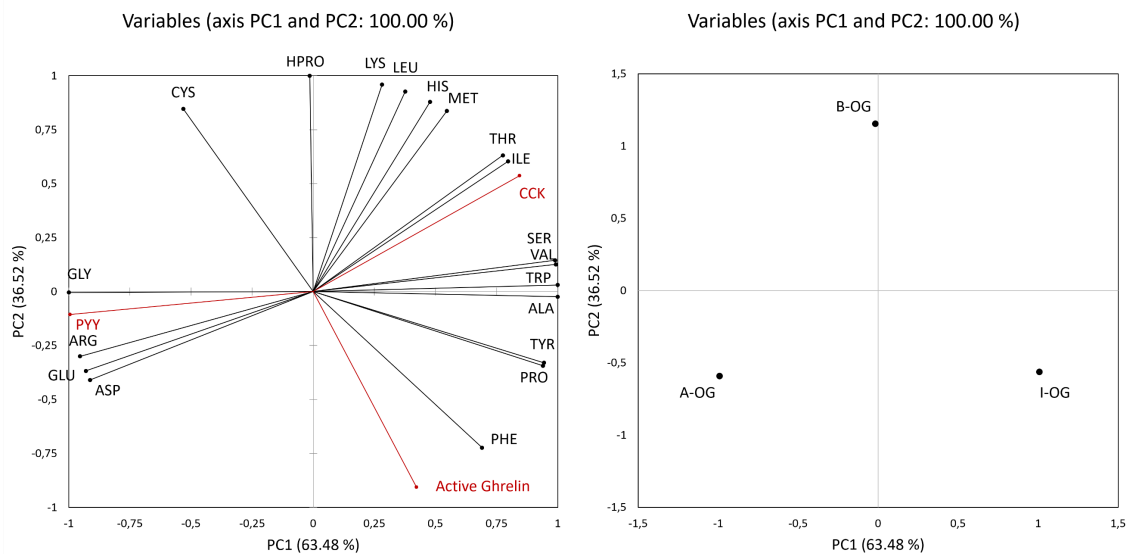
Figure 4. Principal components obtained for the amino acid composition of the in vitro oral-gastric digested almond (A-OG), beef (B-OG) and *Alphitobius diaperinus* insect (I-OG) and the enterohormone secretion of active ghrelin (pg/ml), PYY (pg/ml) and CCK (ng/ml) after treating pig duodenum with these digestions. Pig proximal duodenum is the first segment reached after OG digestion where no pancreatin action occurs. Thus, this duodenum segment was treated with the OG digesta.

Next, a PCA analysis with the data from the amino acid composition of the OGD digestions and the enterohormone secretion data (obtained after the treatment of human colon explants with these OGD digestions) was run (**Figure 5**). In that case, axis 1 and 2 were responsible for explaining around 50% of the total variance. Like in gastric digestions, the PC1 discriminates between insect and almond digestions. PC1 showed that PYY was associated with Asp, Glu, Cys, Arg and Gly and negatively with Val, Ala and Ser. This amino acid profile corresponds to the composition of the protein fraction coming from OGD digestion in almonds. Total ghrelin was positively associated with Gly, Arg and Cys and negatively associated with Tyr, Pro and Phe, some of the most abundant amino acids in the protein fraction from OGD digested lesser mealworm. PC2 still explains the different compositions observed for almonds and insects as compared to beef. Here total GLP-1 is positively associated with Leu, Lys, Met, His, HPro, Ile, Thr, Trp and Cys, the amino acids more abundant in the protein fraction from OGD digested beef. Since GLUTag cells produce GLP1, this

possible correlation was tested experimentally. GLUTag cells were treated with the most abundant amino acids present in the protein fraction of OGD digested beef, to see if they could exert the same result as the digested protein. His, HPro, Lys, Leu, Met, Cys were used, with an equivalent concentration as the one present in the protein fraction from OGD digested beef. Indeed, these amino acids induced a 1.7-fold increase in total GLP-1 secretion (82.86 ± 10.63 and 49.1 ± 5.69 pM for beef amino acids and vehicle respectively; p<0.05), indicating that the more abundant amino acids in the OGD digested beef participate in the stimulation of the total GLP-1 secretion previously published in human colon [20].
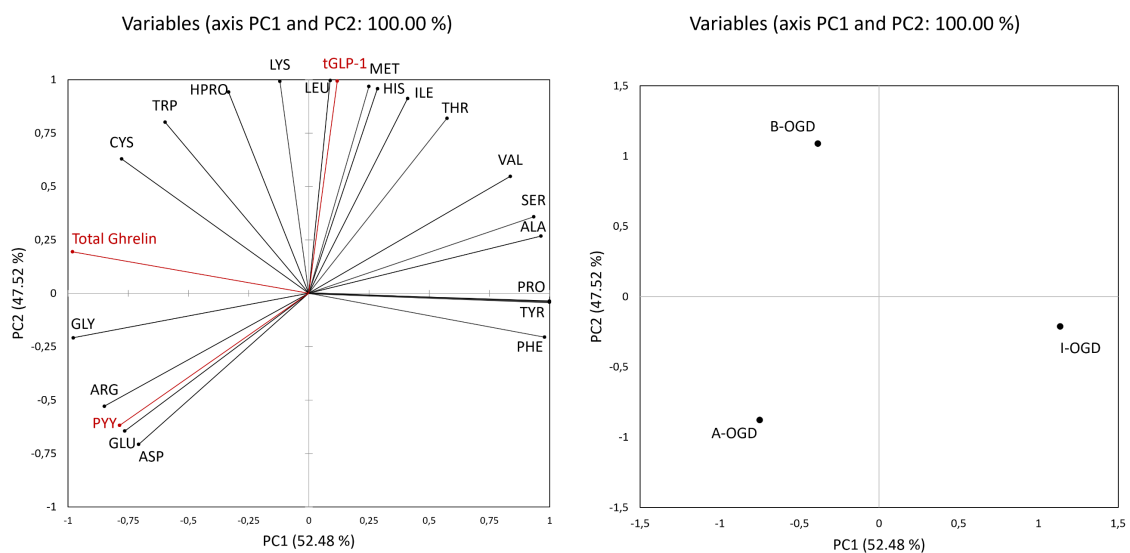

Figure 5. Principal components obtained for the amino acid composition of the *in vitro* oral-gastric-duodenal (OGD) digested almond (A), beef (B) and *Alphitobius diaperinus* insect (I) and the enterohormone secretion of total ghrelin, PYY, tGLP-1 (pg/ml) after treating human colon with these digestions.

Finally, a PCA was run with the amino acid composition of the raw samples and the effects on food intake observed after the administration of these samples to rats. Axis 1 explained 54,47% of the total variance, and similar to what occurred with the PCA of the OG and OGD digestions, it contributed to the differences between almond and lesser mealworm. **Figure 6** shows that food intake, measured either at 3 or 20 hours after food replacement (beginning of the dark cycle), was directly associated with Ser, Ala, Tyr, Val and Pro and negatively

associated with Gly, Arg and Glu, the more abundant amino acids in the protein fraction of raw insect and almond respectively (**Figure 3**).
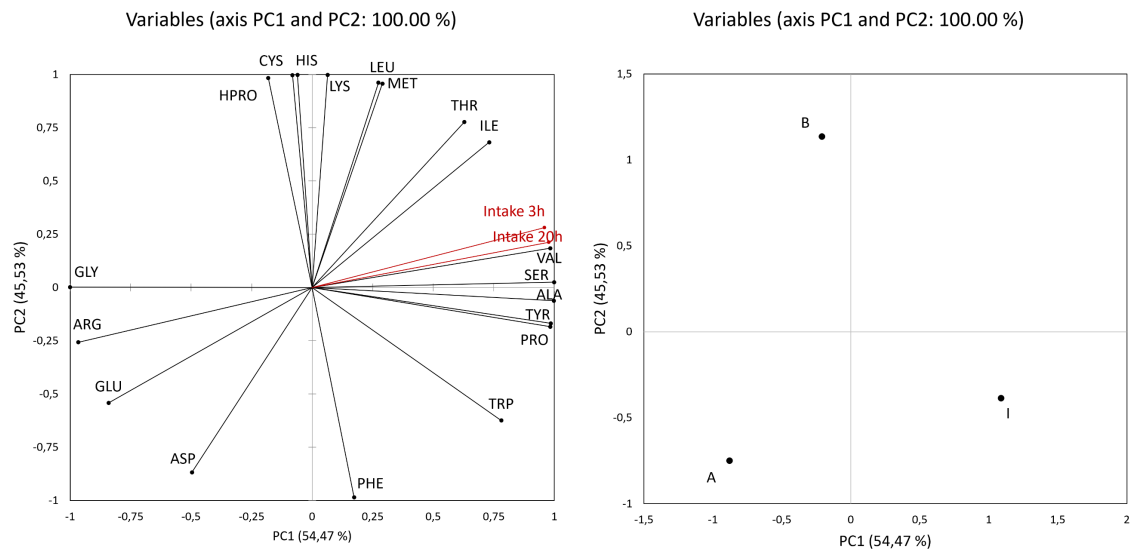

Figure 6. Principal components obtained for the amino acid composition of the raw almond (A), beef (B) and *Alphitobius diaperinus* insect (I) samples and 3 and 20 hours food intake (kcal) after administer to the rats a 300 mg protein/ Kg body weight dose of these samples.

Overall, the present data on total amino acid content indicates that *Alphitobius diaperinus* and beef are more similar as compared to almonds, as described before [47]. In our previous study [20], the secretome of intestinal segments (both pig duodenum or human colon) yielded different results with different protein sources, albeit at the same concentration, which suggested that the total amount of protein was not the only factor responsible for the secretion of these enterohormones. In addition, no relation was observed between enterohormone release and the amount of energy present in each digested sample, suggesting that the amino acid composition or the specific peptides present were the primary factors responsible for the observed results. With the present complete amino acid characterization of the protein samples, together with the PCA analysis, we can now better explain our previous results.

The positive and negative associations described between the amino acids and CCK secretion agreed with the high or low content of the respective amino acids

in the protein fraction from OG digested lesser mealworm. The aromatic amino acids L-Phenylalanine and Tryptophan, are considered potent stimuli for CCK secretion [48]. Daly et. al, observed an increase in CCK secretion after stimulation with L-Phe ex vivo with proximal intestinal tissue in mice and in vitro with STC-1 cells [49]. The same effect was observed when working with isolated intestinal cells from mice [50]. On the other hand, Valine has been reported to not stimulate CCK secretion in vivo in men [51] or ex vivo in pigs [52], but in this last experiment the authors also reported CCK secretion after treatment with Isoleucine. No information has been found for similar experiments about Ser, Ala, Tyr and Pro amino acids and their CCK secretory capacity. Then, these results suggest that CCK secretion in pig duodenum by OG digested insects could be mainly triggered by Phe, Trp and Ile.

When the same tissue was treated with OG digested beef, an increase in the CCK secretion was also observed, but this increase may be due to the presence of other components, as the amino acid profile here is not positively associated with increased secretion of this hormone. A clear positive association was also observed between the amino acid composition of the protein fraction of the digested almond and the PYY secretion. Finally, active ghrelin seemed to be negatively related to the main amino acids present in the protein fraction of OG digested beef (HPro, Lys, Leu, His, Cys, Met, and Thr). In humans, Leucine and Cysteine have shown a reducing effect in ghrelin plasmatic levels [53,54]. No information in similar models has been found regarding the rest of the amino acids negatively associated with ghrelin. Nevertheless, this observation confirms our previous results indicating that beef was the most active source at reducing the active ghrelin secretion in pig duodenum and that it could be caused at least by Leu and Cys. On the other hand, the positive association between ghrelin and Phe was surprising, as none of the samples stimulated ghrelin secretion. The commonly described effect is a reduction of active ghrelin by Phenylalanine, *in vivo* in rats [55], and ex vivo with mouse gastric mucosa [56].

Total GLP-1 secretion measured in the human colon can be explained mostly for the amino acid composition of the OGD beef, being Leu, Lys, Met, His, HPro, Ile, Thr, and Cys the more abundant ones in this sample. We checked this in vitro treating GLUTag cells with a mix of some of the most abundant amino acids in OGD beef, obtaining a substantial increase in secretion compared to the vehicle. Rigamonti *et al.* found a positive correlation between Leu, Lys, Met and Ile plasmatic levels in humans and GLP-1 [57]. Then, with these results and our findings, we can say that the OGD beef amino acid composition is probably responsible for the GLP-1 secretion observed in the human colon *ex vivo*, even though Leucine was shown to not elicit any increase in GLP-1 after an intragastric infusion in lean participants [58].

On the other hand, the amino acid composition of the OGD digested lesser mealworm could be responsible for the secretion of the total ghrelin in the human colon. Phenylalanine is described to exert a reduction of active ghrelin secretion in vivo in rats [55], and *ex vivo* with mouse gastric mucosa [56]. No other comparable studies have been found for other amino acids [59].

Regarding the effect of these protein sources in the modulation of food intake in rats, we previously observed that the lesser mealworm increased it, almond reduced it and beef did not modify it after an acute administration of 300 mg protein/kg body weight [20]. It should be mentioned that rat food intake at 3 and 20 hours was directly associated with the amino acids mostly present in the lesser mealworm and inversely associated with those mostly present in almonds as shown in **Figure 4** and **Figure 7**. These results agree with other authors that have demonstrated that Arg and Glu (in our case mostly abundant in almond) are two of the most anorectic amino acids in rats [60].

A positive association between food intake and the amino acids Val, Ser and Ala and a negative association between the secretion of the anorexigenic enterohormone PYY and these amino acids was found. In humans, an increased food intake in children consuming more Val has been suggested [61]. On the other hand, Val was one of the 8 amino acids implied in the appetite-suppressant

effects of whey proteins through GLP-1 stimulatory effects [57]. In piglets under a low protein diet, Leu and Val balance markedly increased the feed intake [62]. In mice fed a high-fat diet supplemented with Val, it did not increase food intake [63]. These discrepancies strengthen that the effects of the amino acid Val on food intake depend on the experimental model and design. In this sense, in mice, Ser supplementation led to reduced food intake [64,65], but this was observed only after chronic supplementation and linked to hypothalamic effects, while our results showed a positive association of Ser and food intake refer to measures at 3 and 20 hours after an acute protein source administration. Also, a negative association with food intake and a positive for PYY was found with Arg, Gly and Glu. In agreement, acute administration of Arg to rodents led to reduced food intake and increased PYY secretion [60,66]. Also in healthy human volunteers Arg in combination with a meal can significantly elevate PYY, although no reduction in food intake was found [67]. Other authors have often reported food intake modulation in vivo without correlations with intestinal hormone release [68]. In the same study, they found no effects of Gly on PYY secretion. Thus, the present results show that the previously observed effects of different protein sources on food intake are mostly associated with the amino acid profile of these sources, being a higher % of Val, Ser and Ala and lower % of Arg, Glu and Gly related to an increased food intake in rats and inversely associated with the PYY intestinal release.

4. CONCLUSION

Beef and insect were proven to have high similarities in amino acid composition and protein solubilization after digestion (considering the proper corrections for the chitin content) and peptide composition of the digested mixture proved all major proteins being accessible for the digestive enzymes. Lipid fraction from insects appeared to be as accessible as the one of almonds, but with a fastest kinetics. Thus, lesser mealworms are a good source of both lipids and highly nutritional proteins.

Looking beyond pure nutrition, the secretion of CCK in pig duodenum was positively correlated to amino acids mostly present in the protein fraction of gastric digested insects, while PYY secretion was positively associated with the amino acid composition of the protein fraction coming from gastric digested almond. A major positive correlation with the amino acid composition of the digested protein fractions was found between GLP-1 secretion and beef, and total ghrelin in the human colon and insect. The modulation of food intake in rats, which was differently modified by the three protein sources, was also associated with their amino acid profile. Besides the nutritional value, insect proteins seem to have specific effects on enterohormone release and food intake, which might even increase their value as a new source of food and feed.

Acknowledgments

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SUPPLEMENTARY MATERIAL

Table S2. Loadings for the two principal components.

Variables	Raw samples		OG digestions		OGD digestions	
	PC1	PC2	PC1	PC2	PC1	PC2
ALA	0,998	-0,062	1,000	-0,024	0,963	0,268
ARG	-0,966	-0,258	-0,954	-0,300	-0,849	-0,528
ASP	-0,496	-0,868	-0,912	-0,409	-0,707	-0,707
CYS	-0,082	0,997	-0,532	0,847	-0,777	0,629
GLU	-0,840	-0,542	-0,930	-0,367	-0,764	-0,645
GLY	-1,000	0,002	-1,000	-0,004	-0,978	-0,208
HIS	-0,061	0,998	0,477	0,879	0,285	0,958
HPRO	-0,181	0,983	-0,015	1,000	-0,333	0,943
ILE	0,731	0,682	0,797	0,604	0,410	0,912
LEU	0,290	0,957	0,376	0,927	0,089	0,996
LYS	0,064	0,998	0,281	0,960	-0,120	0,993
MET	0,274	0,962	0,547	0,837	0,249	0,968
PHE	0,173	-0,985	0,691	-0,723	0,979	-0,205
PRO	0,983	-0,184	0,939	-0,344	0,999	-0,042
SER	1,000	0,024	0,989	0,145	0,934	0,358
THR	0,629	0,778	0,776	0,631	0,573	0,819
TRP	0,781	-0,625	1,000	0,031	-0,597	0,802
TYR	0,986	-0,169	0,944	-0,330	0,999	-0,036
VAL	0,983	0,184	0,992	0,126	0,837	0,548
Intake 3h	0,960	0,281				
Intake 20h	0,977	0,212				
Active Ghrelin		0,422	-0,906			
CCK			0,844	0,536		
PYY			-0,994	-0,106		
tGLP-1					0,118	0,993
PYY					-0,786	-0,618
Total Ghrelin					-0,981	0,195

CCK, cholecystokinin; PYY, peptide YY; tGLP-1, total glucagon-like peptide 1.

PART 2

Evaluation of the acute effects of an insect protein preload on food intake in human subjects.

Manuscript 3

Effect of an Acute Insect Protein Preload vs an Almond Preload on Energy Intake, Subjective Food Consumption and Intestinal Health in Healthy Young Adults

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Effect of an Acute Insect Protein Preload vs an Almond Preload on Energy Intake, Subjective Food Consumption and Intestinal Health in Healthy Young Adults

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ABSTRACT

Protein is considered the most satiating macronutrient, and its effect on satiety and food intake is source dependent. For the first time, we evaluated the effect of an acute dose of insect protein on appetite and food intake compared with the same dose of almond protein in human subjects. Participants consumed both proteins and vehicle as a liquid preload on three separate days. They were then offered a breakfast and lunch buffet meal at which food intake was measured. VAS questionnaires were completed following the three preloads to assess appetite and other sensations. At breakfast, reduced energy intake was observed for both protein preloads compared with vehicle. At lunch, food intake only differed in the insect group, which consumed more than the vehicle. Insect protein increased the total amount of protein ingested with a slight increase in total energy consumed differently than almond, which significantly increased total protein and energy consumed. There was no correlation between indigestion-sensation ratings and food intake. Moreover, the insect preload resulted in lower sleepiness and tiredness ratings compared with the almond preload. Thus, insect protein may be suitable as a safe ingredient for snacks intended for elderly or infirm patients who require increased protein intake.

Keywords: protein, satiety, insect, almond, appetite, food energy intake.

1. INTRODUCTION

Protein is regarded as the most satiating macronutrient, and high-protein diets can potentially greatly suppress hunger [1,2]. Dairy protein has been the focus of most human-study research in this area. Several studies have indicated that whey protein has a greater effect on appetite than other proteins like egg, tuna, turkey or casein. Pal and Ellis [3] found that energy intake at an *ad libitum* meal was significantly lower four hours after a whey preload compared to the same dose of protein (50.8 g) from tuna, egg or turkey. Other authors have also reported that whey proteins reduce food intake more effectively than soya or egg albumin [4] and that a whey preload performs better than casein, in addition to yielding greater subjective satiety ratings [5] 60 min and 90 min after the preload (50 g of protein). However, other studies have reported an increase in food intake one hour after a preload of 20 g of whey protein compared to the same dose of pea and casein protein [6]. In comparisons of whey and soya and gluten [7] or whey and casein [8] preloads of about 50 g each, other authors have found no differences in appetite or food intake three hours later. These discordant results may be due to the fact that the source of the protein may play a role in its satiating effect, as well as the dose, time of consumption, the characteristics of the participants and the experimental design [4,6,9,10].

The most widely consumed protein today is animal protein and, consequently, it has received the most research attention. The demand for animal protein is expected to increase globally, despite the fact that its production carries numerous negative implications for the environment [11]. The search for new protein sources with environmentally friendly production processes, like insects [12,13], is therefore taking on increasing importance. Some authors have described insects as a promising new food source that is rich in high-quality protein [13–15]. Insects also contain a substantial proportion of good quality fats as well as numerous minerals and vitamins [16–19]. In addition, some evidence, mostly from *in vitro* studies or based on traditional medicinal uses, suggests that edible insects may have beneficial effects on human health related to aspects such

as the interaction with microbiota [20], antihypertensive peptides [21] and the antimicrobial function [22], among other benefits [23–26]. However, to our knowledge, the possible effect of insect consumption on food intake has never been studied. Until now, research has focused only on evaluating the nutritional value and the potential allergic and toxicological risks of this new protein source [27,28]. Previously, our group compared the acute effect of the same dose of three different proteins – insect (*Alphitobius diaperinus*), almond and beef [29] – on food intake in rats. Our results showed a higher caloric intake in the groups treated with the insect protein and a lower intake in the groups treated with the almond protein compared to the control group (water).

The purpose of this study is to test the acute effect of two of these protein sources, insect and almond, on satiety in humans. For this purpose, we measured the food intake of healthy young participants at subsequent *ad libitum* breakfast and lunch meals and their subjective ratings of appetite as well as the effects of the two protein sources on intestinal transit. Our aim was also to compare these proteins in whole foods instead of as isolated proteins and to keep the texture, appearance and taste as similar as possible.

2. METHODS

Subjects

Healthy men (*n*=17) and women (*n*=12) between ages of 22 and 33 years and with a BMI lower than 40 kg/m^2 participated in the study. Volunteers were excluded if they had food allergies, had a BMI exceeding 40 kg/m^2, did not follow the study guidelines, had a major systemic illness, used drugs to treat metabolic related syndrome pathologies, had any intestinal related problem, or were experiencing any COVID-19 symptoms. This study was approved by the ethical research committee on medicines of the Pere Virgili Health Research Institute (CEIM 172/2020). All participants provided informed, written consent before participating in the study.

Experimental design

This was a single blind, cross-over design study. The participants came to the study room on three separate days with a six- to seven-day period between each visit. Subjects were asked to arrive at 8:45 a.m. after a minimum fasting period of 10 hours. On the first day of the study, the participants were weighed and their heights recorded. They were asked to follow their normal dietary habits and not to take part in any intensive physical activity on the days before study days. On each of the three occasions, the subjects received one of the preloads at 9:05 a.m. Five minutes before the preload, participants were asked to complete questionnaires, using visual analogue scales (VAS), to rate their appetite. They were asked to complete the questionnaire again every hour until the end of the study (9:00 a.m. – 7:00 p.m.), as detailed in **Figure 1**.
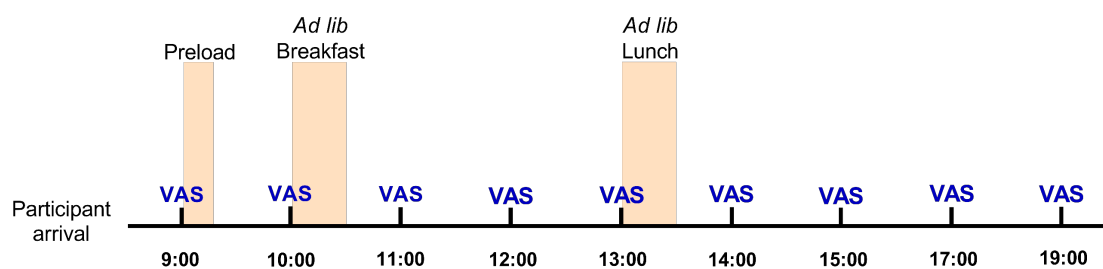

Figure 1. Timeline of the experimental design for each day of the study.

The participants consumed the preload after completing the appetite questionnaires. One hour later, subjects were offered a buffet breakfast to be consumed *ad libitum* consisting of orange juice; chocolate-filled or plain croissants; cheese, turkey or dry sausage sandwiches; black coffee with optional milk and sugar; and bananas. Four hours after the preload (1:00 p.m.), subjects were offered a buffet lunch consisting of four varieties of pizza (barbeque, vegetarian, five-cheese or carbonara, supplied by Telepizza Group, S.A.). We presented the buffets in identical trays containing eight portions of pizza based on the participants' preferences. A piece of fruit, tangerine or pear, the consumption of which was mandatory, was also included. Participants were asked to eat until they felt comfortably full and were given 30 min to consume

the meals. All the food was weighed before each subject started eating the buffet meals and then again afterwards. The energy (kJ) content of the food consumed was determined using the information provided by the manufacturers and the Spanish food composition database BEDCA for the fruit. The volunteers were allowed to continue with their daily routine and leave the building with a commitment to return on time for the scheduled meals. Throughout the duration of the experiment, the participants were allowed to drink as much water as they wanted.

Preloads

On each of the three days of the study, the subjects received one of the preloads containing either insect (buffalo larvae, *Alphitobius diaperinus* powder; Protifarm NV, Ermelo, the Netherlands), almond (*Prunus dulcis* flour; Borges Agricultural & Industrial Nuts (BAIN), Spain) or no protein source (vehicle). The three preloads were prepared using a blender to mix 15 g of protein flour (only for the protein preloads), 100% cocoa powder (Chocolates Valor, S.A., Spain), skimmed milk, sucralose, and a food colorant (brilliant blue, E133). The nutritional composition of the preloads is described in **Table 1**. The test meals were matched as closely as possible in terms of appearance, texture and taste, and were tested for palatability by the laboratory staff. Subjects consumed test meals as a blended drink, flavoured with chocolate powder to blind the subjects to the source of the protein. They were required finish the milkshake within 5 min. They were also asked to report the time at which they first detected the colorant in their faeces in order to monitor the excretion rate of the preloads.

Subjective ratings of appetite

Appetite and other sensations were assessed using a method based on VAS [30,31]. Participants were instructed to move the cursor along a horizontal line using the mouse to indicate how they felt about each individual variable with 'not at all' on the left and 'very/a lot' on the right [32]. Questions about 'motivation to eat' (desire to eat, hunger, prospective food consumption, fullness) alternated with questions about other sensations (thirst, stress, sleepiness,

tiredness, indigestion and tummy rumbling). The complete list of questions is available in **Supplementary Table S1**.

To assess the palatability of the three preloads, the subjects were asked to rate, using something similar to the VAS questionnaires, the overall likeability, pleasantness of taste and likeability of texture after finishing the study.

Table 1. Nutritional composition and content of the three preloads.

	Vehicle	Almond	Insect
Nutritional analysis			
Amount (g)	145	205	170
Energy (kJ)	279.2	1854.2	812.2
Fat (g)	1	34	8.2
Carbohydrate (g)	7.7	10.3	8.4
Fibre (g)		8.6	0.9
Protein (g)	5.7	20.1	20.7
Content			
Test food (g)		60	25
Cocoa powder (g)	5	5	5
Sucralose (g)	0.025	0.025	0.025
Milk (g)	140	140	140
Food colorant (ml)	0.5	0.5	0.5

Statistical analysis

The effect of the preloads on food intake measurements was tested by univariate one-factor repeated-measures analysis of variance (ANOVA) followed by the Bonferroni adjustment for multiple comparisons. To test the effect of preload, time, and their interaction with VAS questionnaires, we used a univariate two-factor repeated-measures ANOVA. When a preload by time interaction was statistically significant, a Bonferroni adjustment for multiple comparisons was assessed to investigate differences between preloads. Gender was introduced as a between-subject factor in both analyses. The area under the curve (AUC) was calculated using the trapezoidal rule. Significance was set at $p < 0.05$, unless

otherwise indicated. Data are presented as means ± SEMs. All the calculations were performed using XLSTAT 2021.2.1 software (Addinsoft, New York, USA).

VAS questionnaires missing values because of delayed or unreported answers were estimated using a Markov Chain Monte Carlo (MCMC) multiple imputation algorithm.

3. RESULTS

3.1 Energy and protein intakes

We evaluated the effect of the quality of a 20 g protein preload on food intake, and analysed the effect of protein source and, subject gender subject, and their interaction with the different outcomes. *Ad libitum* breakfast and lunch test meal intake (without including the energy of the preload) was affected by subject gender subject sex ($p < 0.0001$) and preload ($p = 0.007$ and $p = 0.02$ respectively). Since there was no interaction between preload and gender ($p = 0.781$ and $p = 0.870$, respectively), the results for both genders are presented together. Breakfast test meal intake from both the almond and insect treated groups was significantly lower than the vehicle treated group (**Figure 2A**). At lunch, four hours after preload administration, we observed a different profile: the insect administered group ate more than the almond or vehicle groups (**Figure 2B**). Cumulative energy intake, calculated as the sum of energy from breakfast and lunch, was not significantly different among the treatments (**Figure 2C**).

We observed a gender ($p < 0.001$) and a preload interaction ($p < 0.0001$) with total energy intake, calculated as the sum of energy from the preload and the *ad libitum* meal, for the breakfast test meal and cumulative intake. However, since no interaction was found between preload and subject sex ($p > 0.5$), we again combined the results for both genders. Total energy intake for the almond administered group was significantly higher than that of both the vehicle and insect groups at breakfast (**Figure 2D**). A preload effect was observed in the total cumulative energy intake after lunch. The almond administered group exhibited

the highest energy intake, followed by the insect and vehicle groups (**Figure 2E**). No differences were observed between the vehicle and insect groups, but the difference between the insect and almond groups was significant.
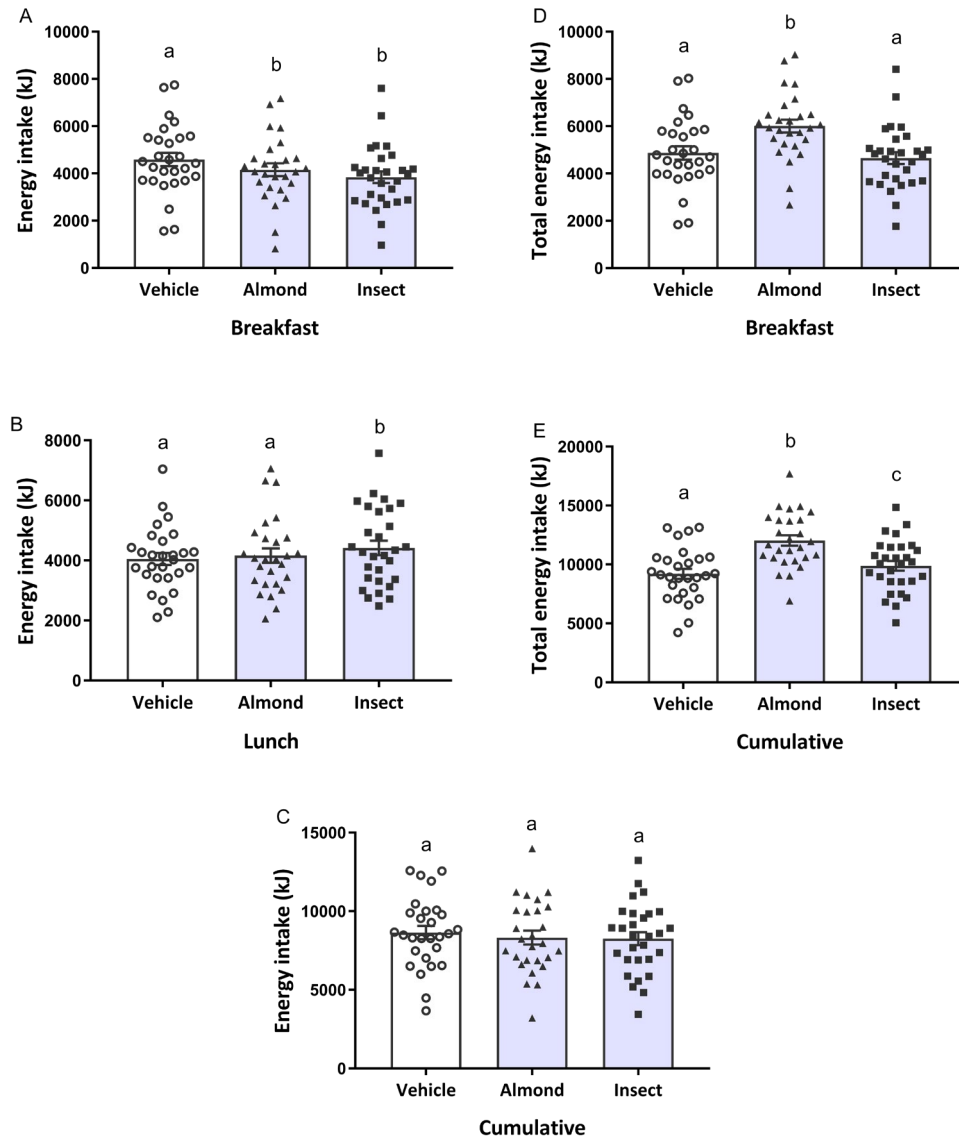

Figure 2. *Ad libitum* energy intake at the offered breakfast (A), lunch (B), and cumulative intake (C), which represents the energy intake from both meals together. Total energy intake, calculated as the sum of the energy from the preload and the *ad libitum* meal, at breakfast (D) and cumulative total energy intake (E). Participants received a dose of 20 g of protein from almond and insect preloads, and 5.7 g of protein from the vehicle preload, on three different occasions. Subsequent energy intake was measured at breakfast and lunch. Data are expressed as participant means (dots) and mean ± SEM of the participants from each group (bars). Different letters indicate significant differences,

$p < 0.05$ (one-factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons).

Since we administered the same amount of protein in both protein preloads, we compared their effect on protein intake. Again, we found no interaction between preload and subject gender ($p > 0.05$). For the *ad libitum* protein intake, a different profile was observed compared to the energy intake at breakfast. We detected a preload effect ($p = 0.012$) in that the insect administered group ate less protein than the almond or vehicle groups (**Figure 3A**). In this case, the almond administered group consumed the same amount of protein as the vehicle group, despite having lower energy consumption. At lunch, we observed the same profile as for energy intake: a higher protein intake only in the insect administered group (**Figure 3B**). No preload effect was found in the cumulated protein intake (**Figure 3C**), calculated as the sum of protein grams eaten at breakfast and at lunch.

We also observed a preload effect at breakfast ($p < 0.0001$) with regard to total protein intake, calculated by adding the protein present in the preloads (20 and 5 g of protein for the protein administered groups and for the vehicle group respectively) to the *ad libitum* protein intake. Here, the almond group had a higher protein intake than the vehicle group (**Figure 3D**). A preload effect was also observed ($p < 0.0001$) for the accumulated total protein intake, since both the insect and almond administered groups presented a higher total protein intake compared than the vehicle group. No differences were found between the two protein groups (**Figure 3E**).
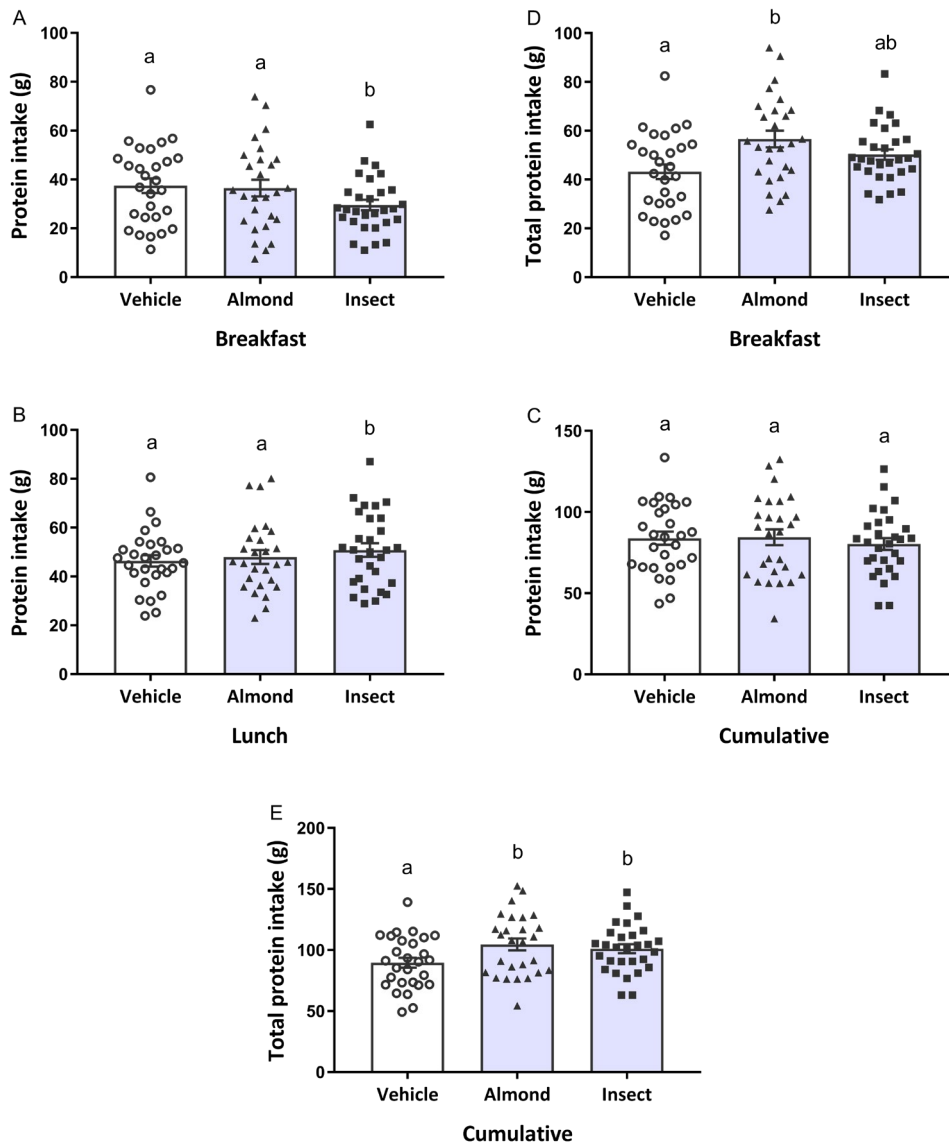
Figure 3. *Ad libitum* protein intake at breakfast (A), lunch (B) and cumulative protein intake (C), calculated as the sum of the protein grams eaten at breakfast and at lunch. Total protein intake, calculated as the sum of the protein from the preload and the *ad libitum* meal, at breakfast (E) and the total cumulative protein intake (E). Data are expressed as participant means (dots) and mean ± SEM of the participants from each group (bars). Different letters indicate significant differences, $p < 0.05$ (one-factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons).

3.2 Subjective ratings of appetite

Complementary to energy and protein intake measurements, subjective ratings of appetite (measured with VAS questionnaires) were collected over the course of each test day at the times indicated in the section on our experimental design (**Figure 1**). As in other analyses, none of the results obtained showed an interaction between preload and subject gender, so the data presented here are combined for both genders.

As expected, one hour after the *ad libitum* test meals (both breakfast and lunch), the desire to eat and prospective food consumption ratings (**Figure 4**) were lower for all three groups compared with the time points just before the meals were given, that is three and six hours after preload administration (time effect, $p < 0.001$).

The temporal profile for the desire to eat is shown in **Figure 4A**. The type of protein ingested had an effect on this factor ($p = 0.035$): the desire to eat was lower after the insect preload than after vehicle consumption. A strong interaction between time and preload by time ($p < 0.006$) was also observed. The desire to eat was significantly lower one hour after preload administration (just before breakfast) for both the insect and almond groups. These ratings predicted what we observed during breakfast intake, where both protein-administered groups consumed fewer calories than the vehicle group. Only the insect administered group reported lower ratings about the desire to eat one hour after breakfast (two hours after preload administration) than the vehicle group.
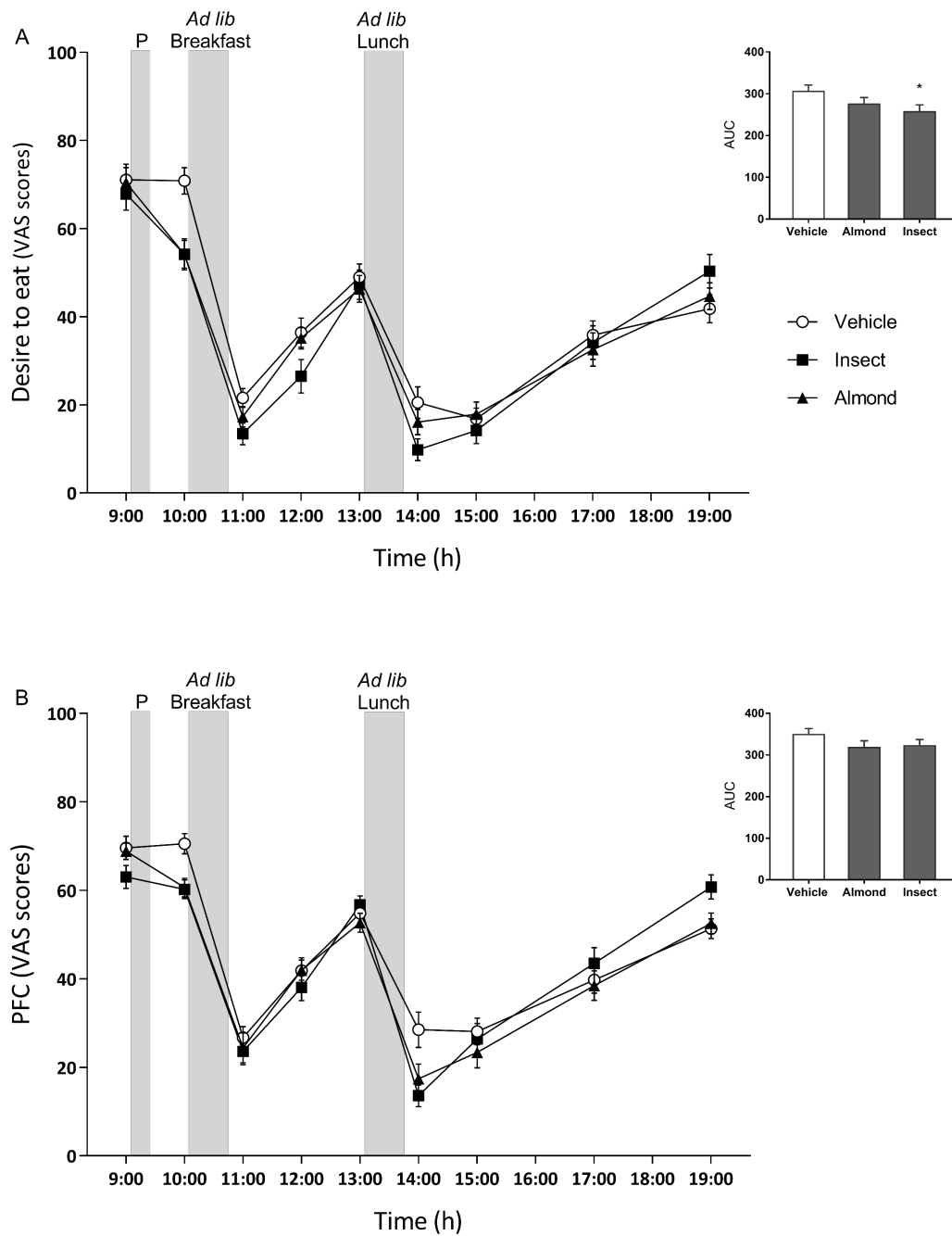
Figure 4. Desire to eat (A) and prospective food consumption (B) ratings during the three test days. Grey bars indicate the times of the preload (P), breakfast, and lunch intake. Participants were asked to complete VAS questionnaires to rate their motivation to eat five minutes before P and at the subsequent time points indicated in the graph. Data are expressed as mean ± SEM. Two factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons were used.

Ratings for prospective food consumption (**Figure 4B**) were independent of the type of preload administered ($p = 0.076$), but they were affected by time and preload by time interaction ($p < 0.0001$ and $p = 0.001$, respectively). Both the insect and almond administered groups reported that they could eat less one and five hours after the preload than the vehicle group did. These differences were statistically significant. At the end of the study, ten hours after the administration of the preload, the insect administered group thought they could eat more than did the almond or vehicle administered groups.

The participants were asked to answer additional questions about their motivation to eat as well as other sensations in the VAS questionnaires. We analysed stress, tiredness, sleepiness, thirst, and tummy rumbling. Only the tummy rumbling question showed a preload by subject gender interaction. Therefore, all the data for both genders are presented together except for this question. These results are shown in **Figures S1-S5** of the supplementary material. The hunger and fullness questions were excluded from the analysis as they were not statistically valid.

A preload effect was observed ($p = 0.042$) for the stress ratings: the insect group felt less stressed than the vehicle group. One hour after the breakfast and lunch test meals, both the insect and almond administered groups felt significantly less stressed than the vehicle group. A preload interaction was observed ($p < 0.0001$) for the feeling of tiredness, with lower tiredness ratings reported for the insect administered group than the almond or vehicle administered groups. One hour after breakfast and one hour after lunch, both the vehicle and almond administered groups felt significantly more tired than the insect group. Also, the almond group felt sleepier one hour after the breakfast and lunch test meals than the insect group. For the sleepiness ratings, we observed a preload effect ($p = 0.003$) in which the insect group felt less sleepy than the almond group. We also observed a preload interaction ($p = 0.012$) in participants' thirst ratings after the preload intake. The insect administered group reported greater thirst than the vehicle group. Finally, we observed a preload interaction ($p < 0.001$) in the men's

tummy rumbling scores that showed that the insect group had lower ratings than the vehicle or almond groups. No preload effect was observed for the women's scores.

Although specific time point differences between the three preloads were observed for some questions (detailed in the supplementary material, **Figures S1-S5**), no preload by time interaction was observed for any of the answered questions.

3.3 Preload palatability and correlation with food intake

The participants were offered a cocoa milkshake containing almond, insect or no protein source (vehicle). The flavour and texture of the three drinks was as similar as possible. Nonetheless, we wanted to check the potential effect of the palatability of the preload on food intake. To this end, the participants were asked to rate the overall likeability, pleasantness of taste and the likeability of the texture by completing VAS type questionnaires scaled from 0 to 10. We found that the preload affected the ratings of overall likeability, taste, and texture of the preloads ($p = 0.001$, $p = 0.006$ and $p < 0.0001$, respectively). Nevertheless, there were no significant differences between the two protein preloads regarding these three parameters (**Table 2**). The vehicle preload obtained significantly higher ratings for texture and overall likeability compared with the two protein preloads and slightly higher ratings for pleasantness of taste compared to the insect preload.

The relationship between the palatability of the preloads and subsequent energy intake was tested using Pearson's correlation analysis. Food energy intake during the test meals, at breakfast and lunch, was not correlated with the measures of pleasantness of taste ($r = 0.152$, $r = 0.104$ respectively), overall likeability ($r = 0.158$, $r = -0.002$ respectively) or likeability of texture ($r = 0.196$, $r = -0.061$ respectively) for any of the three preloads ($p > 0.05$ in all cases).

Table 2. Palatability scores (0-10) for the three preloads containing either insect, almond or no protein source (vehicle).

Preload type	Vehicle	Insect	Almond
Overall likeability of preload meal	7.98 ± 0.24 [a]	5.34 ± 0.28 [b]	5.36 ± 0.28 [b]
Pleasantness of taste	7.61 ± 0.40 [a]	5. 34 ± 0.49 [b]	6.14 ± 0.39 [a,b]
Likeability of texture	8.46 ± 0.38 [a]	4.00 ± 0.45 [b]	3.21 ± 0.45 [b]

All values are means ± SEMs. Means in a row with different superscript letters were significantly different (one factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons, $p < 0.01$).

3.4 Digestive health and its correlation with food intake

As we are testing a non-conventional food product, we wanted to monitor possible side effects at the gastrointestinal level. First, we worked with the VAS questionnaire in which participants were asked to indicate any feeling of indigestion (**Figure 5**). The questionnaire monitored any side effects following the consumption of the preload and test meals, at each time point when the appetite VAS questionnaires were completed. After the consumption of each preload, we observed a significant effect of time ($p < 0.001$) and preload by time interaction ($p = 0.038$) throughout the study period. We also observed a preload effect ($p = 0.007$) in which the insect preload gave rise to a greater sensation of indigestion than the vehicle. Nevertheless, Pearson's correlation analysis indicated that there was no relationship between the intake at breakfast and the indigestion scores just before that meal (r = -0.034, $p = 0.756$). However, we observed a positive correlation between the intake at lunch and the indigestion scores just before that meal (r = 0.236, $p = 0.031$).
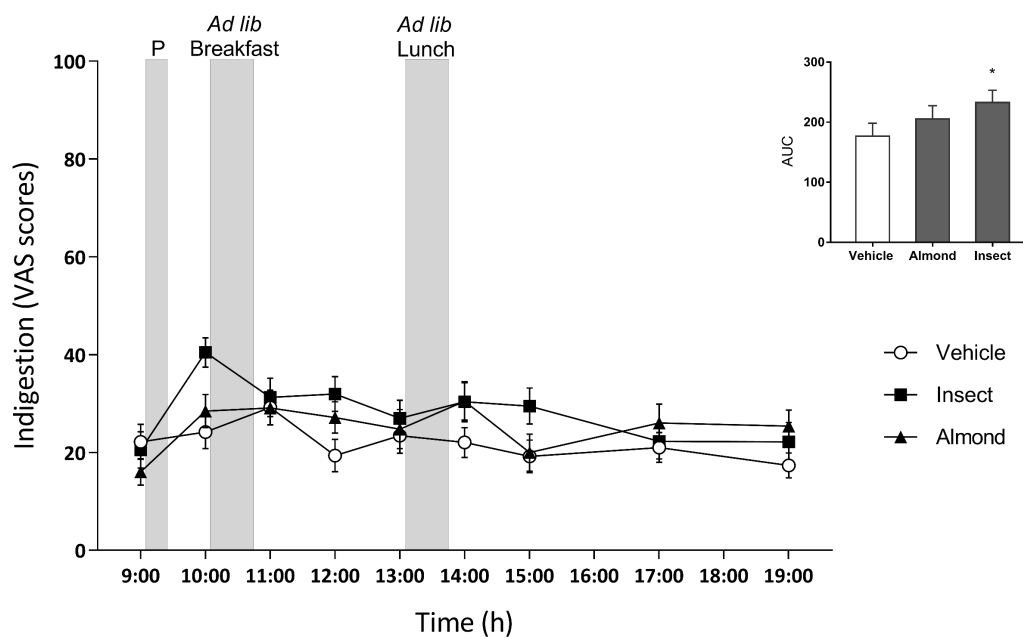
Figure 5. Indigestion ratings during the three test days. Grey bars indicate the time of the preload (P), breakfast, and lunch intake. Data are expressed as mean ± SEM. Two factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons were used.

Secondly, since we are comparing two protein sources with different fibre matrices and protein qualities (Accardo, F. *et al*., 2022, submitted), we also measured how the preload might affect intestinal tract mobility. As with other variables, there was no interaction between gender and the preload (p = 0.347), so data for both genders are presented together. In this case, we observed a subject sex effect (p = 0.026). **Figure 6** shows that there was no preload effect in relation to gender (p = 0.936). What we found was a strong negative correlation (r = -0.345, p = 0.009) between intestinal mobility after the preload intake and the indigestion AUC, suggesting that a greater sensation of indigestion induces shorter intestinal transit.
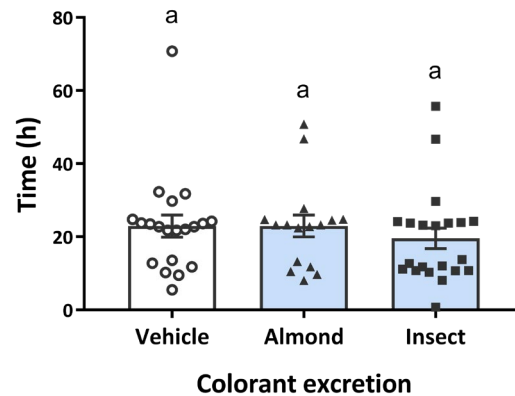
Figure 6. Colorant excretion in faeces. Participants were asked to report the time at which they noticed colorant in their stool. Data are expressed as participant means (dots) and mean ± SEM of the participants from each group (bars). Same letters indicate no significant differences, $p > 0.05$ (one-factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons).

4. DISCUSSION

This is the first study to evaluate the effect of insect consumption on food intake in humans. As expected, both almond and insect preloads (20 g) reduced food intake more than the vehicle (non-protein preload, 5.7 g) one hour after consumption at breakfast (**Figure 2A**). Nevertheless, no differences were found between the two protein preloads even though the almond preload contained 2.3 times more energy than the insect preload. The same occurred at lunch, when the almond group ate the same amount as the vehicle administered group despite having 6.6 times more energy content (**Figure 2B**). This might be explained by the low energy absorption [33] and low protein bioavailability [34] described for almonds. Meanwhile, the preload containing insect exhibited a different profile at lunch, increasing food intake at that time point compared to the vehicle and almond groups. This increased food intake was previously observed in rats 20 hours after the oral administration of an insect protein dose [29], and in humans when comparing a high energy dense preload with a low energy dense preload of the same weight [35]. It is thought that the chitin present in insects affects protein digestibility [36], and may interfere with the absorption of other

nutrients. However, in a previous analysis by our group in which we worked with the same samples used in this study, we found that the digestibility and bioaccessibility of insect protein is better than those of almond (Accardo, F. *et al.*, 2022, submitted). Another explanation might be compensation for the lower energy intake at breakfast. In fact, when we quantified the cumulative energy intake (breakfast and lunch together), the differences in food intake during the two meals disappeared. This indicates that the participants compensated for the energy consumed within the preload, for both preloads, similarly to that reported in Hull, S. *et al* [37] after a mid-morning almond snack.

Several studies have shown that palatability affects satiation, with an increasing intake as palatability increases [38,39]. In our study, the palatability of both protein preloads obtained lower ratings than the vehicle. However, these ratings were not correlated with food intake at breakfast or at lunch, suggesting that palatability did not influence the effects of the preloads on food intake. We also wanted to rule out the possibility that food intake was affected by any side effects caused by the preload. However, as we have shown, although the insect preload obtained higher ratings for the sensation of indigestion (at 10:00 a.m.) than the vehicle did, these were not correlated with food intake at breakfast (r = -0.034, p = 0.756). On the other hand, a positive correlation was observed between indigestion scores at 1:00 p.m. and food intake at lunch, where we observed increased food intake (r = 0.236, p = 0.031). A strong negative correlation was also observed between the colorant excretion rate and the indigestion ratings. Nevertheless, no differences between the three preloads were found regarding this aspect (**Figure 6**), and together with the absence of correlation between indigestion or food intake, we can say that the preloads did not cause any side effects, as reported by other authors[20] for cricket consumption.

The mechanism underlying the satiety process have not yet been fully described. Different pathways are involved and how they work together is still under debate. For that reason, discrepancies in food intake after an equivalent protein dose administration are not new in satiety studies. As an example, whey has been

reported to reduce food intake compared with tuna, egg and, turkey [3], when compared with soya and gluten [4] and with casein [5]. However, an increased food intake after a whey preload has been also reported compared with pea and casein [6]. Taken together, this evidence, along with the scarce information about insects and their effect after consumption, reinforce the hypothesis that the effects on satiation of a protein dose does not depend only on the quantity, but also on the quality, dose, administration time, bioavailability, and the experimental design.

In this study we decided to include a VAS questionnaire, a reproducible and established method to gather data on appetite-related sensations, thus allowing us to conduct a more complete analysis. 'Desire to eat' and 'prospective food consumption' ratings at 10:00 a.m. were in accordance with the reduced energy intake at breakfast after both protein preloads compared with the vehicle (**Figure 4**). VAS ratings of appetite and their ability to predict subsequent food intake in young subjects has been demonstrated in several studies [30,31]. Nevertheless, in our study, this approach failed to predict the subsequent food intake at lunch. The non-motivation to eat questions were given lower ratings by the insect group for: stress, compared with the vehicle group; tiredness, compared with the almond and vehicle groups; sleepiness, compared with the almond group; tummy rumbling (only in men), compared with both the almond and the vehicle groups. These results indicate that the almond preload seemed to make the participants feel more tired and sleepier than the insect preload, probably because of the greater amount of energy consumed, as reported by Wells, A.S. *et al.* for subjects two to three hours after a high-fat meal [40].

Other authors have found that the consumption of extra protein reduced the appeal of subsequent high-protein foods, causing an aversion or a regulation of protein intake [41,42]. Griffioen-Rosse *et al*. [43] reported this effect in a subsequent meal when comparing the protein content between their high- and low-protein meals of 13 g, similar to the 15 g difference between our protein preloads and the vehicle, 30 min after preload administration. We reproduced

this effect at breakfast, but only with the insect preload. After the insect preload, participants had lower protein intakes than participants in either the almond or vehicle groups (**Figure 3A**). As we were administering the same amount of protein to the insect and almond groups, we did not expect to find differences between them in the amount of protein consumed at breakfast. This effect was compensated for at lunch time, resulting in no differences in the cumulative amount of ingested protein for either preload.

Taken together, these results suggest that these two protein preloads present different profiles. An almond-derived protein preload increases both total protein and energy intake after an acute administration. Nevertheless, many studies support the endorsement of almonds as a suitable healthy snack for weight management, as no body weight or food intake increase have been reported after chronic almond consumption [44,45]. In addition to the high protein content of almonds, they also contain a substantial amount of healthy fatty acids and fibre [46]. Meanwhile, an insect-derived protein preload (*A. diaperinus*) could be used as a product to increase protein intake, since we have described its ability to increase total protein intake with a slight increase in the total energy intake (**Figures 2E, 3E**). Insect protein is a new food, and little information has been published about its effect when consumed. It has high nutritional value [14–19], and no adverse side effects were reported after the consumption of insect protein in this study, which is supported by its extensive traditional consumption in other areas of the world, such as Latin America, Asia and Africa. Since it has been described as healthy as meat products [47], and given the results obtained in this study, it may serve as a promising ingredient for snacks suitable for elderly or infirm patients who would benefit from increased protein intake.

In conclusion, our results showed that almond and *A. diaperinus* protein preloads modify food intake differently. Both were useful in increasing total protein intake, but the insect source was able to maintain that intake with only a slight increase in energy intake.

Conflicts of Interest: The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Questions included in the VAS questionnaire.

Motivation to eat	Other sensations
How strong is your desire to eat?	*I feel anxious*
How hungry do you feel?	*I am tired*
How much do you think you could eat?	*I have indigestion*
How full do you feel?	*I feel sleepy*
	How thirsty do you feel?
	My tummy is rumbling

VAS, Visual Analogue Scale
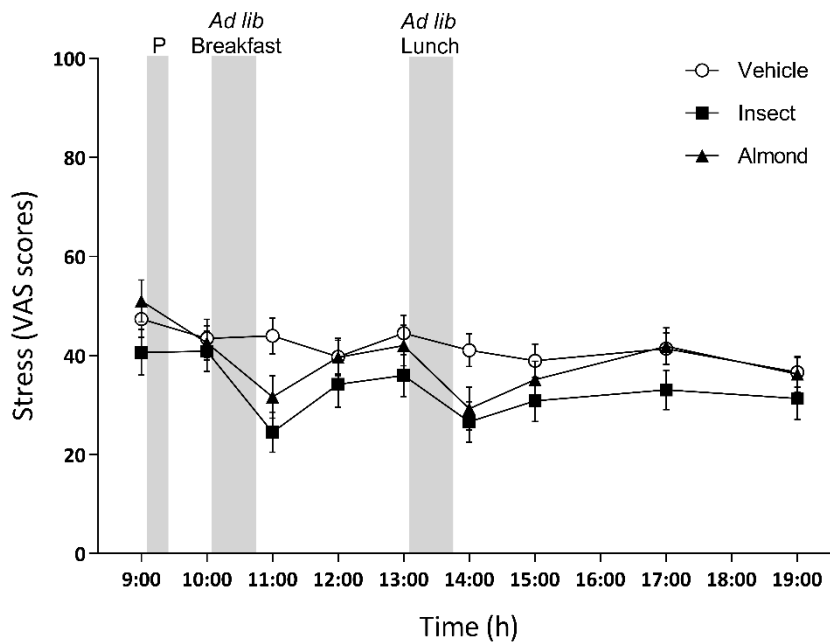
Figure S1. Stress ratings during the three test days. Grey bars indicate the times of the preload (P), breakfast, and lunch intake. There was a preload interaction (p = 0.042), where the insect group felt less stressed compared with the vehicle group. No differences were observed between almond and the other groups. One hour after breakfast and one hour after lunch, both insect and almond administered groups significantly felt less stressed compared with the vehicle group. Nevertheless, there was no preload by time interaction (p = 0.122) but there was a strong time interaction (p < 0.0001). Data are expressed as mean ± SEM. Two factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons were used.
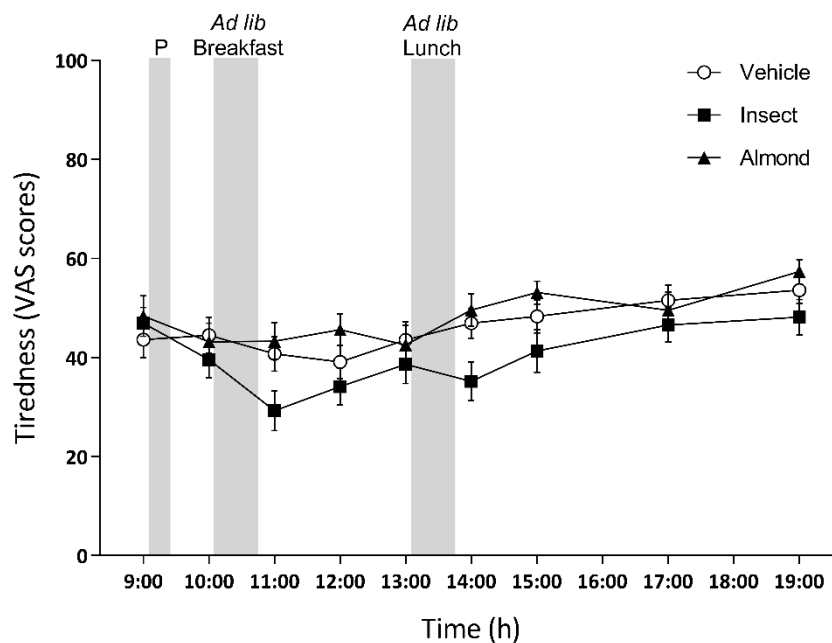
Figure S2. Tiredness ratings during the three test days. Grey bars indicate the times of the preload (P), breakfast, and lunch intake. There was a preload interaction ($p < 0.0001$) with higher tiredness ratings for almond and vehicle administered groups compared with insect administered group. No differences were observed between vehicle and almond groups. One hour after breakfast and one hour after lunch, both vehicle and almond administered groups significantly felt more tired compared with the insect group. Three and six hours after the preload administration, just almond administered group felt more tired compared with the insect group. Nevertheless, there was no preload by time interaction ($p = 0.184$) but there was a strong time interaction ($p < 0.0001$). Data are expressed as mean ± SEM. Two factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons were used.
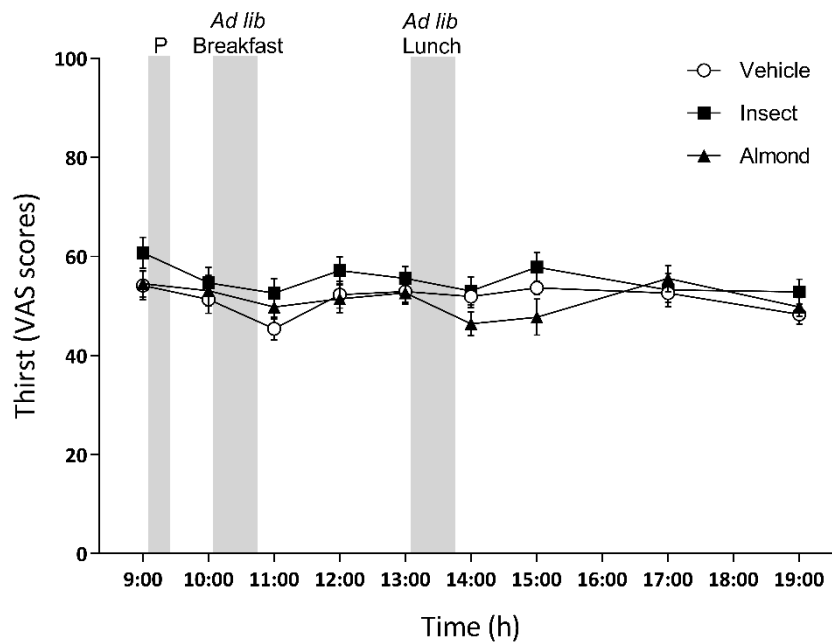
Figure S3. Thirst ratings during the three test days. Grey bars indicate the times of the preload (P), breakfast, and lunch intake. There was a preload interaction ($p = 0.012$) with the insect administered group being the one with higher ratings compared with the vehicle group. A time interaction ($p < 0.0001$), but no a preload by time interaction ($p = 0.232$), was also observed. Data are expressed as mean ± SEM. Two factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons were used.
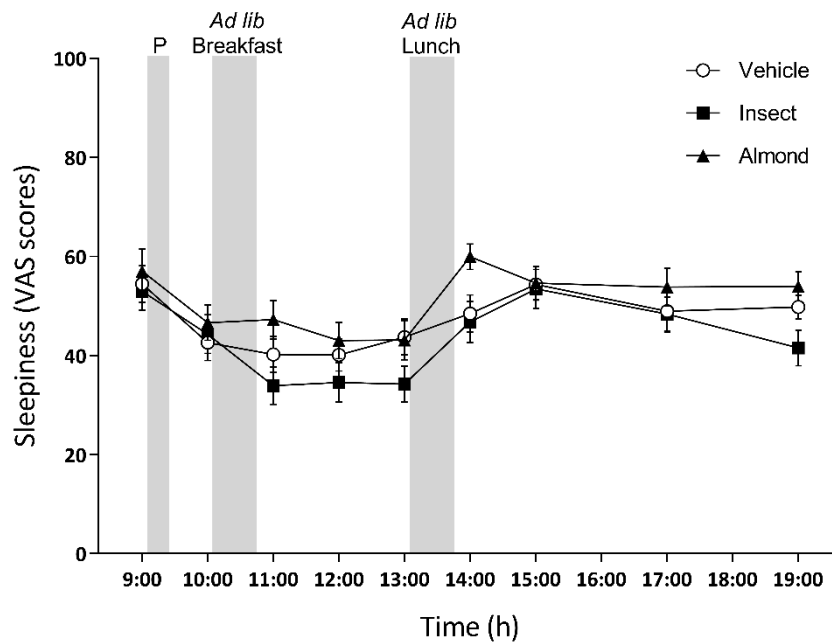
Figure S4. Sleepiness ratings during the three test days. Grey bars indicate the times of the preload (P), breakfast, and lunch intake. There was a preload interaction ($p = 0.003$) whereby insect administered group felt less sleepy than the almond group. One hour after the test meals (both breakfast and lunch) and at the end of the study (10 hours after the preload administration) the insect administered group significantly felt less sleepy compared with almond group. The insect group was also feeling less sleepy compared with the vehicle group just before lunch. Nevertheless, there was no preload by time interaction ($p = 0.238$). In contrast, there was a strong time interaction ($p < 0.0001$). Data are expressed as mean ± SEM. Two factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons were used.
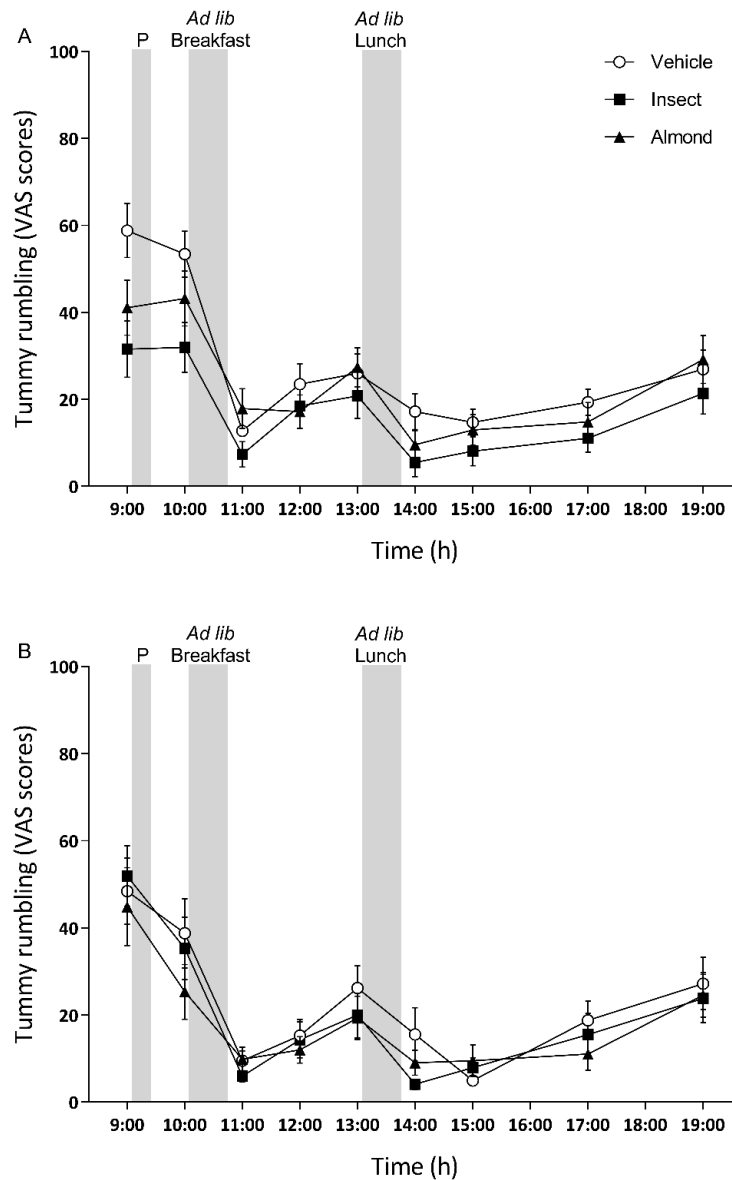
Figure S5. Tummy rumbling ratings during the three test days in men (A) and women (B). Grey bars indicate the times of the preload (P), breakfast, and lunch intake. There was no gender effect ($p = 0.426$) but there was a preload by gender interaction ($p = 0.025$). Thus, we present these results separated for both genders. In men we observed a preload interaction ($p < 0.001$) that showed that the insect administered group had the lower ratings compared with vehicle and almond groups. We also observed a time interaction ($p < 0.0001$) but no preload by time interaction ($p = 0.123$). In women we also observed a time interaction ($p < 0.0001$), as in the men group, but no preload or preload by time interaction was observed ($p = 0.204$ and $p = 0.581$ respectively). Data are expressed as mean ± SEM. Two factor repeated-measures ANOVA followed by Bonferroni adjustment for multiple comparisons were used

PART 3

Analysis of the effects of a chronic administration of insect protein in the enteroendocrine system and food intake in rats.

Manuscript 4

Administration of *Alphitobius diaperinus* or *Tenebrio molitor* Before Meals Transiently Increases Food Intake through Enterohormone Regulation in Female Rats

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Administration of *Alphitobius diaperinus* or *Tenebrio molitor* before Meals Transiently Increases Food Intake through Enterohormone Regulation in Female Rats

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ABSTRACT

It has been previously shown that acutely administered insect *A. diaperinus* protein increases food intake in rats and modifies the *ex vivo* enterohormone secretory profile differently than beef or almond proteins. In this study, we aimed to evaluate whether these effects could be maintained for a longer period and determine the underlying mechanisms. We administered two different insect species to rats for 26 days and measured food intake at different time points. Both insect species increased food intake in the first week, but the effect was later lost. GLP-1 and ghrelin were measured in plasma and *ex vivo*, and no chronic effects on their secretion or desensitization were found. Nevertheless, digested *A. diaperinus* acutely modified GLP-1 and ghrelin secretion *ex vivo*. Our results suggest that increases in food intake could be explained by a local ghrelin reduction acting in the small intestine.

Keywords: dietary protein; gut; enterohormones; food intake; insect

1. INTRODUCTION

Loss of appetite is a common symptom of several health conditions, and it is a frequent problem in the elderly [1], cancer patients [2], especially those undergoing chemotherapy [3], and also in children [4]. The malnutrition caused by loss of appetite is an increasing cause of death in the population aged older than 75 years; however, it is a cause of health loss in the entire population. In all these cases the loss of appetite is always an aggravating factor for the diseases or situations that cause it because reduced food intake leads to weight loss and the deterioration of general health.

Food intake is regulated through a series of complex signalling pathways involving central regulation and peripheral and intestinal signalling. In the intestine, nutrient and food components interact with the epithelium receptors and transporters to signal and regulate the feeding process. The enteroendocrine cells present in the gastrointestinal tract interact with nutrients and secrete hormones in response to food ingestion, acting as chemosensors of the lumen content [5]. Enteroendocrine cells are scattered throughout the gastrointestinal tract, and the hormones they produce are concentrated in specific regions of the gut according to the role they play in regulating these physiological functions [6,7]. Ingested food triggers the release of different enterohormones. Cholecystokinin (CCK) is a hormone secreted in the proximal small intestine that plays an important role in the digestion process that regulates gallbladder contraction and pancreatic enzyme secretion and also modulates intestinal motility and gastric emptying. It responds strongly to the presence of fat and protein in the lumen [8,9]. Glucagon-like peptide 1 (GLP-1) is an incretin hormone released in the distal small intestine and colon, and carbohydrates are a strong stimulus for its release. It is co-localized in the colonic mucosa with peptide YY (PYY). The postprandial levels of these two hormones increase in plasma within 15-30 min in response to nutrients: carbohydrates and lipids are a strong stimulus for GLP-1 secretion while PYY is secreted in proportion to the caloric intake [9,10]. Moreover, ghrelin also modulates food intake. Its secretion

rises in the preprandial state, promoting food intake, and falls rapidly after the ingestion of nutrients [10].

We previously compared the effects of an acute administration of animal protein from insect (*Alphitobius diaperinus)* and beef, and plant protein from almonds on the enterohormone secretion and food intake [11]. We showed that insect, beef and almond proteins modulate food intake differently when administered in a dosage with the same amount of protein one hour before refeeding (beginning of the dark cycle). However, *A. diaperinus* is the only one of these proteins that increased food intake in rats. Using *ex vivo* pig and human intestinal samples we also showed that digested *A. diaperinus* modulated enterohormone release, resulting in a different secretome profile than beef and almond.

The search for new environmentally friendly produced protein sources is becoming more important. Therefore, according to our latest results working with insect protein, in this study we aimed to analyse whether the previously observed orexigenic effects of *Alphitobius diaperinus* were maintained for a longer period and whether they translated to body weight modulation. We also aimed to determine the mechanisms involved in these effects. In addition, we analysed whether the effect was species specific or whether it can be reproduced with other insect proteins.

2. MATERIALS AND METHODS

Chemicals and reagents

Buffalo (*Alphitobius diaperinus* larvae powder) was provided by Protifarm NV (Ermelo, the Netherlands). *Tenebrio molitor* flour of subadult insects was obtained from Iberinsect, S.L (Tarragona, Spain), and processed by FoodIE Research Group, University Rovira i Virgili (URV), Spain. The nutritional composition of these samples is shown in the supplementary materials (**Table S1**).

Chemicals, porcine digestive enzymes (α-amylase, pepsin and pancreatin), bile salts, BSA fatty acid free, D-glucose, D-mannitol, amino acids, aprotinin, protease

inhibitor cocktail (cOmplete™ ULTRA Tablets, Roche) and foetal bovine serum were purchased from Sigma-Aldrich (Madrid, Spain). Amastatin was from Enzo Life Sciences (Madrid, Spain). Glutamine, penicillin, streptomycin and Matrigel from Lonza (O Porriño, Spain).

The ELISA kits for total ghrelin (catalogue no. EZGRT-91K) and total GLP-1 (catalogue no. EZGLPT1-36K) were purchased from Millipore (Billerica, MA, USA). Plasmatic parameters were measured with commercial kits according to manufacturer's instructions: insulin with an Insulin ELISA kit (catalog no. EZRMI-13K) from Millipore (Madrid, Spain), and glucose triglycerides and cholesterol from QCA (Amposta, Spain). The Pierce BCA Protein Assay kit was from ThermoFisher (Waltham, MA, USA).

Animals

We used 24 female RccHan:WIST rats (8 weeks old, 220-240 g) purchased from Janvier (Castellar del Vallés, Spain) and 6 male Wistar rats (used for the *ex vivo* experiment with duodenum). Most of these animals were housed at the animal housing facility of the University Rovira i Virgili. All rats were housed under standard conditions. For the RccHan:WIST rats, upon arrival, the animals were housed in pairs for a week and then individually in animal quarters for another week to get them used to the voluntary oral administration. The rats had free access to food (standard chow, Teklad2014 from ENVIGO), containing (by energy) 20% protein, 13% fat and 67% carbohydrates, and tap water. The room temperature was kept at 22 °C with a 12 h light/12 h dark cycle (lights from 7:00 a.m. to 7:00 p.m.). The animals were used in the experiments after this two-week acclimation period. All procedures were approved by The Animal Ethics Committee of University Rovira i Virgili (Tarragona, Spain) (CEA-OH/10715/3).

Experimental protocol for animal treatments

Animals were divided into three groups (n = 8 each). We tested two different insect sources, *Alphitobius diaperinus* (*A. diaperinus*) and *Tenebrio molitor* (*T. molitor*), at a dose of 300 mg of protein/kg of body weight (BW) dissolved in

water. The control group was administered an equivalent volume of tap water. To perform the treatments, animals were food deprived starting three hours before the treatment, and at 6:00 p.m. animals received the insect protein load or water (control) by controlled voluntary oral intake with a syringe. At 7:00 p.m., when the dark cycle began, they had free access to food again. Treatments started at day 0 and were performed daily for 26 days, with two short breaks in between, of 2 days each, at days 12 and 20. Food intake was measured daily 20 hours after the dark cycle began. Body Weight was measured once a week. At day 13, a plasma sample 20 minutes after the daily insect dose was obtained from the saphenous and immediately frozen for further analysis of the biochemical parameters. At the end of the study the animals were fasted for 12 hours and euthanized by exsanguination (pentobarbital dose of 100 mg/kg of BW for previous sedation). The blood was collected using EDTA covered tubes (Deltalab, Barcelona, Spain) as anticoagulant. Plasma was obtained by centrifugation (4500g, 15 minutes, 4 °C) and stored at −80 °C until analysis. A 0.5 mL blood aliquot to measure ghrelin was collected in a separate tube containing HCl (0.1 M in the final concentration) and protease inhibitors and then centrifuged to obtain the plasma (15.000g, 15 minutes, 4°C). The intestine was carefully excised, washed with cold PBS buffer and the different segments were measured and identified. Pieces of duodenum and distal jejunum were cut and used for *ex vivo* experiments. The rest of the tissue was cut into segments, immediately placed in liquid nitrogen and then stored at -80 °C for further analysis. The different white adipose tissue depots were weighted.

Ex vivo experiments with intestinal segments

Two *ex vivo* experiments were performed: i) a study of the distal jejunum secretory response obtained from the animals treated with *A. diaperinus* as well as the control animals, as mentioned above; ii) another experiment with duodenum from another set of rats (mentioned above) to analyse the secretory response of this segment to acute *A. diaperinus* stimulation.

i) After washing the intestinal tube, the outer muscular layer of the distal jejunum segments was removed from the serosa layer with a scalpel. The tube was then sliced longitudinally and circles of tissue with a diameter of 5 mm were taken using a biopsy punch. The sample was kept at a low temperature with cold PBS buffer and an ice-cold bath during the entire procedure. We then started the secretion study. We placed each distal jejunum circle in a well (48-well plate) containing 0.4 mL of Krebs–Ringer bicarbonate (KRB) buffer with D-mannitol 10 mM pre-warmed to 37 °C for 15 min. After this pre-incubation period, the buffer was replaced by KRB buffer with glucose 10 mM to study baseline secretion, or by pre-warmed intestinally digested *A. diaperinus* dissolved in KRB buffer containing glucose 10 mM to study the response to an acute insect stimulation. Samples were digested as previously described [11] according to the INFOGEST harmonized protocol [12] first published in 2014 by Minekus *et al.* [13]. The protein content of the digested sample was adjusted to a dose of 10 mg protein/mL. The incubation period was 30 min. All the buffers used to incubate the tissue were previously oxygenated (95% O_2, 5% CO_2) for at least 15 minutes. Two replicates of each treatment were performed for each rat. All the treatments contained a cocktail of protease inhibitors, aprotinin 100 KIU, amastatin 10 µM and 0.1% fatty acid free BSA. After the incubation period, the medium was collected in different aliquots and stored at -80 °C for enterohormone quantification.

ii) After a short fasting period (1–3 h), the rats were euthanized by exsanguination (pentobarbital dose of 100 mg/kg of BW for previous sedation) and their intestines were excised. Duodenum segments were collected and the experiment performed as in i), except that the insect treatment was performed this time with 10 mg protein/mL of gastric digested *A. diaperinus.*

GLP-1 secretion test in GLUTag Cells

The GLUTag cells used in the present work were kindly donated by Prof. Staels (University Lille, Institut Pasteur de Lille, Lille, France) with permission from

Prof. Drucker (Lunenfeld-Tanenbaum Research Institute, Toronto, Canada). The medium where the cells were cultured was composed by DMEM (Dulbecco's modified Eagle's medium) containing 1g L−1 D-glucose, supplemented with 10% foetal bovine serum, 1% of 100U mL−1 / 100 mg L−1 Penicillin / Streptomycin and 1% of Glutamine (final concentration 2mM). The cells were incubated under a 5% CO2-humidified atmosphere at 37 °C.

For the treatments, GLUTag cells were plated onto 24-well plates precoated with Matrigel at a density of 200.000 cells/mL 24 h before the secretion study. Cells were then washed twice with PBS buffer and treated for 2 h with *A. diaperinus*, a mix of amino acids or vehicle. To test the effect of *A. diaperinus* on GLP-1 secretion, the intestinally digested *A. diaperinus* sample was dissolved in HEPES buffer (1.25 mM) and tested at a concentration of 5 mg protein/mL. To test the effects of the most abundant amino acids found in the intestinally digested *A. diaperinus* (Accardo, F. *et al*. 2022, Submitted), we prepared a solution in HEPES buffer containing Ala, Phe, Pro, Tyr, Val and Ser at a concentration equivalent to that found in 5 mg/mL of the intestinally digested *A. diaperinus* (0.36 mg /mL, 0.31 mg /mL, 0.35 mg/mL, 0.46 mg/mL, 0.28 mg/mL and 0.29 mg/mL, respectively). The vehicle (HEPES buffer) was used to measure baseline secretion. All the treatments were performed in duplicate in each cell plate and repeated for three passages. After the treatment, the medium of each well was collected and stored at -80 °C in aliquots of 200 µL until the determination of total GLP-1. Next, the cells were lysed with RIPA buffer and lysates were stored at -80 °C. They were then used to analyse total protein content with a BCA kit.

Quantitative Real-Time RT-PCR Analysis

Total RNA and cDNA were obtained as previously defined [14]. Quantitative PCR amplification was performed using specific TaqMan probes from Applied Biosystems (Waltham, USA): Rn00562406_m1 for the GLP-1 receptor gene (*Glp1r*), and Rn00821417_m1 for the ghrelin receptor gene, also known as Growth Hormone Secretagogue Receptor (*Ghsr*). The relative expression of each gene was

compared with the control group using the 2-ΔΔCt method, with PPIA gene expression (Rn00690933_m1) as a reference.

Statistical analysis

The results are expressed as the mean ± the standard error of the mean (SEM). The sample size (*n*) for each variable is indicated in the corresponding figure description. Student's t-test was used to compare the treatments with the control or vehicle group. *p* values < 0.05 were considered statistically significant. These calculations were performed using the XLSTAT 2021.2.1 software (Addinsoft, NY, USA).

3. RESULTS

3.1 The food-intake promoting effects of *Alphitobius diaperinus* are limited in time

To study the effects of a long administration of *A. diaperinus* in rats we administered 300 mg protein/kg BW daily, with two short breaks in between (two days). To test whether the previously shown acute effects of *A. diaperinus* on increasing food intake were maintained after a more prolonged administration, food intake was measured at different time points during the experiment. **Figure 1** shows that there is a tendency (p = 0.058) to increase the 3-day cumulative food intake during the first week of the treatment. However, these effects were lost afterwards, since 3-days cumulative food intake in the second week did not show significant differences. The results for the group treated with a different insect species, *T. molitor*, at an equivalent protein concentration, showed a similar profile to the group treated with *A. diaperinus*, suggesting that the effects were not species specific.
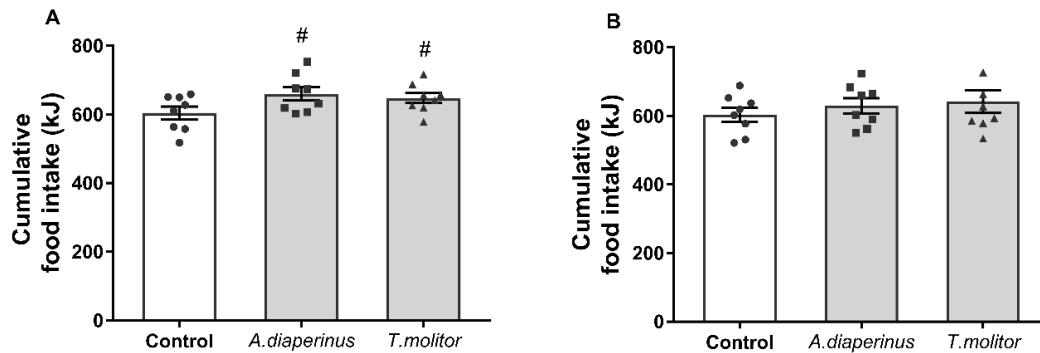
Figure 1. Three-day cumulative food intake within the first (A) and the second (B) week after daily oral administration of an *A. diaperinus* or *T. molitor* dose (300 mg protein/kg BW). Twenty hours food intake was measured on different days during the experiment after the beginning of the dark cycle. The control dose was an equivalent volume of tap water. The sample size was n = 8 rats per group. The results are expressed as the mean ± SEM. # indicates p values < 0.1, T-test.

This increased food intake was accompanied by a significantly higher percentage of body weight gain during the first week in the case of *T. molitor* (p = 0.008), but not for *A. diaperinus* (p = 0.5) (**1.834** ± 0.39, **2.693** ± 1.16, **3.827** ± 0.51 % of BW gain in the control, *A. diaperinus* and *T. molitor*, respectively). The feed efficiency ratio at this time point was also significantly increased in *T. molitor* but not in *A. diaperinus* (**0.034** ± 0.005, **0.059** ± 0.007, **0.040** ± 0.017 g BW increase /kcal in control, *A. diaperinus* and *T. molitor*, respectively). Moreover, in the *T. Molitor* group, body weight gain was still significantly increased at week two, and a tendency remained at the end of the experiment (**Table 1**). However, at sacrifice, the visceral adiposity of the control and insect-fed animals was not significantly different. Fasting biochemical parameters on the sacrifice day (glucose, triglycerides and cholesterol) showed no differences among the different groups (**Table 1**).

3.2 The *Alphitobius diaperinus* treatment increases GLP-1 secretion acutely but does not alter enteroendocrine signalling chronically

We tested the *in vivo* effects of an *A. diaperinus* treatment on the enterohormone signalling system. The effect of both insect loads on total plasmatic GLP-1 were

assayed at day 13. **Figure 2A** shows that 20 minutes after the insect load, the total GLP-1 significantly increased compared to the water-loaded controls. At this time point, the insulin was not modified in these animals (**Figure 2B**), and nor was glucose (**3.86** ± 0.03, **3.85** ± 0.07, **3.82** ± 0.05 mM for the control, *A. diaperinus* and *T. molitor* groups respectively).

Table 1. Parameters at sacrifice.

	Control	*A. diaperinus*	*T. molitor*
Body weight gain (%)	9.42 ± 1.0	9.80 ± 1.0	13.98 ± 2.8 [#]
Visceral adiposity (%)	4.38 ± 0.14	4.26 ± 0.25	5.12 ± 0.64
Glucose (mM)	7.17 ± 0.15	7.34 ± 0.14	7.21 ± 0.38
Triglycerides (mM)	0.18 ± 0.03	0.17 ± 0.01	0.22 ± 0.05
Cholesterol (mM)	0.44 ± 0.01	0.48 ± 0.03	0.46 ± 0.03

Values represent mean ± SEM of eight animals per group. # $p \leq 0.1$ versus Control rats.

The effects of the chronic treatment with *A. diaperinus* were analysed using distal jejunum explants. The day of the sacrifice, the explants were obtained to measure baseline secretion, as well as the acute response to intestinally digested *A. diaperinus.* **Figure 3A** shows that baseline tGLP-1 secretion did not change between the control and insect-fed animals. However, the acute stimulation of the explants with intestinally digested insect, significantly increased the tGLP-1 secretion to the medium both in the control and in the rats fed *A. diaperinus* for 26 days. These results are in accordance with the fact that we did not find any modifications of the mRNA levels from the GLP-1 receptor in the ileum (**1.02** ± 0.1 and **1.49** ± 0.5 for control and *A. diaperinus*, respectively) or hypothalamus (**1.11** ± 0.21 and **1.42** ± 0.18 for control and *A. diaperinus,* respectively). This suggests that there were no desensitization effects due to the chronic treatment.
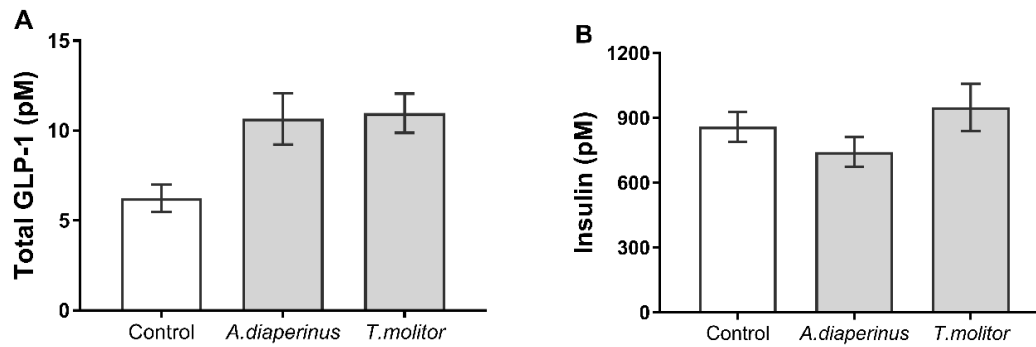
Figure 2. (**A**) Total GLP-1 and (**B**) insulin were measured in plasma collected from safena after 20 minutes of the *A. diaperinus* or *T. molitor* (300 mg protein/kg BW) load. These samples were obtained at day 13 of the experiment. The control was an equivalent volume load of tap water. The sample size was n = 8 rats per group. The results are expressed as the mean ± SEM. * indicates p values < 0.05, T-test.

We worked with GLUTag cells to test whether the acute GLP-1 stimulation was due to direct effects on enteroendocrine cells. **Figure 3B** shows that digested *A. diaperinus* at a concentration of 5 mg protein/mL significantly increases tGLP-1 release. We then tried to decipher whether the observed effects were mainly caused by the protein. To do so we compared the effects of the digested sample with that of a mixture of the most abundant amino acids found in this digested sample (Accardo, F. *et al*. 2022, Submitted), maintaining their concentrations (as described in the materials and methods). **Figure 3B** shows that this mixture of amino acids alone was not responsible for the GLP-1 secretory effect observed for *A. diaperinus*.
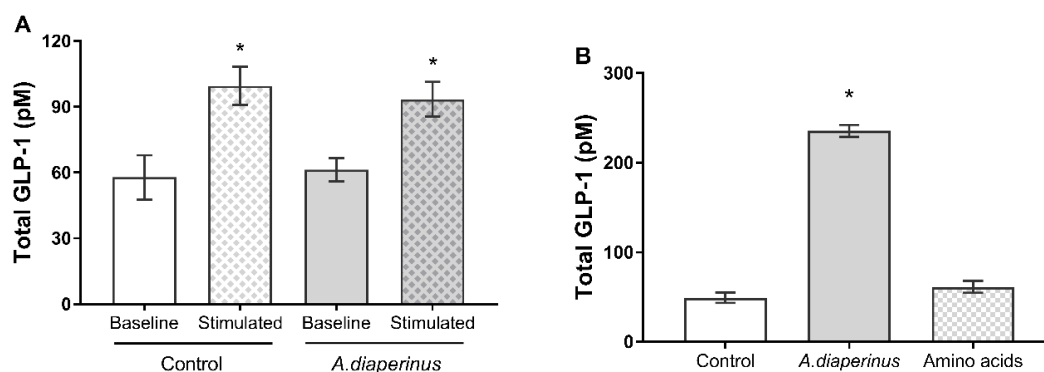

Figure 3. Total GLP-1 secreted to the medium from (**A**) distal jejunum explants obtained

at sacrifice from control and *A. diaperinus* rats in baseline condition (vehicle treated) or stimulated condition (treated with 10 mg protein/mL of intestinally digested *A. diaperinus* for 30 minutes), n = 8 rats each condition, or (**B**) GLUTag cells, treated with 5 mg protein/mL of *A. diaperinus* or amino acid mixture for 2 hours, n = 6, from three different passages. Results are expressed as the mean ± SEM. * indicates p values < 0.05 vs control.

3.3 *Alphitobius diaperinus* reduces acute ghrelin secretion in rat jejunum, showing no chronic effects on ghrelin release

Next, the effects on ghrelin secretion were tested. Ghrelin levels were analysed at day 13, 20 minutes after the daily insect load. There were no differences between the control and *A. diaperinus* groups (**Table 2**). At sacrifice, under fasting conditions, ghrelin levels remained the same in both groups.

Total ghrelin release was measured in the media of the distal jejunum explants obtained at sacrifice, both under baseline conditions and after stimulation with digested *A. diaperinus*. Similarly to what was observed for GLP-1, we found no difference in baseline ghrelin secretion between the control and the rats treated with the insect for 26 days (**0.08** ± 0.02 and **0.06** ± 0.01 ng/mL in control and *A. diaperinus*, respectively). However, explant stimulation with digested *A. diaperinus* led to a reduction in ghrelin release, so that levels were not detectable in either of the two rat groups. To understand the sensitivity to ghrelin we analysed ghrelin receptor expression in the hypothalamus and ileum. **Table 2** shows that while there were no central effects of *A. diaperinus*, the ileal ghrelin receptor tended to be increased in rats administered *A. diaperinus* compared to the controls.

Given the low levels of ghrelin obtained after treating the distal jejunum with the digested *A. diaperinus,* we aimed to reproduce this acute secretion study in an intestine fragment where ghrelin is more abundantly expressed, the duodenum [15]. We obtained *ex vivo* duodenal samples from six different rats and treated them with 10 mg protein/mL of gastric digested *A. diaperinus* for 90 minutes.

Surprisingly, in this case we did not find an inhibition of ghrelin secretion (**1.31** ± 0.3 and **2.87** ± 0.8 ng/mL in the vehicle and *A. diaperinus* treated explants, respectively, p = 0.12, T-test).

Table 2. Total ghrelin plasmatic levels and mRNA levels of the ghrelin receptor, in the ileum and hypothalamus, for control and *A. diaperinus* rats. On day 13, blood samples were collected 20 minutes after the treatments.

	Control	*A. diaperinus*
Total ghrelin (ng/mL)		
Day 13	2.36 ± 0.1	2.63 ± 0.2
Day of sacrifice	1.81 ± 0.1	1.74 ± 0.1
mRNA levels for Ghrelin-R		
Hypothalamus	1.08 ± 0.19	1.13 ± 0.13
Ileum	1.07 ± 0.2	2.94 ± 0.9[#]

Results are expressed as the mean ± SEM. T-test. # $p \leq 0.1$ versus control rats.

4. DISCUSSION

Previously, we compared the effects of an equivalent protein load of beef, insect (*A. diaperinus)* and almond and found that they exerted a different enterohormone secretome, which led to differences in food intake [11]. Our results showed that, surprisingly, only *A. diaperinus,* which reduced ghrelin secretion and increased GLP1 in pig and human intestinal segments, was able to stimulate energy intake when administered to rats as an acute load one hour before refeeding. In the present study we aimed to analyse whether these effects could be extended for several days and determine the mechanisms behind them.

Here we show that the eating-promoting effects of the 300 mg/kg of BW of an *A. diaperinus* protein load are observed during the initial days of the treatment, but later on these effects are lost. Our data on weekly body weight gain and the food efficiency ratio during the first week were not statistically different from the control group. In a small human study, in subjects under resistance training, supplementation with a bar containing *A. diaperinus* protein led to a trend

towards a reduced total energy intake compared to supplementation with an isoenergetic carbohydrate-rich bar; however, these results are based on a short (3 days) weighed-dietary record period, and the administration protocol did not establish any relationship with food intake [16]. *T. molitor*, from the same family as *A. diaperinus* (Tenebrionidae), is a more studied insect, and was therefore also included in the present study. Our results show that it behaved very similarly to *A. diaperinus* in terms of promoting food intake, but it also resulted in significantly increased body weight gain and feed efficiency. The body weight of rats treated with different doses of *T. molitor* for 28 days, was not modified by the 300 mg/kg of BW dose but showed a tendency to increase with the 1000 and 3000 mg/kg of BW dose. This seemed to be related to the food consumption in males, but not females [17]. In Wistar Kyoto strain male rats, treatment for four weeks with defatted *T. molitor* meal showed no effects on food intake or body weight [18]. A longer treatment (90 days) in female rats, treated with 300-3000 mg/kg of BW, also showed no effects on body weight [19]. Instead, increased food intake and body weight were observed in obese Zucker rats fed a meal enriched in *T. molitor*, and this was remarkably accompanied by lipid-lowering effects in the liver [20]. Taken together there is no clear consensus about the effects of *T. molitor* on food intake in rats. The varying results could be explained by different dosages and administration modes. Diverse results have also been found in farm animal species in relation to food intake, which either increased or decreased, although body weight increase has often been reported [21–25]. Further research to clarify the effects of *T. molitor* on food efficiency and the mechanisms behind it would be of interest in order to determine its potential use as an alternative protein source in situations of appetite lost.

To analyse the possible mechanisms for the food intake-promoting properties we studied the effect of insects on the enterohormone system. We previously found that secretion of GLP-1 was stimulated by *A. diaperinus* in the human colon [11]. Now, we have found that in rat distal jejunum explants, treatment with *A. diaperinus* also stimulates GLP-1 secretion. In the current study, we not only

reproduced the GLP-1 secretion in rat explants, but we also found an increase in plasmatic tGLP-1 after administering the insect to the rats. This shows that the GLP-1 promoting effects also take place *in vivo.* Actually, *T. molitor* exerted the same GLP-1 release, suggesting that this effect is not specific to the *A. diaperinus* species. Using the GLUTag cell line, we have shown that *A. diaperinus* acts directly on enterohormone cells to promote GLP-1 release. Since the most abundant component of *A. diaperinus* is protein, we tested whether the GLP-1 releasing effects were due to the most abundant amino acids found in *A. diaperinus.* Our results showed that it was not. The effects then could be mediated by other amino acids, peptides or fatty acids also found in the intestinally digested samples (detailed *A. diaperinus* composition, Accardo, F. *et al.* 2022, Submitted), which could be tested in the future. The lower amount of carbohydrates in this insect powder makes it very unlikely that the effects were due to glucose. In any case, GLP-1 is a well described anorexigenic hormone, and its secretion does not explain the increase in food intake observed in *A. diaperinus*-fed rats. In our experimental protocol we administered the protein load to food-deprived animals at 6:00 p.m. in the evening, and food was replaced one hour after treatment. It has been previously shown that GLP-1 does not induce satiety in fasted rats [26], so this would explain why the secretion of an anorexigenic hormone did not lead to a reduced food intake.

Our previous results showed that beef protein also stimulated GLP-1 secretion in human explants; however, it differed from insect protein in that it did not modify colonic ghrelin secretion, while *A. diaperinus* significantly reduced it [11]. Now we have shown a reduction in ghrelin secretion in rat distal jejunum after treatment with digested *A. diaperinus.* This result agrees with the fact that most metabolite GPCRs controlling ghrelin secretion are inhibitory, whereas all metabolite receptors controlling GLP-1 secretion are stimulatory [27]. This result is striking since ghrelin is a well-known orexigenic hormone. However, it has been shown in mice that when there is a 60 minutes difference between ghrelin and GLP-1 administration, the prevailing action on vagal afferents and feeding

response is that of the second hormone administered [28]. Thus, the GLP-1 secretion induced by food replacement might have overcome the effects of the lowered ghrelin levels 60 minutes earlier, annulling the effect of a reduced orexigenic signalling. Furthermore, it has been proposed that when ghrelin peaks before meals, as a food anticipatory hormonal response [29], this acts as a priming for glucose-stimulated GLP-1 secretion [30] and sensitizes gut neurons to the actions of GLP-1 [31]. According to this hypothesis, acute administration of insect protein before meals would reduce this ghrelin priming. Therefore, after food replacement, intestinal glucose-stimulated GLP-1 secretion and/or GLP-1 sensing would be lower in insect treated rats than in control animals, and this reduced satiating signalling could lead to increased food intake, as we observed until the second week. In the present study we did not find a reduction in plasma ghrelin. This could be explained by a local effect of ghrelin priming in the small intestine that does not involve changes in peripheric ghrelin levels. However, another possible explanation could be the time points when it was analysed. Plasmatic levels at sacrifice were measured 22 hours after the last insect dose, so the acute effects were not observed. Before the sacrifice, at week 13, we also measured ghrelin levels 20 minutes after the *A. diaperinus* dose. This is the time point at which the effect on GLP-1 could be seen but when the effect on ghrelin was still not observed. Treatment with peptone, a meat hydrolysate that reduces ghrelin secretion in mice jejunum *ex vivo*, reduced plasma ghrelin levels 40 minutes after oral gavage [32]. A similar profile was observed for ghrelin with other treatments 60 minutes after their administration [33]. Actually, in another set of rats we found that an acute treatment with *in vitro* digested *A. diaperinus* did not reduce ghrelin secretion in duodenum, as it did in the distal jejunum of the control and *A. diaperinus*-fed rats. This is not unusual since amino acids and small peptides are sensed by the ghrelin cells that respond by activating or inhibiting ghrelin-release pathways depending on the region in the gut [32]. It has been shown that while the primary source of ghrelin is the gastric mucosa, small intestinal nutrient exposure is sufficient to decrease postprandial ghrelin levels [34]. It has also been suggested that glucose, lipids and a mixture of

essential and non-essential amino acids reduced ghrelin secretion through distal effects to the stomach and duodenum [35]. Thus, it is possible that some components of *A. diaperinus* reached the jejunum *in vivo*, reducing intestinal ghrelin secretion, as observed *ex vivo*. Phenylalanine, which is abundant in *A. diaperinus*, has been reported to reduce active ghrelin *in vivo* in rats [36], and *ex vivo* in mouse gastric mucosa [37].

Although we did not find chronic effects on baseline or *A. diaperinus*-stimulated enterohormone secretion, our results showed that the effects on stimulating food intake were lost after one week. We then hypothesized that the enterohormone sensing was altered after this week. Our results showed that there was no central effect on the GLP-1 or ghrelin receptor gene expression. However, in the ileum, there was a tendency towards an increased mRNA of the ghrelin receptor, which makes sense with the hypothesis that the reduction in ghrelin might have local effects on the GLP-1 priming system. Thus, there might be increased intestinal ghrelin sensing that compensates for the *A. diaperinus*-lowered ghrelin levels, leading to a loss in effects on food intake.

In conclusion, we showed for the first time the chronic effects of feeding rats with *A. diaperinus*. With our experimental administration protocol in a fasted state, one hour before refeeding, *A. diaperinus* initially increased food intake. This could be explained by a local ghrelin reduction acting in the small intestine. These results indicate that insect protein could be useful for treating loss of appetite, although compensatory effects should be characterized further to avoid the loss of effects that we found in this study.

Conflicts of Interest: The authors declare that they have no known conflicting financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY MATERIAL

Table S1. Nutritional composition of raw insects (values per 100 g insect flour).

Composition	*A. diaperinus*	*T. molitor*
Energy (kJ)	2550	2604
Protein (g)	56.31	56.1
Total lipids (g)	18.82	26.31
Starch (g)	1.30	3.34
Fibre (g)	7.44	7.78

GENERAL DISCUSSION

GENERAL DISCUSSION

In this thesis, we aimed to characterize the ability of the insect protein to modulate the enterohormone secretion and subsequent food intake and to determine if it can have long-term effects on this enteroendocrine function. For this purpose, we worked with a high-quality animal protein (beef), a vegetal protein-rich in fibre (almond) and insect protein *(Alphitobius diaperinus)*, that is considered as a high-quality protein source with potential low digestibility (which refers to the nutrient absorption of an aliment), similar to almond.

First, we assayed the effects of these three different protein sources on the secretome in *ex vivo* models of pig (for the duodenal secretions) and human intestine (for the colonic secretions). Due to the limited access to fresh human intestinal samples, especially from some intestinal locations, it is common to use animal models for research purposes. Besides, the pig is considered a close and valid model for human studies [1] and it has been reported to produce high secretions of PYY, together with CCK secretions and measurable amounts of acylated ghrelin, in the duodenum [2].

Our first results showed that gastric digested insect, almond and beef significantly modulated the pig duodenal enterohormone profile: they increased CCK and PYY while reducing ghrelin secretion (**Manuscript 1**). The strength of this modulation was different for each sample, even though the protein quantity was adjusted for the experiment, which suggests that the total amount of protein was not the only factor responsible for the secretion of these hormones. Considering the composition of the three treatments, the most abundant component is protein, followed by lipids, in a different degree among the samples, and glucose in a much smaller proportion. Lipids and protein have been reported as a strong stimulus for CCK secretion [3,4] and PYY is secreted in proportion to caloric intake [5,6]. The reduction of ghrelin levels is proportional to the energy load and macronutrient content, and its suppression is greatly promoted by protein and lipids [7,8]. The differences in the lipidic content of the

digested samples imply that their energy load is different, insect being the most caloric, followed by almond and beef. However, our results showed that the increased secretion of PYY and CCK or the inhibition of ghrelin secretion does not correlate with the caloric content of the different food sources, suggesting that the quality of the protein could be the primary factor responsible for the observed differences. Thus, we analysed the possible relationship between the AA composition of the samples and the effects observed *ex vivo* through a Principal Component Analysis (PCA) (**Manuscript 2**).

CCK secretion was positively associated with the AAs more abundant in the digested insect, herein SER, VAL, TRP, ALA, TYR, PRO, ILE, THR and PHE. PHE and TRP are considered potent stimuli for CCK secretion [9]. L-PHE stimulation *ex vivo* with proximal intestinal tissue in mice increased CCK secretion, as well as *in vitro* with STC-1 cells [10]. Wang *et al*. reported the same effect in isolated intestinal cells from mice [11]. On the other hand, VAL has been reported to not stimulate CCK secretion *in vivo* in men [12] or *ex vivo* in pig [13]. However, in this last experiment, treatment with ILE showed a increased secretion of CCK. No information has been found for similar experiments about SER, ALA, TYR and PRO and their CCK secretory capacity. Then, these correlations suggest that CCK secretion in pig duodenum by gastric digested insect could be mainly triggered by PHE, TRP and ILE. Contrary, the CCK release triggered by the digested beef seemed to be responsible for the presence of other compounds, but not affected by its AA composition, since the AA profile of this sample was not associated with the increased secretion observed for this hormone.

Active ghrelin seemed to be negatively related to the main AAs present in the gastric digested beef (HPRO, LYS, LEU, HIS, CYS, MET, and THR). In humans, LEU and CYS have shown a reducing effect in ghrelin plasmatic levels [14,15]. This supports our previous results *ex vivo* with pig duodenum, indicating that beef was the most active source at reducing the active ghrelin secretion, which could be caused at least by LEU and CYS. On the other hand, a clear positive association was also observed between the AA composition of the digested

almond (GLY, ARG, GLU and ASP) and PYY secretion. We also observed a positive association between ghrelin and PHE, which was unexpected since neither beef nor the other tested samples stimulated ghrelin secretion. The commonly described effect for PHE is a reduction of active ghrelin *in vivo* in rats [16], and *ex vivo* with mouse gastric mucosa [17].

As observed with the duodenum, we also found differences among the protein treatments in their effectiveness on modulating PYY, ghrelin and GLP-1 secretions in human colon explants. Here again, neither the energy load nor the macronutrient composition of the intestinally digested samples explained the effects on the enterohormone secretion. However, the AA composition of the samples was correlated with most of the results. Total GLP-1 secretion can be explained in part because of the AA composition of the intestinally digested beef. We checked this *in vitro* by treating intestinal cells (GLUTag) with a mix of some of the most abundant AAs in this sample (HIS, HPRO, LYS, LEU, MET, CYS), obtaining a significant increase in GLP-1 secretion compared to the vehicle. Meyer-Gerspach *et al.* showed that an intragastric infusion of LEU did not elicit any increase in GLP-1 in lean participants [18]. Contrary, and in agreement with our results, a positive correlation between LEU, LYS, MET and ILE plasmatic levels in humans and GLP-1 levels has been previously reported by other authors [19]. Then, we can conclude that the AA composition of the intestinally digested beef is probably responsible for the GLP-1 secretion observed in human colon *ex vivo*. On the other hand, some of the most abundant AAs in the intestinally digested insect (TYR, PRO and PHE), were negatively associated with the secretion of total ghrelin in human colon. As mentioned above, PHE is described to exert a reduction of active ghrelin secretion in rodents [16,17]. No other comparable studies have been found for other amino acids [20]. Again for PYY, a positive association was observed with the more abundant AAs in the digested almond. Therefore, the secretory profile observed in both pig duodenum and human colon samples, triggered by the different digested foods, can be partially explained by their protein quality, determined by the AA composition.

We also wanted to test if the differences between the secretomes obtained *ex vivo* could give rise to different physiological effects, such as changes in food intake patterns. Protein has been reported to have a good satiating capacity compared to other macronutrients [21,22]. The three different protein sources produced a satiating secretory profile. Nevertheless, the totality of these results was not translated to rats or humans *in vivo*.

We observed that almond was the most satiating while insect surprisingly increased food intake in female rats 20 hours after the administration of a 300 mg/kg BW protein dose, 1 hour after refeeding. The beef treatment did not modulate energy intake compared with the vehicle. Here again, the AA composition of the raw samples was in accordance with the observed results. On the one hand, we observed that food intake was inversely associated with the AAs predominant in almond (GLY, ARG and GLU). These results are in accordance with results from other authors that have demonstrated that ARG and GLU are two of the most anorectic AAs in rats [23]. Nonetheless, the satiating effect of almond disappeared in the overall energy intake, including the energy from the preload. This is similar to that observed in other experiments where, although it had the highest satiety quotient, almond total energy intake did not differ from the other treatments [21]. On the other hand, food intake was positively associated with the AAs predominant in the insect *A. diaperinus* (SER, ALA, TYR, VAL and PRO). Jordi *et al.* reported an increased food intake in children consuming higher amounts of VAL [24]. In piglets under a low protein diet, LEU and VAL balance markedly increased the feed intake [25]. Contrary, VAL was one of the 8 AAs implicated in the appetite-suppressant effects of whey proteins [19]. In mice fed a high-fat diet supplemented with VAL, this effect was not shown [26]. On the other hand, SER supplementation in mice led to a reduced food intake [27,28], but this was observed only after chronic supplementation. Other authors have reported that an acute administration of ARG to rodents led to reduced food intake and increased PYY secretion [23,29]. This supports our results, which negatively correlated ARG, GLY and GLU with food intake and

positively with PYY secreted levels. Also in healthy human volunteers, ARG in combination with a meal can significantly elevate PYY, although no reduction in food intake was found [30].

The *ex vivo* secretions triggered by the different protein sources were not in accordance with the effects later observed on food intake in humans (**Manuscript 3**). We tested only almond and insect proteins, since beef was not effective in modulating food intake in rats. Both protein preloads, containing 20 g of protein, reduced food intake more than the vehicle one hour after their consumption, at breakfast. Nevertheless, this reduction was not different between both treatments even though the almond preload had more energy than the insect. The same occurred at lunch when the almond group ate the same as the vehicle administered group despite receiving substantially more energy than vehicle group. On the other hand, the preload containing insect showed a different profile at lunch since at this time point it increased food intake compared with vehicle and almond groups, similarly to what we observed previously in rats. Since our analysis (**Manuscript 2**) showed that the bioavailability of insect protein was higher than the almond one, we discarded any interferences on nutrient absorption caused by chitin for this group. Thus, the high food intake that we observed at lunch for the insect group may be explained by the compensation of the low energy consumed during breakfast, as reported in similar experiments from other authors [31]. Differences in food intake after the administration of an equivalent protein dose from different protein sources have been previously reported in the literature. In a study with rats, a high protein meal composed of albumin suppressed food intake while a wheat gluten-based meal, with the same composition in macronutrients, increased food intake [33]. Other examples are the different effects of a whey protein dose compared with the same dose of tuna, egg, and turkey [34], whey compared with soya and gluten [35], whey and casein [36] or whey, pea, and casein [37]. These varying results could be explained by the different dosages, protein status and experimental designs.

We also wanted to discard that food intake was affected by any side effects caused by the preload, as we are testing a not common protein source. We observed higher indigestion ratings for the insect administered group compared with the vehicle group. However, these ratings, just before breakfast, were not correlated with food intake at this time point. On the other hand, a positive correlation was observed between indigestion scores and food intake at lunch, where we observed an increased food intake. Thus, we can say that the preloads did not cause any side effects as reported by other authors after cricket consumption [32].

Once the first screening about the effects of these protein sources was made, *in vivo* and *ex vivo*, we aimed to analyse whether the previously acutely observed orexigenic effects of *Alphitobius diaperinus* could be maintained for a longer period. To rats we administered a protein dose of 300 mg/kg of BW of *A. diaperinus* – the same dose we used in **Manuscript 1**- for 28 days together with the maintenance diet. We also included a group treated with a more studied insect, *T. molitor*, that belongs to the same family as *A. diaperinus*, in order to see in these effects were species-specific. As discussed in **Manuscript 4**, we observed that the chronic administration of *A. diaperinus* to rats in a fasted state, 1 hour before refeeding, showed an increase in food intake but only during the first week of treatment. The same effect was described for *T. molitor*, although this group also showed an increase in body weight. We hypothesized that these effects on food intake could be mediated by a local ghrelin reduction acting in the small intestine. We observed a reduction in ghrelin secretion in rat distal jejunum after treatment with digested *A. diaperinus*. When GLP-1 is administered 1 hour after ghrelin, the effect that persists is that of the second hormone, that is GLP-1 [38]. Here, although we observed a reduced ghrelin secretion when examined *ex vivo* after the treatment with *A. diaperinus*, the increased GLP-1 plasma levels caused by the refeeding of the animals could have masked the effects of the potentially reduced ghrelin levels triggered by the insect preload 1 hour before this refeeding (parameter not measured). Besides, when ghrelin peaks before meals, it acts as priming for glucose-stimulated GLP-1 secretion [39]

and sensitizes gut neurons to GLP-1 [40]. Therefore, this priming could not be happening in these animals because of the reduced ghrelin levels caused by the insect administration. This, together with the fact that GLP-1 has been shown to not induce satiety in a fasted state, leads to non-effects from this satiating hormone. On the other hand, the loss of effect on food intake modulation could be explained by an altered local ghrelin sensing since we observed a subtle increase in the ghrelin receptor mRNA in the ileum. Hence, this increased ghrelin sensing might compensate for the reduction of the release of this hormone after the *A. diaperinus* administration, being responsible for the later loss of the orexigenic effects.

With all the results presented in this thesis, we have partially described the effects that a non-commonly consumed protein source, of high quality and environmentally friendly production, has on the enteroendocrine system. Concluding, first, we showed that insect, almond and beef significantly modulated the enteroendocrine secretion *ex vivo* in intestinal samples producing a satiating secretome. Nevertheless, when administered acutely to rats, we observed a different effect between them, although being administered in equal amounts of protein. *A. diaperinus* increased food intake while almond limited it, with no differences observed between beef and the vehicle. These effects on food intake were mostly related to the AA profile of these protein sources. A high presence of VAL, SER and ALA and a low presence of ARG, GLU and GLY in the samples were positively associated with an increased food intake in rats. When administered chronically, *A. diaperinus* initially increased food intake, an effect that was lost after one week of treatment probably due to a local ghrelin reduction acting in the small intestine. When we administered these protein sources, insect and almond, to human subjects, we observed again that they modulated food intake. Both increased protein intake with a subtle increase in energy intake after the insect protein preload. Therefore, these properties made insect a good candidate that could be considered as a promising ingredient for snacks suitable for elderly or sick patients where increased food intake is desired.

As we have exposed before, the satiety effect of food is the combination of multiple factors and signalling pathways that together with the experimental design could lead to different results even when we compare the same amount of macronutrient proceeding from the same source. Therefore, further studies will be useful to complete the mechanistic landscape surrounding this alternative protein source and its effects on the enteroendocrine system.

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CONCLUSIONS

CONCLUSIONS

1. Insect protein from *Alphitobius diaperinus* modulates the enteroendocrine system and food intake acutely in rats.

–   Insect protein acts at the intestinal level modulating the enterohormone secretion with some differences with almond and beef.

–   The enterohormone secretion is partially correlated with the amino acid composition of the samples.

–   Food intake is modulated in a different way by the three protein sources: insect increases food intake while almond limits it when administered at a dose of 300 mg/kg body weight in a fasted state one hour previous to food access.

2. An *Alphitobius diaperinus* preload administered to healthy subjects modulates food intake different to almond.

–   One hour after the administration both protein preloads reduce food intake. Four hours after the administration, the insect group eats more than the almond and the vehicle groups.

–   Both protein preloads increase total protein intake while insect one causes a lower increase in total energy intake than almond.

3. The orexigenic effects of a chronic insect protein administration declined over time.

-   The effects in the modulation of food intake are not species-specific since *Tenebrio molitor* also exerts similar effects.

-   The orexigenic effects of *A. diaperinus* are maintained for one week, likely mediated through ghrelin reduction in the intestine. Later on, this condition is compensated by changes in the local ghrelin sensing.

The mode of administration of the insect protein, i.e. in a fasted state one hour previously to refeeding, seems to be relevant for the effects of insect protein on the enterohormone interplay and their effect on food intake modulation.

ANNEX

ANNEX

Published Papers

Miguéns-Gómez, A.; Grau-Bové, C.; Sierra-Cruz, M.; Jorba-Martín, R.; Caro, A.; Rodríguez-Gallego, E.; Beltrán-Debón, R.; Blay, M.T.; Terra, X.; Ardévol, A.; et al. Gastrointestinally digested protein from the insect alphitobius diaperinus stimulates a different intestinal secretome than beef or almond, producing a differential response in food intake in rats. *Nutrients* 2020, *12*, 1–15, doi:10.3390/nu12082366.

Grau-Bové, C.; Sierra-Cruz, M.; **Miguéns-Gómez, A**.; Rodríguez-Gallego, E.; Beltrán-Debón, R.; Blay, M.; Terra, X.; Pinent, M.; Ardévol, A. A ten-day grape seed procyanidin treatment prevents certain ageing processes in female rats over the long term. *Nutrients* 2020, *12*, 1–12, doi:10.3390/nu12123647.

Grau-Bové, C.; **Miguéns-Gómez, A.**; González-Quilen, C.; Fernández-López, J.A.; Remesar, X.; Torres-Fuentes, C.; Ávila-Román, J.; Rodríguez-Gallego, E.; Beltrán-Debón, R.; Blay, M.T.; et al. Modulation of food intake by differential TAS2R stimulation in rat. *Nutrients* 2020, *12*, 1–13, doi:10.3390/nu12123784.

Miguéns-Gómez, A.; Casanova-Martí, À.; Blay, M.T.; Terra, X.; Beltrán-Debón, R.; Rodríguez-Gallego, E.; Ardévol, A.; Pinent, M. GLP-1 regulation by food proteins and protein hydrolysates. *Nutr. Res. Rev.* 2021, *34*, 1–17, doi:10.1017/S0954422421000019.

Sierra-Cruz, M.; **Miguéns-Gómez, A**.; Grau-Bové, C.; Rodríguez-Gallego, E.; Blay, M.; Pinent, M.; Ardévol, A.; Terra, X.; Beltrán-Debón, R. Grape-seed proanthocyanidin extract reverts obesity-related metabolic derangements in aged female rats. *Nutrients* 2021, *13*, 1–16, doi:10.3390/nu13062059.

Submitted Papers

Sierra-Cruz M.; **Miguéns-Gómez A**.; Rodríguez-Gallego E.; D'Addario C.; Di Bartolomeo M.; Blay M.; Pinent M.; Beltrán-Debón R.; Terra X. Effects of grape seed proanthocyanidin extract on lipopolysaccharide translocation and trafficking from the gut to tissues. Submitted to Food Chemistry, 18 January 2022.

Miguéns-Gómez A.; Sierra-Cruz M.; Rodríguez-Gallego E.; Beltrán-Debón R.; Blay M.; Terra X.; Pinent M.; Ardèvol A. Molecular Composition of Lipid and Protein Fraction of Almond, Beef and Lesser Mealworm After in vitro Simulated Gastrointestinal Digestion and Correlation with the Hormone-Stimulating

Properties of the Digesta. Submitted to Food Research International, 3 February 2022.

Miguéns-Gómez A.; Sierra-Cruz M.; Segú H.; Beltrán-Debón R.; Rodríguez-Gallego E.; Terra X.; Blay M.; Pérez-Vendrel AM.; Pinent M.; Ardèvol A. Administration of Alphitobius diaperinus or Tenebrio molitor before meals transiently increases food intake through enterohormone regulation in female rats. Submitted to Journal of Agricultural and Food Chemistry, 28 February 2022.

Miguéns-Gómez A.; Sierra-Cruz M.; Rodríguez-Gallego E.; Beltrán-Debón R.; Blay M.; Terra X.; Pinent M.; Ardèvol A. Effect of an acute insect protein preload vs an almond preload on energy intake, subjective food consumption and intestinal health in healthy young adults. Submitted to Nutrients, 7 March 2022.

Papers under Preparation

Miguéns-Gómez A., Sierra-Cruz M., Pérez-Vendrel AM., Rodríguez-Gallego E., Beltrán-Debón R., Terra X., Ardévol A., Pinent M. Preventive effects of grape seed procyanidins in enteroendocrine system in 21-months-old female rats treated with cafeteria diet.

Segú H., **Miguéns-Gómez A.,** Jalševac F., Rodríguez-Gallego E., Beltrán-Debón R., Terra X., Ardévol A., Pinent M., Blay M. Effects in the immunological response of high protein-insect flour supplementation to healthy and endotoxin treated-Wistar rats.

Sierra-Cruz M.; **Miguéns-Gómez A.;** Grau-Bové C.; Rodríguez-Gallego E.; Blay M.; Ardévol A.; Pinent M.; Terra X.; Beltrán-Debón R. Maintenance of intestinal barrier function in aged rats fed with grape seed proanthocyanidin extract supplemented cafeteria diet.

Poster Communications

Miguéns-Gómez A.; Grau-Bové C.; Sierra-Cruz M.; Beltran-Debon R.; Terra X.; Blay M.; Pinent M.; and Ardévol A. Insect proteins induce a secretion profile intermediate between almond and beef protein. Presented at the International Platform of Insects for Food and Feed (IPIFF) congress, December 2019.

Miguéns-Gómez A.; Grau-Bové C.; Sierra-Cruz M.; Rodríguez-Gallego E.; Beltrán-Debón R.; Blay M., Terra X.; Pinent M.; and Ardévol A. Long term differential effects of procyanidins on CCK secretion in young versus 21-month-old female rats under cafeteria diet. Presented at the 4th European Summer School on Nutrigenomics, University of Camerino, June 2021.

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vivido un poco de todo durante este doctorado, a nivel profesional y personal. Una montaña rusa, por ambas partes, con todas las palabras, pero creo que con todas las fatalidades y todo lo bueno ha sido *amazing*. Echar unas 38 horas al día en la universidad, rescatar muestras de un congelador roto, aprender la técnica de las *ussing chambers* (que tanto nos gusta), el baileoteo en el Highland, mucho llaollao y playa… todo eso y mucho más ha forjado amistad muy fuerte, que seguro ya se queda para siempre. Me ha encantado vivir esta etapa de vuestra mano. A las dos, mil gracias por todo.

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Nowadays, animal protein and derived products are the most extended ones for human consumption as high-quality protein sources. Due to the overpopulation that the world is facing, its demand is expected to increase globally in the next years. However, meat production carries numerous negative implications for the environment. For this reason, the search for alternative environmentally friendly produced protein sources is becoming more important. Insects have been characterized as a good source of high-quality protein, also containing a significant amount of good quality fats, minerals and vitamins. Although many beneficial effects on health have been characterized, scarce information is available about their bioactivity at the intestinal level. Hence, in this thesis we aimed to characterize the ability of the insect protein to modulate the enterohormone secretion and subsequent food intake, comparing it with more traditional protein sources, and to determine if it can have long-term effects on this enteroendocrine function.

We showed that insect protein, from *Alphitobius diaperinus*, can modulate the enterohormone secretion *ex vivo* in intestinal samples producing secretory profile with some differences compared with the secretome obtained from almond and beef proteins. Some of the secretions have been related to the amino acid composition of the respective protein sources. When the different protein sources were acutely administered to rats, we observed that the food intake from the insect group was increased compared with the almond and beef groups, although the protein dose was adjusted for the three treatments. After a chronic administration, this increase in food intake was lost after a week of treatment. The same occurred with *Tenebrio molitor*, which meant that the observed effect was not species-specific. In human subjects, the administration of a protein preload from the insect *A. diaperinus* modulated food intake similarly to an almond preload. We observed an increased protein intake after both preloads with a lower increase in energy intake after the insect protein preload.

In conclusion, insect protein is a promising candidate to be considered as a bioactive ingredient intended for people who need to increase their food intake.